Parental Binge Alcohol Abuse Alters F1 Generation Hypothalamic Gene Expression in the Absence of Direct Fetal Alcohol Exposure

Magdalena M. Przybycien-Szymanska, Yathindar S. Rao, Sarah A. Prins, Toni R. Pak

Loyola University Chicago Health Science Division, Department of Cell and Molecular Physiology, Maywood, Illinois, United States of America

Abstract

Adolescent binge alcohol exposure has long-lasting effects on the expression of hypothalamic genes that regulate the stress response, even in the absence of subsequent adult alcohol exposure. This suggests that alcohol can induce permanent gene expression changes, potentially through epigenetic modifications to specific genes. Epigenetic modifications can be transmitted to future generations therefore, and in these studies we investigated the effects of adolescent binge alcohol exposure on hypothalamic gene expression patterns in the F1 generation offspring. It has been well documented that maternal alcohol exposure during fetal development can have devastating neurological consequences. However, less is known about the consequences of maternal and/or paternal alcohol exposure outside of the gestational time frame. Here, we exposed adolescent male and female rats to a repeated binge EtOH exposure paradigm and then mated them in adulthood. Hypothalamic samples were taken from the offspring of these animals at postnatal day (PND) 7 and subjected to a genome-wide microarray analysis followed by qRT-PCR for selected genes. Importantly, the parents were not intoxicated at the time of mating and were not exposed to EtOH at any time during gestation therefore the offspring were never directly exposed to EtOH. Our results showed that the offspring of alcohol-exposed parents had significant differences compared to offspring from alcohol-naïve parents. Specifically, major differences were observed in the expression of genes that mediate neurogenesis and synaptic plasticity during neurodevelopment, genes important for directing chromatin remodeling, posttranslational modifications or transcription regulation, as well as genes involved in regulation of obesity and reproductive function. These data demonstrate that repeated binge alcohol exposure during pubertal development can potentially have detrimental effects on future offspring even in the absence of direct fetal alcohol exposure.

Introduction

Several decades of research have established that maternal alcohol consumption during critical periods of fetal brain development leads to devastating long-term consequences on cognitive function and social behavior. However, less is known about the consequences of parental alcohol consumption, outside of gestational periods, on alcohol-dependent epigenetic modifications that could subsequently manifest as detrimental phenotypic changes in the offspring. Importantly, transgenerational effects, presumably transmitted through the germline, have been reported after exposure to a variety of physiological insults including endocrine disruptors [1,2], stress [3,4,5] and fetal alcohol exposure [6]. In this study, we examined gene expression profiles in the hypothalamus of offspring (F1, never alcohol exposed) whose parents were exposed to alcohol during a restricted period of time (adolescence) and then compared them to animals whose parents were never exposed to alcohol.

Alcohol is the most commonly abused drug by youth under 21 years of age and, according to the Center for Disease Control and Prevention (CDC), 11% of all alcohol in the United States is consumed by youth between the ages of 12 and 21. More importantly, greater than 90% of alcohol consumed by youth is achieved through “binge” drinking, which is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as consuming enough alcohol in two hour period to raise the blood alcohol concentration (BAC) above the 0.08% legal driving limit. Binge drinking during adolescence has been linked with a greater risk for alcoholism, drug abuse, depression, and suicide [7].

Extensive neurological changes are manifested during adolescence including increased neurogenesis and neuronal spine density, synaptic pruning, and altered neuronal activity patterns [8,9,10,11,12,13], thereby making it a particularly vulnerable developmental time period for the negative effects of alcohol. Previous studies have demonstrated that adolescent brain development is impaired by both physical and psychological stressors, including alcohol [14,15,16]. Our lab and others have shown that binge alcohol exposure, specifically during adolescence, had long-lasting effects on the expression of genes that regulate the stress response, even in the absence of subsequent adult alcohol exposure [14,17,18]. These results raised the possibility that alcohol exposure during adolescence can induce epigenetic modifications...
that result in permanent phenotypic changes in adult gene expression patterns and could potentially be transmitted to future generations.

In these studies we examined the gene expression profile in the hypothalamus of F1 generation offspring whose parents were exposed to binge alcohol during adolescence. The hypothalamus is a critical central regulator of multiple physiological processes including the stress response, appetite/satiety, circadian rhythms, osmoregulation, blood pressure, and reproduction. Strikingly, our results showed that the hypothalamus of offspring from alcohol-exposed parents had significant differences in genes that mediate neurogenesis and synaptic plasticity during neurodevelopment, genes important for directing chromatin remodeling, posttranslational modifications or transcription regulation, as well as genes involved in regulation of obesity and reproductive function. These results were highly compelling and, to our knowledge, are the first to demonstrate that adolescent binge alcohol exposure outside of gestational periods has an effect on hypothalamic gene expression in the F1 offspring. Taken together, these data illustrate the important novel concept that adolescent alcohol exposure can have detrimental effects on future offspring even in the absence of direct fetal alcohol exposure.

**Methods**

**Ethics Statement**

All animal procedures were approved by the Loyola University Medical Center Institutional Animal Care and Use Committee (IACUC), Loyola University Chicago permit #2011002.

**Animals**

Male and female Wistar rats were purchased from Charles River Laboratories (Wilmington, MA) at weaning (postnatal day (PND) 23) and allowed to acclimate for 7 days after arrival. Animals were handled for 5 min./once/day beginning at PND 30. Pubertal EtOH exposure began on PND 37, which is defined as peri-puberty [19,20,21]. Animals were undisturbed following the first exposure of our binge EtOH exposure paradigm until PND 68 (late puberty/early adult) at which time they received a second exposure to the same treatment paradigm (Fig. 1). During the duration of the experiment, males and females were separately housed in pairs on a 12:12 light/dark cycle with lights on at 0700 h with food and water available ad libitum.

