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Research Article

Hotdogs and Beer: Dietary Nitrosamine Exposure Exacerbates Neurodevelopmental Effects of Ethanol in Fetal Alcohol Spectrum Disorder

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Abstract In the immature brain, ethanol impairs insulin, insulin-like growth factor (IGF) and other trophic factor signaling, increases cellular stress, and impairs neuronal and glial functions. However, given the phenotypic heterogeneity of fetal alcohol spectrum disorder (FASD), which is not always ethanol dose-dependent, we hypothesized that cofactors modulate spectrum and severity of disease. Herein, we determined if N-nitrosodiethylamine (NDEA) exposures could mimic or exacerbate ethanol's effects on cerebellar development; NDEA is present in processed foods, inhibits insulin signaling, and promotes oxidative injury. Rat pups were binge ethanol (2 g/kg) or vehicle exposed on postnatal day 3 (P3), P5, P7, and P9; and treated with vehicle or NDEA (2 mg/kg) on P7. Ethanol and NDEA disrupted cerebellar foliation and white matter integrity, inhibited expression of oligodendrocyte and astrocyte proteins, and increased expression of ER stress genes. Combined exposures had qualitatively additive effects. Therefore, cofactor exposures can either mimic or influence the phenotypic features of FASD.

Keywords fetal alcohol spectrum disorder; nitrosamines; diet; myelin; glia; ER stress; ethanol; neuronal migration; development; experimental model

1. Introduction

Fetal alcohol spectrum disorder (FASD) encompasses a wide range of birth defects caused by maternal consumption of alcohol during pregnancy [25]. The most severe abnormalities in FASD include low birth weight, craniofacial defects, and cognitive impairment [25]. The nature and extent of developmental abnormalities in the central nervous system (CNS) vary with timing, duration, and dose of alcohol exposure. For example, first trimester exposures can cause microcephaly, whereas third trimester exposures produce defects in neuronal proliferation, survival, migration, synaptogenesis, and plasticity [16, 17, 18, 29]. The teratogenic effects of alcohol are mediated in part by impairments in trophic factor signaling, such as through insulin and insulin-like growth factor (IGF) pathways [7], and increased oxidative stress [4]. Oxidative injury is due to direct effects of ethanol, and acetaldehyde build-up with attendant poly-molecular damage and adduct formation with lipids, proteins, and nucleic acids which render the cellular machinery nonfunctional [15]. Impairments in insulin/IGF signaling, together with oxidative injury promote mitochondrial dysfunction, inflammation, endoplasmic reticulum (ER) stress, and cell death [1, 4, 33].

Despite extensive experimental data about pathogenic mechanisms of FASD, in humans, the phenotypic heterogeneity in the nature and severity of FASD in relation to alcohol dose and timing of exposure are puzzling [2]; this suggests that other factors may influence neurodevelopmental outcomes of prenatal alcohol exposure. This concept is intriguing because in previous studies, we showed that early exposures to submutagenic doses of dietary nitrosamines (R1N(-R2)-N=O), in particular N-nitrosodiethylamine (NDEA), also cause brain insulin/IGF resistance, oxidative stress, and CNS developmental abnormalities, including cognitive-motor deficits that overlap with the effects of alcohol [7, 35]. Moreover, dual exposures to NDEA and high fat diets lead to visceral obesity, hepatic steatosis, brain insulin resistance, and cognitive-motor deficits that were partly additive [8, 35]. Nitrosamines are derived from reactions between nitrites and secondary amines [5]. Human nitrosamine exposures occur through contaminated water sources, beer, and processed/preserved foods, including meats, fish, and cheeses [5].

The present work tests the hypothesis that NDEA exposures can modulate structural and molecular phenotypic

features of FASD. We considered the concept that dietary nitrosamine exposures during development may modulate or mimic the toxic and metabolic effects of alcohol because (1) like alcohol, NDEA causes cognitive-motor deficits, (2) metabolism of NDEA generates free radicals and impairs the insulin and IGF signaling [1,8], and (3) trends in consumption of low cost, highly processed and preserved foods have increased over time [5].

2. Methods

2.1. Experimental model

Long-Evans (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) female rats ($\sim$ 200–250 g) were maintained on a regular chow diet (Research Diets, Inc., New Brunswick, NJ, USA) throughout pregnancy. Pups from 4 liters were each divided into 4 experimental groups: vehicle (saline)-treated controls, binge ethanol-exposed, NDEA exposed, and binge ethanol+NDEA. Group assignments were indicated by ear tags. Pups were administered saline (control) or 2.0 g/kg of ethanol in saline in 50 μL by intraperitoneal (IP) injection on postnatal day 3 (P3), P5, P7, and P9. In addition, half of the control and ethanol-exposed pups were treated with NDEA (2 mg/kg) or saline by a single i.p. injection (50 μL) on P7. Pups were monitored daily to detect signs failure to thrive.

