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Changes in Gene Expression within the Ventral Tegmental Area Following Repeated Excessive Binge-Like Alcohol Drinking by Alcohol-Preferring (P) Rats

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Abstract

The objective of this study was to detect changes in gene expression in the ventral tegmental area (VTA) following repeated excessive binge-like ('loss-of-control') alcohol drinking by alcohol-preferring (P) rats. Adult female P rats (n = 7) were given concurrent access to 10, 20, and 30% EtOH for 4 1-hour sessions daily for 10 weeks followed by 2 cycles of 2 weeks of abstinence and 2 weeks of EtOH access. Rats were sacrificed by decapitation 3 hours after the 4th daily EtOH-access session at the end of the second 2-week relapse period. A water-control group of female P rats (n = 8) was also sacrificed. RNA was prepared from micro-punch samples of the VTA from individual rats; analyses were conducted with Affymetrix Rat 230.2 GeneChips. Ethanol intakes were 1.2–1.7 g/kg per session, resulting in blood levels > 200 mg% at the end of the 4th session. There were 211 unique named genes that significantly differed (FDR = 0.1) between the water and EtOH groups. Bioinformatics analyses indicated alterations in a) transcription factors that reduced excitation-coupled transcription and promoted excitotoxic neuronal damage involving clusters of genes associated with *Nfkbia*, *Fos,* and *Srebf1*, b) genes that reduced cholesterol and fatty acid synthesis, and increased protein degradation, and c) genes involved in cell-to-cell interactions and regulation of the actin cytoskeleton. Among the named genes, there were 62 genes that showed differences between alcohol-naïve P and non-preferring (NP) rats, with 43 of the genes changing toward NP-like expression levels following excessive binge-like drinking in the P rats. These genes are involved in a pro-inflammatory response, and enhanced response to glucocorticoids and steroid hormones. Overall, the results of this study indicate that the repeated excessive binge-like alcohol drinking can change the expression of genes that may alter neuronal function in several ways, some of which may be deleterious.

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Keywords

alcohol-preferring rat; binge-like alcohol drinking; ventral tegmental area; gene expression

Introduction

Examining changes in gene expression resulting from chronic ethanol drinking could provide clues to identifying genes and gene networks involved in maintaining high alcohol drinking behavior, as well as identifying genes involved in the neurotoxic consequences of chronic alcohol consumption. There have been several studies that applied genomic and proteomic analyses to examine the effects of ethanol in rodent models (Bell et al., 2006, 2009; Kerns et al., 2005; McBride et al., 2010; Mulligan et al., 2006, 2011; Rodd et al., 2008; Saito et al., 2002, 2004; Tabakoff et al., 2009; Treadwell and Singh, 2004). Studies conducted on post-mortem human tissue have examined the effects of chronic alcohol consumption (Alexander-Kaufman et al., 2006, 2007; Flatscher-Bader et al., 2005, 2010; Lewohl et al., 2000, 2004; Liu et al., 2004, 2006; Matsumoto et al., 2007; Mayfield et al., 2002). Collectively, these studies indicate that differences between alcoholics and controls could be detected in several brain regions, and these differences may represent altered neuronal function.

Alterations in gene expression produced by exposure to alcohol have been reported in several studies with rats and mice. Acute ethanol injections (6 g/kg; i.p.) produced changes in whole brain of C57BL/6J and DBA/2J mice (high- and low-alcohol drinkers, respectively) in expression of genes involved in regulating cell signaling, gene regulation, and homeostasis/stress response (Treadwell and Singh, 2004). Kerns et al. (2005) reported that acute i.p. ethanol injections altered expression of genes involved in glucocorticoid signaling, neurogenesis, myelination, neuropeptide signaling, and retinoic acid signaling in the nucleus accumbens (Acb), prefrontal cortex, and ventral tegmental area (VTA) of C57BL/6J and DBA/2J mice. Differences in expression of genes coding for oxido-reductases and ADP-ribosylation factors were found in the dorsal hippocampus of Lewis rats given 12% ethanol or water for 15 months (Saito et al., 2002). In contrast, Saito et al. (2004) found no statistically significant effects of chronic free-choice alcohol drinking on gene expression in the striatum of C57BL/6By mice. The above rodent studies were conducted using ethanol injections or 24-hour free-choice drinking. Moreover, other than the study of Kerns et al. (2005) using i.p. ethanol injections, none of the above rodent studies reported data on limbic regions that are involved in mediating alcohol drinking.

Bell et al. (2006) examined the effects of chronic alcohol drinking by alcohol-preferring (P) rats on protein levels in the Acb and amygdala, and reported a small number of changes in each region following chronic alcohol drinking. Rodd et al. (2008) examined the effects of operant ethanol self-administration on changes in gene expression in the Acb and amygdala of inbred P rats 24 hours after the last 1-hour drinking session. In the Acb, approximately 200 genes differed significantly between the ethanol and water-control groups, whereas in the amygdala, few significant differences were observed. In another study (Bell et al., 2009), gene expression changes were detected in the Acb of P rats following 2 different alcohol-drinking conditions, i.e., continuous 24-hour access and multiple 1-hour daily scheduled access periods. Significant differences in expression of genes involved in intracellular signaling pathways and transcription factors were found between the continuous-access and water-control groups, whereas no significant differences were observed in the Acb between the multiple scheduled-access and the water-control groups, when measured the day after the last drinking episode. McBride et al. (2010) reported significant changes in gene expression in the Acb-shell and central nucleus of the amygdala (CeA) of P rats after binge-like alcohol

drinking (8 weeks of access to 15% and 30% ethanol in 3 1-hour daily scheduled-access sessions). Although there were some categories of biological processes in common between the two regions (e.g., synaptic transmission, neurite development), there were few genes in common between the two regions.

Few studies on changes in gene expression in the VTA following chronic alcohol drinking have been undertaken. In human studies, Flatscher-Bader et al. (2008, 2010) reported that changes in genes involved with neurotransmission and signal transduction in the VTA were associated with alcohol abuse.

The VTA is a critical part of the brain reward system and has been implicated in mediating the rewarding actions of ethanol (Gatto et al, 1994; McBride et al., 1999; Rodd et al., 2004; Rodd-Henricks et al., 2000) and in regulating alcohol drinking (Hodge et al., 1993; Hwa et al., 2012; Rodd et al., 2010) and seeking (Hauser et al., 2011). Other studies indicated that chronic alcohol drinking or repeated local administration of ethanol increased the sensitivity of the VTA to the rewarding (Rodd et al. 2005a, b) and dopamine stimulating (Ding et al., 2009) effects of ethanol.

Thus far, studies on the effects of alcohol drinking on changes in gene expression in the VTA have not been examined in an accepted animal model of alcoholism. The present study uses a unique binge-like ethanol drinking protocol that produces daily blood ethanol concentrations (BECs) in excess of 200 mg%, and examines the effects of this dangerous level of alcohol drinking on gene expression in the VTA 3 hours after the last drinking episode.

Materials and methods

Subjects were adult (approximately 90 days old at the start of the experiment) female selectively bred P rats from the 61st generation. The rats were single-housed on a reverse 12-h/12-h dark-light cycle (lights off at 9:00 AM). Animals had *ad libitum* access to food and water. The 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 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).

