Gene expression changes in the ventral hippocampus and medial prefrontal cortex of adolescent alcohol-preferring (P) rats following binge-like-alcohol drinking.

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ABSTRACT

Binge drinking of alcohol during adolescence is a serious public health concern with long-term consequences, including decreased hippocampal and prefrontal cortex volume and defects in memory. We used RNA sequencing to assess the effects of adolescent binge drinking on gene expression in these regions. Male adolescent alcohol-preferring (P) rats were exposed to repeated binge drinking (three 1-hour sessions/d during the dark/cycle, 5 days/week for 3 weeks starting at 28 days of age; ethanol intakes of 2.5 to 3 g/kg/session). Ethanol significantly altered the expression of 416 of 11,727 genes expressed in the ventral hippocampus. Genes and pathways involved in neurogenesis, long-term potentiation and axonal guidance were decreased, which could relate to the impaired memory function found in subjects with adolescent alcohol binge-like exposure. The decreased expression of myelin and cholesterol genes and apparent decrease in oligodendrocytes in P rats could result in decreased myelination. In the medial prefrontal cortex, 638 of 11,579 genes were altered; genes in cellular stress and inflammatory pathways were increased, as were genes involved in oxidative phosphorylation. Overall, the results of this study suggest that adolescent binge-like alcohol drinking may alter the development of the ventral hippocampus and medial prefrontal cortex and produce long-term consequences on learning and memory, and on control of impulsive behaviors.

Keywords: hippocampus, prefrontal cortex, binge drinking, alcohol

Abbreviations:

P: alcohol preferring

vHip; ventral hippocampus

mPFC; medial prefrontal cortex

DRN: dorsal raphe nucleus

PAG: periaqueductal gray

CeA: central core of the amygdala

Acbshell: nucleus accumbens shell

LTP: long term potentiation

RPKM: reads per kilobase per million reads

FDR: false discovery rate

INTRODUCTION

Alcohol (ethanol) use is typically initiated during adolescence. More than 25% of 8th graders (13 years old) and 50% of 10th graders have used alcohol in the past year. Drinking, especially binge drinking, escalates during adolescence; 20% of 12th graders report consuming ≥ 5 drinks per occasion, and 10.5% consumed ≥ 10 drinks per occasion within the past 2 weeks (Spear, 2015). Adolescents are especially vulnerable to brain impairment by excessive ethanol exposure (Geil et al., 2014; Jacobus & Tapert, 2013; Spear, 2015).

College students given a memory task while intoxicated showed that those students with a previous history of binge drinking performed more poorly than other students (Weissenborn & Duka, 2003). Rats exposed to chronic intermittent ethanol as adolescents showed impaired memory when re-exposed to acute alcohol, whereas rats similarly exposed as adults did not (White, Ghia, Levin, & Swartzwelder, 2000). The hippocampus plays an essential role in episodic memory formation; the ventral hippocampus (vHip) communicates with multiple nuclei of the mesocorticolimbic and extended amygdala systems in this process (Alberini, 2013; Martinez J, 1998). Optogenetic techniques have demonstrated that the hippocampus, particularly the ventral portion of the dentate gyrus, is also involved in anxiety-like behavior (Kheirbek, Klemenhagen, Sahay, & Hen, 2012). Significant maturation of the brain occurs during adolescence, including neurogenesis, myelination, and selective pruning. In rodents, hippocampal neurogenesis is higher in adolescents than adults (He & Crews, 2007). Imaging studies have shown that adolescents with alcohol use disorders have smaller hippocampi (De Bellis et al., 2000; Nagel, Schweinsburg, Phan, & Tapert, 2005) and that the effect of alcohol on hippocampal volume is greater in adolescents than in adults (Geil et al., 2014; Jacobus & Tapert, 2013). Ethanol-induced reductions in hippocampal volume are due in part to inhibition of neurogenesis by alcohol (Morris, Eaves, Smith, & Nixon, 2010), particularly at the higher concentrations of alcohol experienced during alcohol binge exposure (Crews, Mdzinarishvili, Kim, He, & Nixon, 2006).

The medial prefrontal cortex (mPFC) plays many critical functions. It receives inputs from sensory areas of the brain, limbic systems and the hippocampus, which allows context specific decisions using these inputs to guide adaptive behavior (Euston, Gruber, & McNaughton, 2012). The maturation of the PFC continues from adolescence into early adulthood. This delayed maturation plays a role in the thrill-, risk- and novelty-seeking behavior seen in adolescence (Crews, Vetreno, Broadwater, & Robinson, 2016; Ernst & Fudge, 2009). Adolescents with alcohol use disorders have decreased white matter and grey matter in the prefrontal cortex (De

Bellis et al., 2005). The reduced prefrontal volume is associated with increased impulsivity, which can lead to poor decision-making and control (Crews & Nixon, 2009; Dalwani et al., 2011). Adolescent binge drinking in rats reduces prefrontal myelin (Vargas, Bengston, Gilpin, Whitcomb, & Richardson, 2014), and leads to a disruption of dopaminergic and GABAergic transmission in the adult mPFC, which can contribute to deficits in decision making in adults (Trantham-Davidson et al., 2016). Adult P rats exhibit higher impulsive-like behavior compared to non-selected rats (Beckwith & Czachowski, 2014, 2016).

We have used a selectively bred rat model of alcoholism that voluntarily drinks large quantities of ethanol to study the effects of binge ethanol drinking on adolescent neurobiology. The alcohol-preferring P rats consume alcohol for its CNS pharmacologic effects rather than for calories and meet criteria proposed for an animal model of alcoholism (reviewed in (McBride, Rodd, Bell, Lumeng, & Li, 2014). Studies using these animals have revealed important information on behaviors, brain function and transcriptomes affected by drinking ethanol (R. L. Bell, Rodd, Engleman, Toalston, & McBride, 2014; McBride, Kimpel, et al., 2014; McBride, Rodd, et al., 2014; McClintick et al., 2015, 2016). We have previously studied the effects of repeated binge-like alcohol drinking during adolescence on the nucleus accumbens shell (Acbshell) and central nucleus of the amygdala (CeA) (McBride, Kimpel, et al., 2014), the dorsal raphe nucleus (DRN) (McClintick et al., 2015), and the periaqueductal gray (PAG) (McClintick et al., 2016) of these animals. Given the decreases in volume of both hippocampus (De Bellis et al., 2000) and prefrontal cortex (De Bellis et al., 2005) after heavy alcohol use in adolescence, the effects of chronic drinking on memory (Weissenborn & Duka, 2003; White et al., 2000), and the increased impulsivity of P rats (Beckwith & Czachowski, 2014, 2016), here we examined the transcriptome of the ventral hippocampus and medial prefrontal cortex following adolescent binge-like alcohol drinking by alcohol-preferring (P) rats.

MATERIALS and METHODS

Ethanol binge drinking

Adolescent male P rats were allowed to binge drink as described previously (McBride, Kimpel, et al., 2014). 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 available) in 3 x 1 h sessions per day for 5 consecutive days/week, while 10 control animals were treated identically except without access to ethanol (Bell et al., 2014; Bell et al., 2011). As

reported previously (Bell et al., 2011), this procedure leads to BACs of 80 - 100 mg% and motor impairment, criteria established by National Institute for Alcoholism and Alcohol Abuse (NIAAA) for binge-drinking (NIAAA, 2004). Animals were euthanized at 49 days of age, 3 h after the first access period on the 15th day of ethanol drinking. This 3-h time-point was selected in an attempt to maximize the response to alcohol on the expression of genes. 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 Research & Council, 1996)). As previously reported (McBride et al., 2014a), adolescent male P rats had an average ethanol intake of 10 g/kg in the three 1-hr scheduled access sessions during the first week, with average intakes of 3 – 4 g/kg in each of the 3 sessions. In the second and third weeks, the P rats had an average ethanol intake of 8 g/kg in the three 1-hr sessions, with average intakes of 2.5 – 3 g/kg in each of the three 1-hr sessions.

