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Gene expression changes in glutamate and GABA-A receptors, neuropeptides, ion channels and cholesterol synthesis in the periaqueductal gray following binge-like alcohol drinking by adolescent alcohol-preferring (P) rats

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Abstract

Background—Binge-drinking of alcohol during adolescence is a serious public health concern with long-term consequences, including increased pain, fear and anxiety. The periaqueductal gray (PAG) is involved in processing pain, fear and anxiety. The effects of adolescent binge drinking on gene expression in this region have yet to be studied.

Methods—Male adolescent P (alcohol preferring) rats were exposed to repeated binge-drinking (three 1-h sessions/day during the dark-cycle, 5 days/week for 3 weeks starting at 28 days of age; ethanol intakes of 2.5 – 3 g/kg/session). We used RNA sequencing to assess the effects of ethanol intake on gene expression.

Results—Ethanol significantly altered expression of 1670 of the 12,123 detected genes: 877 (53%) decreased. In the glutamate system, 23 genes were altered, including reduction in 7 of 10 genes for metabotropic and NMDA receptors. Subunit changes in the NMDA receptor may make it less sensitive to ethanol. Changes in $GABA_A$ genes would most likely increase the ability of the PAG to produce tonic inhibition. Five serotonin receptor genes, 6 acetylcholine receptor genes and 4 glycine receptor genes showed decreased expression in the alcohol drinking rats. Opioid genes (e.g., *Oprk1*, *Oprm1*) and genes for neuropeptides linked to anxiety and panic behaviors (e.g., *Npy1r*) had mostly decreased expression. Genes for 27 potassium, 10 sodium and 5 calcium ion channels were differentially expressed. Nine genes in the cholesterol synthesis pathway had

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Conflict of Interests

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decreased expression, including *Hmgcr*, encoding the rate limiting enzyme. Genes involved in the production of myelin also had decreased expression.

Conclusion—The results demonstrate that binge-alcohol drinking during adolescence produces developmental changes in the expression of key genes within the PAG; many of these changes point to increased susceptibility to pain, fear and anxiety, which could contribute to excessive drinking to relieve these negative effects.

Keywords

alcoholism; adolescent binge drinking; brain; cholesterol; anxiety

INTRODUCTION

Alcohol (ethanol) consumption by adolescents and young adults continues to be a problem. A multi-national study estimated that between 20% and 40% of adolescents have engaged in binge drinking (Marshall, 2014), defined by NIAAA as “a pattern of drinking that brings blood alcohol concentration (BAC) levels to 0.08 g/dL.” Early age of first use and binge drinking both predict increased risk for alcohol use disorders in adulthood (reviewed in Spear, 2015). Extreme binge drinking is of particular concern, with 10.5% of 12th graders reporting consumption of 10 or more drinks on one occasion (Spear, 2015). Two-thirds of the alcohol consumed by college students is consumed by the 20% of them who are frequent binge drinkers (Spear, 2015). A review by Jacobus and Tapert (2013) reports differences in gray and white matter in adolescents exposed to alcohol; white matter volume decreases and integrity is poorer in cortical and subcortical projections. Neurocognitive performance is poorer, with impaired attention, executive functions and memory (Jacobus and Tapert, 2013).

Binge-like behavior has been observed in selectively bred alcohol preferring (P) rats, with adults and peri-adolescents of both sexes readily achieving blood ethanol levels ≥ 80 mg% (Bell et al., 2011, Bell, et al., 2014). Intermittent access to ethanol using multiple scheduled access protocols enhances this binge-like drinking (Bell, et al., 2014). Peri-adolescent selectively bred rats consume more alcohol than their adult counterparts (Bell et al., 2014). A study of adult and adolescent P rats using a multiple scheduled access protocol reported that adult male rats drank 1.5–2.5 g/kg per session while adolescent males consumed an average of 2.7 g/kg, achieving blood ethanol levels of 80 mg% and 100 mg% respectively (Bell et al., 2011). At comparable blood alcohol levels, both human and animal adolescents show less sedation and better motor coordination than adults, and the rewarding and reinforcing properties of alcohol are higher in adolescents (Bell et al., 2014, Spear 2015).

The effects of adolescent binge-like alcohol exposure on several brain regions have been studied. There was reduced basal α-MSH immune-reactivity in the central nucleus of the amygdala (CeA; Lerma-Cabrera et al., 2013). Binge-like ethanol administration to adolescent rats led to lower c-fos immune-reactivity in the nucleus accumbens (Alaux-Cantin et al., 2013). Binge-like alcohol drinking by adolescent P rats led to many changes within the extended amygdala (McBride et al., 2014) and in the dorsal raphe nucleus (DRN) (McClintick et al., 2015). In the DRN, the serotonin system was most significantly altered with decreased expression in receptors, transporters, and enzymes that synthesize serotonin

(McClintick et al., 2015). $GABA_A$ receptors were also decreased in the DRN, as were many genes in neuropeptide systems (McClintick et al., 2015). Both the extended amygdala and DRN showed changes in cAMP and protein kinase A signaling. Receptors for NPY were increased in the extended amygdala but decreased in the DRN (McBride et al., 2014, McClintick et al., 2015).

The PAG plays important roles in the processing of pain, fear and anxiety (reviewed in Behbehani, 1995). Anxiety and fear are commonly associated with alcohol withdrawal (Pandey et al., 2015, Koob, 2013). Animals exposed to ethanol during adolescence were, as adults, more anxious and drank more than the animals not previously exposed to ethanol (Pandey et al., 2015). Furthermore, the PAG receives significant serotonergic innervation from the DRN involved in fight-or-flight behavioral responses (Johnson et al., 2004). Li et al. (2013) reported that acute ethanol produced a robust enhancement of glutamatergic synaptic transmission in the PAG. $GABA_A$ and μ opioid receptors (Silva and Nobre, 2014) and glutamate receptors (Ezequiel Leite and Nobre, 2012; Long et al., 2007) within the PAG are affected in ethanol withdrawal. Microinjection of NMDA or AMPA antagonists into the PAG reduced ethanol intake during withdrawal (Ezequiel Leite and Nobre, 2012). In addition, there may be an association between chronic pain and alcohol dependence, suggesting overlapping neural mechanisms (Apkarian et al., 2013). But thus far, the global effects of ethanol on changes in gene expression within the PAG have not been studied.

Alcohol dependence is a relapsing disorder that has been conceptualized as three stages, *binge/intoxication, withdrawal/negative affect and preoccupation/anticipation* (Koob, 2013). Increasingly, the negative consequences of not drinking rather than the rewarding or positive aspects of drinking are responsible for relapses. The PAG is associated with anxiety, fear and pain (Bebehani, 1995). Therefore, we examined the PAG gene expression profile in binge-drinking male adolescent P rats to identify developmental changes in the PAG that could play a role in the transition from positive to negative reinforcement as the motivator for drinking.

MATERIALS AND METHODS

Ethanol exposure and RNA extraction

Adolescent male P (alcohol preferring) rats were allowed to binge drink as described previously (McBride et al., 2014). Gene expression changes have been reported in 3 regions of these same rats: accumbens shell, central core of the amygdala (McBride et al., 2014) and dorsal raphe (McClintick et al. 2015). Briefly, starting at 28 days of age, 11 male P rats were given *ad libitum* access to food and water, and access to ethanol (15 and 30% ethanol solutions concurrently) in 3 × 1 h sessions per day for 5 consecutive days/week, while 10 control animals were treated identically except without access to ethanol. This free-choice multiple-scheduled-access to ethanol procedure (Bell et al., 2014, Bell et al., 2011) resulted in average daily ethanol intakes of approximately 8 g/kg/day, with intakes of 2–3 g/kg for each of the 3 daily 1 h sessions (McBride et al., 2014). These levels of intake lead to BAC of 100 mg% (Bell et al., 2011), and therefore meet the criterion for binge-drinking put forth by the National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2004). The rats were sacrificed at 49 days of age, 3 h after the 1st access session on their 15th day of drinking. All

research protocols were approved by the Indiana University School of Medicine 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).

Brains were rapidly extracted and flash-frozen in isopentane in dry ice and stored at −80 C until sectioning. Brains were sectioned (300 μm) and the PAG was micropunched from 6.04 mm to 7.30 mm post bregma, using procedures previously described (McBride et al., 2014). Other brain regions of these animals have been studied (McBride et al., 2014, McClintick et al., 2015). The yield, concentration and purity of the RNA were measured by Nanodrop (Thermo Fisher Scientific, Waltham, MA) spectrum from 220 nm to 340 nm. Quality was further assessed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, Ca); RNA integrity numbers (RIN) averaged 8.4 for the samples.

