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Gene expression changes in serotonin, GABA-A receptors, neuropeptides and ion channels in the dorsal raphe nucleus of adolescent alcohol-preferring (P) rats following binge-like alcohol drinking

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Abstract

Alcohol binge-drinking during adolescence is a serious public health concern with long-term consequences. We used RNA sequencing to assess the effects of excessive adolescent ethanol binge-drinking on gene expression in the dorsal raphe nucleus (DRN) of alcohol preferring (P) rats. Repeated binges across adolescence (three 1h sessions across the dark-cycle per day, 5 days per week for 3 weeks starting at 28 days of age; ethanol intakes of 2.5 – 3 g/kg/session) significantly altered the expression of approximately one-third of the detected genes. Multiple neurotransmitter systems were altered, with the largest changes in the serotonin system (21 of 23 serotonin-related genes showed decreased expression) and GABA-A receptors (8 decreased and 2 increased). Multiple neuropeptide systems were also altered, with changes in the neuropeptide Y and corticotropin-releasing hormone systems similar to those associated with increased drinking and decreased resistance to stress. There was increased expression of 21 of 32 genes for potassium channels. Expression of downstream targets of CREB signaling was increased. There were also changes in expression of genes involved in inflammatory processes, axonal guidance, growth factors, transcription factors, and several intracellular signaling pathways. These widespread changes indicate that excessive binge drinking during adolescence alters the functioning of the

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DRN and likely its modulation of many regions of the central nervous system, including the mesocorticolimbic system.

Keywords

serotonin; GABA-A receptors; neuropeptides; CREB; ion channels; inflammation; RNA sequencing; alcoholism

1. Introduction/Background

A multi-national review of studies of adolescent drinking indicates that about ¾ of adolescents have consumed alcohol by late adolescence, and 20–40% had engaged in binge drinking, the consumption of 5 or more drinks on one occasion (Marshall, 2014). In the US, adolescents (ages 12–20) drink 11 percent of all alcohol consumed, 90% of which is consumed during binge drinking (NIAAA, 2013).

Adolescence is a time in which brain connections mature and are remodeled, and alcohol could alter these processes. Given the legal and ethical prohibition of using adolescents in alcohol research, animal models have been used to examine the effects of ethanol on adolescents. Studies have examined the effects of chronic low dose exposure (Evrard et al., 2006) and different models of binge drinking (McBride et al., 2014; Pascual et al., 2007; Pascual et al., 2014; Ward et al., 2014). Selectively bred alcohol preferring (P) and high-alcohol-drinking (HAD) rats engage in binge-like drinking, and adults and peri-adolescents of both sexes readily achieve blood ethanol levels ≥ 80 mg% (Bell et al., 2014). Multiple scheduled access protocols (usually three 1 h periods of access to alcohol during the dark phase) enhance this binge-like drinking, and under these conditions peri-adolescent selectively bred rats consume more alcohol than their adult counterparts (Bell et al., 2014). In general, animal studies and human observational studies during adolescence indicate that adolescents, relative to adults, are less affected by ethanol-induced sedation and motor incoordination but more affected by its rewarding and reinforcing properties (Bell et al., 2013; Spear, 2010). Greater disturbances in some cognitive functions have also been reported (Bell et al., 2013; Spear, 2010).

Alcohol dependence has two basic components: positive effects primarily attributed to ethanol stimulated release of dopamine in the reward centers of the brain and negative effects of anxiety and depression that occur after cessation of drinking (Koob, 2013). The reward system in the brain includes the mesocorticolimbic dopamine system and extended amygdala, which intersect in the nucleus accumbens shell. Within this system, ethanol increases the release of dopamine in the shell of the nucleus accumbens, which is directly associated with the rewarding effects of ethanol consumption (McBride, 2002; McBride and Li, 1998) reviewed in Koob (2013). Serotonin modulates the effect of ethanol on dopamine release via the 5-HT3 receptor (Campbell et al., 1996; Campbell and McBride, 1995; Engleman et al., 2008; Wozniak et al., 1990).

In rats, a genetic predisposition for excessive ethanol consumption is associated with alterations in central serotonergic activity. For example, ethanol-naïve P and HAD rats have

significantly lower serotonin and/or 5HIAA5-hydroxyindoleacetic acid levels in the frontal cortex, nucleus accumbens, caudate putamen, hippocampus and hypothalamus compared with ethanol-naïve NP and LAD rats (Bell et al., 2012; Gongwer et al., 1989; Murphy et al., 1987). In addition, ethanol-naïve P, and in some cases ethanol-naïve HAD, rats have altered levels of 5HT1A, 5HT1B, 5HT2, 5HT2C and 5HT3 receptors in multiple brain regions compared with ethanol-naïve NP and LAD rats (McBride et al., 1997a; McBride et al., 1997b). Thus, alterations in receptor expression may be compensating for reduced serotonin function. Selective agonists and/or antagonists for various serotonin receptors affect the alcohol-consuming behavior of rats selected for high alcohol preference (Ding et al., 2012; Lankford et al., 1996; Lankford and Myers, 1996; Long et al., 1996; Overstreet et al., 1997; Rodd-Henricks et al., 2000; Rodd et al., 2010). Other selectively bred high alcohol-consuming rat lines (Alko Alcohol preferring and Sardinian alcohol preferring rats) had higher levels of serotonin in whole brain or selected regions than their low alcohol-consuming counterparts (Alko Non-Alcohol and Sardinian non-preferring rats) (Bell, Sable et al. 2012).

The dorsal raphe nucleus (DRN) is an origin of the central serotonergic system. About 50% of the serotonin neurons in the rat brain are in the DRN, although fewer than 50% of the neurons in the DRN are serotonergic (Waselus et al., 2011). The DRN has projections into numerous brain regions, including the mesolimbic regions important in reward and addiction (amygdala, hippocampus, caudate putamen, substantia nigra and ventral tegmental area). Projections from the raphe nuclei mediate dopamine release in the ventral tegmental area (Liu et al., 2006b; Rodd et al., 2007; Sari et al., 2011). Ethanol-naïve P rats have fewer serotonergic neurons in the DRN than ethanol-naïve NP rats and decreased serotonergic projections into terminal regions (Zhou et al., 1994). Postmortem studies of the DRN of alcoholics (Underwood et al., 2007) found less serotonin transporter in the brainstem of alcoholics.

The links between the DRN and the reward centers of the brain, its role in anxiety, stress and depression, and the changes that occur during adolescence make it an important region in which to study the effects of heavy alcohol exposure, particularly during the vulnerable period of adolescence. This study examines the effects of binge drinking by adolescent P rats on the gene expression profile of the DRN.

2. Materials and Methods

2.1 Animal Binge drinking protocol

Peri-adolescent male P (alcohol preferring) rats were maintained on a reverse light-dark cycle and given free-choice access to ethanol using a multiple-scheduled-access procedure, as described in Bell et al. (2011). There were 10 animals in the water control group and 11 in the ethanol binge-drinking group. Animals were given *ad libitum* access to food and water and those in the drinking group were given access to ethanol (15 and 30% ethanol solutions concurrently) in 3 x 1-h sessions per day for 5 consecutive days/week, starting at 28 days of age. The three 1hr ethanol access sessions were scheduled so that the first 1hr session started at dark onset (1000 h), the second 1 h session began 2 h after the end of the first session (1300 h) and the third/last 1hr session began 2 h after the end of the second session (1600 h).