The rats were self-trained in 3-lever operant chambers using essentially the same procedure previously described for a 2-lever operant paradigm (Rodd et al, 2008; Rodd-Henricks et al., 2002a, b). The P rats (n = 7) were given 4 1-hour sessions in the 3-lever operant chambers to respond for 10, 20, and 30% ethanol on an FR5 schedule of reinforcement. Water was freely available during these sessions. Water and food were freely available in the home cage. The 1-hour operant sessions were conducted during the dark cycle; there was a 1-hour interval between each operant session. Sessions were conducted 5 consecutive days each week. Following 10 weeks of ethanol access, rats were taken through 2 cycles of 2 weeks of alcohol deprivation and 2 weeks of ethanol access. Ethanol intakes were similar during the relapse periods as were observed during weeks 9 and 10. Rats were killed by decapitation 3 hours after the 4th session at the end of the second 2-week relapse period. This 3-hour time point was selected in an attempt to maximize the response to alcohol on the expression of genes in tissue from rats that have had a history of repeated excessive binge drinking. The brains were quickly removed and frozen in isopentane on solid frozen CO_2. Age-matched

alcohol-naïve water-control female P rats (n = 8) were killed and brains were removed in a similar manner. Brains were stored at −70°C until sectioned.

Tail-blood samples were taken following the 1st, 2nd and 4th daily-access sessions, and prior to the 3rd session to provide BECs across the drinking period. Blood samples were taken from multiple rats on different occasions to minimize the impact on overall drinking. Ethanol concentrations were determined with an Analox Analyzer.

Sample collection and microarray procedure

On the day of preparation of micro-punch samples, brains were transferred to a cryostat set at −6 to −10°C at least 2 hours prior to sectioning. 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 and Watson (1998) was used to identify the VTA. Micro-dissection needles (Fisher Scientific) with an inner diameter of 0.77 mm were used to obtain the VTA. 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. A second trained individual independently verified the quality of the micro-punch dissections.

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.

Separate preparations of total RNA were made for the VTA from each animal. Samples were not pooled. Standard Affymetrix protocols (GeneChip Expression Analysis Technical Manual, Rev. 5 and updates) were used to synthesize biotinylated cRNA, using the Affymetrix kits for cDNA synthesis, in vitro transcription, and sample cleanup. The fragmented, biotinylated cRNA from each independent sample was mixed into 300 μl of hybridization cocktail, of which 200 μl were used for each sample. Hybridization was carried out for 17 hours at 42°C. Samples were hybridized to the Affymetrix Rat Genome 230 2.0 GeneChips. Washing and scanning of the GeneChips were carried out according to standard protocols, as previously described (Edenberg et al., 2005; McClintick et al., 2003).

To minimize potential systematic errors, all stages of the experiment were balanced across experimental groups. That is, equal numbers of animals in each group were sacrificed within the same 2-hour time frame each day, and equal numbers of RNA preparations from the 2 groups were processed through the labeling, hybridization, washing, and scanning protocols on a given day, in a counterbalanced order, using pre-mixed reagents.

Statistical and neuroinformatics analysis of microarray data

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 under accession GSE42578.

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, 2005) for further analysis with tools available from the Bioconductor Project (Gentleman et al., 2004). Expression data of the 15 arrays from the VTA were normalized and converted to log(2) using the Robust Multi-chip Average (RMA) method (Irizarry et al., 2003) implemented in the Bioconductor package RMA. As a standardization step to facilitate later comparisons with other experiments, expression levels were scaled such that the mean expression of all arrays was $\log_2(1000)$. As we were primarily concerned with identifying genes that could be subjected to further bioinformatic analysis, all probe sets currently annotated by Affymetrix as "expressed sequence tags" or whose gene names contain the words "riken," "predicted," or "similar to" were filtered out. We next filtered out probe sets that were not detectable above background in our samples; this has been shown to reduce noise in microarray experiments (McClintick and Edenberg, 2006). Probe sets that did not have at least 25% of samples with normalized scaled expression greater than 64 were not analyzed. Linear modeling to calculate gene-wise *p* values for the contrasts of the ethanol group versus water group was performed using the package Limma (Smyth, 2004). Probe sets were considered to be statistically significant at FDR = 0.1 (calculated according to Storey et al., 2004).

Testing for over-representation of Gene Ontology (Ashburner et al., 2000; Harris et al., 2004; GO) biological processes (BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories was performed using the Bioconductor package GOstats (Gentleman, 2004). Briefly, for each gene set tested, a list of unique Entrez-Gene identifiers was constructed. This list was then compared to the list of all known Entrez-Gene identifiers that are represented on the Affymetrix chipset Rat Genome 230 2.0. Identification of over-represented GO categories was then accomplished within GOstats using the hypergeometric distribution. GO and KEGG categories were called significant at $p < .05$.

Genes differentially expressed at FDR = 0.1 were uploaded 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.

To provide a more global network analysis, a weighted gene co-expression network analysis (WGCNA) was also conducted (Zhang and Horvath, 2005), using the Bioconductor (Gentleman et al., 2004) package WGCNA (Langfelder and Horvath, 2008, 2012) within R (R: A language and environment for statistical computing Ver 2.15.0; R Foundation for Statistical Computing, 2013). Briefly, gene expression data of named genes were rank-ordered according to their ascending *p* values obtained from traditional *t* testing of the two experimental groups. 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. Various *p*- and FDR-value cutoffs between FDR ≤ 0.10 and $p \leq .10$ were tried in an attempt to select a set of genes whose resultant networks met the criteria of legitimacy for scale free topology (Zhang and Horvath, 2005). Resultant modules were tested for enrichment of various categories of genes using Fisher's Exact Test. Categories tested included GO biologic process (Ashburner et al., 2000; Harris et al., 2004) and location by cell type (Cahoy et al., 2008).

Results

Ethanol intakes and BECs

Figure 1 shows the average daily ethanol intakes during the first 10 weeks of concurrent access to 10, 20, and 30% ethanol and ethanol intakes during each of the 4 sessions during weeks 7–10. P rats readily acquired ethanol self-administration during the 2nd day of concurrent access to the 3 ethanol solutions (similar to Rodd-Henricks et al., 2002a, b). During the initial 5 weeks of responding, overall ethanol intakes were consistent (3.2 ± 0.2 g/kg for the 4 1-hour sessions; Fig. 1, top panel). Elevated ethanol intakes were observed during week 6, when total daily amount increased 48% (two-tailed *t* test indicated significant increase from average intake of weeks 3–5; $p < .001$). Ethanol intakes gradually increased until week 9 (92% increase compared to average intake of weeks 3–5; $p < .001$) and stabilized between weeks 9–10.

During the individual sessions, ethanol intakes were slightly higher during the 1st and 4th session compared to sessions 2 and 3 (Fig. 1, bottom panel); the 1-hour session average was approximately 1.2 to 1.7 g/kg during the last two weeks of access.

BECs were sampled across multiple sessions during weeks 8–10. Samples were obtained following the 1st and 2nd session, immediately prior to the 3rd session (60 minutes post-2nd session), and following the 4th session (see legend of Fig. 1 for values). BECs were approximately 120 mg% following the 1st session, and exceeded 150 mg% following the 2nd session. Prior to the 3rd session, BECs were still higher than 100 mg% even after 60 minutes without access to ethanol. BECs following the 4th session exceeded 200 mg%. The estimated total ethanol intakes were significantly correlated with BECs obtained following the 4th session ($r = 0.89$).

Changes in gene expression in the VTA

There were a total of 335 probe sets that significantly differed between the ethanol and water groups in the VTA: 102 ESTs and 233 named genes (Table 1 shows a list of named genes comprised of 211 unique genes). Over 75% of the fold changes were 1.2 or higher (Table 1).

GO analysis indicated there were 8 biological processes categories with 10 or more genes that differed significantly between the water and ethanol groups (Table 2). Three categories involved metabolic processes, the first for steroids, the second for alcohol and the third for proteins. Two other categories (cellular adhesion and transcription) contain genes that could influence neuronal function.

