Original Paper

Developmental Neuroscience

Dev Neurosci
DOI: 10.1159/000365549

Received: April 22, 2014
Accepted after revision: June 26, 2014
Published online: September 30, 2014

Third Trimester-Equivalent Ethanol Exposure Is Characterized by an Acute Cellular Stress Response and an Ontogenetic Disruption of Genes Critical for Synaptic Establishment and Function in Mice

Morgan L. Kleiber Benjamin I. Laufer Randa L. Stringer Shiva M. Singh

Molecular Genetics Unit, Department of Biology, The University of Western Ontario, London, Ont., Canada

Key Words
Fetal alcohol spectrum disorders · Mouse · Neurodevelopment · Gene expression · Epigenetics · Synaptogenesis · MicroRNA

Abstract
The developing brain is remarkably sensitive to alcohol exposure, resulting in the wide range of cognitive and neurobehavioral characteristics categorized under the term fetal alcohol spectrum disorders (FASD). The brain is particularly susceptible to alcohol during synaptogenesis, a process that occurs heavily during the third trimester and is characterized by the establishment and pruning of neural circuitry; however, the molecular response of the brain to ethanol during synaptogenesis has not been documented. To model a binge-like exposure during the third-trimester neurodevelopmental equivalent, neonate mice were given a high (5 g/kg over 2 h) dose of ethanol at postnatal day 7. Acute transcript changes within the brain were assessed using expression arrays and analyzed for associations with gene ontology functional categories, canonical pathways, and gene network interactions. The short-term effect of ethanol was characterized by an acute stress response and a downregulation of energetically costly cellular processes. Further, alterations to a number of genes with roles in synaptic transmission and hormonal signaling, particularly those associated with the neuroendocrine development and function, were evident. Ethanol exposure during synaptogenesis was also associated with altered histone deacetylase and microRNA transcript levels, suggesting that abnormal epigenetic patterning may maintain some of the persistent molecular consequences of developmental ethanol exposure. The results shed insight into the sensitivity of the brain to ethanol during the third-trimester equivalent and outline how ethanol-induced alterations to genes associated with neural connectivity may contribute to FASD phenotypes.

Introduction

Alcohol is a potent neuroactive molecule that is able to exert variable and often detrimental effects on the developing brain. The ultimate consequences of these effects are diagnostically characterized under the umbrella of fetal alcohol spectrum disorders (FASD) and display as a broad range of physiological, behavioral, and cognitive abnormalities [1, 2]. These range from the craniofacial malformations and severe central nervous system dysfunction evident in fetal alcohol syndrome to more subtle psychopathologies such as hyperactivity and attention deficits, increased risk for anxiety and mood disorders, and learning disabilities with no evidence of physical abnormalities [3].

KARGER

E-Mail karger@karger.com
www.karger.com/dne

0378–5866/14/0000–0000$39.50/0

Dr. Shiva M. Singh
Molecular Genetics Unit, Department of Biology
The University of Western Ontario
London, ON N6A 5B7 (Canada)
E-Mail ssingh@uwo.ca

The ability of ethanol to disrupt early developmental regulation of processes such as organogenesis and neurogenesis has been extensively reported [4–7]. The molecular effects of late-pregnancy alcohol consumption, however, are not nearly as well explored. During the third trimester of pregnancy, the brain undergoes a rapid period of growth and development during which much of the physiological and molecular basis of neural connections is established [8, 9]. This process of synaptogenesis requires the careful regulation of molecular cues to establish appropriate synaptic connectivity, the alteration of which has been implicated in numerous neurological disorders [10, 11]. Synaptogenesis, which occurs during the first postnatal weeks of development in mice, may be one of the most exquisitely ethanol-sensitive periods of brain development [12–14]. Behavioral studies have shown that ethanol exposure during synaptogenesis leads to pronounced cognitive and behavioral FASD-related phenotypes including delayed early neuromuscular coordination, hyperactivity, increased risk for depression, anxiety-related traits and stress vulnerability, and poor spatial learning acquisition and memory [14–16].

It is known that ethanol is able to disrupt neurodevelopmental gene expression through its interactions with a number of signaling pathways [4, 5, 7]. This can lead to long-term patterns of compromised gene expression, molecular function, and brain structure into adulthood [17–19]. The acute effects of ethanol on the brain undergoing synaptogenesis, however, are not known. Also, how ethanol exposure at this stage can establish a life-long pattern of abnormal molecular brain function is unclear. As such, binge-equivalent ethanol treatment models have been developed to evaluate the effects of a single day of ethanol intoxication during synaptogenesis on phenotypic and neuromolecular outcomes [12, 15, 17].

Previous studies conducted in our laboratory have determined that a binge-like ethanol treatment during synaptogenesis results in the appearance of multiple FASD-relevant phenotypes that are apparent in juvenile, adolescent, and adult mice [15]. These phenotypic results coincide with genome-wide alterations in adult brain gene expression [17]. It is pertinent, however, to evaluate the molecular changes that occur immediately following ethanol exposure in order to assess the molecular triggers of the persistent cognitive and molecular changes associated with FASD and third-trimester ethanol consumption. This study evaluates the genome-wide neurotranscriptomic changes induced by a binge-like ethanol treatment paradigm at postnatal day (P)7. We then compare these results to those previously reported by Kleiber et al. [17] with the attempt to discern commonly affected genes, pathways, and biological processes that may indicate a dynamic connection between initiating molecular events and long-term neural dysfunction. Finally, we consider the ability of ethanol to alter epigenetic patterning – specifically histone modification and microRNA (miRNA) regulation – as a primary effect of ethanol exposure that may provide a mechanism maintaining long-term gene alterations.

Materials and Methods

Animal Treatments

Male and female C57BL/6J (B6) mice were originally obtained from Jackson Laboratories (Bar Harbour, Maine, USA) and subsequently maintained in the Health Sciences Animal Care Facility at the University of Western Ontario. Mice were housed in same-sex colonies on a 14-hour light/10-hour dark schedule at 21–24°C and 40–60% humidity with ad libitum access to food and water. Environmental factors such as bedding, cage type, nestlets, and environmental enrichment were standardized between cages. All procedures were approved by the Animal Use Subcommittee at the University of Western Ontario and complied with the ethical standards established by the Canadian Council on Animal Care.

Female mice of 8–10 weeks of age were mated overnight with 8- to 12-week-old males. Pregnant females were housed individually throughout gestation. At P7, male B6 offspring were weighed and given subcutaneous injections of 2.5 g/kg of ethanol in 0.15 M saline at 0 h and again at 2 h (total dose of 5 g/kg) or saline. Ethanol and saline treatments were divided as evenly as possible across pups within a litter, so that each litter contained at least 1 ethanol- and 1 saline-treated male of approximately equivalent weight. This treatment has been previously shown to induce peak blood alcohol levels of over 0.3 g/dl for 4–5 h and be sufficient to induce neuronal apoptosis and result in FASD-related behaviors [12, 14, 15, 20]. This dose resulted in sedation but not loss of consciousness of the pups, lasting approximately 4 h. Pups (control and ethanol treated) remained separated from the dam until sacrificed by carbon dioxide asphyxiation 4 h following the initial injection.

Methods for the long-term samples are described in Kleiber et al. [17]. Briefly, mice were treated using the same paradigm as above with 2 doses of 2.5 g/kg of ethanol or saline spaced 2 h apart at both P4 and P7. These mice were reared by their biological mother until weaning into standard caging with 2–4 same-sex littermates at P21–P22. Male mice were sacrificed at P60 using carbon dioxide asphyxiation followed by cervical dislocation.

The short-term samples (4 h after injection) generated in this study were used to compare acute gene expression changes to those changes that were detectable in the adult brain following ethanol exposure during synaptogenesis (P4 and P7), reported in Kleiber et al. [17], as well as to evaluate changes to miRNA expression in the adult brain following neurodevelopmental ethanol exposure, which are included in the current report. Male mice only were used for assessment of the effect of ethanol on gene expression as it has been shown that the female estrous cycle has an impact on gene expression, including those genes that are affected by ethanol [21].

Sample Collection and RNA Isolation

Whole-brain tissue was isolated, snap-frozen in liquid nitrogen, and stored at –80°C until RNA isolation. Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions and cleaned using the RNeasy Mini Kit (Qiagen, Valencia, Calif., USA). The quality and quantity of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, Calif., USA) and a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Del., USA). All RNA samples had OD_{260}/OD_{280} absorption ratios of 1.8–2.1 nm.

mRNA Expression Array Hybridization

Two control biological replicates (arrays) and 4 ethanol replicates were used in total. The variation in control and ethanol chips increased the statistical power to more specifically determine differentially expressed genes between the two conditions. Each array consisted of pooled RNA from 3 nonlittermate males to reduce litter-specific noise. In total, males from 6 different litters were used, with 1 control and ethanol-treated pup from each of the 6 litters included to account for potential litter effects. Further, each ethanol-treated sample was matched with a saline-treated littermate used in the control samples.

These pooled whole-brain RNA samples were hybridized to Affymetrix Mouse Gene 1.0 ST arrays at the London Regional Genomics Centre (Robarts Research Institute, London, Ont., Canada). Briefly, single-stranded complementary DNA was synthesized using 200 ng of total RNA using the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript WT Expression Arrays (Applied Biosystems, Carlsbad, Calif., USA) and the Affymetrix GeneChip WT Terminal Labeling Kit and hybridization manual (Affymetrix, Santa Clara, Calif., USA). First-cycle cDNA was transcribed in vitro to cRNA and used to synthesize 5.5 μg of single-stranded complementary DNA that was subsequently end-labeled and hybridized for 16 h at 45°C to Affymetrix Mouse Gene 1.0 ST arrays. For miRNA expression studies, 2 control and 2 ethanol-treated samples were used, each consisting of RNA from 3 pooled nonlittermate adult (P60) males. One microgram of total RNA from each long-term replicate was labeled using the FlashTag Biotin HSR kit (Genisphere, Hatfield, Pa., USA) and hybridized to Affymetrix miRNA 2.0 arrays for 16 h at 45°C. All liquid-handling steps were performed by a GeneChip Fluidics Station 450, and arrays were scanned using the GeneChip Scanner 3000 using Command Console version 1.1 (Affymetrix).

Expression Array Data Analysis

Raw signal intensities from the array scans (.cel files) were generated using Affymetrix Command Console version 1.1, and probes were summarized to gene-level data using Partek Genomics Suite software version 6.6 (Partek Inc., St. Louis, Mo., USA). Principal component analysis was performed using Partek software to determine the clustering of the probe intensities that differed between control and ethanol arrays. The data were background corrected, quantile normalized, summarized using the GeneChip-Robust Multiarray Average (mRNA arrays) or Robust Multiarray Average (miRNA arrays) [22] and $\log_2$ transformed. Partek software was also used to determine gene-level one-way ANOVA values, fold changes and false-discovery-rate (FDR)-corrected p values. Genes meeting the criteria of 1.2-fold change and an FDR-corrected significance level of $p = 0.05$ were used to generate a gene lists for further analyses. Unannotated genes, redundant probes, and standards were removed from gene lists used for gene clustering and bioinformatic analyses. Significant genes were also analyzed by hierarchical clustering using euclidean distance and average linkage to assess the consistency across replicates.

Gene Ontology and Pathway Analysis

Gene clusters identified by mRNA fold change (up- or downregulated) based on hierarchical clustering analysis were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [23] for enrichment of gene ontology (GO) functional annotations. Statistically filtered transcripts were also analyzed using Ingenuity® Pathway Analysis™ (IPA; Ingenuity Systems, Redwood, Calif., USA; www.ingenuity.com). Using the Core Analysis feature, genes were analyzed based on biological function and canonical pathway involvement. The average fold change and p value of each gene were included in the analysis, and up- and downregulated genes were assessed concurrently. Redundant GO categories (those containing the same molecules with closely related functions) were removed. Biological functions, annotations, and pathways meeting a significance level of $p < 0.05$ were included. IPA was also used to generate gene networks between genes possessing annotated functional relationships with other genes, proteins, and molecules. Networks showing a cutoff significance score of 3 or more ($p < 0.001$) were reported.

