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FETAL REGIONAL BRAIN PROTEIN SIGNATURE IN FASD RAT MODEL

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Abstract

Fetal alcohol spectrum disorders (FASD) describe neurodevelopmental deficits in children exposed to alcohol *in utero*. We hypothesized that gestational alcohol significantly alters fetal brain regional protein signature. Pregnant rats were binge-treated with alcohol or pair-fed and nutritionally-controlled. Mass spectrometry identified 1,806, 2,077, and 1,456 quantifiable proteins in the fetal hippocampus, cortex, and cerebellum, respectively. A stronger effect of alcohol exposure on the hippocampal proteome was noted: over 600 hippocampal proteins were significantly ($P<0.05$) altered, including annexin A2, nucleobindin-1, and glypican-4, regulators of cellular growth and developmental morphogenesis. In the cerebellum, cadherin-13, reticulocalbin-2, and ankyrin-2 (axonal growth regulators) were significantly ($P<0.05$) altered; altered cortical proteins were involved in autophagy (endophilin-B1, synaptotagmin-1). Ingenuity analysis identified proteins involved in protein homeostasis, oxidative stress, mitochondrial dysfunction, and mTOR as major pathways in the cortex and hippocampus significantly ($P<0.05$) affected by alcohol. Thus, neurodevelopmental protein changes may directly relate to FASD neuropathology.

Graphical abstract

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Keywords

Alcohol; FASD; Teratology; Fetal

1. Introduction

Gestational alcohol exposure is one of the leading causes of intellectual disability in the United States. Fetal Alcohol Spectrum Disorders (FASD), a range of growth deficits and brain abnormalities due to prenatal alcohol exposure, affect 2–5% of children in the United States (1–3). At the extreme end of the spectrum, fetal alcohol syndrome (FAS) is identified by facial dysmorphia, growth restriction, and central nervous system deficits; FAS is reported in 0.2 to 7 cases per 1,000 United States children (4). Alcohol-induced deficits can be severe and may persist for a lifetime.

A recent survey stated that during pregnancy, 1 in 10 women consume alcohol and 1 in 33 women binge drink (5). From the maternal blood, alcohol passes through the placenta to perfuse the fetus. Gestational alcohol exposure impairs the development of nearly all fetal organ systems (6–8), including a profound effect on neurodevelopment (9–12). Alcohol differentially affects a variety of brain regions and neuronal populations (9); specifically, structural anomalies have been observed in highly vulnerable regions including the hippocampus, cerebellum, and cortex (13). Heavy prenatal alcohol exposure can result in functional deficits relating to poor memory, motor dysfunction, and attention deficit disorders, which may go undiagnosed until a child is in elementary school (14). Microcephaly and microencephaly are thought to be the underlying causes of multiple secondary developmental issues such as mental health disorders, disrupted educational experiences, and increased risk for substance abuse problems in humans (15–17).

Animal models of FASD have allowed for specific observations of neuroanatomic and behavioral deficits, contributing to mechanistic discernment of FASD pathogenesis (18–21). Using these models, along with recent advances in the field of proteomics, new insights into alcohol-induced protein profile changes have been observed (22). One study detected reduced alpha-fetoprotein (AFP), a serum protein involved in the pathogenesis of various developmental deficits, in the amniotic fluid following gestational alcohol exposure (23). Another 2D SDS-PAGE-based proteomic study revealed alcohol-induced glucose metabolism in the cerebral cortex of weanling rats following prenatal alcohol exposure,

which was interpreted to indicate that energy utilization is increased to compensate for delayed neurodevelopment (24). We have previously reported maternal uterine artery proteomic changes following gestational alcohol exposure (25–28). Together these findings help interrogate FASD neuropathogenesis, and are necessary to develop novel screening and therapeutic approaches. However, the mechanisms of the alcohol-induced neuropathology in regions of high vulnerability remain to be determined. Herein, our study of gestational alcohol exposure-induced differential protein changes in brain regions aims to elucidate potential mechanisms by identifying specifically altered cellular processes and pathways.

As prenatal alcohol is a neurotoxicant, we hypothesize that alcohol will differentially alter the protein profiles of fetal brain regions. Here, we evaluate the altered protein signature profile in the fetal hippocampus, cortex, and cerebellum following a binge paradigm in a rat model utilizing a state-of-the-art Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer.