KEGG analysis of the VTA gene data indicated 11 significant categories containing 6 or more genes each (Table 3), some of which could alter neuronal function. Two categories, steroid biosynthesis and extracellular matrix- (ECM) receptor interaction, are consistent with the results of the GO categories of ‘steroid metabolic process’ and ‘cell adhesion.’ Genes in the category of ‘regulation of actin cytoskeleton’ could influence dendritic structure and function, including synaptic plasticity.

Ingenuity pathways analysis indicated different networks were altered by ethanol drinking, one of which had clusters around *Fos* and *Srebf1* and a second which had clusters of genes around *Nfkb1a* (Table 4). There were 16 genes clustered around *Fos* and 15 genes clustered around *Srebf1*; 11 of the 16 genes clustered around *Fos* and 13 of the 15 genes clustered around *Srebf1* had lower expression levels in the ethanol group than in the water group. In contrast, 10 of the 14 genes clustered around *Nfkb1a* had higher expression levels in the ethanol drinking group vs. water controls.

For the WGCNA, only by using a very liberal *p* value of .10 as the cutoff for gene inclusion (2137 genes) did the resultant network meet the criteria for scale free topology (Zhang and Horvath, 2005). With $\beta = 12$, the soft-threshold R^2 value was 0.85 with a slope of −1.79. Median connectivity was 3.18 and maximum connectivity was 38.63. Not including the grey module of uncorrelated genes, there were 4 modules with 100 genes or more (Yellow, Blue, Turquoise, and Brown). The Yellow module is a neuron-enriched module and contained 350 genes. The Blue module is an astrocyte- and oligodendrocyte-enriched module and contained 597 genes. The Yellow module had several GO categories (Table 5) that did not appear in the other GO analysis, e.g., G-protein coupled receptor signaling, transmission of nerve impulses, and regulation of membrane potential. The Blue module had some significant categories that were consistent with the results of the GO, KEGG, and Ingenuity analyses of significant (FDR = 0.10) genes (Tables 2–4), e.g., steroid biosynthetic process, sterol metabolic process. In addition, the Blue module had significant GO categories associated with lipid, fatty acid, and phospholipid metabolism (Table 5).

Among the 211 unique named genes that differed (FDR = 0.1) between the ethanol and water groups in the VTA, there were 26 genes located within rat ethanol-preference QTLs (Table 1). There were 4 rat ethanol-preference QTLs that contained 4 or more genes: Alc18 (*Ezh2, Gstk1. Gng12, Arhgap25, Tmem176a*, *Tmem176b*); Alc11 (*Aadat, Dctd, Sc4mol, Scrg1*); Alc15 (*Celsr2, Cd53, Lppr5, Ugt8*); and Alc5 (*Dusp1, Mpg, Slc9a3r2, Tekt4*).

Changes in expression of genes that were altered by alcohol drinking and also significantly differed between alcohol-naïve P and NP rats

There were 62 genes that significantly differed between the ethanol and water groups and also differed between naïve P and NP rats (McBride et al., 2012) in the VTA (Table 6). Among these 62 genes, 7 were located in 4 different rat QTLs: a) *Dctd, Sc4mol, Scrg1* in Alc11, b) *Tmem176a, Tmem176b* in Alc18, c) *Cd53* in Alc15, and (d) *Ralgds* in Alc8.

KEGG analysis of the 62 genes indicated only an antigen-presentation category and an immune category, whereas the GO biological processes gene enrichment analysis revealed several significant categories with 5 or more genes (Table 7). Prominent among these GO categories were those involved in immune and inflammatory responses, as well as responses to glucocorticoid and steroid hormone stimuli.

Among the 62 genes, 43 differed between the alcohol-naïve P and NP rats and changed toward NP expression levels as a result of binge drinking in the P rats (Table 6). There were 28 genes lower in the P than NP rats that were up-regulated in the ethanol group compared to the water group, and 15 genes higher in the VTA of the P than NP rats that were down-regulated with alcohol drinking.

There were 16 genes down-regulated with alcohol drinking that were also lower in the VTA of the P compared to the NP rats (Table 6). Only 3 genes were up-regulated with alcohol drinking and were also higher in the P than NP rats.

Although Ingenuity pathway analysis did not reveal significant networks when all 62 genes were used in the analysis, one network emerged using only genes that changed in the opposite direction in the alcohol drinking group compared to the difference between the alcohol-naïve P vs. NP rats. There were several genes associated with *Ubc* (ubiquitin conjugating gene) that were lower in the P vs. NP rats and were higher in the ethanol vs. water group (listed in protein metabolic processes, cell adhesion, and inflammatory response categories; Table 8). Within this same network, there were several genes associated with *Il6* (Interleuken 6) that were also significantly changed in the opposite direction with alcohol drinking relative to the naïve group (listed in inflammatory response category; Table 8).

Discussion

This study examined changes in gene expression in response to alcohol in the VTA of P rats with a history of excessive binge drinking. The changes reflect the effects of the combination of a high brain ethanol concentration plus a history of binge drinking on gene expression in the VTA examined 3 hours after the last drinking episode. Samples taken at later time points after the last binge drinking episode will likely show different patterns of genes being expressed between the ethanol and water groups.

The major findings of this study are that multiple scheduled (4 × 1 hour sessions during the dark cycle) sessions of concurrent access to 10, 20 and 30% ethanol a) resulted in 'loss-of-control' alcohol drinking (Fig. 1), defined as drinking that routinely produces BECs exceeding 200 mg%, and b) produced major changes (mainly 1.2-fold and higher in 211 unique genes) in expression of genes in the VTA (Table 1). Genes that are involved in transcription, metabolic processes, cell-to-cell interactions, inflammatory response, and response to hormone stimuli were particularly affected (Tables 7 and 8). The overall results support the idea that this level of drinking is producing major changes in gene expression within the VTA that could promote neuroadaptations mediating excessive alcohol drinking and alcohol-induced neuronal damage.

The reduction in *Fos* (FBJ osteosarcoma oncogene) expression with alcohol drinking along with the reduction in gene expression of 11 of the 16 genes associated with the *Fos* (Table 4) supports the idea that there is reduced excitation-coupled transcription in the alcohol group compared to controls (George et al., 2012; Schiavone et al., 2011). The findings that 10 of 14 genes are up-regulated in the *Nfkbia* (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) cluster (Table 4) of the alcohol group suggest promotion of excitotoxic neuronal damage (Himadri et al., 2010; Koltsova et al., 2012). It is noteworthy that glucocorticoid receptor activity, implicated in the present study, modulates transcription of *Nfkbia* and *Tsc22d3* (e.g., D'Adamio et al., 1997; Scheinman et al., 1995). Moreover, there is some evidence that morphine may co-regulate *Tsc22d3*, *Sgk*, *Klf15,* and *Nfkbia* (Korostynski et al., 2007); paralleling a similar pattern of *Tsc22d3*, *Sgk1*, *Klf9,* and *Nfkbia* all being down-regulated by ethanol in the present study.

The reduced expression of 13 of 15 genes (Table 4) associated with *Srebf1* (sterol regulatory element binding transcription factor 1) suggests reduced cholesterol and fatty acid synthesis (Ong et al., 2000; Tabernero et al., 2002), which is also consistent with excessive binge drinking having a negative impact on neuronal function. In addition, *Srebf1*, *Hmgcr*, and *Hmgcs1* are all implicated in white matter integrity (e.g., Carter, 2007; Xiang et al., 2011), which is disrupted after chronic alcohol abuse (Harris et al., 2008). Neither the GO (Table 2) nor the KEGG (Table 3) analysis provided a clear answer on the effects of repeated excessive binge drinking on neuronal function in the VTA, since both positive (e.g., regulation of actin cytoskeleton and cell adhesion) and negative (e.g., killer cell mediated cytotoxicity, negative regulation of transcription, and steroid biosynthesis with all genes down-regulated) effects on cellular function are evident.