Gene Expression Confirmation

Quantitative RT-PCR was performed on RNA extracts not used for array hybridization to confirm the expression of select short-term mRNA transcripts. Long-term transcripts have previously been confirmed [17]. cDNA was synthesized from 2 μg of total brain RNA isolated from control and ethanol-exposed P7 mice using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's protocol. Gene-specific TaqMan® Assay reagents and TaqMan Gene Expression Assay products were used in reactions, which were performed using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, Calif., USA). The genes assayed were selected on the basis of their functional relevance and significance within key pathways affected by ethanol treatment as identified by bioinformatic analysis (see below).

Primers and FAM-labeled probes for *dual specificity phosphatase 1 (Dusp1)*, *Jun protooncogene (Jun)*, *nuclear receptor subfamily 4, group A, member 1 (Nr4a1)*, and *proopiomelanocortin (Pomc)* were obtained from Applied Biosystems Inventoried Assays and used according to the manufacturer's instructions. Multiplex reactions were performed using *Actb* as a control housekeeping gene using gene-specific primers and VIC-labeled probes. Reactions were run using 10 μl of cDNA following the standard ramp speed protocol of a 10-min initiation at 95°C, followed by 40 cycles consisting of a 15-second denaturation at 95°C and annealing and extention at 60°C for 60 s. All experiments included 6 biological replicates per treatment and 3 technical replicates per sample. Relative expression was calculated according to the comparative C_T method [24] using StepOne™ version 2.0 software (Applied Biosystems). All RT-PCR data are described as mean ± standard error of the mean relative expression values. Significant differences were assessed using a two-tailed Student t test, assessed using SPSS version 16 (SPSS Inc., Chicago, Ill., USA).

miRNA Target Filtering and IPA

miRNA transcripts identified as differentially expressed in the brain of P60 mice exposed to neonatal (P4 and P7) ethanol exposure were analyzed concurrently with the mRNA transcripts identified from the expression array studies [17] using Ingenuity microRNA Target Filter™ (Ingenuity Systems) to develop a list of potential regulatory interactions between identified gene and miRNA transcripts. Only moderate- to high-confidence or experimentally demonstrated interactions were included. Results were further filtered based on brain-specific expression and an inverse miRNA-to-target mRNA expression relationship. The subsequent mRNA transcript list meeting these criteria was then subject to IPA.

Results

Short-Term Acute Gene Expression Response to Ethanol

Ethanol exposure at P7 resulted in the acute alteration of 315 transcripts in the brain 4 h following exposure, with 138 (44%) showing upregulation 4 h after ethanol exposure (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000365549). Changes ranged from –1.73-fold *(Dusp6)* to 2.63-fold *(thioredoxin-interacting protein)*, though most gene expression changes were subtle, with 78% (245/315) of gene expression changes showing less than a 1.3-fold difference. Quantitative RT-PCR validation of 4 genes, *Dusp1, Jun, Nr4a1*, and *Pomc*, are shown in figure 1.

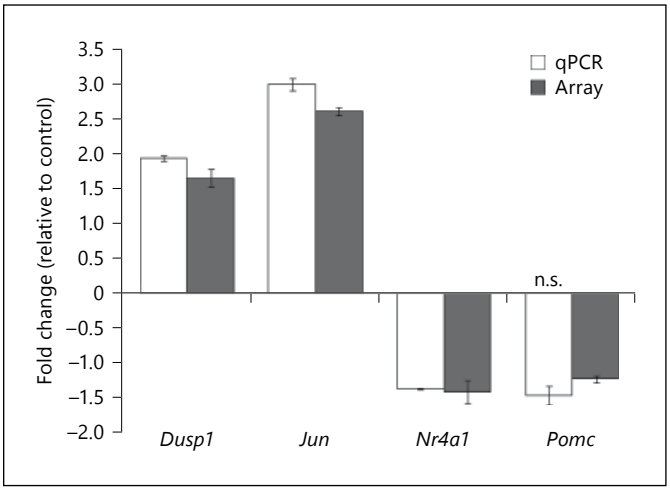


Fig. 1. Quantitative RT-PCR (qPCR) confirmation of genes altered by ethanol exposure at P7. Expression values are shown as a ratio of the control value ± SEM. Microarray values represent the results of an ANOVA comparison of ethanol versus control arrays, each array consisting of pooled RNA from 3 nonlittermate male mice. qPCR values were generated using 6 biological replicates. Significance was determined using unpaired t tests ($p < 0.05$). n.s. = Not significant.

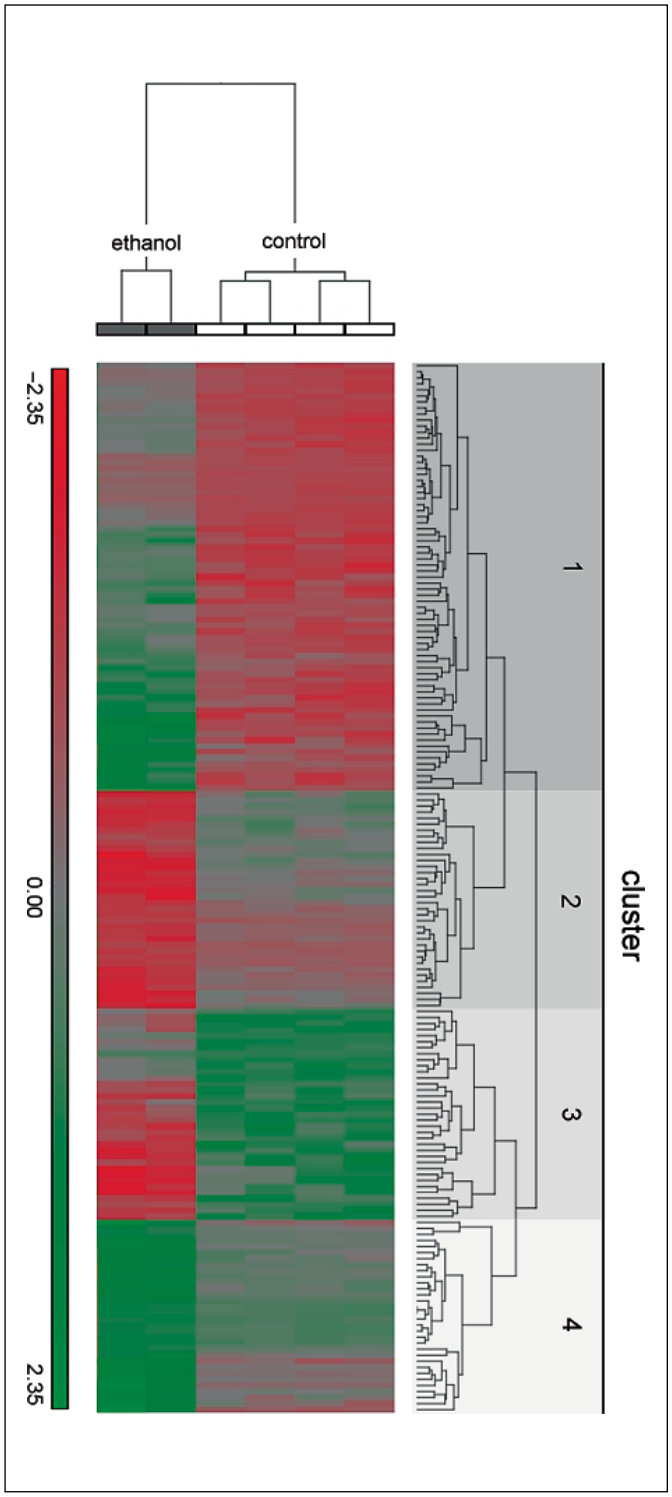
Array Clustering and Functional Annotation Analysis of Up- and Downregulated Genes

Hierarchical clustering of the probe signal intensity showed good consistency between arrays, with the 2 ethanol treatment arrays clustering together distinctly from the 4 control arrays (fig. 2). The clustering also revealed 4 subsets of genes that responded similarly to ethanol treatment. Some of these groups were not large enough to be subjected to meaningful gene functional clustering analysis, so the groups that showed upregulation (groups 1 and 4) or downregulation (groups 2 and 3) in response to ethanol treatment were submitted to DAVID [23] to assess for any GO biological process, molecular function, or cellular component terms common between similarly responsive genes.

Genes showing upregulation 4 h following ethanol exposure at P7 were significantly enriched for GO terms associated with a positive regulation of apoptosis, which included genes such as *cysteine-serine-rich nuclear protein 3 (Csrnp3)*, a transcriptional activator involved in apoptotic genes, *glutamate (NMDA) receptor subunit epsilon-2 (Grin2b)*, growth factors [*transforming growth factor, beta 3 (Tgfb3)* and *Igfbp3*], and *Bcl2-like 11 (apoptosis facilitator)* (*Bcl2l11*; table 1). Interestingly, a large cluster of genes (59 transcripts) were associated with cell membrane integrity. Also significant were genes with GO terms associated with postsynaptic density, amino acid dephosphorylation, glycosylation, and the regulation of synaptic transmission.

Downregulated genes showed enrichment for GO terms related to ribosome biogenesis, mitotic cell cycle regulation, MAP kinase (MAPK) phosphatase activity, transcriptional regulation, and hormone activity, including a significant downregulation in genes associated with steroid hormone receptor activity (table 1). Other significantly reduced transcripts were associated with chromosomal remodeling or a constituent of chromatin or DNA structure, such as *Ube2a*, required for DNA damage repair, *histone deacetylase 1 (Hdac1)*, and *nuclear FMRP-interacting protein 1 (Nufip1)*.

The up- and downregulated transcripts were also evaluated independently for biological function clusters using IPA (table 2). IPA identified abnormal expression of genes associated with dopaminergic neuron development and neuroglial function. Also, this analysis identified alterations to genes associated with the appropriate development of endocrine system development and function,



Color version available online

Fig. 2. Heat map hierarchical clustering of control and ethanol treatment arrays based on the normalized intensity of the probe sets. The heat map was generated by Partek® Genomics Suite software based on ANOVA-calculated significance levels at a fold change cutoff of 1.2 and an FDR-corrected p value <0.05.

including the hydroxylation of melatonin, corticosterone secretion, and phospholipid metabolism. Interestingly, pathways associated with calcium mobilization and influx were also affected, which, given their pleiotropic roles in the development of synapse development and survival, suggests that the immediate effect of ethanol exposure at P7 may be initiated by disruption of the active cellular processes occurring at this developmental stage.

Canonical Signaling Pathways and Gene Networks Affected Acutely following Ethanol Exposure

To assess biological pathways that may be affected immediately following ethanol treatment at P7, the genes identified by the array analysis were subjected to an IPA canonical pathway analysis to identify pathways associated with altered gene expression and the identified biological functions (table 3). The most significantly affected pathways included p53 signaling (p = 0.0002), circadian rhythm signaling (p = 0.0002), and cell cycle G1/S checkpoint regulation (p = 0.0008). Other pathways of note included phosphoinositide biosynthesis (associated with membrane integrity), thyroid hormone receptor/retinoid X receptor signaling (TR/RXR) activation, and PTEN signaling.

