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Gene Expression within the Extended Amygdala of 5 Pairs of Rat Lines Selectively Bred for High or Low Ethanol Consumption

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Abstract

The objectives of this study were to determine innate differences in gene expression in 2 regions of the extended amygdala between 5 different pairs of lines of male rats selectively bred for high or low ethanol consumption: a) alcohol-preferring (P) vs. alcohol-non-preferring (NP) rats, b) high-alcohol-drinking (HAD) vs. low-alcohol-drinking (LAD) rats (replicate line-pairs 1 and 2), c) ALKO alcohol (AA) vs. nonalcohol (ANA) rats, and d) Sardinian alcohol-preferring (sP) vs. Sardinian alcohol-nonpreferring (sNP) rats, and then to determine if these differences are common across the line-pairs. Microarray analysis revealed up to 1,772 unique named genes in the nucleus accumbens shell (AcbSh) and 494 unique named genes in the central nucleus of the amygdala (CeA) that significantly differed [False Discovery Rate (FDR) = 0.10; fold-change at least 1.2] in expression between the individual line-pairs. Analysis using Gene Ontology (GO) and Ingenuity Pathways information indicated significant categories and networks in common for up to 3 or 4 line-pairs, but not for all 5 line-pairs. However, there were almost no individual genes in common within these categories and networks. ANOVAs of the combined data for the 5 line-pairs indicated 1,014 and 731 significant ($p < 0.01$) differences in expression of named genes in the AcbSh and CeA, respectively. There were 4–6 individual named genes that significantly differed across up to 3 line-pairs in both regions; only 1 gene (*Gsta4* in the CeA) differed in as many as 4 line-pairs. Overall, the findings suggest that a) some biological categories or networks (e.g., cell-to-cell signaling, cellular stress response, cellular organization, etc.) may be in common for subsets of line-pairs within either the AcbSh or CeA, and b) regulation of different genes and/or combinations of multiple biological systems may be contributing to the disparate alcohol drinking behaviors of these line-pairs.

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Keywords

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Introduction

Gene expression studies can potentially contribute to the identification of genes associated with the risk for excessive alcohol drinking and help elucidate mechanisms underlying this behavior. Gene expression studies with human tissue used autopsy samples from individuals with a previous history of alcohol abuse versus controls without this history (Flatscher-Bader et al., 2005; 2008; Lewohl et al., 2000; Mayfield et al., 2002). To differentiate genetic differences related to susceptibility from those resulting from long-term alcohol abuse, it is important to conduct studies on subjects who are genetically susceptible to high alcohol drinking but have not had any previous exposure to ethanol. Since this is not feasible in humans, animal studies offer an alternative. To that end, multiple rat and mouse lines, selected on the basis of various criteria for high (HEC) or low (LEC) ethanol consumption or displaying these phenotypes innately, have been studied.

Kimpel et al. (2007) reported several differences in gene expression between inbred alcohol-preferring (iP) and inbred alcohol non-preferring (iNP) rats in the nucleus accumbens (Acb) and amygdala. Arlinde et al. (2004) reported gene expression differences between ALKO alcohol (AA), ALKO non-alcohol (ANA), and Wistar rats in the Acb and amygdala. However, these studies did not distinguish between the Acb shell (Sh) and core, or attempt to delineate any of the amygdala nuclei. It is important to distinguish subregions of the Acb because the shell (Sh), but not the core, is involved in processing dopamine-mediated rewards (Ikemoto et al., 1997) and the reinforcing actions of ethanol (Engleman et al., 2009). It is also important to distinguish nuclei within the amygdala, because each has a different function, especially the central nucleus of the amygdala (CeA), which may be involved in regulating dependence-induced alcohol drinking (Roberts et al., 1996).

Tabakoff et al. (2009) reported results of gene expression levels in HXB/BXH recombinant inbred rats that linked candidate genes involved in GABA release, activation of dopamine neurons, and postsynaptic GABA receptor trafficking in the hypothalamus, ventral tegmentum, and amygdala to alcohol consumption. In another study, Kerns et al. (2005) reported the effects of acute ethanol administration on gene expression in the Acb, prefrontal cortex, and ventral tegmental area (VTA) of DBA/2J and C57BL/6J mice, and reported region-specific changes in ethanol-responsive genes.

Tabakoff et al. (2008) performed a meta-analysis across 3 types of mouse populations (high-alcohol-preference [HAP], low-alcohol-preference [LAP], and BXD recombinant inbred) and 22 inbred strains, to correlate innate whole brain gene expression with previously reported levels of alcohol intake to identify candidate genes associated with the variance in alcohol consumption across the mouse lines and strains. These authors reported that the 8 candidate genes identified play important roles in neuronal migration and differentiation, as well as synaptic remodeling. However, this study was conducted using whole brains, and not all of the mice were selected for high alcohol consumption or low alcohol consumption.

A recent study (McBride et al., 2012) examined gene expression in the VTA of 5 pairs of rat lines selectively bred for HEC or LEC. The results of this study indicated that there were no common gene differences across all 5 line-pairs, but rather there were biological processes

(e.g., transcription, synaptic function, intracellular signaling, and protection against oxidative stress) or gene networks that were in common for subsets of the line-pairs.

The AcbSh is a region mediating the rewarding effects of ethanol (Engleman et al., 2009) and alcohol drinking behavior (reviewed in Koob et al., 1998; McBride & Li, 1998). The CeA appears to be involved in regulating dependence-induced alcohol drinking (Roberts et al., 1996). Changes in gene expression in the AcbSh and CeA have been reported following binge-drinking by P rats (McBride et al., 2010). Therefore, comparing innate gene expression differences in these 2 regions of the extended amygdala across 5 line-pairs of rats selectively bred for high or low ethanol intake may yield important information on common differences that could contribute to their disparate alcohol drinking characteristics.

The present study was undertaken to better delineate innate differences in gene expression in the AcbSh and CeA between 5 pairs of independent, divergently selected HEC and LEC rat line-pairs: a) the alcohol-preferring (P) and alcohol-non-preferring (NP) rats (Murphy et al., 2002), b) two replicate lines of high-alcohol-drinking (HAD) and low-alcohol-drinking (LAD) rats (Murphy et al., 2002), c) ALKO alcohol (AA) and nonalcohol (ANA) rats (Eriksson, 1968), and d) Sardinian alcohol-preferring (sP) and Sardinian non-preferring (sNP) rats (Colombo, 1997). Characteristics of these 5 selectively bred line-pairs have recently been reviewed (Bell et al., 2012). Undoubtedly, genes unrelated to alcohol preference were fixed during the selection process of these lines. By analyzing differential gene expression both within and across line-pairs, it is hoped that any gene expression differences present by chance would be minimized and gene expression differences present because of the high alcohol drinking phenotype would be maximized. It is also possible that different genes may contribute to alcohol drinking via the same underlying mechanism if they are in the same biologic pathway. For this reason, it is important to also analyze pathways that may be affected within line-pairs.

Because of the involvement of the AcbSh and CeA in regulating alcohol drinking, it is important to examine gene expression in these regions of alcohol-naïve subjects with a genetic predisposition for HEC or LEC. The hypothesis to be tested is that there will be common differences across the 5 pairs of selectively bred lines of male rats in gene expression or within biological systems in the AcbSh and/or CeA that could influence neuronal function, contribute to the reinforcing actions of ethanol, and promote high alcohol consumption.

Materials and methods

Animals

Animals used for this study were ethanol-naïve, male adult selectively bred rats (n = 9–10/line). The alcohol-preferring (P), alcohol-non-preferring (NP), high-alcohol-drinking lines 1 & 2 (HAD1, HAD2), and low-alcohol-drinking lines 1 & 2 (LAD1, LAD2) were bred at the Indiana University School of Medicine. Animals were pair-housed in normal 12-h light/dark cycle rooms (lights on at 8:00 A.M.). The ALKO alcohol (AA) and ALKO nonalcohol (ANA) were housed in a similar manner in animal facilities in Helsinki, Finland. The Sardinian alcohol-preferring (sP) and Sardinian alcohol-nonpreferring (sNP) rats were also similarly housed in animal facilities in Cagliari, Italy.

The P and NP lines of rats were derived by selective breeding from an outbred stock of Wistar rats maintained at Walter Reed Army Institute of Research (Lumeng et al., 1977). The HAD and LAD replicate lines were derived from the N/Nih heterogeneous stock rats (Hansen & Spuhler, 1984; Li et al., 1993). The sP and sNP rats were selectively bred from a base population of Wistar rats obtained from a vendor in Italy (Fadda et al., 1989). The AA

and ANA lines were originally derived from a foundation stock that included Wistar and Sprague-Dawley strains; these lines were subsequently crossed with F1 hybrids from Lewis and Brown Norway rats (Erikson, 1981; Kiianmaa et al., 1992; Sinclair et al., 1989; Sommer et al., 2006). The selection criteria were similar for all the lines. The selection criteria for the HEC lines were ethanol intakes of > 5 g/kg/day and a preference ratio of 10% ethanol to water ≥ 2:1, using a 24-h free-choice drinking procedure. The LEC lines were selected for ethanol intakes less than 1 g/kg/day.

The P, NP, HAD1,2 and LAD1,2 animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the institutional animal care and use committee and are in accordance with the guidelines of a) the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council 1996), and b) the European Communities Council Directive (86/609/EEC) on the "Protection of animals used for experimental and other scientific reasons".