2. Material and methods

2.1 Animals

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at Texas A&M University. Timed pregnant Sprague-Dawley rats were purchased from Charles River, and housed in a temperature-controlled room (23°C) with a 12:12-hour light–dark cycle. Rats were assigned to a pair-fed control group (n = 8 dams) or an alcohol treatment group (n = 8 dams). The prenatal model of binge alcohol exposure used in this study is based on previously established rodent models of FASD (29, 30). The alcohol group animals were acclimatized via a dosing regimen of 22.5% (wt/v) ethanol (4.5 g/kg) between GD 5–10 (31), followed by 28.5% ethanol (6 g/kg) on GD 11–20. In a separate set of dams, blood from the tail vein was collected for BAC measurement following the 6 g/kg alcohol dose on GD 11. The plasma was separated (3,000 RPM, 4°C, 15 min) and the alcohol concentration was measured using EnzyChrom™ Ethanol Assay Kit (ECET-100, BioAssay Systems). A separate set of dams were utilized for BAC measurements in this study as we wanted to avoid any confounding effect of repeated blood-draws for BACs on proteomic data. The peak BAC following the 6 g/kg alcohol dose was 288 mg/dl. The peak BAC for the 4.5 g/kg dose (216 mg/dl) was reported in our previous publication (31), where a separate cohort of rats were again utilized for BAC measurement. The pair-fed controls were isocalorically matched to alcohol rats by dosing with maltose dextrin to account for calories from alcohol, and the amount of diet consumed by the pair-fed animals was matched with the alcohol-fed animals. Animals received a once daily orogastric gavage in a binge paradigm. Animals were sacrificed on GD 21, one day after the last alcohol exposure. One female and one male fetus were collected from each dam. Principal component analysis based on the individual protein abundances revealed that there were no sex differences in the proteomes of male and female samples. Therefore, both male (n=8) and female (n=8) samples were included in the mass spectrometry evaluation, and thus a total n=16 tissues were used per treatment group. The fetal brain was isolated, meninges removed, and the hippocampus, cortex, and cerebellum were dissected. The dissections were done in a 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) bath and blood-free tissue was immediately flash frozen and stored at -80°C .

2.2 Sample Preparation

Blood-free tissue was collected with 400 μ l proteome lysis buffer (QIAGEN, Hilden, Germany) supplemented with protease inhibitor cocktail (Complete Mini, Roche) and directly transferred to a pre-chilled bead-beater tube with 1.4 mm zirconium oxide beads (~100 μ l in the tube). Samples were lysed using a cycle of 20 b Precellys bead beater 1-mm zirconium beads; ~100 μ l in the tube 40 sec per cycle, cooled on ice; 40 sec per further cycle; 6,500 Hz speed for each cycle; chilled on dry ice. The mixture was centrifuged at 10,000g for 10 min at 4°C. Clear lysates were collected, transferred to new tubes and protein concentrations were estimated using a BCA assay kit (Thermo Scientific) (32). The protein concentrations in the cell extracts are adjusted by adding lysis buffer. In brief, undiluted neat urine (150 μ L, i.e., ~15 μ g of protein) was added to a mixture of 150 μ g of urea and 30 μ L of dithiothreitol (DTT; 100 mM in 1 M Tris/HCl pH 8.5). The samples were incubated for 20 min and the cysteine residues were blocked with 50 mM iodoacetamide for 20 min in the dark.

2.3 Filter assisted sample preparation (FASP)

The filter assisted sample preparation method was carried out as previously described (33, 34). In short: 100 µg proteins were denatured and reduced by adding 400 µl 8M urea supplemented with 200 mM tris (2-carboxyethyl) phosphine (TCEP) for 30 min at 60°C. Samples were then loaded on a 10 kDa MWCO spin filter column (Milipore) and spun 14,000 × g for 15 min at 23°C. Two wash steps with 200 µl 8M urea solution were carried out and proteins were alkylated with 100 µl 0.05 M iodoacetamide solution in 8 M urea, shaking at 600 rpm for one minute and incubated in the dark at (23°C) for 20 min before centrifugation at 14,000 × g for 15 min. Two further washes with 8 M urea solution were carried out and subsequently, three washes with 100 µl 50 mM ammonium bicarbonate solutions were performed. Protein digestion was performed with sequencing grade trypsin (Promega) at a nominal enzyme to substrate ratio of 1:50. After incubation for 16 h at 600 rpm, the resulting peptides were eluted in two wash steps with 50 µl ABC and a final spin with 50 µl 0.5 M sodium chloride (NaCl). Peptide elutes were acidified and desalted using reversed phase C-18 microspin columns (SEMSS18R, Nest Group) and eluted in two fractions (30% Acetonitrile and 50%–70% Acetonitrile fractions). Samples were vacuum dried and frozen at −20°C prior to LC-MS/MS analysis.

2.4 LC-MS/MS Analysis

Randomized samples were analyzed using a Q Exactive mass spectrometer (Thermo) coupled to a micro-autosampler AS2 and a nanoflow HPLC pump (Eksigent). Peptides were separated using an in-house packed C18 analytical column (Magic C18 particles, 3 μ m, 300 Å, 150 mm, Michrom Bioresource) by a linear 120 min gradient starting from 95% buffer A (0.1% (v/v) formic acid in HPLC-H₂O) and 5% buffer B (0.2% (v/v) formic acid in acetonitrile) to 35% buffer B. A full mass spectrum with resolution of 70,000 (relative to an m/z of 200) was acquired in a mass range of 300–1,500 m/z (AGC target 3×10^6 , maximum injection time 20 ms). The 10 most intense ions were selected for fragmentation via higher-energy c-

trap dissociation (HCD, resolution 17,500, AGC target 2×10^5 , maximum injection time 250 ms, isolation window 1.6 m/z, normalized collision energy 27%).

