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The allostatic impact of chronic ethanol on gene expression: A genetic analysis of chronic intermittent ethanol treatment in the BXD cohort

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Abstract

The transition from acute to chronic ethanol exposure leads to lasting behavioral and physiological changes such as increased consumption, dependence, and withdrawal. Changes in brain gene expression are hypothesized to underlie these adaptive responses to ethanol. Previous studies on acute ethanol identified genetic variation in brain gene expression networks and behavioral responses to ethanol across the BXD panel of recombinant inbred mice. In this work, we have performed the first joint genetic and genomic analysis of transcriptome shifts in response to chronic intermittent ethanol (CIE) by vapor chamber exposure in a BXD cohort. CIE treatment is known to produce significant and sustained changes in ethanol consumption with repeated cycles of ethanol vapor. Using Affymetrix microarray analysis of prefrontal cortex (PFC) and nucleus accumbens (NAC) RNA, we compared CIE expression responses to those seen following acute ethanol treatment, and to voluntary ethanol consumption. Gene expression changes in PFC and NAC after CIE overlapped significantly across brain regions and with previously published expression following acute ethanol. Genes highly modulated by CIE were enriched for specific biological processes including synaptic transmission, neuron ensheathment, intracellular signaling, and neuronal projection development. Expression quantitative trait locus (eQTL) analyses identified genomic loci associated with ethanol-induced transcriptional changes with largely distinct loci identified between brain regions. Correlating CIE-regulated genes to ethanol

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alcohol.2016.07.010>.

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consumption data identified specific genes highly associated with variation in the increase in drinking seen with repeated cycles of CIE. In particular, multiple myelin-related genes were identified. Furthermore, genetic variance in or near dynamin3 (*Dnm3*) on Chr1 at ~164 Mb may have a major regulatory role in CIE-responsive gene expression. *Dnm3* expression correlates significantly with ethanol consumption, is contained in a highly ranked functional group of CIE-regulated genes in the NAC, and has a *cis*-eQTL within a genomic region linked with multiple CIE-responsive genes.

Keywords

Chronic intermittent ethanol; Bioinformatics; Genomics

1. Introduction

Alcohol use disorder (AUD) is the complex result of a multitude of central nervous system (CNS) adaptations following long-term, repeated episodes of heavy ethanol consumption and withdrawal. Although AUD is a uniquely human trait possibly requiring decades to develop, key facets of ethanol-induced behaviors and cognate molecular adaptations following acute ethanol exposure can be studied across a spectrum of animal models including monkeys, mice, rats, flies, and worms. (Becker & Hale, 1993; Bettinger, Leung, Bolling, Goldsmith, & Davies, 2012; Bhandari, Kendler, Bettinger, Davies, & Grotewiel, 2009). Moreover, human genetic studies have shown that acute behavioral responses to ethanol have predictive ability in terms of long-term risks for developing AUD (Schuckit, 1994). Gene targeting studies in animal models also frequently show correlations between alterations in acute behavioral responses and ethanol consumption behaviors (Crabbe, 2012).

The molecular and cellular mechanisms underlying the transition from acute ethanol exposure to abusive behaviors in AUD are unknown. Changes in stress reactivity, gene expression, and neuronal signaling all accompany acute ethanol exposure and have been postulated to lead to chronic adaptations—essentially an allostatic imprint on the CNS (Costin, Wolen, Fitting, Shelton, & Miles, 2013; McBride et al., 2005). Proving causality between molecular changes and long-lasting behaviors has not yet been achieved (Heilig & Egli, 2006; Higley, Koob, & Mason, 2012).

As an approach to identifying causal relationships between molecular effects and chronic ethanol consumption, we have exploited a mouse genetic model of chronic ethanol exposure and progressive consumption, together with a genomic analysis of regional changes in gene expression in the brain. Our goal is to identify possible transcripts and shared processes underlying the transition in the brain from acute ethanol exposure to chronic intermittent ethanol exposure and withdrawal.

The chronic intermittent ethanol vapor model (CIE) has been widely used in rodent studies (Lopez & Becker, 2005, 2012; O'Dell, Roberts, Smith, & Koob, 2004; Roberts, Heyser, Cole, Griffin, & Koob, 2000) as a tool to approximate the repeated cycles of heavy consumption and withdrawal that are seen in humans during development of AUD. CIE exposed rats or mice will show alterations in the amount and pattern of ethanol

consumption, generally increasing their ethanol consumption following CIE vapor (Becker, 2013; Griffin, Lopez, Yanke, Middaugh, & Becker, 2009; Lopez & Becker, 2005; O'Dell et al., 2004). Genomic studies have correlated patterns of gene expression following CIE with coincident changes in ethanol consumption (Osterndorff-Kahanek, Ponomarev, Blednov, & Harris, 2013). For example, recent CIE studies on brain derived neurotrophic factor (BDNF) signaling events and miRNA regulatory mechanisms hint at one elegant potential mechanism for neuro-adaptation to chronic ethanol (Darcq et al., 2015; Logrip, Janak, & Ron, 2009; Smith et al., 2016). The CIE model provides a powerful tool for discovery and hypothesis testing of processes and mechanisms underlying progressive ethanol consumption. However, to identify more causal links between CIE behavioral adaptations and specific molecular mechanisms, we combined the CIE model with both a genetic and genomic analysis. Such a “genetical genomics” approach has been widely used with other genetic studies on ethanol behaviors (Hitzemann et al., 2014; Tabakoff et al., 2009), but only rarely with a matched analysis of ethanol-evoked changes in gene expression (Putman et al., 2016; Wolen et al., 2012).

Here, we report an initial analysis of CIE influences on ethanol consumption and gene expression changes in two brain regions of mice from the BXD recombinant inbred panel. Our results identify significant similarities and differences between acute and CIE genomic responses to ethanol. We characterized the functional groups associated with CIE genomic responses and highlight possible genetic intervals crucial for both CIE-evoked changes in gene expression and ethanol consumption. Our findings highlight the promise of this integrated behavioral, genetic and genomic analysis of CIE and suggest future work that may identify novel targets for therapeutic development in AUD.

2. Materials and methods

2.1. Animals

Male and female C57BL/6J and DBA/2J mice for CIE experiments were purchased from Jackson Laboratory at 10 weeks old (Bar Harbor, ME). After 1 week acclimation to the animal facility, mice were singly housed for 72 h prior to the drinking experiments. Male and female BXD RI strains for CIE (n = 43 strains) at 12–16 weeks old were supplied by the University of Tennessee Health Sciences Center (Memphis, TN). BXD mice were single housed immediately, and began drinking experiments after 72 h acclimation to single housing. Please refer to Supplemental Table 1 and Lopez, Miles, Williams, and Becker (2016) in this Special Issue for additional details regarding strain number and attrition during the CIE protocol. All mice were housed individual in an AALAC-accredited facility under 12 h light/dark cycles with free access to food and water. All animal housing and care was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Council, 2011).

2.2. Chronic intermittent ethanol (CIE)

Chronic intermittent ethanol procedures were performed at Medical University of South Carolina with approval by the Institutional Animal Care and Use Committee, according to well-established procedures shown to cause increased ethanol consumption (Lopez &

Becker, 2005). Since the CIE model is a complex and time consuming behavioral analysis, as an initial genetic analysis of CIE-evoked behaviors and gene expression alterations, we utilized a design where single animals per strain/treatment were used for most strains to maximize the number of strains for genetic and genomic analysis. After 6 weeks of limited access (2 h/day) baseline drinking with 2-bottle choice 15% v/v ethanol and water, mice (n = 119) representing 43 BXD RI strains and progenitors were divided into two groups: CIE and control. CIE mice received ethanol vapor in Plexiglass inhalation chambers (60 × 36 × 60 cm) for 16 h/day for 4 days. Control mice were also placed in the inhalation chambers for 16 h/day for 4 days, but did not receive ethanol vapor. After 4 days in the inhalation chamber, mice underwent 72 h of complete ethanol abstinence, followed by 5 days limited access drinking (2-bottle choice 15% v/v ethanol and water, 2 h/day) (Lopez & Becker, 2005). This cycle was repeated such that BXD mice underwent 4 sessions of inter-cycle ethanol consumption and 5 sessions of inhalation chamber exposure. Ethanol levels in the inhalation chambers were set to produce blood ethanol concentrations of 200–300 mg/dl. Prior to each vapor chamber session, mice were injected intraperitoneally with 1 mmol/kg pyrazole, an alcohol dehydrogenase inhibitor used to stabilize blood ethanol concentration. Blood was collected from mice after the third day in the inhalation chamber during each inhalation chamber cycle. Mice were sacrificed 72 h after the 5th inhalation chamber session. A schematic of the CIE protocol employed here is given in Fig. 1. A table of the BXD strains used in this analysis is included in Supplemental Table 1.