**Binge Exposure Paradigm and Treatment Design**

Rats were handled 5 min./once/day for 7 d prior to treatment to control for nonspecific stress responses. At 37 d, animals were given 3 g/kg EtOH (20% v/v in tap water; N = 3/sex), or tap water alone (N = 3/sex), once/day via oral gavage at 10:00 AM to avoid disrupting normal feeding patterns. This process was repeated according to the following schedule for a total duration of 8 consecutive days: 3 d EtOH, 2 d tap water, 3 d EtOH. Control animals were given tap water alone once/day for 8 consecutive days (Fig. 1). Our previous studies showed that this repeated binge-pattern EtOH paradigm does not affect body weight/growth curves and consistently results in blood alcohol concentrations (BAC) of 150–180 mg/dl in males and 210–240 mg/dl in females [14,22,23]. We and others have previously used this paradigm as a model for the pattern of binge alcohol consumption observed in adolescents [14,22,24,25] and BAC achieved are similar to those observed in humans following a binge drinking episode [26,27]. After peri-pubertal treatments, animals were left undisturbed in their home cage until PND 68 when each group was again exposed to their respective treatment (i.e. control or binge EtOH, Fig. 1). We waited 24 hours after the last dose of EtOH to ensure that blood alcohol concentrations in the parents were undetectable at the time of mating (data not shown). Animals were grouped into mating pairs: binge male×binge female (N = 3 pairs); water male×water female (N = 3 pairs). All of the females gave birth to 12–16 pups approximately 28 d after being housed with a male, indicating that conception took place approximately 6 d after pairing; therefore, the pups were never directly exposed to alcohol at any time. The pups were culled to 10 pups/litter immediately after birth in an equivalent sex ratio. The pups were then returned to their biological mothers until PND 7 at which time they were deeply anesthetized on ice and sacrificed (Fig. 1). Brains were rapidly removed, the hypothalamus microdissected on ice, and then stored in −80°C until further processing for a genome-wide analysis on hypothalamic total RNA samples using a chip-based microarray (Southern California Genotyping Consortium, SCGCC, Illumina Rat Ref-12). The PND 7 time point was chosen because the extent of rat neurodevelopment at PND 7 is roughly equivalent to that of a human infant at birth [28].

---

**Figure 1. Adolescent binge EtOH exposure paradigm.** Diagram depicting the experimental design. Male and female Wistar rats were purchased from Charles River Laboratories at weaning (PND 23). Animals were handled for 5 min./1x/day beginning on PND 30. Hatched box indicates treatment days for the binge EtOH exposure paradigm. Control rats were given tap water once/day during the 8-day treatment period. Rats were mated according to treatment groups on PND 75. Offspring were untreated and sacrificed at PND 7.

doi:10.1371/journal.pone.0089320.g001
Sample Processing, Microarray Analysis and Bioinformatics

Total RNA from PND 7 rat pup hypothalami (N = 6/treatment/sex) was isolated using RNeasy Plus Mini Kit (Qiagen, Inc.) according to manufacturer’s instructions and submitted to the Southern California Genotyping Consortium (SCGC) for microarray processing. The samples were loaded onto a RatRef-12 expression BeadChip (Illumina) which contained 22,523 probes targeted to 21,910 unique genes that were selected from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq – Release 16) database. Total RNA purity and integrity were verified by SCGC. Multiple control probes on the chip were used to account for variation in chip production, sample labelling and assay conditions. Raw data were processed by subtraction of background signals and signal intensities above threshold (>500) were analyzed using GenomeStudio V2010.3 software. A differential score was calculated for each gene in order to determine statistical significance between treatment groups (p<0.05 by one-way ANOVA). All of the statistical analyses were performed by the Biostatistics Core Facility at Loyola University Stritch School of Medicine in consultation with Dr. James Sinacore. Of the 21,910 genes targeted on the microarray chip, 11,494 and 11,503 were detected above threshold in the male and female hypothalamus, respectively. Further analysis of these genes was performed by the Biostatistics Core Facility at Loyola University Stritch School of Medicine in consultation with Dr. James Sinacore.

Global Microarray Analysis

We used a genome-wide microarray approach to determine the effects of parental adolescent binge EtOH exposure on transgenerational (F1, EtOH naïve) gene expression in the hypothalamus. This approach was used as an initial screen to identify specific genes of interest, which were then validated using qRT-PCR on hypothalamic samples from the same animals. In this report, qRT-PCR data rather than raw microarray data are described. The complete microarray dataset was submitted to the Gene Expression Omnibus (GEO).

Results

Table 1. Primer sequences for selected genes.

| GENE NAME | UPPER PRIMER | LOWER PRIMER |
|-----------|--------------|--------------|
| APOE      | 5′TTGTTCGCGAAGAGGACCTGGT | 5′CCTGTCAGCAATGGGACCAA |
| BMP1      | 5′ACAGTGCGACGGACGACCCTTC | 5′TGCCAAATTGTTCCACGAG |
| Dicer1    | 5′GGGAAAGCTGTGCAGAACAAC | 5′GGCTGCTCTAGGTCTTAGTC |
| Dnmt1     | 5′CCCTGTACATTGCTGTGGT | 5′TCTGCCCCTTCTGCTTCC |
| Egr2      | 5′ACTACATCGACCACTCTTGGGC | 5′GCTCTTGGCGCTACATTGG |
| Fgf13     | 5′TCTCGGGGTGGTGCTGGTATCC | 5′ATCGCGGAAGATCTCCGAG |
| Fgfr3     | 5′GAGACTTGGCTGCCGAGAAC | 5′GGAAAGCCACCAAAAGACCA |
| GnRH      | 5′CTCTGTCTGCTCCTGAC | 5′CTTGCCCCCTCCTCAAATCA |
| Hdac3     | 5′ACACCGGGTGAACCCCCATC | 5′TCAGATGGAAAGCCGACCAT |
| Igf2r     | 5′CAGATGGTGTGGGAGACCGT | 5′GAAAGTGCGAGGGGAGCAC |
| Pak3      | 5′CTCTGGGGTGGATCTGTCT | 5′AGCAGAACGTGCTCCTGGAT |
| Reln      | 5′ACACACCTGACAGCCGAGG | 5′CACACAGCTCTCCTGGACG |
| Serpin1   | 5′AGCCGATGTGGTACAGAATG | 5′ACTCTGTAAGTCGCACACC |
| Sirt2     | 5′GCACCGACTCGTACCTTAT | 5′AGCGAGCGTGCTGACAGG |
| Sumo2     | 5′CTTCAGGGTGGGAGGGCAGG | 5′TCACGATGTGCTGACAGG |
| Vamp3     | 5′CCCGCGAATAATGCTCAG | 5′TGTTCCTAACCCTGGGAGG |
| Wbp4      | 5′GAGTGCCCGGACTCTTCAA | 5′CTGGAAAGAGGACGCGCTCA |

Intron-spanning primers were designed to detect selected genes that were initially identified by microarray analysis.
doi:10.1371/journal.pone.0089320.t001
database (accession #GSE53028). Mating pairs (both male and female partners) were exposed to water alone or to our binge EtOH paradigm. Importantly, the mating pairs (parental generation) had undetectable BAC at the time of mating and the females were not exposed to EtOH during gestation; therefore, the offspring were never directly exposed to EtOH at any time. There were no differences in the time to fertilization (approx. 6 days), total number of pups (12–16), birth weights, or male:female sex ratios in either treatment group (data not shown). Moreover, all pups were viable and did not exhibit any overt abnormalities within the first week of postnatal life. Overall, 16.8% more genes were significantly altered in females (767 genes) compared to males (638 genes), despite only a 0.7% sex difference in the total number of genes detected (11,503 female and 11,494 male). Interestingly, gene expression patterns in the offspring were overall decreased (77.2%) due to parental adolescent binge EtOH exposure, compared with 22.8% of genes that were increased. Specifically, parental adolescent binge EtOH exposure significantly decreased 602 genes in female pups and 482 in males, while only 165 and 156 genes were increased in females and males, respectively.