Many of the genes, which differed between the alcohol-naive P vs. NP rats and changed in the opposite direction with alcohol drinking (Table 6), promoted pro-inflammatory responses and induced programmed cell death (Table 7). The innate differences in the lower expression of genes involved in the immune and inflammatory responses in the VTA of the P compared to the NP line (e.g., *C3, Serpinb9, B2m, C1qa, Fcer1g, Itgb2, Pycard, Aif1*; Tables 6 and 7) could be factors contributing to the vulnerability of the P line to high alcohol drinking behavior. Recent findings (Crews et al., 2013) are compatible with the present results of increased brain neuroimmune activation in alcohol dependence. These results

(Tables 6 and 7) are in agreement with the overall analysis of the data indicating a negative impact of this excessive binge-like drinking protocol by P rats (Table 8). On the other hand, the complement system has been implicated in synaptic plasticity (Stephan et al., 2012), and some of the changes observed in the immune process and inflammatory response may result in positive alterations in synaptic function. The results with WGCNA (Table 5, Yellow module) support the contention that positive alterations in synaptic function may have occurred under the excessive binge-like drinking conditions utilized in the present study. However, with few exceptions, the individual genes in this module were not significantly different at a modest p value of .01.

Previous studies indicated that chronic 24-hour free-choice alcohol drinking increased the sensitivity and response of the posterior VTA to the rewarding effects of ethanol (Rodd et al., 2005a, b). These results suggest that ethanol drinking is producing positive effects on neuronal function within the VTA. In addition, repeated binge-like ethanol intakes of 1.5–2 g/kg/session (3 × 1-hour daily sessions) produced changes in expression of genes that could alter transcription, synaptic function, and neuronal plasticity in a generally positive manner in the Acb-shell and central nucleus of the amygdala (McBride et al., 2010). In contrast to the above results, the findings of the present study suggest a more negative effect of alcohol drinking on cellular function. Main differences between the current study and the previous 3 reports are that BECs in excess of 200 mg% were attained on a daily basis over several weeks in the present study, that different time points after drinking were measured, and that different tissues were assayed. Such chronically high brain levels of alcohol may produce excessive cellular oxidative stress and eventually cause neuronal damage within the VTA. The WGCNA provided additional evidence that excessive binge drinking produces alterations in glia cells within the VTA (Table 5; Blue module GO categories). Alterations in fatty acid, steroid, cholesterol, and phospholipid biosynthetic processes could be indicators of the damaging effects of the very high daily concentrations of ethanol on brain function.

The effects of binge-like alcohol drinking on gene expression have been studied in the Acb of inbred P rats (Rodd et al., 2008), the extended amygdala of P rats (McBride et al., 2010), and several brain regions (olfactory bulb, frontal cortex, striatum, cerebellum, ventral midbrain, and hippocampus) of C57 mice using a single-day procedure (Mulligan et al., 2011). Comparison of the present results with those obtained with the C57 mice or the 2 rat binge-drinking studies did not indicate any overlap in the top annotated genes, with one exception: *B2m*; beta-2 microglobin was common in the present study and that of Rodd et al., 2008. The lack of overlap likely reflects a combination of factors, such as different regions analyzed, duration and level of binge-like alcohol drinking, time of sampling after ethanol drinking, and, in the case of mice, species differences.

There were no genes in common between those identified as possible candidate genes in the expression profiling of congenic rat strains (Carr et al., 2007) and the present study (Table 1). In addition, there were no genes in common between the present study (Table 1) and the candidate genes within the chromosome 10 QTL (Bice et al., 2010).

Flatscher-Bader et al. (2010) reported on differences in gene expression in the VTA of alcoholics vs. control subjects. There was a general overlap (see Table 7) in a canonical pathway related to “regulation of the actin skeleton”. In both studies, the changes in gene expression may reflect altered cellular organization and structure.

QTL Alc18 contained 6 significant genes (*Ezh2, Gstk1, Gng12, Arhgap25, Tmem176a,* and *Tmem176b*) altered by excessive binge drinking (Table 2), 2 of which (*Tmem176a, Tmem176b*) were also common in the differences between the alcohol-naïve P vs. NP rats (Table 5). *Tmem176a* and *Tmem176b* are trans-membrane proteins involved in the immune

system (Cuajungco et al., 2012). *Ezh2* is the catalytic subunit of Polycomb repressive complex, which is a highly conserved histone methyltransferase that targets lysine-27 of histone H3 (Simon and Lange, 2008). *Gstk1* (glutathione S-transferase kappa 1), located in mitochondria and peroxisomes, is involved in energy and lipid metabolism (Morel and Aninat, 2011). *Gng12* (guanine nucleotide binding protein, gamma 12) is a negative regulator of the inflammatory response (Larson et al., 2010). *Arhgap25* (Rho GTPase activating protein 25) is a member of the RhoGAP family, which are negative regulators of Rho family GTPases implicated in actin remodeling (Katoh and Katoh, 2004). Further research is required to determine how these genes could impact a predisposition for high alcohol drinking or be involved in maintaining high alcohol consumption.

All 3 genes within Alc 11 (*Dctd, Sc4mol,* Scrg1; Table 1) were also common in the differences between alcohol- naïve P vs. NP rats (Table 5). *Dctd* (cCMP [deoxycytidine-5′-monophosphate] deaminase) provides the main nucleotide substrate for thymidylate synthase, which is important for DNA synthesis (Hou et al., 2008). *Sc4mol* (sterol-C4-methyl oxidase-like) is involved in cholesterol synthesis (He et al., 2011) and the growth and guidance of axons (Yu et al., 2008). *Scrg1* (stimulator of chondrogenesis 1) encodes a highly conserved cysteine-rich protein, is principally expressed in the CNS and is associated with large dense-core vesicles in neurons (Dandoy-Dron et al., 2003). Overall, the findings may point to segments of QTLs Alc11 and Alc18 being important for containing genes involved in vulnerability to alcohol abuse and contributing to the maintenance of high alcohol intake.

The study of Rodd et al. (2008) examined the effects of operant responding for a 0.0125% saccharin solution (approximately 500 lever presses/session for 10 weeks) and did not observe any significant effects on gene expression in the Acb of inbred P rats, suggesting that instrument responding *per se* is not producing alterations in gene expression. The results of the current study are consistent with the observed effects being due to repeated excessive binge-like alcohol drinking, and not mainly a result of instrument responding.

Validation studies on key genes, using qRT-PCR, could not be conducted because there was not sufficient sample remaining after the microarray procedure. Previous studies from our laboratory indicated good agreement 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).

In summary, the overall results of the bioinformatic analyses (Table 8) indicated the up-regulation of a number of genes that could produce a pro-inflammatory response, promote excitotoxic neuronal damage, and increase protein degradation. These results suggest that the high BECs repeatedly attained in the present study could be causing neuronal damage in the VTA. Consistent with this interpretation are the findings that several genes involved in cholesterol and fatty acid synthesis were down-regulated (Table 8). Also, alterations in expression of genes around 3 transcription factors suggested reduced excitation-coupled transcription. Thus, the combination of alterations in transcription factors, metabolism of proteins and steroid, and pro-inflammatory response are indicators that this level of alcohol drinking may be repeatedly producing brain ethanol levels that could cause cellular damage.