RNA sequencing and analysis

RNA sequencing and analysis were carried out as previously reported (McClintick et al., 2015). We first used SOLiD™ Instrument Control Software and SOLiD™ Experiment Tracking System Software for the read quality recalibration. Sequences containing more than two 'N' were discarded. If a 5 base sliding window had an average quality score less than 20, the read was truncated at the beginning of that 5-base window. Reads with fewer than 35 bases were discarded. Reads that passed these filters were mapped to the rat genome (rn4) using the BFAST algorithm (Homer et al., 2009). We used a Tophat-like strategy (Trapnell et al., 2009) to align the sequencing reads on both exonic regions and across junctions. The expression levels of each isoform were counted using NGSUtils (Breese and Liu, 2013), normalized to the total number of sequencing reads falling into annotated gene regions in each sample, and further scaled based on a trimmed mean of log transformed counts per million (CPM) value to correct for the variability of RNA composition in each sample (Robinson and Oshlack, 2010). The scaled CPM was used as gene level quantification in each sample. We used the edgeR package to identify the genes that are differentially expressed between alcohol drinking and water groups (Robinson et al., 2010). FDR was calculated according to Benjamini and Hochberg (1995). RPKM (Reads per Kilobase per Million Reads), which adjusts expression relative to transcript length, is reported in the tables and supplemental tables.

Qiagen Ingenuity Pathway Analysis (IPA) was performed on the genes significant at FDR ≤ 0.05. Pathways that contained fewer than 5 differentially expressed genes were dropped. Pathways with the same list of genes were collapsed (e.g. multiple cholesterol synthesis pathways).

RESULTS

Differential gene expression

We used RNA sequencing to examine changes in gene expression in the periaqueductal gray (PAG) of adolescent male P rats that had consumed high levels of ethanol in a repeated binge-drinking pattern over a 3-week period (post-natal days 28 to day 49). The average consumption was approximately 8 g/kg per day for the 5 drinking days of each week, with average intakes of 2–3 g/kg in each 1 h session (McBride et al., 2014). Although blood alcohol levels were not measured in these animals, similarly treated adolescent animals reached blood alcohol levels of 100 mg% at the end of a 1 h session (Bell et al., 2011). There were 12,123 genes detectably expressed, of which 1,670 genes (14%) were differentially expressed between ethanol exposed and control animals; 877 (53%) were decreased (at FDR ≤ 0.05; Supplementary Table 1). Among the differentially expressed genes, 815 (49%) had absolute fold changes >1.5; 51% of genes with > 1.5 fold changes were decreased.

Genes encoding receptor subunits and transporters for several neurotransmitters were differentially expressed (Table 1). The glutamate system had the largest number of genes with altered expression, with nearly equal numbers increased and decreased. Three of the four NMDA receptor subunits and four of the 6 metabotropic receptors had decreased expression with alcohol drinking. Expression of the vesicular transporter gene *Slc17a6* (encoding VGLUT2) was decreased, whereas *Slc17a7* (encoding VGLUT1) was increased. Two glutamate reuptake transporters, *Slc1a3* (astrocytes) and *Slc1a6* (neuronal), had increased expression in the alcohol group, while another astrocyte-associated transporter, *Slc1a2*, had decreased expression.

Most $GABA_A$ receptor subunit genes (6 of 8) showed decreased expression in the alcohol group, but *Gabra6*, which is expressed more highly than other $GABA_A$ subunits in this tissue, showed increased expression. The 5 serotonin-related genes that were differentially expressed all had reduced expression. Similarly, all 6 differentially expressed acetylcholine receptor-related genes and all 4 of the glycine receptor-related genes had reduced expression in the drinking animals. Expression of dopamine receptors *Drd2* and *Drd5* was decreased in the alcohol drinking group. One highly expressed adenosine receptor (*Adora1*) was increased 1.4-fold. Four purinergic receptors also had altered gene expression.

Expression of genes for some neuropeptides and their receptors was altered (Table 2); most (17/21) were expressed at lower levels after repeated binge drinking. The five genes in the opioid system, the 2 NPY receptors and the 2 galanin receptors that were altered by drinking all had decreased expression. The hypocretin (orexin) neuropeptide precursor (*Hcrt*) had increased expression but its receptor *Hcrtr2* had reduced expression. The tachykinin precursor gene (*Tac1*) and the *Tacr1* receptor had decreased expression, while *Tacr3* had increased expression. Two of the 3 somatostatin receptor genes had decreased expression, whereas *Sstr3* had increased expression.

Many ion channel genes were differentially expressed (Table 3). Among the K+-channels that were differentially expressed, 11 of 15 K+ voltage-gated channels had reduced

expression in the drinking group. In contrast, 3 of 4 K+-inward rectifying channels had increased expression. Among the differentially expressed Na+-channels, 6 of 8 voltage-gated channels had reduced gene expression. The two voltage-dependent calcium channels expressed at highest levels (*Cacna1a*, *Cacna1g*) were both increased, while 3 expressed at lower levels were decreased. Both intracellular chloride channels that were differentially expressed were increased.

Nine genes in the cholesterol synthesis pathway (Table 4) had decreased expression in the binge drinking animals including the rate limiting enzyme for cholesterol production, *Hmgcr* (3-hydroxy-3-methylglutaryl-CoA synthase 1; down 1.5 fold). Many genes involved in myelin production were decreased by 1.3 to 1.8 fold (Table 4).

Pathway Analysis

Qiagen Ingenuity pathway analysis (IPA) was performed using the 1,670 differentially expressed genes. IPA Pathway analysis showed 143 pathways with FDR ≤ 0.05 (Supplemental Table 2). Many of these pathways were not independent: 23 genes (mostly kinases and other signaling molecules) were found in between 25 and 87 of the pathways, and 119 of the pathways contained at least 5 of these genes. There is a cluster of PI3 kinases (*Pik3r2, Pik3r3* and *Pik3c2g*) that are found in 86–87 of the pathways. Three protein kinase C genes (*Prkcd, Prkcg* and *Prkar2b*) are found in 42–58 pathways, some overlapping with the PI3 kinase-containing pathways. Other groups of genes with disproportionate impact are adenylate cyclases (*Adcy1, Adcy10, Adcy7*) and G protein subunits that can be coupled to many different G-protein coupled receptors (*Gng7, Gnb3, Gng13, Gnas*).

Upstream regulator analysis identified 201 putative upstream regulators that could contribute to the observed changes in gene expression (Supplemental Table 3). Glucagon, glutamate, somatostatin and norepinephrine are identified as active, as were ILR1, IL17A, IL2 and LPS. Also noted as possible activators are CREBBP/CREM and protein kinases A and C. PPAR α and γ, HDAC4, mifepristone, cannabinol, morphine, taurine, testosterone, RXR, PXR, Insulin receptor, and steroid regulatory binding proteins Srebf1 and Srebf2 all appear to show reduced activity. Many circulating molecules and drugs are predicted to be active (e.g. have effects similar to those found after ethanol) or to be inactive (and potentially oppose those effects) (Table 5).

DISCUSSION

The PAG plays a role in processing fear and anxiety, which are characteristic of alcohol withdrawal (Bebehani et al., 1995). We examined the effect of repeated binge drinking of ethanol during adolescence on gene expression profiles in the periaqueductal gray (PAG). Adolescent P rats consumed quantities of ethanol that are known to result in BACs of 100 mg% (McBride et al., 2014). Of the detectably expressed genes, 14% were differentially expressed 3 hours after the last drinking episode. These results demonstrate that repeated binge drinking has a significant effect on the function of the PAG.

Gene expression in other brain regions of these same animals has previously been studied (McBride et al, 2014; McClintick et al., 2015). In the dorsal raphe nucleus (DRN), 12,047

genes were detected, of which 3567 (30%) were differentially expressed; 1648 (46%) of these had decreased expression (McClintick et al., 2015). The PAG and DRN had 1280 differentially expressed genes in common with same direction of change (Supplemental Table 1). Although technical differences greatly affect the comparisons, microarray studies showed 182 named genes differentially expressed in central amygdala and 154 in the accumbens shell of these same animals (McBride et al., 2014). Only 4 genes were differentially expressed in the same direction (all increased) in all 4 of these regions: *Cyr61* (cysteine-Rich, angiogenic inducer, 61), *Dusp1* (dual specificity phosphatase 1), *Ier2* (immediate early response 2) and *Kif15* (kinesin family member 15).

