Sustained Impairments in Brain Insulin/IGF Signaling in Adolescent Rats Subjected to Binge Alcohol Exposures during Development

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Abstract

Background: Chronic or binge ethanol exposures during development can cause fetal alcohol spectrum disorder (FASD) which consists of an array of neurobehavioral deficits, together with structural, molecular, biochemical, and neurotransmitter abnormalities in the brain. Previous studies showed that perinatal neurodevelopmental defects in FASD are associated with inhibition of brain insulin and insulin-like growth factor (IGF) signaling. However, it is not known whether sustained abnormalities in adolescent brain structure and function are mediated by the same phenomena.

Aims: Using an early postnatal (3rd trimester equivalent) binge ethanol exposure model, we assessed neurobehavioral function, structure, and the integrity of insulin/IGF signaling in young adolescent cerebellum. Methods: Long Evans male rats were treated with 50 µl of saline (vehicle) or 2 mg/kg of ethanol by i.p. injection on postnatal days (P) 2, 4, 6, and 8. On P19-20, rats were subjected to rotarod testing of motor function, and on P30, they were sacrificed to harvest cerebella for histological, molecular, and biochemical studies.

Results: Binge ethanol exposures impaired motor function, caused sustained cerebellar hypocellularity, and reduced neuronal and oligodendrocyte gene expression. These effects were associated with significant deficits in insulin and IGF signaling, including impaired receptor binding, reduced Akt, and increased GSK-3β activation.

Conclusions: FASD-associated neurobehavioral, structural, and functional abnormalities in young adolescent brains may be mediated by sustained inhibition of insulin/IGF-1 signaling needed for cell survival, neuronal plasticity, and myelin maintenance.

Keywords: Fetal alcohol syndrome; Adolescence; Brain development; Motor function; Insulin signaling; Central nervous system; Receptor binding; Brain insulin resistance; Cerebellum

Introduction

Alcohol misuse during pregnancy causes significant neurodevelopmental abnormalities including microcephaly, cerebellar hypoplasia, motor deficits, and neuro-cognitive impairments ranging from attention deficit hyperactivity disorder to mental retardation. This pathology, combined with various stereotypical craniofacial defects is termed, 'fetal alcohol spectrum disorders' (FASD) [1,2]. Long-term consequences of ethanol's selective targeting of the mediators of such responses are poorly understood. Since chronic or binge ethanol exposures during development can have significant long-term adverse consequences with respect to neurobehavioral function in adolescents [2,29], yet the mediators of such responses are poorly understood. Since chronic ethanol exposures in adult humans and experimental animals also cause brain insulin/IGF resistance with reduced signaling downstream of insulin receptor tyrosine kinases (RTKs), and their immediate down-stream effector molecules, including insulin receptor substrate (IRS) proteins [23,24]; 2) inhibiting phosphorylation and activation of corresponding receptor tyrosine kinases (RTKs), and their immediate downstream effector molecules, including insulin receptor substrate (IRS) proteins [23,24]; 2) inhibiting phosphorylation and activation of corresponding receptor tyrosine kinases (RTKs), and their immediate downstream effector molecules, including insulin receptor substrate (IRS) proteins [23,24]; 2) inhibiting phosphorylation and activation of corresponding receptor tyrosine kinases (RTKs), and their immediate downstream effector molecules, including insulin receptor substrate (IRS) proteins [23,24];

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through IRS-PI3K-Akt, neuronal loss, impaired mitochondrial and neurotransmitter functions, and increased oxidative stress [31,35], we hypothesized that similar abnormalities might persist in young adolescent brains, even in the absence of subsequent developmental exposures to ethanol. Herein, using a binge ethanol exposure model in which rat pups were exposed to ethanol in the early postnatal period, we assessed the potential role of persistent insulin/IGF resistance as a mediator of impaired cerebellar motor function in the early adolescent period.

Materials and Methods

Materials

Qiagen reagent, EZI RNA universal tissue kit, QuantiTect SYBR Green polymerase chain reaction (PCR) master mix, and the BIO Robot Z1 were from Qiagen Inc (Valencia, CA). Histofix was purchased from Histochoice (Armoreco, Solon, OH). The AMV first strand cDNA synthesis kit was obtained from Roche Diagnostics Corporation (Indianapolis, IN). The Akt Pathway Total and Phospho 7-Plex panels were purchased from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) reagents were from Pierce Chemical Corp. (Rockford, IL). All other fine chemicals were purchased from CalBiochem (Carlsbad, CA), Pierce (Rockford, IL), or Sigma (St. Louis, MO).