On P10, the pups were sacrificed by isoflurane inhalation to harvest cerebella for histological and molecular studies. Fresh cerebella were divided in the mid-sagittal plane; one hemisphere was snap-frozen and stored at -80 °C, and the other was fixed in Histochoice and embedded in paraffin. Histological sections (8-microns thick) were stained with Hematoxylin and Eosin (H&E) and examined by light microscopy. Throughout the experiment, rats were housed under humane conditions and kept on a 12-h light/dark cycle with free access to food. The protocol was approved by the institutional Animal Care and Use Committee at the Lifespan Rhode Island Hospital and conformed to guidelines set by the National Institutes of Health.

2.2. Protein expression studies

See Supplementary Methods for sources of reagents. Immunoreactivity was measured using duplex enzyme-linked immunosorbent assays (ELISAs) in which immunoreactivity was normalized to large ribosomal protein (RPLPO) measured in the same wells as previously described [15]. Fresh frozen cerebella were homogenized in lysis buffer containing protease and phosphatase inhibitors [15]. Protein concentration was measured using the bicinchoninic acid (BCA) assay. Protein samples (100 ng/50 μL buffer) were absorbed (over night, 4 °C) to well bottoms (quadruplicate) of 96-well MaxiSorb plates, then blocked for 3 h at room temperature with 3% bovine serum albumin in Tris buffered saline (TBS), and incubated (over night, 4 °C) with 0.1–0.4 μg/mL of primary antibody [35]. Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibody and Amplex UltraRed soluble fluorophore. Fluorescence intensity was measured (Ex 565 nm/Em 595 nm) in a SpectraMax M5 reader (Molecular Devices, Sunnyvale, CA, USA). Subsequently, the samples were incubated with biotin-conjugated antibodies to large ribosomal protein (RPLPO) as a loading control, and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase and the 4-methylumbelliferyl phosphate (4-MUP) fluorophore. Fluorescence (Ex360/Em450) was measured in a SpectraMax M5. Binding specificity was assessed in parallel negative control incubations with primary or secondary antibody omitted. The ratios of specific protein/RPLPO immunoreactivity were calculated and used for intergroup comparisons.

2.3. Gene expression studies

Gene expression was measured with duplex, probe hydrolysis-based quantitative reverse transcriptase polymerase chain reaction assays (qRT-PCR) as described [24]. In brief, RNA was extracted using the RN easy Mini Kit. cDNA templates were generated with the AMV 1st Strand cDNA Synthesis Kit. Gene expression was measured in hydrolysis, FAM-labeled probe-based duplex qRT-PCR assays in which β-actin (Y555-labeled) served as the internal control. PCR amplifications were performed in a LightCycler 480 PCR machine and results were analyzed using the LightCycler Software 4.0.

2.4. Statistical analysis

Box plots reflect the median (horizontal bar), 95% confidence interval limits (upper and lower boundaries of boxes), and range (whiskers). Intergroup comparisons were made by two-way analysis of variance (ANOVA) with Tukey's post-hoc test. Statistics were performed with GraphPad Prism 6 software.

3. Results

3.1. Effects of ethanol and NDEA on growth

This study begins to examine early independent and potentially additive effects of alcohol and NDEA exposures on cerebellar structure and protein and gene expression. P10 cerebella were studied because in rodents, the first 10 postnatal days correspond to the third trimester of human pregnancy, during which most of the cellular differentiation and neuronal migration occur [34]. Therefore, the model used corresponds to binge drinking, exposure to highly processed and preserved foods, or both in the third trimester of pregnancy. Growth, behavior, and weight gain were similar among the groups, and postmortem examinations failed to detect any evidence of tumor formation or pathology related to the injections (data not shown).

3.2. Ethanol and/or NDEA exposures impair cerebellar structural development

The effects of early developmental exposures to ethanol and/or NDEA on cerebellar structure were examined in H&E stained histological section (Figure 1). Control cerebella exhibited the normal 4-layered cortex composed of uniformly laminated external granule, molecular, Purkinje, and internal granule cell layers, and cellular white matter cores (Figures 1(a), 1(e), and 1(i)). NDEA treatment caused mild cerebellar hypotrophy with irregular and somewhat simplified patterns of foliation (Figure 1(b)), with thinning of the molecular layer (Figure 1(f)) and white matter cores (Figure 1(j)). Ethanol exposure caused pronounced cerebellar hypotrophy with blunting and simplification of folia (Figure 1(c)) and marked thinning of white matter cores (Figure 1(k)). Combined exposures to ethanol and NDEA produced composite effects of both with prominent simplification of the folia (Figure 1(d)), reduced thickness of the external granule cell layer (Figure 1(h)), overall thinning of the cortex and hypoplasia (reduced cell density within the white matter cores (Figure 1(l)).