To evaluate how the ethanol-responsive genes may interact with one another, we utilized the gene network analysis tool within the Ingenuity software platform to predict interacting molecular networks (algorithmically generated pathways). Network analysis identified 3 significant networks, shown in figures 3–6: 'cellular development, cellular growth and proliferation, tumor morphology' (CDCG; focus molecules = 17; score = 20; fig. 3), 'tissue morphology, cell-to-cell signaling and interaction, cellular growth and proliferation' (TMCS; focus molecules = 13; score = 13; fig. 4), and 'behavior, cell-to-cell signaling and interaction, nervous system development and function' (BCSN; focus molecules = 11; score = 10; fig. 5). As the network descriptors suggest, these networks were highly interrelated. The CDCG network centralized on 3 highly connected 'hub' molecules: *Jun, cAMP-responsive element-binding protein 1 (Creb1)*, and *FBJ osteosarcoma oncogene* (*Fos*; fig. 3). The TMCS network also included *Jun*, and contained *tumor necrosis factor (Tnf)* and *Tgfb1* as hub molecules, as well as *interleukin 6 (Il6)* and *Il15* (fig. 4). Also relevant to this network was *adenylate cyclase-activating polypeptide 1 (Adcyap1)*, which was downregulated and acted as a molecular hub connecting the downregulated *Pomc* to *Tnf*. Finally, the BCSN network was characterized by the presence of multiple glutamate receptor subunits, including *glutamate receptor,*

Table 1. GO functional annotation clusters of genes up- and downregulated in the brain by acute ethanol exposure at P7

GO term	GO ID	Genes, n	p value	Upregulated genes
Positive regulation of apoptosis	0043065	10	0.000170	CSRNP3, GRIN2A, TGFB3, TRP53INP1, FOXO3, SP110, PERP, IGFBP3, BCL2L11, JMY
Integral to membrane	0016021	61	0.00643	MCHR1, SLC16A14, B3GALT5, OSMR, SLC44A5, INTS2, SDC4, MEGF10, PIGM, CLEC4E, SLC24A4, GRIN2B, SLC25A24, KCNK6, SLC2A1, ATP8B2, HTR1D, HTR5A, SLC43A2, GPR139, VMN2R33, PIGV, LIFR, GRIN2A, VMN2R37, NTSR1, PIK3IP1, PARP16, FOLH1, CLDN1, MFSD11, STEAP2, VMN2R43, GCNT2, TMEM194B, ENPP3, APH1B, ST8SIA1, GPR6, CSMD3, IGSF10, IGF1R, PRRG4, B3GNT5, PCDHB18, SCN9A, TMC7, FAM26E, RNF144B, FKTN, ST6GAL1, ABCA8B, ACER2, BCG1, CDHB21, KCTD6, SLC7A3, SLC16A9, ITPRIPL2, MERTK, PERP
Postsynaptic density	0014069	4	0.00761	GRIN2B, GRIN2A, PSD3, CPEB1
Protein amino acid dephosphorylation	0006470	5	0.0139	DUSP1, PPM1K, PTPN4, DUSP16, DUSP8
Glycosylation	0070085	4	0.0366	ST6GAL1, B3GNT5, B3GALT5, ST8SIA1
Regulation of synaptic transmission	0050804	4	0.0464	GRIN2B, EDN1, GRIN2A, CPEB1
				Downregulated genes
Ribosome biogenesis	0042254	7	0.0000618	UTP11L, TBL3, GTPBP4, NOP2, UTP18, MPHOSPH10, NMD3
Mitotic cell cycle	0000278	8	0.000719	CCND1, E2F4, INCENP, AURKA, PMF1, SIRT7, NUP43, RBBP8
MAP kinase phosphatase activity	0033549	3	0.00280	DUSP4, DUSP6, DUSP7
Regulation of transcription	0045449	25	0.00293	E2F4, NUFIP1, ZBTB17, CITED4, MED27, PER2, OLIG2, CRY1, ETV5, ETV4, KLF6, EGR3, EGR4, SNAPC1, RXRG, NR4A1, MCM2, PMF1, SIRT7, NR0B1, INHBA, HDAC1, VEGFA, ZFP143, HDAC9
Chromosomal part	0044427	8	0.00363	UBE2A, HDAC1, INCENP, NUFIP1, PMF1, MCM2, SIRT7, NUP43
Hormone activity	0005179	4	0.0401	INHBA, POMC, APLN, ADCYAP1
Steroid hormone receptor activity	0003707	3	0.0425	RXRG, NR4A1, NROB1

ionotropic, NMDA 2A (Grin2a) and *Grin2b*, both upregulated (fig. 5).

To identify molecules that may be key effectors of the ethanol-induced changes in gene expression, the 3 networks were merged to identify hub molecules that may unify their interactions (fig. 6). Early immediate transcriptional regulators controlling cell cycle and proliferation, such as *Jun, Fos, early growth response 1 (Egr1), Egr2*, and *Creb1*, are prominent as central hubs. Also highly represented are signaling molecules such as cytokines and growth hormones, including *growth hormone 1 (Gh1), nerve growth factor (Ngf), Tnf, Tgfb1*, and *Il6*. The glutamate receptors (*Grin2a*, *Grin2b*, and *Grin1*) prominent in the BCSN network remain as hubs in the merged model of molecular interactions, as does *Htt*. Finally, an interesting interaction emerges between *Adcyap1, Pomc*, and *Nr4a1*, all of which were downregulated. Taken together, these genes and their interactions may be viewed as the primary response of developing brain cells to ethanol exposure at synaptogenesis.

Genes Affected both Acutely (4 h) and Persistently (at P60) following Ethanol Exposure

This section deals with the genes and pathways that may represent the molecular adaptation of neural cells to developmental ethanol exposure. Eleven transcripts that were altered acutely following ethanol exposure at P7 remained altered into early adulthood without additional ethanol treatment (table 4). These included 5 genes that remained downregulated [*Heatr1, hemicentin (Hmcn), Pomc, seizure threshold 2 (Szt2)*, and *XRCC6-binding protein 1 (Xrcc6bp1)*] and 6 transcripts that showed an inverse relationship (upregulated 4 h following exposure but downregu-

Table 2. IPA of biological functions associated with ethanol-induced gene expression changes

Category	Function annotation	p value	Molecules, n	Molecules	
				upregulated	downregulated
Cell death and survival	apoptosis of neurons	0.000497	17	AGT, BCL2L11, BTG2, DUSP1, GRIN2B, IGF1R, JUN, MT1E, MT1H, PERP, ST8SIA1, TGFB3, Zfp110/Zfp369	ADCYAP1, HDAC1, KLF6, VEGFA
Nervous system development and function	proliferation of pituitary cells	0.00220	3	TGFB3	INHBA, SRSF2
	proliferation of neuroglia	0.0155	5	CPEB1, FOXO3	ADCYAP1, CCND1, ETV5
	abnormal morphology of astrocytes	0.0252	3	MT1E, MT1H	LDLR
	quantity of neurons	0.0334	10	DUSP1, IGF1R	ADCYAP1, EGR3, INHBA, LIFR, OLIG2, POMC, TRPA1, VEGFA
	plasticity of neuronal synapse	0.0371	2	CPEB1, GRIN2B	–
Tissue development	regeneration of nerves	0.000544	3	AGT, JUN, ST8SIA1	–
Cell cycle	mitosis	0.00175	3	EDN1, TGFB3	CCND1
	mitogenesis of central nervous system cells	0.00216	2	EDN1, TGFB3	–
Lipid metabolism	hydrolysis of phospholipid	0.0181	2	AGT, EDN1	–
	turnover of phospholipid	0.00216	2	EDN1	ADCYAP1
	metabolism of membrane lipid derivative	0.0462	2	ST8SIA1	POMC
Neurological disease	neuromuscular disease	0.0116	15	AGT, MERTK, MT1E, MT1H, SDC4, SLC16A9, Zfp932 (includes others)	EGR4, ETV5, GABRA5, HLA-B, LIFR, NR4A1, RGS4, RXRG
	disorder of basal ganglia	0.0171	14	MERTK, MT1E, MT1H, SDC4, SLC16A9, Zfp932 (includes others)	EGR4, ETV5, GABRA5, HLA-B, LIFR, NR4A1, RGS4, RXRG
	neuropathy of the brain	0.00216	2	MT1E, MT1H	–
	damage to the brain	0.0479	4	EDN1, FOLH1, GRIN2A, GRIN2B	–
Cellular growth and proliferation	proliferation of cells	0.0197	13	CPEB1, FOXO3, TGFB3	ADCYAP1, CCND1, ETV5, GNL3, GTPBP4, INHBA, KLF6, POMC, SRSF3, VEGFA
Vitamin and mineral metabolism	mobilization of Ca^{2+}	0.0122	2	–	ADCYAP1, TRPA1
	influx of Ca^{2+}	0.0371	2	GRIN2B	TRPA1
Cell-to-cell signaling and interaction	damage to dopaminergic neurons	0.00356	2	MT1E, MT1H	–
	plasticity of neuronal synapse	0.0371	2	CPEB1, GRIN2B	–
Endocrine system development and function	proliferation of pituitary cells	0.00220	3	TGFB3	INHBA, SRSF2
	hydroxylation of melatonin	0.0193	1	CYP1B1	–
	production of L-triiodothyronine	0.0193	1	–	DIO2
	secretion of corticosterone	0.0193	1	–	POMC

Table 3. Ingenuity canonical pathways associated with transcripts altered by ethanol exposure at P7

Canonical pathway	p value	Ratio of molecules altered	Molecules	
			upregulated	downregulated
p53 signaling	0.000225	8/89	JMY, JUN, PERP, PIK3R1, TP53INP1	CCND1, GNL3, HDAC1
Circadian rhythm signaling	0.000244	6/33	GRIN2A, GRIN2B, PER1	ADCYAP1, CRY1, PER2
Cell cycle G1/S checkpoint regulation	0.000765	6/60	TGFB3	CCND1, E2F4, GNL3, HDAC1, HDAC9
Chronic myeloid leukemia signaling	0.00698	9/90	PIK3R1, TGFB3	CCDN1, E2F4, HDAC1, HDAC9
3-Phosphoinositide biosynthesis	0.00927	7/125	DUSP1, DUSP8, DUSP16. NUDT11, PIK3R1, PPM1K	PPTC7
D-Myoinositol tetraphosphate biosynthesis	0.011	6/100	DUSP1, DUSP8, DUSP16, NUDT11, PPM1K	PPTC7
Cyclins and cell cycle regulation	0.0132	5/77	TGFB3	CCND1, E2F4, HDAC1, HDAC9
ErbB2-ErbB3 signaling	0.0165	4/53	JUN, PIK3R1	CCND1, ETV4
TR/RXR activation	0.0172	5/80	PIK3R1, SLC2A1	DIO2, LDLR, RXRG
PTEN signaling	0.0203	5/113	PIK3R1, SLC2A1	DIO2, LDLR, RXRG

lated in adulthood). Interestingly, these transcripts also included genes critical for glutamate regulation (*folate hydrolase 1* and *Grin2b*), cellular organization (*Hmcn1* and *protocadherin beta 18*), and hormonal signaling *(Pomc)*, as well as genes that have been previously implicated with neurological phenotypes such as *Szt2* and *Xrcc6bp1*.

We also compared the biological functions identified in the short- and long-term analyses to identify commonly targeted pathways that may represent a continuity of consequences of ethanol exposure during synaptogenesis. The results of this analysis are shown in table 4. This analysis revealed that, while the number of overlapping transcripts identified by both the short- and long-term gene arrays was limited, there were a number of closely associated biological functions and canonical pathways represented within both gene lists. Genes associated with synaptic transmission and plasticity were present in both the short- and long-term gene lists (table 4), with both showing a number of glutamate receptor subunit transcripts including *Grin2a*, *Grin2b*, and *Grik1*. Also common between the short- and long-term lists were GO functions related to hormone activity and signaling, specifically steroid and corticosterone secretion and, interestingly, circadian rhythm signaling. Finally, retinoic acid signaling and the canonical PTEN/ATK/mTOR signaling pathway were identified as associated with transcripts within both the short- and long-term gene lists.