Early postnatal binge ethanol exposure model

Long Evans rats were used to generate a human 3rd trimester-equivalent binge ethanol exposure model. At birth, litters were culled to 8 pups per dam. Pups from 12 different litters were administered intra-peritoneal (i.p.) injections (50 µl) of sterile saline or 2 mg/kg to 8 pups per dam. Pups from 12 different litters were administered equivalent binge ethanol exposure model. At birth, litters were culled for astrocytes, allograft inflammatory factor 1 (AIF1) for activated microglia, acetyl cholinesterase (AChE), choline acetyltransferase (ChAT), insulin, IGF-1, and IGF-2 polypeptides and receptors, and insulin receptor substrates (IRS), types 1, 2, and 4 [39-41]. Gene specific primer pairs were designed using MacVector 10 software (MacVector, Inc., Cary, NC) and target specificity was verified using NCBI-BLAST (Basic Local Alignment Search Tool). The amplified signals were detected and analyzed using the Master Cycler ep reaLplex instrument and software (Eppendorf AG, Hamburg, Germany). Relative mRNA abundance was calculated from the ng ratios of specific mRNA to 18S RNA measured in the same samples. Assays were performed in triplicate.

Multiplex ELISA

We used bead-based multiplex ELISAs to examine the integrity of insulin and IGF-1 signaling networks by measuring immunoreactivity to the insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, glycogen synthase kinase 3β (GSK-3β), pYpY1162/1163-IR, pYpY1135/1136-IGF-1R, pS307-IRS-1, pS637-Akt, and pS6-GSK-3β according to the manufacturer’s protocol. Brain tissue samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 2 mM EDTA, 100 mM NaF, 1 mM Na4P2O7, 2 mM Na3VO4) containing protease and phosphatase inhibitors [42]. 200 µg proteins in 100 µl of lysis buffer were incubated with antibody-bound beads. Captured antigens were detected with biotinylated secondary antibody and phycoerythrin-conjugated Streptavidin. Plates were read in a Bio-Plex 200 system (Bio-Rad, Hercules, CA). Data are expressed as fluorescence light units (FLU) corrected for protein concentration.

Receptor binding assays

Insulin, IGF-1, and IGF-2 receptor binding in the brain was measured using competitive saturation assays [41]. Membrane proteins extracted from fresh frozen tissue were incubated in 100 µl reactions containing binding buffer and 0.0031 to 1 µCi/ml of 125I (2000 Ci/mmol) insulin, IGF-1, or IGF-2. Non-specific binding was measured in duplicate reactions containing excess (0.1 µM) cold ligand. Radioactivity was measured in polyethylene glycol 8000 precipitates (bound ligand) and the corresponding supernatants (free ligand) in an LKB CompuGamma CS Gamma counter. Specific binding was calculated by subtracting fmols bound in the presence of excess cold ligand (non-specific), from fmols bound in the absence of cold ligand (total). Best-fit analysis predicted a one-site model, and Scatchard analysis was used to calculate saturation binding (BMAX) and binding affinity (Kd). Binding assay results were analyzed using the Graph Pad Prism 5 software (Graph Pad Software, Inc., San Diego, CA).

Statistics

The experimental model was generated with 12 litters (8 pups each) in which 2 each were treated with vehicle or ethanol. All assays were performed with 12 samples per group. Data corresponding to levels of gene expression or immunoreactivity are depicted in boxplot graphs representing the medians (horizontal bars), 95% confidence intervals (box limits), and range (whiskers) for each group. Inter-group comparisons were made using Student t-test. Statistical analyses were performed using the Graph Pad Prism 5 software (San Diego, CA) and significant P-values (<0.05) are indicated within the graph panels.

Results

Early postnatal binge ethanol exposures lead to impaired motor function in young adolescent rats

Data from rotarod tests of cerebellar function were grouped into trials according to the speed of the rotating bar. For all 3 trial clusters,
the ethanol-exposed group exhibited significantly reduced latencies to fall relative to control (Figure 1). In Trials 1-3, which were the least challenging, the differences between the control and ethanol-exposed group were modest but statistically significant (Figure 1A). For Trials 4-6, performance among controls was similar to that observed for Trials 1-3, whereas for the ethanol-exposed group, mean latency to fall was further reduced (Figure 1B). Finally, for Trials 7-10, which were the most challenging, although performance among controls declined relative to earlier trials, the ethanol-exposed group exhibited its worst performance, and the shortest mean latency to fall (Figure 1C).