Sample preparation

Rats were killed by decapitation, and the brains were quickly removed and frozen in isopentane in frozen carbon dioxide (dry ice). Brains were stored at −80 °C until sectioned. Whole brains from AA, ANA, sP, and sNP rats were shipped overnight to Indianapolis in dry ice. On the day of preparation of micro-punch samples, brains were transferred, at least 2 h prior to sectioning, to a cryostat set at −6 to −10 °C. Sections (300 μm) were obtained and transferred to glass slides that had been pre-cooled in the cryostat. Micro-punch sampling was done on a frozen stage (−25 to −35 °C) with an anatomic microscope equipped with a cool microscope lamp. The stereotaxic atlas of Paxinos & Watson (1998) was used to identify the AcbSh and CeA. Micro-dissection needles (Fisher Scientific) with an inner diameter of 0.77 mm were used to obtain samples. This inner diameter fits within the entire region and minimizes contamination from adjacent tissue. Punches were taken bilaterally from 2–3 sections. A different fresh sterile micro-punch needle was used for each animal. After withdrawing the micro-punch sample, a distinct demarcated hole remained; this hole was used to validate the micro-dissection method. All equipment used to obtain tissue was treated with RNAse Zap (Ambion, Inc., Austin, TX) to prevent RNA degradation. To minimize batch and order effects within each line-pair, micro-punch samples were obtained from each line-pair of HEC and LEC rats in a counterbalanced design over a 2–3 day period, such that equal numbers within each line-pair were obtained on the same day. However, micro-punch samples from the 5 line-pairs were processed at different times, with intervals up to several months between line-pairs.

The micro-punched samples were immediately homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and processed according to the manufacturer's protocol, but with twice the suggested ratio of Trizol to tissue (Edenberg et al., 2005). Ethanol-precipitated RNA was further purified through RNeasy® columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. The yield, concentration, and purity of the RNA were determined by running a spectrum from 210 to 350 nm, and analyzing the ratio of large and small ribosomal RNA bands using an Agilent Bioanalyzer. Yields, purity, and quality of the RNA were excellent; RNA integrity numbers (RIN) averaged 8.5 for the samples, showing little or no degradation.

Microarray procedures

Separate preparations of total RNA were made for each animal. Samples from different animals were not pooled at any stage Thus, 9–10 microarrays were analyzed for each line per region. RNA extraction, sample processing, and array hybridization/scanning were completed at different times for each line-pair. Within each line-pair, all of the samples from each brain region were processed in a single batch, to eliminate batch effects within each brain region. This approach was used to keep the technical variability for each brain region as low as possible, because between-region comparisons within a line-pair were not the focus of this study. Because the primary comparisons are between the 2 lines within each pair, samples from each line-pair were processed in 1 batch for labeling. Samples from the 2 HAD/LAD line-pairs were labeled using the Affymetrix 2-cycle protocol. Due to the discontinuation of that kit, the remaining line-pairs were labeled using the 3′ IVT Express Protocol, which uses only 1 round of IVT amplification. Although amplification biases have been reported between PCR and IVT protocols (Degrelle et al., 2008), this is not likely to be a factor in the present study since the PCR protocol was not used. Moreover, the primary comparisons are within line-pairs and were done using identical protocols at the same time. Hybridization and scanning of samples from each line-pair were in 2 evenly distributed batches across the HEC and LEC animals. The fragmented, biotinylated cRNA from each independent sample was mixed into 300 μL of hybridization cocktail, of which 200 μL were used for hybridization to Affymetrix Rat Genome 230 2.0 GeneChips. Hybridization was carried out for 17 h at 42 °C. Washing and scanning of the GeneChips were carried out according to standard protocols, as previously described (Edenberg et al., 2005; McClintick et al., 2003). Each GeneChip® was scanned using an Affymetrix Model 3000 scanner and underwent image analysis using Affymetrix GCOS software. Microarray data are available from the National Center for Biotechnology Information's Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/ under accession SuperSeries GSE31709 (AcbSh subseries GSE31705, CeA subseries GSE31708).

Statistical and neuroinformatics analysis of microarray data

Raw CEL files were imported into the statistical programming environment R (R: A language and environment for statistical computing Ver 2.13.0; R Foundation for Statistical Computing, 2011) for further analysis with tools available from the Bioconductor Project (Gentleman et al., 2004). Expression data from the arrays of each experiment were normalized and converted to log(2) values using the Robust Multi-chip Average (RMA) method (Irizarry et al., 2003). As a standardization step to facilitate later comparisons with other experiments, expression levels were scaled such that the mean expression of all arrays was the raw RMA signal scaled to 1000. After the initial statistical analysis (Table 1), all probe sets currently annotated by Affymetrix as “expressed sequence tags” or whose gene names contain the words “riken”, “predicted”, or “similar to” were not included in any further analyses; only the remaining probe sets deemed “named genes” were further analyzed. Probe sets that were not detectable above background were filtered out to reduce noise (McClintick & Edenberg, 2006). Probe sets that did not have a median expression across experiments of at least $\log_2$ (100) were therefore excluded. To obtain lists of significant genes within each line-pair, a simple t test was performed and the resultant *p* values were used to calculate the FDR q value for each gene according to the method of Storey et al. (2004). Genes with a q value ≤ 0.10 were deemed significant. Because this approach resulted in a very high number of statistically significant genes between P vs. NP rats in the AcbSh, an additional filter was applied to eliminate genes with a fold-change less than 1.2. This filter was applied to data for all other line-pairs in the AcbSh, as well as to all line-pairs in the CeA. To test for significance of genes across line-pairs, 2-factor linear modeling including the interaction term using ANOVA ($p < 0.01$) was conducted. One factor was line-pair (5 levels) and the other factor was ethanol consumption phenotype (2

levels). FDR was calculated using the same method as for the within-line t tests and significance was again set at FDR ≤ 0.10.

Testing for over-representation of GO (Ashburner et al., 2000; Harris et al., 2004) biological processes (BP) and KEGG categories was performed using the Bioconductor package GOstats (Gentleman, 2004). Briefly, for each set of significant genes tested, a list of unique Entrez-Gene identifiers was constructed. This list was then compared to the list of all Entrez-Gene identifiers for the named genes that were called "present" using the filtering methods described above. Identification of over-represented categories was then accomplished using the hypergeometric distribution. Categories were called significant at $p < 0.05$.

Genes differentially expressed at FDR 10% and 1.2 fold-change cutoff for each individual line-pair and for those significant at FDR 10% in the ANOVA analyses were uploaded separately into Ingenuity Pathway Analysis® (Ingenuity® Systems, www.ingenuity.com). Genes were identified by their Affymetrix probe set ID and then mapped to their corresponding objects in the Ingenuity® Knowledge Base. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Canonical Pathway Analysis results are reported. Pathways were deemed significant if the genes in the pathway were over-represented in the list of genes used for the analysis.

Weighted gene co-expression network analysis (WGCNA) was conducted (Zhang & Horvath, 2005) using the Bioconductor (Gentleman et al., 2004) package WGCNA (Langfelder & Horvath, 2008, 2012) within R (R: A language and environment for statistical computing Ver 3.0.0; R Foundation for Statistical Computing, 2013). Analyses were conducted separately within each brain region but across all line-pairs, using the consensus module approach to build modules with consensus across line-pairs. For WGCNA, default values, including the use of the power function with power β, were used for all functions with the exception that signed correlation coefficients were used. All "present" probe sets were included to achieve the best scale-free topology. A power β equal to 8 met the criteria for scale-free topology in each brain region and was used in the construction of modules. Resultant modules were tested for enrichment with differentially expressed genes by line-pair using Gene Set Enrichment Analysis (GSEA, Subramanian et al., 2005) with significance set at $p < 0.05$. For modules found significant with GSEA in at least 3 line-pairs, further testing for enrichment was performed using the hyper-geometric distribution using sets of genes by GO biologic process (Ashburner et al., 2000; Harris et al., 2004) and location by cell type (Cahoy et al., 2008).

Present probe sets were analyzed for enrichment in regions of established rat QTLs for alcohol consumption in P × NP (Bice et al., 1998; Carr et al., 1998, 2007; Foroud et al., 2002) and HAD × LAD replicates (Bice et al., 2010; Carr et al., 2003; Foroud et al., 2003) using the methodology of Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) accompanied by linear modeling (Oron et al., 2008) with the Bioconductor packages GSEABase (R package version 1.17.1) and GSEAlm (R package version 1.15.0). For the across line-pairs analysis, the same linear model was used, as described above for the ANOVA analysis, and GSEA was conducted using the statistic for the ethanol consumption phenotype factor. For the within line-pair analyses, ethanol consumption phenotype was the only factor in the model. The aggregate gene set statistic used was the J-G statistic described in Jiang & Gentleman (2007).

Results

Individual line-pair analysis

In the AcbSh (Table 1), there is a 14-fold difference in the number of significant named genes across the line-pairs, with the highest number in the P-NP line-pair (1,772) and the lowest number in the AA-ANA line-pair (127; see Table 1). Supplemental Tables A–E list all named genes that significantly differed for each line-pair. Supplemental Tables G–K list unnamed probe sets that significantly differed for each line-pair.

In the CeA (Table 1), there was a 3-fold difference in significant unique named genes across 4 of the line-pairs with the highest number in the HAD2-LAD2 line-pair (518) and a lower number in the HAD1-LAD1 line-pair (158); the AA and ANA lines showed only 1 difference (*Mosc2*: MOCO sulphurase C-terminal domain containing 2). Supplemental Tables AA–DD list unique named genes that significantly differed for each line-pair. Supplemental Tables FF–II list unnamed probe sets that significantly differed for each line-pair.