Relationship between miRNA Changes and Target mRNA Transcripts Altered in the Adult Brain by Ethanol Exposure during Synaptogenesis

We utilized the IPA Target Filter software to compare the long-term mRNA expression array data to miRNA array data and to assess potential regulatory interactions based on literature and sequence analysis. The miRNA array identified 33 different miRNA transcripts that were altered in the brain of adult male mice that had been exposed to alcohol during early neonatal development (online suppl. table 2). Fold changes ranged from –4.17 (mmu-miR-704) to 2.44 (mmu-miR-721). Most of the miRNAs were downregulated, with 73% (24/33) showing a negative fold change as compared to the saline-treated controls.

Target Filter analysis matched 5 transcripts with 36 genes (table 5) using high-confidence criteria based on miRNA sequence and annotated interactions and showing an inverse expression relationship. Interestingly, a given miRNA species was matched with multiple altered mRNA transcripts, suggesting that the alteration of a single miRNA can alter a number of different biological processes and exert widespread effects. Further, each altered

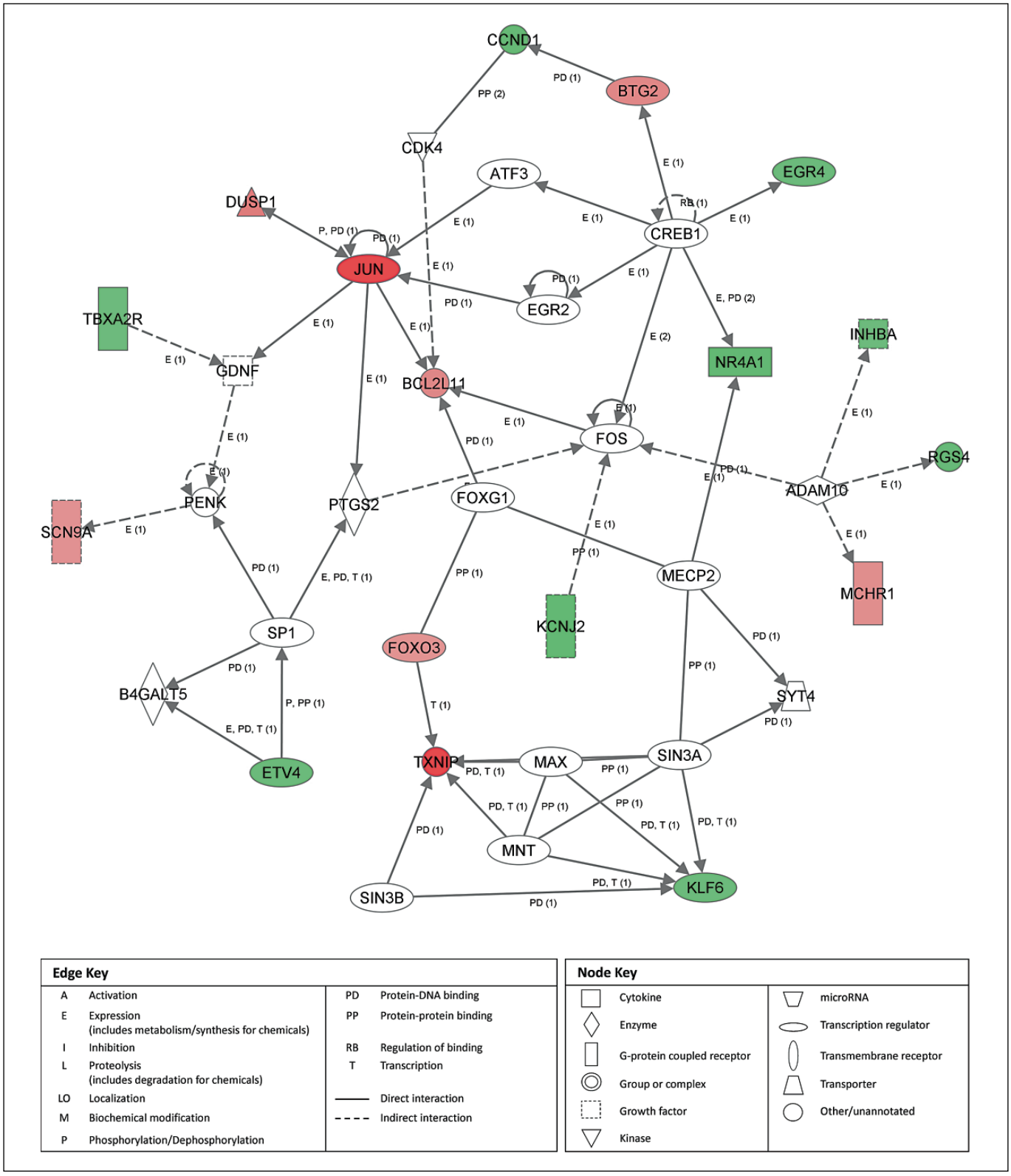
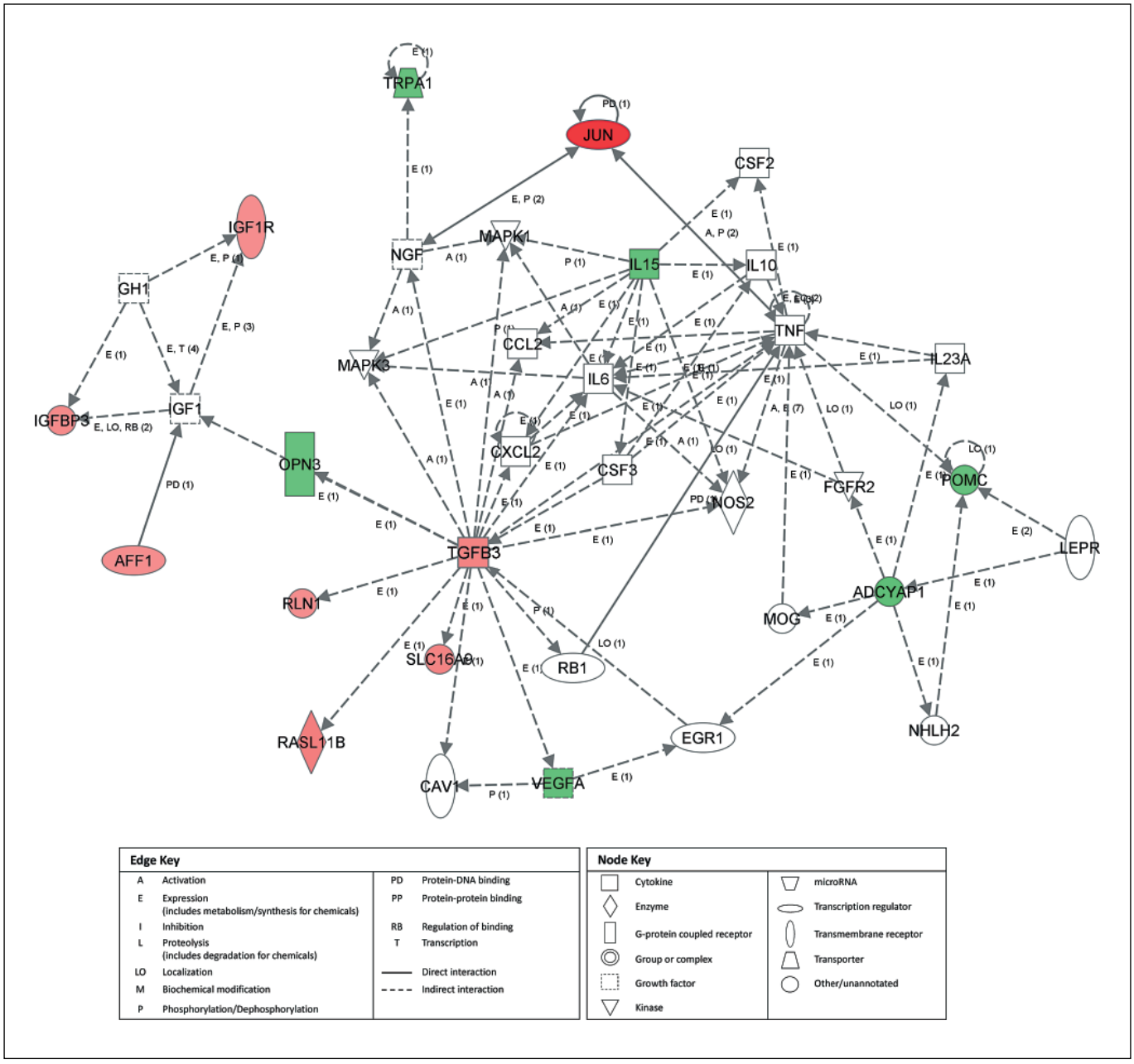


Fig. 3. IPA network analysis indicating annotated interactions between genes affected in the adult brain by ethanol exposure at P7 associated with CDCG. Upregulated (red; colors refer to the online version only) and downregulated genes (green) are indicated. Centralized hub molecules linking multiple interacting genes are enlarged.



Color version available online

Fig. 4. IPA network analysis indicating annotated interactions between genes affected in the adult brain by ethanol exposure at P7 associated with TMCS. Upregulated (red) and downregulated genes (green) are indicated. Centralized hub molecules linking multiple interacting genes are enlarged.

gene was associated with a number of biological pathways (table 5). Affected pathways that showed consistency with previous DAVID analysis and IPA included alterations to axonal guidance (*Adam9*, *Lingo1*, *Sema6d*, *Shank2*, *Prkcb*, and *Efnb2*), cell cycle and cell migration mechanisms [*Mras*, *Rhou*, *Tab3*, *Tdrd7*, *Kitl*, *Ncoa3*, and *tubular sclerosis 1 (Tsc1)*], and inositol phosphate metabolism *(Fam20b)*. Interestingly, multiple hormonal signaling mechanisms were affected that were consistent with previously identified pathways. These included corticotrophin-releasing hormone signaling, glucocorticoid receptor signaling, Huntington's disease signaling, and estrogen and androgen receptor signaling (*Cnr1*, *Mras*, *Ncor2*, *Ncoa3*, *Prkcb*, *Ppargc1a*, *Rbm9*, and *Spen*). Lastly, mechanisms associat-