2.3. Tissue harvesting and RNA isolation

Surviving mice (n = 72) were sacrificed 72 h after CIE procedures by decapitation. Brains were immediately removed, and specific regions dissected using a brain punch micro-dissection and snap frozen in liquid nitrogen. Tissue samples were shipped on dry ice to Virginia Commonwealth University, and stored at –80 °C until RNA isolation. Total RNA was isolated from prefrontal cortex (PFC) and nucleus accumbens (NAC), as described (Kerns et al., 2005; Wolen et al., 2012). Briefly, brain tissue from individual mice (n = 72) was ground with a glass homogenizer, and RNA extracted using Stat 60 (AMS Biotechnology, Abingdon, UK). RNA quality was assessed by capillary gel electrophoresis with the Experion™ Automated Electrophoresis System (BioRad Laboratories, Hercules, CA). Samples showing poor RNA quality (i.e. RIN less than 8, n = 2 for NAC, n = 3 for PFC, Supplemental Table 1) were eliminated from analysis.

For qPCR validation of Dmn3, NAC brain tissue from separate cohorts of C57BL/6J and DBA2/J mice having undergone the same CIE with drinking procedure described were used. Brain regions were harvested using brain punch micro-dissection as previously described (Kerns et al., 2005). Brain tissues were immediately snap frozen in liquid nitrogen, and stored long-term at –80 °C. NAC tissue was lysed using Stat 60 and RNA was extracted with the Qiagen miRNeasy Mini Kit. RNA quality was assessed with the Experion™ System and RNA yield was determined using a Nano-Drop 2000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

2.4. CIE gene expression microarray analysis

Gene expression was quantified with Affymetrix GeneChip® Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Arrays were run on PFC and NAC from 70 mice comprised of 19 BXD RI strains, C57BL/6J and DBA/2J progenitors, and C57BL/6J $\pm$ DBA/2J F1 (n = 1—2 per strain per treatment group for BXDs, B6D2F1, C57BL/6UT and DBA/2J; n = 6 CIE C57BL/6J; n = 8 CTL C57BL/6J; Supplemental Table 1). RNA samples from PFC and NAC were processed separately using standard protocols outlined by Affymetrix. RNA samples were randomized for cRNA preparation, and re-randomized before hybridization and scanning to minimize batch effects.

Mouse Genome 430 2.0 arrays were initially analyzed using Affymetrix Expression Console software. Array quality was assessed based on average background, scaling factor, present probesets, and 3'/5' ratios of *Actin* and *Gapdh*. Robust multichip analysis (RMA) with quantile normalization was performed within the R statistical package for generation of normalized expression values from control and CIE treated animals (Irizarry et al., 2003). CIE responsive genes were identified within each brain region by using the Significance-score (S-score) algorithm (GeneNetwork Accession: GN:299 for PFC and GN:407 for NAC) (Kennedy, Kerns, Kong, Archer, & Miles, 2006; Kerns, Zhang, & Miles, 2003; Zhang, Wang, Ravindranathan, & Miles, 2002). The S-Score is a method developed to measure change in expression between compared oligonucleotide microarrays using probe-level data, and so is particularly suited to comparisons of smaller numbers of chips. The algorithm uses relative changes in probe pair intensities to essentially create z-scores centered around zero for individual probesets, with values reflecting significance of expression change in the positive or negative direction. Thus, while not technically a measure of magnitude of expression change, S-Scores generally correlate to fold-change. S-scores were generated using the S-score R package from each BXD RI strain, C57BL/6J, DBA/2J, and C57BL/6J $\pm$ DBA/2J F1 mice separately. Because only 1 CIE and 1 control sample was available for most BXD RI strains, significance of gene expression response across the BXD cohort was determined using Fisher's Combined Probability Test with S-scores as previously described (Wolen et al., 2012). Ethanol “responsive” genes were identified as probesets with a q-value < 0.05.

2.5. Comparison of CIE and acute ethanol genomic responses in BXD mice

CIE responsive genes identified by Fisher's Combined S-scores in the PFC and NAC, were compared to probesets previously identified as responsive to acute ethanol exposure (4 h, 1.8 g/kg i.p., 6—8/strain) across 29 or 36 strains of the BXD RI cohort in PFC and NAC, respectively (GEO: GSE28515, GeneNetwork Accession: GN:137 for PFC and GN:154 for NAC) (Wolen et al., 2012). Overlap analyses were performed using GeneWeaver (Baker, Jay, Bubier, Langston, & Chesler, 2012) and visualized with Venny (Oliveros, 2007). Overlaps of interest were Acute PFC vs. CIE PFC, Acute NAC vs. CIE NAC, and overlap between all four groups (two brain regions each with two treatment paradigms). Probesets exclusively regulated in the PFC with CIE, and exclusively regulated in the NAC with CIE were also examined. Significance of overlap between pairwise combinations were determined using Fisher's Test for Count Data (Fisher, 1922).

2.6. Pathway enrichment and gene ontology analyses of ethanol-responsive gene sets

ToppGene (<http://toppgene.cchmc.org/>), an open-source gene ontology analysis tool, was used for gene set enrichment analysis for Gene Ontology (GO) terms among the Biological Process, Molecular Function and Cellular Component categories. GO analyses were performed in June 2016. Results were filtered for $FDR < 0.05$ for all the comparisons to increase stringency of the analysis. Results were further filtered to remove terms containing fewer than 2 genes or greater than 1000 genes, and terms with similar definitions and gene lists were trimmed for clarity. Gene Ontology results were then summarized using REVIGO (<http://revigo.irb.hr/>). REVIGO reduces and summarizes GO results by clustering redundant terms into overarching categories representing biological functions (Supek, Bošnjak, Škunca, & Šmuc, 2011). GO Biological Processes were then visualized as tag clouds (Supplemental Fig. 1—5). Ingenuity Pathways Analysis (Ingenuity Systems Inc., Redwood City, CA) was used to identify gene networks coordinately regulated exclusively by CIE in the PFC and NAC, or by acute ethanol and CIE within the PFC or NAC. Ingenuity Pathway Analysis (IPA) was also used to identify over-represented potential upstream regulators such as transcription factors that may be responsible for regulating expression of genes in the set. Networks were limited to 35 molecules, and scored by IPA based on the hypergeometric distribution, calculated using the right-tailed Fisher's Exact test. Results were reported as the $-\log$ of this value, such that a score of 20 indicates that there is a 1 in 10^{20} chance of producing a network containing at least the same number of genes of interest from 35 randomly chosen genes.

2.7. eQTL analyses of ethanol-responsive gene sets

Gene expression data in the form of S-score values for significant ethanol-responsive probesets (acute and CIE), were used as the quantitative trait values to be analyzed across the BXD cohort and are accessible in GeneNetwork (GN299 for PFC and GN407 for NAC, www.genenetwork.org). GeneNetwork web-based tool sets were used for genetic mapping and correlation of quantitative traits such as gene expression data and behavioral parameters (Wang, Williams, & Manly, 2003). GeneNetwork employs genotype data from 3809 markers, selected based on their being informative (i.e. different between progenitor strains). GeneNetwork outputs peak “likelihood ratio statistic” (LRS) locations for each trait, which can be directly converted to log odds ratios (LOD) by dividing by 4.61. In our analysis, we defined “suggestive” eQTLs as markers with an $LRS > 10$ ($LOD > 2.17$) (Williams, 2012). This lenient threshold allowed for a high number of associations between genomic loci and ethanol-responsive transcripts, useful for an exploratory genetical genomics analysis. Within the subsets of suggestive eQTLs for a given brain region and treatment paradigm, significance of individual eQTLs was determined using permutation testing, wherein 5000 random reassignments of genotypes and trait values are compared to the actual observation. Genes were considered significant in the permuted data analysis at $p < 0.05$. (Williams & Broman, 2010). Cis eQTLs were defined as loci within 5 Mb of the gene showing associated ethanol-responsive expression. To identify genomic regions regulating large numbers of ethanol responsive genes, the genome was split into 10 Mb bins and the numbers of significant or suggestive eQTLs were counted within each bin. In the absence of cis eQTLs, QTLminer (Alberts & Schughart, 2010), which integrates gene annotations, expression, and single nucleotide polymorphisms (SNPs), was used to identify candidate regulatory genes.