The animals used in this study are the same ones described in McBride et al. (2014). A graph of the 15-day ethanol drinking data is given in McBride et al. (2014). In general, during week 1, ethanol intakes ranged from 3–4 g/kg/1hr session with a total intake of approximately 10 g/kg/day; during weeks 2 and 3, ethanol intakes ranged from 2–3 g/kg/1hr session with a total intake of approximately 8 g/kg/day.

The rats were sacrificed 3 h after the 1st access session on their 15th day of drinking. 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 DRN micro-punched using procedures previously described (McBride et al., 2014). The DRN was punched from coronal sections between −7.30 mm to −8.30 mm post bregma according to the rat brain atlas by Paxinos and Watson (1998). All equipment used to obtain tissue was treated with RNase Zap (Life Technologies, Carlsbad, CA). Other brain regions of these animals have been studied (McBride et al., 2014). 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).

2.2 Sample collection and quality

To extract the RNA, punches were immediately homogenized in Trizol (Life Technologies, Carlsbad, CA) and processed according to manufacturer's protocol but with twice the suggested ratio of Trizol to tissue (Edenberg et al., 2005). Ethanol-precipitated total RNA was further purified through Qiagen RNeasy columns (Qiagen, Hilden Germany) according to the manufacturer's protocol. 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 also assessed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, Ca); RNA integrity numbers (RIN) averaged 8.6 for the samples, showing little degradation.

2.3 RNA sequencing

Standard methods were used for RNA-seq library construction, EZBead preparation and Next-Gen sequencing using the Life Technologies SOLiD system. Briefly, total RNA (≤ 400 ng per sample) was first ribosome-depleted using Ribominus™ Eukaryote Kit for RNA-Seq (Ambion, CA), and whole transcriptome library was prepared and barcoded per sample using the standard protocol of SOLiD Total RNA-seq Kit (Life Technologies, Carlsbad, CA). Each barcoded library was quantified by quantitative PCR using SOLiD Library Taqman qPCR Module (Life Technologies, Carlsbad, CA), and pooled in equal molarity. EZBead preparation, bead library amplification, and bead enrichment were then conducted using Life Technologies EZ Bead™ E80 System (Life Technologies, Carlsbad, CA). And finally sequencing by ligation was carried out using standard single-read, 5′-3′ strand-specific sequencing procedure on SOLiD4™ Sequencer (50b-read), as well as on SOLiD™

5500xl Sequencer (75 b read). The average number of mapped reads per sample was 20.8 million.

2.4 Data Processing and Quality Assessment

We used SOLiD Instrument Control Software and SOLiD Experiment Tracking System software for the read quality recalibration. Each sequence read was scanned for low-quality regions, and if a 5-base sliding window had an average quality score less than 20, the read was truncated at that position. Any read with a length of less than 35 bases was discarded. Our experience suggests that this strategy effectively eliminates low-quality reads while retaining high-quality regions (Breese and Liu, 2013; Juan et al., 2013; Todd et al., 2013).

2.5 Sequence Alignment

We used BFAST (http://bfast.sourceforge.net) (Homer et al., 2009) as our primary alignment algorithm because it has high sensitivity for aligning the reads on the loci containing small insertions and deletions compared to the reference genome (rn4). We used a TopHat-like strategy (Trapnell et al., 2009) to align the sequencing reads that cross splicing junctions using NGSUtils (http://ngsutils.org/) (Breese and Liu, 2013). After aligning the sequence reads to a filtering index including repeats, ribosome RNA, and other sequences that are not of interest, we conducted a sequence alignment for three levels: genome, known junctions (University of California Santa Cruz Genome Browser), and novel junctions (based on the enriched regions identified in the genomic alignment). We restricted our analysis to the uniquely aligned sequences with no more than two mismatches.

2.6 Analysis

Differential Expression between the drinking and water groups was determined using the R package EdgeR with continuous dispersion (Robinson et al., 2010). FDR was calculated according to Benjamini and Hochberg (1995).

Differentially expressed genes were analyzed using the Winter 2013 Release of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Pathway, Functional and Upstream analysis were performed.

2.7 Convergence Analysis

We assembled a list of genes that were identified in GWAS studies (Bierut et al., 2010; Edenberg et al., 2010; Hack et al., 2011; Johnson et al., 2011; Kendler et al., 2011; Lind et al., 2010; Treutlein et al., 2009; Wang et al., 2013; Zlojutro et al., 2011; Zuo et al., 2012) and postmortem studies of several brain regions. The human post-mortem studies examined superior frontal cortex (Lewohl et al., 2000; Liu et al., 2007; Liu et al., 2006), frontal cortex (Liu et al., 2007; Mayfield et al., 2002), prefrontal cortex (Flatscher-Bader et al., 2005; Iwamoto et al., 2004), temporal cortex (Sokolov et al., 2003), motor cortex (Mayfield et al., 2002) nucleus accumbens and ventral tegmental area (Flatscher-Bader et al., 2010; Flatscher-Bader et al., 2005), basolateral amygdala (Kryger and Wilce, 2010) and hippocampus (McClintick et al., 2013; Zhou et al., 2011).

The animal gene expression studies used for comparison were all P/NP studies done by our group. These included comparisons between naïve P and NP animals, as well as P animals exposed (or not) to alcohol using various models of drinking, different ages and gender. These previous studies predominantly used the hippocampus, ventral tegmental area, accumbens, amygdala or sub-regions of these areas. (Bell et al., 2009; Kimpel et al., 2007; McBride et al., 2013a; McBride et al., 2012; McBride et al., 2013b; McBride et al., 2010; Rodd et al., 2008). None examined the DRN. Genes from microarray studies using the central amygdala and the accumbens shell from these same animals (McBride et al., 2014) are noted specifically. Genes from these 3 types of analyses (GWAS, post-mortem human and P/NP animal studies) were matched to the DRN data by gene symbol.

3. Results

3.1 RNA-seq results

We used RNA-seq to examine the pattern of gene expression in the DRN of peri-adolescent male P rats that had consumed large amounts of ethanol in a repeated binge pattern over a 3-week period (post-natal days 28 to day 49). Post-natal day 28 coincides with the beginning of adolescence, which ends at approximately day 42 in female rats and day 45 in male rats (Bell et al., 2013; Spear, 2010). The average consumption was approximately 8 g/kg per day for the 5 drinking days of each week (McBride et al., 2014). These levels of intake meet the criterion for binge-drinking put forth by the National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2004), blood ethanol levels ≥ 80mg%. There were 12,047 genes detectably expressed in the DRN, of which 3,567 genes (30%) were differentially expressed between ethanol exposed and control animals (at FDR ≤ 0.05; Supplementary Table 1 lists all genes with differential expression). Among the differentially expressed genes, 1391 (39%) had absolute fold changes >1.5 (Figure 1).