Principal Components Analysis (PCA) by line-pair for the AcbSh and CeA (Fig. 1) indicated that, in both regions, the HAD-LAD replicate lines clustered separately from the other 3 lines. However, replicate line 1 clustered separately from replicate line 2. Because the HAD-LAD replicates clustered separately from the other 3 line-pairs in the 1st PCA (Fig. 1, left panels), separate PCA plots were made for the HEC and LEC lines (Fig. 1, middle and right panels). The second PCA indicated that genes for the HEC lines did not cluster separately from genes for the LEC lines for both regions (Fig. 1). Heat maps (clustergrams; not shown) of genes that were significant between individual line-pairs did not reveal any clear pattern of genes associated with high or low ethanol consumption in either region.

Intersections of lists of unique named genes indicated that there were no overlapping common genes across 4 or 5 line-pairs in the AcbSh (see Supplemental Tables A–E) or across 4 line-pairs in the CeA (see Supplemental Tables AA–DD). However, in the AcbSh, there were 23 genes that were in common across 3 of the 5 line-pairs (e.g., *Acaa2*, *Cbr4*, *Nek1*, *Pls1*, *Sp110*, *Tmem14a* and *Zcchc9*). In the CeA, there were 6 genes in common across 3 of the 5 line-pairs (*Acaa2*, *Exosc7*, *Mcee*, *Ncaph*, *Snx10*, and *Svep1*). No single gene was differentially expressed across the 5 line-pairs of selectively bred rats.

GSEA was used to determine enrichment of probe sets in established rat QTLs for ethanol consumption. No enrichment was found for any of the QTLs within a line-pair or across line-pairs for either region.

Gene Ontology (GO) analysis of individual line-pairs

Within the AcbSh, there were 8 significant biological categories that were in common across 3 line-pairs, but there were none in common across 4 or 5 line-pairs (Table 2). The P-NP and sP-sNP line-pairs were most similar, with all 8 categories being found in both. In the AcbSh, the HAD1-LAD1 and HAD2-LAD2 line-pairs were not similar to each other. There were 4 GO categories (regulation of neurotransmitter levels, and responses to glucocorticoid, retinoic acid, and steroid hormone stimuli) that were common across the P-NP, HAD2-LAD2, and sP-sNP line-pairs in the AcbSh. However, among these, there was only 1 gene in common (*Mef2c*; myocyte enhancer factor 2c).

Within the CeA, there were 7 significant GO categories that were common across 3 or 4 line-pairs, with only the AA-ANA line-pair having no significant GO categories (Table 2). The ‘circadian rhythm’ category (along with ‘leukocyte chemotaxis’ and ‘wound healing’ categories) was observed in 4 line-pairs; there was only 1 gene (*Pf4*; platelet factor 4) in

common across the 4 line-pairs. An intracellular signaling cascade category was observed for the P-NP, HAD1-LAD1 and HAD2-LAD2 line-pairs, but there were no genes in common within this category.

Ingenuity Pathway Analyses for individual line-pairs

Within the AcbSh, there were 22 significant canonical pathways that contained 15 or more genes (Table 3). Twenty-one of the pathways were observed for the P-NP line-pair, which reflects the high number of significant unique named genes found in this line-pair compared to the others (Table 1). The HAD2-LAD2 line-pair was the only other line-pair that exhibited a significant canonical pathway with at least 15 genes, the ILK (integrin-linked kinase) signaling pathway. This pathway was also significant for the P-NP line-pair; however, there were no genes in common in this pathway between the P-NP and HAD2-LAD2 line-pairs.

In the AcbSh, several pathways had a noticeable imbalance in the number of genes with significantly lower expression, compared to the number with higher expression in the P vs. NP line. The cAMP-mediated signaling, synaptic long-term potentiation, glutamate receptor signaling, CREB signaling in neurons, synaptic long-term depression, alpha-adrenergic signaling, and gap junction signaling pathways all had differences of 6 to 17 genes, and a 1.7- to 2.7-fold higher number with lower expression than with higher expression.

In the CeA, there were 5 significant pathways with 10 or more genes observed for the HAD2-LAD2 line-pair (Table 3). In addition, there was 1 canonical pathway observed for the HAD1-LAD1 line-pair in the CeA. Both replicate line-pairs of HAD-LAD rats had a significant IL-8 signaling pathway; however, there were no genes in common in this pathway between the 2 line-pairs. There were no significant canonical pathways in the CeA of the P-NP and sP-sNP line-pairs, even though both line-pairs had large numbers of unique named genes that significantly differed (Table 1).

Overall ANOVA for all line-pairs combined

ANOVAs, using ethanol consumption phenotype (HEC and LEC) and line-pair (5 categories) as factors, were conducted for each probe set. In the AcbSh, there were 1,014 individual named genes that were significantly ($p < 0.01$) different between the HEC and LEC lines; in the CeA, there were 731 individual named genes that differed (see Supplemental Tables F and EE for named genes in the AcbSh and CeA, respectively, and Supplemental Tables L and JJ for un-named probe sets that significantly differed in these 2 regions).

Ingenuity Pathway Analysis (IPA) identified 5 major networks each in the AcbSh and CeA (Table 4). For the AcbSh, 1 network involved general development and function and had 1.7-fold more genes with higher than lower expression in the HEC vs. LEC lines. In contrast, 2 other networks (general category of Cellular Growth and Proliferation, and Nervous System Development and Function) had 1.7-fold more genes with lower than higher expression in the HEC vs. LEC lines. The remaining 2 categories (Cell-to-Cell Signaling and Interaction, and Cellular Assembly and Organization) had approximately equal numbers of genes with higher and lower expression levels in the HEC vs. LEC lines.

In the CeA, networks 3, 4, and 5 had 1.4-fold more genes with lower than higher expression in the HEC vs. LEC lines (Table 4). Although both regions had networks involved in Cellular Assembly and Cell-to-Cell Signaling, there was only 1 gene in common between the 2 regions (i.e., *Dhx36*).

Several KEGG categories emerged from the overall ANOVA that were evident in at least 3 line-pairs in the CeA (Table 5). All of the categories were significant in the P-NP and sP-sNP line-pairs, and 1 category was significant across 4 line-pairs. However, none of the 3 categories had any clear role in neuronal function. There were no significant KEGG categories observed for the AcbSh that were evident in more than 2 line-pairs.

The WGCNA identified several GO biological categories that were significant in at least 3 line-pairs (Table 6). In the AcbSh, these categories were relatively general. In the CeA, there were 2 categories, involving protein kinase activity and Wnt receptor signaling, that were slightly less general. Overall, the WGNCA did not provide much information toward defining any common biological systems among all 5 line-pairs.

There were 6 genes differentially expressed (in the same direction) in at least 3 of the line-pairs in the overall ANOVAs for the AcbSh (Table 7). The HAD2-LAD2 line-pair exhibited significant differences in all 6 genes; the P-NP line-pair exhibited significant differences in 5 of the 6 genes. AA-ANA differences were observed for only 1 gene, i.e., *Avil*.

In the CeA, there were 5 genes differentially expressed in at least 3 line-pairs from the overall ANOVAs (Table 7). All 5 genes were differentially expressed in both replicate line-pairs of HAD-LAD rats. *Gsta4* was the only gene differentially expressed in 4 line-pairs in the CeA. None of the genes differentially expressed in the CeA were differentially expressed in the AcbSh.

Sufficient material was available from several of the micro-punched samples of the AcbSh to conduct a qRT-PCR validation on some of the genes in Table 7. Three genes (*Avil*, *Mef2c*, and *Zcchc9*) were selected that had a fold-change of at least 1.4 in the AcbSh of the P vs. NP rats. The results with the qRT-PCR were in excellent agreement with the microarray data (Table 8).

Discussion

Several significant findings emerged from this study using male rats from the 5 line-pairs selectively bred for disparate alcohol drinking. However, the overall hypothesis that there are common biological pathways within the AcbSh and/or CeA across the 5 line-pairs that contribute to their disparate alcohol drinking characteristics was not supported. The PCA plots by line-pair for both regions (Fig. 1, left panels) indicated a separation of the HAD-LAD replicates from the other 3 line-pairs. This separation is likely due to a combination of factors, such as differences in microarray protocols, batch effects, and genetic background. However, there was also separate clustering of each replicate line-pair, suggesting some differences between the replicates. Examining the data with ethanol consumption as a factor (Fig. 1, middle and right panels) does not indicate any clear separation between the HEC vs. LEC groups, suggesting that there may be few genes in common across the 5 line-pairs that could influence alcohol drinking, and/or other factors (i.e., technical) may predominate over any possible clusters of genes associated with high or low ethanol consumption. Other factors may also play a role in not finding more genes in common across the 5 line-pairs. For example, the origins of the line-pairs are different, the environment in which the line-pairs were raised is different, and other factors not adequately studied across line-pairs may influence the gene expression data. Only the HAD-LAD replicate lines were derived from the same foundation stock (Hansen & Spuhler, 1984; Li et al., 1993), which was not the case with the other 3 line-pairs. The foundation stocks of these 3 line-pairs were also different from each other (Eriksson, 1981; Fadda et al., 1989; Kiianmaa et al., 1992; Lumeng et al., 1977; Sinclair et al., 1989). Although the selection criteria were similar for each of the line-pairs, there were differences in the environments in which the line-pairs were raised that

could influence gene expression. Finally, even though the selection criteria were similar, other traits (e.g., anxiety, metabolism, etc.) may have been differentially carried through in the selection process depending upon the line-pair. Furthermore, essentially no overlap of significant categories was obtained across line-pairs using a general FDR enrichment cut-off set at either 0.1 or 0.2.