Neurotransmitters

The glutamate signaling system was broadly affected, with trends similar to those seen in the DRN (McClintick et al., 2015). The overall effect suggests a change in the composition of the glutamate receptors rather than a major reduction in receptor numbers. There are offsetting changes in expression for type I metabotropic receptors (*Grm1* and *Grm5*). Grm1 and Grm5 have downstream effects on phosphoinositide 3-kinase (PI3K). In the PAG, three PI3K associated genes were increased by binge drinking (Pik3c2g, Pik3r2, Pik3r3), along with Akt2. These genes are associated with numerous pathways that were identified by the IPA analysis. Cozzoli et al. (2016) found that male adolescent mice exposed to binge drinking had decreased PI3K activity in the nucleus accumbens; the difference in direction could be related to either brain region or species differences. Among the type III metabotropic receptors (*Grm4*, *Grm7* and *Grm8*), which are generally pre-synaptic, the increase in the highly expressed *Grm4* could offset the decreased expression of *Grm7* and *Grm8* in terms of total receptor number.

For NMDA receptors, again the changes are offsetting: *Grin2c* is the most highly expressed NMDAR2 receptor and was the only NMDA subunit with increased expression; *Grin2b*, *Grin2d* and *Grin3a* had decreased expression. Alcohol acts as an allosteric modulator of NMDA receptors, and can decrease glutamate signaling by decreasing the mean open time of the channel. *Grin1*, *Grin2a* and *Grin2b* all contain ethanol-sensitive domains (Zhao et al., 2015), but of these only *Grin2b* expression was decreased. Replacing Grin2b subunits by Grin2c subunits could result in NMDA receptors that are less sensitive to ethanol. *Gria4*, the most highly expressed AMPA receptor, was increased 1.4 fold and *Grid2* was increased 1.7-fold in the PAG; these were the only 2 glutamate receptors with no offsetting changes in other subunits of the same class. AMPA receptors are also inhibited by ethanol (Wirkner et al., 2000), so the increase in *Gria4* may be compensatory.

Overall GABA transmission may be reduced because the upstream regulator analysis (Table 5) identified bicuculine, a $GABA_A$ antagonist, as being capable of producing some of the changes seen with ethanol. Changes in $GABA_A$ subunit expression may therefore be compensatory for this loss. One of the main aspects of $GABA_A$ receptors in the PAG is tonic inhibition (Behbehani, 1995). $GABA_A$ receptors composed of *Gabrd* along with the α4 or α6 subunit are primarily found extrasynaptically (Lovinger and Roberto, 2013); these receptors generate tonic inhibitory conductance (Hanchar et al., 2005). These extra synaptic receptors are sensitive to ethanol, which potentiates this tonic current in a protein kinase C

delta dependent manner (Lovinger and Roberto, 2013). Given this potentiation, it is notable that *Gabrd* and *Gabra6*, both highly expressed, were both increased along with *Prkcd*. The most highly expressed GABA transporter (*Slc6a11*, GAT2) was decreased, which could leave more GABA in extracellular spaces, where it could activate phasic or tonic GABA conductance. Three of the genes downstream of bicuculine (*Ier2*, *Cyr61* and *Dusp1*) were increased in the other 3 brain regions studied (McBride et al., 2014, McClintick et al., 2015).

Glycine receptors are ligand gated ion channels, which, when activated, reduce firing. Ethanol is a positive allosteric modulator of the glycine receptor (Farley and Mihic, 2015). Four subunits, α1, α2, α3 and β, had decreased expression, which may compensate for the positive modulation by ethanol. *Glrb* (encoding the gly-B receptor) is the most abundantly expressed glycine receptor in the PAG, and has been implicated in hypo-nociception (Martins et al., 2008), thus the reduction in this receptor could increase nociception. Expression of the glycine reuptake transporter gene, *Slc6a5*, was also decreased, which may moderate the effect.

Receptor subunits for 3 other neurotransmitter systems, dopamine, acetylcholine and serotonin, all had reduced gene expression. Acetylcholine injected into the ventral lateral PAG causes hypotension (Delindo, et al., 2010), indicating that acetylcholine signaling can limit physiological changes associated with anxiety and stress. Genes for serotonin signaling were also largely decreased in the DRN of adolescent alcohol drinking P rats (McClintick et al., 2015). Release of serotonin in the dorsal lateral PAG inhibits stress induced sympathetic activity via the *Htr1a* receptor (Johnson et al., 2004), which was decreased −1.5 fold in these binge drinking animals. *Adra1a*, an alpha-adrenergic receptor subunit gene, also had decreased expression. Since norepinephrine injected into the PAG has an anxiolytic effect (Pelosi, et al., 2009) the decrease in *Adra1a* could make these animals more prone to anxiety in stressful situations.

The highly expressed adenosine receptor *Adora1* had 1.4-fold increased expression. Ethanol has been shown to increase the expression of the α1 adenosine receptor, especially after multiple withdrawal periods (Butler and Prendergast, 2012). Ethanol also increases extracellular adenosine (Butler and Prendergast, 2012). *Adora1* is linked to $G_{i/o}$ proteins, which inhibit adenylate cyclase, and thus decrease cAMP production. Adenosine may be responsible for some of the sedating/sleep inducing effects of ethanol (Butler and Prendergast, 2012). P2RX type purinergic receptors had mixed changes but the overall effect was increased expression for these receptors.

Nitric oxide (NO) in the PAG is involved in anxiety like behaviors. NO scavengers and antagonists of neuronal nitric oxide synthase (*Nos1*) injected into the PAG have been shown to reduce anxiogenic effects of alcohol withdrawal in rats (Bonassoli et al., 2012). *Nos1* increased 1.75 fold in the PAG. Nitric oxide works by increasing the production of the second messenger cGMP (Meyer and Queszner, 2013). Phosphodiesterase 5A (*Pde5a*), which breaks down cGMP, was up 1.75 fold. The drug sildenafil, a Pde5a inhibitor (Table 5), could reverse some of the effects.

Neuropeptides

OPRK1, PDYN and *OPRM1* have been associated with alcohol use disorders (Edenberg et al., 2008, Schwantes-An et al., 2015, Xuei et al., 2006). Opioid receptors Oprk1 (κ), Oprm1 (μ) and the nociception receptor Oprl1 as well as the prodynorphin gene all had decreased expression (Table 2). These results suggest an increase in pain processing and a reduction in the effectiveness of opioid agonists such as morphine to treat pain. Morphine which can also have anxiolytic effects, has no effect on alcohol withdrawn animals (Silva and Nobre, 2014).

Stimulation of the dorsal PAG evokes defensive behavior and physiological responses (tachycardia and increased blood pressure) (Paul et al., 2014). Cholecystokinin peptide CCK-4 can enhance this response (Paul et al., 2014). Both the cholecystokinin precursor (*Cck*) and the Cck B receptor (*Cckbr*) had decreased expression, suggesting reduced autonomic response to threat. Decreased expression of two NPY receptors, *Npy5r* and *Npy1r* could increase anxiety-like behavior.

The tachykinin 3 receptor has been reported to be associated with alcohol and cocaine dependence (Foroud et al., 2008). *Tacr3* expression was increased in the binge drinking animals. The tachykinin receptor *Tacr1* had decreased expression, as did *Tac1,* the precursor for its ligand, Substance P. Substance P is associated with anxiety, stress and addiction (Schank et al., 2014). Antagonists of *Tacr1* have been shown to decrease escalated drinking in P rats but not in Wistar rats (Schank et al., 2013). *Tacr1* is more highly expressed in the accumbens shell (McBride et. al, 2013b) and CeA (Schank et al., 2014) of naïve P rats compared to NP or Wistar rats. The decrease in both *Tac1* and *Tacr1* could result in decreased anxiety and response to stress in the drinking animals.

Three of the genes for the somatostatin family of receptors were altered: *Sstr1* (−1.7), *Sstr3* (+1.5) and *Sstr4* (−1.4). Somatostatin is decreased in several neuropsychiatric disorders, including major depressive disorder, schizophrenia and bipolar disorder (Lin and Sibille, 2013). Activation of somatostatin receptors generally opposes stress related behaviors including anxiety and autonomic effects (Stengel et al., 2013). Activation of Sstr1 mediates the blocking of stress induced colonic stimulation (Lin and Sibille, 2013). The reduction in gene expression for 2 of the 3 Sst receptors could also contribute to potentially higher levels of response to stress in adolescent binge drinking rats. The higher expression levels of *Crhr1* in the PAG of the binge drinking rats may also predict a higher level of stress reactivity.