Overall Analysis of Functional Gene Clusters

To determine the functional significance of the genes that were detected by microarray analysis as being altered by parental adolescent binge EtOH exposure, we performed a functional gene cluster analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources 6.7 software [29,31]. Using this software, we were able to group genes that have related biological functions into distinct clusters. This allowed us to make predictions about the possible functions of the hypothalamus that could be altered due to parental binge EtOH exposure. The gene groups that came up (increased/decreased) with the analysis as a result of parental binge EtOH exposure fell broadly into 19 functional gene cluster categories. Notably, genes that were significantly decreased by parental binge EtOH exposure were restricted to fewer functional clusters (8 clusters) than the genes that were increased (14 clusters, Table 2), despite the fact that the majority of genes significantly altered according to the microarray analysis were decreased (77.2% compared to 22.8% increased). Moreover, distinct functional gene clusters for genes that were decreased were more widely represented in female pups compared to males. For instance, there were no gene clusters unique to males for the downregulated genes, yet 5 were unique to females and 3 were common between both males and females. By contrast in the 14 clusters representing genes that were upregulated, 5 were unique to males, 2 were unique to females, and 7 were common between both sexes. In addition, several multifunctional genes in both males and females could not be grouped into a distinct functional cluster.

Functional Cluster Analysis in Male Offspring

There were a total of 3 functional gene clusters that included all of the genes downregulated in male offspring (Table 2). The most highly represented of these was a cluster of genes that encoded ribosomal proteins (109 genes). The other 2 clusters contained genes encoding molecules involved in cell cycle (15 genes) and mRNA processing/splicing (10 genes). In general, there were more widely diverse gene clusters associated with the genes that were upregulated in males compared to those that were downregulated (Table 2). Highly represented in this group were clusters that included genes encoding nuclear proteins (57 genes), kinases (48 genes) and molecules involved in metal ion binding (35 genes). A minor representation of clusters included genes for ribosomal proteins (14 genes), disulfide bonds (5 genes), glycoproteins (5 genes), signaling molecules (7 genes), secreted proteins (3 genes) and EGF-like domains (3 genes).

Functional Cluster Analysis in Female Offspring

Overall, more total genes were altered by parental binge EtOH exposure in the F1 generation PND 7 females compared to males (767 genes and 638 genes, respectively). Global gene changes in females could be clustered into 13 distinct functional groups, of which 8 contained genes that were significantly downregulated in females (Table 2). These clusters contained genes encoding ribosomal proteins (87 genes), cell cycle proteins (80 genes), signaling proteins (43 genes), G protein-coupled receptors (11 genes) and proteins involved in transcriptional regulation (60 genes), mRNA processing/splicing (11 genes), disulfide bonds (26 genes) and glycoproteins (35 genes). Genes that were upregulated in females could be grouped into 9 clusters (Table 2) including nuclear proteins (57 genes), metal ion binding (35 genes), kinases (48 genes), ribosomal proteins (14 genes), EGF-like domains (3 genes) secreted proteins (3 genes), signalling molecules (7 genes), disulfide bonds (3 genes) and glycoproteins (5 genes).

qRT-PCR Validation of Selected Genes from Functional Clusters Identified using DAVID Software

Quantitative real-time RT-PCR was used to validate a subset of genes that were significantly altered according to the microarray analysis in PND 7 offspring of binge EtOH-exposed parents. Importantly, the qRT-PCR method was chosen to validate these genes because the sensitivity of detection for qRT-PCR is superior to a chip-based microarray platform. Out of those 17 selected genes, 70% showed the same results using both methods. More specifically, 65% showed similar results in males and 76% in females. The genes chosen for further analysis were selected based on their well described roles in neurodevelopment, neuroplasticity, and epigenetic processes including: [Fig. 2: fibroblast growth factor 13 (FGF13), bone morphogenic protein 1 (BMP1), reelin (RELN), serpin peptidase inhibitor 1 (SERPIN1); [Fig. 3: p21-activated kinase 3 (PAK3), insulin-like growth factor receptor 2 (IGF2R), vesicle associated membrane protein 3 (VAMP3), fibroblast growth factor receptor 3 (FGFR3); [Fig. 4: gonadotropin-releasing hormone (GnRH), apolipoprotein E (ApoE); [Fig. 5: early growth response 2 (Egr2), DlCER1, WW domain binding protein 4 (WBP4), and small ubiquitin-like modifier protein 2 (SUMO2)]; [Fig. 6: histone deacetylase 3 (HDAC3), Siruin 2 (SIRT2), DNA methyltransferase 1 (DNMT1).

Selected Genes in the Neurodevelopment Functional Cluster

Fibroblast growth factor 13 (FGF13) is an important regulator of neuronal polarization and migration [32]. Our results showed that parental adolescent binge EtOH exposure significantly decreased Fgf13 hypothalamic mRNA expression in both male and female PND7 offspring (Fig. 2A). Another gene involved in neurodevelopment is BMP1, which interacts with other proteins to control neuronal stem cell differentiation [33]. Our qRT-PCR analysis showed a significant upregulation of Bmp1 mRNA in male, but not female, offspring (Fig. 2B). Reelin (Reln), a large extracellular matrix protein that when secreted serves as a signalling molecule in cell-cell interactions, has been shown to play a role in the lamination of the hippocampal dentate gyrus [34,35]. Our results revealed that there was a significant increase in Reln mRNA expression in female, but not male, offspring of parents exposed to adolescent binge alcohol (Fig. 2C). Finally, we analyzed the expression of Serpin1, which is a member of serpin
superfamily of serine proteinase inhibitors that is secreted by axons and plays a role in regulation of axonal growth and synaptic plasticity [36,37,38]. Our qRT-PCR analysis revealed that Serpini1 mRNA was significantly upregulated in F1 generation males due to parental adolescent binge EtOH exposure (Fig. 2D) and there was a similar trend in females. Interestingly, a number of genes important for regulating synaptic plasticity were also identified by the microarray analysis as significantly altered due to paternal adolescent binge EtOH exposure including PAK3, IGF2R, VAMP 3, and FGFR3. According to our results, Pak3 mRNA was significantly decreased by parental adolescent alcohol exposure in both F1 generation PND 7 males and females (Fig. 3A). Together with Pak3, IGF2R is also involved in spine maturation. There was a statistically significant increase in the expression of Igf2R mRNA in F1 generation PND 7 males (Fig. 3B) and a strong trend towards an increase in the expression of Igf2R mRNA in females (Fig. 4B, p = 0.099) due to parental adolescent binge EtOH exposure. Another protein important for synaptic plasticity is VAMP3, a SNARE complex interacting protein responsible for vesicle docking at the synapse [39]. Our qRT-PCR analysis showed that Vamp3 mRNA was significantly increased in male F1 generation offspring pups after parental adolescent binge EtOH exposure (Fig. 3C). By contrast, there was an opposite effect in females which showed a decreased trend for Vamp3 mRNA expression, although this result was not statistically significant (Fig. 3C). Finally, we analyzed the hypothalamic expression of FgfR3 based on our initial microarray results. Previous work has shown that activation of FgfR3 influences development of the occipitotemporal cortex during early postnatal life by overproduction of intermediate neuronal progenitors and therefore prolonged neurogenesis [40]. However, our qRT-PCR analysis did not show any significant changes in FgfR3 mRNA levels in male or female offspring as a result of parental binge EtOH exposure (Fig. 3D).