Raw data were analyzed by MaxQuant software version 1.5.2.10 (35) and peptide list searched against the Uniprot protein sequence database (February 2016, only reviewed entries appended with common laboratory contaminants [cRAP database, 247 entries]) using the Andromeda search engine (36). The following settings were applied: trypsin (specificity set as C-terminal to arginine and lysine) with up to two missed cleavages, mass tolerances set to 20 ppm for the first search and 4.5 ppm for the second search. Oxidation of M- and N-terminal acetylation were chosen as dynamic modifications and propionylation of cysteine as static modification. False discovery rate (FDR) was set to 1% on peptide and protein levels with a minimum length of seven amino acids and was determined by searching a reverse database. Peptide identification was performed with an allowed initial precursor mass deviation up to 7 ppm and an allowed fragment mass deviation of 20 ppm. For all other search parameters, the default settings were used. Label-free quantification was done using the XIC-based in-built label-free quantification (LFQ) algorithm (37) integrated into MaxQuant. Data analysis was performed with the Perseus software in the MaxQuant computation platform and in the R statistical computing environment. Therefore, label-free intensities were logarithmized and were normalized using median intensities for each sample to account for differences in protein loading. Two-sided t-tests were performed comparing either hippocampal, cortical, or cerebellar proteomes from 16 pair-fed controls (8 female, 8 male) to 16 alcohol-exposed (8 female, 8 male) fetuses. Significant proteins were determined with a permutation-based False Discovery Rate (FDR) calculation. Fold expression differences of the proteins between the comparison groups were calculated from the individual median averaged protein LFQ intensities for the two comparison groups. Pathway membership information and categorical annotation was obtained from Gene ontology (GO) for biological process, molecular function and cellular compartment and functional enrichment was tested using Fisher's exact test. Furthermore, Ingenuity Pathway Analysis (IPA) was used to identify overrepresented functions and canonical pathways. Altered proteins ($P < 0.05$) were used for network analysis based on criteria annotated in the IPA database. The IPA database contains molecular information available in the scientific literature. Biological functions and canonical pathways are identified overlaying the significantly altered molecules onto predefined maps containing functional or pathway information of the IPA database. Right-tailed Fisher's exact test was used to calculate P -values for the assigned pathways.

3. Results

Mass spectrometry analysis stipulated that quantifiable proteins were identified in 50% of all the samples, and in at least 4 samples of one comparison group. A full list of identified and differentially regulated proteins in the fetal hippocampus, cortex, and cerebellum following gestational alcohol exposure, can be found in the Supplemental Information.

Proteomic changes in the hippocampus following prenatal alcohol exposure

A total of 3,799 fetal hippocampal proteins were identified, of which 2,077 were used for the statistical analysis after filtering (see above). 634 proteins were significantly ($P < 0.05$) altered following gestational alcohol exposure: 188 were upregulated and 446 were downregulated. Volcano plots diagramed the statistically significant ($P < 0.05$) differences of the protein abundance changes between the pair-fed control and alcohol groups (Figure 1A). Significantly altered proteins were involved in cellular growth, differentiation and morphogenesis, such as annexin A2 ($P = 0.005$), nucleobindin-1 ($P = 0.0001$), and glypican-4 ($P = 0.003$). A Gene ontology (GO) enrichment analysis evaluated the distinct biological pathways and cellular compartments of the dysregulated hippocampal proteins. This analysis revealed an overrepresentation of dysregulated proteins in the “nuclear chromosome part”, “chromosomal part”, “nucleoplasm” and in the cellular compartment GO categories, and “chromosome organization”, “chromatin modification”, “chromatin organization”, “RNA metabolic process”, “organelle organization”, and “nucleobase-containing compound metabolic process” for biological pathways (Figure 1B). Bioinformatic pathway analysis of the differentially regulated hippocampal proteins reinforced these findings by identifying pathways related to growth, translation, homeostasis, and oxidative stress. The dysregulated fetal hippocampal proteins were associated with “EIF2 signaling”, “mechanistic target of rapamycin (mTOR) signaling”, included downstream “EIF4 and p70S6K signaling”, “protein ubiquitination” and mitochondrial stress as identified by “sirtuin signaling pathway” and “mitochondrial dysfunction” (Figure 1C). Toxicity analysis revealed that alcohol exposure primarily affected mitochondrial function and oxidative stress response, leading to necrosis (Figure 1D).

Proteomic changes in the cortex following prenatal alcohol exposure

In the fetal cortex, a total of 2,764 protein were identified and the abundance levels of 1,806 proteins were used for statistical analysis. 49 cortical proteins were found to be significantly ($P < 0.05$) downregulated, whereas 32 proteins were significantly ($P < 0.05$) upregulated following gestational alcohol exposure (Figure 2A). However, significantly altered proteins in the cortex, did not survive multiple hypothesis correction as these proteins did not reach the statistical threshold ($P < 0.05$, FDR-adjusted). Interesting candidates include endophilin-B1 ($P = 0.001$), a membrane protein involved in autophagy; synaptotagmin-1 ($P = 0.002$), a calcium sensor protein critical for vesicle fusion; and stathmin ($P = 0.011$), a phosphoprotein involved in learning were significantly downregulated in the cortex following gestational alcohol exposure. Bioinformatic pathway analysis classified many of the dysregulated proteins as having a role in translational control and protein synthesis; specifically “EIF2 signaling”, “regulation of eIF4 and p70S6K signaling”, and “mTOR signaling” were identified (Figure 2B). Metabolic pathways such as N-acetylglucosamine degradation I and II, Acetyl-CoA biosynthesis, and glutamine degradation were also included in the IPA (Figure 2B). Toxicity analysis of the fetal cortex shows similarities to the fetal hippocampus with functional abnormalities in oxidative stress and mitochondria dysfunction (Figure 2C; Fisher’s exact test; $P < 0.05$).