The expression levels of genes encoding biosynthesis and transport of many neurotransmitters were altered, along with genes encoding their receptors (Table 1; Supplementary Table 1). Genes for the serotonin pathway were changed the most (Table 1), with 21 of 23 expressed genes showing decreased expression, including tryptophan hydroxylase, dopa decarboxylase, serotonin transporter *Slc6a4*, vesicular monoamine transporter *Slc18a2* and organic ion transporter *Slc22a3*. Expression of genes for other neurotransmitter systems was also altered (Table 1). Among the 12 $GABA_A$ receptors expressed in the DRN, 8 were decreased and 2 were increased. GABA transporters *Slc6a1* and *Slc6a13* were also increased. The glutamate system showed decreases in many receptor genes, but large increases in the expression of *Grin2c* (2.6-fold), *Grm1* (2.0-fold) and *Grm4* (3.0-fold) as well as increases in glutamate transporters *Slc1a6* and *Slc1a3*. *Drd1a* was decreased, as were the genes for synthesizing dopamine, the vesicular monoamine transporter VMAT2 (*Slc18a2*) and several protein phosphatases downstream of dopamine receptors.

Expression of genes for neuropeptides and their receptors was also altered (Table 2; Supplementary Table 1). In many cases the expression of the neuropeptide and receptors had parallel changes. *Pdyn* (prodynorphin) and its receptor *Oprk1* were both decreased, as was *Pnoc* (prepronociceptin) and its receptor *Oprl1*, galanin and two of its receptors, and

Neuromedin B and its receptor. For others, there appeared to be compensatory changes, with the expression of the peptide and its receptors changing in opposite directions. Neuropeptide Y (*Npy*) was increased (+1.6 fold) but its receptors *Npy1r* and *Npy5r* were both decreased (−1.6 fold). There was a 3-fold increase in the gene expression for hypocretin (orexin), but expression of both hypocretin receptors was decreased about 2-fold. Expression of *Cck* (cholecystokinin) was increased but its B receptor gene was decreased. There was a small decrease in expression of *Sst* (somatostatin) with decreases in two of its receptors (*Sstr1* and *Sstr2*) and a large increase in a third receptor, *Sstr3*. The two corticotropin-releasing hormone (Crh) receptors were differentially altered, with *Crhr1* increased and *Crhr2* decreased.

Expression of many ion channel genes was altered (Table 3): 32 of 67 potassium channels were differentially expressed, with 21 increased and 11 decreased; *Kcnj6* was increased 30%. Twelve calcium channels showed altered expression, with equal numbers increased and decreased. Three sodium channels were increased and 6 decreased. Two chloride channel genes were increased and 4 decreased.

Many genes involved in myelin production were decreased by 30 to 60% (Supplementary Table 1). Similar decreases in multiple brain regions have been reported multiple times in human post-mortem studies (Liu et al., 2006a; McClintick et al., 2013) and animal models (Alfonso-Loeches et al., 2012).

3.2 Pathway Analysis

Ingenuity pathway analysis (IPA) identified 153 pathways that changed significantly as a result of adolescent ethanol binge-drinking (at FDR ≤ 0.05; Supplementary Table 2; selected subset in Table 4). Neurotransmitter pathways were significantly altered, especially the serotonin pathway (Table 4). Pathways related to cAMP and CREB signaling were also altered, along with pathways involved in axonal guidance. Inflammatory pathways and pathways related to oxidative stress, such as Hif1α and NRF2-mediated stress responses, were altered. Pathways related to neuropeptides *Crh* and cholecystokinin/gastrin and to several growth factors *(Bmp, Igf1, Ngf, Hgf, Pedf, Fgf* and *Egf*) were altered, as were pathways for transcription factors related to PPAR2, RAR, LXR/RXR, and TR/RXR (Table 4). Several genes were found in a large percentage of the significantly altered pathways, including *Crebbp*, *Jun* and *Fo*s, MAP kinases, a group of protein kinase A and C genes, and two NFκB subunits (*Rela* and *Nfkb2)* (Supplementary Table 3).

IPA analysis of upstream regulators provides further evidence for a strong pro-inflammatory response: NFκB and 11 cytokines genes were activated and *Il1rn* (an antagonist of the IL1 receptor) was decreased (Supplementary Table 4). This analysis also provided additional evidence for increased signaling by CREB and cAMP, and evidence for increased calcium signaling. Growth factors *Vegfa*, TGFβ1, *Bmp4, Kitlg, and Grp* were all activated. Stearate biosynthesis and multiple cholesterol pathways, particularly those downstream of *Srebf1* and *Srebf2*, all had decreased expression (Supplementary Table 4).

3.3 Converging evidence

To look for converging evidence from genetic and genomic studies, we compiled a list of genes whose expression is altered in humans, genes implicated by genome wide association studies (GWAS), and genes whose expression was altered in previous studies of alcohol preferring and non-preferring rats (P and NP, including both naïve states and P animals exposed to alcohol). Many genes in the present study overlapped with the previously implicated genes (Figure 2); specific genes are marked in Supplementary Table 1, which has references for all 29 studies used. Since we were interested in how the present data could inform human studies, we examined which pathways were significantly overrepresented from the 713 genes found in the intersection between this study and the human studies, using IPA. 36 pathways were altered (FDR ≤ 0.10, Supplementary Table 5). These include signaling pathways related to serotonin, cAMP, CREB, oxidative stress, dopamine, Crh and axonal guidance. Fos and Jun, which were included in many of the altered pathways, have also been frequently identified in other experiments using P animals and alcohol (Supplementary Table 1). Among the genes in the intersection, 564 were found in the human post-mortem studies even though most examined frontal cortex and none measured the DRN. Among these are several genes related to myelin, which have decreased expression in the DRN as they had in multiple post-mortem human studies (e.g. Mayfield et al., 2002; McClintick et al., 2013).

4. Discussion

We used RNA sequencing to examine the effect of binge drinking of ethanol during adolescence on gene expression profiles in the dorsal raphe nucleus (DRN), which has not previously been studied in a binge drinking adolescent model. The DRN is a central serotonergic region of the brain (Waselus et al., 2011). It has serotonergic efferents to the reward centers in the brain and is implicated in stress, anxiety and depression (Hale et al., 2012; Waselus et al., 2011). Adolescent P rats consumed 8–10 g ethanol/kg of body weight/day during the three 1-hour sessions, and did this repeatedly for 5 consecutive days during 3 weeks, with a 2-day withdrawal period after each of the initial two weeks (McBride et al., 2014). This repeated binge drinking of ethanol had a profound effect on gene expression in the DRN, with 30% of the identified genes differentially expressed between the ethanol drinking and control animals (Supplementary Table 1). Key functional groups of genes that were altered in expression included neurotransmitter systems (Table 1), neuropeptide systems (Table 2), ion channels (Table 3), and genes related to cAMP, CREB, inflammation, oxidative stress, axonal guidance as well as many growth factors and transcription factors (Table 4, Supplementary Table 2). Pathways identified from genes overlapping with human studies (Supplementary Table 5) highlight pathways related to oxidative stress, inflammation, cAMP, CREB, axonal guidance, dopamine and serotonin.