Selected Genes in the Metabolism Functional Cluster

One of the primary functions of the hypothalamus is the coordination of physiological homeostasis through regulation of feeding behaviors, reproduction, thermoregulation, and water balance. Two genes identified by microarray and cluster analyses were GnRH and ApoE. GnRH is the most upstream regulator of the hypothalamo-pituitary gonadal axis and is absolutely required for reproductive function. Moreover, the synthesis and release of GnRH is significantly elevated during pubertal maturation [41,42,43,44,45]. Our qRT-PCR results showed that GnRH was significantly increased, but only in F1 generation males due to parental adolescent binge alcohol exposure (Fig. 4A), and there was no effect in females. Perhaps the most striking results were observed with ApoE, a component of plasma lipoprotein that is involved in cholesterol metabolism and lipid transport [46]. ApoE is synthesized in the hypothalamus and plays a major role in regulation of feeding behavior [47].

Table 2. Gene cluster analysis using DAVID software.

| GENE CLUSTER          | # GENES DECREASED MALE OFFSPRING | # GENES DECREASED FEMALE OFFSPRING |
|-----------------------|---------------------------------|-----------------------------------|
| Ribosomal proteins    | 109                             | 87                                |
| Cell cycle            | 15                              | 80                                |
| mRNA processing/splicing | 10                           | 11                                |
| Transcriptional regulation | -                           | 60                                |
| Cell signaling/kinases | -                              | 43                                |
| Disulfide bonds       | -                               | 26                                |
| Glycoproteins         | -                               | 33                                |
| G-protein coupled receptors | -                           | 11                                |

| GENE CLUSTER          | # GENES INCREASED MALE OFFSPRING | # GENES INCREASED FEMALE OFFSPRING |
|-----------------------|---------------------------------|-----------------------------------|
| Ribosomal proteins    | 16                              | 14                                |
| Cell cycle            | -                               | -                                 |
| mRNA processing/splicing | -                           | -                                 |
| Transcriptional regulation | 31                           | -                                 |
| Cell signaling/kinases | 13                             | 55                                |
| Disulfide bonds       | 7                               | 3                                 |
| Glycoproteins         | 10                              | 5                                 |
| G-protein coupled receptors | -                           | -                                 |
| Metal ion binding     | 28                              | 35                                |
| Secreted proteins     | 4                               | 3                                 |
| Cell adhesion         | 10                              | -                                 |
| EGF-like domains      | -                               | 3                                 |
| ATP-binding           | 11                              | -                                 |
| Nucleotide binding    | 13                              | -                                 |
showed that parental adolescent binge EtOH exposure almost completely abolished ApoE mRNA expression in both the male and female F1 generation offspring (Fig. 4B).

**Selected Genes in Transcriptional and Translational Regulation Cluster**

Genes tested from this functional cluster included EGR2, DICER1, WBP4, and SUMO2. The early growth responsive 2 (Egr2) gene is a transcription factor that targets a number of genes involved in myelin formation and maintenance. Our results showed that Egr2 mRNA expression was significantly decreased in female F1 offspring as a result of parental adolescent binge ETOH exposure (Fig. 5A). A similar sex difference was observed with Dicer1, which is important for posttranscriptional processing of small non-coding RNAs. Parental adolescent binge EtOH exposure significantly increased Dicer1 in F1 male (Fig. 5B), but not female, offspring. Next, we measured Wbp4, a gene involved in alternative mRNA splicing [48]. Parental adolescent binge EtOH exposure significantly decreased Wbp4 mRNA in the hypothalamus of F1 generation females (Fig. 5C), but males were unaffected. Finally, the posttranslational modifier, Sumo2, was increased by more than 2-fold in male offspring, but decreased by 30% in female offspring of parents exposed to adolescent binge EtOH (Fig. 5D).

**Genes in the Chromatin Modification Cluster**

The last set of genes that we chose to further analyze using qRT-PCR were those involved in chromatin modifications. These genes are critical for regulating global gene expression patterns through alterations of histone proteins and nucleotides within gene promoter regions. For instance, HDAC3 is a protein that removes acetyl groups from histones, thereby making the DNA more accessible for transcriptional regulation [49]. The microarray analysis revealed that Hdac3 was downregulated in both F1 generation males and females as a result of parental adolescent binge EtOH exposure, and our follow-up qRT-PCR analysis showed the same direction of change, in Hdac3 mRNA expression, although it did not reach statistical significance (Fig. 6A). Another interesting histone deacetylase that came up on the microarray was the class III histone deacetylase SIRT2. Similar to HDAC3, Sirt2 mRNA was downregulated in both F1 generation males and females as a result of parental adolescent binge EtOH exposure according to the microarray results. By contrast, however, our qRT-PCR analysis showed the same direction of change in mRNA expression of female offspring only (Fig. 6B). Lastly, DNMT1 is a maintenance methyltransferase and it is responsible for long-term gene methylation and therefore gene suppression. The microarray analysis showed that Dnmt1 mRNA was upregulated in both F1 generation males and females however,
this result could not be confirmed using qRT-PCR (Fig. 6C). Although there was no significant change in the expression of Dnmt1 mRNA in the offspring of either sex, there was a 20% decrease in the expression observed in males and a strong trend towards a significant decrease in females (Fig. 6C, p = 0.086).

