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Regional Dysregulation of Taurine and Related Amino Acids in the Fetal Rat Brain Following Gestational Alcohol Exposure

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Abstract

The fetal brain exhibits exquisite alcohol-induced regional neuronal vulnerability. A candidate mechanism for alcohol-mediated brain deficits is disruption of amino acid (AA) bioavailability. AAs are vitally important for proper neurodevelopment, as they comprise the most abundant neurotransmitters in the brain and act as neurotransmitter precursors, nitric oxide donors, antioxidants, and neurotrophic factors, which induce synaptogenesis, neuronal proliferation, and migration. We hypothesized that gestational alcohol alters brain AA concentrations, disrupts AAs associated with neuropathogenesis, and that alterations are region-specific. We assigned pregnant Sprague-Dawley rats to either a pair-fed control or a binge alcohol treatment group on gestational day (GD) 4. Alcohol animals acclimatized via a once daily orogastric gavage of a 4.5 g/kg alcohol dose from GD 5-10, and progressed to a 6 g/kg alcohol dose from GD 11-20. Pair-fed animals received isocaloric maltose dextrin (once daily; GD 5-20). Fetal cerebral cortex, cerebellum, and hippocampus were collected on GD 21. Following collection, Fluorometric High Performance Liquid Chromatography (HPLC) involving pre-column derivatization with *o*-phthaldialdehyde quantified regional content of 22 AAs. Chronic binge alcohol administration to pregnant dams regionally altered AA concentrations in all three structures, with the cerebral cortex exhibiting least vulnerability and the hippocampus exhibiting maximal vulnerability. We conjecture that the AA imbalances observed in this study are critically implicated in pathological and compensatory processes occurring in the brain in response to gestational alcohol exposure.

Keywords

Alcohol; Teratology; Gestation

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Introduction

Alcohol is an established teratogen responsible for a range of physical, physiological, neuroanatomical, and behavioral deficits collectively termed fetal alcohol spectrum disorders (FASD; Riley & McGee, 2005; Sokol, Delaney-Black, & Nordstrom, 2003). In the United States, it is currently estimated that 1 in 10 pregnant women consume alcohol, and 1 in 33 pregnant women report binge drinking in the past 30 days (Tan, Denny, Cheal, Sniezek, & Kanny, 2015). Alcohol consumption during pregnancy affects virtually every developing fetal organ system, of which the most-studied target is the developing brain. The deficits reported include whole and regional brain volume reductions, cortical dysmorphology, neuronal depletion, disruption of neuronal differentiation and migration, and neurobehavioral deficits including learning, memory, and attention impairments (Gautam et al., 2015; Lebel, Roussotte, & Sowell, 2011). Since 1968, an extensive body of work has catalogued the impact of alcohol on the developing brain (Berman & Hannigan, 2000; Burd, 2004; Lebel et al., 2011; Lemione, Harasseau, Borteryu, & Menuet, 1968). However, delineation of candidate mechanisms underlying alcohol-induced neurological deficits remain indeterminate, attributed in part to the complexity of alcohol's cellular targets and pharmacokinetics, as well as varying temporal and regional vulnerability observed in the brain throughout gestation (G. F. Hamilton, Whitcher, & Klintsova, 2010; Livy, Miller, Maier, & West, 2003; Ramadoss, Lunde, Chen, West, & Cudd, 2007).

Gestational alcohol exposure significantly impairs AA bioavailability in both the mother and the developing fetus. In rodent models, maternal plasma threonine, serine, glutamine, glycine, alanine, and methionine are reduced following an acute alcohol exposure (Padmanabhan, Ibrahim, & Bener, 2002), and plasma proline diminishes following chronic exposure (Marquis, Leichter, & Lee, 1984). Our group, demonstrated maternal and fetal plasma AA dysregulation including glutamine and glutamate in response to alcohol (Ramadoss, Wu, & Cudd, 2008; Washburn, Sawant, Lunde, Wu, & Cudd, 2013). In addition, impairment of maternal uterine artery blood flow and placental uptake of AAs are observed following maternal alcohol consumption, and these factors all critically regulate fetal AA bioavailability (Chung, Teng, Timmerman, Meschia, & Battaglia, 1998). Maternal alcohol consumption and AA disruption are directly associated with intrauterine growth restriction (IUGR), which may potentiate the risk for a myriad of adult-onset diseases (Lunde et al., 2016; Ramsay, 2010; Guoyao Wu, Bazer, Cudd, Meininger, & Spencer, 2004). Despite this evidence regarding the effects of alcohol on AA homeostasis, to the best of our knowledge, no information is available on the AA profile in the alcohol-sensitive developing brain, regionally or holistically in FASD.