The AcbSh and CeA are 2 regions that are key parts of the extended amygdala. Both regions are important in regulating alcohol drinking behavior and both regions respond to ethanol administration (reviewed in Koob et al., 1998; McBride, 2002; McBride & Li, 1998). Because of the importance of these two distinct regions in the effects of ethanol and alcohol drinking behavior, the expectation was that some innate common gene expression differences would be found within a region across 5 line-pairs of rats selectively bred for high or low alcohol consumption. However, because these 2 regions receive different inputs and have different intrinsic neuronal circuitries (Cassell et al., 1999; Meredith, 1999), it is possible that any common differences in gene expression found across the line-pairs in one region may not necessarily be the same common differences found in another region. On the other hand, some similarities exist between the 2 regions, e.g., both contain GABAergic medium spiny neurons (Cassell et al., 1999; Meredith, 1999), and some common differences might be expected. Apparently, the unique features of the CeA vs. the AcbSh outweigh the similarities since there were so few common differences in gene expression between these 2 regions.

Individual line-pair analysis

In neither the AcbSh nor the CeA were there any common genes that significantly differed across all 5 individual line-pairs, suggesting that differential expression of various combinations of genes may contribute to the disparate alcohol drinking characteristics of each line-pair. This could be due to the way selection works. Selection acts on the existing genetic variations in the founder stock, which differ among the starting lines and increases the frequency of alleles that contribute to the phenotype (high or low preference) as well as nearby alleles. Thus, differences in the selectable alleles in each line will lead to differences in the genes and pathways that emerge. And, if there are multiple pathways that can lead to the selected phenotype, an early divergence in one pathway is likely to be reinforced by continued selection. The lack of common genes across all 5 line-pairs also implies that there are multiple mechanisms that can contribute to innate differences in responses to alcohol that, in turn, contribute to high ethanol intake. It could also be that other brain regions involved in regulating alcohol drinking are more important sites of innate differences in gene expression. The lack of common genes could also be due to technical reasons: accumulated effects of several small differences may not be detected with the microarray procedure, and/or common differences in as yet unnamed genes are important. It is also possible that common differences in gene expression across the 5 line-pairs within a given region may not be detected unless the rats are first exposed to ethanol.

The lack of differentially expressed genes in the CeA between the AA and ANA lines suggests that this region may not play a major role in determining alcohol drinking behavior in these lines. However, innate differences between the AA and ANA line could still exist (e.g., post-translational modifications) but are not detected with the current technique.

Within the AcbSh, there were no GO biological categories that differed across more than 3 line-pairs. Furthermore, there was only 1 gene in common within any of these categories across the line-pairs. These results support the idea that various combinations of biological pathways in this region may contribute to the alcohol drinking characteristics of each line-pair.

In the CeA, there were 3 GO biological categories (Circadian Rhythm, Leukocyte Chemotaxis, and Wound Healing) that were significant across all the line-pairs, except the AA-ANA line-pair, suggesting that, in this region, there may be some common biological pathways that could influence alcohol drinking in most of the line-pairs. However, since there was only 1 gene in common across the 4 line-pairs in these categories, the mechanisms underlying these biological categories may all be different.

The high number of significant biological canonical pathways identified by IPA (Table 3) suggests that the AcbSh may strongly influence a predisposition for high alcohol drinking in the P rats, whereas the CeA may strongly influence a predisposition for high alcohol drinking in the HAD2 rats. In the AcbSh of the P-NP line-pair, there were several significant notable canonical pathways that could influence intracellular signaling pathways, as well as dopamine and glutamate receptor signaling, all of which could influence synaptic plasticity. However, similar pathways were not evident in the CeA of the HAD2-LAD2 line-pair (Table 3).

Overall ANOVA for all the line-pairs combined

The overall ANOVA revealed some possible common biological networks involving cellular assembly and organization, and cell-to-cell signaling in both the AcbSh and CeA across the 5 line-pairs (Table 4). However, there were few genes in common between the 2 regions or across the 5 line-pairs, further supporting the idea that various combinations of multiple cellular mechanisms may be involved in mediating the disparate alcohol-drinking behaviors of the 5 selectively bred rat line-pairs. Networks involved in growth and proliferation, cell-to-cell signaling and interaction, as well as cellular assembly and organization were observed (Table 4). The genes within these networks and the networks alone had little in common with the networks and genes in the AcbSh and CeA altered by alcohol binge drinking (McBride et al., 2010).

In the CeA, but not in the AcbSh, there were several significant KEGG categories in the overall ANOVA that were also significant in 3 or more individual line-pairs (Table 5). Interestingly, the complement system appears to be involved to some degree in differences between the high vs. low ethanol consuming lines. Complement components have been identified within a list of candidate genes that influence alcohol consumption in mice (Saba et al., 2011). Moreover, the complement system may have a role in synaptic reorganization (see review by Stephan et al., 2012).

In the AcbSh, there were 6 genes that significantly differed in the overall ANOVA and were also significantly changed in at least 3 line-pairs (Table 7). These included: a) Advillin (*Avil*), a member of the gelsolin/villin family of actin regulatory proteins (Marks et al., 1998) that is involved in neurite-like outgrowth (Shibata et al., 2004); b) 5-azacytidine induced 2 (*Azi2* also known as *Nap1*), which produces a protein that i) activates IKK-related kinases and by extension NF-kappa B-dependent gene expression (Fujita et al., 2003), ii) activates TLR3/TLR4-mediated IFN-beta induction (Sasai et al., 2005), and iii) regulates histone 3 (H3K9) acetylation during transcription elongation (Xue et al., 2013); c) myocyte enhancer factor 2C (*Mef2c*), a transcription factor involved in short-term synaptic plasticity (Akhtar et al., 2012) and neuronal response to cellular stress (She et al., 2012); d) NIMA-related expressed kinase 1 (*Nek1*), which plays an important role in preventing cell death induced by DNA damage (Chen et al., 2010; Pelegrini et al., 2010); e) RT1 class 1, locus T24, gene 4 (*RT1-T24-4*), which produces a protein that is primarily located in the cell membrane of astrocytes where it processes immune responses (Muotri et al., 2005); and f) zinc finger, CCHC domain containing 9 (*Zcchc9*), a nuclear protein involved in regulating MAPK signaling pathways (Zhou et al., 2008). Overall, the sum effects of these differences

in gene expression may promote neurite outgrowth, modify gene transcription, and process multiple cellular, immune, and stress responses.

In the CeA, there were 5 genes that significantly differed in the overall ANOVA and were also significantly changed in at least 3 line-pairs (Table 7). These included: a) ankyrin repeat domain (ARD) 12 (*Ankrd12*), which may facilitate the formation of the I-kappa-B-alpha-NF-kappa B complex in the nucleus (Ferreiro & Komives, 2010; Zhang et al., 2004), as well as modulating the cellular stress response (Miller et al., 2003); b) glutathione S-transferase alpha 4 (*Gsta4*), involved in reduction of oxidative stress (Raza, 2011) and other cellular stresses (Ström et al., 2012), with some evidence that *Gst* expression may play a role in ethanol preference and longevity (Björk et al., 2006); c) pleckstrin homology domain containing family H member 1 (*Plekhh1*), involved in regulation of guanine nucleotide-exchange activity (Baumeister et al., 2006; Cheng et al., 2004); d) RT1 class Ia, locus A2 (*RT1-A2*), which processes cellular immune responses in a brain-region dependent and rat-line dependent manner (Blaveri et al., 2010); and e) sushi, van Willebrand factor type A, EGF and pentraxin domain containing 1 (*Svep1*), a cell adhesive molecule (Shefer & Benayahu, 2010). Overall, these results suggest that gene expression differences between the HEC and LEC rats may alter gene transcription, as well as processing multiple cellular, immune, and stress responses, along with cell-to-cell and altered guanine nucleotide cellular signaling.

None of the genes in Table 7 were observed among the list of candidate genes reported for alcohol consumption in HXB/BXH RI rats (Tabakoff et al., 2009), among the candidate genes within chromosome 10 QTL of the high and low alcohol-drinking rats (Bice et al., 2010), or among candidate genes for alcohol preference expressed in congenic rat strains (Carr et al., 2007). In addition, none of these genes were among the list of candidate genes for alcohol drinking identified through transcriptome meta-analysis (Mulligan et al., 2006), and none appeared to be related to GABAergic transmission identified in a systems genetic analysis of alcohol drinking (Saba et al., 2011). In addition, none of the genes in Table 7 appeared in any of the GWAS results (Edenberg et al., 2010; Johnson et al., 2011; Kendler et al., 2011). On the other hand, genes associated with alcohol abuse produced changes in the human Acb in general categories of transcription and DNA repair, proliferation and development, and signaling and cell structure and adhesion (Flatscher-Bader et al., 2010). All of these categories were also evident in the overall ANOVA of the AcbSh of the 5 line-pairs (Table 4). These results suggest that either some of the differences in gene expression attributed to alcohol abuse may be evident before exposure to alcohol, and/or these biological systems are innately vulnerable to the effects of alcohol in alcoholic populations.