**Discussion**

Adolescent binge alcohol abuse is an increasing societal burden. While it has been well documented that maternal alcohol abuse during pregnancy results in detrimental effects in the developing offspring, less is understood about heavy and repetitive alcohol abuse prior to, but outside of the gestational period, on neurodevelopment in the offspring. Moreover, the contribution of prior paternal (male) binge alcohol abuse on gene expression in the developing fetal brain is unclear. On the other hand, maternal exposure to environmental factors such as stress, drugs of abuse, and endocrine disruptors have been shown to affect gene expression patterns and behavioral responses in the offspring [1,4,5,6,50,51,52] and alcohol is both a drug of abuse and a physiological stressor, since it potently activates the hypothalamo-pituitary-adrenal (HPA) axis [14,15,18,53]. For example, early life stress in female mice has been associated with the development of a depressive-like phenotype in adulthood; a phenotype which persists in the future offspring, despite normal rearing conditions [4]. Moreover, child abuse in humans leads to an altered adult neuroendocrine response of the HPA axis that is perpetuated in the babies of female child abuse victims [54]. There is also some evidence that paternal exposure to other drugs of abuse can also adversely affect future offspring brain development. For instance, He and colleagues demonstrated significant impaired memory formation in the offspring of mice whose male, but not female, parent was exposed daily to cocaine beginning during early adolescence and continued daily until mating. Interestingly, the effect on memory impairment was permanent throughout the life of the offspring and was significantly greater in the female offspring [55]. The data presented in this report provide evidence that parental (both maternal and paternal) adolescent binge EtOH exposure, outside of fertilization or gestation periods, can lead to aberrant gene expression in the hypothalamus of the offspring. Specifically, these data show that parental adolescent binge EtOH exposure changed the expression of genes that are critical for neurodevelopment and synaptic plasticity (FGF13, BMP1, SERPIN1, RELN, PAK3, IGF2R, VAMP3, FGFR3), metabolic functions (GNRH, APOE), transcriptional regulation and post-translational processing (EGR2, DICER1, WBP4, SUMO2) and...
chromatin modifications (HDAC3, SIRT2, DNMT1). Alteration in the expression of these genes during the fetal and early postnatal periods may lead to abnormal hypothalamic development and function, as well as increase the potential risk for mood disorders, stress-induced obesity, and addiction in adulthood.

**Paternal Adolescent Binge EtOH Exposure Significantly Altered Genes Critical for Neurodevelopment in the F1 Generation Offspring**

Proper neurogenesis and neural differentiation during embryonic and early postnatal life is critical for healthy brain development and adult cognitive functions. We observed in these studies that parental adolescent binge EtOH exposure resulted in alterations of the expression of several genes critical for neurodevelopment (BMP1, FGF13, SERPINI1 and RELN) and synaptic functioning in the hypothalamus (IGF2R, FGFR3, PAK3, VAMP3). Growth factors, in particular, are critical for the formation and maintenance of the brain during embryogenesis. In our study, parental adolescent binge EtOH exposure significantly decreased the expression of Fgf13 mRNA in the F1 generation offspring leading us to speculate that parental binge EtOH consumption may lead to abnormal hypothalamic development by decreasing critical growth factors important for embryonic brain development. Importantly, FGF13 is highly expressed in the brain and involved in tyrosine phosphorylation of mitogen-activated protein kinase (MAPK), c-RAF activation, phosphorylation of phospholipase C-gamma (PLCγ), increased number of glutamic acid decarboxylase positive neurons, increased GABA uptake, and increased choline acetyltransferase enzyme activity [56], all of which are important for successful neurodevelopment during embryogenesis. In addition, it has recently been shown that FGF13 is crucial for neuronal polarization and migration and that it acts as microtubule stabilizing protein during early development [32].

Similarly, another growth factor that was significantly altered in the F1 generation offspring in our study was BMP1. Our results showed that Bmp1 mRNA was upregulated in PND 7 males as a result of parental adolescent binge EtOH exposure. The significance of increased Bmp1 mRNA is currently unclear, however, one possibility might be that Bmp1 upregulation is a compensatory effect to minimize neuronal damage. For instance, one of the primary functions for BMP1 is to regulate neural stem cell proliferation, differentiation and maturation of the developing brain, as well as stem cell maintenance in the subventricular zone of the adult brain [33,57,58].

Growth factor receptors are central components of growth factor signalling pathways and altered receptor complement can be equally deleterious as changes in ligand for growth factor activity. Our data revealed significant parental alcohol-induced increases in Igf2R mRNA expression in both male and female offspring, as well as decreased expression of Fgfr3. Interestingly, IGF2R is important for regulating axonal spine maturation and synapse formation [59]. One possibility is that high IGF2R levels may be an indication of decreased IGF2R ligand availability, as many receptor:ligand concentrations are inversely correlated. In that case, it can be inferred that synapse formation in the offspring might be impaired as a consequence of paternal adolescent binge EtOH exposure. Similarly, FGFR3 has also been implicated in coordinating growth and development of the occipitotemporal cortex [40] and our data showed a significant decrease in Fgfr3 mRNA expression in female pups after parental adolescent binge EtOH exposure. These data further support our hypothesis that binge drinking during adolescence could disrupt normal hypothalamic neurodevelopment in future offspring. Moreover, our data also demonstrate the interesting observation that parental adolescent exposure to EtOH induced sexually dimorphic changes in offspring gene expression profiles.

Our data revealed EtOH-induced changes in several other genes important for neurodevelopment in the offspring, which are not categorized specifically as growth factors. These include Serpin1 (also known as neuroserpin) and Reln. SERPIN1 is expressed in the brain where it inhibits tissue plasminogen and regulates axonal growth [60,61]. Our data revealed that Serpin1 mRNA was increased in the hypothalamus of both male and female F1 generation PND 7 pups whose parents were prior exposed to adolescent binge EtOH. Interestingly, upregulation of Serpin1 has also been linked with the pathogenicity of AD [37]. Similarly, our data showed that Reln mRNA expression was increased in female pups after parental adolescent binge EtOH exposure. REELIN is an extracellular matrix protein that controls neuronal migration and lamination of the hippocampus [34,62,63]. REELIN exerts its actions through α3β1 integrin to inhibit neuronal migration [65], suggesting that increased expression of Reln mRNA may lead to
increased inhibition of neuronal migration in the hypothalamus and therefore, disrupted hypothalamic neuronal connectivity.

Successful neurodevelopment also requires the formation of appropriate synaptic connections and maintenance of coordinated synaptic activity. We found that parental adolescent binge EtOH exposure results in altered expression of a variety of genes that regulate synaptic plasticity, including PAK3. The p21-activated kinase 3 (PAK3) is known to act through the CDC42/Rho pathway and is implicated in regulating various neuronal functions including synaptic plasticity and spine morphogenesis [64], cytoskeletal dynamics, as well as cell proliferation and differentiation [65]. Mutations in the expression of the PAK3 gene and abnormalities in spine maturation have been correlated with mental retardation [64,66,67,68]. Our data showed that there was a significant decrease in \textit{Pak3} mRNA levels after parental adolescent binge EtOH exposure in both male and female F1 generation pups, suggesting that the fine-tuning processes associated with brain maturation could be adversely affected. The DAVID functional cluster analysis also showed that genes involved in SNARE interactions with vesicular and secreted proteins, such as VAMP3, were also altered by parental binge EtOH exposure. VAMP3 is a fusion protein associated with SNARE complexes, highly expressed in astrocytes, and facilitates vesicle docking at the membrane [39]. Activation of VAMP3 has been shown to influence glutamate and D-serine release [69] therefore, increased Vamp3 mRNA expression as observed in our study may indicate a potential for abnormal synaptic functioning.