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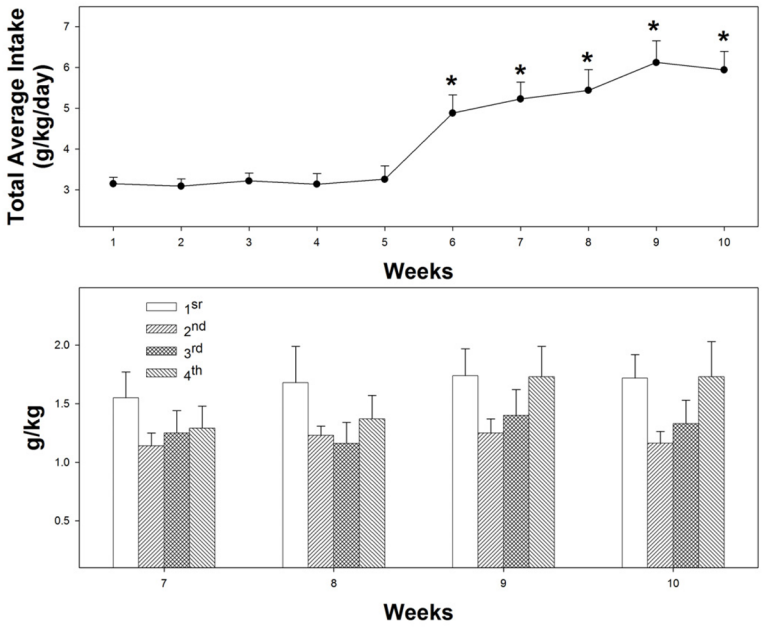

Fig. 1.

Estimated total ethanol intakes (g/kg/day; top panel) by P rats given concurrent access to 10, 20, and 30% ethanol (with water freely available) for 4 1-hour sessions each day during the dark cycle. Estimated intakes (g/kg) for each of the 4 1-hour access sessions during weeks 7–10 are given in the bottom panel. Ethanol was given 5 consecutive days each week (no ethanol on weekends) over the 10 weeks of access. *Indicates significantly ($p < .001$) higher ethanol intakes compared to sessions 3–5. BECs sampled across multiple sessions during weeks 8–10 indicated: 1) 124 ± 14 mg% following 1st session; 2) 177 ± 19 mg% following the 2nd session; 3) 128 ± 16 mg% immediately prior to the 3rd session; and 4) 237 ± 20 mg% following the 4th session. Data are the means ± SEM (n = 7).

Table 1

List of named genes in the VTA of P rats that were significantly different (FDR = 0.1) between the ethanol and water groups

Symbol	Gene Description	F- C	QTL
Aadat	aminoadipate aminotransferase	1.29	Alc11
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	1.28	
Acat2	acetyl-Coenzyme A acetyltransferase 2	−1.29	
Aco1	aconitase 1, soluble	−1.13	
Acot4	acyl-CoA thioesterase 4	1.31	
Acss2	acyl-CoA synthetase short-chain family member 2	−1.40	
Aif1	allograft inflammatory factor 1	1.32	
Aifm3	apoptosis-inducing factor, mitochondrion-associated 3	−1.20	
Anxa3	annexin A3	1.27	
Anxa3	annexin A3	1.34	
Anxa3	Annexin A3	1.32	
Apln	apelin	−1.34	
Apln	apelin	−1.42	
Arhgap25	Rho GTPase activating protein 25	1.28	Alc18
Arl11	ADP-ribosylation factor-like 11	1.29	
Azin1	antizyme inhibitor 1	−1.20	
Azin1	antizyme inhibitor 1	−1.42	
B2m	beta-2 microglobulin	1.14	
B2m	Beta-2 microglobulin	1.17	
Baiap2	BAI1-associated protein 2	−1.17	
Baiap2	BAI1-associated protein 2	−1.21	
Bin2	bridging integrator 2	1.20	
Birc7	baculoviral IAP repeat-containing 7	1.39	
C1qa	complement component 1, q subcomponent, A chain	1.29	
C1qtnf5	C1q and tumor necrosis factor related protein 5	−1.20	
C3	complement component 3	2.56	
Casp1	caspase 1	1.20	
Ccdc125	coiled-coil domain containing 125	1.19	
Ccdc28b	coiled coil domain containing 28B	−1.18	
Ccl6	chemokine (C-C motif) ligand 6	−1.71	
Ccne2	cyclin E2	−1.29	
Cd53	Cd53 molecule	1.28	Alc15
Cd9	CD9 molecule	−1.20	
Cdh23	cadherin 23 (otocadherin)	1.24	
Cdk2ap1	CDK2-associated protein 1	−1.15	Alc6
Celsr2	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	1.21	Alc15
Chfr	checkpoint with forkhead and ring finger domains	−1.14	
Chmp1b	chromatin modifying protein 1B	−1.12	
Chordc1	cysteine and histidine-rich domain (CHORD)-containing 1	−1.23	

Symbol	Gene Description	F- C	QTL
Cldn11	claudin 11	−1.37	
Cldn11	claudin 11	−1.21	
Cmip	c-Maf-inducing protein	1.13	
Col14a1	collagen, type XIV, alpha 1	1.25	
Col1a1	collagen, type I, alpha 1	−1.40	
Col3a1	collagen, type III, alpha 1	−1.60	
Col4a5	collagen, type IV, alpha 5	−1.30	
Crcp	CGRP receptor component	1.20	Alc10
Csrp2	cysteine and glycine-rich protein 2	−1.37	
Ctse	cathepsin E	1.22	
Ctsl1	cathepsin L1	−1.17	
Ctss	cathepsin S	1.28	
Ctsz	cathepsin Z	1.27	
Cxcr4	chemokine (C-X-C motif) receptor 4	1.21	
Cyp51	cytochrome P450, family 51	−1.40	
Cyp51	cytochrome P450, family 51	−1.35	
Dctd	dCMP deaminase	1.30	Alc11
Dcxr	dicarbonyl L-xylulose reductase	1.15	
Ddit4	DNA-damage-inducible transcript 4	−1.56	
Decr1	2,4-dienoyl CoA reductase 1, mitochondrial	1.14	
Dhcr7	7-dehydrocholesterol reductase	−1.23	
Dock6	dedicator of cytokinesis 6	−1.23	
Dock8	dedicator of cytokinesis 8	1.20	
Dusp1	dual specificity phosphatase 1	−1.40	Alc5
Ece2	endothelin-converting enzyme 2	1.16	
Egr1	early growth response 1	−1.54	
Eif4b	eukaryotic translation initiation factor 4B	1.15	
Emr1	EGF-like module containing, mucin-like, hormone receptor-like 1	1.57	
Errfi1	ERBB receptor feedback inhibitor 1	−1.35	
Ezh2	enhancer of zeste homolog 2 (Drosophila)	−1.33	Alc18
Ezh2	enhancer of zeste homolog 2 (Drosophila)	−1.27	Alc18
Fa2h	fatty acid 2-hydroxylase	−1.36	
Fads1	fatty acid desaturase 1	−1.15	
Fam181b	family with sequence similarity 181, member B	−1.16	
Fcer1g	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	1.33	
Fcgr2a	Fc fragment of IgG, low affinity IIa, receptor (CD32)	1.32	
Fcgr2a	Fc fragment of IgG, low affinity IIa, receptor (CD32)	1.69	
Fcgr2a	Fc fragment of IgG, low affinity IIa, receptor (CD32)	1.33	
Fcgr3a	Fc fragment of IgG, low affinity IIIa, receptor	1.28	
Fgfr3	fibroblast growth factor receptor 3	−1.17	
Fgfr3	Fibroblast growth factor receptor 3	−1.13	
Fos	FBJ osteosarcoma oncogene	−1.54	