No enrichment of probe sets in established rat QTLs for ethanol consumption was found. This may reflect the complexity of factors (and brain regions) that contribute to alcohol drinking and the multiple biological systems involved in regulating gene expression. However, a search for potentially interesting genes was conducted using significant WGCNA modules; several genes associated with neurons were selected that were within a rat alcohol QTL and were significant (FDR = 0.10) in at least 2 line-pairs. In the AcbSh, these genes were *Gsn* (gelsolin), *A2bp1* (ataxin 2 binding protein 1), *Ankrd28* (ankyrin repeat domain 28), *Siae* (sialic acid acetylesterase), *Ppp3ca* (protein phosphatase 3, catalytic subunit, alpha isoform), *Plvap* (plasmalemma vesicle associated protein), and *Dph3* (DPH3, KYI11 homolog). In the CeA, only 1 gene was identified, i.e., *Cxxc4* (CXXC finger 4). Differences in expression of *Gsn*, *Ankrd28*, and *Ppp3ca* could alter formation of proteins involved in transmitter release (Kim & Ryan, 2013), cell-cell interaction (Tachibana et al., 2009), and dendrite formation (Khaitlina et al., 2013).

Both the AcbSh and CeA displayed altered protection against oxidative or other cellular stress factors, and cell-to-cell signaling in the HEC vs. LEC lines. In contrast, the complement system may be a significant factor in the CeA but not AcbSh. However, these differences appear to be too general to relate to a predisposition toward high alcohol drinking behavior.

A recent study reporting on gene expression differences in the VTA of these same 5 line-pairs found that the interactions of different combinations of multiple biological systems mediating transcription, oxidative stress protection, synaptic function, and intracellular signaling were observed between the HEC and LEC line-pairs (McBride et al., 2012). The oxidative stress category for the VTA overlaps somewhat with the CeA, with 1 gene in common, i.e., *Gsta4*. The synaptic function category for the VTA would fall within the cell-to-cell signaling category observed for both the AcbSh and CeA, but there were no genes in common. The intracellular signaling system in the VTA involved cAMP-PKA and CREB, whereas the system in the CeA involved guanine nucleotide exchange activities. Thus, there were no apparent biological categories or genes that were common across all 3 regions for the 5 HEC-LEC line-pairs. This latter finding likely reflects the impact of the unique neurobiology and functional interactions of each of the regions on gene expression.

Validation studies were conducted on a small number of genes in the AcbSh (Table 8). The 3 genes (*Avil*, *Mef2c*, and *Zcchc9*) were selected because they were significantly different in the ANOVAs of the 5 line-pairs, were significantly changed in the same direction in at least 3 line-pairs, and the fold difference was at least 1.4 (Tables 7 and 8). There was excellent agreement between the microarray and qRT-PCR results. In addition, previous studies from our laboratory reported good agreement, in 30 of 32 genes tested, between the data obtained with microarrays and the results found with qRT-PCR (Bell et al., 2009; Kimpel et al., 2007; Rodd et al., 2008). For example, *Gsta4* (Table 7) has been previously validated with qRT-PCR in 3 different brain regions of inbred P vs. inbred NP rats (Kimpel et al., 2007).

Conclusions

In summary, as measured with the present microarray procedure, no single gene or pathway appears to account for the disparate alcohol drinking characteristics of all 5 line-pairs of male HEC and LEC rats within the AcbSh or CeA. Instead, the interactions of different combinations of multiple biological systems within the AcbSh and CeA appear to be contributing to the disparate alcohol drinking characteristics of the 5 line-pairs. Although there were some common general categories between the 2 regions (Table 9), there were no genes in common within these categories. The cell-to-cell signaling and intracellular signaling pathways, as well as cellular organization, could produce innate high sensitivity to the reinforcing actions of ethanol in the AcbSh and/or CeA of the HEC rats, which would result in increased acquisition and maintenance of high alcohol drinking. The cellular mechanisms that respond to oxidative stress and other cellular stress factors may be protective and allow the HEC rats to consume high amounts of alcohol over a long period. Therefore, the combination of these and other biological processes (Table 9) within each region of the HEC rats may respond to the initial and repeated exposure to ethanol to promote its continued use and abuse. The current analysis is not likely to be sensitive to SNP artifacts since the array technology uses multiple probe-sets to measure each gene, which minimizes the effect of an SNP that happens to fall within a probe-set. The likelihood that SNPs influence the measurement of a gene in multiple line-pairs in the same direction is small. Additional studies, using more advanced technology to address alternative splicing and examining other CNS regions, may be needed to provide more complete information on molecular mechanisms and biological pathways that contribute to the alcohol drinking behaviors of the HEC and LEC rats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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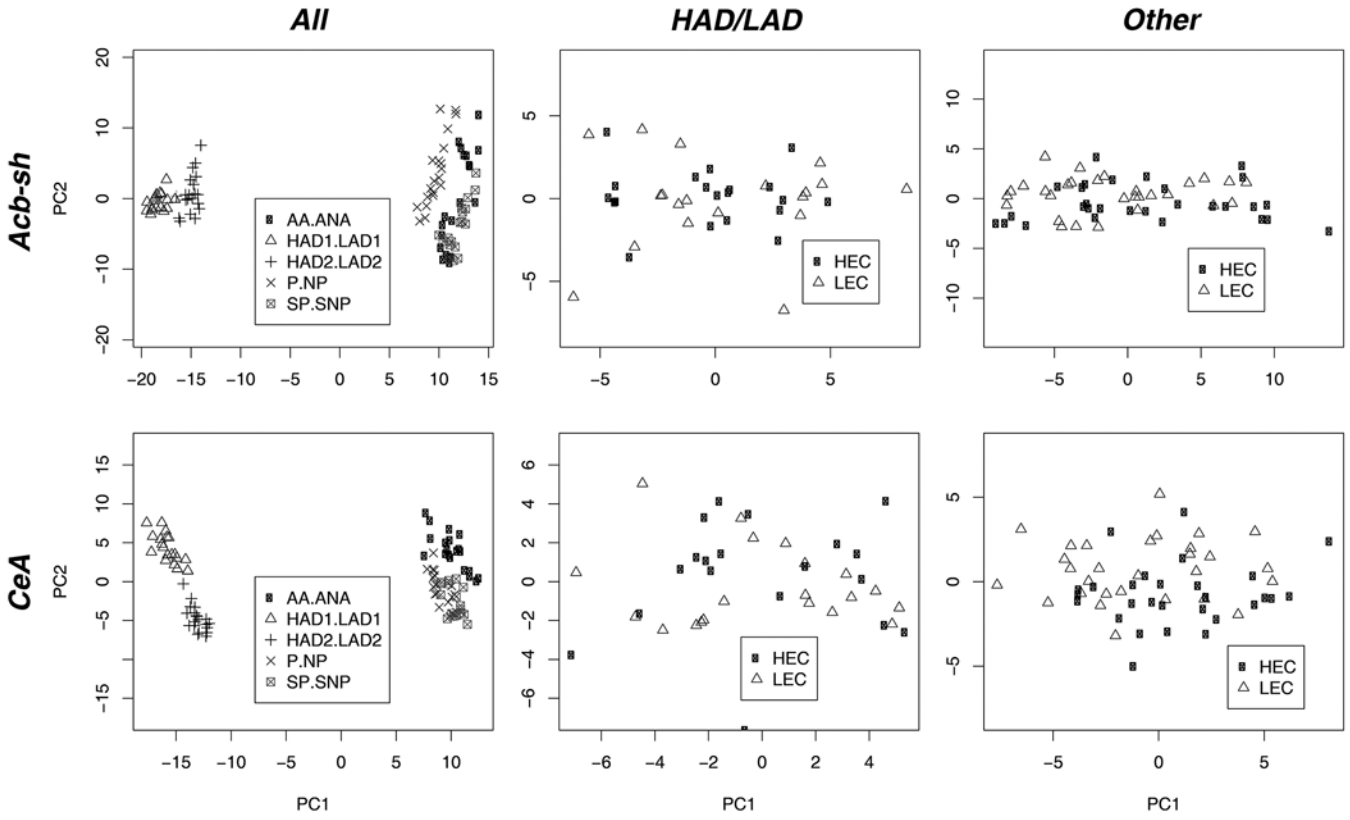

Figure 1.

Principal components analyses (PCA) for genes found significant in the ANOVA of the 5 line-pairs for the AcbSh (upper 3 panels) and CeA (lower 3 panels). Plots on far left are of processed expression data with animals labeled by line-pair for all 5 line-pairs. The middle and far right plots are derived from the residuals after linear modeling taking into account line-pair only, which allows for a view of the data with only ethanol consumption as a factor. Data for the replicate HAD-LAD lines are plotted in the middle panels; data for other 3 line-pairs are plotted in the far right panels.

Table 1

Number of probe sets that significantly differed (FDR = 0.1; fold-change ≥ 1.2) in expression in the AcbSh and CeA between each of the selectively bred pairs of high (HEC) and low (LEC) ethanol-consuming lines of rats

	P-NP	sP-sNP	HAD1-LAD1	HAD2-LAD2	AA-ANA
AcbSh					
Named genes[a]	1772	402	157	565	127
Un-named genes	286	78	23	91	30
CeA					
Named genes[a]	293	494	158	518	1
Un-named genes	51	102	29	87	0

Probe-sets remaining after removing those not detectably expressed was 20,745 in the AcbSh and 20,596 in the CeA.

[a]Omits any annotated as “riken”, “predicted” or “similar to”.