Dissection and RNA extraction

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 mPFC was micro-punched from +3.2 mm to +2.2 mm from bregma, including both prelimbic and infralimbic cortices, and the ventral hippocampus was micro-punched from -5.3 mm to -6.3 mm from bregma, using procedures previously described (McBride, Kimpel, et al., 2014). RNA was extracted using twice the suggested ratio of TRIzol (Life Technologies, Carlsbad, CA) to tissue (Edenberg et al., 2005), followed by additional purification using RNeasy columns (Qiagen, Hilden Germany). 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 8.7 for vHip and 8.4 for mPFC samples. Gene expression changes have previously been reported in four brain regions of these same animals: nucleus accumbens shell and central nucleus of the amygdala (McBride, Kimpel, et al., 2014), dorsal raphe nucleus (McClintick et al., 2015) and periaqueductal gray (McClintick et al., 2016).

RNA sequencing and analysis

RNA sequencing and analysis were carried out as previously reported (McClintick et al., 2015). Briefly, strand-specific libraries were prepared after ribo-reduction using Life Technologies SOLiD^{TM} Total RNAseq kit (Life Technology, Carlsbad, CA). Library preparations

were done in balanced batches. All samples for each brain region were pooled in equal molarity before EZbead preparation, followed by sequencing on a combination of SOLiD4™ and SOLiD™ 5500xl sequencers (50 base reads and 75 base reads, respectively). Aliquots of the same library preparations were sequenced on both machines. An average of 22.7 M (vHip) and 24.1 M (mPFC) reads per sample were mapped to Rn4 (Table 1). The edgeR package (Robinson, McCarthy, & Smyth, 2010) was used to identify genes differentially expressed between control and alcohol groups. FDR was calculated within edgeR according to Benjamini and Hochberg (Benjamini & Hochberg, 1995). Analysis was limited to those genes with ≥ 1 count per million in at least three samples. Library preparation batch was included as a factor in the analysis.

Qiagen Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, version Winter 2015) was used to identify pathways that are significantly enriched in differentially expressed (FDR ≤ 0.05) genes. Upstream regulator analysis uses the curated knowledge base to identify molecules that could possibly be responsible for the observed changes in gene expression. Upstream regulators can be proteins and other endogenous factors or exogenous factors such as drugs. A positive z-score suggests the regulator is active and a negative z-score suggests the regulator is inhibited; the magnitude indicates the strength of the predicted effect. In the case of exogenous factors, a positive z-score indicates the effect expected if the factor were added and a negative z-scores indicates that changes in expression of downstream targets are opposite what would be expected if the factor is added, thus addition of the factor might be able to reverse the effects. Putative target genes of upstream regulators are also identified. A white paper description of the z-score calculation is available at: http://pages.ingenuity.com/rs/ingenuity/images/0812%20upstream_regulator_analysis_whitepaper.pdf . IPA comparison analysis was used to compare pathway and upstream results from the four brain regions in which RNA sequencing has been done: vHIP and mPFC (this manuscript), DRN (McClintick et al., 2015) and PAG (McClintick et al., 2016). For this 4-region analysis, pathways were analyzed if p ≤ 0.05. Hierarchical clustering in Partek Genomics Suite version 6.6 (Partek, St. Louis, Mo) was used to identify pathways that contain similar sets of genes.

GeneMANIA (Warde-Farley et al., 2010) (genemaina.org), a tool for functional gene analysis, was used for co-expression analysis. Default parameter values were used except "max resultant genes" was set to zero to limit the analysis to those genes supplied in the list. GeneMANIA identifies the genes most related to a query gene set using a guilt-by-association approach, based upon a large database of publicly available functional interaction networks. For

co-expression networks, two genes are linked if their expression levels are similar across conditions in a gene expression study; GeneMANIA weights this across data from multiple organisms, cell-types and tissues. Data from Cahoy et al., 2008, was used to identify genes enriched in astrocytes, oligodendrocytes and neurons. Designation as "enriched" was limited to genes having at least a 1.5-fold enrichment in one of the three cells types compared to the other cells. The proportions test in R (https://cran.r-project.org/) was used to determine if there was a significant difference in the percentage of genes decreased in each of the three cell types compared to the percentage decreased among all differentially expressed genes.

RESULTS

We examined the effects of repeated binge drinking by adolescent rats on gene expression in the ventral Hippocampus (vHip) and medial prefrontal cortex (mPFC). The repeated binge exposures between post-natal days 28 to 49 resulted in high average daily ethanol intakes, approximately 8 g/kg/day, with intakes of 2-3 g/kg for each of the 3 daily 1 h sessions for the 5 drinking days each week (McBride, Kimpel, et al., 2014). Blood alcohol levels were not measured in these animals but similarly treated animals reached blood alcohol levels of 80-100 mg% at the end of each 1-hour session (Bell et al., 2011).

Ventral Hippocampus

RNA sequencing detected 11,727 genes expressed in the vHip, among which 416 (3.5%) were differentially expressed at FDR < 0.05 (Table 1; genes listed in Supplemental Table 1, Table 2 lists genes with at least 2-fold difference plus selected genes discussed here). Many of the differentially expressed genes were expressed at low levels (i.e., have small RPKMs), but the distribution of expression levels for differentially expressed genes did not differ from that of all detected genes (Supplemental Figure 1). Fold changes were small: only 18% of the differentially expressed genes changed by > 1.5 fold (absolute values) (Figure 1). There are genes with large fold changes that are not found in enriched pathways, including *Atf3*, *Cyr61*, *Apold1*, *Shank1*, *Btg2*, *Nts* and *Npas4* (Table 2). In the vHip, approximately 4/5 of the differentially expressed genes were expressed at lower levels in the binge drinking animals. These included genes enriched in neurons (70%), astrocytes (77%) and oligodendrocytes (86%); the additional bias in oligodendrocytes is suggestive but not significant (p=0.09).

The changes in gene expression in the vHip significantly altered 22 biological pathways (Table 3). Most of the genes differentially expressed in these pathways had decreased expression in the binge-drinking animals. Many of these pathways contain sets of overlapping genes, and in some cases may have related functions such as inflammation. Hierarchical clustering based on the differentially expressed genes within the pathways identified groups of related pathways, highlighted in Table 3. Axonal Guidance is affected, with most genes (including *Tuba4a*, *Plxd1*, *Plxb3, Shank2, Mmp9* and *Sema3c*) expressed at lower levels (Tables 2, 3). Wnt/β-catenin signaling is the most significant pathway, and is clustered with 2 cancer related pathways, 2 rheumatoid arthritis pathways and regulation of the epithelial-mesenchymal transition; these all share *Tcf7l2, Axin1, Tcf4* and *Gsk3b* (Tables 2, 3). The Wnt/β catenin pathway is decreased, with most of the affected genes having lower expression. A second cluster of pathways includes synaptic long term potentiation (LTP) and several signaling pathways, including those related to dopamine regulation of cAMP signaling and nNOS; these pathways also show reduced activity. Very closely related to these pathways, with some key overlapping genes, are pathways of cAMP and G-Protein signaling, which contain a cluster of protein kinases and phosphatases (*Prkcg*, *Gsk3b*, *Ppp3r2*, *Ppp1r14a)* that are decreased in expression. Protein kinase A signaling, which overlaps with both the Wnt and cAMP groups, has an overall neutral z-score, but most differentially expressed genes in that pathway are decreased. Protein kinase A signaling is initiated by multiple G-protein coupled receptors and has many different downstream targets, some of which are increased (e.g. tyrosine hydroxylase) and others decreased (e.g. eNOS / *Nos3* and Gsk3β) (Tables 2, 3). Four pathways involved in phosphoinositide metabolism have a completely overlapping set of genes, all decreased in expression; these include a different group of phosphatases *(Ppp1r12c, Ppp1r13b, Ptpn23*). Pathways related to fibrosis contain a set of collagens that are all expressed at lower levels in the binge-drinking animals. Some genes, including *Crebbp, Prkcg, Gsk3b, Ppp3r2, Nfkbia, Dusp1* and *Calm1,* are shared across the different groups of pathways (Table 3).

Medial Prefrontal Cortex

In the mPFC 11,579 genes were detected, among which 638 (5.5%) (Table 1) were differentially expressed (Supplemental Table 1). As in the vHip, many of the differentially expressed genes were expressed at low levels (Supplemental Figure 2), and only 10% of the differentially expressed genes changed by > 1.5 fold (Figure 1). In contrast to the vHip, only 35% of the differentially expressed genes in the mPFC were lower in the binge drinkers (Table 1). Astrocyte enriched genes had a significantly larger percentage of genes decreased (62%. *p*

= 1.4e-7) than did neurons (25%) or oligodendrocytes (36%). Genes with large fold changes in the mPFC include *Atf3*, *Cyr61*, *Apold1*, *Btg2*, and *Npas4* (Table 4) all increased; these genes are also increased in the vHip. Also increased in the mPFC are *Ier2, Gadd45g* and *Klf4* (Table 4).