Proteomic changes in the cerebellum following prenatal alcohol exposure

A total of 3,294 fetal cerebellar proteins were identified and 1,456 were quantified for statistical analysis: 30 were significantly ($P < 0.05$) upregulated and 12 were significantly ($P < 0.05$) downregulated following gestational alcohol exposure (Figure 3A). Similarly to the cortex, significant cerebellar protein alterations did not survive multiple comparison corrections. Nevertheless, proteins of interest include cadherin-13 ($P = 0.019$), a membrane protein which regulates axonal growth during neural differentiation; reticulocalbin-2 ($P = 0.002$), a calcium binding protein located in the endoplasmic reticulum; and ankyrin-2 ($P = 0.041$), a membrane protein involved in ion transportation and implicated in intellectual disability (Figure 3A). Due to the small number of altered proteins, dysregulated proteins in the cerebellum have very weak associations with canonical pathways (Figure 3B) and toxicity functions ($P > 0.05$, not shown).

Comparison across the different brain regions

Comparing the three regional proteomic analyses, the hippocampus is identified as the most vulnerable brain region in terms of protein changes following prenatal chronic binge alcohol exposure in our model (Figure 4). Only one protein, ras-related protein Rab-21, is found to be dysregulated in all three fetal brain regions after alcohol exposure, which is involved in endosomal trafficking and is implicated in autophagy and protein homeostasis (38). 22 proteins overlap between the hippocampus and cortex, and 10 proteins between the hippocampus and cerebellum. Major candidate proteins which survive the multiple comparison correction ($P < 0.05$, FDR-adj.) in the hippocampus and are differentially dysregulated ($P < 0.05$) in the cortex include nucleobindin, citrate ATPase and profilin-1. Pathway overlap in these brain regions point towards mTOR signaling and translation dysregulation following prenatal gestational alcohol exposure.

4. Discussion

To the best of our knowledge, this is the first proteomic study in the FASD field to identify changes in the fetal hippocampus, cortex, and cerebellum following gestational alcohol exposure. We herein demonstrate that chronic binge alcohol exposure has specific and substantial effects on fetal brain regions highly vulnerable to the teratogenic effects of gestational alcohol exposure, particularly in the hippocampal region, in alignment with prior studies utilizing comparable rodent models of gestational alcohol exposure (39–41). Previous rat models of gestational alcohol exposure have implicated hippocampal deficits which manifested as altered synaptic plasticity (42) and functional connectivity (39) and was evidenced through impaired behavioral tasks in memory, learning, and spatial navigation. Our analysis of alcohol-induced changes in the fetal hippocampus identified regulators of cellular growth and developmental morphogenesis. In the cortex some of the dysregulated proteins were involved in autophagy, a key player in cellular development and differentiation, which has been hypothesized to be affected by ethanol neurotoxicity. Our data also demonstrate that alcohol alters expression of proteins involved in neuronal growth, differentiation, and neurodegenerative diseases, which may directly relate to the neurobehavioral delays and disabilities associated with FASD.

The severity of FASD neuropathology is largely dependent on the timing and dosing of alcohol exposure during pregnancy. Regional neurogenesis and temporal vulnerabilities contribute to the complexity of notable alcohol-induced deficits, specifically in the hippocampus, cortex, and cerebellum (9). Prenatal rodent models of gestational alcohol exposure, like the one in this study, model the first two trimesters of human brain development. Acute prenatal exposure alcohol exposure, defined as a single day of alcohol treatment, alters regional brain shapes (43) and maternal plasma amino acid bioavailability (44). Chronic prenatal alcohol exposure, administered as liquid diet or as repeated binges, leads to disrupted cell migration and synaptogenesis (45) and altered amino acid bioavailability and fetal brain regions (46). The functional consequences of these anatomical changes detrimentally impacts memory (47, 48), spatial navigation, and nociceptive responses (43, 49, 50). In rodent models, the early postnatal period approximates the third trimester of human brain development, which includes the ‘brain growth spurt’. Alcohol exposure during the postnatal period leads to reduced brain volume, loss of frontal cortical neurons (51, 52), impairment of hippocampal neurogenesis (53), loss of hippocampal and cerebellar neurons (54, 55), and apoptotic neurodegeneration (56). These long-term neural circuit disruptions are associated with neurobehavioral deficits which persist into adulthood (57). Postnatal models of alcohol exposure are linked to deficits in spatial memory retention (58) and learning and memory (59), as well as object place memory impairments (60). Despite these observations, questions remain regarding the underlying mechanism of the alcohol-induced neuropathology, especially in brain regions highly vulnerable to the teratogenic effects of alcohol.