Table 2

Significant Gene Ontology (GO) biological categories in the AcbSh and CeA of the individual line-pairs

Biological category	P-NP	sP-sNP	HAD1-LAD1	HAD2-LAD2	AA-ANA
AcbSh					
Gland morphogenesis	X	X			X
Muscle cell proliferation	X	X	X		
Regulation of neurotransmitter levels	X	X		X	
Response to glucocorticoid stimulus	X	X		X	
Response to retinoic acid	X	X		X	
Response to steroid hormone stimulus	X	X		X	
Tissue morphogenesis	X	X	X		
Vascular process in circulatory system	X	X	X		
CeA					
Circadian rhythm	X	X	X	X	
Leukocyte chemotaxis	X	X	X	X	
Wound healing	X	X	X	X	
Diol metabolic process	X	X	X		
Positive regulation protein kinase B signaling cascade	X		X	X	
Response to molecule of bacterial origin		X	X	X	
Response to other organism		X	X	X	

GO categories significant at $p < 0.05$ and observed in 3 or more line-pairs

Table 3

Networks identified by Ingenuity Pathway Analysis of significant differences in genes with higher or lower expression in the AcbSh and CeA of the P, HAD1, HAD2, AA and sP lines compared to their respective low ethanol consuming lines

AcbSh			
Line-pair	**Canonical Pathway**	**Higher gene expression**	**Lower gene expression**
HAD2-LAD2	ILK Signaling	9 genes: *Map2k6, Pik3ca, Pik3c2a, Pik3r1, Vim, Myl9, Vegfb, Ppp1r12a, Rps6ka5*	6 genes: *Mapk8, Rhog, Rnd3, Pik3c3, Rsu1, Acta1*
P-NP	cAMP-mediated signaling	19 genes: *Camk2d, Htr7, Atf4, Cnr1, Rapgef3, Pde8a, Pkib, Rps6ka1, Camk2g, Akap12, Pde4a, Chrm3, Ppp3cb, Adcy8, Adcy2, Adcy6, Crhr1, Gabbr2, Pth1r*	36 genes: *Mapk1, Grm3, Camk2a, Adcy5, Ppp3r1, Rgs14, Pde2a, Grm8, Pde10a, Rgs4, Grm4, Drd2, Mc4r, Grm7, Pde1b, Pkig, Grm2, Camk4, Htr4, Chrm4, Htr1d, Pde1a, Hrh3, Akap11, Vipr1, Map2k1, Ppp3ca, Akap5, Npy1r, Prkar2b, Oprk1, Adra2c, Htr1f, Pde8b, Adora2a, Akap1*
P-NP	G-Protein Coupled Receptor Signaling	40 genes: *Gpr37, Htr7, Atf4, Cnr1, Sstr1, Npy5r, Pde8a, Hcrtr1, Rps6ka1, Camk2g, Fzd1, Gprc5b, Pik3c3, Adcy6, Crhr1, Gpr64, Pth1r, Ntsr1, Gpr162, Casr, Gpr116, Camk2d, Adra1b, Grm1, Rapgef3, Hcrtr2, Cxcr7, Nfkbie, Pde4a, Nfkb1, Chrm3, Tacr1, Nfkbia, Smo, Adcy8, Adcy2, Trhr, Ednrb, Gabbr2, Plcb4*	52 genes: *Grm3, Gpr149, Camk2a, Pde2a, Grm8, Lphn3, Grm7, Pde1b, Fyn, Gpr12, Htr4, Ptk2b, Chrm4, Htr1d, Hrh3, Vipr1, Gpr155, Pik3r2, Bai2, Htr2a, Prkcg, Grm5, Rasgrp1, Adora2a, Prkcb, Mapk1, Pthlh, Pdpk1, Adcy5, Mras, Rgs14, Bai3, Pde10a, Rgs4, Grm4, Drd2, Mc4r, P2ry1, Grm2, Camk4, Pde1a, Map2k1, Npy1r, Gpr88, Gipr, Prkar2b, Sstr2, Oprk1, Calcr, Adra2c, Htr1f, Pde8b*
P-NP	Synaptic Long Term Potentiation	12 genes: *Gria4, Camk2d, Ppp3cb, Atf4, Adcy8, Ppp1r14c, Grm1, Ppp1r14a, Rapgef3, Plcb4, Rps6ka1, Camk2g*	24 genes: *Grm2, Camk4, Mapk1, Grm3, Gria1, Camk2b, Camk2a, Ppp1r7, Ppp3r1, Mras, Map2k1, Ppp3ca, Grm8, Gria2, Itpr1, Grm4, Prkcg, Grm7, Grm5, Prkar2b, Ppp1r12a, Prkch, Gria3, Prkcb*
P-NP	Glutamate Receptor Signaling	6 genes: *Slc17a6, Grm1, Grip1, Gria4, Gng2, Grik1*	16 genes: *Grm2, Camk4, Grm3, Grm8, Gls, Gria1, Gria2, Slc1a3, Grm4, Gng7, Grm5, Grm7, Dlg4, Grik2, Homer1, Gria3*
P-NP	Protein Kinase A Signaling	28 genes: *Dusp15, Camk2d, Atf4, Cdc16, Cdc25a, Ppp1r14c, Ptprd, Ppp1r14a, Gng3, Pde8a, Pygm, Dusp19, Gng2, Camk2g, Anapc2, Akap12, Nfkbie, Pde4a, Nfkb1, Nfkbia, Ppp3cb, Smo, Tgfb2, Adcy8, Adcy2, Adcy6, Pygb, Plcb4*	37 genes: *Mapk1, Ptpla, Ptk2, Gnb4, Camk2b, Camk2a, Ppp1r7, Adcy5, Ppp3r1, Ryr3, Ptpre, Pde2a, Pde10a, Ppp1r1b, Ptpn18, Itpr1, Pde1b, Tgfb3, Ppp1r12a, Prkch, Camk4, Ptk2b, Pde1a, Ptpn5, Gng7, Akap11, Dusp14, Map2k1, Ppp3ca, Akap5, Pygl, Prkcg, Prkar2b, Pde8b, Ptgs2, Prkcb, Akap1*
P-NP	CREB Signaling in Neurons	14 genes: *Gria4, Camk2d, Pik3c3, Atf4, Adcy8, Grik1, Adcy2, Grm1, Adcy6, Gng3, Plcb4, Gng2, Rps6ka1, Camk2g*	26 genes: *Grm2, Camk4, Mapk1, Grm3, Gria1, Gng7, Gnb4, Camk2b, Camk2a, Adcy5, Mras, Grik2, Pik3r2, Map2k1, Grm8, Gria2, Grm4, Itpr1, Prkcg, Grm5, Grm7, Prkar2b, Prkch, Gnal, Gria3, Prkcb*
P-NP	Calcium Signaling	14 genes: *Tnnc1, Atp2a2, Gria4, Camk2d, Ppp3cb, Atf4, Grik1, Hdac4, Myh14, Tpm2, Trpc4, Atp2b2, Tpm4, Camk2g*	21 genes: *Tnnt1, Camk4, Mapk1, Atp2b1, Gria1, Tpm1, Camk2b, Camk2a, Ppp3r1, Ryr3, Ppp3ca, Casq2, Akap5, Tnnc2, Gria2, Itpr1, Prkar2b, Htr3a, Mef2c, Camkk2, Gria3*
P-NP	Relaxin Signaling	12 genes: *Nfkbie, Pde4a, Nfkb1, Nfkbia, Pik3c3, Adcy8, Adcy2, Adcy6, Gng3, Pde8a, Gng2, Npr2*	16 genes: *Mapk1, Pde1a, Gng7, Gnb4, Adcy5, Mras, Pik3r2, Map2k1, Gucy1b3, Pde2a, Gucy1a3, Pde10a, Prkar2b, Pde1b, Pde8b, Gnal*
P-NP	Phospholipase C Signaling	20 genes: *Nfkb1, Rhog, Ppp3cb, Gpld1, Atf4, Adcy8, Adcy2, Hdac4, Pld3, Adcy6, Ppp1r14a, Arhgef17, Rapgef3, Gng3, Rhov, Plcb4, Rhoq, Rnd3, Gng2, Fnbp1*	20 genes: *Fyn, Camk4, Mapk1, Rps6ka3, Gng7, Gnb4, Adcy5, Ppp3r1, Mras, Arhgef2, Map2k1, Ppp3ca, Itpr1, Prkcg, Ppp1r12b, Ppp1r12a, Mef2c, Prkch, Arhgef9, Prkcb*
P-NP	Signaling by Rho	21 genes: *Pip5k1b, Sept9, Nfkb1, Limk1,*	20 genes: *Mapk1, Ptk2b, Gng7, Ptk2,*