The significantly altered genes clustered into 22 biological pathways (Table 5; Supplemental Table 1). The oxidative phosphorylation and the mitochondrial dysfunction pathways nearly completely overlap: both contain 17 genes involved in oxidative phosphorylation. These include genes from complexes I, III, IV and V, including *Atp5i, Atp5c1* and *Atp5o* (Tables 4, 5), and most of the genes showed 20-30%higher expression in the binge-drinking animals. EIF2 signaling was increased, with *Atf3* (the gene with the second largest fold-change detected), *Eif3g*, and *Eif2d* all increased. The AMPK pathway is one of the central regulators of ATP levels and may play a role in the increased expression of genes involved in oxidative phosphorylation. Two large, related groups of pathways include signaling in the acute phase response and by many cytokines, including IL-6, IL-17A and TNFr1. Common genes in these pathways include transcription factors *Fos* and *Jun*, P21 protein activated kinases *Pak6* and *Pak3*, insulin signaling *IGF1* and *Irs2*, and *Nfκbib*. There are two Sertoli cell signaling pathways that have some of these same genes but also include tubulin genes *Tuba1a* and *Tuba4a*.

DISCUSSION

Binge drinking escalates during adolescence, a time when significant maturation of the brain occurs (Spear, 2015). Perhaps because of that, adolescents are especially vulnerable to brain impairment by excessive ethanol exposure (Crews et al., 2016; Jacobus & Tapert, 2013; Welch, Carson, & Lawrie, 2013). To better understand how repeated binge drinking affects key brain regions, we modeled this behavior in rats. Male adolescent P rats were exposed to binge drinking for 3 weeks. The repeated voluntary binges altered gene expression in both the ventral hippocampus (vHip) and the medial prefrontal cortex (mPFC).

The hippocampus plays a role in both episodic memory formation and anxiety-like behavior (Kheirbek et al., 2012). It is one of the main sites for neurogenesis in the brain (Zhou, Borello, Rubenstein, & Pleasure, 2006), and in rodents, neurogenesis occurs in the hippocampus at higher levels during adolescence than in adulthood (He & Crews, 2007). Ethanol is known to decrease neurogenesis in the hippocampus (Geil et al., 2014). The patterns of gene expression observed in this study (Table 3) provide potential mechanistic explanations for ethanol's deleterious effects. The Wnt/β catenin pathway is necessary for hippocampal neurogenesis (Lie et al., 2005). Wnt signaling rescues β catenin from proteasomal degradation and allows it to

move into the nucleus, where it works with co-regulators in the LEF/TCF family to activate the transcription of multiple genes necessary for neurogenesis (Varela-Nallar & Inestrosa, 2013). Many genes within this pathway had reduced expression in the binge-drinking rats, including disheveled (*Dvl2*), *Lrp1* (lipoprotein receptor 1), *GSK3β* and *Tcf4* (Tables 2, 3). *Tcf7l2*, a member of the T-cell factor/lymphoid enhancer-binding factor family of high mobility group (HMG) box transcriptional activators, is decreased in expression in the vHip of the alcohol exposed animals; it is also the most inhibited gene in the upstream regulator analysis (Supplemental Table 2). Downstream targets of *Tcf7l2* include genes involved in myelin production *(*e.g. *Plp1, Mbp, Mag) (Lees & Brostoff, 1984; Norton & Cammer, 1984)* and in synthesis of cholesterol (e.g. *Hmgcs1,* and indirectly, *Hmgcr* via its effect on the expression of *Srepbf2*). *Hmgcr* has decreased expression in the vHip (Table 2), which is necessary for myelination (Zhao et al., 2016). When β catenin combines with Tcf7l2, it controls the development and maturation of oligodendrocytes, and their production of myelin (Zhao et al. 2016). The somewhat larger fraction of differentially expressed genes characteristically enriched in oligodendrocytes that were decreased in the vHip (86%) compared to 79% overall, although only suggestive (p=0.09), may hint at a relative decrease in the number or activity of these critical cells. Previous work showed that expression of genes responsible for serotonin production and signaling, also required for neurogenesis (Brezun & Daszuta, 1999), were greatly decreased in the DRN of these animals (McClintick et al., 2015). The decreased Wnt signaling in the hippocampus, combined with the lower serotonin input from the DRN, together would be expected to reduce neurogenesis in the vHip. Reduced neurogenesis would likely result in retarded development of the vHip, and potentially produce long-term consequences on learning and memory in adulthood; however, these animals were not tested for effects on memory.

Synaptic long term potentiation is decreased in adolescents by acute alcohol, and adults exposed to binge drinking as adolescents continue to be affected more strongly by acute alcohol (Markwiese, Acheson, Levin, Wilson, & Swartzwelder, 1998; Weissenborn & Duka, 2003). The LTP pathway and key genes within it were decreased in expression in the vHip (Tables 2, 3). The reduction in capability of producing LTP following adolescent binge drinking would seriously impact memory formation if such a reduction persisted into adulthood. Research with the P rat animal model indicates this rat line displays innate expression differences compared to its selectively bred low ethanol-consuming NP counterpart, in excitatory synaptic genes, including glutamate receptors and scaffolding proteins, which can be exacerbated by ethanol binge drinking (Bell et al., 2016).

Connections between brain regions continue to be made during adolescence (Spear, 2010). Axonal guidance, important in this process, is decreased in the hippocampus: 14 of the 17 altered genes in this pathway have decreased expression, including *Tuba4a*, *Plxd1*, *Plxb3, Shank2, Mmp9* and *Sema3c* (Table 2). Tubulins are necessary for axonal outgrowth. *Tuba4a*, when mutated, is associated with cortical malformations (Romaniello, Arrigoni, Bassi, & Borgatti, 2015). Plexin D1 interacts with semaphorin 3E and is important in vascular and neural development (Oh & Gu, 2013). Plexin-B3, found in dendrites, promotes inhibitory synapse formation in the hippocampus and suppresses excitatory synapse production (Laht et al., 2015). A decrease in Plexin-B3 may reverse this trend, allowing more excitatory synapses to form. The three genes increased in the axonal guidance pathway are Semaphorin 3c, *Adamts1* and neuropilin 2 (Tables 2, 3). *Adamts1* cleaves semaphorin 3C from the extracellular matrix so that it can act in axon guidance and promote cell migration, (Esselens et al., 2010). Neuropilin 2 is a receptor for semaphorin 3F (which is expressed in the hippocampus) and functions similarly to the plexins. These results suggest that adolescent binge drinking has retarded the formation of normal synapsis within the vHip; if this persisted into adulthood it would seriously impair the normal functioning of this region.

Very few neurotransmitter genes have altered expression in the vHip. Although changes in glutamate receptors themselves were quite limited, decreases were seen in all 3 of the Shank post-synaptic scaffolding genes (Table 2). Shank proteins link the various glutamate receptors together and are necessary for functional spine formation and proper maturation and functioning of excitatory synapses (reviewed in (O'Connor, Bariselli, & Bellone, 2014). Decreased expression of Shank1 decreases the size of neuron spines and decreased recruitment of endoplasmic reticulum Ca^{2+} stores.