**Paternal Adolescent Binge EtOH Exposure Altered Genes that Regulate Metabolism in the F1 Generation Offspring**

The hypothalamus is the primary brain region mediating homeostasis through its central regulation of circadian rhythms, reproduction, feeding behaviors, thermoregulation, osmotic balance and stress responses. One of the most striking observations in our study was the complete abolition of \textit{ApoE} mRNA expression in both male and female F1 generation offspring as a result of parental adolescent binge EtOH exposure. \textit{ApoE} is involved in lipid metabolism and cholesterol homeostasis [46]. Recent data has shown that \textit{ApoE} is regulated in the arcuate nucleus of the hypothalamus by the hormone leptin, which regulates feeding behavior. [70]. Although we did not observe any birth weight differences in the offspring from control and EtOH-exposed parents, a significant decrease in \textit{ApoE} expression in the offspring could potentially predispose them for an increased risk of developing obesity later in life. Another significant finding was the observed increase in \textit{GnRH} mRNA expression in male offspring as a result of parental adolescent binge EtOH exposure. \textit{GnRH} has long been known for stimulation of gonadotropin release from the pituitary gland in both males and females and therefore, is absolutely required for pubertal onset and fertility [43,44,45,71,72,73,74,75,76,77,78,79,80]. Recently, a role for \textit{GnRH} in metabolic functions has been demonstrated by Harris and colleagues, who showed that \textit{GnRH} increased the expression of the glucose transporter, GLU-1, in pituitary gonadotrophs and stimulated glucose uptake in these cells [81]. The observed
significant increase in GnRH expression in the male offspring could lead to abnormal pituitary glucose regulation, thereby raising the potential for a potential host of neuroendocrine dysfunctions.

**Paternal Adolescent Binge EtOH Exposure Altered Genes Important for Modifying Chromatin, Transcriptional Regulation, and Posttranslational Processing in the F1 Generation Offspring**

Altered gene expression can result from modifications in the transcriptional machinery, such as through chromatin modifications and transcription factors, or posttranslational processing. In this study we did not analyze specific epigenetic modifications of chromatin for any of the genes tested. However, an epigenetic transfer from the parental generation is the most parsimonious explanation for the effects observed in the offspring, which were not exposed to any differential conditions in utero. In this study, we demonstrate that the F1 generation offspring of parents exposed to binge EtOH during adolescence had significantly different gene expression profiles according to the microarray analysis for genes that are capable of modifying chromatin structure (HDAC3, SIRT2, DNMT1) genes that regulate transcription and RNA processing (EGR2, WBP4, DICER1), and genes that regulate posttranslational modifications of proteins (SUMO2). Importantly, however, only Egr2 and Wbp4 were statistically significant when validated using qRT-PCR. This could be due to amplification of individual variance that was observed using the highly sensitive qRT-PCR method and our relatively low sample size (N = 6). Nevertheless, the results obtained using qRT-PCR had the same trends and direction of change as the results using microarray analysis, suggesting that these results could be biologically relevant. HDAC3 is a histone deacetylase that removes acetyl groups from the histone making the DNA more tightly bound to the histone proteins and therefore, less accessible for transcriptional regulation. In general, it is well accepted that HDAC3 suppresses global gene transcription in a wide variety of systems. However, it has also been shown to have very specific roles in the regulation of normal brain function. For instance, HDAC3 acts in concert with nuclear receptor corepressors (such as NCor and SMRT), to regulate thyroid hormone receptor [82], neurogenesis [83], as well as for the suppression of long term memory formation [84]. Our data showed that Hdac3 mRNA was downregulated in the hypothalami of both male and female PND 7 pups whose parents were exposed to adolescent binge EtOH. A decrease in Hdac3 expression could have an overall positive impact on gene transcription and might explain the increased expression levels we observed in several other genes. In addition to alterations in Hdac3 expression, we also observed gene expression changes in another type of histone deacetylase, SIRT2, which is a NAD-dependent class III histone deacetylase. Similar to other deacetylases, SIRT2 is primarily involved in transcriptional silencing [85,86,87]. Chronic cocaine exposure increased the catalytic activity of SIRT2 in the nucleus accumbens and a pharmacological block of SIRT2 activity dramatically reduced...
coca-seeking behavior, suggesting that SIRT is an important component of the reward pathways for drugs of abuse [96].

Our studies showed a significant decrease in Egr2 mRNA expression in female F1 offspring of parents exposed to binge EtOH. The potential consequences of this for female offspring is unclear however, mutations in this gene cause severe sensory deficits in human patients and is associated with Charcot Marie Tooth Syndrome [88]. EGR2 is a transcription factor that regulates transcription of a variety of genes, particularly those involved with axonal myelination. Interestingly, increased Egr2 mRNA expression in Fos-positive neurons of the prefrontal cortex has also been linked with cue-induced relapse to drug addiction [89]. One of the more interesting genes that we found altered in our study was Dicer1, which regulates small noncoding RNA processing including microRNAs [90], microRNAs have recently come to the forefront as a critical molecular mechanism underlying virtually all cellular process. Notably for our study, DICER1 has been shown to mediate stress-induced anxiety through its regulation of microRNA-34 in the amygdala [91]. These data raise the interesting possibility that changes in Dicer1 mRNA expression in male offspring after parental binge EtOH exposure may have far-reaching effects on posttranscriptional gene regulation. Two other interesting genes that we investigated regulate gene expression through transcriptional or posttranslational modifications (WBP4 and SUMO2). Wbp4 encodes a splicosomal domain that is involved in binding various proline rich ligands and influences pre-mRNA splicing [48,92,93,94]. SUMO2, on the other hand, is a protein that conjugates lysine residues of target proteins and modulates protein activity, stability and cellular localization. Interestingly, the main targets for SUMO proteins are transcription factors that regulate gene transcription and expression [95,96,97]. Changes in these genes after parental binge EtOH consumption point to yet another mode of possible mechanisms responsible for altered gene transcription in the offspring.