Symbol	Gene Description	F- C	QTL
Gadd45b	growth arrest and DNA-damage-inducible, beta	1.24	
Gamt	guanidinoacetate N-methyltransferase	−1.24	
Gng12	guanine nucleotide binding protein (G protein), gamma 12	−1.34	Alc18
Gpatch4	G patch domain containing 4	−1.31	
Gpatch4	G patch domain containing 4	−1.35	
Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble)	−1.42	
Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble)	−1.76	
Gpr34	G protein-coupled receptor 34	1.81	
Gpr84	G protein-coupled receptor 84	1.30	
Gramd3	GRAM domain containing 3	−1.22	
Gramd3	GRAM domain containing 3	−1.29	
Grxcr1	glutaredoxin, cysteine rich 1	2.64	
Gstk1	glutathione S-transferase kappa 1	1.13	Alc18
Hhip	Hedgehog-interacting protein	−1.27	
Hip1	huntingtin interacting protein 1	−1.25	Alc10
Hip1	huntingtin interacting protein 1	−1.29	Alc10
Hist1h2bh	histone cluster 1, H2bh	−1.28	
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	−1.20	
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	−1.34	
Hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7	−1.28	
Idi1	isopentenyl-diphosphate delta isomerase 1	−1.31	
Ifrd1	interferon-related developmental regulator 1	−1.16	
Igf2bp2	insulin-like growth factor 2 mRNA binding protein 2	−1.16	
Il10rb	interleukin 10 receptor, beta	1.18	
Irf1	interferon regulatory factor 1	1.18	
Irf8	Interferon regulatory factor 8	1.33	
Itgb1	integrin, beta 1	−1.15	
Itgb2	integrin, beta 2	1.26	
Kcnj8	potassium inwardly-rectifying channel, subfamily J, member 8	1.31	
Klf9	Kruppel-like factor 9	−1.24	
Laptm5	lysosomal protein transmembrane 5	1.25	
Laptm5	lysosomal protein transmembrane 5	1.24	
Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	1.29	
Lims2	LIM and senescent cell antigen like domains 2	−1.29	
LOC100362458	RG23949-like	1.21	
LOC290595	hypothetical gene supported by AF152002	1.48	Alc13
LOC64038	Sertolin	1.11	
LOC691777	hypothetical protein LOC691777	1.16	
Lppr5	lipid phosphate phosphatase-related protein type 5	−1.22	Alc15
Lrpap1	low density lipoprotein receptor-related protein associated protein 1	1.17	
Lrrc33	leucine rich repeat containing 33	1.23	
Ly86	lymphocyte antigen 86	1.24	

Symbol	Gene Description	F- C	QTL
Mal	mal, T-cell differentiation protein	−1.33	
Mfsd2	major facilitator superfamily domain containing 2	−1.29	
Mog	myelin oligodendrocyte glycoprotein	−1.23	
Mpg	N-methylpurine-DNA glycosylase	1.15	Alc5
Mthfd1l	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	−1.26	
Mtmr2	myotubularin related protein 2	−1.27	
Mysm1	myb-like, SWIRM and MPN domains 1	−1.18	
Naaa	N-acylethanolamine acid amidase	1.15	
Ndfip2	Nedd4 family interacting protein 2	−1.12	
Nfasc	neurofascin	−1.23	
Nfasc	neurofascin	−1.25	
Nfil3	nuclear factor, interleukin 3 regulated	−1.40	
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	−1.32	
Nol3	nucleolar protein 3 (apoptosis repressor with CARD domain)	1.11	
Npc2	Niemann-Pick disease, type C2	1.20	
Nr1d1	nuclear receptor subfamily 1, group D, member 1	1.25	
Nsdhl	NAD(P) dependent steroid dehydrogenase-like	−1.24	
Olig1	oligodendrocyte transcription factor 1	−1.22	
Pald	paladin	1.18	
Pcdh20	protocadherin 20	−1.46	
Pcdh20	protocadherin 20	−1.47	
Pcdhb9	protocadherin beta 9	1.33	
Pck2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	1.16	
Pex11a	peroxisomal biogenesis factor 11 alpha	−1.44	
Phactr3	phosphatase and actin regulator 3	−1.34	
Plcd4	phospholipase C, delta 4	−1.21	
Plekha1	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	−1.16	
Plekhf1	pleckstrin homology domain containing, family F (with FYVE domain) member 1	−1.34	
Plod1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	−1.14	
Pnlip	pancreatic lipase	−1.52	
Pppde2	PPPDE peptidase domain containing 2	−1.18	
Prickle1	prickle homolog 1 (Drosophila)	−1.16	
Prkcd	protein kinase C, delta	1.19	Alc13
Prkd2	protein kinase D2	−1.26	
Psmb9	proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	1.30	
Psme1	proteasome (prosome, macropain) activator subunit 1	1.18	
Ptpla	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a	−1.29	
Ptpla	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a	−1.22	
Ptprc	protein tyrosine phosphatase, receptor type, C	1.29	
Pycard	PYD and CARD domain containing	1.23	
Rab27a	RAB27A, member RAS oncogene family	1.20	

Symbol	Gene Description	F- C	QTL
Rac2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	1.21	
Ralgds	ral guanine nucleotide dissociation stimulator	−1.22	Alc8
Rem2	RAS (RAD and GEM) like GTP binding 2	−1.22	
RGD1566254	RGD1566254	1.29	
Rhobtb3	Rho-related BTB domain containing 3	−1.22	
Ril	reversion induced LIM gene	1.15	
Rnasel	Ribonuclease L (2′,5′-oligoisoadenylate synthetase-dependent)	1.17	
Rnaset2	ribonuclease T2	1.21	
Rpe	ribulose-5-phosphate-3-epimerase	−1.12	
Rras2	related RAS viral (r-ras) oncogene homolog 2	−1.21	
Rras2	related RAS viral (r-ras) oncogene homolog 2	−1.25	
Sc4mol	sterol-C4-methyl oxidase-like	−1.32	Alc11
Sc5dl	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like	−1.19	
Sc5dl	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like	−1.17	
Scarb2	scavenger receptor class B, member 2	−1.15	
Scd1	stearoyl-Coenzyme A desaturase 1	−1.48	
Scrg1	stimulator of chondrogenesis 1	−1.26	Alc11
Sephs2	selenophosphate synthetase 2	−1.15	
Serpinb9	serine (or cysteine) peptidase inhibitor, clade B, member 9	1.22	
Serpinb9	Serine (or cysteine) peptidase inhibitor, clade B, member 9	1.17	
Serpinh1	serine (or cysteine) peptidase inhibitor, clade H, member 1	−1.28	
Sfrs7	splicing factor, arginine/serine-rich 7	1.12	Alc17
Sgk1	serum/glucocorticoid regulated kinase 1	−1.60	
Sh3tc2	SH3 domain and tetratricopeptide repeats 2	1.19	
Slain2	SLAIN motif family, member 2	−1.18	
Slc15a3	solute carrier family 15, member 3	1.38	
Slc22a23	solute carrier family 22, member 23	−1.12	
Slc22a4	solute carrier family 22 (organic cation transporter), member 4	1.59	
Slc25a1	solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1	−1.21	
Slc29a2	solute carrier family 29 (nucleoside transporters), member 2	−1.12	
Slc38a2	solute carrier family 38, member 2	−1.16	
Slc6a8	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	−1.17	
Slc9a3	solute carrier family 9 (sodium/hydrogen exchanger), member 3	1.31	
Slc9a3r2	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2	−1.21	Alc5
Smagp	small trans-membrane and glycosylated protein	1.31	
Smarcd2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	−1.22	
Smc4	structural maintenance of chromosomes 4	−1.18	
Spint2	serine peptidase inhibitor, Kunitz type, 2	1.29	
Sqle	squalene epoxidase	−1.35	
Srebf1	sterol regulatory element binding transcription factor 1	1.30	
Svep1	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	−1.29	