The DRN is a main origin of the serotonin system, so it is significant that all of the 21 differentially expressed genes in the serotonin pathway are decreased after the repeated binge drinking. This could reflect direct down-regulation of the serotonin system, or the retarded development of the serotonin system. Rodents prenatally exposed to alcohol have decreased serotonin and serotonin metabolism that persists into adulthood resulting in

impaired impulse control and changes in other serotonin dependent related behaviors (Macri et al., 2007). If the decrease in serotonin function for these binge drinking adolescents persists into adulthood, these animals could have similar outcomes.

Among the 12 $GABA_A$ genes expressed in the DRN, 8 $GABA_A$ subunits showed reduced expression. Two subunits, *Gabra6* and *Gabrd*, showed >4-fold increases. Thus, the balance of $GABA_A$ subunits was significantly altered, suggesting altered local inhibitory regulation within the DRN. The *Gabrd* subunit is found in extrasynaptic $GABA_A$ receptors composed primarily of α4 or α6 subunits along with β subunits (Lovinger and Roberto, 2013), which generate tonic inhibitory conductance that results in spontaneous low firing rates (Hanchar et al., 2005). These receptors are sensitive to ethanol, which potentiates this tonic current in a protein kinase C delta dependent manner (Lovinger and Roberto, 2013). *Prkcd* is also increased 2.4 fold. *Gad1* (encoding glutamate decarboxylase 1, a rate-limiting enzyme for GABA synthesis) showed significant increases in expression following binge-like drinking; polymorphisms in *Gad1* are associated with age of onset for alcohol dependence and initial alcohol sensitivity (Kuo et al., 2009).

NMDA receptors vary in their response to ethanol depending on which subunits make up the receptor, reviewed in (Lovinger and Roberto, 2013). Binge drinking decreased the expression of *Grin2b* (encoding NR2B; −1.6-fold) and increased *Grin2c* (encoding NR2C, +2.6-fold) (Table 1). NR2C-containing receptors are less sensitive to inhibition by ethanol than those that contain NR2B (Lovinger and Roberto, 2013), so this shift in ratio should result in decreased sensitivity of the NMDA receptors to ethanol in the DRN, decreasing its inhibitory effects and potentially increasing tolerance to ethanol's intoxicating effects. Furthermore, the net increased expression of glutamate receptor genes in combination with a net decreased expression of $GABA_A$ receptor genes (Table 1) could result in a higher excitatory tone regulating the DRN.

Many neuropeptide systems in the DRN, particularly those related to anxiety and stress (*Npy*, *Crh*, galanin, hypocretin/oxytocin – reviewed in Kormos and Gaszner (2013), were altered by binge drinking (Table 2). *Npy* and *Crh* work in opposition and must be balanced to maintain homeostasis and resistance to stress (Gilpin, 2012; Sajdyk et al., 2004; Valdez and Koob, 2004). *Npy* is increased but receptors *Npy1r* and *Npy5r* are decreased, perhaps as an adaptation to increased *Npy*. In contrast to the DRN, *Npy5r* was found to be increased in both the accumbens shell (Acb-sh) and central nucleus of the amygdala (CeA) of these same animals (McBride et al., 2014). *Crh* is anxiogenic (Valdez and Koob, 2004). P rats have lower *Crh* in multiple brain regions, increased *Crhr1* receptors (Ehlers et al., 1992), and decreased levels of *Crhr2* in several regions (Yong et al., 2014). These two receptors have different affinities for *Crh*, with *Crhr1* binding *Crh* at low concentrations and *Crhr2* binding only at high concentrations. *Crhr1* is activated by acute stress and initiates decreased serotonin release and active coping, while *Crhr2* is activated by repeated stress and increases serotonin release and passive coping (Valentino, Lucki et al. 2010). In the DRN of these binge drinking adolescent animals, *Crhr1* increased 3 fold and *Crhr2* decreased 1.6 fold, which could contribute to decreased serotonin levels in these animals.

Galanin mainly has a hyperpolarizing inhibitory effect and is implicated in anxiety, stress, depression and drug addiction, reviewed in Holmes and Picciotto (2006). Galanin and its receptors were decreased about 2 fold indicating a decreased function for this peptide in the DRN. Galanin receptors on serotonin neurons in the DRN can have an inhibitory effect on serotonin signaling (Holmes and Picciotto, 2006). *Galr1* is linked to Gi/Go proteins which inhibit adenylyl cyclase. The decrease in Galanin and the receptor may contribute to the overall increase in CREB signaling seen in the DRN of these animals.

Hypocretin is mainly expressed in the lateral hypothalamus. Unexpectedly, the mRNA for hypocretin was detected in the DRN and was 3 fold higher in the binge-drinking rats. The hypocretin mRNA may be in those axons that project from the hypothalamus to innervate the DRN (Xu et al., 2013). Although there is no difference in hypocretin mRNA levels between naïve iP and iNP animals, alcohol drinking increased the hypocretin mRNA levels in iP rats (Lawrence et al., 2006). A hypocretin antagonist (SB-334867) caused a significant decrease in response to ethanol in a cue induced reinstatement experiment (Lawrence et al., 2006). The DRN provides negative feedback via serotonergic projections to the lateral hypothalamus (Xu et al., 2013), so decreased serotonin signaling from the DRN could be expected to lead to decreased inhibition of hypocretin signaling. Both hypocretin receptors (*Hcrtr1* and *Hcrtr2*) were decreased about 2 fold, which could intensify this effect.

There is strong evidence for a neuroinflammatory response, with NFκB and multiple cytokines activated, altering expression of many of their downstream targets including the acute phase response pathway. Chronic alcoholics are known to have highly activated NFκB signaling in the hippocampus (McClintick et al., 2013) and NFκB can be instrumental in the addiction process (Crews et al., 2011). There is evidence for increased oxidative stress which can accompany inflammatory responses (Table 4). There were also indications of an inflammatory response in the Acb-sh of these animals but not the CeA (McBride et al., 2014) with acute phase signaling and the IL-2 pathway altered in Acb-sh. The overlap analysis with the human data (Figure 2, Supplementary Tables 1 & 5) supports previous findings that both oxidative stress and inflammation are important factors in alcohol use disorders in humans.