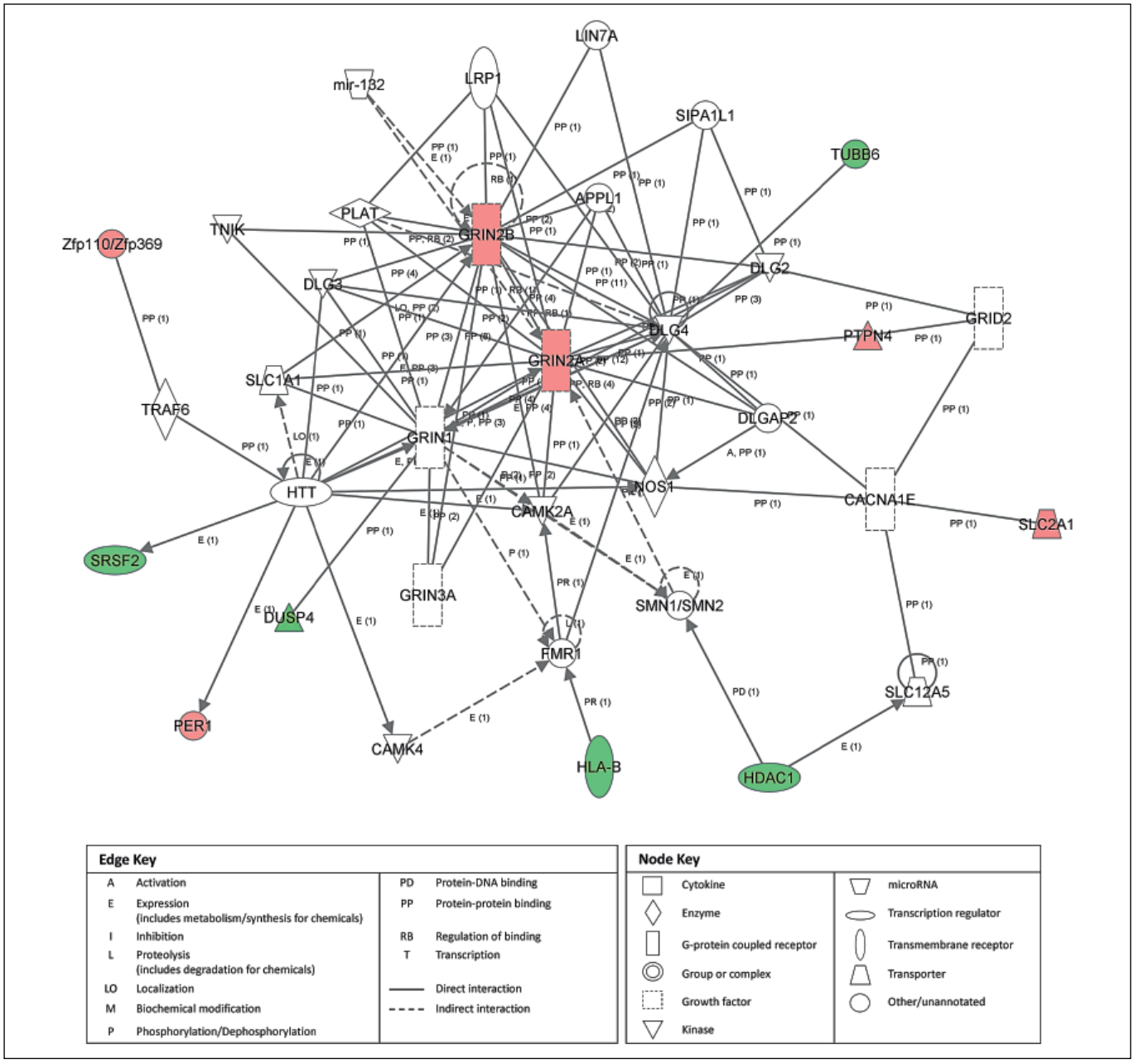
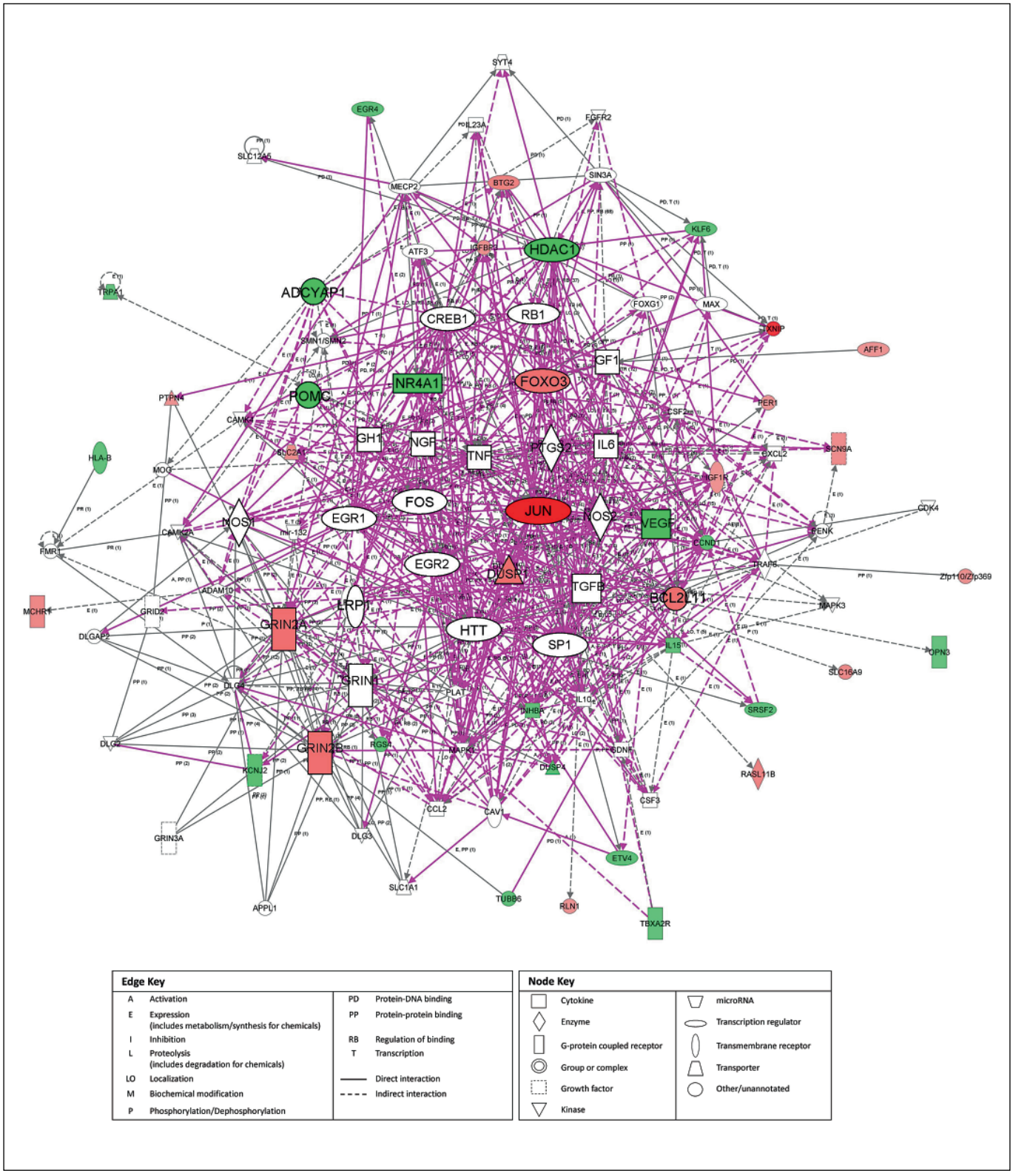


Fig. 5. IPA network analysis indicating annotated interactions between genes affected in the adult brain by ethanol exposure at P7 associated with BCSN. Upregulated (red) and downregulated genes (green) are indicated. Centralized hub molecules linking multiple interacting genes are enlarged.

ed with retinoic acid receptor signaling [TR/RXR, peroxisome proliferator-activated receptor (PPAR), and retinoic acid receptor (RAR) activation] and the highly related developmental PI3K/AKT/mTOR pathway signaling were well represented, they and were associated with miR-721, which was implicated in the regulation of multiple genes (*Irf1*, *Ncoa3*, *Ppargc1*, and *Tsc1*).

Discussion

This study characterizes the gene expression changes that occur in the developing brain acutely (4 h) following a binge-like dose of ethanol during synaptogenesis in B6 mice. We argue that the genes and pathways identified represent an in vivo initial response to ethanol within a



Edge Key			
A	Activation	PD	Protein-DNA binding
E	Expression (includes metabolism/synthesis for chemicals)	PP	Protein-protein binding
I	Inhibition	RB	Regulation of binding
L	Proteolysis (includes degradation for chemicals)	T	Transcription
LO	Localization	——	Direct interaction
M	Biochemical modification	- - -	Indirect interaction
P	Phosphorylation/Dephosphorylation		

Node Key			
□	Cytokine	▽	microRNA
◇	Enzyme	⬭	Transcription regulator
▯	G-protein coupled receptor	⬮	Transmembrane receptor
◎	Group or complex	⏢	Transporter
⬚	Growth factor	○	Other/unannotated
▽	Kinase		

Fig. 6. Merged IPA networks showing interactions between the most significant gene networks (see fig. 3, 4, 5) affected in the adult brain by ethanol exposure at P7. Upregulated (red) and downregulated genes (green) are indicated. Centralized hub molecules linking multiple interacting genes are enlarged.

Table 4. Genes altered by alcohol exposure acutely following treatment (acute) that remain altered during early adulthood (long-term)

Accession No.	Gene symbol	Gene name	Chromosome location	Fold change	
				acute (P7)	long-term (P60)
NM_016770	Folh1	folate hydrolase 1	7 D1–D2	1.44	–1.28
NM_008171	Grin2b	glutamate (NMDA) receptor subunit epsilon-2	6 G1	1.25	–1.27
NM_144835	Heatr1	HEAT repeat-containing 1	13 A1	–1.31	–1.33
NM_001024720	Hmcn1	hemicentin 1	1 G1	–1.20	–1.28
NM_053143	Pcdhb18	protocadherin beta 18	18 B3	1.21	–1.43
NM_008895	Pomc	proopiomelanocortin-alpha	12 A1	–1.21	–1.57
NM_001029842	Slc16a6	solute carrier family 16 (monocarboxylic acid transporters), member 6	11 E1	1.35	–1.45
NM_198170	Szt2	seizure threshold 2	4 D2	–1.22	–1.24
NM_026858	Xrcc6bp1	XRCC6-binding protein 1	10 D3	–1.20	–1.36
NM_177660	Zbtb10	zinc finger and BTB domain-containing 10	3 A1	1.38	–1.30
NM_011763	Zfp9	zinc finger protein 9	6 F1	1.25	–1.29

highly dynamic and sensitive biological system. This response represents a discontinuity in normal developmental processes that, depending on the balance of cellular processes active at the time of insult, may result in apoptosis [12, 20] or, in surviving cells, a 'molecular footprint' of neurodevelopmental disruption [15, 17].

Transcripts Associated with Acute Ethanol Exposure during Synaptogenesis Indicate a Balance between Cellular Apoptosis, Survival, and Stress Response

Expression array analysis identified 315 ethanol-responsive transcripts, with a number of genes that were downregulated in response to ethanol performing biological functions that are notoriously energy costly, such as ribosome synthesis and mitotic progression (tables 1, 2; fig. 3, 4) [25, 26]. The downregulation of genes that drive mitotic progression and activate MAPK phosphatases – and, subsequently, increase the presence of active MAPKs – has previously been implicated in cellular stress response, including that associated with hyperosmotic conditions such as high extracellular ethanol concentrations [25, 27]. The arrest of prodevelopmental cues is further supported by the downregulation of transcription and growth factors *Etv4*, *E2f4*, *Egr3*, *Egr4*, and *Vegfa* (tables 1, 2). These molecules have well-established roles in multiple developmental processes, and their reduced expression may alter the maturation of neurons, the extent of dendritic branching, synapse formation, and neuronal plasticity [28–30].

In contrast to the functions affiliated with the downregulated transcripts, upregulated transcripts were associated with a positive regulation of apoptosis and cell membrane integrity (tables 1, 2). These results corroborate physiological evidence that this binge-like ethanol treatment at synaptogenesis results in widespread cell death across the cortex, hippocampus, cerebellum, and other brain regions [14, 20, 31]. The cell membrane composition can strongly affect the function of intrinsic membrane proteins, and high alcohol concentrations have been shown to force the transmembrane proteins into unfavorable conformations, affecting the chemical and physical functioning of the cell, including at synaptic plasma membranes [32]. Coinciding with this, the upregulation of genes associated with glycosylation may be related to reinstating membrane integrity as glycoproteins are developmentally integral to cell membrane stability and cell-cell recognition and interaction [33], as well as regulating excitatory synapse development and the modulation of synaptic efficacy [34]. These findings agree with previous reports that ethanol targets the glycosylation machinery [35] and that this likely contributes to the behavioral and cognitive impairments observed in FASD [36].