Symbol	Gene Description	F- C	QTL
Tc2n	tandem C2 domains, nuclear	1.25	
Tekt4	tektin 4	−1.32	Alc5
Timm8a1	translocase of inner mitochondrial membrane 8 homolog a1 (yeast)	−1.31	
Timm8a1	translocase of inner mitochondrial membrane 8 homolog a1 (yeast)	−1.26	
Timp2	TIMP metallopeptidase inhibitor 2	1.23	
Tinagl1	tubulointerstitial nephritis antigen-like 1	−1.30	
Tjap1	tight junction associated protein 1	−1.19	
Tm7sf2	transmembrane 7 superfamily member 2	−1.47	
Tmem176a	transmembrane protein 176A	1.24	Alc18
Tmem176b	transmembrane protein 176B	1.27	Alc18
Tmem81	transmembrane protein 81	1.14	
Tnfaip6	tumor necrosis factor alpha induced protein 6	−1.35	
Trem2	triggering receptor expressed on myeloid cells 2	1.43	
Tsc22d1	TSC22 domain family, member 1	−1.10	
Tsc22d3	TSC22 domain family, member 3	−1.27	
Ttk	Ttk protein kinase	1.21	
Tyrobp	Tyro protein tyrosine kinase binding protein	1.34	
Ube2d2	ubiquitin-conjugating enzyme E2D 2	−1.14	
Ube2g1	ubiquitin-conjugating enzyme E2G 1	−1.12	
Ugt8	UDP glycosyltransferase 8	−1.64	Alc15
Usp2	ubiquitin specific peptidase 2	−1.41	
Vav1	vav 1 guanine nucleotide exchange factor	1.14	
Vcl	vinculin	1.20	
Wfdc1	WAP four-disulfide core domain 1	−1.22	
Xdh	xanthine dehydrogenase	−1.24	Alc17
Xylt2	xylosyltransferase II	1.14	
Zfp189	zinc finger protein 189	−1.20	
Zfp90	zinc finger protein 90	1.12	

F-C = fold change ethanol/water; positive sign – ethanol > water; negative sign – ethanol < water QTLs are for rat.

Table 2

List of significant GO biological processes categories containing 10 or more genes in the VTA of the ethanol vs. water group

Category_ID	Term	P-value	OddsRatio	ExpCount	Count	Size
GO:0008202	steroid metabolic process	0	7.09	1.9	11	73
GO:0045321	leukocyte activation	0	4.66	3.7	15	144
GO:0006066	alcohol metabolic process	0.00012	4.05	3.3	12	128
GO:0007155	cell adhesion	0.00087	2.67	6.6	16	258
GO:0032268	regulation of cellular protein metabolic process	0.01403	2.05	7.3	14	287
GO:0003008	system process	0.01659	2.32	4.6	10	180
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	0.0182	2.1	6.1	12	233
GO:0001568	blood vessel development	0.02795	1.97	6.4	12	249

Table 3

List of significant KEGG categories with 6 or more genes in the VTA of the ethanol vs. water group

Category_ID	Term	Pvalue	OddsRatio	ExpCount	Count	Size
00100	Steroid biosynthesis	0	24.43	0.5	7	16
05140	Leishmaniasis	0.00214	5.15	1.4	6	42
04512	ECM-receptor interaction	0.00473	4.3	1.6	6	49
04510	Focal adhesion	0.00514	2.71	4.5	11	139
05146	Amoebiasis	0.00523	3.67	2.2	7	66
04810	Regulation of actin cytoskeleton	0.00603	2.64	4.6	11	142
04142	Lysosome	0.01022	2.92	3	8	93
04380	Osteoclast differentiation	0.01122	3.13	2.5	7	76
04670	Leukocyte transendothelial migration	0.01561	2.92	2.6	7	81
04650	Natural killer cell mediated cytotoxicity	0.01589	3.23	2.1	6	63
04145	Phagosome	0.03419	2.44	3.1	7	95

Table 4

Summary of Ingenuity Pathway Analysis on effects of excessive binge-like drinking in the VTA of P rats with genes clustering around transcription factors involved with steroid metabolism, excitation-coupled transcription and excitotoxicity

Transcription factor	Gene Cluster
Sterol regulatory element binding transcription factor 1 (*Srebf1*)	**UP**: *Abca1, Decr1*
	DOWN: *Acss2, Cyp51a1, Dhcr7, Fads1, Hmgcr, Hmgcs1, Idi1, Msmo1, Nsdhl, Sc5dl, Scd, Sqle, Tm7sf2*
FBJ osteosarcoma oncogene (*Fos*)	**UP**: *Git2, Golm1, Rac2, Serpinb9, Vav1*
	DOWN: *Apln, Cry1, Ctsl, Fstl1, Gamt, Gjb6, Mfil3, Nsdhl, Scd, Smarcd2, Xdh*
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (*Nfkb1a*)	**UP**: *B2m, C3, Cxcr4, Fcer1g, Fcgr2a, Irf1, Itgb2, Psmb9, Pycard, Tmem76b*
	DOWN: *Ccne2, Col3a1, Errf1, Tnfrsf11a*

UP: Indicates higher gene expression in the ethanol than water group; DOWN: Indicates lower gene expression in the ethanol than water group.

See Table 1 for descriptions of gene symbols.

Table 5

WGCNA list of significant GO biological categories containing 10 or more genes in the Yellow and Blue modules for the VTA of the ethanol vs. water group

Category_ID	Term	P-value	OddsRatio	ExpCount	Count	Size
	YELLOW MODULE (neuron enriched)					
GO:0007186	G-protein coupled receptor signaling pathway	0.0012	2.31	9.4	20	268
GO:0008284	positive regulation of cell proliferation	0.0065	1.86	13.2	23	375
GO:0019226	transmission of nerve impulse	0.0062	1.82	14.7	25	417
GO:0019725	cellular homeostasis	0.0087	1.73	16.7	27	474
GO:0042391	regulation of membrane potential	0.0084	2.19	6.8	14	194
GO:0050801	ion homeostasis	0.0027	1.93	14.5	26	413
GO:0071702	organic substance transport	0.0094	1.92	10.6	19	300
	BLUE MODULE (glia enriched)					
GO:0001508	regulation of action potential	0.0085	2.21	6.9	14	113
GO:0006629	lipid metabolic process	0.0000	3.07	8.7	23	147
GO:0006633	fatty acid biosynthetic process	0.0016	2.87	5.1	13	84
GO:0006694	steroid biosynthetic process	0.0000	4.76	4.8	18	78
GO:0006695	cholesterol biosynthetic process	0.0000	8.59	2.1	12	34
GO:0007272	ensheathment of neurons	0.0005	3.35	4.5	13	74
GO:0008285	negative regulation of cell proliferation	0.0034	1.83	17.1	29	280
GO:0008654	phospholipid biosynthetic process	0.0075	2.44	5.4	12	89
GO:0016125	sterol metabolic process	0.0000	4.01	4.5	15	74
GO:0030099	myeloid cell differentiation	0.0004	2.57	8.7	20	143
GO:0042552	myelination	0.0012	3.13	4.4	12	72
GO:0044255	cellular lipid metabolic process	0.0033	1.65	27.4	42	449
GO:0044283	small molecule biosynthetic process	0.0041	1.92	13.5	24	221
GO:0048634	regulation of muscle organ development	0.0045	2.95	3.8	10	63
GO:0048741	skeletal muscle fiber development	0.0018	3.4	3.4	10	56
GO:0055002	striated muscle cell development	0.0031	2.76	4.9	12	80
GO:0061061	muscle structure development	0.0015	1.95	16.2	29	265
GO:0070507	regulation microtubule cytoskeleton organization	0.0012	3.64	3.2	10	53