Overall, the results with the neuropeptides suggest that repeated binge-like alcohol drinking in these adolescent rats resulted in alterations in the expression of genes within the PAG that could increase anxiety and reactivity to stress.

Ion Channels

Voltage gated sodium channels are made up of α and β subunits. The β subunits are multifunctional and can modulate gating, voltage dependence and the kinetics of the α subunits, which form the pore. There is an overall decrease in α subunit expression and an increase in β subunit expression; the reduction in α subunit expression suggests reduced neuronal excitability.

Twenty-seven potassium channels had altered expression. Three of four inwardly-rectifying channels had increased expression. Eleven of 15 voltage-gated channels had decreased expression, which may indicate altered thresholds for neuronal excitability, although the most highly expressed channel, *Kcnd2,* was increased. Although 3 of 5 voltage gated calcium channel genes had decreased expression, two channels that were more highly expressed had increased expression, so the total effect may have been a net increase in voltage gated calcium channel activity. This possible increase is mirrored in the upstream analysis (Supplemental Table 3), which suggests that calcium signaling could be more active in the alcohol group.

Decreased cholesterol and myelin synthesis

Cholesterol synthesis pathways in the PAG (and also in the DRN; McClintick et al., 2015) were altered by binge drinking (Table 4), with many genes having decreased expression. Hmgcr (3-hydroxy-3-methylglutaryl-CoA synthase 1, down 1.5-fold), catalyzes the rate limiting step for cholesterol production. Eight additional genes in the cholesterol pathway expression decreased by 1.3 to 1.5 fold. Alcohol consumption by P rats also decreased expression of cholesterol synthesis genes in the liver (Klein et al., 2014). Cholesterol is important for brain function, playing a major role in synaptogenesis, membranes, synaptic vesicles and myelin sheaths; 70–80% of the cholesterol in the brain is in the myelin sheaths. Since cholesterol cannot cross the blood-brain barrier, all cholesterol in the brain is synthesized there (Zhang and Liu, 2015). Cholesterol production peaks during development when myelination is heaviest. Cholesterol levels are tightly regulated, and levels are sensed by the sterol regulatory-element binding proteins (Srebp); the upstream regulator analysis indicates that Srebp and insulin signaling (which can also control expression of genes for cholesterol synthesis, uptake and transport) appear to be reduced. Decreases in brain cholesterol can have deleterious effects. Lovastatin applied to primary hippocampus neurons, leading to decreases in cholesterol, impaired synaptic vesicle release and decreased neurite growth (Mailman et al., 2011).

The binge drinking animals also had decreased expression of genes involved in myelin formation, which has been noted in other tissues (Lewohl et al., 2000, Mayfield et al., 2002, McClintick et al., 2013, Sokolov et al., 2003, and in pre-clinical models of adolescent binge drinking (Vargas et al., 2014). Decreased myelination and poorer myelin integrity have also been found in adolescents with extensive alcohol use (Jacobus and Tapert, 2013). Decreased myelination during adolescent development could have a major impact on brain function. It is unknown whether decreased cholesterol production is the cause of decreased myelination in the brains of alcoholics but *Hmgcs1* and *Hmgcr* in the biosynthetic pathway also had decreased expression in the hippocampus of alcoholics, who also had decreased expression of myelin forming genes (McClintick et al., 2013). Expression of both myelin-related genes and Hmgcr is decreased in post-mortem studies of frontal and motor cortex and temporal cortex (Mayfield et al., 2002; Sokolov, et al., 2003). *Hmgcr, Hmgcs1, Dhcr7* and *Sqle* all had decreased expression in the VTA of binge drinking adult female P rats (McBride et al., 2013a).

Potential alterations in cellular composition of the PAG

Cahoy et al. (2008) identified genes enriched in different cell types that could serve as characteristic markers of those cells. We examined the differentially expressed genes for evidence of patterns that might reflect a change in relative numbers of several cell types in the PAG of these animals. Genes that are characteristic of neurons and astrocytes (Cahoy et al., 2008) showed a similar ratio of over- and under-expression as the full set of genes, close to 50%, indicating that there was no substantial change in the contribution of those cells to the PAG. But of the genes characteristic of oligodendrocytes, 80% were decreased, suggesting a decrease in the number and/or function of oligodendrocytes as a result of the alcohol exposure.

Drugs that may reverse transcriptional effects of alcohol

The upstream analysis performed with IPA looks for endogenous genes and molecules that could be responsible for the alterations in gene expression, and also for drugs that could have similar or opposing effects. Negative z-scores in Supplementary Table 3 mean that the molecule causes expression of the subset of genes it regulates to go in the opposite direction to those caused by the ethanol exposure. The drugs that have negative z-scores (Table 5) might, therefore, reverse some of the effects of this repeated high level of alcohol exposure. Many of these are drugs already have FDA approval for another indication. One of them, mifepristone, is in early clinical trials (Vendruscolo et al., 2015); early indications are that it may decrease alcohol seeking in alcohol dependent individuals. PPARα agonists (fibrates and thiozolidinediones) have already been used in pre-clinical studies and shown to reduce ethanol intake in mice (Blednov et al., 2015). Isoquercitrin, which is found in medicinal herbs like St. John's Wort, appears to increase the expression of many genes in the cholesterol pathway (Soundararajan et al., 2008) that were decreased by ethanol exposure (Table 4). Taurine, which is found in many energy drinks, is a glycine receptor agonist. The FDA has warned that mixing of those energy drinks with alcohol should be avoided because it is associated with higher levels of alcohol consumption, especially in adolescents and young adults (Food and Drug Administration, 2010); this might be in part because it reduces the negative effects of alcohol. The upstream regulator analysis also indicates that norepinephrine is active; two drugs that block α1 adrenergic receptors, prazosin and doxazosin, are in randomized trials for alcohol dependence and alcohol dependence plus PTSD (Kenna et al., 2015; Simpson et al., 2015). The positive score for morphine (Table 5) indicates that an opioid antagaonist like Naltrexone may be effective in countering some of the effects of the excessive drinking.

Conclusions

We have shown that gene expression in the PAG is strongly affected by binge drinking in adolescent rats, including alterations in the composition of NMDA, AMPA and $GABA_A$ receptors. Many serotonin, glycine and acetylcholine receptors had decreased expression after adolescent binge drinking, as did many neuropeptides and their receptors including the opioid systems and receptors that are linked to anxiety and panic behavior. Taken together, these changes in the development of these transmitter systems suggest increased susceptibility to stress and anxiety, which could increase relapse drinking to relieve these

symptoms (the “dark side” of addiction – Koob, 2013). Genes for cholesterol production had reduced expression, as were genes involved with myelination, which could have lasting effects on the connectivity between brain regions. The overall pattern of altered gene expression suggests marked behavioral changes would occur if these alterations in gene expression persisted into adulthood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Neurotransmitter-related genes