In addition to their basic role as protein building blocks, AAs also function as precursors for neurotransmitters, nucleotides, sphingolipids, polyamines, as donors for nitric oxide, (Kwon, Spencer, Bazer, & Wu, 2003) and as potent antioxidants, regulators of hormone secretion, signaling modulators (Kwon et al., 2003). AAs are the most abundant neurotransmitters in the brain, and act as neurotrophic factors, playing major roles in synaptogenesis, neuronal proliferation, and migration (Herlenius & Lagercrantz, 2004). Outside the FASD field, AA analysis in developing brain regions has led to significant advances in understanding the mechanistic pathways leading to neuropathology.

The purpose of this study was to explore the effect of alcohol on region-specific patterns of AA abundance. We chose to explore the cerebral cortex, the hippocampus, and the cerebellum, as these regions have previously shown high alcohol-induced vulnerability, manifesting as a myriad of developmental deficits (Berman & Hannigan, 2000; D. A. Hamilton et al., 2014; Livy et al., 2003). We hypothesized that gestational alcohol exposure will alter brain AA concentrations, causing disruption to key AAs associated with neuropathogenesis, and that these alterations will be region-specific.

Materials and Methods

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 (Wilmington, MA), and were 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 = 6) or an alcohol treatment group (n = 6) on GD 4. The alcohol animals acclimatized via a once daily orogastric gavage of a 4.5 g/kg (22.5% wt/v) alcohol dose from GD 5-10, and progressed to a 6 g/kg alcohol dose from GD 11-20 (28.5% wt/v). The pair-fed control rats were isocalorically matched to alcohol rats by daily dosing with a gavage of maltose dextrin to account for calories from alcohol. The regimen of exposure utilized in this study is based on both reported binge alcohol consumption patterns in pregnant women and binge exposure patterns implemented across FASD animal models (Caetano, Ramisetty-Mikler, Floyd, & McGrath, 2006; Church & Gerkin, 1988; Cudd, Chen, & West, 2002; May et al., 2013; Ryan, Williams, & Thomas, 2008; Thomas, Idrus, Monk, & Dominguez, 2010). All rats were weighed prior to the start of the study, and each treatment animal was yoked with a control animal of similar weight throughout the duration of the study. Feed intake in both groups was measured daily and the amount of diet consumed by the pair-fed animals was matched with the alcohol-fed animals. There was no significant maternal weight difference between the groups on GD 21. Animals were sacrificed on GD 21, one day after the last alcohol exposure. Litter size between treatment groups was not different (P = 0.77).

Fetal brain region isolation

Fetal brain tissue was collected from an equal number of male and female offspring within each treatment group. A single fetal brain per dam was utilized for sample analysis. Samples were serially washed in cold phosphate buffered saline (PBS), meninges were removed, and the bilateral hippocampi, cerebellum, and whole cerebral cortex were micro-dissected in ice-cold HEPES buffer. Individual samples were then flash frozen and stored at −80°C.

Sample Preparation

Fetal brain tissue was weighed, subsequently acidified with 50 μl of 1.5 mM $HClO_4$, homogenized in 925 μl H_2O, and then neutralized 25 μl of 2 mM K_2CO_3 (Go et al., 2012). The supernatant fluid was used for AA analysis by HPLC, as described previously (G. Wu, Davis, Flynn, Knabe, & Davidson, 1997). Concentrations of AAs in samples were quantified

on the basis of authentic standards from Sigma Chemicals (St. Louis, MO, USA) using the Waters Millenium-32 workstation (Dai et al., 2012a, 2012b).

Statistical Analysis

The concentrations of AAs in the cortex, cerebellum, and hippocampus of control and alcohol animals were analyzed by Student's t-test. Statistical significance was established *a priori* at $P < 0.05$.