3.3. Effects of ethanol, NDEA, and both exposures on cerebellar neuronal and glial cell protein expression

Cerebellar protein homogenates were used to measure Hu (neuronal), myelin-associated glycoprotein 1 (MAG-1; oligodendrocyte), glial fibrillary acidic protein (GFAP; astrocyte), Tau (neuronal cytoskeleton), phospho-Tau (pTau), choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and 4-hydroxy-2-nonenal (HNE; lipid peroxidation adduct) protein expression by duplex ELISAs with results normalized to RPLPO [15]. Two-way ANOVA tests demonstrated no significant effects of ethanol, NDEA, or combined exposures on the expression levels of Hu, Tau, pTau, ChAT, or AChE, or relative levels of Tau phosphorylation (pTau/Tau) (Table 1). Correspondingly, the mean levels of Hu, Tau, pTau, pTau/Tau, ChAT, and AChE were similar among the four groups (Figures 2(a), 2(d)–2(h)). In contrast, ethanol significantly altered MAG-1 ($P = .0015$), GFAP ($P < .0001$), and HNE ($P = .0042$) expression, NDEA significantly altered MAG-1 ($P = .0002$) and GFAP ($P = .01$) expression, and ethanol×NDEA had significant interactive effects on GFAP and HNE (both $P < .05$) (Table 1). Post hoc significance tests demonstrated that the main effects of ethanol and/or NDEA were to reduce expression of MAG-1 and GFAP relative to vehicle (control) (Figures 2(b) and 2(c)). The further lowering of MAG-1 and GFAP expression by ethanol+NDEA relative to ethanol or NDEA treatment alone suggests the exposures had additive effects. HNE expression was significantly lower in the ethanol+vehicle group compared with all others (Figure 2(i)). However, the significantly higher levels of HNE in the NDEA-treated relative to ethanol alone suggests that NDEA treatment promoted lipid peroxidation.

3.4. Effects of ethanol, NDEA, and both exposures on cerebellar ER stress gene expression

We used cerebellar mRNA to measure ER stress gene expression by duplex probe-based qRT-PCR analysis (see Supplementary Table 1 for functions of the genes examined). Two-way ANOVA tests demonstrated significant effects of ethanol on protein kinase inhibitor p58 ($p58^{IPK}$; $P = .03$), transcription factor ATF4 ($P = .001$), and apoptosis-associated protein BAX ($P = .03$) expression, significant effects of NDEA on cerebellar expression of the molecular chaperones, glucose regulated protein 78 (BiP/GRP78; $P < .0001$) and $p58^{IPK}$; $P = .0004$), and a significant trend with respect to ethanol×NDEA interactive effects on ATF4 ($P = .083$) (Table 2). Post-hoc significance testing demonstrated higher levels of BiP/GRP78 and $p58^{IPK}$ in NDEA-treated control and ethanol-exposed groups relative to corresponding vehicle treated cerebella (Figures 3(a) and 3(c)). ATF4 expression was significantly increased by ethanol, but not NDEA treatment (Figure 3(d)). Similarly, ethanol increased expression of BAX whereas NDEA did not. BAX corresponds to unfolded protein (UPR)-driven pathological responses that promote apoptosis. Tryptophanyl-tRNA synthetase (WARS) expression was marginally but significantly higher in the ethanol+NDEA group relative to control (Figure 3(h)). In contrast, no significant intergroup differences were observed with respect to ER degradation-enhancing α-mannosidase-like 1 (EDEM) (Figure 3(b)), CCAAT/-enhancer-binding protein homologous protein (CHOP; Figure 3(e)), protein disulphur isomerase (PDI) (Figure 3(f)), or homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member (HERPUD) (Figure 3(i)).