Conclusions

Taken together, our data herein describe highly novel results showing significant changes in hypothalamic gene expression in offspring born to parents exposed to adolescent binge EtOH. Children of alcoholic parents are more prone to develop psychological disorders, such as anxiety, major depression, conduct abnormalities, and attention deficit disorder [98,99,100,101,102,103,104]. Moreover, adoption and twin studies have demonstrated that the propensity to develop these psychological disorders has a strong genetic component [100,105] however, a clear genetic mark has yet to be identified. Those findings raise the possibility that EtOH exposure could induce epigenetic modifications in the parents resulting in an epigenetic "signature" that is transmitted to the offspring. However, we acknowledge that caution must be exercised when drawing conclusions based on gene expression studies. For instance, changes in gene expression can be a predisposing factor for the development of neurological and behavioral disorders, although both the positive and negative effects of environment cannot be discounted. In our study, the mothers were not intoxicated at any point during mating or pregnancy leading to the assumption that their behavior during pregnancy, and also postnatally, was similar to controls. Despite this assumption, physiology ultimately dictates behavior therefore, identifying the molecular basis of the physiological changes in the parent is critical to understanding the transgenerational effects of adolescent binge alcohol exposure. In addition, the maternal uterine environment might have also been a factor. The experimental approach for these studies exposed both the male and female parents to our binge EtOH paradigm making it impossible to determine whether one parent conferred more epigenetic information than the other and to date, we understand very little about the mechanisms for how epigenetic marks are conferred to offspring. In addition, we used a "two-hit" paradigm in which animals were exposed to repeated binge EtOH during both the early (37–44 days of age) and late (67–72 days of age) pubertal period, which was designed to mimic early and late human adolescent exposure (13–16 & 18–21 yrs). Therefore, the precise window of vulnerability during adolescence remains unclear. Our previous studies showed that perturbations in the HPA axis were observed only with an early adolescent binge and not with a single late adolescent “binge”, indicating the early adolescent binge was critical [14]. Therefore, we would predict that a single binge episode during the early adolescent period would be sufficient to elicit the observed altered gene expression in the offspring, however, the importance of late adolescent binge EtOH exposure cannot be ruled out at this time. Overall, these data are a first step towards fully understanding the long-term and transgenerational consequences of adolescent binge alcohol abuse.

Author Contributions

Conceived and designed the experiments: MMPS TRP. Performed the experiments: MMPS YSR SAP TRP. Analyzed the data: MMPS YSR TRP. Contributed reagents/materials/analysis tools: TRP. Wrote the paper: MMPS YSR SAP TRP.