Within QTLminer, our interval was set as 1 Mb on either side of the peak LRS of interest, the strains for nsSNP count as DBA/2J and C57BL/6J. Input datasets were expression data from treatment of interest (acute or CIE). Dataset 1 used S-score, while Datasets 2 and 3 used RMA values of ethanol treated and control animals, respectively (<http://www.genenetwork.org/webqtl/main.py?FormID=qtlminer>).

2.8. Correlation of gene expression with ethanol drinking behavior

To assess the relationship between ethanol-responsive gene expression and ethanol-drinking behavior across the BXD cohort, we correlated the average ethanol intake over the 5 day period of 2-bottle choice following the 3rd and 4th cycles of ethanol or air vapor exposure to gene expression (RMA values) in ethanol-vapor-exposed (CIE, PFC GN:791, NAC GN:795) or air-exposed (control, PFC GN:789, NAC GN:793) mice. RMA values were generated using a SNP mask to remove probes and probesets that target regions of known SNPs between the D2 and B6 strains. Additionally, the RMA files were corrected for possible batch effects using the ComBat function (Johnson, 2007). These RMA files allowed us to identify probesets whose expression correlated with ethanol drinking behavior in the ethanol-exposed animals, but not in controls. In each comparison, Spearman correlations were used, with p-values for each probeset's correlation coefficient calculated using a two-tailed t-test. The probesets were then ranked in ascending order from the smallest p-value. Spearman correlations with a p-value < 0.05 were considered significant. GO analysis was performed using the ToppGene Suite (as described above) on the lists of significantly correlated genes in the air-exposed or the CIE-exposed mice in both the PFC and the NAC.

We also queried GeneNetwork for other BXD phenotypes related to ethanol consumption after the third cycle of ethanol vapor treatment (GN:12967). The top 500 Spearman correlations were filtered for the term “ethanol” and p-values < 0.05 were considered significant.

2.9. Quantitative real-time reverse transcription e qPCR

Total RNA (1 mg) from 18 DBA/2J and 20 C57BL/6J mice (n = 4—5/group) was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR performed using iQ SYBR Green Supermix and the CFX system (Bio-Rad) according to manufacturer's instructions. Three technical replicates were performed for each cDNA and relative abundance of target transcripts were normalized to *Ppp2a* and *Ublcp1* as reference genes using GeneNorm in the CFX software. Primers were designed to minimize secondary structure formation and cross intron-exon boundaries to reduce genomic DNA amplification. Primers were validated to produce a single PCR product via DNA gel electrophoresis (not shown).

3. Results

3.1. Identification of significantly ethanol-responsive gene expression following CIE

Chronic intermittent ethanol by vapor chamber significantly affected gene expression across the BXD cohort in both the PFC and NAC. In all, the expression of 759 genes in the PFC and 867 genes in NAC were ethanol-responsive by CIE at a significance level of $q < 0.05$

(Supplemental Table 2). We previously found that an acute dose of ethanol significantly altered 1479 genes in PFC and 1373 genes in NAC (Wolen et al., 2012) using a similar genomic and statistical approach. In this study, we compared the probesets altered by acute ethanol to our CIE findings (Fig. 2, Supplemental Table 3) to gain potential insights into conserved vs. unique mechanisms of CIE induced expression changes. The degree of overlap between ethanol-responsive probesets was significant ($p < 0.05$) by Fisher's exact test, when comparing treatment paradigms (Acute PFC vs. CIE PFC, Acute NAC vs. CIE NAC). In the PFC, ~35% of CIE responsive probesets were also significantly responsive to acute ethanol ($n = 269$ probesets), while in NAC ~26% of CIE responsive probesets were also responsive to acute ethanol ($n = 225$ probesets). We found 224 probesets unique to the CIE PFC, and 378 unique to CIE NAC, representing effects specific to brain region and to chronic ethanol. Of interest, 88 probesets were regulated by both acute ethanol and CIE in both brain regions.

3.2. Bioinformatic analyses of gene sets in the PFC and NAC following CIE or acute ethanol

Bioinformatic analysis using ToppGene or Ingenuity Pathway Analysis was performed to identify significantly over-represented biological themes within gene sets (see Supplemental Tables 4-8). Gene Ontology categories were further summarized using REVIGO (see Supplemental Fig. 1—5 (Supek et al., 2011)). Five gene sets developed from our overlap analyses were queried: (1) genes unique to CIE PFC, (2) genes unique to CIE NAC, (3) overlapping genes between acute PFC and CIE PFC (4) overlapping genes between acute NAC and CIE NAC, and (5) overlapping genes between both treatment conditions in PFC and NAC.

3.2.1. Genes exclusively regulated by ethanol in the PFC following CIE—224

probesets were modulated by only CIE and exclusively in the PFC (Supplemental Fig. 1 and Supplemental Table 4). Five molecular functions were significantly overrepresented: protein complex binding, cytoskeletal protein binding, MHC II receptor activity, cell adhesion molecule binding and calmodulin binding. Of the fifty-one Biological Process categories identified, the major themes are involved in action potentials and synaptic transmission, antigen processing and presentation, dopamine receptor signaling, and brain developmental processes including locomotor behavior and learning.

Ingenuity Pathway Analysis (IPA) generated several novel networks, one of which (Fig. 3) contained CaM kinase II and two myelin-associated genes (*Mag* and *Mal*), with Akt as the major integration point. Two types of collagen and N-Cadherin were also in this pathway. The top upstream regulators of this gene set were Huntingtin (HTT) which regulates 32 of the 193 genes analyzed ($p = 1.22 \times 10^{-15}$), and β -estradiol which may regulate 39 out of 193 genes in the set ($p = 4.06 \times 10^{-10}$).

3.2.2. Genes regulated by ethanol in the NAC following CIE—

Three hundred seventy-eight probesets were exclusively altered by ethanol in the NAC only following CIE (Supplemental Fig. 2 and Table 5). Only three Molecular Function categories were significantly over represented: neuropeptide hormone activity, phorbol ester receptor activity and protein heterodimerization activity. Substantially more Biological Processes were

identified (55 categories) and can be described by the main themes of synaptic transmission, negative regulation of cell death, neuron projection development, behavior, including behavioral fear response, learning or memory, and regulation of nervous system development. The ten significant Cellular Component categories reflect many parts of the neuron or synapse including the synapse, axon, dendrite and synaptic vesicles. An additional category from ToppFun is the Mouse Phenotype. For genes altered by ethanol in NAC of CIE BXD mice, significant results in the Mouse Phenotype category highlight potential problems in neurological function: abnormal fear/anxiety-related behavior, synaptic transmission, increased susceptibility to induction of seizure, abnormal learning/memory/conditioning, and locomotor activation. These phenotypic categories are consistent with the gene ontology findings discussed above.

IPA analysis identified gene networks that involved genes within the synapse, BDNF and GABA receptor subunits. CREB1 was identified as the top upstream regulator with 34 genes potentially regulated out of 304 in the analysis ($p = 1 \times 10^{-14}$).

3.2.3. Genes regulated by ethanol in the PFC by CIE and acute ethanol—In the PFC, 269 probesets were in common after overlapping gene sets from the CIE protocol and acute ethanol (Supplemental Fig. 3 and Table 6). Thirty-eight identified Molecular Functions included some rather diverse categories including mRNA binding, syntaxin binding, GABA-A receptor activity, calcium dependent protein binding activity, voltage gated potassium channel activity, toll-like receptor 4 binding and structural constituent of the myelin sheath. Over represented Biological Processes (109 categories) were complementary to the Molecular Function categories and included synaptic transmission, mRNA processing, learning or memory and cognition, neurotransmitter secretion and axon development. Similarly, the 43 categories from the Cellular Component analysis identified many specific parts of the neuron (somatodendritic compartment, synapse, dendrite, neuronal body, dendritic spine, and post synaptic membrane). It also included ion channel complexes, voltage gated potassium channel, SWI/SNF family complex, GABA-A receptor, nuclear chromatin and myelin sheath and axonal region. Mouse Phenotypes identified abnormalities in synaptic transmission, specifically miniature EPSCs, learning, memory and cognition and abnormal gait.

Novel networks based on IPA curated data sets identified networks associated with GABA receptor subunits, synaptic structural elements or voltage gated potassium channels. IPA analysis revealed many similarities between the CIE PFC and NAC overlap analysis. For example, three of the top upstream regulators in the PFC CIE vs. acute ethanol overlap were also found in the top upstream regulators in the CIE PFC vs. NAC overlap: BDNF (28 genes/227 analyzed, $p = 1 \times 10^{-19}$), HTT (39 genes/227, $p = 2.25 \times 10^{-19}$), and APP (36 genes/227, $p = 3.39 \times 10^{-14}$).