Results

There was no significant difference in the maternal weight between the pair-fed control and alcohol-fed dams on GD 20 (Pair-fed control, 309 ± 8 g; Alcohol, 308 ± 15 g). Fetal weight was significantly decreased in the alcohol group (2.12 ± 0.11 g), compared with that in the pair-fed control (2.53 ± 0.06 g).

Concentrations of 22 AAs within each of the three brain regions of interest (cerebral cortex, hippocampus, and cerebellum) for both control and alcohol animals are summarized in Table 1 (supplementary information). The most notable changes in AA concentrations were observed in the fetal cerebellum and hippocampus, two structures established as exquisitely sensitive to prenatal alcohol exposure (Livy et al., 2003).

Fetal cerebral cortical AA dysregulation

In the cerebral cortex (Figure 1), taurine was the most abundant AA, followed by glutamate, glutamine, alanine, and serine. Of the 22 AAs analyzed, concentrations of 8 AAs were significantly different ($P < 0.05$) between the pair-fed control and alcohol groups. Cortical isoleucine ($P = 0.0093$), serine ($P = 0.0416$), valine ($P = 0.0205$), leucine ($P = 0.0170$), and ornithine ($P = 0.0174$) concentrations significantly *increased* in the cerebral cortex of gestational alcohol exposed offspring compared with those in pair-fed controls. In contrast, glutamine ($P = 0.0057$), taurine ($P = 0.0113$), and tryptophan ($P = 0.0078$) were significantly *decreased* in the alcohol group compared to pair-fed controls. Of the three structures analyzed, the cerebral cortex had the fewest number of significantly different AA concentrations.

Fetal cerebellar AA dysregulation

Within the cerebellum (Figure 2), taurine was the most abundant AA, followed by glutamate, glutamine, and alanine in both treatment groups. Interestingly, the fifth most abundant AA was glycine in the control group and serine in the alcohol group. Of the 22 AAs analyzed, 20 (~91%) AA concentrations were significantly different ($P < .05$) between the pair-fed control and alcohol groups. All 20 of these AA concentrations were *increased* in alcohol-exposed cerebella compared with those from pair-fed control animals. These AAs were alanine ($P = 0.000069$), arginine ($P = 0.0000096$), asparagine ($P = 0.0000099$), aspartate ($P = 0.00026$), glutamine ($P = 0.0026$), glutamate ($P = 0.0013$), glycine ($P = 0.000027$), histidine ($P = 0.000042$), isoleucine ($P = 0.0000061$), leucine ($P = 0.0000061$), lysine ($P = 0.000017$), methionine ($P = 0.000018$), ornithine ($P = 0.0000033$), phenylalanine ($P = 0.0000068$), serine ($P = 0.0000059$), taurine ($P = 0.034$), threonine ($P = 0.0000046$),

tryptophan (P = 0.00071), tyrosine (P = 0.0000023), and valine (P = 0.0000056). The cerebellum showed more alteration than the cerebral cortex, but less than that in the hippocampus.

Fetal hippocampal AA dysregulation

Taurine was the most abundant AA in the hippocampus (Figure 3), followed by glutamate, glutamine, alanine, and serine in both treatment groups. Representative chromatogram traces are shown from (A) standards, (B) pair-fed control, and (C) alcohol treatment groups (Figure 4). All 22 AAs analyzed were significantly different between treatment groups (P < .01). Every hippocampal AA concentration *increased* in the alcohol group compared with those in the pair-fed control group. Of the three structures analyzed, the hippocampus had the highest percentage of significantly different AA concentrations between treatment groups. These AAs were alanine (P = 0.00011), arginine (P = 0.0000043), asparagine (P = 0.000013), aspartate (P = 0.000033), β-alanine (P = 0.0015), citrulline (P = 0.0012), glutamine (P = 0.00052), glutamate (P = 0.00035), histidine (P = 0.000011), isoleucine (P = 0.0000039), leucine (P = 0.0000047), lysine (P = 0.000010), methionine (P = 0.000013), ornithine (P = 0.0012), phenylalanine (P = 0.0000091), serine (P = 0.0000074), taurine (P = 0.0038), threonine (P = 0.000022), tryptophan (P = 0.0052), tyrosine (P = 0.000019), and valine (P = 0.0000071).