References

1. Anway MD, Cupp AS, Uzunova M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308: 1466–1469.
2. Skinner MK, Anway MD, Savenkova MI, Gore AC, Crews D (2008) Transgenerational epigenetic programming of the brain transcriptome and anxiety behavior. PLoS One 3: e3745.
3. Crews D, Gillette R, Scarpino SV, Manikkam M, Savenkova MI, et al. (2012) Epigenetic transgenerational inheritance of altered stress responses. Proc Natl Acad Sci U S A 109: 9143–9148.
4. Franklin TB, Russig H, Weiss IC, Graff J, Linder N, et al. (2010) Epigenetic transmission of the impact of early stress across generations. Biol Psychiatry 68: 408–415.
5. Roth TL, Lubin FD, Funk AJ, Sweatt JD (2009) Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry 65: 760–769.
6. Govorko D, Bekdash RA, Zhang C, Sarkar DK (2012) Male germ line transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. Biol Psychiatry 72: 378–389.
7. Miller JW, Nauni TS, Brewer RD, Jones SE (2007) Binge drinking and associated health risk behaviors among high school students. Pediatrics 119: 76–85.
8. Ahmed EI, Zehr JL, Schulz KM, Lorenz BH, DonCarlos LL, et al. (2008) Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat Neurosci 11: 993–997.
9. Rankin SL, Parлiss GD, McCurdy RD, Giles ED, Fisher KR (2003) Postnatal neurogenesis in the vasopressin and oxytocin-containing nucleus of the pig hypothalamus. Brain Res 971: 189–196.
10. Raymond AD, Kucherepa NN, Fisher KR, Halina WG, Parлiss GD (2006) Neurogenesis of oxytocin-containing neurons in the paraventricular nucleus (PVN) of the female pig in 3 reproductive states: puberty gilts, adult gilts and lactating sows. Brain Res 1102: 44–51.
11. Woo TU, Pucak ML, Kye CH, Matus CV, Lewis DA (1997) Peripubertal refinement of the intrinsic and associational circuitry in monkey prefrontal cortex. Neuroscience 80: 1149–1156.
12. Zehr JL, Todd BJ, Schulz KM, McCurdy MM, Sisk CL (2006) Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster. J Neurobiol 66: 578–590.
13. Vian V, Bingham B, Davis J, Lee P, Wong M (2003) Gender and puberty interact on the stress-induced activation of parvocellular neurosecretory neurons and corticotropin-releasing hormone messenger ribonucleic acid expression in the rat. Endocrinology 146: 137–146.
14. Przybyszewski-Szymanska MM, Mott NN, Paul CR, Gillespie RA, Pak TR (2011) Binge-pattern alcohol exposure during puberty induces long-term changes in HPA axis reactivity. PLoS ONE 6: e18350.
15. Gilpin NW, Karanikas CA, Richardson HH (2012) Adolescent binge drinking leads to changes in alcohol dehydrogenase and acetylcholinesterase activity and promoter methylation. Alcohol 46: 163–173.
16. Allen CD, Rivier CL, Lee SY Adolescent alcohol exposure alters the central brain circuits known to regulate the stress response. Neuroscience.
17. Przybyszewski-Szymanska MM, Gillespie RA, Pak TR (2012) 17beta-Estradiol is required for the sexually dimorphic effects of repeated binge-pattern alcohol exposure on the HPA axis during adolescence. PLoS One 7: e32263.
18. Przybyszewski-Szymanska MM, Rao YS, Pak TR (2010) Binge-pattern alcohol exposure during puberty induces sexually dimorphic changes in genes regulating the HPA axis. Am J Physiol Endocrinol Metab 298: E320–328.
19. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 64–75.
20. Viswanath S, Kanthasamy A, Prasad BS, Arumugam TV, Cha KJ (2011) Expression and functional characterization of the serine protease inhibitor neuroserpin is up-regulated in the Alzheimer disease brain. J Neurochem 109: 236–345.
21. Huang da W, Sherman BT, Lempicki RA (2009) Comparative analysis of the factors affecting auxological response to GnRH agonist treatment with a gonadotropin-releasing hormone antagonist on sexual development in female rams. Horm Behav 60: 307–317.
22. Przybyszewski-Szymanska MM, Gillespie RA, Pak TR (2012) 17beta-Estradiol is required for the sexually dimorphic effects of repeated binge-pattern alcohol exposure on the HPA axis during adolescence. PLoS One 7: e32263.
23. Kodama P, Darnell RE (1999) Reeler/Disabled-like disruption of neuronal migration in knockout mice with site-specific differences in bone loss and incomplete recovery of bone mass and strength. Alcohol 42: 649–656.
24. Przybyszewski-Szymanska MM, Rao YS, Pak TR (2009) Binge-pattern alcohol exposure during puberty induces sexually dimorphic changes in genes regulating the HPA axis. Am J Physiol Endocrinol Metab.
25. Read JP, Beattie M, Chamberlain R, Merril JE (2008) Beyond the “Binge” threshold: heavy drinking patterns and their association with alcohol involvement indices in college students. Addict Behav 33: 225–234.
26. Donovan JE (2009) Estimated blood alcohol concentrations for child and adolescent drinking and their implications for screening instruments. Pediatrics 123: e973–981.
27. Dobbing J, Sands J (1979) Comparative aspects of the brain growth spurt. Early Hum Dev 3: 79–83.
28. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 64–75.
29. Viswanath S, Kanthasamy A, Prasad BS, Arumugam TV, Cha KJ (2011) Expression and functional characterization of the serine protease inhibitor neuroserpin is up-regulated in the Alzheimer disease brain. J Neurochem 109: 236–345.
the GnRH antagonist cetrorelix using a female rat model. Pediatr Res 48: 468–474.
73. McGivern RF, Yellon SM (1992) Delayed onset of puberty and subtle alterations in GnRH neuronal morphology in female rats exposed prenatally to ethanol. Alcohol 9: 335–340.
74. Pinilla L, Castellano J, Romero M, Tena-Sempere M, Gaytan F, et al. (2009) Delayed puberty in spontaneously hypertensive rats involves a primary oварian failure independent of the hypothalamic KiSS-1/GPR54/GnRH system. Endocrinology 150: 2898–2907.
75. Whitlock KE, Illing N, Bridae NJ, Smith KM, Twomey S (2006) Development of GnRH cells: Setting the stage for puberty. Mol Cell Endocrinol 254–255: 39–50.
76. Diaval SA, Williams TR, Carver SE, Koch L, Bruning JC, et al. (2010) Divergent roles of growth factors in the GnRH regulation of puberty in mice. J Clin Invest 120: 2900–2909.
77. Sim CL, Richardson HN, Chappell PE, Levine JE (2001) In vivo gonadotropin-releasing hormone secretion in female rats during peripubertal development and on proestrus. Endocrinology 142: 2929–2936.
78. Graesemann C, Wessels HT, Knauer-Fischer S, Richter-Unruh A, Hauffa BP (2004) Increase of serum leptin after short-term pulsatile GnRH administration in children with delayed puberty. Eur J Endocrinol 150: 691–696.
79. Bajpai A, Sharma J, Kabra M, Gupta AK, Menon PS (2002) Long-acting GnRH analogue triptorelin therapy in central isosexual precocious puberty. Indian Pediatr 39: 633–639.
80. Krajnik K, Kasthorn ML, Rosewell KL, Wise PM (1998) Sex differences in the daily rhythm of vasoactive intestinal polypeptide but not arginine vasopressin messenger ribonucleic acid in the suprachiasmatic nuclei. Endocrinology 139: 4109–4116.
81. Harris VM, Bendre SV, Gonzalez De Los Santos F, Fite A, El-Yaman El-Dandachi A, et al. (2012) GnRH increases glucose transporter-1 expression and stimulates glucose uptake in the gonadotroph. J Endocrinol 212: 139–147.
82. You SH, Liao X, Weiss RE, Lucia MA (2010) The interaction between nuclear receptor corepressor and histone deacetylase 3 regulates both positive and negative thyroid hormone action in vivo. Mol Endocrinol 24: 1359–1367.
83. Soriano FX, Hardingham GE (2011) In cortical neurons HDAC3 activity suppresses Rho-dependent SIRT2 export. PLoS One 6: e21056.
84. McQuown SC, Barrett RM, Matheos DP, Post RJ, Rogge GA, et al. (2011) HDAC3 is a critical negative regulator of long-term memory formation. J Neurosci 31: 764–774.
85. Finnin MS, Donajian JR, Pavletich NP (2001) Structure of the histone deacetylase SIRT2. Nat Struct Biol 8: 621–625.
86. Renthal W, Kumar A, Xiao G, Wilkinson M, Covington HE, 3rd, et al. (2009) Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. Neuron 62: 335–348.
87. Gal J, Bang Y, Choi HJ (2012) SIRT2 interferes with autophagy-mediated degradation of protein aggregates in neuronal cells under proteasome inhibition. Neurochem Int 61: 992–1000.
88. Jones EA, Brewer MH, Smoothan R, Krueger G, Sun G, et al. (2012) Divalent enhancers upstream of the Charcot-Marie-Tooth type 1A disease gene MPP2. Human molecular genetics 21: 1501–1509.
89. Fanous S, Guez-Barber DH, Goldhart EM, Schrama R, Theberge FR, et al. (2013) Unique gene alterations are induced in FACS-purified Fos-positive neurons activated during cue-induced relapse to heroin seeking. J Neurochem 124: 100–108.
90. Krol J, Loedige I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11: 597–610.
91. Knopf A, Nacon I, Heo E, Jang H, Lee S, et al. (2011) MicroRNA as repressors of stress-induced anxiety: the case of amygdalar m4R. J Neurosci 31: 14191–14203.
92. Bedford MT, Chan DC, Leder P (1997) FBP WW domains and the Ali SH3 domain bind to a specific class of proline-rich ligands. EMBO J 16: 2376–2383.
93. Einbond A, Sudol M (1996) Towards prediction of cognate complexes between the WW domain and proline-rich ligands. FEBS Lett 384: 1–8.
94. Huang X, Beilin A, Zhang J, Zhou Y, Nicolaescu E, et al. (2009) Structure and function of the two tandem WW domains of the pre-mRNA splicing factor FBP21 (formin-binding protein 21). J Biol Chem 284: 25373–25387.
95. Bergink S, Jentsch S (2009) Principles of ubiquitin and SUMO modifications in DNA repair. Nature 458: 461–467.
96. Yang W, Wang L, Roehn G, Pearlstein RD, Ali-Osman F, et al. (2013) Small ubiquitin-like modifier 1–3 conjugation [corrected] is activated in human astrocytic brain tumors and is required for glioblastoma cell survival. Cancer Sci 104: 70–77.
97. Dorval V, Fraser PE (2006) Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. J Biol Chem 281: 9919–9924.
98. Hellemans KG, Verma P, Yoon E, Yu W, Weinberg J (2008) Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. Annals of the New York Academy of Sciences 1144: 154–175.
99. Hill SY, Tesner KD, McDermott MD (2011) Psychopathology in offspring from families of alcohol dependent female probands: a prospective study. Journal of psychiatric research 45: 285–294.
100. Sorensen HJ, Manzardo AM, Knop J, Penick EC, Madarasz W, et al. (2011) Paternal alcoholism and offspring ADHD problems: a children of twins design. Twin research and human genetics : the official journal of the International Society for Twin Studies 12: 53–62.
101. King SM, Keyes M, Malone SM, Elkins I, Legrand LN, et al. (2009) Parental alcohol dependence and the transmission of adolescent behavioral disinhibition: a study of adoptive and non-adoptive families. Addiction 104: 578–586.