3.2.4. Genes regulated by ethanol in the NAC by CIE and acute ethanol—In the NAC, 225 probesets were similarly regulated by ethanol following acute ethanol and the CIE protocol (Supplemental Fig. 4 and Supplemental Table 7). Surprisingly, no Molecular Functions were significantly over represented in the ToppFun analysis. However, 91 categories in the Biological Processes were significantly over-represented. These categories

included such processes as synaptic transmission, neurotransmitter secretion, behavior, CNS development and glutamate receptor signaling. Again, categories identified in the Cellular Component analysis (29 categories) identified many parts of the neuron including neuron projection, synapse, clathrin-coated vesicles, GABA and glutamate transport vesicles, dendritic spine and neuronal cell body. Similar to the PFC, Mouse Phenotypes identified were primarily abnormalities in learning and memory, synaptic transmission, miniature EPSCs and synaptic depression.

The novel networks identified by IPA were again similar in theme and generated networks containing calcium channel and glutamate receptor subunits along with synapse structural components (Fig. 4). Upstream regulators of these genes were similar to the regulators of PFC genes regulated by CIE and acute ethanol: HTT (28 genes/191 analyzed, $p = 2.6 \times 10^{-12}$), BDNF (18 genes/191, $p = 5.8 \times 10^{-11}$) and HDAC4 (11 genes/191, $p = 1.12 \times 10^{-9}$).

3.2.5. Genes similarly regulated by ethanol in PFC and NAC following CIE or acute ethanol—We were also interested in examining the genes which were in common between both PFC and NAC in regulation by acute ethanol or CIE (Supplemental Fig. 5 and Table 8). We reasoned that this group might reflect particularly ethanol-sensitive gene regulatory events that persist following chronic ethanol exposure. Eighty-eight probesets were similarly regulated by both ethanol protocols in both brain regions (Fig. 2). GO analysis identified 16 Biological Process categories (enzyme regulator activity, protein domain specific binding, SMAD binding, voltage gated cation channel activity, calmodulin binding and GTPase regulator activity). The 56 Molecular Function categories were similar in theme to the previous gene set comparisons and included synaptic transmission, neurotransmitter transport, signal release, regulation of calcium ion transport, and regulation of hormone levels as well as learning and memory. However, there were unique categories that included receptor clustering and DNA methylation. Mouse Phenotypes found abnormalities in synaptic transmission, miniature EPSCs and learning and memory.

One of the top novel networks generated by IPA contained either CREBBP, FOS and NEDD4, or voltage gated potassium channel subunits and structural components of the synapse. Importantly, the genes regulated by CIE and acute ethanol in the PFC and NAC shared several upstream regulators with the PFC or NAC alone and included HTT (15 genes/78 analyzed, $p = 9.5 \times 10^{-9}$), BDNF (10 genes/78, $p = 7.35 \times 10^{-8}$), and HDAC4 (6 genes/78, $p = 1.64 \times 10^{-6}$).

3.3. eQTL analyses of ethanol-responsive genes

3.3.1. PFC—When S-scores were studied as a quantitative genetic trait, we found that 633 probesets with significant ethanol responses in the CIE PFC showed at least a suggestive (LRS 10) QTL with 15 of these reaching empirical significance ($p < 0.05$, Supplemental Table 9). S-scores avoid the potential false-positive *cis*-eQTLs that can result from genetic polymorphisms affecting probeset hybridization performance (Wolen et al., 2012). In PFC, none of the observed eQTLs were *cis*-regulated. Top eQTLs in PFC from CIE or acute ethanol (Wolen et al., 2012) treatment are shown in Table 1 ranked by LRS. There was no overlap between the two lists of top ranked eQTLs.

Histograms of the number of ethanol responsive genes with suggestive or significant eQTLs in 10 Mb chromosomal bins are shown in Fig. 5. In the CIE PFC experiment, large peaks representing *trans*-bands, were seen on Chr3 (peak LRS: 39.9 Mb) and Chr16 (peak LRS: 97.8 Mb). Highly suggestive (LRS>18) *trans*-regulated genes at the Chr3 locus were *Ube2b*, *Cplx2*, and *Smarca4*. Significant *trans*-regulated genes at the Chr16 locus were *Ppm1l*, *Ilf3*, *Shk3kbp1*, *Endod1*, and *Syn2*. Analysis by QTLminer within GeneNetwork suggested that the highest ranked candidate regulatory gene within 1 Mb of the Chr3 peak was the cell adhesion molecule, FAT tumor suppressor homolog 4 (*Fat4*). The highest ranked candidate gene for the Chr16 peak was Down syndrome cell adhesion molecule (*Dscam*). There was minimal overlap between the acute PFC eQTL bands and the CIE PFC bands, which may indicate distinct sites of genomic regulation of ethanol responsive transcripts in acute vs. chronic ethanol in the PFC. All ethanol-responsive eQTLs in PFC with LRS > 10 following acute or CIE ethanol treatment, are given in Supplemental Tables 9-10.

3.3.2. NAC—In CIE NAC, 699 probesets with significant ethanol response showed at least a suggestive QTL with 69 of these reaching empirical significance ($p < 0.05$, Supplemental Table 11). There was one significant *cis* eQTL, Nuclear Receptor Subfamily 2, Group C, Member 2 (*Nr2c2*) (eQTL map shown in Supplemental Fig. 6). There were 4 suggestive *cis* eQTLs: *Pcolce*, *Dnm3*, *Gfap*, and *Fbxo39*. Top ranked eQTLs from CIE treatment are compared to those from acute ethanol (Wolen et al., 2012) in Table 2.

Histograms of the number of ethanol responsive genes with suggestive or significant eQTLs in 10 Mb bins are shown in Fig. 6. In CIE NAC, a large peak, corresponding to 109 ethanol-responsive transcripts was observed on Chr6:90–100 Mb. This locus was the site of the significant *Nr2c2* *cis* eQTL, indicating that this gene may play a role in regulating the ethanol-induced expression changes of a large number of genes. Thirty-four *trans*-eQTLs were significantly linked to this region including *Apc*, *Gabra2*, *Slc1a2*, and *Homer1*. All significant eQTLs at this locus are shown in Table 3. Between the acute and chronic treatments, a *trans*-band was observed at Chr1:160–170 Mb; however, the ethanol-responsive transcripts regulated at this site differed between the treatment conditions. The suggestive *cis* eQTL *Kidins220* was located at this site following acute ethanol, whereas the suggestive *cis* eQTL *Dnm3* was found at this site in the CIE NAC data. All ethanol-responsive eQTLs in NAC with LRS > 10 following acute or CIE ethanol treatment, are given in Supplemental Tables 11-12.

3.4. Correlation of gene expression with ethanol drinking behavior

As an initial effort to genetically correlate gene expression to behavioral outcomes of CIE treatment, we generated Spearman correlation analyses between ethanol consumption (g/kg averaged over a 5 day drinking period) following the 3rd and 4th cycles of air or CIE treatment versus RMA expression values from the same treatment groups. Our premise was that expression correlated with ethanol consumption following CIE should reveal a different biological function “signature” than seen with air treated controls. PFC gene lists for correlations from CIE- or air-treated animals are contained in Supplemental Table 13. Only four genes were in common between the CIE ($n = 58$) and air ($n = 170$) gene lists. Table 4 shows the top fifteen correlated genes in PFC for ethanol consumption in CIE-treated and

air-treated animals. Bioinformatic (GO) analysis of PFC genes significantly correlated to 3rd and 4th cycle drinking after CIE-exposure revealed enrichment for Biological Processes related to neuron ensheathment, sodium ion channel activity, and neuron projection development (Supplemental Table 14). Highly correlated genes after CIE-exposure included *Mobp* and *Mbp* (components of the myelin sheath), *Scn2b*, *Scn4b*, *Kcnma1*, and *Gad1*. In contrast, bioinformatic (GO) analysis of PFC genes significantly correlated to 3rd and 4th cycle drinking after air-exposure revealed enrichment for Molecular Processes related to DNA and RNA polymerase binding, biological processes related to synaptic transmission and neurotransmitter secretion, cellular processes related to the synapse and neuronal projection, and mRNA splicing and processing (Supplemental Table 15). Highly correlated genes after air-exposure included *Hook3*, *Trpm7*, *Clasp1* and *Kcnc1*.