Discussion

To our knowledge, this is the first study distinguishing dynamic, region-specific, alterations of AA concentrations in the developing fetal brain in response to gestational alcohol exposure. The cerebral cortex showed the fewest altered AA concentrations, in contrast to the cerebellum and hippocampus, which exhibited dramatic AA dysregulation. Additionally, our data demonstrate that prenatal alcohol exposure regionally increases excitatory amino acids in the fetal brain, and also increases taurine levels in these same regions. These AA imbalances may provide insight into both alcohol-mediated neuropathogenesis and the brain's compensatory neuroprotective response to prenatal alcohol exposure.

Alcohol significantly increases hippocampal and cerebellar excitatory AAs

In alcohol-exposed offspring, considerable increases in *glutamate* were detected in the cerebellum (177.40%) and hippocampus (149.00%). *Aspartate* also increased in the cerebellum (218.54%) and hippocampus (186.73%) in the alcohol group compared with pair-fed controls. Excitatory AAs, which include glutamate and aspartate, act on voltage gated channels in plasma membranes throughout cell populations in the brain (Hsu, Chou, Chang, Chou, & Wong, 2001). Studies show increases of these AAs in the developing brain in response to traumatic brain injury (Ruppel et al., 2001), hypoxia-ischemia (Silverstein, Naik, & Simpson, 1991), and maternal stress (Peters, 1990). Excitatory AA disruption has also been observed previously in offspring brain regions following intrauterine inflammation (Lesniak et al., 2013). In the developing brain, acute increases in concentrations of excitatory neurotransmitters lead to acute neurotoxicity, as well as impairment in neurotransmitter programming, receptor expression, neuronal migration, synapse maturation, and have been linked with numerous long-term behavioral deficits and

psychiatric illnesses (Herlenius & Lagercrantz, 2004). Our data indicate that alcohol induces significant regional increases in glutamate and aspartate, and increases of these AAs may be responsible for neuronal death observed in the hippocampus and cerebellum following alcohol exposure, two regions with well-documented acute alcohol vulnerability. Interestingly, branched chain AAs, *valine, leucine,* and *isoleucine* exhibited significant increases in the cerebellum and hippocampus respectively following alcohol exposure. Together, these branched chain AAs function as primary nitrogen donors in glutamate synthesis, and thus may be integral in the marked increase we observed in the excitatory AA glutamate (Daikhin & Yudkoff, 2000).

Alcohol regionally alters taurine levels, the most abundant AA in the developing brain

Our data illustrates that gestational alcohol regionally alters *taurine* distribution in the cerebral cortex (-16.31%, cerebellum (+88.85%, and hippocampus (+126.71%. Alcohol also increased *methionine*, a direct taurine precursor, in both of these structures (cerebellum: 550.88%; hippocampus: 230.95%. Taurine is the most abundant AA in the developing brain, exhibiting region-specific abundance and significantly higher levels in the developing versus mature brain (Pasantes-Morales & Hernandez-Benitez, 2010). By region, taurine concentration is highest in the olfactory bulbs (an area of sustained neurogenesis), followed by the cerebellum and then cerebral cortex. By cell type, taurine concentration is highest in the cerebellar Purkinje cells, an established target exquisitely vulnerable to prenatal alcohol exposure. Taurine is the highest free AA in milk, further suggesting a role in neuronal maturation. Across species, taurine is critically implicated in brain development and in maintaining neuronal homeostasis by acting as a model osmoregulator and intracellular calcium modulator (El Idrissi & Trenkner, 2004; Pasantes-Morales & Hernandez-Benitez, 2010). Taurine deficiency during development correlates with impaired neuronal migration, proliferation, and organization, yet the mechanisms of taurine requirements for nervous system maturation are not yet fully understood (Pasantes-Morales & Hernandez-Benitez, 2010). Taurine disruption in the developing brain correlates with impaired sensory integration and cortical processing, yet not with somatic growth impairments (Sturman, 1993; Uauy, Mena, & Peirano, 2001). This phenotype is a hallmark for many children affected with FASDs, and our working hypothesis is that taurine is a key mechanistic component involved in neuropathology underlying behavioral deficits of FASD.