Dopamine signaling appears to be increased in the hippocampus, as indicated by large increases in tyrosine hydroxylase (*Th*) and VMAT2 (*Slc18a2*), both increased more than 4 fold, and the dopamine receptor subunit *Drd2* (increased by 50%) (Table 2). These three genes are expressed at low levels. The upstream regulator analysis also suggests dopamine is more active in the alcohol-drinking animals (Supplemental Table 2). While the hippocampus is not thought to contain dopaminergic neurons, the VTA has dopaminergic projections to the hippocampus and these three mRNAs might be contained in the axons of these projections. These mRNAs were also detected in the hippocampus of human post-mortem brains in a comparison of alcoholics to controls (McClintick et al., 2013),

The delayed maturation of the PFC, which continues from adolescence into early adulthood, is thought to play a role in the increased impulsivity, thrill-, risk- and novelty-seeking behavior seen in adolescence (Crews & Nixon, 2009; Crews et al., 2016; Dalwani et al., 2011; Ernst & Fudge, 2009; Petanjek et al., 2011). Impulsivity was not measured in these animals. A study using a binge-drinking model (via alcohol laced gelatin) in Sprague Dawley rats showed that the animals that consumed high amounts of ethanol showed increased risk preference (McMurray, Amodeo, & Roitman, 2016). Our data suggest that there are fewer astrocytes in the mPFC of the binge drinking animals; genes that are characteristically enriched in astrocytes are decreased far more than the remaining genes (Table 1). The oxidative phosphorylation pathway was the most significantly affected in the mPFC (Table 5); many of the genes in this pathway were increased in expression (Tables 4, 5). This suggests increased energy utilization in the mPFC. Many of the oxidative phosphorylation genes are downstream of both *Igf1r* and *Vegfa*, and their downstream targets show increased expression (Supplemental Table 3). *Vegfa* is itself downstream of many of the transcription factors and receptors that appear to be activated in the mPFC, including *Ppargc1a,* which is sensitive to energy needs (Finck & Kelly, 2006). *Vegfa* is also downstream of *Atf4*, involved in the cellular stress response. The increase in oxidative phosphorylation in the mPFC was not seen in the vHip (Table 3), nor in prior studies of the DRN (McClintick et al., 2015) and the PAG (McClintick et al., 2016).

Pathways related to cellular stress response and inflammation showed increased activity in the mPFC (Table 5). Immune related genes and pathways were also reported to be increased in the prefrontal cortex of CIE-exposed C57BL/6J mice examined 0 h and 8 h post-exposure (Osterndorff-Kahanek et al., 2015). The upstream regulator analysis supports this, with indications that interferons are active (Supplemental Table 3). MiR132 expression in the mPFC is increased by the alcohol binges (Table 4); it was previously shown to be increased in the livers of alcohol fed mice (Bala & Szabo, 2012). MiR132 is important in mouse PFC development during adolescence (Miller et al., 2012), so dysregulation could interfere with PFC development in the alcohol exposed adolescent animals. MiR132 also plays a role in neuro-inflammatory responses: increased expression can help block inflammation by targeting acetylcholinesterase (Shaked et al., 2009). The increased expression of miR132 may be an attempt to dampen neuro-inflammation.

Osterndorff-Kahanek et al. (2015) reported that in the prefrontal cortex of CIE-exposed C57BL/6J mice, neuron- and astrocyte-related genes were differentially expressed. We saw no enrichment of astrocyte genes, but did see enrichment of neuron-related differentially expressed

genes in the mPFC, vHip, DRN and PAG of these animals. A study by Meinhardt et al. (2013) of Wistar rats after 3 weeks of recovery from chronic intermittent exposure to ethanol vapor (CIE) reported decreased expression of genes related to glutamtergic neurons in the mPFC. At 3h post-exposure we saw increases in 7 of the genes related to glutamate transmission, *Egr4, Stx1a, Lmo4, Nrgn, Nr4a1* and *Zfp238*.

The two regions studied here came from animals in which gene expression in the DRN (McClintick et al., 2015) and PAG (McClintick et al., 2016) was previously examined. Eleven genes have significant (FDR< 0.05) expression changes in the same direction in all 4 sequenced regions (Table 6). *Dgkb*, diacylglycerol kinase beta, the only gene with decreased expression in all 4 regions, has been identified by GWAS as associated with alcoholism (Kendler et al., 2011). Ten of these 11 genes are increased in all four regions, and five were also enriched in the nucleus accumbens shell and the central nucleus of the amygdala of these animals (McBride, Kimpel, et al., 2014). Five of these genes, noted in Table 6, were also increased in the nucleus accumbens of binge drinking adult rats in a previous study by our group (Bell et al., 2009). A GeneMania (Warde-Farley et al., 2010) analysis of these 11 genes indicates that all 10 genes with positive fold changes are highly co-expressed. The two main regulators appear to be cAMP responsive element binding protein 1 (*CREB1*) and Tumor protein P53 (*TP53*). *CREB1* targets 7 of the 10 upregulated genes; upstream analysis indicates *CREB1* is active in vHip and mPFC (Supplemental Tables 2, 3), and the DRN (McClintick et al., 2015) and PAG (McClintick et al., 2016). *TP53* targets 6 of the genes; three (*Atf3*, *Btg2*, and *Npas4),* along with *Nr4a1*and *Gadd45γ,* have been shown to be upregulated by calcium signaling and CREB (Tan, Zhang, Hoffmann, & Bading, 2012), and are neuroprotective (Zhang et al., 2009). Both *GADD45γ* and *Nr4a1* are increased in all 4 brain regions, but the increases met significance in only 1 and 3 regions respectively. *Atf3* is expressed at low levels in the nervous system but is induced following seizures and other stressors in the brain (Moore & Goldberg, 2011). Seven of the 10 co-expressed genes, are enriched in astrocytes (Cahoy et al., 2008). Five of these 7 (Table 6) were also increased in binge exposed adult rats (Bell et al., 2009). This could be a potentially protective response to the insult of repeated binges.

Some pathways are altered across multiple regions (vHip, mPFC, DRN, PAG; Supplemental Table 4). Those affected in all 4 regions include axonal guidance signaling, Hif1α signaling, signaling by Rho family GTPases, 14-3-3-mediated signaling (*Ywhag* is a 14-3-3 protein), pathways related to rheumatoid arthritis (perhaps through TNF related molecules), hepatic fibrosis signaling (*Tnfrsf11b* + collagens), ERK/MAPK signaling, pathways dealing with inositol

phosphates, and growth hormone signaling. Glucocorticoid signaling is affected in vHip, mPFC and DRN. Overall, these results suggest widespread effects of adolescent binge drinking that altered the development of these 4 regions and would negatively impact a wide variety of behaviors throughout life.

The vHip, but not the mPFC, shares some pathways with the DRN and PAG (Supplemental Table 4), including cAMP-mediated signaling, protein kinase A signaling, CREB signaling in neurons, Gαq signaling, and calcium signaling. The decrease in expression of *Hmgcr* and *Srepbf2* (genes important in cholesterol synthesis) in the vHip was paralleled by decreases in genes important in cholesterol synthesis and myelination in both the DRN and PAG of these animals (McClintick et al., 2015, 2016), and also in human post-mortem tissue (Liu et al., 2006; Mayfield et al., 2002; McClintick et al., 2013). *Tcf7l2,* which can control cholesterol production along with oligodendrocyte maturation, was decreased in vHip and PAG, and both of these regions have a disproportionate number of oligodendrocyte enriched genes with decreased expression. Genes necessary for cholesterol production were also decreased in expression. While myelin was not measured in these animals, these three lines of evidence suggest decreases in myelination in the binge-drinking animals. Alcohol consumption has resulted in decreased or poor myelination in other rat lines and in humans (Vargas et al., 2014, Jacobus & Tapert, 2013).

The mPFC shares several pathways with the DRN and PAG that the vHip does not; these include stress responsive and immune responsive pathways, such as NRF2-mediated oxidative stress response, acute phase response, IL1-signaling, LPS-stimulated MAPK signaling, IL-6 signaling (Supplemental Table 4). In addition to these stress and immune pathways, some signaling pathways are also shared by mPFC, DRN and PAG: GABA receptor signaling, RAR activation, Rac and RhoGDI signaling, P2Y purinergic receptor signaling pathway, GDNF family ligand-receptor, IGF1 signaling, and PPAR signaling. Norepinephrine also appears to be active in all three regions (Supplemental Table 4).

IPA comparison analysis of the 4 regions indicates that the vHip is the only region with decreases in the Wnt/B catenin, ILK signaling, synaptic long term potentiation and depression, corticotropin releasing hormone signaling, endothelin-1 signaling, and AMPK signaling (Table 3).

There is interest in drugs with the potential to block or reverse some of the damage caused by binge drinking. Our upstream regulator analysis (Supplemental Tables 2, 3) suggests that Mifepristone and fulvestrant (an estrogen receptor antagonist) might reverse some of the effects

of alcohol in all 4 brain regions (see also McClintick et al., 2015, 2016). In the hypothalamus, ethanol has the same effects as estrogen (Sarkar & Boyadjieva, 2007). Most other drugs identified by upstream analysis show differences among the 4 brain regions.