CREB signaling is increased in the DRN, with the expression of 67 downstream targets altered (Supplementary Table 4). CREB must be phosphorylated to be active and acute alcohol increases phosphorylated CREB (p-CREB) in the CeA and medial amygdala in P but not NP rats (Pandey et al., 2005). Increasing p-CREB in the CeA of P rats, by activating protein kinase A, decreases anxiety and ethanol consumption (Pandey et al., 2005). CREB signaling activates genes necessary for the survival of neurons under stress (Volakakis et al., 2010). CREB can be activated by multiple kinases, several of which are themselves altered, e.g. *Camk4* (3.0-fold), *Camk2b* (1.3-fold), and Protein Kinase A (*Prkaca* −1.3-fold). Genes induced by CREB include NR4A nuclear receptors (*Nr4a1*, *Nr4a2* and *Nr4a3*), with expression increased 3- to 6-fold in the binge-drinking adolescents. These receptors are necessary for the increased transcription of some of the neuroprotective genes downstream of CREB (Volakakis et al., 2010). *Ppargc1a* expression is also increased, and it is responsible for inducing another set of neuroprotective genes that respond to oxidative stress (Volakakis et al., 2010). The increased CREB signaling could in part be responding to

oxidative or excitotoxic stress in these animals. The acumbens shell also showed increased CREB signaling in these animals, but the central amygdala did not (McBride et al., 2014). Chronic alcohol has been shown to decrease the phosphorylation of CREB, in other brain regions (Crews and Nixon, 2009). cAMP-mediated signaling was affected in both the acumbens shell and central amygdala of these animals(McBride et al., 2014). The overlap analysis with human data (Supplementary Table 1 & 5, Figure 2) suggests that cAMP and CREB signaling also play an important role in alcohol use disorders in humans.

There were 34 K+ channel subunits that had differential gene expression with a 2 to 1 ratio of increased expression with adolescent binge drinking. A double knockout of *Kcnc1* (+1.5) and *Kcnc3* (+3.1) in mice is related to hyperactivity and alcohol hypersensitivity (Espinosa et al., 2001). Variants in *Kcnj6* (+1.3), an inwardly rectifying potassium channel, were found to be associated with an electrophysiological endophentype related to alcohol dependence (Kang et al., 2012). *Kcnh3* had a 4.5 fold increase; a knockout of this gene in mice is associated with hippocampal hyperexciteability and seizures (Zhang et al., 2010). On the other hand, 6 of 9 Na+ channel subunit genes had reduced expression with adolescent binge drinking. Overall, these results suggest changes in neuronal firing and interactions within the DRN that could alter the function of regions receiving serotonin inputs from the DRN.

The repeated binge paradigm had its major impact on the DRN. The CeA and Acb-sh of these same animals showed many fewer genes with altered expression, 309 in the central amygdala and 154 in the Acb-sh (McBride et al., 2014). Sixteen of the 22 altered pathways in the Acb-sh and 3 of the 4 in CeA were also altered in the DRN (noted in Table 4 and Supplementary Table 2). The large difference in numbers of altered genes might be in part attributable to the differences in the cell populations of these 3 regions; the DRN is more focused on the serotonin system, with nearly 50% of the neurons serotonergic (Waselus et al., 2011). More heterogeneity in the other regions could dilute the changes and make them more difficult to identify. Also, differences in the postnatal development of the DRN compared to the other regions may make it more vulnerable to ethanol. Another potential contributor to the difference among the regions is the different technology used to analyze those regions; the RNA sequencing used here may be more sensitive to changes. Technical differences cannot, however, be the primary reason because additional regions also examined by RNA sequencing showed many fewer alterations than the DRN (unpublished data).

5. Conclusion

The DRN is an important region that integrates inputs from many systems, and can have far-reaching effects via the serotonergic projections. Binge drinking in adolescent P rats altered the expression of nearly 30% of the genes in the DRN, suggesting major alterations in DRN functioning. Numerous systems are affected, including multiple neurotransmitter systems, prominent among them the serotonin system (Table 1). Because the DRN has efferents to many brain regions, including the reward systems, the down-regulation of the serotonin system could have far-reaching effects on the brain and its response to alcohol. Neuropeptide systems (Table 2) are also prominently altered, in ways that indicate increased anxiety and decreased tolerance to stress at this time point, 3 h after the last exposure to

alcohol. Stress and oxidative damage responses were also greatly affected. The large effects of repeated binges on gene expression in this key region could have major impacts on responses to alcohol and other stressors, which might persist into adulthood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Acb-sh	nucleus accumbens shell
CeA	central nucleus of the amygdala
CREB	cAMP response element-binding protein
DRN	dorsal raphe nucleus
HAD/LAD	High Alcohol and Low Alcohol Drinking rats
p-CREB	phosphorylated CREB
P/NP	Alcohol Preferring and Non-Preferring rats

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Highlights

- Adolescent binge drinking altered gene expression in the rat Dorsal Raphe Nucleus.
- Serotonin gene expression in the DRN was decreased by adolescent binge drinking.
- Expression of 8 GABA receptor subunits was decreased by adolescent binge drinking.
- Neuropeptide systems related to stress/anxiety are dysregulated by binge drinking.
- CREB signaling is activated by adolescent binge drinking.