Differentially expressed neurotransmitter genes

gene	FC	PValue	FDR	control RPKM	alcohol RPKM	gene title
Glutamate						
Gria4	1.4	1.5E-04	2.4E-03	129.1	178.8	glutamate receptor, ionotropic, AMPA 4
Grid2	1.7	5.1E-09	4.1E-07	63.4	105.3	glutamate receptor, ionotropic, delta 2
Grid2ip	1.8	2.3E-09	2.0E-07	6.2	11.4	glutamate receptor, ionotropic, delta 2 (Grid2) interacting protein
Grik4	−1.3	3.8E-03	3.2E-02	13.6	10.2	glutamate receptor, ionotropic, kainate 4
Grin2b	−1.4	1.5E-04	2.4E-03	21.7	15.4	glutamate receptor, ionotropic, N-methyl D-aspartate 2B
Grin2c	1.6	1.3E-08	9.6E-07	37.7	62.1	glutamate receptor, ionotropic, N-methyl D-aspartate 2C
Grin2d	−1.4	4.9E-04	6.3E-03	8.2	5.8	glutamate receptor, ionotropic, N-methyl D-aspartate 2D
Grin3a	−1.4	1.5E-04	2.4E-03	19.7	13.9	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A
Grip2	−1.3	1.5E-03	1.6E-02	17.6	13.2	glutamate receptor interacting protein 2
Grm1	1.6	5.0E-07	2.1E-05	29.9	46.4	glutamate receptor, metabotropic 1
Grm3	−1.3	4.2E-03	3.4E-02	38.1	29.5	glutamate receptor, metabotropic 3
Grm4	1.5	1.5E-06	5.1E-05	45.7	69.9	glutamate receptor, metabotropic 4
Grm5	−1.4	7.5E-05	1.3E-03	30.4	21.3	glutamate receptor, metabotropic 5
Grm7	−1.3	2.5E-03	2.3E-02	22.2	16.8	glutamate receptor, metabotropic 7
Grm8	−1.3	3.8E-03	3.2E-02	25.2	19.2	glutamate receptor, metabotropic 8
Camk4	1.5	2.1E-05	4.7E-04	74.6	108.8	calcium/calmodulin-dependent protein kinase IV
Homer3	2.3	5.1E-20	2.5E-17	39.6	92.9	homer homolog 3 (Drosophila)
Slc17a6	−1.8	1.1E-11	1.8E-09	125.3	69.7	solute carrier family 17 (vesicular glutamate transporter), member 6
Slc17a7	1.9	3.7E-13	7.8E-11	165.8	311.3	solute carrier family 17 (vesicular glutamate transporter), member 7
Slc1a2	−1.3	8.3E-04	9.7E-03	338.6	254.6	solute carrier family 1 (glial high affinity glutamate transporter), member 2
Slc1a3	1.5	1.4E-06	5.0E-05	250.9	379.0	solute carrier family 1 (glial high affinity glutamate transporter), member 3
Slc1a6	2.1	2.7E-15	8.0E-13	31.3	64.6	solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6
Slc25a22	1.3	3.7E-03	3.1E-02	87.6	112.9	solute carrier family 25 (mitochondrial carrier: glutamate), member 22
GABA						
Gabra2	−1.6	3.2E-07	1.5E-05	66.8	42.3	gamma-aminobutyric acid (GABA) A receptor, alpha 2
Gabra3	−1.5	4.2E-05	8.4E-04	25.8	16.9	gamma-aminobutyric acid (GABA) A receptor, alpha 3

gene	FC	PValue	FDR	control RPKM	alcohol RPKM	gene title
Gabra5	−1.7	3.1E-07	1.4E-05	19.4	11.7	gamma-aminobutyric acid (GABA) A receptor, alpha 5
Gabra6	1.6	2.2E-08	1.5E-06	322.9	523.0	gamma-aminobutyric acid (GABA) A receptor, alpha 6
Gabre	−1.8	3.4E-03	2.9E-02	1.2	0.6	gamma-aminobutyric acid (GABA) A receptor, epsilon
Gabrg1	−1.3	4.6E-03	3.7E-02	65.8	50.9	gamma-aminobutyric acid (GABA) A receptor, gamma 1
Gabrg3	−1.8	9.0E-08	4.9E-06	14.3	7.9	gamma-aminobutyric acid (GABA) A receptor, gamma 3
Gabrd	2.0	1.1E-14	3.1E-12	59.8	119.0	gamma-aminobutyric acid (GABA) A receptor, delta
Gad2	−1.3	6.6E-04	8.0E-03	129.6	96.3	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)
Slc6a11	−1.4	4.0E-05	8.2E-04	260.2	183.2	solute carrier family 6 (neurotransmitter transporter, GABA), member 11 - GAT3
Slc6a13	1.4	4.6E-03	3.7E-02	7.2	9.9	solute carrier family 6 (neurotransmitter transporter, GABA), member 13 - GAT2
Serotonin						
Htr1a	−1.5	3.3E-03	2.9E-02	7.9	5.3	5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled
Htr2a	−1.9	9.3E-06	2.4E-04	6.2	3.3	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled
Htr2c	−1.8	4.8E-12	8.5E-10	79.6	43.6	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled
Htr3a	−1.6	3.2E-03	2.8E-02	2.8	1.7	5-hydroxytryptamine (serotonin) receptor 3A, ionotropic
Htr4	−1.5	1.6E-03	1.7E-02	10.9	7.4	5-hydroxytryptamine (serotonin) receptor 4, G protein-coupled
Qdpr	−1.6	4.5E-08	2.7E-06	165.5	102.1	quinoid dihydropteridine reductase
Acetylcholine						
Chrm2	−1.6	3.8E-06	1.1E-04	16.6	10.4	cholinergic receptor, muscarinic 2
Chrm2	−1.7	6.2E-04	7.6E-03	2.7	1.6	cholinergic receptor, muscarinic 2
Chrm3	−1.4	2.5E-04	3.7E-03	16.9	12.0	cholinergic receptor, muscarinic 3
Chrna4	−1.6	4.7E-06	1.3E-04	25.9	16.6	cholinergic receptor, nicotinic, alpha 4 (neuronal)
Chrna7	−1.9	1.2E-08	8.9E-07	17.3	9.2	cholinergic receptor, nicotinic, alpha 7 (neuronal)
Chrnb3	−1.5	4.5E-03	3.6E-02	2.9	1.9	cholinergic receptor, nicotinic, beta 3 (neuronal)
Glycine						
Glra1	−1.9	2.4E-11	3.8E-09	35.2	18.7	glycine receptor, alpha 1
Glra2	−1.5	7.3E-05	1.3E-03	21.9	15.0	glycine receptor, alpha 2
Glra3	−1.6	6.0E-05	1.1E-03	19.5	12.1	glycine receptor, alpha 3
Glrb	−1.4	5.0E-04	6.4E-03	169.6	125.2	glycine receptor, beta
Slc6a5	−1.3	3.5E-03	3.0E-02	7.5	5.7	solute carrier family 6 (neurotransmitter transporter, glycine), member 5

Alcohol Clin Exp Res. Author manuscript; available in PMC 2017 May 01.

gene	FC	PValue	FDR	control RPKM	alcohol RPKM	gene title
Dopamine						
Drd2	−1.3	6.0E-03	4.5E-02	11.3	8.5	dopamine receptor D2
Drd5	−1.7	1.2E-03	1.3E-02	3.6	2.1	dopamine receptor D5
Th	1.6	6.9E-03	5.0E-02	1.6	2.5	tyrosine hydroxylase
Purinergic						
P2rx2	−2.1	4.0E-05	8.2E-04	2.6	1.3	purinergic receptor P2X, ligand-gated ion channel, 2
P2rx5	−2.1	2.2E-06	7.1E-05	3.1	1.5	purinergic receptor P2X, ligand-gated ion channel, 5
P2rx4	1.4	6.2E-04	7.6E-03	25.5	35.2	purinergic receptor P2X, ligand-gated ion channel, 4
P2rx6	1.4	7.0E-04	8.4E-03	23.5	32.3	purinergic receptor P2X, ligand-gated ion channel, 6
Adrenergic						
Adra1a	−1.4	5.9E-03	4.5E-02	8.5	6.0	adrenoceptor alpha 1A
Adrbk2	1.3	6.0E-03	4.5E-02	27.1	35.1	adrenergic, beta, receptor kinase 2
Adenosine						
Adora1	1.4	1.5E-04	2.4E-03	95.2	133.7	adenosine A1 receptor

Neurotransmitter-related genes whose expression is altered by adolescent ethanol binge-drinking (FDR<0.05) FC – fold change, FDR – false discovery rate, average RPKM (reads per kilobase per million mapped reads) for control and alcohol exposed animals.

Alcohol Clin Exp Res. Author manuscript; available in PMC 2017 May 01.