CONCLUSION

This study has pointed to changes in gene expression that might underlie some of the harmful long-term effects of binge drinking during adolescence. Repeated binge drinking decreased expression of genes involved in neurogenesis, e.g. in the WNT/β catenin pathway, long term potentiation and axonal guidance in the ventral Hippocampus. Genes involved in cholesterol production and myelin formation are decreased and a disproportionate number of genes enriched in oligodendrocytes may indicate a decrease in oligodendrocytes in the vHip. Changes in expression of genes in axonal guidance in the vHip may decrease inhibitory synapse formation and allow increased glutamatergic synapse formation, although decreased expression of shank genes might moderate the formation of excitatory synapses. Together with earlier data showing decreased expression of most genes in the serotonin pathway in the DRN of these animals (McClintick, 2015), this might partially explain reduced neurogenesis in this region.

In the mPFC, cellular stress and inflammation pathways were activated, as are many genes for oxidative phosphorylation. MiR132, which moderates neuro-inflammatory responses and is necessary for mouse PFC development, is increased in expression, perhaps in partial compensation for the increased expression of genes involved in neuro-inflammation. A disproportionate number of genes enriched in astrocytes had decreased expression, which may indicate a loss or decreased production of astrocytes in the mPFC. Multiple genes that are upregulated in response to cellular stress are highly increased in four brain regions of these animals, indicating a global response to the stress of repeated binge-like drinking of high amounts of alcohol.

In summary, adolescent binge-like alcohol drinking by P rats produces widespread changes in the expression of genes within the vHip and mPFC, which likely alters their normal development and could produce long-lasting deficits in neuronal functioning within these regions.

CONFLICT OF INTEREST

None

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TABLES and FIGURES.

Figure 1. Distribution of fold changes for ventral Hippocampus and medial Prefrontal Cortex.

Table 1. Number of differentially expressed genes per region. Mapped reads is the average per sample. *Detection limit set at ≥ 1 count per million in at least 3 samples. **% of genes enriched in astrocytes that were decreased. *** % of genes enriched in neurons that were decreased. **** % of genes enriched in oligodendrocytes that were decreased.

Table 2. Selected genes differentially expressed in ventral hippocampus. *FC: fold change; **FDR: false discovery rate; ***RPKM; reads per thousand bp per million reads. This table contains genes with absolute fold change ≥ 2 and all genes discussed in the text.

Table 3. Pathway analysis for ventral Hippocampus, with FDR≤ 0.05. Highlighted and boxed clusters identify pathways that contain many genes in common. *Bolded genes have increased expression, non-bold indicates decreased expression. **Z-score indicates whether the pathway is activated (positive z-score) or decreased (negative z-score); blank where IPA did not return a z-score**.**

Table 4. Selected genes differentially expressed in medial prefrontal cortex. *FC: fold change; **FDR: false discovery rate; ***RPKM; reads per thousand bp per million reads. This table contains genes with absolute fold change ≥ 2 and all genes discussed in the text.

Table 5. Pathway analysis for medial Prefrontal Cortex. FDR≤ 0.05. Colored clusters identify pathways that contain many genes in common. ***Bolded genes** have increased expression, non-bold indicates decreased expression. **Z-score indicates whether the pathway is activated (positive z-score) or decreased (negative z-score); blank where IPA did not return a z-score.

Table 6. Genes differentially expressed in at least 4 brain regions. FC: fold change, vHip: ventral hippocampus, mPFC: medial prefrontal cortex, DRN: dorsal raphe nucleus (McClintick et al., 2015), PAG: periaqueductal gray (McClintick et al. 2016), AcbS: nucleus accumbens shell, CeA central core of the amygdala (McBride et al., 2014b). Adult: significant fold change in nucleus accumbens of adult P rats exposed to binge drinking (Bell et al., 2009).

Supplemental Materials:

Supplemental Figure 1. Distribution of RPKMs in vHip for all genes and those differentially expressed.

Supplemental Figure 2. Distribution of RPKMs in mPFC for all genes and those differentially expressed.

Supplemental Table 1. Differentially expressed genes in ventral Hippocampus and medial Prefrontal Cortex of binge drinking adolescent P rats. False Discovery Rate ≤ 0.05 for at least one of the 2 brain regions. Data "grayed" for those results not meeting FDR significance threshold.

Supplemental Table 2. Upstream Regulator analysis for ventral Hippocampus. List of genes, drug, or molecules that could be responsible for observed changes in expression. Fold lists the fold change for genes in the dataset. Z-score indicates whether this regulator is active or not (list limited to those with an absolute score ≥ 1.5. Positive score: direction of changes observed in affected genes indicate this regulator is active. Negative score: direction of changes observed are opposite of what would be observed if this regulator is active. P value of overlap indicates significance regardless of fold direction. Data is sorted so that endogenous effectors are listed first followed by drugs and other chemicals that would be exogenous.

Supplemental Table 3. Upstream Regulator analysis for medial Prefrontal Cortex. List of genes, drug, or molecules that could be responsible for observed changes in expression. Fold lists the fold change for genes in the dataset. Z-score indicates whether this regulator is active or not (list limited to those with an absolute score ≥ 1.5. Positive score: direction of changes observed in affected genes indicate this regulator is active. Negative score: direction of changes observed are opposite of what would be observed if this regulator is active. P value of overlap indicates significance regardless of fold direction. Data is sorted so that endogenous effectors are listed first followed by drugs and other chemicals that would be exogenous.

741 **Supplemental Table 4**. Pathways affected in multiple brain regions. Limited to those with p<
742 0.01 in either medial prefrontal cortex or ventral hippocampus. Pathway name, number of genes
743 affected in the pathway, brain region, P value of significance of changes, ratio or fraction of
744 genes in the pathway affected, z-score: a measure of whether the pathway is active (positive) or
745 inhibited (negative), not all pathways have a z-score. List of genes in the pathway differentially
746 expressed in the named brain region.

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Table 1. Number of differentially expressed genes

	# mapped reads (M)	# genes detected*	# FDR < 0.05	% down	% down astrocytes**	% down neurons***	% down oligo****
vHip	22.7	11,727	416	79%	77%	70%	86%
mPFC	24.1	11,679	638	35%	62%	25%	36%

Table 1. Number of differentially expressed genes per region.
Mapped reads is the average per sample. *Detection limit set at ≥ 1 count per million in at least 3 samples. **% of genes enriched in astrocytes that were decreased. *** % of genes enriched in neurons that were decreased. **** % of gene enriched in oligodendrocytes that were decreased.

Table 2.

Gene	Gene title	*FC	**FDR	Control ***RPKM	Alcohol ***RPKM
Genes with absolute fold change ≥2					
Apold1	apolipoprotein L domain containing 1	2.71	9.1E-54	8.0	21.6
Atf3	activating transcription factor 3	7.84	6.5E-32	0.5	3.9
Btg2	BTG family, member 2	2.25	5.2E-35	9.6	21.7
Cftr	cystic fibrosis transmembrane conductance regulator (ATP-bin	-2.04	3.2E-07	1.0	0.5
Col8a1	collagen, type VIII, alpha 1	-2.39	1.5E-02	0.6	0.2
Cym	chymosin	-3.42	1.8E-04	1.1	0.3
Cyr61	cysteine-rich, angiogenic inducer, 61	11.32	1.7E-106	1.1	12.8
Dlk1	delta-like 1 homolog (Drosophila)	2.25	3.0E-04	1.0	2.4
F5	coagulation factor V (proaccelerin, labile factor)	-2.46	1.7E-02	0.2	0.1
Fermt1	fermitin family member 1	2.12	2.9E-03	0.3	0.6
Gprc5a	G protein-coupled receptor, family C, group 5, member A	-2.15	2.2E-04	1.5	0.7
Klf2	Kruppel-like factor 2 (lung)	2.11	2.6E-04	1.7	3.6
Mttp	microsomal triglyceride transfer protein	3.40	5.8E-10	0.3	1.1
Mx1	myxovirus (influenza virus) resistance 1, interferon-inducible prote	2.11	2.3E-06	0.8	1.8
Pzp	pregnancy-zone protein	2.41	1.3E-02	0.1	0.4
RGD1562229		2.05	2.0E-02	1.1	2.3
Rps28	ribosomal protein S28	-2.73	8.0E-03	3.5	1.3
Scn11a	sodium channel, voltage-gated, type XI, alpha subunit	-3.69	2.4E-09	0.5	0.1
Shank1	SH3 and multiple ankyrin repeat domains 1	-2.05	4.0E-49	58.7	28.7
Slc18a2	solute carrier family 18 (vesicular monoamine), member 2	4.33	8.6E-16	0.4	1.8
Synpo2	synaptopodin 2	-2.08	2.5E-05	1.0	0.5
Th	tyrosine hydroxylase	4.05	1.5E-27	1.5	5.9
Tmem26	transmembrane protein 26	-2.97	7.9E-07	0.8	0.3
Trhr2	thyrotropin releasing hormone receptor 2	-2.06	2.1E-04	3.2	1.6