AcbSh			
Line-pair	**Canonical Pathway**	**Higher gene expression**	**Lower gene expression**
	Family GTPases	*Stmn1, Rhog, Pik3c3, Gfap, Cdc42ep1, Mapk8, Rdx, Arhgef17, Gng3, Mapk12, Rhov, Rhoq, Rnd3, Gng2, Pip4k2a, Sept6, Fnbp1*	*Gnb4, Ezr, Baiap2, Mras, Arhgef2, Arpc1a, Pik3r2, Map2k1, Cdc42ep3, Wasf1, Pip5k1a, Ppp1r12b, Ppp1r12a, Pard3, Arhgef9, Gnal*
P-NP	Axonal Guidance Signaling	28 genes: *Bmp4, Ephb2, Adam11, Limk1, Tubb3, Sema5a, Gng3, Sema6d, Gng2, Rgs3, Slit1, Pdgfa, Fzd1, Tubb2b, Ppp3cb, Igf1, Tuba8, Pik3c3, Ntrk1, Smo, Rassf5, Plcb4, Ntrk2, Sema4d, Mag, Epha5, Sema3c, Gli1*	34 genes: *Slit3, Mapk1, Itsn1, Ptk2, Gnb4, Ephb1, Ppp3r1, Baiap2, Mras, Arpc1a, Ablim2, Epha7, Ngef, Kalrn, Vegfc, Prkch, Gnal, Fyn, Bmp2, Epha4, Plxna2, Gng7, Wnt7a, Pik3r2, Robo2, Shank2, Map2k1, Ppp3ca, Plxnc1, Slit2, Plxnd1, Prkcg, Prkar2b, Prkcb*
P-NP	Synaptic Long Term Depression	8 genes: *Gria4, Lcat, Igf1, Ppm1l, Grm1, Crhr1, Plcb4, Npr2*	20 genes: *Grm2, Mapk1, Grm3, Gria1, Ryr3, Mras, Map2k1, Gucy1b3, Gucy1a3, Grm8, Gria2, Itpr1, Grm4, Prkcg, Grm5, Grm7, Prkch, Gnal, Prkcb, Gria3*
P-NP	α-Adrenergic Signaling	7 genes: *Adcy2, Adcy6, Pygb, Gng3, Pygm, Gng2, Adcy8*	13 genes: *Camk4, Mapk1, Pygl, Itpr1, Gng7, Prkcg, Gnb4, Prkar2b, Adcy5, Mras, Prkch, Map2k1, Prkcb*
P-NP	Gap Junction Signaling	10 genes: *Tubb2b, Ppp3cb, Tuba8, Pik3c3, Adcy8, Adcy2, Tubb3, Adcy6, Plcb4, Npr2*	17 genes: *Csnk1g1, Mapk1, Adcy5, Ppp3r1, Mras, Pik3r2, Map2k1, Ppp3ca, Gucy1b3, Htr2a, Gucy1a3, Itpr1, Drd2, Prkcg, Prkar2b, Prkch, Prkcb*
P-NP	Dopamine Receptor Signaling	10 genes: *Ppp1r14c, Adcy2, Adcy6, Ppp1r14a, Slc18a3, Ppm1l, Smox, Adcy8, Pcbd1, Qdpr*	6 genes: *Ppp1r1b, Drd2, Prkar2b, Ppp1r7, Adcy5, Ppp1r12a*
P-NP	Corticotropin Releasing Hormone Signaling	10 genes: *Smo, Atf4, Adcy8, Adcy2, Cnr1, Adcy6, Crhr1, Mapk12, Gli1, Npr2*	13 genes: *Camk4, Mapk1, Adcy5, Map2k1, Gucy1b3, Gucy1a3, Itpr1, Prkcg, Prkar2b, Mef2c, Prkch, Ptgs2, Prkcb*
P-NP	Dopamine-DARPP32 Feedback in cAMP Signaling	13 genes: *Kcnj16, Atp2a2, Ppp3cb, Ppm1l, Atf4, Adcy8, Ppp1r14c, Adcy2, Adcy6, Ppp1r14a, Plcb4, Kcnj5, Kcnj6*	19 genes: *Camk4, Csnk1g1, Kcnj4, Adcy5, Ppp1r7, Ppp3r1, Ppp3ca, Gucy1b3, Cacna1d, Gucy1a3, Ppp1r1b, Itpr1, Drd2, Prkcg, Prkar2b, Ppp1r12a, Prkch, Camkk2, Prkcb*
P-NP	CXCR4 Signaling*	14 genes: *Rhog, Pik3c3, Adcy8, Adcy2, Mapk8, Adcy6, Gng3, Mapk12, Plcb4, Rhov, Rhoq, Rnd3, Gng2, Fnbp1*	14 genes: *Mapk1, Gng7, Ptk2, Gnb4, Adcy5, Mras, Pik3r2, Map2k1, Egr1, Itpr1, Prkcg, Prkch, Gnal, Prkcb*
P-NP	ILK Signaling	18 genes: *Nfkb1, Ccnd1, Tgfb1i1, Rhog, Pik3c3, Ppm1l, Atf4, Itgb5, Myh14, Fermt2, Mapk8, Mapk12, Rhov, Rhoq, Rnd3, Sh2b2, Rsu1, Fnbp1*	14 genes: *Map2k6, Mapk1, Bmp2, Pdpk1, Ptk2, Myc, Pik3r2, Actn1, Casp3, Actn2, Vegfc, Ppp1r12a, Rps6ka5, Ptgs2*
P-NP	Renin-Angiotensin Signaling*	10 genes: *Adcy2, Adcy6, Mapk8, Nfkb1, Mapk12, Pik3c3, Stat1, Adcy8, Ace, Agt*	13 genes: *Ptk2b, Mapk1, Itpr1, Jak2, Prkcg, Ptk2, Prkar2b, Adcy5, Mras, Prkch, Pik3r2, Map2k1, Prkcb*
P-NP	IL-1 Signaling	10 genes: *Adcy2, Nfkbie, Mapk8, Adcy6, Gng3, Mapk12, Nfkb1, Nfkbia, Gng2, Adcy8*	9 genes: *Map2k6, Mapk1, Gng7, Gnb4, Prkar2b, Adcy5, Mras, Gnal, Irak2*
CeA			
Line-pair	Canonical Pathway	Higher gene expression	Lower gene expression
HAD1-LAD1	IL-8 Signaling	6 genes: *Ccnd2, Mapk1, Mapk8, Nfkb1, Ccnd1, Tek*	4 genes: *Itgb2, Pik3ca, Pik3c2a, Mmp2*
HAD2-LAD2	IGF-1 Signaling	7 genes: *Csnk2a2, Igfbp6, Pik3ca, Jun, Pik3c2a, Stat3, Igfbp2*	3 genes: *Pik3c3, Mapk8, Igfbp5*
HAD2-LAD2	Cardiac Hypertrophy Signaling	8 genes: *Adcy9, Pik3ca, Pik3c2a, Gng13, Myl9, Jun, Eif2b1, Hspb1*	6 genes: *Mapk8, Eif4e, Rock2, Pik3c3, Eif2b5, Mef2c*

AcbSh			
Line-pair	**Canonical Pathway**	**Higher gene expression**	**Lower gene expression**
HAD2-LAD2	Breast Cancer Regulation by Stathmin1	*Adcy9, Pik3ca, Pik3c2a, Gng13, Rb1cc1, Arhgef18, Prkd3*	*Rock2, Camk2a, Camk2d, Pik3c3, Arhgef9*
HAD2-LAD2	IL-8 Signaling	8 genes: *Pik3ca, Pik3c2a, Gng13, Myl9, Jun, Vegfb, Prkd3, Itgb5*	6 genes: *Mapk8, Irak3, Rock2, Traf6, Pik3c3, Tek*
HAD2-LAD2	Role of NFAT in Cardiac Hypertrophy	6 genes: *Adcy9, Akap5, Pik3ca, Pik3c2a, Gng13, Prkd3*	5 genes: *Mapk8, Camk2d, Camk2a, Pik3c3, Mef2c*

There were no significant canonical pathways in the AcbSh or CeA for the AA-ANA and sP-sNP line-pairs. For the AcbSh, only pathways with 15 or more genes are listed. For the CeA, only pathways with 10 or more genes are listed.

* Indicates that similar pathways were found in the AcbSh of the HAD2-LAD2 line-pair, but are not listed because there were less than 15 genes in each pathway. There were no genes in common within these pathways between the P-NP and HAD2-LAD2 line-pairs.

Table 4

Networks identified by Ingenuity Pathway Analysis of significant differences in higher or lower gene expression in the AcbSh and CeA of the HEC compared to the LEC lines