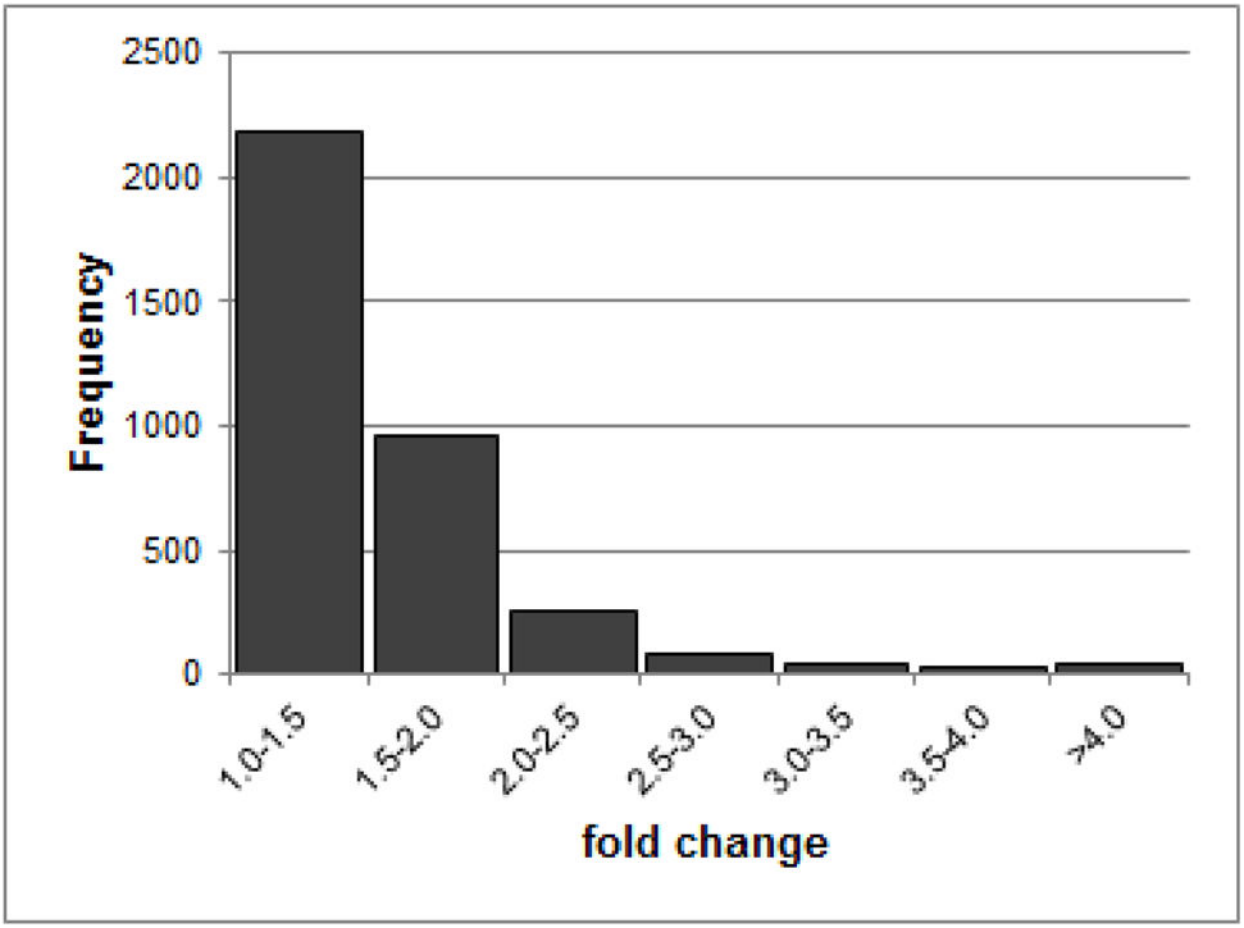

Figure 1.
Distribution of fold changes of genes with an FDR ≤ 0.05.

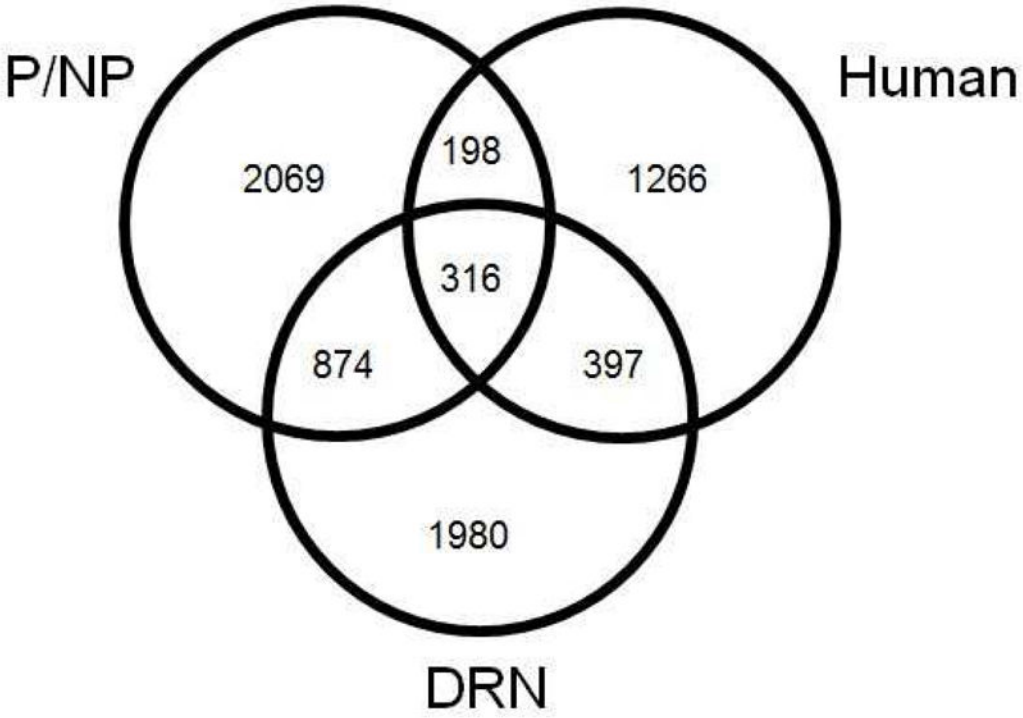

Figure 2. Overlap of present data with human and other studies using P rats

Venn diagram indicating overlap of differentially expressed genes from this study (within the DRN of adolescent binge ethanol-drinking P rats) with previous P/NP animal studies (includes expression studies of naïve P vs. naïve NP and alcohol treated P vs. controls) and human studies (postmortem expression and GWAS). Specific genes and studies are noted in Supplementary Table 1.

Table 1
Neurotransmitter genes differentially expressed

Neurotransmitter related genes whose expression is altered by adolescent ethanol binge-drinking (FDR<0.05)

GENE	Fold Change	Gene Name
Serotonin		
Htr1a	−1.6	5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled
Htr1b	−1.28	5-hydroxytryptamine (serotonin) receptor 1B, G protein-coupled
Htr1d	−1.65	5-hydroxytryptamine (serotonin) receptor 1D, G protein-coupled
Htr2a	−1.4	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled
Htr2c	−1.7	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled
Htr3a	−1.58	5-hydroxytryptamine (serotonin) receptor 3A, ionotropic
Htr4	−1.8	5-hydroxytryptamine (serotonin) receptor 4, G protein-coupled
Htr5a	−1.48	5-hydroxytryptamine (serotonin) receptor 5A, G protein-coupled
Htr5b	−2.15	5-hydroxytryptamine (serotonin) receptor 5B, G protein-coupled
Slc6a4	−2.06	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4, also 5-HTT and SERT1
Slc18a2	−2.1	solute carrier family 18 (vesicular monoamine), member 2, also VMAT2
Slc22a3	−2.26	solute carrier family 22 (extraneuronal monoamine transporter), member 3 (post-synaptic)
Maoa	−1.7	monoamine oxidase A
Maob	−1.48	monoamine oxidase B
Tph2	−2.59	tryptophan hydroxylase 2
Tph1	−2.33	tryptophan hydroxylase 1
Ddc	−2.06	dopa decarboxylase (aromatic L-amino acid decarboxylase)
Gch1	−2.17	GTP cyclohydrolase 1
Pcbd1	−1.68	pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha
Pts	-−1.51	6-pyruvoyltetrahydropterin synthase
Qdpr	−1.56	quinoid dihydropteridine reductase
GABA		
Gabarapl1	−1.33	GABA(A) receptor-associated protein like 1
Gabra2	−1.62	gamma-aminobutyric acid (GABA) A receptor, alpha 2
Gabra3	−1.69	gamma-aminobutyric acid (GABA) A receptor, alpha 3
Gabra5	−1.8	gamma-aminobutyric acid (GABA) A receptor, alpha 5
Gabra6	4.53	gamma-aminobutyric acid (GABA) A receptor, alpha 6
Gabrb1	−1.32	gamma-aminobutyric acid (GABA) A receptor, beta 1
Gabrd	4.34	gamma-aminobutyric acid (GABA) A receptor, delta
Gabre	−2.27	gamma-aminobutyric acid (GABA) A receptor, epsilon
Gabrg1	−1.22	gamma-aminobutyric acid (GABA) A receptor, gamma 1
Gabrg3	−1.42	gamma-aminobutyric acid (GABA) A receptor, gamma 3
Gabrq	−2.45	gamma-aminobutyric acid (GABA) A receptor, theta
Gad1	1.33	glutamate decarboxylase 1 (brain, 67kDa)
Slc6a1	1.27	solute carrier family 6 (neurotransmitter transporter, GABA
Slc6a13	1.8	solute carrier family 6 (neurotransmitter transporter) member 13, also GAT2