Our findings detailing the proteomic changes observed in the fetal hippocampus, cortex, and cerebellum aid in our understanding of FASD pathogenesis and the mechanistic teratogenic effects of gestational alcohol. In our model, the most vulnerable region was the fetal hippocampus, where proteins significantly altered by alcohol exposure include regulators of cellular growth and developmental morphogenesis. Interestingly, there were 22 proteins which were significantly dysregulated in both the hippocampus and the cortex following gestational alcohol exposure. For example, nucleobindin-1, a multi-domain protein with calcium-binding and DNA-binding domains which functions to regulate calcium homeostasis (61), cellular growth and differentiation (62), and T cell activation (63) is dysregulated in both the hippocampus and the cortex. Profilin-1, a small actin-binding protein has a role in dendritic spine morphological changes relating to the synaptic responsiveness associated with memory formation (64, 65), is also dysregulated in both regions. The susceptibility of the brain regions analyzed in this study to developmental alcohol exposure is well established, however it is important to note that our prenatal exposure period is limited in that it does not include the third trimester-equivalent, the period in which these regions are exquisitely vulnerable to alcohol as evidenced by neuronal loss (66–69). Thus, protein changes observed herein may not represent those observed following an exposure period comprising this critical developmental window susceptible to neuronal loss. As such, our assessment of protein changes in these regions is not the same as comparison with previous studies on alcohol-induced neuronal loss.

Autophagy is critical for maintaining cellular homeostasis and has a protective role in the central nervous system (CNS). Ethanol neurotoxicity activates autophagy, possibly through

the modulation of mTOR signaling, nutrient deprivation, and other stresses (70). In the cortex, endophilin-B1, a membrane protein which interacts with Beclin-1 to induce autophagy and may be involved in the early stages of autophagosome formation (71), was significantly downregulated. In the hippocampus, annexin A2, a pleiotropic calcium-dependent phospholipid (72), was significantly upregulated following gestational alcohol exposure. Annexin A2 has a role in exocytosis and is involved in with phagocytosis of apoptotic cells (73, 74). In glioblastomas, Annexin A2 expression is considered a prognostic factor, and possible therapeutic target (75).

Several of the differentially regulated proteins detected in this study have previously been linked to Alzheimer's, Parkinson's, and Huntington's diseases. The link between the gestational alcohol exposure and neurodegenerative diseases reinforces that neurotoxic effects of gestational alcohol exposure to have substantial and prolonged effects which may contribute to the alcohol-induced deficits. For example, disruption of autophagy has been linked to multiple neurodegenerative diseases (70), patients with Alzheimer's disease typically have improper phosphorylation of stathmin (76), and nuclebindin-1 inhibits amyloid peptides or proteins associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease (77).

The Pathway Ingenuity Analysis of cortical and hippocampal tissues identified mTOR as an upstream regulator and one of the canonical pathways significantly altered following gestational alcohol exposure. mTOR mediates cellular development by influencing the cell cycle, cell growth, and metabolism through several signaling pathways. Additionally, it is a repressor of autophagy. The neuroprotectant functions of autophagy previously described may be unable to mitigate alcohol-induced FASD neuropathology. Therefore, activating autophagy and blocking mTOR repressor function may have potential therapeutic effects following gestational alcohol exposure.

5. Conclusion

Our data demonstrate that alcohol alters expression of proteins involved in neuronal growth and differentiation, which may directly relate to the neurobehavioral delays and disabilities associated with FASD. The proteomic changes observed in this study aid in our understanding of the mechanistic teratogenic effects of gestation alcohol. This study will narrow the focus of future studies aimed at drug discovery targeting proteins and pathways dysregulated by gestational alcohol exposure.

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References

1. Sokol RJ, Delaney-Black V, Nordstrom B. Fetal alcohol spectrum disorder. JAMA. 2003; 290:2996–2999. [PubMed: 14665662]
2. May PA, et al. Prevalence and characteristics of fetal alcohol spectrum disorders. Pediatrics. 2014; 134:855–866. [PubMed: 25349310]