Effects of early postnatal binge ethanol exposures on cerebellar structure in young adolescent rats

Histological sections of P30 rat cerebella demonstrated slender folia with deep and complex sulci (grooves), uniform molecular layers and white matter cores, and well-populated Purkinje and granule cell layers in the cortex (Figures 2A, 2C). In contrast, cerebella of ethanol-exposed rats exhibited conspicuous blunting and simplification of the folia with irregular white matter cores and thicknesses of the molecular and granule cell layers, and numerous gaps corresponding to neuronal loss in the Purkinje cell layer (Figures 2B, 2D). In addition, many residual Purkinje cells had eosinophilic cytoplasms with pyknotic nuclei, corresponding to changes associated with early necrosis.

Reduced neuronal and oligodendrocyte gene expression following early postnatal binge ethanol exposures

mRNA transcripts corresponding to Hu (neurons), myelin-associated glycoprotein 1 (MAG-1; oligodendrocytes), glial fibrillary acidic protein (GFAP; astrocytes), allograft inflammatory factor 1 (AIF-1; activated microglia), acetyl cholinesterase (AChE), and choline acetyltransferase (CHAT) were measured by qRT-PCR analysis with results normalized to 18S rRNA (Figure 3). These genes were selected for study to help gauge the long-term impact of developmental exposure to ethanol on cerebellar structure, particularly with regard to survival of neurons and oligodendrocytes. Cholinergic neurotransmitter function was also assessed because acetylcholine is a major neurotransmitter utilized for cerebellar motor functions [43,44]. Corresponding with the ethanol-associated motor impairments, cerebellar atrophy, cell loss in Purkinje and granule cell cortical layers, and irregular structure of the subcortical white matter, Hu, MAG-1, and CHAT mRNA levels were significantly reduced in the ethanol-exposed cerebella (Figures 3A, 3B, 3F). In contrast, GFAP (Figure 3C), AIF-1 (Figure 3D), and AChE (Figure 3E) mRNA levels were similar in control and ethanol-exposed cerebella. These findings suggest that early postnatal binge ethanol exposure-induced impairments in young adolescent motor function were mediated in part by reduced populations of cholinergic neurons rather than increased degradation of acetylcholine.

Early postnatal binge ethanol exposure impairs insulin/IGF signaling in young adolescent cerebella

We used qRT-PCR analysis to measure expression of insulin and IGF trophic factors and receptors, and IRS molecules as previously described [24,28,35]. Due to their inter-relatedness, data corresponding to the trophic factors, receptors, or IRS genes were grouped and analyzed by two-way ANOVA with the Bonferroni post-hoc significance test (Figure 4). Insulin and IGF-1 receptors were similarly expressed in control and ethanol-exposed cerebella.
ELISAs demonstrated that early postnatal binge ethanol exposures significantly reduced cerebellar insulin receptor (Figure 5A), IGF-1 receptor, IRS-1 (pSer312), and Akt (pSer473), and GSK-3β (pSer9), and calculated the relative levels of phosphorylation from the ratios of phospho-/total protein (Figures 5B-F) and pSer312/total IRS-1 (Figure 5I) to total insulin receptor (Figure 5G) or IRS molecules were made by repeated measures two-way ANOVA tests. Significant P-values are shown over the graphs.

We used multiplex ELISAs to further interrogate ethanol’s long-term effects on the integrity of insulin/IGF signaling in the cerebellum. We measured total and phosphorylated levels of insulin receptor (pY1162/pY1163), IGF-1 receptor (pY1135/pY1136), IRS-1 (pS312), Akt (pSer473), and GSK-3β (pSer9), and calculated the relative levels of phosphorylation from the ratios of phospho-/total protein (Figures 5 and 6). Early postnatal binge ethanol exposures significantly increased cerebellar insulin receptor (Figure 5A), IGF-1 receptor, IRS-1 (Figure 5C), and pYpY1135/1136-IGF-1R (Figure 5E), and reduced the relative levels (ratios) of pYpY1112/1116 to total insulin receptor (Figure 5G) and pS312/total IRS-1 (Figure 5I) relative to control. In contrast, no significant inter-group differences were observed with respect to pYpY1112/1116 insulin receptor (Figure 5D) and pS312 IRS-1 (Figure 5F).

Insulin, IGF-1 and IRS-1 signal downstream to activate Akt and inhibit GSK-3β through phosphorylation of specific Ser residues on these proteins. In addition, signaling through Akt and GSK-3β can be regulated by altering the expression levels of these proteins. Multiplex ELISAs demonstrated that early postnatal binge ethanol exposures significantly reduced cerebellar levels of pS473-Akt (Figure 6C), pS473/total Akt (Figure 6E), and pS9-GSK-3β (Figure 6D). In contrast, the mean levels of total Akt (Figure 6A), GSK-3β (Figure 6B), and pS9/total GSK-3β (Figure 6F) were similar in control and ethanol-exposed cerebella.