The Effect of Neurodevelopmental Ethanol Exposure on the Formation and Function of Neural Circuits

In addition to the prominent effect of ethanol on cell survival and stress response mechanisms, a number of transcripts were identified that, while they may have roles in cellular stress, also have demonstrated roles in neurodevelopment and have been implicated in long-term risk for neurological disorders. Particularly notable is the alteration of genes related to hormonal signaling and endocrine system development and synaptic transmission (tables 1–3; fig. 5).

Table 5. miRNAs and putative mRNA transcript targets showing altered expression in the adult brain following early neonatal ethanol exposure

miRNA ID	miRNA fold change	Target gene ID	Target fold change	Associated pathways
mmu-miR-26b	1.284	Adam9	–1.114	axonal guidance signaling
mmu-miR-26b	1.284	Chsy1	–1.128	chondroitin sulfate biosynthesis
mmu-miR-26b	1.284	Cnr1	–1.332	corticotropin-releasing hormone signaling, G protein-coupled receptor signaling, reelin signaling in neurons, cAMP-mediated signaling
mmu-miR-26b	1.284	Exoc8	–1.221	Cdc42 signaling
mmu-miR-26b	1.284	Hs6st1	–1.523	chondroitin sulfate biosynthesis, cysteine metabolism, keratan sulfate biosynthesis, LPS/IL-1-mediated inhibition of RXR function, xenobiotic metabolism signaling
mmu-miR-26b	1.284	Lingo1	–1.153	axonal guidance signaling
mmu-miR-26b	1.284	Map3k7	–1.193	acute-phase response signaling, BMP signaling pathway
mmu-miR-26b	1.284	Mras	–1.200	14-3-3-mediated signaling, AMPK signaling, actin cytoskeleton signaling, acute myeloid leukemia signaling, acute-phase response signaling, agrin interactions at neuromuscular junction, androgen signaling
mmu-miR-26b	1.284	Pfkfb3	–1.143	AMPK signaling, fructose and mannose metabolism
mmu-miR-26b	1.284	Ppm1b	–1.429	AMPK signaling
mmu-miR-26b	1.284	Rhou	–1.250	CXCR4 signaling, glioblastoma multiforme signaling, glioma invasiveness signaling, HMGB1 signaling
mmu-miR-26b	1.284	Sema6d	–1.181	axonal guidance signaling
mmu-miR-26b	1.284	Shank2	–1.291	axonal guidance signaling
mmu-miR-26b	1.284	Tab3	–1.253	NF-κB signaling
mmu-miR-26b	1.284	Tdrd7	–1.568	role of Oct4 in mammalian embryonic stem cell pluripotency
mmu-miR-26b	1.284	Ube2j1	–1.151	hypoxia signaling, protein ubiquitination pathway
mmu-miR-34b-5p	–1.292	Kitl	1.322	acute myeloid leukemia signaling
mmu-miR-184	1.485	Ncor2	–1.273	aryl hydrocarbon receptor signaling, estrogen receptor signaling, glucocorticoid receptor signaling, Huntington's disease signaling, liver X receptor/RXR activation, PPAR signaling, PPAR-α/RXR-α activation, RAR activation

Table 5. (continued)

miRNA ID	miRNA fold change	Target gene ID	Target fold change	Associated pathways
mmu-miR-184	1.485	Prkcb	–1.115	14-3-3-mediated signaling, aldosterone signaling, androgen signaling, axonal guidance signaling
mmu-miR-721	2.437	Akap11	–1.111	β-adrenergic signaling, protein kinase A signaling, cAMP-mediated signaling
mmu-miR-721	2.437	B4galt5	–1.257	keratan sulfate biosynthesis, N-glycan biosynthesis
mmu-miR-721	2.437	Cnr1	–1.332	corticotropin-releasing hormone signaling, G protein-coupled receptor signaling, reelin signaling in neurons, cAMP-mediated signaling
mmu-miR-721	2.437	Efnb2	–1.261	axonal guidance signaling, ephrin receptor signaling
mmu-miR-721	2.437	Fam20b	–1.271	inositol phosphate metabolism, nicotinate and nicotinamide metabolism
mmu-miR-721	2.437	Ino80	–1.247	purine metabolism
mmu-miR-721	2.437	Irf1	–1.323	IL-15 production, interferon signaling, production of nitric oxide and reactive oxygen species, retinoic acid-mediated apoptosis signaling, role of PKR in interferon induction
mmu-miR-721	2.437	Lrrk2	–1.196	mitochondrial dysfunction
mmu-miR-721	2.437	Ncoa3	–1.247	aryl hydrocarbon receptor signaling, estrogen receptor signaling, glucocorticoid receptor signaling, PPAR-α/RXR-α activation, role of Wnt/GSK-3β signaling, TR/RXR activation, vitamin D receptor/RXR activation
mmu-miR-721	2.437	Pfkfb3	–1.143	AMPK signaling, fructose and mannose metabolism
mmu-miR-721	2.437	Ppargc1a	–1.123	estrogen receptor signaling, farnesoid X receptor/RXR activation, PPAR signaling, PPAR-α/RXR-α activation, pregnane X receptor/RXR activation, RAR activation, TR/RXR activation, xenobiotic metabolism signaling
mmu-miR-721	2.437	Rbm9	–1.311	estrogen receptor signaling
mmu-miR-721	2.437	Shank2	–1.291	axonal guidance signaling
mmu-miR-721	2.437	Spen	–1.140	estrogen receptor signaling
mmu-miR-721	2.437	Sphk2	–1.304	ceramide signaling, PDGF signaling, sphingolipid metabolism

Table 5. (continued)

miRNA ID	miRNA fold change	Target gene ID	Target fold change	Associated pathways
mmu-miR-721	2.437	Tsc1	–1.381	14-3-3-mediated signaling, AMPK signaling, glioblastoma multiforme signaling, PI3K/AKT signaling, mTOR signaling
mmu-miR-721	2.437	Wdfy3	–1.327	keratan sulfate biosynthesis, N-glycan biosynthesis
mmu-miR-1970	–1.694	Arhgap6	1.188	leukocyte extravasation signaling, RhoA signaling

Hormone Activity and Hypothalamic-Pituitary-Adrenal Axis Development

The DAVID analyses identified hormone activity and hormone receptor activity as biological functions with a significantly overrepresented proportion of altered transcripts. Most were significantly downregulated following ethanol exposure. These genes included *Adcyap1* and *Pomc* as well as *Nr4a1*, *nuclear receptor subfamily 0, group B, member 1 (Nr0b1)*, and *retinoid X receptor gamma* (*Rxrg*; table 1). These genes play varying roles in the development of the brain, most notably in the proper function of the hypothalamic-pituitary-adrenal (HPA) axis [37] including altered stress reactivity, a consistent endophenotype associated with FASD [38–40]. Indeed, we have previously reported increased anxiety-related traits in young adult mice following ethanol exposure during synaptogenesis [15]. It is possible that this period of neural development is critically susceptible to molecular changes that affect HPA connectivity and maturation and lead to a risk for these pathologies in prenatally exposed adolescents and adults [41]. The downregulation of *Nr4a1* is interesting, given that it is able to mediate both pro- and antiapoptotic effects [42] as well as mediating thyrotropin-releasing hormone stimulation within the pituitary [43]. Also relevant to HPA axis function is the reduced expression of *Rxrg*. This gene is part of the family of retinoic acid receptors that mediate the antiproliferative effects of retinoic acid and act as ligand-dependent transcriptional regulators [44]. These results follow other studies that have implicated retinoic acid signaling in the effects of prenatal alcohol exposure (PAE) [45–47]: the reduced expression of retinoic acid can delay cellular differentiation and myelination [48], most notably in the HPA axis structures, but also in the cerebellum and hippocampus, affecting developmental and behavioral phenotypes [49].

Neurotransmitter Signaling and Synaptic Connectivity and Plasticity

Among the genes affected by ethanol treatment were *Cpeb1*, *gamma-aminobutyric acid (GABA) A receptor, subunit alpha 5 (Gabra5)*, *Grin2a*, and *Grin2b*, all of which are canonically critical to synaptic formation and maintenance (table 2). *Cpeb1* regulates translation at the synapses, and reduced – or elimination of – expression during development results in poor synaptic efficacy, leading to reduced long-term potentiation [50] and, in adulthood, rapid extinction of hippocampus-dependent spatial learning memories [51]. CPEB1 protein is activated in response to NMDA ionotropic glutamate receptor stimulation to facilitate activity-dependent gene transcription [52]. Further, during early postnatal development, the formation of functional circuit formation is dependent on the synchronous activity of depolarization of $GABA_A$ receptors and the activation of NMDA receptors [53, 54].

In this study, *Gabra5*, *Grin2a*, and *Grin2b* transcript levels were identified as altered following ethanol exposure, with *Gabra5* downregulated and the glutamate subunits upregulated (fig. 5). While these genes are associated with the major inhibitory (GABA) and excitatory (glutamate) neurotransmitter systems in the adult brain, it has also been established that they play key roles in the neurodevelopmental patterning of synaptic networks [55]. Both NR2A, encoded by *Grin2a*, and NR2B, encoded by *Grin2b* are modulatory NMDA receptor subunits, with *Grin2a* widely expressed in the postnatal brain and *Grin2b* expressed in multiple regions embryonically but restricted to the forebrain during postnatal stages [55], the transition of which occurs during synaptogenesis [56]. Further, the subunit composition (NR2A vs. NR2B) of NMDA receptors has been shown to control dendritic spine motility as well as synapse formation and stabiliza-

tion [57]. The *Gabra5* subunit is expressed in the brain throughout pre- and postnatal development [58]. The role of NMDA and GABA receptors in the formation, maturation, and pruning of synapses has been well established [9, 53, 55, 59]. Disruption of the regulatory control of subunit expression by ethanol at this developmental stage has potent consequences for neural network connectivity, consequently not only acutely affecting brain function but also establishing an abnormal foundation for adult cellular communication and plasticity [60], a crucial impairment associated with PAE.

Molecular Pathways Acutely Affected by Ethanol Exposure (at P7) that Remain Modified in Adulthood (at P60)

While the present study focuses on the immediate effects of ethanol on the transcriptome of the brain at synaptogenesis, the results also shed insight into the long-term effects of alcohol exposure on the cellular function of the adult brain that have been previously reported [17]. The genes that remain altered into adulthood may be an adaptive response involving the reorganization of gene expression in surviving cells undergoing development. Ultimately, this modified expression profile results in the molecular 'footprint' of third trimester-equivalent ethanol exposure observed in adult mice that may, in part, account for the behavioral deficits presented by these animals [15, 17].

Genes that remain altered in adulthood following a binge-like ethanol exposure during the third-trimester equivalent suggest a continued trend of altered synaptic signaling, with both long- and short-term pathways involving glutamate transmission and alterations to synaptic plasticity (table 5). Further, genes associated with steroid hormone signaling also remain altered, potentially maintaining an abnormal HPA axis function in the adult brain. Interestingly, this is associated with altered circadian rhythm signaling, as identified in both the short- and long-term canonical pathways. We observed an altered expression of the *period* genes *Per1* and *Per2* as well as a long-term altered cyclical expression of *Pomc* [61]. Altered sleep-wake cycles have previously been observed in human infants and adolescents exposed to alcohol during neurodevelopment [61]. Consistent with this study and others, these effects are most pronounced in animal models when exposure occurs during the brain growth spurt period [62, 63]. Altered circadian rhythm and, correspondingly, altered HPA axis corticosterone signaling are further associated in FASD individuals or models with increased vulnerability to stress, depression, hyperactivity, and diminished cognitive function [41, 62–64].