Table 2

neuropeptides and receptors

Differentially expressed neuropeptides

gene	fold	PValue	FDR	control RPKM	alcohol RPKM	Name
Cck	−1.9	1.3E-07	6.5E-06	25.2	13.4	cholecystokinin
Cckbr	−1.8	5.9E-05	1.1E-03	4.2	2.4	cholecystokinin B receptor
Crhr1	1.4	2.7E-03	2.5E-02	19.2	26.1	corticotropin releasing hormone receptor 1
Galr1	−1.6	4.6E-04	6.0E-03	4.1	2.5	galanin receptor 1
Galr2	−1.5	5.5E-03	4.2E-02	6.9	4.7	galanin receptor 2
Hcrt	1.8	8.0E-05	1.4E-03	9.7	17.3	hypocretin (orexin) neuropeptide precursor
Hcrtr2	−1.5	2.8E-03	2.5E-02	2.8	1.8	hypocretin (orexin) receptor 2
Npy5r	−2.4	3.2E-09	2.7E-07	3.6	1.5	neuropeptide Y receptor Y5
Npy1r	−2.0	2.1E-08	1.4E-06	6.1	3.1	neuropeptide Y receptor Y1
Ntsr1	−1.8	2.4E-04	3.6E-03	1.6	0.9	neurotensin receptor 1 (high affinity)
Oprk1	−1.8	7.4E-06	2.0E-04	9.6	5.4	opioid receptor, kappa 1
Pdyn	−1.6	1.3E-05	3.1E-04	8.4	5.1	prodynorphin
Oprl1	−1.6	8.8E-07	3.4E-05	18.6	11.5	opiate receptor-like 1
Oprm1	−1.8	1.6E-08	1.1E-06	9.7	5.3	opioid receptor, mu 1
Penk	−1.4	2.2E-04	3.4E-03	99.3	71.4	proenkephalin
Sstr1	−1.7	1.1E-06	4.0E-05	8.7	5.2	somatostatin receptor 1
Sstr3	1.5	2.4E-05	5.2E-04	8.8	13.2	somatostatin receptor 3
Sstr4	−1.4	7.5E-03	5.3E-02	5.6	3.9	somatostatin receptor 4
Tac1	−1.4	2.0E-04	3.1E-03	69.7	49.2	tachykinin, precursor 1
Tacr1	−1.7	2.6E-07	1.2E-05	7.8	4.5	tachykinin receptor 1
Tacr3	1.4	2.1E-03	2.0E-02	12.2	16.7	tachykinin receptor 3

Neuropeptide and receptor genes whose expression is altered by adolescent ethanol binge-drinking (FDR<0.05) FC – fold change, FDR – false discovery rate, average RPKM (reads per kilobase per million mapped reads) for control and alcohol exposed animals.

Table 3

Ion Channels

Differentially expressed Ion Channels

gene	FC	PValue	FDR	control RPKM	Alcohol RPKM	gene title
Potassium	27 of 70					
Kcnip3	1.4	2.2E-04	3.3E-03	34.3	49.5	Kv channel interacting protein 3, calsenilin
Kcnip4	1.4	7.8E-04	9.3E-03	32.1	44.3	Kv channel interacting protein 4
Kcnk12	1.6	6.9E-06	1.8E-04	7.4	12.2	potassium channel, subfamily K, member 12
Kcnk16	2.0	2.9E-08	1.8E-06	3.7	7.3	potassium channel, subfamily K, member 16
Kcnk2	−1.4	6.2E-04	7.6E-03	16.0	11.5	potassium channel, subfamily K, member 2
Kcnt1	1.3	2.0E-03	2.0E-02	63.6	83.2	potassium channel, subfamily T, member 1
Kcnt2	−1.7	1.3E-06	4.4E-05	8.6	5.1	potassium channel, subfamily T, member 2
Kcnj12	1.7	3.4E-09	2.9E-07	27.3	47.0	potassium inwardly-rectifying channel, subfamily J, member 12
Kcnj2	−1.5	2.8E-03	2.5E-02	6.4	4.2	potassium inwardly-rectifying channel, subfamily J, member 2
Kcnj3	1.4	9.5E-05	1.6E-03	74.5	105.3	potassium inwardly-rectifying channel, subfamily J, member 3
Kcnj9	1.5	9.0E-05	1.6E-03	9.8	14.8	potassium inwardly-rectifying channel, subfamily J, member 9
Kcnmb4	1.5	1.5E-03	1.6E-02	10.4	15.2	potassium large conductance calcium-activated channel, subfamily
Kcns3	−2.0	5.7E-07	2.4E-05	4.5	2.2	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3
Kcnq3	−1.3	1.1E-03	1.2E-02	19.1	14.2	potassium voltage-gated channel, KQT-like subfamily, member 3
Kcnb2	−1.4	2.2E-04	3.3E-03	15.3	10.7	potassium voltage-gated channel, Shab-related subfamily, member 2
Kcnab2	−1.4	7.2E-04	8.7E-03	79.9	59.0	potassium voltage-gated channel, shaker-related subfamily, beta m
Kcna6	−1.3	6.8E-04	8.2E-03	47.2	35.2	potassium voltage-gated channel, shaker-related subfamily, member
Kcnd1	−1.8	3.5E-06	1.1E-04	4.1	2.3	potassium voltage-gated channel, Shal-related subfamily, member 1
Kcnd2	1.3	8.1E-04	9.5E-03	112.6	150.2	potassium voltage-gated channel, Shal-related subfamily, member 2
Kcnc2	−1.3	1.0E-03	1.1E-02	50.1	37.5	potassium voltage-gated channel, Shaw-related subfamily, member 2
Kcnf1	−1.5	3.8E-03	3.2E-02	3.0	2.0	potassium voltage-gated channel, subfamily F, member 1
Kcng2	−1.8	5.6E-03	4.2E-02	1.7	0.9	potassium voltage-gated channel, subfamily G, member 2
Kcng4	1.5	1.7E-06	5.7E-05	21.5	33.1	potassium voltage-gated channel, subfamily G, member 4
Kcnh3	1.8	7.4E-11	1.1E-08	12.9	23.6	potassium voltage-gated channel, subfamily H (eag-related), member
Kcnh5	−1.5	3.9E-06	1.1E-04	20.9	13.5	potassium voltage-gated channel, subfamily H (eag-related), member
Kcnh7	−1.5	1.4E-05	3.2E-04	19.4	12.9	potassium voltage-gated channel, subfamily H (eag-related), member

gene	FC	PValue	FDR	control RPKM	Alcohol RPKM	gene title
Kcnh1	1.4	1.3E-04	2.1E-03	20.0	28.2	potassium voltage-gated channel, subfamily H (eag-related), member
Sodium	10 of 14					
Scn1a	−1.4	2.5E-04	3.6E-03	85.6	62.6	sodium channel, voltage-gated, type I, alpha subunit
Scn1b	1.5	8.8E-07	3.4E-05	163.5	250.5	sodium channel, voltage-gated, type I, beta subunit
Scn2b	1.3	1.5E-03	1.6E-02	180.6	238.5	sodium channel, voltage-gated, type II, beta subunit
Scn3b	−1.5	7.6E-06	2.0E-04	63.7	43.1	sodium channel, voltage-gated, type III, beta subunit
Scn4b	−2.0	5.0E-14	1.3E-11	35.7	18.1	sodium channel, voltage-gated, type IV, beta subunit
Scn9a	−1.3	5.0E-03	3.9E-02	6.6	5.0	sodium channel, voltage-gated, type IX, alpha subunit
Scn5a	−1.6	1.1E-03	1.2E-02	0.8	0.5	sodium channel, voltage-gated, type V, alpha subunit
Scn7a	−1.9	1.3E-07	6.5E-06	2.9	1.6	sodium channel, voltage-gated, type VII, alpha subunit
Scnn1a	−1.6	5.4E-03	4.1E-02	1.4	0.9	sodium channel, non-voltage-gated 1 alpha subunit
Scnm1	1.5	1.3E-03	1.4E-02	11.7	17.0	sodium channel modifier 1
Calcium	5 of 21					
Cacna1g	1.5	6.9E-06	1.8E-04	35.7	52.8	calcium channel, voltage-dependent, T type, alpha 1G subunit
Cacna1h	−1.5	5.6E-05	1.1E-03	9.1	6.2	calcium channel, voltage-dependent, T type, alpha 1H subu
Cacng3	−1.6	5.2E-04	6.6E-03	11.8	7.5	calcium channel, voltage-dependent, gamma subunit 3
Cacna1a	1.3	1.5E-03	1.6E-02	55.8	73.3	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit /
Cacng8	−1.4	2.2E-03	2.1E-02	11.6	8.0	calcium channel, voltage-dependent, gamma subunit 8
Chloride	2 of 8					
Clic6	1.8	1.7E-07	8.5E-06	5.1	9.3	chloride intracellular channel 6
Clic1	1.4	3.7E-03	3.1E-02	8.4	12.0	chloride intracellular channel 1

Ion Channel genes whose expression is altered by adolescent ethanol binge-drinking (FDR< 0.05) FC – fold change, FDR– false discovery rate, average RPKM (reads per kilobase per million mapped reads) for control and alcohol exposed animals.