Other genes discussed in text

Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	1.54	9.4E-05	2	3
Atp5g3	ATP Synthase, H+ Transporting, Mitochondrial Fo Complex Subunit C3 (Subunit 9)	-1.29	4.6E-05	231.6	180
Axin1	axin 1	-1.20	3.7E-02	15.3	12.8
Cox6a1	cytochrome c oxidase subunit VIa polypeptide 1	-1.17	3.0E-02	370.9	316.1
Dgkb	diacylglycerol kinase, beta 90kDa	-1.29	8.0E-06	67.4	52.3
Drd2	dopamine receptor D2	1.47	4.3E-02	1.4	2
Dusp1	dual specificity phosphatase 1	1.63	8.8E-16	26.3	42.8
Dvl2	dishevelled, dsh homolog 2 (Drosophila)	-1.30	2.1E-02	6.3	4.8
Fos	FBJ murine osteosarcoma viral oncogene homolog	1.46	4.9E-07	16.4	23.9
Grin2c	glutamate receptor, ionotropic, N-methyl D-aspartate 2C	-1.21	1.1E-02	22.8	18.9
Gsk3b	glycogen synthase kinase 3 beta	-1.20	1.6E-02	73.7	61.6
Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase	-1.29	7.1E-05	30.6	23.8
Ier2	immediate early response 2	1.60	2.6E-05	5.2	8.3
Klf4	Kruppel-like factor 4 (gut)	1.60	2.5E-05	3.3	5.3
Mag	myelin associated glycoprotein	-1.20	6.3E-03	111.3	92.8
Mbp	myelin basic protein	-1.20	2.7E-03	1142.3	951.2
Mmp9	matrix metallopeptidase 9	-1.31	3.2E-02	4.59	3.51
Mobp	myelin-associated oligodendrocyte basic protein	-1.25	1.3E-04	140.5	112.3
Npas4	neuronal PAS domain protein 4	1.84	2.1E-11	4.3	7.9
Nrp2	neuropilin 2	1.18	5.0E-02	19.22	22.72
Nts	neurotensin	-1.66	3.3E-02	3.9	2.4
Plp1	proteolipid protein 1	-1.22	5.5E-04	1473.5	1204.2
Plxnb3	plexin B3	-1.22	7.2E-03	17.56	14.45
Plxnd1	plexin D1	-1.38	2.4E-05	6.21	4.5
Sema3c	sema domain, immunoglobulin domain (Ig), short basic domain, secr	1.27	5.9E-03	6.99	8.85
Shank2	SH3 and multiple ankyrin repeat domains 2	-1.30	4.1E-06	31.47	24.18
Tcf4	transcription factor 4	-1.17	4.1E-02	74.2	63.4
Tcf7l2	transcription factor 7-like 2	-1.38	9.0E-04	5.2	3.8
Tuba4a	tubulin, alpha 4a	-1.49	2.1E-13	127.08	85.18

Table 2. Selected genes differentially expressed in ventral hippocampus. *FC: fold change; **FDR: false discovery rate; ***RPKM; reads per thousand bp per million reads. This table contains genes with absolute fold change ≥ 2 and all genes discussed in the text.

Table 3. Pathways altered in the ventral hippocampus

Ingenuity Canonical Pathways	Genes*	FDR	z-score
Axonal Guidance Signaling	***ADAMTS1***, *GIT1, GNA12, GNAO1, GSK3B, HHIP, KLC1, MAG, MMP9,* ***NRP2****, PLXNB3, PLXND1, PPP3R2, PRKCG,* ***SEMA3C****, SHANK2, TUBA4A*	3.6E-02	
Wnt/β-catenin Signaling	*AXIN1, BCL9, CREBBP, DVL2, GJA1, GNAO1, GSK3B, LRP1, RARA, SOX7, TCF4, TCF7L2,* ***Ubb***	4.2E-03	-1.16
Basal Cell Carcinoma Signaling	*AXIN1, DVL2, GSK3B, HHIP, TCF4, TCF7L2*	3.4E-02	-1.63
Regulation of the Epithelial-Mesenchymal Transition Pathway	*AXIN1, BCL9, DVL2, FGFR2, GSK3B, MMP9, NOTCH1, NOTCH3, TCF4, TCF7L2*	3.4E-02	
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	*AXIN1,* ***Calm1****, COL1A1,* ***FOS****, GSK3B, LRP1, NFKBIA, PPP3R2, TCF4, TCF7L2,* ***TNFRSF11B***	3.4E-02	
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	*AXIN1,* ***Calm1****, CREBBP,* ***FOS****, GNAO1, GSK3B,* ***IL16,*** *LRP1, NFKBIA, PPP3R2, PRKCG, TCF4, TCF7L2,* ***TNFRSF11B***	3.1E-02	
Molecular Mechanisms of Cancer	*Aph1c, AXIN1, CDKN1A, CREBBP,* ***FOS****, GNA12, GNAO1, GSK3B, HIPK2, LRP1,* ***MAX****, NFKBIA, NOTCH1, PRKCG, RAPGEF3, SMAD7, SYNGAP1, TCF4*	1.3E-02	
nNOS Signaling in Neurons	***Calm1****, CAPN2, GRIN2C, PPP3R2, PRKCG*	3.4E-02	
Calcium-induced T Lymphocyte Apoptosis	***Calm1****, CAPN2, ITPR1, PPP3R2, PRKCG, ZAP70*	3.1E-02	-1.63
Synaptic Long Term Potentiation	***Calm1****, CREBBP, GRIN2C, ITPR1, PPP1R14A, PPP3R2, PRKCG, RAPGEF3*	3.4E-02	-1.41
Dopamine-DARPP32 Feedback in cAMP Signaling	***Calm1****, CREBBP,* ***DRD2****, GRIN2C, ITPR1, KCNJ6, PAWR, PPP1R14A, PPP3R2, PRKCG*	2.9E-02	-0.38
cAMP-mediated signaling	***Calm1****, CREBBP,* ***DRD2, DUSP1****, GNAO1, HRH2, HRH3, P2RY13, PDE4A, PPP3R2, RAPGEF3*	3.4E-02	-1.90
G-Protein Coupled Receptor Signaling	*CREBBP,* ***DRD2****,* ***DUSP1****, GNAO1, HRH2, HRH3,* ***HTR2A****, NFKBIA, P2RY13, PDE4A, PRKCG, RAPGEF3, SYNGAP1*	3.0E-02	
Protein Kinase A Signaling	***Calm1****, CREBBP,* ***DUSP1,*** *GSK3B, ITPR1, MYLK, NFKBIA, NOS3, PDE4A, PPP1R14A, PPP3R2, PRKCG, PTPN23, SIRPA, TCF4, TCF7L2,* ***TH****, YWHAB*	1.6E-02	0.00
3-phosphoinositide Biosynthesis	*CILP, DOT1L,* ***DUSP1****, PAWR, PPP1R12C, PPP1R13B, PPP1R14A, PTPN23, SIRPA*	3.4E-02	
3-phosphoinositide Degradation	*CILP, DOT1L,* ***DUSP1****, PAWR, PPP1R12C, PPP1R13B, PPP1R14A, PTPN23, SIRPA*	3.4E-02	

D-myo-inositol-5-phosphate Metabolism	*CILP, DOT1L,* ***DUSP1****, PAWR, PPP1R12C, PPP1R13B, PPP1R14A, PTPN23, SIRPA*	3.4E-02	
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	*CILP, DOT1L,* ***DUSP1****, PAWR, PPP1R12C, PPP1R13B, PPP1R14A, PTPN23, SIRPA*	2.6E-02	
Hepatic Fibrosis / Hepatic Stellate Cell Activation	*COL11A2, COL1A1, COL1A2, COL3A1, COL8A1,* ***EDN1****, FGFR2, IGF2, MMP9, MYH11, SMAD7,* ***TNFRSF11B***	1.3E-02	
Intrinsic Prothrombin Activation Pathway	*COL11A2, COL1A1, COL1A2, COL3A1, F5*	1.3E-02	-2.24
Atherosclerosis Signaling	*COL11A2, COL1A1, COL1A2, COL3A1, F3, MMP9, PLA2G7, SELPLG*	3.4E-02	
Huntington's Disease Signaling	*CAPN2, CREBBP, HDAC5,* ***HSPA1A/HSPA1B****, ITPR1, NCOR2, PACSIN1,* ***POLR2C****, PRKCG, SGK1,* ***Ubb***	4.4E-02	-0.45

Table 3. Pathways altered in the ventral hippocampus. *Genes in normal font were expressed at lower levels in the binge-drinking animals; bold font indicates genes that were expressed at higher levels. z-score indicates whether the pathway is activiate (postivie z) or decreased (negative z). Pathways sharing many genes are boxed.