This is further highlighted with the identification of the TR/RXR pathway within the significantly altered canonical pathways that remain altered in adult mice neurodevelopmentally exposed to ethanol during synaptogenesis (table 4). RXRs are nuclear receptors that mediate retinoic acid-induced gene expression through the formation of homo- or heterodimers with other receptors including TR, among many others [65, 66]. While many data have been published examining the role of retinoic acid in immediate ethanol effects, few studies have reported the role of retinoic acid signaling alterations in the long-term effects associated with FASD. However, retinoic acid receptors and TR have been implicated in the thyroid dysfunction following PAE, resulting in reduced glucocorticoid signaling and multiple phenotypes such as a higher incidence of depressive behavior and spatial learning deficits [67]. This role in spatial learning is consistent in many studies, with retinoic acid signaling implicated in changes to long-term potentiation, hippocampal synaptic function, and impairments to working memory [68, 69]. Interestingly, these impairments in cognition are also correlated with altered circadian clock patterns [70], which has been identified by this study as a potential short-term target of ethanol exposure during synaptogenesis (tables 3, 4; fig. 5).

The PTEN canonical pathway was also identified as significantly affected in the short- and long-term effects of ethanol (tables 3, 5). Given the broad intracellular signaling functions of the PTEN and associated PI3K/AKT/mTOR pathway and its ability to respond to nutrient availability, stress, hormones, and growth factors to modulate protein synthesis, a comprehensive discussion of this pathway with relevance to PAE would be assumptive. The pathway is worth attention, however, in light of recent findings implicating *Pten* and *Akt1* regulation in other mouse models of neurodevelopmental ethanol exposure [71].

Establishment of the Long-Term Footprint of Ethanol Exposure May Include Altered miRNA Regulation

The results included in this study offer an evaluation of potential epigenetic mechanisms that may help establish a long-term footprint of altered gene expression following ethanol exposure during synaptogenesis. There has been increasing evidence that epigenetic processes, such as the establishment of DNA methylation, chromatin structure, and miRNA expression, are integral to normal neurodevelopment [72–74]. Histone deacetylases (*Hdac1* and *Hdac9*) appear as target molecules within multiple canonical pathways identified as altered acutely (at P7) following ethanol exposure (table 3). These genes

play an important role in controlling gene expression and cell signaling events via histone modifications and chromatin remodeling and play a large role in a number of neurodegenerative disorders including FASD [71, 75, 76]. These genes act not only by altering the chromatin structure but also in interacting with tissue-specific transcription factors to control apoptosis and differentiation [77, 78].

The evaluation of miRNA changes in the adult brain of mice exposed to alcohol at P4 and P7, within the brain growth spurt period, identified a number of miRNAs as altered at P60 following ethanol exposure at synaptogenesis (online suppl. table 2). The genes identified as potential targets of some of these miRNAs play important roles in the function of mature neuronal communication and signaling (table 5). Interestingly, many of the biological pathways associated with these miRNAs and their targets showed a similarity with the pathways affiliated with the modified short-term (P7) transcripts and a relevance to synaptogenesis. Further, these pathways showed a higher relevance to synaptic function and neural function than to cell survival processes, as may be expected given that these results highlight the residual effects of exposure at synaptogenesis rather than an immediate stress response. Of note are the miRNAs posed to regulate genes associated with HPA axis-associated corticotrophin and retinoic acid signaling (table 5). Correspondingly, the Target Filter results suggested that PI3K/AKT/mTOR signaling may be altered via aberrant miRNA regulation of the tumor suppressor protein *Tsc1* (table 5). The upregulation of miR-721 and subsequent downregulation of *Tsc1* is significant with relevance to FASD-related abnormalities, given current evidence that knockdown of *Tsc1* or its functional partner, *huntingtin-associated protein 1 (Hap1)*, profoundly impairs the positioning of pyramidal neurons in the hippocampus [79], leading to long-term neurological impairment including cognitive deficits and learning impairments [80]. These results argue that altered epigenetic mechanisms, including histone modifications and miRNA regulatory control, may reflect consequences of neurodevelopmental ethanol exposure that do not have a specific relationship to stress response but nonetheless exert long-term functional changes within the adult brain.

Caveats

There are some limitations to these studies which should be noted. First, the gene expression changes were evaluated using whole-brain homogenates. The authors recognize that the developing brain is a complex tissue with a number of regions undergoing neurodevelopmental processes at varying time frames. While synapse formation may be the predominant process occurring during the first 2 weeks of postnatal mouse development (corresponding to the processes occurring during the third-trimester equivalent), there are also a number of other critical processes occurring at this time depending on the region, including neurogenesis, glial development, apoptotic pruning, and myelination. The results we have generated have relevance to synapse-related processes, particularly in regions such as the corpus callosum, the HPA axis, the cerebellum, and the cortices. Coincidentally, these are the regions that are particularly vulnerable to ethanol-induced neurodegeneration and structural changes resulting from third trimester-associated processes [81, 82]. We hypothesize that the brain regions most vulnerable to ethanol will be those cells that are actively undergoing developmental processes and that are responsive to external cues, either internal or external (such as ethanol). As such, the results generated in this study provide an overview of the pathways that may be affected at a broad scale on biological processes that are occurring in multiple brain regions simultaneously. Region-specific analysis may provide a more detailed perspective on those genes and pathways that may be affected within those regions.

Second, while it is likely uncommon that a pregnant female will ingest a high dose of alcohol during the third trimester only, this paradigm models a binge-like exposure at a specific neurodevelopmental time. Neurodevelopment is a lengthy and complex process, and without controlled models of exposures at specific times, it is difficult to differentiate the resulting molecular and, subsequently, phenotypic effects that result from exposures at each stage of development. Evaluations of third trimester-equivalent models of FASD are becoming increasingly prevalent in the literature, due to the brain's vulnerability at this stage. Studies have established that ethanol exposure at P7 results in robust phenotypic consequences such as profound learning deficits, altered stress reactivity, and circadian rhythm dysfunction [14, 16, 62]. While it is clear that there is no one exposure time that is independently responsible for the range of phenotypes associated with FASD, it is important to discern the mechanisms that may underlie the results generated from each developmental stage. Also, it would be prudent to complete these studies using female as well as male mice, given the differences in ethanol response between sexes. Specifically, females have been shown to be particularly

vulnerable to ethanol-induced changes in HPA axis reactivity [16, 40].

Finally, given that the gene expression changes from alcohol exposure are subtle and multifactorial, it will be important to investigate the impact of these gene expression changes on protein levels and their relevance to cellular function.

Conclusions

This study reports the gene expression changes, associated biological pathways, and gene interactions altered immediately following ethanol exposure during the neurodevelopmental stage of synaptogenesis. The findings indicate that ethanol induces a strong stress response in developing cells, promoting the activation of specific pathways that orchestrate the balance between cell survival and cell death. This response includes the downregulation of energetically costly biological processes, presumably to reorient their resources to negotiate the immediate ethanol insult.

Additionally, there are a number of affected genes that have critical roles in establishing the connectivity and communication between neurons that will eventually comprise the neural circuitry of the adult brain. This molecular adaptation leads to a discontinuity in the course of normal neurodevelopment that may leave a 'molecular footprint' of neurodevelopmental alcohol exposure. These changes represent transcriptome alterations that may persist into adulthood and may be maintained, at least in part, by altered epigenetic patterning including histone modification and miRNA expression. The results presented in this study suggest that ethanol exposure during the third-trimester equivalent has pervasive consequences that broadly affect synaptic communication, altering HPA axis function and higher cognitive functioning.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), Canadian Institutes of Health Research (CIHR), and the Ontario Mental Health Foundation (OMHF) to S.M.S. The authors thank David Carter and the Robarts Research Institute for their assistance and expertise with array hybridization.

Disclosure Statement

The authors declare no competing financial interests.

References

1 Guerri C, Bazinet A, Riley EP: Foetal alcohol spectrum disorders and alterations in brain and behaviour. Alcohol Alcohol 2009;44:108–114.
2 Riley EP, Infante MA, Warren KR: Fetal alcohol spectrum disorders: an overview. Neuropsychol Rev 2011;21:73–80.
3 Green CR, Mihic AM, Nikkel SM, Stade BC, Rasmussen C, Munoz DP, Reynolds JN: Executive function deficits in children with fetal alcohol spectrum disorders (FASD) measured using the Cambridge Neuropsychological Tests Automated Battery (CANTAB). J Child Psychol Psychiatry 2009;50:688–697.
4 Zhou FC, Zhao Q, Liu Y, Goodlett CR, Liang T, McClintick JN, Edenberg HJ, Li L: Alteration of gene expression by alcohol exposure at early neurulation. BMC Genomics 2011;12:124.
5 Hard ML, Abdolell M, Robinson BH, Koren G: Gene-expression analysis after alcohol exposure in the developing mouse. J Lab Clin Med 2005;145:47–54.
6 Bartlett PF, Dutton R, Likiardopoulos V, Brooker G: Regulation of neurogenesis in the embryonic and adult brain by fibroblast growth factors. Alcohol Alcohol Suppl 1994;2:387–394.
7 Green ML, Singh AV, Zhang Y, Nemeth KA, Sulik KK, Knudsen TB: Reprogramming of genetic networks during initiation of the fetal alcohol syndrome. Dev Dyn 2007;236:613–631.
8 Bayer SA, Altman J, Russo RJ, Zhang X: Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. Neurotoxicology 1993;14:83–144.
9 Cohen-Cory S: The developing synapse: construction and modulation of synaptic structures and circuits. Science 2002;298:770–776.
10 Cline H, Haas K: The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis. J Physiol 2008;586:1509–1517.
11 Wondolowski J, Dickman D: Emerging links between homeostatic synaptic plasticity and neurological disease. Front Cell Neurosci 2013;7:223.
12 Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW: Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 2000;287:1056–1060.
13 Olney JW, Wozniak DF, Farber NB, Jevtovic-Todorovic V, Bittigau P, Ikonomidou C: The enigma of fetal alcohol neurotoxicity. Ann Med 2002;34:109–119.
14 Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ: Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. Neurobiol Dis 2004;17:403–414.
15 Mantha K, Kleiber ML, Singh SM: Neurodevelopmental timing of ethanol exposure may contribute to observed heterogeneity of behavioral deficits in a mouse model of fetal alcohol spectrum disorder (FASD). J Behav Brain Sci 2013;3:85–99.
16 Brocardo PS, Boehme F, Patten A, Cox A, Gil-Mohapel J, Christie BR: Anxiety- and depression-like behaviors are accompanied by an increase in oxidative stress in a rat model of fetal alcohol spectrum disorders: protective effects of voluntary physical exercise. Neuropharmacology 2012;62:1607–1618.