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Table 6

List of genes that were altered in the VTA by alcohol drinking and were also significantly different between naïve alcohol-preferring (P) and –non-preferring (NP) rats

Symbol	Gene Description	Lower/up[a]	Higher/down	Higher/up	Lower/down
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1			X	
Aif1	allograft inflammatory factor 1	X			
B2m	beta-2 microglobulin	X			
C1qa	complement component 1, q subcomponent, A chain	X			
C3	complement component 3	X			
Cd53	Cd53 molecule	X			
Celsr2	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	X			
Chmp1b	chromatin modifying protein 1B		X		
Cldn11	claudin 11				X
Coq6	Coenzyme Q6 homolog (yeast)				X
Cry1	cryptochrome 1 (photolyase-like)		X		
Ctsl1	cathepsin L1				X
Ctss	cathepsin S	X			
Dctd	dCMP deaminase			X	
Dcxr	dicarbonyl L-xylulose reductase	X			
Eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1	X			
Emr1	EGF-like module containing, mucin-like, hormone receptor-like 1	X			
Errfi1	ERBB receptor feedback inhibitor 1		X		
Fcer1g	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	X			
Fcgr3a	Fc fragment of IgG, low affinity IIIa, receptor	X			
Gamt	guanidinoacetate N-methyltransferase				X
Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble)		X		
Gpr84	G protein-coupled receptor 84	X			
Grxcr1	glutaredoxin, cysteine rich 1	X			
Hhip	Hedgehog-interacting protein				X
Hist1h2bh	histone cluster 1, H2bh				X
Hist2h2aa3	histone cluster 2, H2aa3				X
Idi1	isopentenyl-diphosphate delta isomerase 1				X

Symbol	Gene Description	Lower/up[a]	Higher/down	Higher/up	Lower/down
Irf8	Interferon regulatory factor 8	X			
Itgb2	integrin, beta 2	X			
Klf9	Kruppel-like factor 9		X		
Laptm5	lysosomal protein transmembrane 5	X			
Mal	mal, T-cell differentiation protein				X
Mtmr2	myotubularin related protein 2		X		
Mysm1	myb-like, SWIRM and MPN domains 1				X
Npc2	Niemann-Pick disease, type C2	X			
Pald	paladin	X			
Pcdh20	protocadherin 20		X		
Pex11a	peroxisomal biogenesis factor 11 alpha		X		
Plekhf1	pleckstrin homology domain containing, family F (with FYVE domain) member 1		X		
Psme1	proteasome (prosome, macropain) activator subunit 1	X			
Ptpla	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a				X
Pycard	PYD and CARD domain containing	X			
Qdpr	quinoid dihydropteridine reductase			X	
Ralgds	ral guanine nucleotide dissociation stimulator				X
Rcn3	reticulocalbin 3, EF-hand calcium binding domain	X			
Sc4mol	sterol-C4-methyl oxidase-like				X
Sc65	synaptonemal complex protein SC65	X			
Scarb2	scavenger receptor class B, member 2				X
Scrg1	stimulator of chondrogenesis 1				X
Serpinb9	Serine (or cysteine) peptidase inhibitor, clade B, member 9	X			
Sgk1	serum/glucocorticoid regulated kinase 1		X		
Slc22a4	solute carrier family 22 (organic cation transporter), member 4	X			
Slc29a2	solute carrier family 29 (nucleoside transporters), member 2		X		
Slc38a2	solute carrier family 38, member 2		X		
Tjap1	tight junction associated protein 1				X
Tmem176a	transmembrane protein 176A	X			
Tmem176b	transmembrane protein 176B	X			
Tsc22d3	TSC22 domain family, member 3		X		

Symbol	Gene Description	Lower/up[a]	Higher/down	Higher/up	Lower/down
Ube2g1	ubiquitin-conjugating enzyme E2G 1		X		
Unc93b1	unc-93 homolog B1 (C. elegans)	X			
Zfp189	zinc finger protein 189		X		

[a]Lower/up = lower expression levels in P vs. NP and up-regulation of expression in the ethanol vs. water group; Higher/down = higher expression levels in P vs. NP and down-regulation of expression in the ethanol vs. water group; Higher/up = higher expression levels in P vs. NP and up-regulation of expression in the ethanol vs. water group; Lower/down = lower expression levels in P vs. NP and down-regulation of expression in the ethanol vs. water group

Table 7

List of GO biological process categories for common genes that were significantly different between the P vs. NP line and also changed significantly with alcohol drinking

GO Biological Processes	Genes
Immune effector process	**Up**: *C3, Serpinb9, Anax3a, B2m, C1qa, Fcer1g*
Inflammatory response	**Up**: *C3, Serpinb9, Fcer1g, Itgb2, Pycard*
Induction programmed cell death	**Up**: *Serpinb9, B2m, Mal, Pycard*
	Down: *Pleckhf1*
Cellular response to hormone stimulus	**Up**: *Serpinb9, Aif1, Ctss*
	Down: *Klf9, Sgk1, Slc29a2, Ctsl1*
Leukocyte activation	**Up**: *Cxcr4, Aif1, Anxa3, B2m, Fcer1g, Fcgr3a, Itgb2*
Response to steroid hormone stimulus	**Up**: *C3, Serpinb9, Aif1, Anxa3*
	Down: *Sgk1, Ctsl1*
Response to glucocorticoid stimulus	**Up**: *C3, Aif1, Anxa3*
	Down: *Sgk1, Ctsl1*
Response to other organisms	**Up**: *Abca1, Serpinb9, Anxa3, B2m, Fcer1g, Fcgr3a, Npc2*
Organic substance transport	**Up**: *Abca1, Slc38a2, Fcer1g, Npc2, Slc22a4*

Up = genes with higher expression levels in ethanol compared to water group. Down = genes with lower expression levels in ethanol compared to water group

Table 8

Overall summary of bioinformatics analyses of changes in gene expression in the VTA resulting from loss-of-control drinking by P rats

Biological Categories	Genes	Function
Transcription factors	Multiple genes centered around *Nfkbia, Fos, Srebf1*	Reduced excitation-coupled transcription; promotion of excitotoxic neuronal damage
Steroid & protein metabolic processes	**Up** (protein): *Rcn3, Anxa3, Pycard, Serpinb9, Dcxr, Psme1, Laptm5, Eif4ebp1, Unc93b1, Celsr2*	Reduced cholesterol & fatty acid synthesis; increased protein degradation
	Down (steroid): *Acss2, Sc5dl, Sqle, Cyp51a1, Hmgcr, Dhcr7, Msmo1, Scd, Tm7sf2, Hmgcs1, Idl1*	
Cell adhesion; regulation of actin cytoskeleton	**Up**: *Emr1, C3, C1qa, Leprel4, Cd53, Serpinb9, Cxcr4, Laptm5, Ctss, Tmem176b*	Altered cell-to-cell interactions & dendrite development
	Down: *Sgk1, Gpd, Pcdh20, Gpatch4, Klf9, Plekhf1, Errfi1, Ube2g1, Slc38a2, Tsc22d3*	
Inflammatory response & immune process	**Up**: *Emr1, Ctss, Npc2, Eif4ebp1, Psme1, Pycard, Cd53, C3, Serpinb9, Anax3a, B2m, C1qa, Fcer1g, Itgb2*	Pro-inflammatory
Response to glucocorticoid or steroid hormone stimulus	**Up**: *C3, Serpinb9, Aif1, Anxa3*	Altered response to stimulus
	Down: *Sgk1, Ctsl1*	

Up = genes with higher expression levels in ethanol compared to water group. Down = genes with lower expression levels in ethanol compared to water group