3. Roozen S, et al. Worldwide Prevalence of Fetal Alcohol Spectrum Disorders: A Systematic Literature Review Including Meta-Analysis. *Alcohol Clin Exp Res*. 2016; 40:18–32. [PubMed: 26727519]
4. May PA, Gossage JP. Estimating the prevalence of fetal alcohol syndrome. A summary. *Alcohol Res Health*. 2001; 25:159–167. [PubMed: 11810953]
5. Tan CH, Denny CH, Cheal NE, Snizek JE, Kanny D. Alcohol use and binge drinking among women of childbearing age – United States, 2011–2013. *MMWR Morb Mortal Wkly Rep*. 2015; 64:1042–1046. [PubMed: 26401713]
6. Warren KR, Hewitt BG, Thomas JD. Fetal alcohol spectrum disorders: research challenges and opportunities. *Alcohol Res Health*. 2011; 34:4–14. [PubMed: 23580035]
7. Burd L, et al. Congenital heart defects and fetal alcohol spectrum disorders. *Congenit Heart Dis*. 2007; 2:250–255. [PubMed: 18377476]
8. Lewis CE, et al. Verbal learning and memory impairment in children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res*. 2015; 39:724–732. [PubMed: 25833031]
9. Riley EP, McGee CL. Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp Biol Med (Maywood)*. 2005; 230:357–365. [PubMed: 15956765]
10. Lebel C, Roussotte F, Sowell ER. Imaging the impact of prenatal alcohol exposure on the structure of the developing human brain. *Neuropsychol Rev*. 2011; 21:102–118. [PubMed: 21369875]
11. Archibald SL, et al. Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Dev Med Child Neurol*. 2001; 43:148–154. [PubMed: 11263683]
12. Gautam P, et al. Volume changes and brain-behavior relationships in white matter and subcortical gray matter in children with prenatal alcohol exposure. *Hum Brain Mapp*. 2015; 36:2318–2329. [PubMed: 25711175]
13. Norman AL, Crocker N, Mattson SN, Riley EP. Neuroimaging and fetal alcohol spectrum disorders. *Dev Disabil Res Rev*. 2009; 15:209–217. [PubMed: 19731391]
14. Mattson SN, et al. Further development of a neurobehavioral profile of fetal alcohol spectrum disorders. *Alcohol Clin Exp Res*. 2013; 37:517–528. [PubMed: 22974253]
15. Streissguth AP, et al. Risk factors for adverse life outcomes in fetal alcohol syndrome and fetal alcohol effects. *J Dev Behav Pediatr*. 2004; 25:228–238. [PubMed: 15308923]
16. Roebuck TM, Mattson SN, Riley EP. A review of the neuroanatomical findings in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcohol Clin Exp Res*. 1998; 22:339–344. [PubMed: 9581638]
17. Carter RC, et al. Fetal Alcohol Growth Restriction and Cognitive Impairment. *Pediatrics*. 2016; 138
18. Wilson SE, Cudd TA. Focus on: the use of animal models for the study of fetal alcohol spectrum disorders. *Alcohol Res Health*. 2011; 34:92–98. [PubMed: 23580046]
19. Patten AR, Fontaine CJ, Christie BR. A comparison of the different animal models of fetal alcohol spectrum disorders and their use in studying complex behaviors. *Front Pediatr*. 2014; 2:93. [PubMed: 25232537]
20. Parnell SE, et al. Chronic ethanol increases fetal cerebral blood flow specific to the ethanol-sensitive cerebellum under normoxaemic, hypercapnic and acidaemic conditions: ovine model. *Exp Physiol*. 2007; 92:933–943. [PubMed: 17526556]
21. Bake S, Tingling JD, Miranda RC. Ethanol exposure during pregnancy persistently attenuates cranially directed blood flow in the developing fetus: evidence from ultrasound imaging in a murine second trimester equivalent model. *Alcohol Clin Exp Res*. 2012; 36:748–758. [PubMed: 22141380]
22. Sari Y, Zhang M, Mechref Y. Differential expression of proteins in fetal brains of alcohol-treated prenatally C57BL/6 mice: a proteomic investigation. *Electrophoresis*. 2010; 31:483–496. [PubMed: 20119957]
23. Datta S, et al. Fetal alcohol syndrome (FAS) in C57BL/6 mice detected through proteomics screening of the amniotic fluid. *Birth Defects Res A Clin Mol Teratol*. 2008; 82:177–186. [PubMed: 18240165]
24. Canales L, Gambrell C, Chen J, Neal RE. Prenatal alcohol exposure alters the cerebral cortex proteome in weanling rats. *Reprod Toxicol*. 2013; 39:69–75. [PubMed: 23702218]

25. Ramadoss J, Magness RR. Alcohol-induced alterations in maternal uterine endothelial proteome: a quantitative iTRAQ mass spectrometric approach. *Reprod Toxicol*. 2012; 34:538–544. [PubMed: 22960358]
26. Ramadoss J, Magness RR. Vascular effects of maternal alcohol consumption. *Am J Physiol Heart Circ Physiol*. 2012; 303:H414–421. [PubMed: 22730388]
27. Ramadoss J, Magness RR. 2-D DIGE uterine endothelial proteomic profile for maternal chronic binge-like alcohol exposure. *J Proteomics*. 2011; 74:2986–2994. [PubMed: 21839868]
28. Ramadoss J, Liao WX, Chen DB, Magness RR. High-throughput caveolar proteomic signature profile for maternal binge alcohol consumption. *Alcohol*. 2010; 44:691–697. [PubMed: 20053519]
29. Thomas JD, Idrus NM, Monk BR, Dominguez HD. Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. *Birth Defects Res A Clin Mol Teratol*. 2010; 88:827–837. [PubMed: 20706995]
30. Cui ZJ, et al. Prenatal alcohol exposure induces long-term changes in dendritic spines and synapses in the mouse visual cortex. *Alcohol Alcohol*. 2010; 45:312–319. [PubMed: 20543181]
31. Subramanian K, et al. Chronic binge alcohol exposure during pregnancy impairs rat maternal uterine vascular function. *Alcohol Clin Exp Res*. 2014; 38:1832–1838. [PubMed: 24962648]
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*. 1976; 72:248–254. [PubMed: 942051]
33. Berger ST, et al. MStern Blotting-High Throughput Polyvinylidene Fluoride (PVDF) Membrane-Based Proteomic Sample Preparation for 96-Well Plates. *Mol Cell Proteomics*. 2015; 14:2814–2823. [PubMed: 26223766]
34. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009; 6:359–362. [PubMed: 19377485]
35. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008; 26:1367–1372. [PubMed: 19029910]
36. Cox J, et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res*. 2011; 10:1794–1805. [PubMed: 21254760]
37. Cox J, et al. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics*. 2014; 13:2513–2526. [PubMed: 24942700]
38. Pellinen T, et al. Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. *J Cell Biol*. 2006; 173:767–780. [PubMed: 16754960]
39. Rodriguez CI, Davies S, Calhoun V, Savage DD, Hamilton DA. Moderate Prenatal Alcohol Exposure Alters Functional Connectivity in the Adult Rat Brain. *Alcoholism: Clinical and Experimental Research*. 2016; 40:2134–2146.
40. Rice JP, Wallace DG, Hamilton DA. Lesions of the hippocampus or dorsolateral striatum disrupt distinct aspects of spatial navigation strategies based on proximal and distal information in a cued variant of the Morris water task. *Behavioural brain research*. 2015; 289:105–117. [PubMed: 25907746]
41. Raghunathan R, et al. Evaluating the Effects of Maternal Alcohol Consumption on Murine Fetal Brain Vasculature Using Optical Coherence Tomography. *Journal of biophotonics*. 2018
42. Savage DD, Becher M, Torre AJ, Sutherland RJ. Dose-dependent effects of prenatal ethanol exposure on synaptic plasticity and learning in mature offspring. *Alcoholism: Clinical and Experimental Research*. 2002; 26:1752–1758.
43. Fish EW, et al. Acute alcohol exposure during neurulation: Behavioral and brain structural consequences in adolescent C57BL/6J mice. *Behav Brain Res*. 2016; 311:70–80. [PubMed: 27185739]
44. Padmanabhan R, Ibrahim A, Bener A. Effect of maternal methionine pre-treatment on alcohol-induced exencephaly and axial skeletal dysmorphogenesis in mouse fetuses. *Drug Alcohol Depend*. 2002; 65:263–281. [PubMed: 11841898]