17 Kleiber ML, Mantha K, Stringer RL, Singh SM: Neurodevelopmental alcohol exposure elicits long-term changes to gene expression that alter distinct molecular pathways dependent on timing of exposure. J Neurodev Disord 2013;5:6.
18 Galindo R, Zamudio PA, Valenzuela CF: Alcohol is a potent stimulant of immature neuronal networks: implications for fetal alcohol spectrum disorder. J Neurochem 2005;94: 1500–1511.
19 Servais L, Hourez R, Bearzatto B, Gall D, Schiffmann SN, Cheron G: Purkinje cell dysfunction and alteration of long-term synaptic plasticity in fetal alcohol syndrome. Proc Natl Acad Sci USA 2007;104:9858–9863.
20 Olney JW, Tenkova T, Dikranian K, Qin YQ, Labruyere J, Ikonomidou C: Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. Brain Res Dev Brain Res 2002;133:115–126.
21 Jorge JC, McIntyre KL, Henderson LP: The function and the expression of forebrain $GABA_A$ receptors change with hormonal state in the adult mouse. J Neurobiol 2002;50: 137–149.
22 Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP: Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 2003;31:e15.
23 Huang da W, Sherman BT, Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.
24 Schmittgen TD, Livak KJ: Analyzing real-time PCR data by the comparative C_T method. Nat Protoc 2008;3:1101–1108.
25 Moir RD, Willis IM: Regulation of pol III transcription by nutrient and stress signaling pathways. Biochim Biophys Acta 2013;1829: 361–375.
26 Thomas G: An encore for ribosome biogenesis in the control of cell proliferation. Nat Cell Biol 2000;2:E71–E72.
27 Arsenijevic T, Vujovic A, Libert F, Op de Beeck A, Hebrant A, Janssens S, Gregoire F, Lefort A, Bolaky N, Perret J, Caspers L, Willermain F, Delporte C: Hyperosmotic stress induces cell cycle arrest in retinal pigmented epithelial cells. Cell Death Dis 2013;4:e662.
28 Perez-Cadahia B, Drobic B, Davie JR: Activation and function of immediate-early genes in the nervous system. Biochem Cell Biol 2011; 89:61–73.
29 Ruzhynsky VA, McClellan KA, Vanderluit JL, Jeong Y, Furimsky M, Park DS, Epstein DJ, Wallace VA, Slack RS: Cell cycle regulator E2F4 is essential for the development of the ventral telencephalon. J Neurosci 2007;27:5926–5935.
30 Rosenstein JM, Krum JM, Ruhrberg C: VEGF in the nervous system. Organogenesis 2010;6: 107–114.
31 Dikranian K, Qin YQ, Labruyere J, Nemmers B, Olney JW: Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. Brain Res Dev Brain Res 2005;155:1–13.
32 Avdulov NA, Wood WG, Harris RA: Effects of ethanol on structural parameters of rat brain membranes: relationship to genetic differences in ethanol sensitivity. Alcohol Clin Exp Res 1994;18:53–59.
33 Kleene R, Schachner M: Glycans and neural cell interactions. Nat Rev Neurosci 2004;5: 195–208.
34 de Wit J, O'Sullivan ML, Savas JN, Condomitti G, Caccese MC, Vennekens KM, Yates JR 3rd, Ghosh A: Unbiased discovery of glypican as a receptor for LRRTM4 in regulating excitatory synapse development. Neuron 2013;79:696–711.
35 Tomas M, Fornas E, Megias L, Duran JM, Portoles M, Guerri C, Egea G, Renau-Piqueras J: Ethanol impairs monosaccharide uptake and glycosylation in cultured rat astrocytes. J Neurochem 2002;83:601–612.
36 Binkhorst M, Wortmann SB, Funke S, Kozicz T, Wevers RA, Morava E: Glycosylation defects underlying fetal alcohol spectrum disorder: a novel pathogenetic model. 'When the wine goes in, strange things come out' – ST Coleridge, *The Piccolomini*. J Inherit Metab Dis 2012;35:399–405.
37 Vazquez DM: Stress and the developing limbic-hypothalamic-pituitary-adrenal axis. Psychoneuroendocrinology 1998;23:663–700.
38 Hellemans KG, Sliwowska JH, Verma P, Weinberg J: Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. Neurosci Biobehav Rev 2010;34:791–807.
39 Hellemans KG, Verma P, Yoon E, Yu W, Weinberg J: Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. Ann NY Acad Sci 2008;1144:154–175.
40 Weinberg J, Sliwowska JH, Lan N, Hellemans KG: Prenatal alcohol exposure: foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome. J Neuroendocrinol 2008;20:470–488.
41 Weinstock M: Intrauterine factors as determinants of depressive disorder. Isr J Psychiatry Relat Sci 2010;47:36–45.
42 Li QX, Ke N, Sundaram R, Wong-Staal F: NR4A1, 2, 3: an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. Histol Histopathol 2006;21: 533–540.
43 Nakajima Y, Yamada M, Taguchi R, Shibusawa N, Ozawa A, Tomaru T, Hashimoto K, Saito T, Tsuchiya T, Okada S, Satoh T, Mori M: NR4A1 (Nur77) mediates thyrotropin-releasing hormone-induced stimulation of transcription of the thyrotropin β gene: analysis of TRH knockout mice. PLoS One 2012; 7:e40437.
44 Dawson MI, Xia Z: The retinoid X receptors and their ligands. Biochim Biophys Acta 2012;1821:21–56.
45 Grummer MA, Zachman RD: Prenatal ethanol consumption alters the expression of cellular retinol binding protein and retinoic acid receptor mRNA in fetal rat embryo and brain. Alcohol Clin Exp Res 1995;19:1376–1381.
46 Kane MA, Folias AE, Wang C, Napoli JL: Ethanol elevates physiological all-*trans*-retinoic acid levels in select loci through altering retinoid metabolism in multiple loci: a potential mechanism of ethanol toxicity. FASEB J 2010; 24:823–832.
47 Kumar A, Singh CK, DiPette DD, Singh US: Ethanol impairs activation of retinoic acid receptors in cerebellar granule cells in a rodent model of fetal alcohol spectrum disorders. Alcohol Clin Exp Res 2010;34:928–937.
48 Huang JK, Jarjour AA, Nait Oumesmar B, Kerninon C, Williams A, Krezel W, Kagechika H, Bauer J, Zhao C, Baron-Van Evercooren A, Chambon P, Ffrench-Constant C, Franklin RJ: Retinoid X receptor gamma signaling accelerates CNS remyelination. Nat Neurosci 2011;14:45–53.
49 Lu L, Airey DC, Williams RW: Complex trait analysis of the hippocampus: mapping and biometric analysis of two novel gene loci with specific effects on hippocampal structure in mice. J Neurosci 2001;21:3503–3514.
50 Alarcon JM, Hodgman R, Theis M, Huang YS, Kandel ER, Richter JD: Selective modulation of some forms of Schaffer collateral-CA1 synaptic plasticity in mice with a disruption of the *CPEB-1* gene. Learn Mem 2004;11:318–327.
51 Berger-Sweeney J, Zearfoss NR, Richter JD: Reduced extinction of hippocampal-dependent memories in CPEB knockout mice. Learn Mem 2006;13:4–7.
52 Wells DG, Richter JD, Fallon JR: Molecular mechanisms for activity-regulated protein synthesis in the synapto-dendritic compartment. Curr Opin Neurobiol 2000;10:132–137.
53 Cserep C, Szabadits E, Szonyi A, Watanabe M, Freund TF, Nyiri G: NMDA receptors in GABAergic synapses during postnatal development. PLoS One 2012;7:e37753.
54 Wang DD, Kriegstein AR: GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. J Neurosci 2008;28:5547–5558.
55 Lujan R, Shigemoto R, Lopez-Bendito G: Glutamate and GABA receptor signalling in the developing brain. Neuroscience 2005;130: 567–580.
56 Yashiro K, Philpot BD: Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. Neuropharmacology 2008;55:1081–1094.
57 Gambrill AC, Barria A: NMDA receptor subunit composition controls synaptogenesis and synapse stabilization. Proc Natl Acad Sci USA 2011;108:5855–5860.
58 Killisch I, Dotti CG, Laurie DJ, Luddens H, Seeburg PH: Expression patterns of $GABA_A$ receptor subtypes in developing hippocampal neurons. Neuron 1991;7:927–936.
59 Bolton MM, Blanpied TA, Ehlers MD: Localization and stabilization of ionotropic glutamate receptors at synapses. Cell Mol Life Sci 2000;57:1517–1525.

60 Malenka RC, Bear MF: LTP and LTD: an embarrassment of riches. Neuron 2004;44:5–21.
61 Chen CP, Kuhn P, Advis JP, Sarkar DK: Prenatal ethanol exposure alters the expression of period genes governing the circadian function of β-endorphin neurons in the hypothalamus. J Neurochem 2006;97:1026–1033.
62 Earnest DJ, Chen WJ, West JR: Developmental alcohol and circadian clock function. Alcohol Res Health 2001;25:136–140.
63 Sakata-Haga H, Dominguez HD, Sei H, Fukui Y, Riley EP, Thomas JD: Alterations in circadian rhythm phase shifting ability in rats following ethanol exposure during the third trimester brain growth spurt. Alcohol Clin Exp Res 2006;30:899–907.
64 Girotti M, Weinberg MS, Spencer RL: Differential responses of hypothalamus-pituitary-adrenal axis immediate early genes to corticosterone and circadian drive. Endocrinology 2007;148:2542–2552.
65 Bastien J, Rochette-Egly C: Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 2004;328:1–16.
66 Chambon P: A decade of molecular biology of retinoic acid receptors. FASEB J 1996;10:940–954.
67 Wilcoxon JS, Kuo AG, Disterhoft JF, Redei EE: Behavioral deficits associated with fetal alcohol exposure are reversed by prenatal thyroid hormone treatment: a role for maternal thyroid hormone deficiency in FAE. Mol Psychiatry 2005;10:961–971.
68 Lane MA, Bailey SJ: Role of retinoid signalling in the adult brain. Prog Neurobiol 2005;75:275–293.
69 Alfos S, Boucheron C, Pallet V, Higueret D, Enderlin V, Beracochea D, Jaffard R, Higueret P: A retinoic acid receptor antagonist suppresses brain retinoic acid receptor overexpression and reverses a working memory deficit induced by chronic ethanol consumption in mice. Alcohol Clin Exp Res 2001;25:1506–1514.
70 Golini RS, Delgado SM, Navigatore Fonzo LS, Ponce IT, Lacoste MG, Anzulovich AC: Daily patterns of clock and cognition-related factors are modified in the hippocampus of vitamin A-deficient rats. Hippocampus 2012;22:1720–1732.
71 Laufer BI, Mantha K, Kleiber ML, Diehl EJ, Addison SM, Singh SM: Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. Dis Model Mech 2013;6:977–992.
72 Bekdash RA, Zhang C, Sarkar DK: Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation, and POMC gene expression in β-endorphin-producing POMC neurons of the hypothalamus. Alcohol Clin Exp Res 2013;37:1133–1142.
73 Miranda RC: MicroRNAs and fetal brain development: implications for ethanol teratology during the second trimester period of neurogenesis. Front Genet 2011;3:77.
74 Veazey KJ, Carnahan MN, Muller D, Miranda RC, Golding MC: Alcohol-induced epigenetic alterations to developmentally crucial genes regulating neural stemness and differentiation. Alcohol Clin Exp Res 2013;37:1111–1122.
75 Butler R, Bates GP: Histone deacetylase inhibitors as therapeutics for polyglutamine disorders. Nat Rev Neurosci 2006;7:784–796.
76 Vogel-Ciernia A, Wood MA: Neuron-specific chromatin remodeling: a missing link in epigenetic mechanisms underlying synaptic plasticity, memory, and intellectual disability disorders. Neuropharmacology 2014;80:18–27.
77 Conway GD, O'Bara MA, Vedia BH, Pol SU, Sim FJ: Histone deacetylase activity is required for human oligodendrocyte progenitor differentiation. Glia 2012;60:1944–1953.
78 Parra M, Verdin E: Regulatory signal transduction pathways for class IIA histone deacetylases. Curr Opin Pharmacol 2010;10:454–460.
79 Mejia LA, Litterman N, Ikeuchi Y, de la Torre-Ubieta L, Bennett EJ, Zhang C, Harper JW, Bonni A: A novel Hap1-Tsc1 interaction regulates neuronal mTORC1 signaling and morphogenesis in the brain. J Neurosci 2013;33:18015–18021.
80 Orlova KA, Crino PB: The tuberous sclerosis complex. Ann NY Acad Sci 2010;1184:87–105.
81 Olney JW: Fetal alcohol syndrome at the cellular level. Addict Biol 2004;9:137–149, discussion 151.
82 Dobbing J: The later growth of the brain and its vulnerability. Pediatrics 1974;53:2–6.