AcbSh		
Network/main theme	**Gene expression higher[a]**	**Gene expression lower[b]**
Network 1: Development and Function	19 genes: *Acss1, Cpne8, Ext2, Grk6, Grm8, Hsd17b7, Kif1a, Lcat, Maoa, Myh11, Nav2, Nfix, Phf20, Pias2, Sncg, Sp1, Suds3, Tagln, Tnrc6a*	11 genes: *Arid4b, Gas7, Hk2, Igfbp4, Ing2, Lpxn, Nap1l1, Nr2f6, Rps19, Sf3a1, Wdr5*
Network 2: Cellular Growth and Proliferation	11 genes: *Adcy9, Capn1, Cpt1a, Creg1, Dctn6, Gfap, Pou6f1, S100a6, Scg2, Stat3, Tgfa*	19 genes: *Akt1s1, Axl, Blvrb, Bzw2, Epn2, Fkbp1a, Kitlg, Laptm5, Mlxipl, Myo5a, Npy, Ppp3ca, Ppp3r1, Pygl, Rock2, S100b, Sst, Txnl1, Wars*
Network 3: Nervous System Development and Function	12 genes: *Bcan, Dnm1, Grb14, Gria4, Grin3a, Hexim1, Mpp6, Numb, Reln, Rhot2, Rnf41, Zeb2*	20 genes: *Arf4, Camk2a, Cbr1, Efnb3, Egfr, Ephb1, Fyn, Gabbr1, Gda, Grin2b, Grm5, Kars, Lin7a, Map2, Mpp5, Pip5k1a, Scarf2, Sdc2, Shc2, Trak2*
Network 4: Cell-To-Cell Signaling and Interaction	14 genes: *Ankrd12, B4galt1, Bag3, Caprin1, Cdk9, Cxcl12, Dlat, Dnajb1, Dync1h1, Hs2st1, Ppm1b, Sdf4, Tubgcp3, Yme1l1*	17 genes: *Arpc4, Copg, Copz1, Dad1, Dap3, Exosc2, Exosc5, Gapdhs, Lancl1, Mapk3, Mzf1, Pdk1, Phb2, Ptprz1, Ralgds, Sod3, Tmed9*
Network 5: Cellular Assembly and Organization	14 genes: *Dhx36, Hif1a, Lrrc59, Map4, Pank2, Pfkp, Pgk1, Pik3r4, Plod2, Rb1cc1, Sephs1, Sh3glb1, Vps33a, Wdfy3*	17 genes: *Acp6, Aldh2, Atg10, Atg12, Atg4b, Camkk2, Ccs, Echs1, Erh, Fyco1, Gsto1, Mdh1, Prkaa2, Rgs10, Tars, Uba5, Wdr41*
CeA		
Network/main theme	Gene expression higher[a]	Gene expression lower[b]
Network 1: DNA Repair, RNA Damage and Repair	18 genes: *Abce1, Aifm2, Ccnl2, Col14a1, Comt, Creg1, Cul5, Gstm4, Hint1, Iqcb1, Morc3, Ncapd3, Ncaph, Ppm1a, Rnasel, Scn3b, Setd2, Vps33a*	17 genes: *Cstf1, Fech, Mbnl2, Mcam, Nthl1, Phkg1, Polg, Ppp2r2a, Ppp2r5a, Rab40c, Rnf40, Sh3bgrl2, Slc6a6, Spsb1, Tbxas1, Tp53, Trio*
Network 2: Developmental Disorder, Neurological Disease	18 genes: *Adam9, Bbs1, Cep290, Eps8, Ercc8, Grb14, H1f0, Hdgfrp3, Hook3, Ipo5, MAST1, Pcm1, Pldn, Rnf41, Tmco3, Tmod2, Ttc8, Wdyhv1*	16 genes: *Ahnak, Apbb3, Axl, Cbr1, Ccng2, Egfr, Inppl1, Kif5c, Lin7a, Mdh1, Mpp3, Rab31, Shc1, Tmod1, Tpm1, Trak2*
Network 3: Cellular Assembly and Organization, Cell-To-Cell Signaling and Interaction	13 genes: *Dcun1d4, Dhx36, Etv4, Flnb, Ipo4, Mlh1, Nedd9, Nup107, Phf20, Reln, Tgfb1i1, Trim23, Vav2*	18 genes: *Abcd3, C1qa, Fn1, Kdm5b, Map2, Nup93, Pde3b, Pex19, Phyh, Plec, Psd3, Ptp4a3, Rnaset2, Rragd, Swap70, Tmem14a, Tnfrsf11b, Tnfsf13*
Network 4: Molecular Transport, RNA Trafficking	13 genes: *Bmpr1a, Bud13, Crmp1, Fam107b, Hif1a, Hnrnpul1, Ncbp1, Pias2, Plod2, Rad1, Tdg, Tgfb2, Thoc4*	20 genes: *Abcg1, Aldh2, Chd1l, Csf1r, Echs1, Fyco1, Hbegf, Hexb, Hk2, Il33, Nfia, Pias4, Ptafr, Purb, Rad51, Rpa1, S100a4, Sash3, Smad7, Uba5*
Network 5: Cellular Function and Maintenance	11 genes: *Cul2, Eps15l1, Kif1a, Klf5, Lama1, Pcgf2, Rac1, Sap130, Sqstm1,*	16 genes: *E2f6, Efna2, Elmo1, Enpp1, Epn2, Gas7, Kars, Laptm5, Limd1, Nqo1,*

AcbSh		
Network/main theme	**Gene expression higher[a]**	**Gene expression lower[b]**
	Suds3, Usp7	*Slc40a1, Snap23, Stx6, Timm44, Tomm70a, Usf2*

[a] Genes with higher expression in high vs. low ethanol consuming lines

[b] Genes with lower expression in high vs. low ethanol consuming lines

Table 5

Significant KEGG categories ($p < 0.05$) in the overall ANOVA that were also significant in the CeA of 3 or more individual line-pairs

Category	P-NP	sP-sNP	HAD1-LAD1	HAD2-LAD2
Viral myocarditis	*Sgcb* *Fyn* *RT1-CE16* *RT1-EC2* *RT1-S3* *Sgcb*	*Myh11* *Rac1* *RT1-Da* *RT1-DMb* *RT1-EC2* *RT1-S3*	*Ccnd1* *Itgb2* *Myh11* *RT1-EC2*	*Itgal* *Myh11* *RT1-CE16* *RT1-S3* *Sgca* *Sgcb* *Myh9* *RT1-CE12* *RT1-DMb* *RT1-EC2* *RT1-M3-1* *Sgcb*
Antigen processing and presentation	*RT1-CE16* *RT1-EC2* *RT1-S3*	*Cd74* *RT1-Da* *RT1-DMb* *RT1-EC2* *Ctss* *RT1-S3*		*RT1-CE16* *RT1-S3* *RT1-CE12* *RT1-DMb* *RT1-EC2* *RT1-M3-1*
Systemic lupus erythematosus	*C1r* *C1s* *C3* *Hist1h2bh* *Hist1h4b*	*C3* *Fcgr3a* *RGD1562378* *RT1-Da* *RT1-DMb* *Hist2h2be*	*C1qa* *C1qc* *Fcgr1a*	

There were no significant KEGG categories in the AcbSh that were also significant in at least 3 line-pairs. The AA-ANA line-pair had no significant KEGG categories in either region.

Underlined genes indicate lower expression levels in the high ethanol consuming line compared to the low ethanol consuming line.

Table 6

Significant GO biological categories of statistically relevant WGCNA modules*

Category ID	Term	*p* value	OddsRatio	ExpCount	Count	Size
AcbSh (grey60)						
GO:0006605	protein targeting	0.009	4.3	1.3	5	280
GO:0006606	protein import into nucleus	0.001	7.4	0.8	5	167
GO:0006913	nucleocytoplasmic transport	0.005	4.9	1.1	5	248
GO:0032496	response to lipopolysaccharide	0.007	4.5	1.2	5	265
AcbSh (midnight blue)						
GO:0010975	regulation of neuron projection development	0.004	5.4	1.0	5	225
GO:0051960	regulation of nervous system development	0.004	3.8	2.1	7	453
GO:1900542	regulation of purine nucleotide metabolic process	0.009	4.3	1.3	5	276
CeA (light yellow)						
GO:0006469	negative regulation of protein kinase activity	0.002	6.3	0.9	5	117
GO:0006511	ubiquitin-dependent protein catabolic process	0.004	3.9	2.0	7	265
GO:0010563	negative regulation of phosphorus metabolic process	0.005	4.1	1.6	6	215
GO:0030111	regulation of Wnt receptor signaling pathway	0.005	4.9	1.1	5	148
GO:0031400	negative regulation of protein modification process	0.006	4.0	1.6	6	220
GO:0042326	negative regulation of phosphorylation	0.002	4.9	1.3	6	180
GO:0043065	positive regulation of apoptotic process	0.008	3.0	2.9	8	388
GO:0043632	modification-dependent macromolecule catabolic process	0.004	3.8	2.0	7	273
GO:0045862	positive regulation of proteolysis	0.000	12.6	0.5	5	61
GO:0051348	negative regulation of transferase activity	0.003	5.4	1.0	5	136
GO:0051603	proteolysis involved in cellular	0.007	3.4	2.2	7	300

Category ID	Term	*p* value	OddsRatio	ExpCount	Count	Size
	protein catabolic process					

*Consensus, across line-pair, WGCNA modules identified with GSEA were tested within line-pair for enrichment with GO BP category genes. Modules with significant enrichment ($p < 0.05$) in > 3 line-pairs and containing at least 5 genes are presented.

Table 7

Genes that were significantly different in the ANOVAs of the 5 line-pairs and were significantly changed in the same direction in at least 3 line-pairs

Symbol	Description	Direction[a]	P[b]	sP	H1	H2	AA
AcbSh							
Avil	Advillin	higher	X			X	X
Azi2	5-azacytidine induced 2	higher	X	X		X	
Mef2c	myocyte enhancer factor 2C	lower	X	X		X	
Nek1	NIMA-related expressed kinase 1	lower		X	X	X	
RT1-T24-4	RT1 class I, locus T24, gene 4	higher	X		X	X	
Zcchc9	zinc finger, CCHC domain containing 9	lower	X	X		X	
CeA							
Ankrd12	ankyrin repeat domain 12	higher		X	X	X	
Gsta4	glutathione S-transferase alpha 4	lower	X	X	X	X	
Plekhh1	pleckstrin homology domain containing family H (with MyTH4 domain) member 1	higher		X	X	X	
RT1-A2	RT1 class Ia, locus A2	higher	X		X	X	
Svep1	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	lower	X		X	X	

[a] Indicates that values were higher (or lower) in the high ethanol consuming lines compared to the low ethanol consuming lines.

[b] Abbreviations: P = P-NP; H1 = HAD1-LAD1; H2 = HAD2-LAD2; AA = AA-ANA; sP = sP-sNP

Table 8

Validation with qRT-PCR of 3 key genes that were significantly different in the ANOVAs of the 5 line-pairs and were significantly changed in the same direction in at least 3 line-pairs in the AcbSh

	qRT-PCR				Microarray
Gene	**P**	**NP**	**F-C**	***p* value**	**F-C**
Avil	2.6 ± 0.1	2.1 ± 0.1	1.25	0.000	1.36
Mef2c	0.7 ± 0.1	1.0 ± 0.1	1.45	0.008	1.59
Zcchc	4.1 ± 0.2	5.0 ± 0.2	1.23	0.004	1.42

Data are the means ± SEM (*n* = 8/line). F-C = fold-change

Table 9

Summary of key findings from the results of the overall ANOVAs in the AcbSh and CeA of the HEC and LEC line-pairs

AcbSh	CeA
Cell-to-cell signaling	Cell-to-cell signaling
Cellular organization	Cellular organization
Cellular stress factors	Cellular and oxidative stress
Neurite outgrowth	RNA trafficking
	Synaptic pruning (complement)
	Guanine nucleotide cellular signaling