45. Cuzon VC, Yeh PW, Yanagawa Y, Obata K, Yeh HH. Ethanol consumption during early pregnancy alters the disposition of tangentially migrating GABAergic interneurons in the fetal cortex. *J Neurosci.* 2008; 28:1854–1864. [PubMed: 18287502]
46. Lunde-Young R, et al. Regional Dysregulation of Taurine and Related Amino Acids in the Fetal Rat Brain Following Gestational Alcohol Exposure. *Alcohol.* 2017
47. Sutherland RJ, McDonald RJ, Savage DD. Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. *Hippocampus.* 1997; 7:232–238. [PubMed: 9136052]
48. Zimmerberg B, Sukel HL, Stekler JD. Spatial learning of adult rats with fetal alcohol exposure: deficits are sex-dependent. *Behav Brain Res.* 1991; 42:49–56. [PubMed: 2029344]
49. Blanchard BA, Riley EP, Hannigan JH. Deficits on a spatial navigation task following prenatal exposure to ethanol. *Neurotoxicol Teratol.* 1987; 9:253–258. [PubMed: 3627089]
50. Reyes E, Wolfe J, Savage DD. The effects of prenatal alcohol exposure on radial arm maze performance in adult rats. *Physiol Behav.* 1989; 46:45–48. [PubMed: 2813555]
51. Coleman LG Jr, Oguz I, Lee J, Styner M, Crews FT. Postnatal day 7 ethanol treatment causes persistent reductions in adult mouse brain volume and cortical neurons with sex specific effects on neurogenesis. *Alcohol.* 2012; 46:603–612. [PubMed: 22572057]
52. Maier SE, Miller JA, West JR. Prenatal binge-like alcohol exposure in the rat results in region-specific deficits in brain growth. *Neurotoxicol Teratol.* 1999; 21:285–291. [PubMed: 10386832]
53. Klintsova AY, et al. Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. *Alcohol Clin Exp Res.* 2007; 31:2073–2082. [PubMed: 17949464]
54. Bonthius DJ, West JR. Alcohol-induced neuronal loss in developing rats: increased brain damage with binge exposure. *Alcohol Clin Exp Res.* 1990; 14:107–118. [PubMed: 1689970]
55. Livy DJ, Miller EK, Maier SE, West JR. Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. *Neurotoxicol Teratol.* 2003; 25:447–458. [PubMed: 12798962]
56. Ikonomidou C, et al. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science.* 2000; 287:1056–1060. [PubMed: 10669420]
57. Sadrian B, Wilson DA, Saito M. Long-lasting neural circuit dysfunction following developmental ethanol exposure. *Brain Sci.* 2013; 3:704–727. [PubMed: 24027632]
58. Savage DD, et al. Effects of a novel cognition-enhancing agent on fetal ethanol-induced learning deficits. *Alcohol Clin Exp Res.* 2010; 34:1793–1802. [PubMed: 20626729]
59. Wozniak DF, et al. 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. [PubMed: 15571976]
60. Wilson DA, Peterson J, Basavaraj BS, Saito M. Local and regional network function in behaviorally relevant cortical circuits of adult mice following postnatal alcohol exposure. *Alcohol Clin Exp Res.* 2011; 35:1974–1984. [PubMed: 21649667]
61. Kourtis N, Nikolettou V, Tavernarakis N. Small heat-shock proteins protect from heat-stroke-associated neurodegeneration. *Nature.* 2012; 490:213–218. [PubMed: 22972192]
62. Miura K, Titani K, Kurosawa Y, Kanai Y. Molecular cloning of nucleobindin, a novel DNA-binding protein that contains both a signal peptide and a leucine zipper structure. *Biochem Biophys Res Commun.* 1992; 187:375–380. [PubMed: 1520323]
63. Kubota T, et al. Activation of autoreactive T cells that help nucleobindin-injected mice produce anti-DNA antibodies. *Immunol Lett.* 2001; 75:111–115. [PubMed: 11137134]
64. Ackermann M, Matus A. Activity-induced targeting of profilin and stabilization of dendritic spine morphology. *Nat Neurosci.* 2003; 6:1194–1200. [PubMed: 14555951]
65. Brunette KE, Tran PV, Wobken JD, Carlson ES, Georgieff MK. Gestational and neonatal iron deficiency alters apical dendrite structure of CA1 pyramidal neurons in adult rat hippocampus. *Dev Neurosci.* 2010; 32:238–248. [PubMed: 20689287]
66. Maier SE, Miller JA, Blackwell JM, West JR. Fetal Alcohol Exposure and Temporal Vulnerability: Regional Differences in Cell Loss as a Function of the Timing of Binge-Like Alcohol Exposure During Brain Development. *Alcoholism: Clinical and Experimental Research.* 1999; 23:726–734.