Our working model

Interestingly, *taurine* and *glutamate* are linked in a unique cell-signaling pathway within the brain (Figure 5). Previous studies show that excessive glutamate release disrupts intracellular calcium homeostasis, increasing intracellular osmolality (El Idrissi & Trenkner, 1999; El Idrissi & Trenkner, 2004). In response, taurine levels increase to regulate these increases back within homeostatic levels, actively preventing neuronal death induced via excitatory neurotoxicity (Banerjee, Vitvitsky, & Garg, 2008; Nagelhus, Lehmann, & Ottersen, 1993). Based on our findings, we hypothesize that in select developing brain regions, gestational alcohol induces excessive glutamate release, potentially leading to intracellular hypertonicity; in response, taurine increases downstream in an effort to normalize glutamate-induced excitatory neurotoxicity. We further propose taurine may be acting as a compelling neuroprotectant in response to this exposure. We conjecture that AA imbalances observed in

this study are critically implicated in pathological and compensatory processes occurring in the brain in response to gestational alcohol exposure.

Study Limitations

It should be noted that in our chronic binge alcohol paradigm, withdrawal might play an influential role in fetal brain AA homeostasis. Our results may indicate a direct alcohol effect, alcohol withdrawal consequences, or a combined effect of both. Previous studies have ascertained that key brain excitatory amino acids increase in response to alcohol withdrawal (Dahchour, Hoffman, Deitrich, & de Witte, 2000; Dahchour, Quertemont, & De Witte, 1996; Rossetti & Carboni, 1995; Tsai & Coyle, 1998), though this is the first study analyzing an AA profile, including excitatory AAs and their precursors, following a chronic binge gestational exposure. An additional consideration worth noting is that the AA disruption observed in this study may be a reflection of alcohol-induced cellular impairment rather than alcohol teratogenicity. For instance, observed AA alterations may be an accumulative effect, reflective of alcohol-induced impairment of cellular processes, which could in turn affect AA metabolism and utilization, differentiation, and/or migration (Guerri, Bazinet, & Riley, 2009). Future studies of other brain regions and additional developmental time-points are warranted to understand how gestational alcohol exposure affects global AA homeostasis in the developing brain and to discern further mechanistic insights.

Perspectives

In the last half century, vast progress has been made discerning targets of gestational alcohol exposure, yet limited knowledge persists for the mechanisms underlying alcohol-mediated developmental pathology. Understanding foundational building blocks for pathogenesis, such as alcohol-induced disruption of AA homeostasis essential for development, is key for mechanistic discernment. Collectively, our data demonstrate that alcohol-induced dysregulation of AA concentrations in the developing brain are region-specific. Future studies are warranted in identifying mechanisms underlying the observed AA dysregulation and whether these mechanisms are region-specific. Further discernment of these mechanisms would advance the FASD field by providing pivotal insight into alcohol's action on specific regions of the fetal brain, as well as by identifying strategic sites for therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Alcohol exposure regionally dysregulates amino acid homeostasis in the fetal brain
- Alcohol increases excitatory amino acids, which may induce excitatory neurotoxicity
- Regional taurine elevation could indicate a neuroprotective response to alcohol

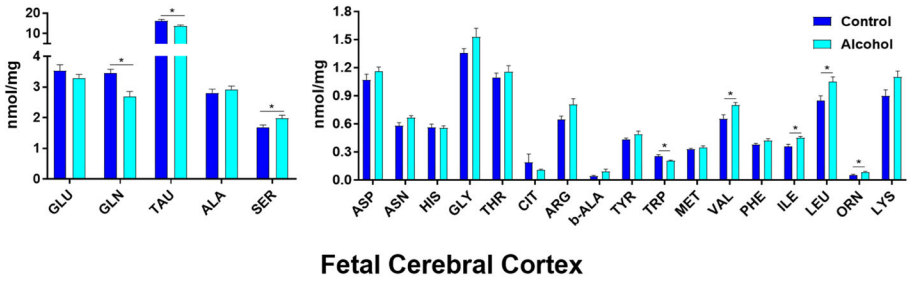

Figure 1. Effect of chronic gestational binge alcohol exposure on fetal cortical amino acid concentrations (nmol/mg)

Left: Most abundant fetal brain amino acids: Levels of glutamine (↓), taurine (↓), and serine (↑) were significantly different within cortical tissue in the alcohol group compared with those in the pair-fed control group. ***Right***: Less abundant fetal brain amino acids: Isoleucine (↑), leucine (↑), valine (↑), ornithine (↑), and tryptophan (↓) were significantly different within cortical tissue in the alcohol group compared with those in the pair-fed control group. * indicates statistically significant difference in amino acid concentration, $P < 0.05$.