GENE	Fold Change	Gene Name
Glutamate		
Gria3	1.29	glutamate receptor, ionotropic, AMPA 3
Gria4	1.31	glutamate receptor, ionotropic, AMPA 4
Grid2	2.31	glutamate receptor, ionotropic, delta 2
Grid2ip	3.49	glutamate receptor, ionotropic, delta 2 (Grid2) interacting protein
Grik3	1.35	glutamate receptor, ionotropic, kainate 3
Grin2b	−1.56	glutamate receptor, ionotropic, N-methyl D-aspartate 2B
Grin2c	2.61	glutamate receptor, ionotropic, N-methyl D-aspartate 2C
Grin2d	−1.4	glutamate receptor, ionotropic, N-methyl D-aspartate 2D
Grin3a	−1.39	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A
Grinl1a	−1.32	GRINL1A downstream protein Gdown1 (Grinl1a)
Grip1	−1.36	glutamate receptor interacting protein 1
Grip2	−1.58	glutamate receptor interacting protein 2
Grm1	2.06	glutamate receptor, metabotropic 1
Grm4	2.98	glutamate receptor, metabotropic 4
Grm6	−1.7	glutamate receptor, metabotropic 6
Slc1a3	1.7	solute carrier family 1 (glial high affinity glutamate transporter), member 3, also EAAT1
Slc1a6	2.79	solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6, also EAAT4
Slc25a18	1.45	solute carrier family 25 (mitochondrial carrier: glutamate), member 22
Slc25a22	1.3	solute carrier family 25 (glutamate carrier), member 18
Glycine		
Glra1	−1.75	glycine receptor, alpha 1
Glra2	−2.02	glycine receptor, alpha 2
Glra3	−1.56	glycine receptor, alpha 3
Glrb	−1.24	glycine receptor, beta
Cholinergic		
Chrna4	−1.48	cholinergic receptor, nicotinic, alpha 4 (neuronal)
Chrna7	−1.37	cholinergic receptor, nicotinic, alpha 7 (neuronal)
Chrnb3	−2.44	cholinergic receptor, nicotinic, beta 3 (neuronal)
Slc18a3	1.61	solute carrier family 18 (vesicular acetylcholine), member 3, also VAChT
Adrenergic		
Adra1b	−1.59	adrenoceptor alpha 1B
Adra1d	3.8	adrenoceptor alpha 1D
Adrb2	1.4	adrenoceptor beta 2, surface
Adrbk2	1.57	adrenergic, beta, receptor kinase 2
Purinergic		
P2rx2	−1.85	purinergic receptor P2X, ligand-gated ion channel, 2

GENE	Fold Change	Gene Name
P2rx4	1.56	purinergic receptor P2X, ligand-gated ion channel, 4
P2rx6	1.86	purinergic receptor P2X, ligand-gated ion channel, 6
P2ry12	−1.36	purinergic receptor P2Y, G-protein coupled, 12
Dopamine		
Drd1a	−1.36	dopamine receptor D1
Canabinoid		
Cnr1	2.16	cannabinoid receptor 1 (brain)

Table 2

Differentially expressed neuropeptides

Neuropeptide and receptor genes whose expression is altered by adolescent ethanol binge-drinking (FDR<0.05)

Gene	Fold Change	Gene Name
Parallel changes		
Pdyn	−1.5	Preprodynorphin
Oprk1	−1.4	Kappa opioid receptor1
Pnoc	−1.3	Prepronociceptin
Oprl1	−1.5	Opioid receptor like 1
Gal	−2.1	Galanin
Galr1	−2	galanin receptor 1
Galr2	−1.8	galanin receptor 2
Nmbr	−2.3	Neuromedin B
Nmbr	−1.7	Neuromedin B receptor
Nts	−1.8	Neurotensin
Ntsr1	−1.4	Neurotensin receptor 1
Compensatory changes		
Npy	1.6	Neuropeptide Y
Npy1r	−1.6	NPY receptor 1
Npy5r	−1.6	NPY receptor 5
Hcrt	3	hypocretin
Hcrtr1	−2	hypocretin receptor 1
Hcrtr2	−2	hypocretin receptor 2
Cck	1.5	Cholecystokinin
Cckbr	−1.9	B receptor for cholecystokinin
Sst	−1.3	Somatostatin
Sstr1	−1.6	Somatostatin receptor 1
Sstr2	−1.6	Somatostatin receptor 2
Sstr3	3	Somatostatin receptor 3
Other		
Oprm1	−1.6	Mu opioid receptor
Oxtr	−1.6	Oxytocin receptor
Tacr1	−1.8	Tachykinin receptor 1
Tacr3	1.3	Tachykinin receptor 3
Nmur2	−1.8	Neuromedin U receptor 2
Crhr1	3	corticotropin releasing hormone receptor1
Crhr2	−1.6	corticotropin releasing hormone receptor2
Mc4r	−2	melanocortin receptor 4

Table 3

Differentially expressed Ion Channels

Ion Channel genes whose expression is altered by adolescent ethanol binge-drinking (FDR< 0.05)

GENE	Fold Change	Gene Name
Potassium channels		34 of 67 identified
Kcna1	1.35	potassium voltage-gated channel, shaker-related subfamily, member
Kcna3	−1.48	potassium voltage-gated channel, shaker-related subfamily, member 3
Kcna4	−1.41	potassium voltage-gated channel, shaker-related subfamily, member 4
Kcna6	−1.35	potassium voltage-gated channel, shaker-related subfamily, member 6
Kcnab1	1.42	potassium voltage-gated channel, shaker-related subfamily, beta member 1
Kcnab3	1.4	potassium voltage-gated channel, shaker-related subfamily, beta member 3
Kcnc1	1.51	potassium voltage-gated channel, Shaw-related subfamily, member 1
Kcnc2	−1.24	potassium voltage-gated channel, Shaw-related subfamily, member 2
Kcnc3	3.08	potassium voltage-gated channel, Shaw-related subfamily, member 3
Kcnd2	1.63	potassium voltage-gated channel, Shal-related subfamily, member 2
Kcng1	1.79	potassium voltage-gated channel, subfamily G, member 1
Kcng4	2.15	potassium voltage-gated channel, subfamily G, member 4
Kcnh1	2.24	potassium voltage-gated channel, subfamily H (eag-related), member 1
Kcnh3	4.54	potassium voltage-gated channel, subfamily H (eag-related), member 3
Kcnh5	−1.4	potassium voltage-gated channel, subfamily H (eag-related), member 5
Kcnj12	2.64	potassium inwardly-rectifying channel, subfamily J, member 12
Kcnj3	1.7	potassium inwardly-rectifying channel, subfamily J, member 3
Kcnj6	1.31	potassium inwardly-rectifying channel, subfamily J, member 6
Kcnk1	1.51	potassium channel, subfamily K, member 1
Kcnk10	1.35	potassium channel, subfamily K, member 10
Kcnk12	3.06	potassium channel, subfamily K, member 12
Kcnk16	2.64	potassium channel, subfamily K, member 16
Kcnk2	−1.51	potassium channel, subfamily K, member 2
Kcnk3	1.6	potassium channel, subfamily K, member 3
Kcnk9	−1.6	potassium channel, subfamily K, member 9
Kcnmb4	1.44	potassium large conductance calcium-activated channel, subfamily M, beta member 4
Kcnn3	−1.73	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3
Kcnq3	−1.39	potassium voltage-gated channel, KQT-like subfamily, member 3
Kcns3	−1.47	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3
Kcnt1	1.74	potassium channel, subfamily T, member 1
Kcnt2	−1.61	potassium channel, subfamily T, member 2
Kcnv1	1.88	potassium channel, subfamily V, member 1
Sodium Channels		9 of 14 identified
Scn1b	2.08	sodium channel, voltage-gated, type I, beta subunit
Scn3a	−1.46	sodium channel, voltage-gated, type III, alpha subunit
Scn3b	−1.63	sodium channel, voltage-gated, type III, beta subunit