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Table 4

Differentially Expressed Cholesterol-related Genes

Differentially expressed Cholesterol Biosynthesis and Myelin-associated Genes

gene	FC	PValue	FDR	control RPKM	alcohol RPKM	gene title
cholesterol						
Dhcr24	−1.4	1.2E-04	2.0E-03	125.3	89.3	24-dehydrocholesterol reductase
Dhcr7	−1.5	5.2E-05	1.0E-03	18.6	12.5	7-dehydrocholesterol reductase
Fdft1	−1.3	2.6E-03	2.4E-02	66.8	51.3	farnesyl-diphosphate farnesyltransferase 1
Fdps	−1.3	1.3E-03	1.4E-02	94.8	70.8	farnesyl diphosphate synthase
Hmgcr	−1.5	2.1E-05	4.8E-04	28.2	19.2	3-hydroxy-3-methylglutaryl-CoA reductase
Hmgcs1	−1.5	1.5E-05	3.5E-04	154.6	106.6	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)
Sc4mol	−1.5	2.2E-06	7.2E-05	120.6	79.5	methylsterol monooxygenase 1
Sqle	−1.3	3.7E-03	3.1E-02	59.0	45.5	squalene epoxidase
Tm7sf2	−1.4	4.7E-03	3.7E-02	13.1	9.5	transmembrane 7 superfamily member 2
Myelin						
Aspa	−1.6	1.7E-06	5.7E-05	25.2	15.4	aspartoacylase
Cnp	−1.3	2.4E-03	2.3E-02	282.8	218.3	2′,3′-cyclic nucleotide 3′ phosphodiesterase
Enpp2	−1.5	3.7E-06	1.1E-04	120.5	80.7	ectonucleotide pyrophosphatase/phosphodiesterase 2
Erbb3	−1.6	1.8E-06	6.1E-05	15.5	9.8	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) //
Gjc2	−1.6	1.0E-06	3.8E-05	25.5	15.9	gap junction protein, gamma 2, 47kDa
Klk6	−1.5	8.0E-05	1.4E-03	29.8	19.8	kallikrein-related peptidase 6
Mag	−1.4	2.0E-05	4.6E-04	169.9	117.7	myelin associated glycoprotein
Mal	−1.5	2.5E-06	8.0E-05	184.7	123.1	mal, T-cell differentiation protein
Mbp	−1.6	8.0E-09	6.2E-07	1805.2	1107.2	myelin basic protein
Mobp	−1.7	1.4E-09	1.3E-07	216.3	128.6	myelin-associated oligodendrocyte basic protein
Mog	−1.6	9.0E-07	3.4E-05	98.9	63.8	myelin oligodendrocyte glycoprotein
Omg	−1.3	1.2E-03	1.3E-02	80.8	60.5	oligodendrocyte myelin glycoprotein
Opalin	−1.7	1.2E-08	9.0E-07	44.8	26.7	oligodendrocytic myelin paranodal and inner loop protein /
Pllp	−1.4	1.7E-03	1.7E-02	37.6	27.8	plasmolipin
Plp1	−1.8	1.4E-12	2.8E-10	2382.8	1306.7	proteolipid protein 1
Ugt8	−1.8	1.0E-10	1.4E-08	96.8	55.2	UDP glycosyltransferase 8

Genes in the Cholesterol biosynthesis pathway and the pathway of myelin synthesis altered by adolescent binge drinking. FC – fold change, FDR – false discovery rate, average RPKM (reads per kilobase per million mapped reads) for control and alcohol exposed animals.

Table 5

Selected small molecule upstream regulators.
Selected ustream regulators.

Upstream Regulator	Activation z-score	Target genes in dataset	target
wortmannin	−2.91	AGTR1, BCL2L11, CKB, COL1A1, COL1A2, COL3A1, E2F1, EIF1AX, ERBB3, FOS, GPD1, IGF1, JUN, MAF, MYH11, NR4A1, PTGER4, PTGS2, PXN, RAP1A, TH, TYMP, VIM	covalent inhibitor of phosphoinositide 3-kinases and mTOR
isoquercitrin	−2.63	DHCR7, FDFT1, FDPS, HMGCR, INSIG1, LDLR, SQLE	plant derived isoflavone, known to induce APOa1 expression, possible antioxidant and anti-inflammatory
PP1	−2.62	ANGPT2, CXCR4, FOS, GJA1, JUN, LHB, PTGS2	kinase inhibitor
sildenafil	−2.24	FGF1, GFAP, PDE5A, PTGS2, VEGFB	cGMP-specific phosphodiesterase inhibitor, inhibits PDE5
pentobarbital	−2.21	FOS, JUN, JUNB, NR4A1, SUMO1	Nembutal, CNS depressant, barbiturate, increases affinity of GABA-A receptor for GABA (GABA-A receptor positive allosteric modulator), increase duration of Cl-channel opening.
nimodipine	−2.18	FOS, LHB, NR4A1, NR4A2, TH	Voltage-dependent L-type calcium channel, mineralcorticoid receptor, aryl hydrocarbon receptor,
4-phenylbutyric acid	−2.17	ATP2A3, CNP, EHHADH, GJA1, MOG, PEX11A, PLP1, SEPP1, SERPINA1, SOD2, STK10, TIMP2, USP29, YPEL5	treatment of urea cycle disorders, histone deacetylase inhibitor and chemical chaperone
phenobarbital	−2.09	CYP2C9, CYP4B1, GSTA2, GSTM5, INSIG1, PAPSS2, RAF1, Sult1a1, THRSP, TRPC3, UGT1A1	barbiturate, possitive modulator of GABA-A receptors
mifepristone	−2.06	Acan, ANXA1, ARG1, ATF3, BCAT1, BMP7, C3, CA2, Cd24a, CHI3L1, COL18A1, DUSP1, FDFT1, FOS, GJA1, HDC, ITGB4, JUN, JUNB, KIT, LDLR, LOX, NDN, NDRG1, NPY1R, NR4A1, OPRM1, PLAT, PRKG2, PTGS2, PTPN5, RELN, RHOB, ROBO1, SERPING1, SFRP2, SLC1A2, SPEG, SPP1, Sult1d1, SUMO1, VCAN	glucocoricoid and progesterone receptor antagonist
bezafibrate	−2.04	ACSL1, ALDH1A2, CAT, CPT1B, DLK1, EHHADH, INSIG1, LGALS3, MGLL, NR1H3, PANK1, PDK4, PEX11A, PTGS2, SCD, SLC27A2, TSPO	Peroxisome proliferator activated receptor alpha, gamma, delta
testosterone	−2.02	A2M, ACSL1, ANXA1, AR, ATP5F1, BMP7, CA3, CAMK4, CAT, CRHR1, CYP26B1, DHCR24, EGFR, FOS, GALR1, GALR2, GDI2, HCN4, HCRTR2, HDC, HMGCR, HSPA1A/HSPA1B, ID3, IGF1, JUN, LHB, MAL, MGST1, Mx1/Mx2, NDRG1, NOS1, PDE5A, PDYN, PRLR, PTGS2, PXN, RAF1, SCNN1A, SDC1, SOD2, SRD5A1, SSTR1, SSTR3, STK11, TF, TGFA, TUBB3, UGT8, VCAN	steroid hormone, androgen receptor agonist
taurine	−2.00	GFAP, PTGS2, SYN1, VIM	“amino acid” exerts positive allosteric modulation of NMDA and voltage-gated calcium channels-acamprosate is related: calcium acetylhomotaurinate
cannabidiol	−1.98	PENK, PTGS2, TAC1, VIM	non-psychoactive component of cannabis, target ?