A similar correlation analysis was conducted for gene expression in NAC of air- or CIE-treated animals (Supplemental Table 16). There was an overlap of 13 genes between the CIE (n = 150) and the air (n = 160) gene lists. Interestingly, multiple genes involved in neurodevelopment (including *Smarca5*, *Ddx6*, *Qk*, and *Ndrp1*) were highly correlated to drinking in the NAC after the 3rd and 4th cycles of air-exposure, but were not significantly correlated with drinking after CIE-exposure. *Dnm3*, a hub gene that emerged from IPA network analysis (Fig. 4) and as a suggestive eQTL (Supplemental Table 12), was also significantly correlated to drinking (Fig. 7). Table 5 shows the top ten genes whose expression in NAC most highly correlates with ethanol consumption in CIE-treated and air-treated animals.

Of interest, ethanol intake during the 3rd cycle (GN:12967) strongly correlated to ethanol consumption after the other cycles of ethanol vapor exposure from this study, giving additional validity to the robustness of the dataset and the reproducibility of drinking data across these BXD strains (Supplemental Table 17). Ethanol intake during the 3rd cycle also correlates inversely with drinking phenotypes performed in other labs using drinking paradigms without ethanol vapor exposure (GN:12624, $R = -0.714$, $p = 0.0012$ and GN:13578, $R = -0.763$, $p = 0.014$). These strong inverse correlations suggest that the chronic exposure to ethanol vapor has a distinctly different biological effect from simple voluntarily ethanol consumption. Importantly, our phenotype of interest was positively correlated with change in ethanol consumption after a chronic mild stressor relative to control (GN:13573, $R = 0.700$, $p = 0.033$) and negatively correlated with ethanol consumption in the non-stressed control group (GN:13578, $R = -0.763$, $p = 0.014$), further supporting the notion that chronic stress dramatically alters ethanol consumption in mice. The phenotype of interest was positively correlated corticosterone levels in plasma 3 days following the 5th and final ethanol vapor exposure (GN:13023, $R = 0.497$, $p = 0.006$), suggesting that increased levels of stress hormones following a chronic stressor may be a potential driving mechanism leading to increased consumption. The phenotype of interest is also inversely correlated with an anxiety assay (GN:12430, $R = -0.499$, $p = 0.0044$). Finally, the third cycle ethanol intake inversely correlated with the difference in acute ataxia in animals previously exposed to ethanol relative to naïve controls, which is a measure of ethanol sensitization and an inverse measure of ethanol tolerance (GN:10497, $R = -0.821$, $p = 0.02$ and GN:10498, $R = -0.821$, $p = 0.02$). This suggests that animals which are more likely to be sensitized to the acute effects of ethanol, and thus have a lower tolerance, are less likely to consume ethanol after

repeated exposures to ethanol vapor. Future behavioral studies are necessary to directly test this relationship.

3.5. Validation of *Dnm3* downregulation by CIE in male DBA/2J mice

Dynamin-3 (*Dnm3*) is a member of the dynamin family which possesses mechanochemical properties involved in actin-membrane processes, predominantly in membrane budding (Smillie, Pawson, Perkins, Jackson, & Cousin, 2013; Wiejak & Wyroba, 2002). In BXD RI strains, *Dnm3* is significantly ethanol responsive in PFC and NAC, in both acute and chronic ethanol treatment paradigms (Supplemental Table 3). The direction of ethanol response with CIE showed wide variation across the BXD cohort (Fig. 8a), with the D2 progenitor strain showing strong down-regulation and B6 mice showing little response. The direction of the *Dnm3* response to ethanol across the BXD strains showed a strong relationship with the genotype at the *Dnm3* gene locus, as expected given the suggestive *Dnm3* cis-eQTL seen in NAc (Table 2). We performed qPCR on a larger cohort of DBA/2J and C57BL/6J mice following CIE. We noted a down-regulation in CIE mice that was led by D2 males (Two-way ANOVA for interaction $p < 0.05$, partial $\eta^2 = 0.249$, Fig. 8b). *Dnm3* expression appeared to be unaffected by CIE in B6 mice (Two-way ANOVA $p > 0.05$, partial $\eta^2 = 0.066$, Fig. 8c), although we acknowledge the possibility that these data may be underpowered.

4. Discussion

We describe an exploratory genetic and genomic analysis of transcriptome responses to chronic ethanol by intermittent vapor chamber and consumption in BXD mice, and compare these changes to acute ethanol transcriptome responses. Our goal was to gain potential insights into conserved vs. unique mechanisms of ethanol induced expression changes. A striking finding in this study was the significant number of ethanol responsive genes regulated by both acute ethanol and CIE across the BXD cohort, indicating that the initial transcriptional response from acute ethanol significantly informs certain long-term effects of ethanol. Furthermore, while we found significant expression changes unique to CIE treatment, our bioinformatics analysis showed a remarkable overall conservation of functional groups amongst the expression responses either unique or shared across CIE vs. acute ethanol and PFC vs. NAC. Finally, these initial studies identified provisional expression/behavioral correlations and eQTLs which may lead to identification of novel targets for future intervention in abusive ethanol consumption.

Our use of the BXD panel across 2 treatment paradigms provided the opportunity to compare both acute and chronic ethanol exposure paradigms on gene regulation in a genetic panel. A major strength of this was that we were able to employ 43 BXD strains exposed to CIE and 29 BXD strains exposed to acute ethanol, providing a wide range of genotypes. However, our overall power was likely reduced since this initial analysis only was able to utilize 1–2 mice per strain/treatment due to the overall complexity of the animal treatment paradigm. As we previously showed for studies on acute ethanol regulated expression networks across the BXD cohort (Wolen et al., 2012), using the S-score algorithm to directly compare control vs. treated samples within strains, together with a statistical comparison of

S-scores across strains, allowed our analysis to identify large groups of ethanol-responsive genes, despite strain-dependent differences in the magnitude or even direction of the ethanol regulation. The number of strains employed here also provided reasonable ability to identify expression/behavioral correlations. However, more definitive analysis of within-strain expression levels or behavioral results will have to await an ongoing enlarged study that will provide a more powerful genetic analysis of both expression networks and behavioral responses to CIE. Despite these caveats, the studies presented here contribute multiple unique findings.

One of our major assumptions in the experimental design of these studies was that by comparing our prior BXD studies on acute ethanol to these CIE-evoked responses, we would be able to define expression changes more explicitly involved in the long-term neuroadaptive events responsive for progressive ethanol consumption with the CIE paradigm.

Unexpectedly, our studies found a striking overlap between CIE and acute ethanol responses, extending even across both PFC and NAC. This occurred despite the studies occurring nearly a decade apart, employing different investigators doing the primary assays, and having very different experimental designs. The CIE treatment entailed a chronic vapor exposure and repeated oral consumption with a 72 h abstinence period prior to harvesting brain tissue. In contrast, the acute ethanol data was a single i.p. injection with brain tissue harvested 4 h later at a time when blood ethanol levels would just be reaching non-detectable levels. While we did indeed identify gene responses unique to CIE, the overlap between acute and chronic ethanol may provide valuable mechanistic clues to major mechanisms of ethanol action on the CNS. It would make intuitive sense that certain reliable ethanol actions, such as direct receptor or signaling molecule interactions (e.g. binding to GABA_A receptor subunits), would produce consistent downstream responses acutely or chronically, barring major temporal changes to the receptor population. Thus our list of 88 genes that are ethanol-responsive across both treatments and in both brain regions studied may represent these particularly “reliable” downstream ethanol effectors. Indeed, when this list was studied for enrichment, synaptic transmission was by far the dominant theme, with voltage gated cation channel activity, and calcium-regulated responses as over-represented functions (see Supplemental Table 8). *Kcnma1* and *Gabra2* were prominent members of the functional groups over-represented in these 88 ubiquitously regulated probesets. These genes both have substantial biochemical, structural, pharmacological and genetic prior validation as prominent target of direct ethanol action (Tapocik et al., 2014; Treistman & Martin, 2009), thus at least partially substantiating our conclusions regarding this group of 88 genes regulated by acute and chronic ethanol.

Genes unique to PFC CIE showed an enrichment of myelin genes, with *Mag* and *Mal* identified in the pathway shown in Fig. 3. The PFC expression of two other major myelin sheath components, *Mobp* and *Mbp*, correlated with drinking data in CIE treated animals. The CIE PFC geneset also showed enrichment for cytoskeletal binding and cell adhesion molecules and overall, showed the greatest functional over-representation differences from any of the other treatment group analyses (see Supplemental Tables 4-8). In the eQTL analysis of CIE PFC, top QTL candidates at the two major *trans*-regulated peaks, Chr3 and Chr16 (Fig. 5) were identified as the cell adhesion molecules *Fat4* and *Dscam*, respectively. Thus, it is conceivable that a distinct mechanism of chronic ethanol response in PFC

operates through altered cellular adhesion and potentially an interaction with CNS myelin to drive resulting behavioral changes.