GENE	Fold Change	Gene Name
Scn4a	−1.67	sodium channel, voltage-gated, type IV, alpha subunit
Scn4b	1.9	sodium channel, voltage-gated, type IV, beta subunit
Scn5a	−1.63	sodium channel, voltage-gated, type V, alpha subunit
Scn8a	1.22	sodium channel, voltage gated, type VIII, alpha subunit
Scn9a	−1.91	sodium channel, voltage-gated, type IX, alpha subunit
Scnn1a	−1.45	sodium channel, non-voltage-gated 1 alpha subunit
Calcium Channels		12 of 21 identified
Cacna1a	1.53	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit/
Cacna1b	−1.25	calcium channel, voltage-dependent, N type, alpha 1B subunit
Cacna1d	1.22	calcium channel, voltage-dependent, L type, alpha 1D subu
Cacna1g	1.76	calcium channel, voltage-dependent, T type, alpha 1G subunit
Cacna1h	−1.39	calcium channel, voltage-dependent, T type, alpha 1H subu
Cacna1i	1.49	calcium channel, voltage-dependent, T type, alpha 1I subunit
Cacna2d1	−1.47	calcium channel, voltage-dependent, alpha 2/delta subunit 1
Cacnb1	−1.26	calcium channel, voltage-dependent, beta 1 subunit
Cacnb4	1.69	calcium channel, voltage-dependent, beta 4 subunit
Cacng3	−1.78	calcium channel, voltage-dependent, gamma subunit 3
Cacng7	1.25	calcium channel, voltage-dependent, gamma subunit 7
Cacng8	−1.39	calcium channel, voltage-dependent, gamma subunit 8
Chloride Channels		6 of 14 identified
Clcn5	−1.55	chloride channel, voltage-sensitive 5
Clcn6	−1.32	chloride channel, voltage-sensitive 6
Clcnkb	−2.46	chloride channel, voltage-sensitive Kb
Clic1	1.61	chloride intracellular channel 1
Clic2	−1.43	chloride intracellular channel 2
Clic6	2.93	chloride intracellular channel 6

Table 4
Selected pathways altered by adolescent ethanol binge-drinking

Genes with FDR ≤ 0.05 (from Supplementary Table 1) were analyzed using Ingenuity Pathway Analysis; the full list of pathways that were significantly altered (FDR ≤ 0.05) and the genes within each pathway is in Supplementary Table 2. This is a subset selected for putative relevance.

Ingenuity Canonical Pathways	FDR	[1] Affected in ACB-sh or CEA
Neurotransmitters		
Serotonin Receptor Signaling	1.70E-07	
Dopamine Receptor Signaling	9.30E-04	
GABA Receptor Signaling	1.70E-03	
Glutamate Receptor Signaling	1.70E-03	
Dopamine Degradation	3.10E-02	
α-Adrenergic Signaling	3.10E-02	
Neuropeptides		
Corticotropin Releasing Hormone Signaling	7.10E-04	
Cholecystokinin/Gastrin-mediated Signaling	1.40E-03	
cAMP, Creb related		
Protein Kinase A Signaling	1.30E-05	Acb-sh
cAMP-mediated signaling	4.30E-05	both
Gα12/13 Signaling	4.30E-05	
Dopamine-DARPP32 Feedback in cAMP Signaling	2.10E-04	
Gαi Signaling	3.50E-04	
CREB Signaling in Neurons	9.10E-04	
P2Y Purigenic Receptor Signaling Pathway	1.10E-03	
Calcium Transport I	1.90E-03	
Calcium Signaling	3.20E-03	
Gαq Signaling	4.40E-03	
Gαs Signaling	4.70E-02	
Inflammation		
LPS/IL-1 Mediated Inhibition of RXR Function	4.30E-05	
CXCR4 Signaling	9.50E-04	
Acute Phase Response Signaling	1.10E-03	Acb-sh
LPS-stimulated MAPK Signaling	2.00E-03	
IL-1 Signaling	2.90E-03	
TNFR1 Signaling	3.70E-03	
IL-6 Signaling	5.50E-03	
CCR3 Signaling in Eosinophils	1.50E-02	
HMGB1 Signaling	1.90E-02	both
IL-10 Signaling	2.90E-02	

Ingenuity Canonical Pathways	FDR	[1] Affected in ACB-sh or CEA
IL-17 Signaling	4.70E-02	
IL-2 Signaling	4.80E-02	
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	2.90E-03	
Axonal Guidance		
Axonal Guidance Signaling	4.60E-09	
Ephrin Receptor Signaling	9.10E-03	
Netrin Signaling	4.70E-02	
Oxidative stress		
NRF2-mediated Oxidative Stress Response	3.20E-04	
Aryl Hydrocarbon Receptor Signaling	1.80E-03	Acb-sh
HIF1α Signaling	6.30E-03	
Angiopoietin Signaling	1.00E-02	
Erythropoietin Signaling	2.60E-02	
Growth factors		
BMP signaling pathway	3.20E-05	
IGF-1 Signaling	9.10E-04	Acb-sh
NGF Signaling	2.90E-03	
HGF Signaling	8.10E-03	
PEDF Signaling	1.30E-02	
FGF Signaling	1.50E-02	
EGF Signaling	2.10E-02	
Neurotrophin/TRK Signaling	2.90E-02	
TGF-β Signaling	2.30E-03	
Transcription Factors		
PPARα/RXRα Activation	1.00E-04	
RAR Activation	2.10E-04	Acb-sh
PPAR Signaling	5.50E-04	
LXR/RXR Activation	1.80E-03	
TR/RXR Activation	3.90E-02	
Signaling pathways		
Signaling by Rho Family GTPases	8.70E-05	
Rac Signaling	1.10E-03	
RhoGDI Signaling	2.90E-03	
p38 MAPK Signaling	3.10E-02	
G-Protein Coupled Receptor Signaling	8.50E-07	Acb-sh

[1] Identifies pathways also significantly altered in the accumbens shell or central amydala of these same animals