Competitive saturation binding assays were used to examine the effects of early postnatal binge ethanol exposures on expression of insulin, insulin-like growth factor-1 (IGF-1), IRS-2, and insulin receptor substrate genes in young adolescent cerebella.

Results were normalized to 18S rRNA measured in parallel reactions. Graphs depict relative levels of gene expression Inter-group comparisons with respect to trophic factors, receptors, or IRS molecules were made by repeated measures two-way ANOVA tests with the Bonferroni post hoc significance threshold. Significant P-values are shown over the graphs.

**Figure 4:** Effects of early postnatal binge ethanol exposures on expression of insulin, insulin-like growth factor-1 (IGF-1), IRS-2, and insulin-receptor substrate genes in young adolescent cerebella.

**Discussion**

Effects of early postnatal binge ethanol exposures on adolescent cerebellar function and structure

This study demonstrates that early postnatal binge ethanol exposures cause sustained structural and functional abnormalities in the cerebellum. The Structural damage was characterized by blunting and simplification of cerebellar folia with reduced thickness of the granule cell layer and reduced neuronal densities in the Purkinje cell layer. The motor deficits were manifested by impaired rotarod test performance. Further studies employing qRT-PCR analysis demonstrated significant reductions in Hu, MAG-1, and ChAT.
expression, reflecting reduced populations of neurons, particularly cholinergic, as well as oligodendrocytes. The reduced levels of Hu and ChAT correlate with the conspicuous reductions in granule and Purkinje cell populations, whereas the reductions in MAG-1 expression correspond to hypotrophy or atrophy of central white matter in ethanol-exposed cerebella.

The loss or impaired development of neurons in the granule and Purkinje cell layers, and reductions in MAG-1 expression in ethanol-exposed cerebella were likely due to sustained inhibition of insulin/IGF signaling [17,20,30,31,46]. Insulin and IGF mediate neuronal and oligodendrocyte survival, growth, and metabolism, in addition to neuronal plasticity, myelin maintenance, and cholinergic function [7,8]. Since the binge ethanol exposures were performed within the critical period of robust postnatal cerebellar growth, cerebellar granule cell proliferation, and myelination [47-51] it is conceivable that the toxic effects of ethanol caused a permanent loss or impairment in function of cerebellar neurons and oligodendrocytes. In essence, our findings support the concept that late gestation binge ethanol exposures cause permanent damage to the program of cerebellar development, and thereby produce several of the well-characterized features of FASD [24,28,30].

Early postnatal binge ethanol exposure causes sustained impairments in cerebellar insulin/IGF signaling

The integrity of upstream signaling through the insulin and IGF-1 receptors was assessed by measuring ligand and receptor gene expression by qRT-PCR analysis, and immunoreactivity corresponding to total and tyrosine phosphorylated insulin and IGF-1 receptors with multiplex ELISAs. The qRT-PCR studies demonstrated similar levels of insulin and IGF-1 polypeptide and receptor gene expression in control and ethanol-exposed cerebella. In contrast, insulin and IGF-1 receptor protein levels were significantly higher in the ethanol-exposed cerebella, marking discordances between mRNA and protein study results. Although tyrosine phosphorylated IGF-1 receptor expression was also increased in ethanol-exposed brains, the relative levels of phosphorylated/total IGR-1 receptor were similar to control. On the other hand, tyrosine phosphorylated insulin receptor expression increased markedly in ethanol-exposed brains, whereas insulin receptor expression was similar to control. On the other hand, tyrosine phosphorylated insulin receptor expression increased markedly in ethanol-exposed brains, whereas insulin receptor expression was similar to control.
reduced by early postnatal binge ethanol exposures. The reduced levels of receptor tyrosine phosphorylation and calculated ratio of phospho-total insulin receptor immunoreactivity reflect a state of brain insulin resistance. With regard to the IGF-1 receptor, the increased levels of IGF-1R protein and tyrosine phosphorylated IGF-1R suggest that signaling through IGF-1R pathways was increased, perhaps as a compensatory response to insulin resistance.

To better understand the mechanisms of sustained insulin resistance in ethanol-exposed brains, we used competitive saturation assays to measure ligand-receptor binding. Those studies demonstrated that binge ethanol exposure in the early postnatal period leads to significantly impaired insulin as well as IGF-1 receptor binding in adolescent brains. This suggests that both insulin and IGF-1 resistance contributed to the ethanol-associated impairments in cerebellar structure and function in adolescent brains. These results correspond with previous observations in chronic prenatal ethanol exposure models in which we demonstrated that impaired insulin and IGF receptor binding and signal transduction in the perinatal period were mediated by alterations in membrane lipid composition [7,24,28].