Except for the gene group uniquely regulated by CIE in PFC as noted above, the other comparison groups showed remarkable similarities in the highest ranked over-represented functional categories (Supplemental Tables 4-8). Although there were differences in the component genes, these categories all concerned synaptic transmission or neuronal structure. This clearly identifies prominent transcriptional adaptation events as important responses to both acute ethanol and CIE, particularly in NAC. Despite the large similarities in functional group over-representations, several gene ontology groups and pathways emerged as unique to each brain region. In the PFC, overlap between acute and chronic treatments showed overrepresentation of genes related to of GABA_A receptor, whereas in the NAC, both treatments altered genes related to GABA and glutamate transport vesicles. It is interesting to consider that ethanol has been shown to potentiate GABA release both through altered receptor expression and altered release (Kumar et al., 2010; Roberto, Madamba, Moore, Tallent, & Siggins, 2003). As preliminary analyses, our data seem to indicate that these alternate mechanisms may show brain-region specific differences in effect (both acutely and chronically), though further studies would be necessary to confirm this possibility.

When comparing gene sets unique to treatment condition, brain region, or both, there remained a striking similarity in the top upstream regulators identified by IPA. This is perhaps not surprising, given the functional group over-representation overlaps noted above. In particular, HTT and BDNF were identified in nearly every set out of the five queried, despite the fact that many of these gene sets were completely distinct (by definition). This may be partially explained by these genes serving as major regulator of neuronal signaling in general, but likely indicates particular relevance of these genes in ethanol-induced signaling across multiple pathways. BDNF signaling has, in particular, been closely linked to the mechanisms underlying progressive ethanol consumption resulting from the CIE model or similar intermittent ethanol access models (Darcq et al., 2015; Smith et al., 2016; Tapocik et al., 2014). This extensive genomic analysis across the BXD strains serves as strong affirmation of BDNF signaling as an important regulatory point for progressive ethanol consumption, and may implicate targets for future therapeutic intervention development.

The eQTL analyses revealed several important points. The first was that there are distinct regulatory hot-spots in the BXD genome that influence groups of CIE-responsive genes, as seen previously with our genetical genomics study on acute ethanol (Wolen et al., 2012). However, the majority of observed eQTLs were remote to the QTL location (*trans*-eQTL). No significant *cis*-eQTLs were seen in PFC with CIE and only several significant or suggestive *cis*-eQTL were found in NAC (see below). It is possible that our experimental design with limited replication and use of the S-score algorithm could have decreased our power to detect *cis*-eQTL. However, the S-score actually is advantageous in that it eliminates confounding false-positive *cis*-eQTL occurring due to SNPs within DNA segments corresponding to microarray probe positions (Wolen et al., 2012). RNA-seq analysis in future studies might be an advantageous approach for *cis*-eQTL identification as well as CIE-induced changes in splicing or non-coding RNA expression.

The large number of *trans*-eQTLs identified in this study and their grouping into regulatory hot-spots, or *trans*-bands (see histograms in Figs. 5—6), may have implications for future identification of the major regulators of CIE-responsive gene networks. Strikingly, we observed minimal overlap in the position of major *trans*-bands between CIE and acute ethanol treatments. This suggests that distinct genetic variation underlies ethanol induced expression changes from acute to chronic treatment, *even if* individual gene expression or function group changes themselves are conserved.

The one significant *cis* eQTL identified in the CIE NAC condition was also found on the largest *trans*-band, in Chr6:90—95 Mb. The *cis* transcript is Nuclear Receptor 2, Group C, Member 2 (*Nr2c2*), located at Chr6:92091390-92173057 on the mouse genome. Its eQTL map is shown in Supplemental Fig. 6; its peak LRS (with S-score) is within 100 Kb of its genomic location. In a genome-wide association study (GWAS) of human alcohol and nicotine co-dependent participants the gene region *SHE3BP5*—*NR2C2* was found to be enriched for risk-conferring SNPs, with the region being the only to reach genome-wide significance in the meta-analysis (Zuo et al., 2012). *Nr2c2*'s expression has also been shown to be significantly altered by subchronic morphine in adult rats (Schwarz & Bilbo, 2013), providing further evidence of its potential role in addiction. In our study, *Nr2c2* expression did not correlate with drinking behavior in the CIE-treated animals; however, its expression did correlate significantly among air-treated controls during both free-drinking cycles analyzed, suggesting that its expression pattern may confer risk before ethanol use becomes chronic. With chronic treatment, its expression is significantly altered, but does not correlate with consumption. Significant *trans*-eQTLs in the *Nr2c2* *trans*-band include *Gabra1*, *Homer1*, and *Slc1a2*, indicating that alterations in synaptic transmission could occur via altered *Nr2c2* regulation; however, such specific mechanisms remain to be elucidated.

Four suggestive *cis* eQTLs were also found in CIE NAC: *Pcolce*, *Dnm3*, *Gfap*, and *Fbxo39*. Of these, *Dnm3*, located at Chr1:160 Mb, was particularly interesting. *Dnm3* expression in the NAC was correlated with ethanol drinking in CIE-treated animals, and appeared as a hub in the top ranked network of CIE NAC and acute NAC overlapping genes (Fig. 4). Its gene expression levels varied considerably across BXD strains and certain strains showed greater differences in expression between CIE and control groups than seen in progenitor strains (Fig. 8a). This finding suggests transgressive or epistatic genetic interactions may be involved in the *Dnm3* transcriptional response to chronic ethanol. In the DBA2/J strain, *Dnm3* showed strong down-regulation with chronic ethanol, a finding validated by q-PCR (Fig. 8). Ethanol-induced expression changes of *Dnm3* (downregulation in the case of the particularly sensitive DBA2/J strain) could represent a major mechanism of ethanol neuroadaptation. The expression of 21 other genes show ethanol-response at the Chr1:160—170 Mb *trans*-band in which *Dnm3* is a suggestive *cis* eQTL. It is thus reasonable to suggest that these expression changes could be mediated via *Dnm3*. The NAC expression for 15 of 21 of these genes also correlated significantly with drinking in CIE-treated animals. This is not overly surprising given that all genes regulated by a common locus would be expected to correlate with each other to some degree, but does illustrate the possibility of a *Dnm3*-regulated network affecting ethanol drinking behavior. As in the case of *Nr2c2*, specific mechanisms by which this candidate gene exerts ethanol-relevant effects are unknown. Our IPA network (Fig. 4) indicates an interaction between dynamin family members, GTPases

involved in endocytosis and vesicular trafficking (Urrutia, Henley, Cook, & McNiven, 1997), and the Homer family, involved in regulation of glutamatergic post-synaptic densities (de Bartolomeis and Iasevoli, 2003). More targeted studies will be necessary to explore this relationship in the context of CIE exposure.

This genetical genomics study on CIE across BXD strains was intended as an initial analysis on genetic factors influencing CIE-regulated behaviors and brain gene expression in brain regions targeted by the mesolimbocortical dopamine pathway. Our findings show remarkable functional genomics overlaps between CIE and acute ethanol treatment, but also have identified brain expression changes unique to CIE. Our genetic analysis of expression results provide initial details on a limited number of genetic loci that may have an important impact on the molecular adaptations to CIE. Future studies on expanding and validating the findings here, including our results on candidates such as *Dnm3*, may lead to identification of novel targets for therapeutic intervention in the progression from acute ethanol exposure to compulsive consumption as seen with AUD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AUD	alcohol use disorder
BXD	B6 and D2 recombinant inbred strains
CIE	chronic intermittent ethanol
PFC	prefrontal cortex
NAC	nucleus accumbens
eQTL	expression quantitative trait locus
CNS	central nervous system
RI	recombinant inbred
GO	gene ontology
IPA	Ingenuity pathway analysis
LRS	likelihood ratio statistic

LOD log odds ratio

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Fig. 1. Chronic Intermittent Ethanol Model
Following 6 weeks baseline drinking, male and female BXD mice and their progenitors (n = 48) underwent 4 cycles of CIE by Plexiglass vapor chamber 16 h/day × 4 days, 72 h ethanol abstinence, then 2 bottle-choice drinking 2 h/day × 5 days. After the final cycle of CIE mice were sacrificed 72 h after a 5th vapor chamber session.