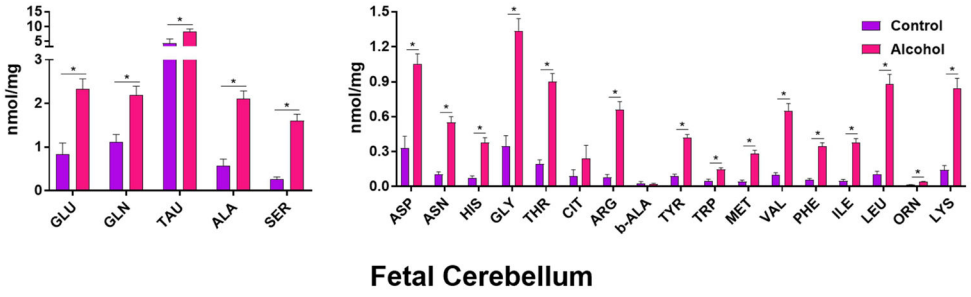

Figure 2. Effect of chronic gestational binge alcohol exposure on fetal cerebellar amino acid concentrations (nmol/mg)

Left: Most abundant fetal brain amino acids: Glutamate, glutamine, taurine, alanine, and serine levels significantly increased (↑) in cerebellar tissue in the alcohol group compared with those in the pair-fed control group. ***Right:*** Less abundant fetal brain amino acids: Asparagine, aspartate, histidine, glycine, threonine, arginine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine, ornithine, and lysine levels significantly increased (↑) in cerebellar tissue in response to gestational alcohol exposure. * indicates statistically significant difference in amino acid concentration, $P < 0.05$.

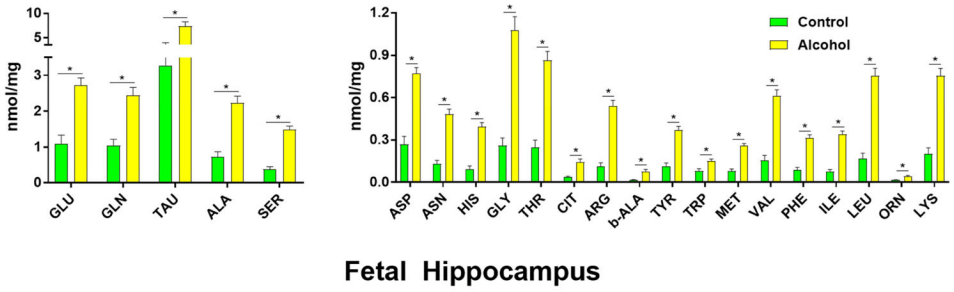

Figure 3. Effect of chronic gestational binge alcohol exposure on fetal hippocampal amino acid concentrations (nmol/mg)

Left: Most abundant fetal brain amino acids: Glutamate, glutamine, taurine, alanine, and serine levels significantly increased (↑) in hippocampal tissue in the alcohol group compared with those in the pair-fed control group. ***Right:*** Less abundant fetal brain amino acids: Asparagine, aspartate, histidine, glycine, threonine, citrulline, arginine, β-alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine, ornithine, and lysine levels significantly increased (↑) in hippocampal tissue in the alcohol group compared with those in the pair-fed control group. * indicates statistically significant difference in amino acid concentration, $P < 0.05$.