With regard to upstream signaling mechanisms, our additional new findings are as follows: 1) ethanol-impaired insulin and IGF-1 signaling persist well beyond the period of exposure and are detectable in young adolescent brains; 2) rather than reducing expression of both the ligands and receptors as occurs in the early postnatal period following chronic prenatal ethanol exposure, postnatal binge ethanol exposures mediate their inhibitory effects on insulin/IGF-1 signaling by impairing ligand-receptor binding, which would likely have resulted in decreased activation of the corresponding receptor tyrosine kinases (at least with respect to the insulin receptor); and 3) although late gestation-equivalent binge ethanol exposures significantly increased IGF-2R and decreased IGF-2 expression, in contrast to the findings in chronic prenatal ethanol exposure models [7,24,28], there were no significant adverse effects of ethanol on IGF-2 receptor binding. It is conceivable that the sustained deficits in brain insulin/IGF-1 signaling might have been compensated for by alternate use of IGF-2 activated networks. However, the effectiveness of this type of response would likely be limited due to reduced levels of IGF-2 polypeptide gene expression in ethanol-exposed brains.

Effects of early postnatal binge ethanol exposures on IRS expression and signaling in adolescent brains

The stimulatory effects of insulin and IGF-1 are mediated by receptor binding and activation of receptor tyrosine kinases (RTKs) [8,52-58] that phosphorylate IRS proteins. IRSs transmit downstream growth, metabolism, survival, myelin synthesis and myelin homeostasis signals by activating Erk MAPK and PI3K/Akt [16]. Previous studies demonstrated that ethanol impairs insulin and IGF-1 signaling through IRS proteins in various models [23,59], including FASD [24]. The present study shows that early postnatal binge ethanol exposures lead to inhibition of IRS1 and IRS2, but not IRS4 gene expression. Reduced expression of IRS1 and IRS2 compromise the brain’s capacity to transmit signals downstream of the insulin and IGF-1 receptors, and thereby exacerbate states of insulin/IGF-1 resistance, irrespective of the integrity of receptor tyrosine kinase phosphorylation of IRS proteins. However, in contrast to the qRT-PCR analyses, the multiplex ELISA studies demonstrated higher levels of IRS1 protein, and reduced relative levels of $S^{34}$-total-IRS-1 in the ethanol exposed brains. Since $S^{34}$ phosphorylation of IRS1 inhibits downstream signaling, reduced inhibition corresponds to increased activation. This phenomenon could reflect a compensatory means of supporting downstream signaling via-insulin/IGF-1 receptor resistance and thereby account for the fact that cerebellar structure and function are maintained in FASD, albeit at lower levels relative to control.

Effects of early postnatal binge ethanol exposures on Akt pathway signaling in the cerebellum

Ethanol has profound inhibitory effects on insulin/IGF signaling through PI3K/Akt in immature neurons and the developing brains [27,30,34,60]. Ethanol mediates these effects by: 1) inhibiting IRS-associated PI3K activity, and subsequent phosphorylation and activation of Akt and phosphorylation/inhibition of GSK-3β [7,19,23-27]; and 2) increasing the activity of phosphatases that negatively regulate receptor tyrosine kinases, e.g. PTP-1b and PI3K (PTEN) [24-26]. Akt promotes cell survival, cell migration, energy metabolism, and neuronal plasticity, and it inhibits GSK-3β activity. Consequently, ethanol inhibition of PI3K-Akt leads to increased GSK-3β-mediated oxidative stress, DNA damage, mitochondrial dysfunction, apoptosis, and disordered neuronal migration [16,27,60,61]. The reduced relative levels of $S^{34}$ phosphorylation of Akt and $S^{34}$ phosphorylation of GSK-3β in ethanol-exposed young adolescent cerebella indicates that the inhibitory effects of ethanol on signaling downstream of the insulin receptor are sustained beyond the period of exposure and likely mediated by persistent brain insulin resistance as discussed earlier [7].

In conclusion, early postnatal binge ethanol exposures cause long-term deficits in motor function associated with structural abnormalities in the cerebellum, including hypocellularity and hypopofilation. These adverse effects were likely due to sustained inhibition of signaling through insulin/IGF-1 pathways, and downstream through IRS and PI3K/Akt. Future studies will determine the degree to which restoration of insulin/IGF signaling with insulin sensitizer agents abrogates structural and functional abnormalities in the cerebellum.

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