Fig. 2. CIE-Responsive Genes and Overlap with Acute Ethanol Response

Gene expression response to CIE or acute ethanol was measured by Fisher's Combined S-scores in the PFC and NAC. Ethanol responsive genes were defined as those with p-values 0.05. CIE responsive genes identified were compared to probesets previously identified as responsive to acute ethanol exposure across the BXD RI cohort in PFC and NAC. Both pairwise and hierarchal overlap was examined. Significance of overlap between each pairwise combination was determined using Fisher's Test for Count Data (Fisher, 1922).

Fig. 3. Network of genes exclusively regulated by CIE in the PFC

Gene network was generated by Ingenuity Pathways Analysis (www.ingenuity.com). Solid arrowheads reflect “acts on” interactions while lines without arrow indicate binding interactions only. Solid and dotted lines indicate, respectively, direct vs. indirect interactions.

Fig. 4. Top ranked network in the NAC from CIE and acute ethanol

Gene network enriched in genes involved in synapse structural components and calcium channels was generated by Ingenuity Pathways Analysis (www.ingenuity.com). Solid arrowheads reflect “acts on” interactions while lines without arrow indicate binding interactions only. Solid and dotted lines indicate, respectively, direct vs. indirect interactions.

Fig. 5. Genetic regulation of ethanol-responsive transcripts in acute and CIE PFC

Histograms of significant or suggestive eQTLs ($LRS > 10$) across the genome, divided into 10 Mb bins. In the eQTL analyses, transcripts were filtered by significant ethanol response ($p < 0.05$) in PFC following acute or chronic ethanol. Ethanol vs. control S-score was used as the trait of interest.

Fig. 6. Genetic regulation of ethanol-responsive transcripts in acute and CIE NAC
 Histograms of significant or suggestive eQTLs ($LRS > 10$) across the genome, divided into 10 Mb bins. In the eQTL analyses, transcripts were filtered by significant ethanol response ($p < 0.05$) in NAC following acute or chronic ethanol. Ethanol vs. control S-score was used as the trait of interest.

Fig. 7. *Dnm3* Expression and Ethanol Drinking

Correlation of RMA expression values for *Dnm3* [1436875_at] in the NAC and total ethanol consumption after the third cycle of ethanol vapor exposure (g/kg). Spearman correlation across microarray expression values for 26 strains of mice from the BXD cohort. The red dots represent the drinking and expression values for each respective strain of mouse. There was a significant ($p = 0.00287$) positive correlation ($\rho = 0.561$) using the Spearman rank order test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Ethanol-vapor induced changes in *Dnm3* expression in the NAC

(A) S-score strain distributions for the ethanol responsive gene, *Dnm3* [1436875_at], in the NAC. A positive S-score indicates that *Dnm3* expression is up-regulated in the NAC in ethanol-vapor exposed animals relative to air-vapor exposed animals of the same strain. BXD strains with a D2 genotype at the *Dnm3* locus are colored red and strains with a B6 genotype are colored black. (B) Quantitative PCR results for *Dnm3* mRNA expression in the NAC of D2 mice from later cohorts (cohorts 3, 4, and 5). *Dnm3* was significantly down-regulated ($p < 0.05$, partial $\eta^2 = 0.249$) in the NAC of D2 males exposed to ethanol-vapor (grey bars, $n = 4-5$ /group) relative to those exposed to air-vapor (black bars, $n = 4-5$ /group). (C) *Dnm3* expression in male and female B6 mice ($n = 5$ /group) exposed to Air or CIE. *Dnm3* expression was not significantly altered by CIE and ethanol drinking in B6 males and females (Two-way ANOVA $p > 0.05$, partial $\eta^2 = 0.06$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

PFC—Ten highest ranked eQTLs (by LRS), CIE and Acute.

Probeset	Gene	Location (Chr: Mb)	Max LRS	Max LRS location (Chr: Mb)
CIE				
1438012_at	Ppm1l	Chr3: 69.358475	23.3	Chr16: 97.799871
1422546_at	Ilf3	Chr9: 21.198197	23.3	Chr16: 11.886515
1432269_a_at	Sh3kbp1	ChrX: 156.266314	23.1	Chr16: 97.617114
1423506_a_at	Nnat	Chr2: 157.387623	22.8	Chr7: 67.179978
1436098_at	Bche	Chr3: 73.439883	22.6	Chr7: 57.048459
1426543_x_at	Endod1	Chr9: 14.160201	22.6	Chr16: 97.799871
1448380_at	Lgals3bp	Chr11: 118.254260	22.2	ChrX: 48.285597
1458263_at	Cugbp2	Chr2: 6.494081	21.9	Chr1: 194.086272
1460218_at	Cd52	Chr4: 133.649510	21.7	ChrX: 56.488673
1418580_at	Rtp4	Chr16: 23.613274	21.6	ChrX: 48.285597
Acute				
1442026_at	Zbtb16	Chr9: 48.460145	26.4	Chr7: 35.483869
1418508_a_at	Grb2	Chr11: 115.505490	26.4	Chr13: 54.980446
1431028_a_at	Pank1	Chr19: 34.886667	23.7	Chr11: 58.057819
1427345_a_at	Sult1a1	Chr7: 133.816669	22.1	Chr2: 162.978274
1448534_at	Ptpns1	Chr2: 129.456079	22	ChrX: 70.273782
1450056_at	Apc	Chr18: 34.477683	21.9	Chr16: 67.888886
1417409_at	Jun	Chr4: 94.716397	21.7	Chr1: 20.623897
1451403_at	Ppp1r37	Chr7: 20.116409	21.7	Chr13: 52.866221
1434661_at	Syng1	Chr15: 79.949377	21	Chr13: 54.980446
1452958_at	Asphd2	Chr5: 112.814574	20.4	Chr7: 25.722934

Table 2

NAC—Ten highest ranked eQTLs (by LRS), CIE and Acute.

Probeset	Gene	Location (Chr: Mb)	Max LRS	Max LRS location (Chr: Mb)
CIE				
1420957_at	Apc	Chr18: 34.472690	31.9	Chr6: 92.514677
1452360_a_at	Jarid1a	Chr6: 120.362354	28.6	Chr6: 94.596952
1419127_at	Npy	Chr6: 49.773622	28.5	ChrX: 147.758346
1456656_at	Lin7a	Chr10: 106.859723	27.4	Chr6: 94.596952
1422164_at	Pou3f4	ChrX: 108.010303	27.1	Chr4: 16.419441
1424504_at	Rab22a	Chr2: 173.530582	26	Chr6: 94.596952
1435635_at	Pcmd1	Chr1: 7.150977	25.3	Chr6: 92.514677
1421738_at	Gabra2	Chr5: 71.352858	24.2	Chr6: 94.596952
1425014_at	Nr2c2 [‡]	Chr6: 92.117447	24.2	Chr6: 92.514677
1431020_a_at	Fgfr1op2	Chr6: 146.546009	24.2	Chr19: 28.477768
Acute				
1440901_at	Dgkb	Chr12: 38.863144	26.9	Chr3: 10.327101
1447454_at	Kif5c	Chr2: 49.485000	26.7	Chr18: 3.516538
1425382_a_at	Aqp4	Chr18: 15.551957	26.2	Chr3: 10.327101
1429882_at	Cdh11	Chr8: 19.687909	26	Chr18: 3.516538
1438069_a_at	Rbm5	Chr9: 107.662114	24.9	Chr3: 10.327101
1421933_at	Cbx5	Chr15: 103.026753	24.8	Chr12: 28.507707
1433413_at	Nrxn1	Chr17: 91.488263	24.2	Chr3: 10.327101
1440439_at	Jazf1	Chr6: 52.961885	23.9	Chr18: 3.516538
1421905_at	Ncoa6ip	Chr4: 3.502142	23.8	Chr3: 10.018672
1445717_at	Luc7l2	Chr6: 38.548642	23.1	Chr17: 27.985169

[‡] = cis eQTL.

Table 3All significant eQTLs in CIE NAC, Chr6:90–95 Mb *trans*-band.