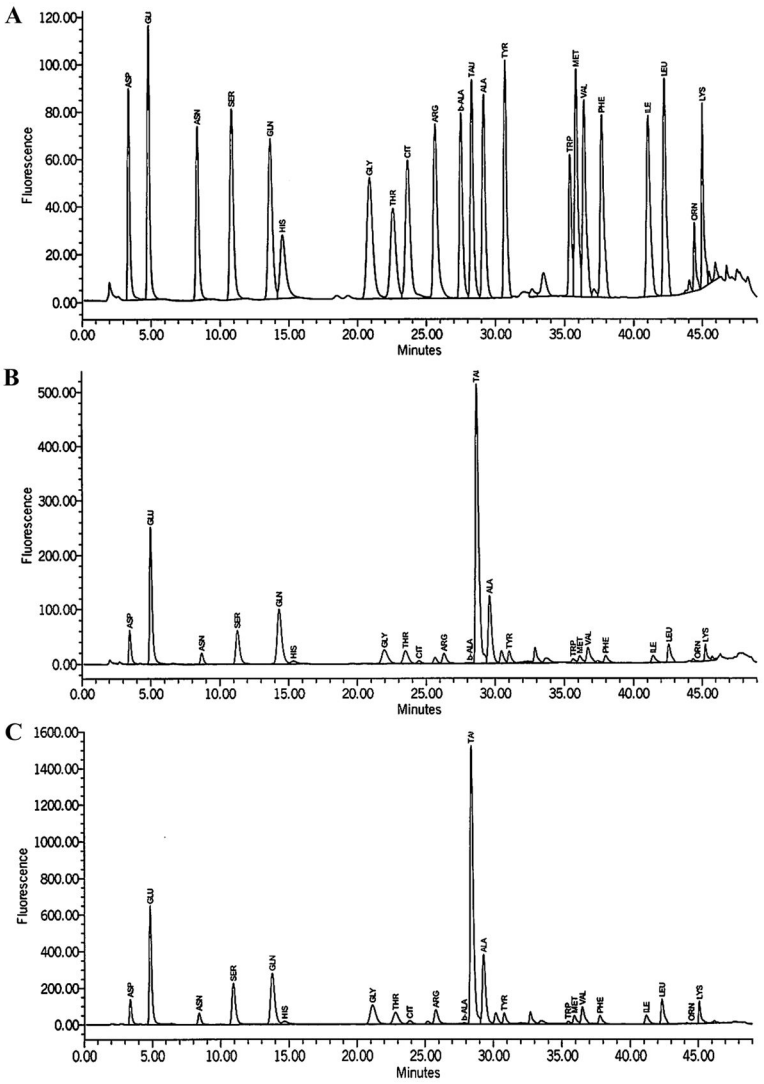

Figure 4. Example chromatogram traces from fetal hippocampal tissue

Representative chromatogram traces are shown from (A) standards, (B) pair-fed control, and (C) alcohol treatment groups.

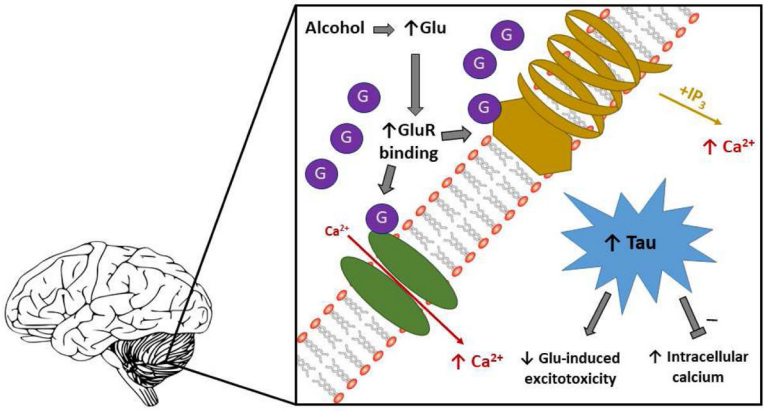

Figure 5. Our working hypothesis

Glutamate and taurine increase in the fetal cerebellum and hippocampus following a chronic, binge, gestational alcohol exposure paradigm. We theorize that this alcohol exposure increases glutamate and subsequent glutamate receptor (NMDA and/or metabotropic) binding in these structures, inducing calcium influx, in turn stimulating excitatory neurotoxicity leading to cell death, and potentially accounting for previously observed neuronal impairment following similar exposure paradigms. We conjecture that taurine, an ideal osmolyte, increases in response to glutamate-induced Calcium influx, counteracting osmotic disruption and offsetting alcohol-mediated damage within these distinctly vulnerable structures.