Table 4.

Gene	Gene title	*FC	**FDR	Control ***RPKM	Alcohol ***RPKM
Genes with absolute fold change ≥2					
Apold1	apolipoprotein L domain containing 1	4.11	1.2E-146	6.7	27.5
Atf3	activating transcription factor 3	16.15	1.0E-57	0.3	4.3
Btg2	BTG family, member 2	3.07	6.7E-129	18.3	56.0
Cyr61	cysteine-rich, angiogenic inducer, 61	9.55	8.5E-138	1.5	14.1
F2rl2	coagulation factor II (thrombin) receptor-like 2	2.52	1.2E-02	0.4	0.9
Fgl2	fibrinogen-like 2	-2.21	9.9E-04	0.7	0.3
Gadd45g	growth arrest and DNA-damage-inducible, gamma	2.12	3.2E-21	8.7	18.5
Grap	GRB2-related adaptor protein	2.12	2.5E-02	0.5	1.0
Ier2	immediate early response 2	2.72	1.6E-45	6.8	18.4
Kif15	kinesin family member 15	2.01	2.8E-02	0.2	0.4
Klf2	Kruppel-like factor 2 (lung)	2.17	8.9E-09	2.9	6.3
Klf4	Kruppel-like factor 4 (gut)	2.07	1.5E-16	3.1	6.5
Mir212	microRNA 212	2.37	1.9E-04	10.3	24.4
Npas4	neuronal PAS domain protein 4	2.63	2.2E-84	13.9	36.6
Tbx2	T-box 2	2.39	4.1E-02	0.4	0.9
Vom2r57	vomeronasal 2 receptor, 57	2.32	9.3E-03	0.3	0.6
Zfp36	zinc finger protein 36, C3H type, homolog (mouse)	2.07	9.0E-08	1.7	3.6
Other genes discussed in text					
Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	1.90	3.5E-14	2	3.8
Atp5c1	ATP Synthase, H+ Transporting, Mitochondrial F1 Complex, Gamma Polypeptide 1	1.19	3.4E-03	83.1	98.8
Atp5e	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	1.35	2.4E-06	100	134.6
Atp5g1	ATP Synthase, H+ Transporting, Mitochondrial Fo Complex Subunit C1 (Subunit 9)	1.27	9.8E-05	85.6	108.7

Atp5g3	ATP Synthase, H+ Transporting, Mitochondrial Fo Complex Subunit C3 (Subunit 9)	1.18	4.1E-03	168.5	198.5
Atp5h	ATP Synthase, H+ Transporting, Mitochondrial Fo Complex Subunit D	1.49	5.0E-06	21	31.1
Atp5i	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit E	1.22	2.7E-02	71.1	86.8
Atp5o	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	1.25	2.0E-05	138.3	173
Cox4i1	cytochrome c oxidase subunit IV isoform 1	1.13	2.5E-02	446.3	505.7
Cox6a1	cytochrome c oxidase subunit VIa polypeptide 1	1.21	1.6E-04	279.2	339.2
Cox8a	cytochrome c oxidase subunit VIIIA (ubiquitous)	1.20	1.7E-03	157.6	189.9
Dgkb	diacylglycerol kinase, beta 90kDa	-1.36	2.2E-12	39.6	29.1
Dusp1	dual specificity phosphatase 1	1.63	1.1E-29	61.5	100.1
Fos	FBJ murine osteosarcoma viral oncogene homolog	1.45	2.2E-15	54.9	79.6
Jun	jun proto-oncogene	1.46	1.3E-16	33.2	48.3
Mir132	microRNA 132	1.90	9.4E-05	25.6	48.6
Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	1.31	9.8E-03	15.7	20.6
Ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa	1.22	6.9E-04	84.1	102.5
Ndufa6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	1.18	3.6E-02	72	84.8
Ndufb10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	1.18	2.3E-02	59.4	70.4
Ndufb9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9, 22kDa	1.16	2.0E-02	116.8	135.9
Nr4a1	nuclear receptor subfamily 4, group A, member 1	1.56	2.8E-25	52.3	81.8
Shank1	SH3 and multiple ankyrin repeat domains 1	1.24	2.0E-06	51.1	63.4

Tuba1a	tubulin, alpha 1a	1.16	4.2E-03	169.6	197.1
Tuba4a	tubulin, alpha 4a	1.12	4.9E-02	138	155.2
Uqcr11	ubiquinol-cytochrome c reductase, complex III subunit XI	1.16	2.2E-02	193.4	223.5
Uqcrb	ubiquinol-cytochrome c reductase binding protein	-1.22	2.6E-02	24.7	20.2
Uqcrq	ubiquinol-cytochrome c reductase, complex III subunit VII, 9.5kDa	1.24	1.3E-03	27.1	33.6

Table 4. Selected genes differentially expressed in medial prefrontal cortex. *FC: fold change; **FDR: false discovery rate; ***RPKM; reads per thousand bp per million reads. This table contains genes with absolute fold change ≥ 2 and all genes discussed in the text.

Table 5. Pathways altered in medial Prefrontal cortex

Pathway	genes	p-value	z-score
Mitochondrial Dysfunction	***ATP5C1, Atp5e, ATP5G1, ATP5G3, ATP5H, ATP5O, COX17, COX4I1, COX6A1, COX8A, CPT1C, FIS1, GPX4, NDUFA1, NDUFA4, NDUFA6, NDUFB10, NDUFB9, PRDX5, PSEN2, UQCR11, UQCRQ***	3.5E-07	
Oxidative Phosphorylation	***ATP5C1, Atp5e, ATP5G1, ATP5G3, ATP5H, ATP5O, COX17, COX4I1, COX6A1, COX8A, NDUFA1, NDUFA4, NDUFA6, NDUFB10, NDUFB9, UQCR11, UQCRQ***	7.6E-07	
EIF2 Signaling	***EIF3G, FAU, MAP2K2,*** *PIK3C2A*, ***RPL14, RPL18, RPL27, RPL28, RPL30,*** *RPL37A*, ***RPL7, RPS15A,*** *RPS29*, ***RPS3***	1.7E-02	1.67
AMPK Signaling	***ADRA1D, CAMKK2,*** *CHRNA5*, ***CPT1C, DPF1,*** *IRS2*, *MAP3K7*, ***PFKL,*** *PIK3C2A*, ***PRKAR1A,*** *SLC2A1*, ***SMARCB1***	5.0E-02	0.00
Acute Phase Response Signaling	*A2M*, ***CEBPB,*** *CP*, ***ECSIT, FN1, FOS,*** *IL1R1, IL6R*, ***JUN, MAP2K2,*** *MAP3K7*, ***NFKBIB,*** *SERPINF1*, ***TNFRSF11B***	1.0E-02	0.28
IL-6 Signaling	*A2M*, ***CEBPB, FOS,*** *IL1R1, IL6R*, ***JUN, MAP2K2,*** *MAP3K7*, ***NFKBIB,*** *PIK3C2A*, ***TNFRSF11B, VEGFA***	5.5E-03	0.58
14-3-3-mediated Signaling	***FOS,*** *GFAP*, ***JUN, MAP2K2,*** *PIK3C2A*, *PLCD4*, ***TUBA1A, TUBA4A,*** *VIM*, ***YWHAG***	4.2E-02	0.38
IGF-1 Signaling	***CTGF, CYR61, FOS,*** *IGF1*, *IRS2*, ***JUN, MAP2K2,*** *PIK3C2A*, ***PRKAR1A, YWHAG***	1.3E-02	0.82
Signaling by Rho Family GTPases	*ARHGEF3*, ***ARPC4,*** *CDH5*, ***FOS,*** *GFAP*, ***GNG10, JUN, MAP2K2, MYL6,*** *PAK3, PAK6*, *PIK3C2A*, ***PPP1R12C, RHOB,*** *VIM*	5.0E-02	-0.54
Hepatic Fibrosis / Hepatic Stellate Cell Activation	*A2M*, *COL1A2, COL3A1*, ***COL5A3,*** *COL6A3*, ***CTGF, EDN1, FN1,*** *IGF1, IGF2*, *IL1R1, IL6R*, ***MYL6, TNFRSF11B, VEGFA***	8.3E-03	
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	***CEBPA, CEBPB, DVL1, FN1, FOS,*** *IL1R1, IL6R*, ***JUN, MAP2K2,*** *MAP3K7*, ***MIF, NFKBIB, PIK3C2A,*** *PLCD4*, *TLR7*, ***TNFRSF11B, VEGFA***	5.0E-02	
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	***ADAMTS5, DVL1, FOS,*** *IGF1*, *IL1R1*, ***JUN,*** *MAP3K7*, *MMP14*, ***NFKBIB,*** *PIK3C2A*, *RUNX2*, *SMAD5*, ***TNFRSF11B,*** *XIAP*	5.0E-02	
Renal Cell Carcinoma Signaling	***FOS, JUN, MAP2K2,*** *PAK3, PAK6*, *PIK3C2A*, *SLC2A1*, ***Ubb,*** *UBC*, ***VEGFA***	2.1E-03	0.00
IL-1 Signaling	***ECSIT, FOS, GNG10,*** *IL1R1*, ***JUN,*** *MAP3K7*, ***NFKBIB, PRKAR1A***	5.0E-02	1.13
IL-17A Signaling in Fibroblasts	***CEBPB, FOS, JUN,*** *MAP3K7*, ***NFKBIB***	5.0E-02	