67. Marcussen BL, Goodlett CR, Mahoney JC, West JR. Developing rat Purkinje cells are more vulnerable to alcohol-induced depletion during differentiation than during neurogenesis. *Alcohol*. 1994; 11:147–156. [PubMed: 8204201]
68. West JR, Chen WJA, Pantazis NJ. Fetal alcohol syndrome: the vulnerability of the developing brain and possible mechanisms of damage. *Metabolic brain disease*. 1994; 9:291–322. [PubMed: 7898398]
69. Livy D, Miller EK, Maier SE, West JR. Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. *Neurotoxicology and teratology*. 2003; 25:447–458. [PubMed: 12798962]
70. Luo J. Autophagy and ethanol neurotoxicity. *Autophagy*. 2014; 10:2099–2108. [PubMed: 25484085]
71. Takahashi Y, Meyerkord CL, Wang HG. Bif-1/endophilin B1: a candidate for crescent driving force in autophagy. *Cell Death Differ*. 2009; 16:947–955. [PubMed: 19265852]
72. Matsunaga T, et al. Regulation of annexin II by cytokine-initiated signaling pathways and E2A-HLF oncoprotein. *Blood*. 2004; 103:3185–3191. [PubMed: 15070701]
73. Weyd H. More than just innate affairs – on the role of annexins in adaptive immunity. *Biol Chem*. 2016; 397:1017–1029. [PubMed: 27467753]
74. Gerke V, Creutz CE, Moss SE. Annexins: linking Ca²⁺ signalling to membrane dynamics. *Nat Rev Mol Cell Biol*. 2005; 6:449–461. [PubMed: 15928709]
75. Kling T, et al. Integrative Modeling Reveals Annexin A2-mediated Epigenetic Control of Mesenchymal Glioblastoma. *EBioMedicine*. 2016; 12:72–85. [PubMed: 27667176]
76. Uchida S, Shumyatsky GP. Deceivingly dynamic: Learning-dependent changes in stathmin and microtubules. *Neurobiol Learn Mem*. 2015; 124:52–61. [PubMed: 26211874]
77. Bonito-Oliva A, Barbash S, Sakmar TP, Graham WV. Nucleobindin 1 binds to multiple types of pre-fibrillar amyloid and inhibits fibrillization. *Sci Rep*. 2017; 7:42880. [PubMed: 28220836]

Highlights

1. Alcohol has specific and substantial effects on vulnerable fetal brain regions
2. Fetal cortex - dysregulated proteins in autophagy
3. Hippocampus - alterations in regulators of cell growth, development morphogenesis
4. Cerebellum - alterations in regulators of axonal growth

Figure 1. Proteomic changes in the fetal hippocampus following prenatal alcohol exposure
(A) Volcano plot of protein abundance and P -values ($n = 16$ per group) following gestational alcohol exposure, shows the distribution of proteins (T-test P value ($\log_2$)) versus the abundance difference ($\log_2$ (EtOH/Pair-fed control)). Colored dots represent significantly ($P < 0.05$) upregulated (red) or downregulated (blue) proteins, black lines represent the significance thresholds post multiple comparison correction (Permutation testing; $P < 0.05$); (B) Functional enrichment analysis of significantly upregulated proteins; (C) INGENUITY pathways analysis (IPA) of canonical pathways altered following gestational alcohol exposure; and (D) IPA of toxicity functions altered following gestational alcohol exposure.

Figure 2. Proteomic changes in the cortex following prenatal alcohol exposure
 (A) Volcano plot of cortical proteins abundance and P -values ($n = 16$ per group) following gestational alcohol exposure. (B) INGENUITY pathway analysis (IPA) of canonical pathways of significantly altered proteins. (C) IPA of toxicity of significantly altered proteins.

B INGENUITY Pathway Analysis – Canonical Pathways

Figure 3. Proteomic changes in the cerebellum following prenatal alcohol exposure
 (A) Volcano plot of cerebellar proteins abundance and P -values ($n = 16$ per group) following gestational alcohol exposure; and (B) INGENUITY pathway analysis (IPA) of canonical pathways altered following gestational alcohol exposure.

Figure 4. Venn diagram comparing the number of proteins which are significantly dysregulated in abundance following alcohol-exposed compared to pair-fed control rats across the three brain proteomic analyses
The protein “ras-related protein Rab-21” was found to be dysregulated in all three fetal brain regions after gestational alcohol exposure. Overlapping regions indicate the number of shared significantly altered proteins (EtOH/Pair-fed control) between the respective brain regions.