Probeset	Gene	Location (Chr, Mb)	Max LRS	Max LRS location (Chr: Mb)
1420957_at	Apc	Chr18: 34.472690	31.9	Chr6: 92.514677
1452360_a_at	Jarid1a	Chr6: 120.362354	28.6	Chr6: 94.596952
1456656_at	Lin7a	Chr10: 106.859723	27.4	Chr6: 94.596952
1424504_at	Rab22a	Chr2: 173.530582	26	Chr6: 94.596952
1435635_at	Pcmd1	Chr1: 7.150977	25.3	Chr6: 92.514677
1421738_at	Gabra2	Chr5: 71.352858	24.2	Chr6: 94.596952
1425014_at	Nr2c2 [‡]	Chr6: 92.117447	24.2	Chr6: 92.514677
1439940_at	Slc1a2	Chr2: 102.623749	24.1	Chr6: 92.570486
1455998_at	G630041M05Rik	Chr1: 135.556378	23.8	Chr6: 92.514677
1417736_at	Smc6l1	Chr12: 11.298458	23.2	Chr6: 94.596952
1426259_at	Pank3	Chr11: 35.599852	23	Chr6: 92.514677
1439450_x_at	Kiaa1033	Chr10: 83.053882	22	Chr6: 92.514677
1457361_at	Zfp804a	Chr2: 82.097416	21.9	Chr6: 92.570486
1436023_at	Bclaf1	Chr10: 20.043434	21.7	Chr6: 92.570486
1453612_at	Nek1	Chr8: 63.533396	21.6	Chr6: 92.514677
1457625_s_at	Cdkl2	Chr5: 92.453971	21.5	Chr6: 92.514677
1419277_at	Usp48	Chr4: 137.172327	21.4	Chr6: 94.596952
1421768_a_at	Homer1	Chr13: 94.119180	21.4	Chr6: 92.514677
1449120_a_at	Pcm1	Chr8: 42.415987	21.2	Chr6: 94.596952
1452470_at	4933409L06Rik	Chr1: 157.791229	21	Chr6: 94.596952
1452708_a_at	Luc7l	Chr17: 26.403234	21	Chr6: 94.596952
1456088_at	Birc4	ChrX: 39.460010	21	Chr6: 92.570486
1429432_at	1810043M20Rik	Chr1: 164.640706	20.9	Chr6: 92.514677
1445081_at	Scai	Chr2: 38.928170	20.9	Chr6: 92.514677
1423184_at	Itsn2	Chr12: 4.642424	20.9	Chr6: 92.514677
1457891_at	Cugbp2	Chr2: 6.487216	20.3	Chr6: 94.596952
1434643_at	Tbl1x	ChrX: 74.900816	20.3	Chr6: 92.570486
1422842_at	Xrn2	Chr2: 146.853329	20.1	Chr6: 92.514677
1424658_at	Taok1	Chr11: 77.349717	20	Chr6: 92.514677
1450051_at	Atrx	ChrX: 103.072338	20	Chr6: 92.514677
1459984_at	Mia3	Chr1: 185.000000	19.9	Chr6: 92.514677
1453414_at	E130113K08Rik	Chr11: 86.752656	19.5	Chr6: 92.514677
1440437_at	Herc1	Chr9: 66.315187	19.1	Chr6: 92.514677
1460729_at	Rock1	Chr18: 10.119892	19	Chr6: 94.596952
1437502_x_at	Cd24a	Chr10: 43.303878	18.4	Chr6: 90.308023

[‡]
= cis eQTL.

Table 4

PFC–Spearman correlations of gene expression (RMA) with ethanol consumption after the 3rd and 4th cycles of vapor chamber exposure.

Probeset	Gene	Pval. corr	Corr. spear	Cycle ^a
CIE-treated				
1458499_at	Pde10a	9.47E-05	0.712173913	BOTH (4th)
1421860_at	Clstn1	0.00091914	0.632173913	BOTH (3rd)
1425833_a_at	Hpca	0.001181409	0.62173913	BOTH (3rd)
1439618_at	Pde10a	0.002053437	0.597391304	BOTH (3rd)
1442019_at	Gas7	0.003023388	0.579130435	BOTH (3rd)
1416562_at	Gad1	0.003799673	0.567826087	BOTH (4th)
1425810_a_at	Csrp1	0.003932856	0.566086957	BOTH (4th)
1430449_at	Kidins220	0.005147076	0.552173913	BOTH (3rd)
1425281_a_at	Tsc22d3	0.005676965	0.546956522	3rd
1429859_a_at	Arl2bp	0.005769573	0.546086957	BOTH (3rd)
1449932_at	Csnk1d	0.005769573	0.546086957	BOTH (3rd)
1420899_at	Rab18	0.00635219	0.540869565	3rd
1436713_s_at	Meg3	0.006874531	0.536521739	4th
1418586_at	Adcy9	0.007548179	0.531304348	BOTH (4th)
1449264_at	Syt11	0.007665626	0.530434783	BOTH (3rd)
Air-treated				
1439196_at	Hook3	0.000319626	−0.696216827	BOTH (4th)
1444435_at	1110014F16Rik	0.001608394	−0.631846414	4th
1427150_at	Mll3	0.001962023	−0.622811971	4th
1427319_at	A230046K03Rik	0.002110577	−0.619424054	4th
1427353_at	Clasp1	0.002868941	−0.604743083	4th
1437020_at	Ep400	0.003364405	−0.596837945	4th
1417754_at	Topors	0.00428699	−0.584415584	4th
1416501_at	Pdpk1	0.004572405	−0.581027668	4th
1452187_at	Rbm5	0.004771353	−0.578769057	4th
1423559_at	Kcnc1	0.005083271	−0.575381141	4th
1430820_a_at	Bbx	0.005190943	−0.574251835	4th
1450401_at	Ncoa6ip	0.005411987	−0.571993224	4th
1426327_s_at	Zfp91	0.005758193	−0.568605308	4th
1453512_at	Mbnl2	0.006248242	−0.564088086	4th
1460417_at	AB041803	0.006248242	−0.564088086	4th

^aCycle column: reveals if the probeset was significantly correlated with ethanol consumption after the 3rd or 4th cycle. If the probeset was significantly correlated after both cycles, then the row is labeled BOTH and the cycle with the strongest correlation is listed and the cycle is denoted in parenthesis.

Table 5

NAC–Spearman correlations of gene expression (RMA) with ethanol consumption after the 3rd and 4th cycles of vapor chamber exposure.

Probeset	Gene	Pval. corr	Corr. spear	Cycle ^a
CIE-treated				
1450769_s_at	Stard5	0.00030775	−0.651965812	4th
1417943_at	Gng4	0.000359169	−0.646495726	3rd
1449571_at	Trhr	0.000531398	−0.632136752	BOTH (4th)
1446176_at	Mrg1	0.001404264	0.593162393	3rd
1437403_at	Samd5	0.001449756	0.591794872	BOTH (3rd)
1419271_at	Pax6	0.002071286	−0.576068376	3rd
1441917_s_at	Tmem40	0.002233124	−0.572649573	3rd
1447669_s_at	Gng4	0.002300826	−0.571282051	3rd
1438782_at	Axcam	0.003169373	−0.556239316	3rd
1439633_at	Syt7	0.003803452	−0.547350427	3rd
1436875_at	Dnm3	0.003964172	0.545299145	3rd
1434779_at	Cbln2	0.004665976	−0.537094017	3rd
1442019_at	Gas7	0.00512194	0.532307692	BOTH (3rd)
1434877_at	Nptx1	0.005398996	−0.52957265	3rd
1453771_at	Gulp1	0.005470202	−0.528888889	4th
Air-treated				
1452161_at	Tiparp	0.000433941	0.672924901	BOTH (4th)
1458830_at	Fgf14	0.00095138	−0.64229249	3rd
1459288_at	Kcnd2	0.001176552	−0.633399209	3rd
1456257_at	Fam126b	0.001232344	0.631422925	3rd
1436713_s_at	Meg3	0.001727521	−0.616600791	BOTH (3rd)
1443237_at	Ptprd	0.002284704	−0.603754941	BOTH (3rd)
1420917_at	Fnbp3	0.002432929	0.600790514	4th
1416180_a_at	Rdx	0.002698052	0.595849802	BOTH (4th)
1427351_s_at	Ighg	0.002810732	0.593873518	3rd
1430979_a_at	Prdx2	0.004016181	0.576086957	3rd
1452444_at	Napb	0.004094228	0.575098814	BOTH (3rd)
1426432_a_at	Slc4a4	0.004094228	0.575098814	4th
1437864_at	Adipor2	0.004173545	0.574110672	BOTH (4th)
1442025_a_at	Zbtb16	0.004254147	0.57312253	3rd
1437869_at	Ppp2r3a	0.004503832	0.570158103	4th

^aCycle column: reveals if the probeset was significantly correlated with ethanol consumption after the 3rd or 4th cycle. If the probeset was significantly correlated after both cycles, then the row is labeled BOTH and the cycle with the strongest correlation is listed and the cycle is denoted in parentheses.