TGF-β Signaling	***FOS, IRF7, JUN, MAP2K2,*** *MAP3K7****, PIAS4,*** *RUNX2****,*** *SMAD5*	5.0E-02	-0.38
TNFR1 Signaling	***FOS, JUN, NFKBIB,*** *PAK3, PAK6****,*** *XIAP*	5.0E-02	0.82
JAK/Stat Signaling	***CEBPB, FOS, JUN, MAP2K2, PIAS4,*** *PIK3C2A****,*** ***STAT6***	5.0E-02	0.38
RANK Signaling in Osteoclasts	***FOS, JUN, MAP2K2,*** *MAP3K7, MAP3K8****, NFKBIB,*** *PIK3C2A****,*** *XIAP*	5.0E-02	-0.38
CD27 Signaling in Lymphocytes	***FOS, JUN, MAP2K2,*** *MAP3K7, MAP3K8****, NFKBIB***	5.0E-02	0.00
Sertoli Cell-Sertoli Cell Junction Signaling	*A2M****, ACTN2, JUN, JUP, MAP2K2,*** *MAP3K7, MAP3K8****, PRKAR1A,*** *TGFBR3****, TUBA1A, TUBA4A,*** *VCL*	5.0E-02	
Germ Cell-Sertoli Cell Junction Signaling	*A2M****, ACTN2, JUP, MAP2K2,*** *MAP3K7, MAP3K8****,*** *PAK3, PAK6****,*** *PIK3C2A****, RHOB, TUBA1A, TUBA4A,*** *VCL*	1.6E-02	

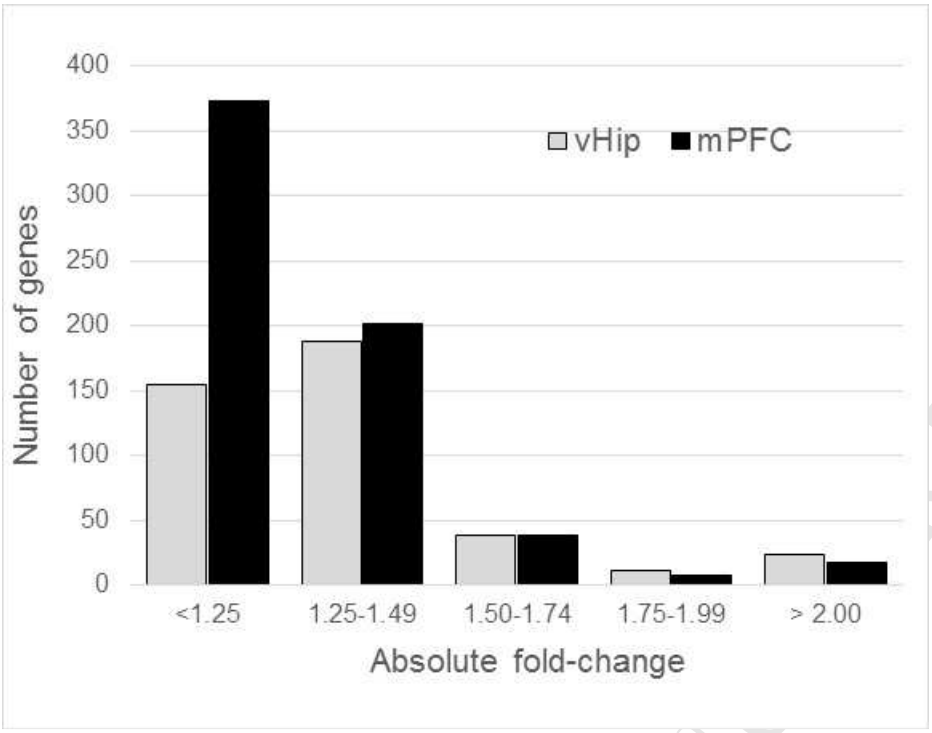
Table 5. Pathways altered in the medial prefrontal cortex.

*Genes in normal font were expressed at lower levels in the binge-drinking animals; bold font indicates genes that were expressed at higher levels. Z-score indicates whether the pathway is activated (positive z) or decreased (negative z). Pathways sharing many genes are boxed.

gene	vHip FC	mPFC FC	DRN FC	PAG FC	AcbS FC	CeA FC	Adult Nac FC	gene title
Adamts1	1.5	1.9	1.7	1.4			1.4	ADAM metallopeptidase with thrombospondin type 1 motif, 1
Atf3	7.8	16.2	3.0	2.2	1.8			activating transcription factor 3
Btg2	2.2	3.1	1.7	2.3			1.7	BTG family, member 2
Cyr61	11.3	9.5	3.6	3.2	3.8	1.6		cysteine-rich, angiogenic inducer, 61
Dusp1	1.6	1.6	2.0	2.2	1.9	1.6	1.4	dual specificity phosphatase 1
Fos	1.5	1.4	3.1	3.1	1.7		1.7	FBJ murine osteosarcoma viral oncogene homolog
Hspa1a	1.3	1.6	1.5	1.6				heat shock 70kDa protein 1A
Hspa1b	1.3	1.6	1.4	1.5				heat shock 70kDa protein 1B
Ier2	1.6	2.7	2.9	1.8	1.7		1.3	immediate early response 2
Npas4	1.8	2.6	4.8	3.8				neuronal PAS domain protein 4
Dgkb	-1.3	-1.4	-1.4	-1.3				diacylglycerol kinase, beta 90kDa

Table 6. Genes differentially expressed in at least 4 brain regions.

FC: fold change, vHip: ventral hippocampus (this study), mPFC: medial prefrontal cortex (this study), DRN: dorsal raphe nucleus (RNAseq, McClintick et al., 2015), PAG: periaqueductal gray (RNAseq, McClintick et al., 2016), AcbS: nucleus accumbens shell, CeA central core of the amygdala (microarrays, McBride et al., 2014b). Adult NAc: significant fold change in nucleus accumbens of adult P rats exposed to binge drinking (microarrays, Bell et al., 2009).



Highlights

- Alcohol binge drinking in decreased Wnt/β catenine pathway
- Long term potentiation and axonal guidance decreased by alcohol
- Myelination & cholesterol gene expression decreased by alcohol
- Alcohol increases cellular response to stress and inflammation