Upstream Regulator	Activation z-score	Target genes in dataset	target
salicylic acid	−1.98	GRIN2B, KITLG, PTGS2, SLC2A4	aspirin metabolite, reduces fever, antipain, anti inflammatory, NSAID, suppresses activity of cyclooxygenase (COX) which produces prostaglandins
PD98059	−1.94	ACOT7, AGTR1, ANGPT2, ANXA1, AR, ATF3, BCL2L11, BCL6, C3, CA2, CCK, CD83, CDC42EP1, COL1A1, COL1A2, COL3A1, CXCR4, DUSP1, DUSP5, E2F1, EGFR, ELK1, ELN, FAH, FOS, FURIN, GJA1, GPC5, GRIN2C, GSK3B, HPCAL1, ID3, IGF1, ITGB5, JUN, JUNB, KRT8, LDLR, LHB, MET, MGLL, MYH11, NR4A1, PCSK6, PER1, PLAGL1, PLAT, PTAFR, PTGER4, PTGS2, RNASE4, RPS6, S100A4, SCNN1A, SDC1, SEPP1, SERPINA1, SPP1, SQLE, ST6GAL1, TF, TH, TIMP2, TYMP, VCAN, VIM	O-methylated flavonoid, ERK signaling pathway inhibitor
ciglitazone	−1.92	CAT, GPD1, JUN, PTGS2	thiazolidinedion similar to pioglitazone. Agonist of PPAR gamma (antihyperglycemic). Also decreases VEGF production.
gemfibrozil	−1.91	BMP7, CNP, CYP2C9, EHHADH, FURIN, HCRT, LDLR, MOG, NR1H3, PLP1, RTN4, SCD	fibrate, PPARalpha agonist
pitavastatin	−1.91	CAT, FDFT1, FDPS, HMGCR, HMGCS1, PTGS2, SLC27A2, SQLE	3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (member of Statins)
methotrexate	−1.84	ALB, Apoc1, ASS1, BTG2, C1R, C3, C6, CASP4, CFB, CFH, DUSP1, DUSP14, EHHADH, FA2H, HMGCS1, IGFBP5, ITIH4, MET, NR4A2, PFN2, PTGS2, SCD, SEPP1, SERPING1, SLC27A2, SPP1, TOB1, UGT1A6	Dihydrofolate reductase inhibitor, so inhibits production of tetra hydrofolate
morphine	1.77	CALB1, CCK, EGFR, ERBB3, FOS, GFAP, GHR, GNAS, GRIN2B, JUNB, KCNAB2, KCNJ9, NR4A1, NTRK3, OPRL1, OPRM1, PDYN, PENK, PLAT, PTGS2, SLC1A2, TH	mu, delta and kappa opioid receptors
etoposide	1.78	ATF3, BCL2L11, CA2, DUSP1, E2F1, IRF7, JUN, NR4A1, PIK3R3, SGK1, SOD2, TP73	DNA topoisomerase 2-alpha & 2-beta
cocaine	1.88	ADCY1, ASMT, DRD2, DUSP1, DUSP14, DUSP5, EGR4, FOS, GABRA6, GABRD, GRM1, GRM5, JUN, JUNB, MNS1, NAB2, NOS1, NR4A1, NR4A3, OPRM1, PDYN, PER1, PPP1R1B, PVALB, SLC1A2, SPAG4, TAC1, TH, TSPO, UBASH3B	sodium channel protein type 10 submint alpha, sodium-dependent dopamine transporter, serotonin transporter, noradrenaline transporter, chrmM1, chrmM2, sodium channel protein type 5 subunit alpha, type 11, submint alpha
nilvadipine	1.89	CD38, CRYAB, GALR2, LGALS8, PRKCD, PRKCG, Tpm2	voltage-dependent L-type calcium channel also, CACNA2D3, Ltype: CACNA1D
n-3 fatty acids	1.90	CAT, ELOVL6, PTGS2, RGN, SCD, SDC1, SLC2A4	
CD 437	1.98	AK2, ATP5G3, CLIC1, DNAJA1, E2F1, FOS, GNAS, ITGA11, JUN, MGST1, NR4A1, SH3BGRL3, SUMO1	synthetic retinoid, RAR gamma selective agonist
3-deazaneplanocin	2.00	AKNA, CLDN11, MVP, NOTCH2	AKA Dznep, inhibits expression of EZH2 Enhancer of zeste homolog-2, drug is an S-adenosyl-l-homocysteine (AdoHcy) hydrolase
colchicine	2.19	ATF3, CYP2C9, FOS, JUN, PTGS2	inhibitor Tubulin beta chain
asoprisnil	2.22	DPP4, EGFR, IGF1, RBP4, SGK1	selective progesterone-receptor modulator

Upstream Regulator	Activation z-score	Target genes in dataset	target
ketoconazole	2.22	CYP2C9, GSTA2, HMGCR, TH, UGT1A1	antifungal, inhibits lanosterol 14-alpha demthylase, binds androgen receptor
anisomycin	2.28	ATF3, DUSP1, FOS, GJA1, JUN, LDLR, NR4A1, PPM1D, SDC1, SLC17A6, SLC17A7, SPP1	inhibits protein synthesis, activates stress-activated protein kinases (antibiotic), projected to remove memories from hippocampus by inhibiting new context-specific long-term memories
isoproterenol	2.29	APLN, ATF3, ATP1A1, BCL2L11, FOS, HS3ST2, JUN, JUNB, MEF2D, NR4A1, PLAGL1, PLP1, PTGS2, SCD, TH, TIMP2, TUBB3, TYRO3, XRCC1	beta adrenergic receptor agonist
L-glutamic acid	2.37	ACLY, CALB1, DLK1, FOS, GRM5, JUN, JUNB, MAP1B, MGLL, NOS1, PER1, PTGS2, SLC1A2, SORL1	glutamate
desmopressin	2.43	ALB, Anp32a, ATP1A1, FLNA, FOS, HSPA1A/HSPA1B, PPP1R1B, PXN, SLC2A4, SLC43A2, SLC9A3, SPTBN2, ST14	agonist vasopressin V1a, V1b abd V2 receptor
cephaloridine	2.43	CYP2C9, E2F1, Folh1, GSTM5, HSPA1A/HSPA1B, KCNH1	antibiotic
methapyrilene	2.52	A2M, ALB, Apoc1, ASS1, ATF3, BTG2, CAT, CP, CPS1, DAO, EHHADH, ENPP2, GFRA1, GJB1, GRB14, GSK3B, HMGCR, IGF1, ITIH4, LCAT, MAPK6, Mx1/Mx2, NFIB, OPLAH, Ppp1r15a, RXRG, SCD, SEPP1, SLC27A2, Sult1a1	antihistamine and anticholinergic (sedative) in OTC sleep aids like Sominex, Nytol, etc.
norepinephrine	2.67	ADRBK2, ATP2A2, CACNA1G, Ccl9, Cd24a, CITED4, CRY1, CRY2, Dos, DUSP1, ELOVL1, ELOVL6, FOS, GPD1, GRID2, GRIN2B, GRM1, HHIP, HS3ST2, MCAM, NAP1L5, NPY1R, NR4A1, NR4A3, PER1, PLAGL1, PTGS2, RBP4, SGK1, SLC17A6, SLC2A4, THRSP, Vof16	neurotransmitter, alpha adrenergic and beta adrenergic receptors
glucagon	3.00	FOS, NR4A1, NR4A2, NR4A3, PPP1R1B, PXN, SLC2A4, SLC43A2, SLC9A3, ST14	opposes effect of insulin
nitrofurantoin	3.08	A2M, ADAMTS1, ALB, Apoc1, ASNS, ASS1, BTG2, C3, CAPN2, CAT, CP, CPS1, EHHADH, GJB1, HMGCR, HMGCS1, HPX, IGF1, ITGB4, ITIH4, LCAT, LGALS3, LOX, MET, PLAT, SDC1, SEPP1, SERPINA1, SLC27A2, SPP1	antibiotic, especially UTI
potassium chloride	3.15	AMIGO2, ATF3, ATP1A1, ATP2B3, ATP2B4, BCL2L11, BTG2, CALB1, E2F1, FOS, GABRD, ID3, IGFBP5, JUN, LHB, NOS1, NPAS4, NPTX1, NR4A1, NR4A2, NR4A3, PDYN, PTGS2, SLC8A2, SPP1, TH, TP73	source of potassium and chloride ions
bicuculline	3.46	ATF3, BTG2, CYR61, DUSP1, EGR4, FOS, IER2, JUN, JUNB, NPAS4, NR4A1, NR4A2, PTGS2	competitive antagonist for GABA-A receptor
dalfampridine	3.61	ATF3, BTG2, CYR61, DUSP1, EGR4, FOS, IER2, JUN, JUNB, NPAS4, NR4A1, NR4A2, PTGS2	potassium voltage-gated channel blocker used to treat multiple sclerosis (Ampyra)

Putative upstream regulators that are drugs or small molecules (a subset from Supplemental Table 3). Activation Z-scores that are positive suggest the molecule causes changes similar to those of the repeated ethanol exposure, on the subset of genes listed as “Target genes in dataset;” negative Z-scores suggest the molecule would prevent or oppose the effects of ethanol on its target genes.