4. Discussion

4.1. Concept

This study was designed to explore a novel concept about the potential contributions of dietary nitrosamine exposures in the pathogenesis of FASD and FASD-like developmental abnormalities in the brain. The rationale for pursuing this line of investigation stemmed from awareness about the escalating exposures to nitrosamines in the diet [7], and the fact that FASD features can be present in children of mothers who had not consumed high levels of alcohol during pregnancy. Therefore, cofactor mediators of FASD should be considered to order to optimize future public health preventive initiatives. NDEA was selected for study because of its widespread presence in highly processed, inexpensive convenience foods that have long shelf lives due to the addition of nitrite-containing preservatives. Furthermore, in previous studies we showed that low, submutagenic doses of NDEA impaired cerebellar development, function,

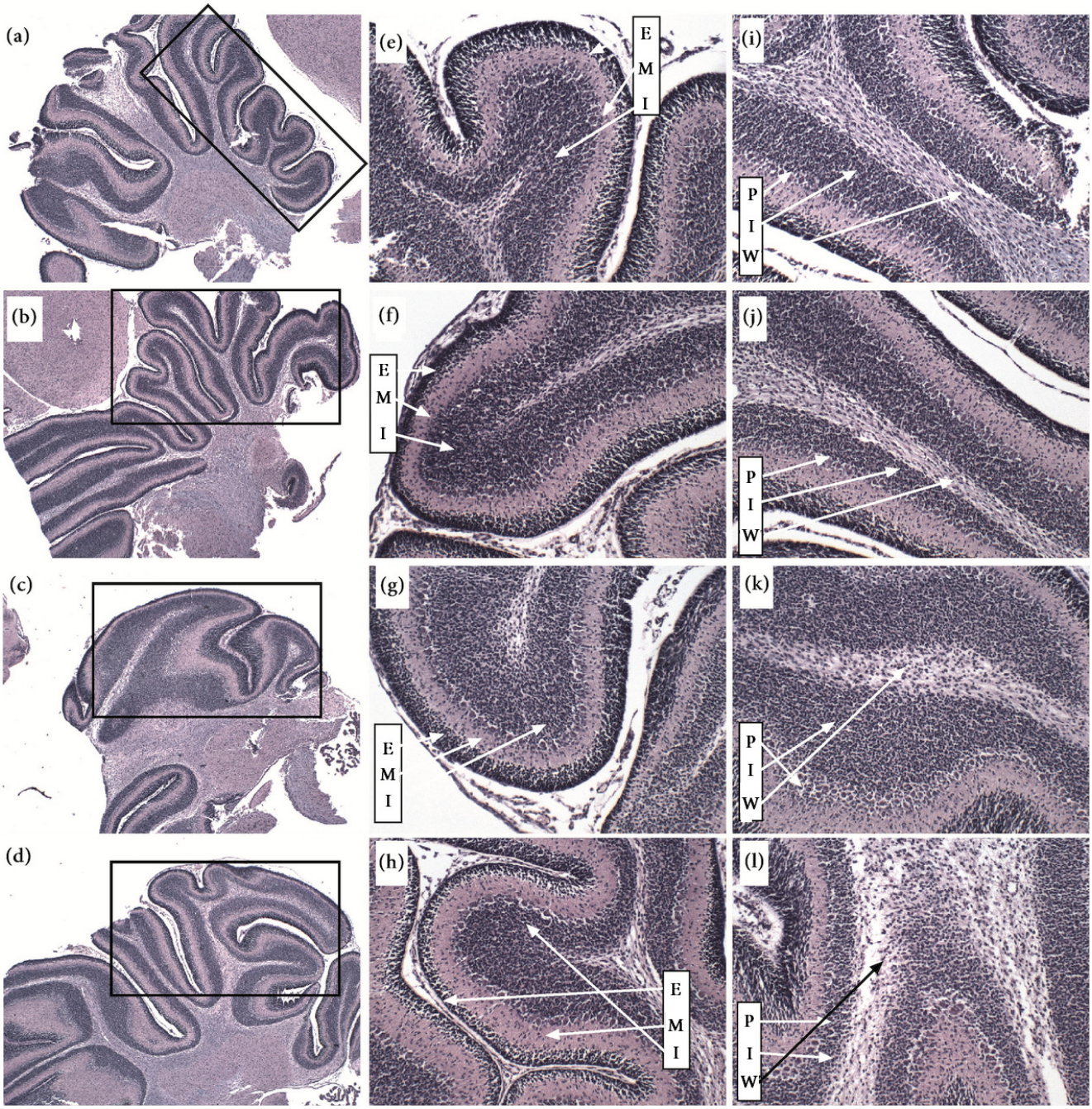

Figure 1: Ethanol and NDEA effects of cerebellar structure. Rat pups were exposed to ethanol, NDEA, or both in the early postnatal period. Half cerebella harvested on P10 were fixed and embedded in paraffin. Histological sections (8 μm thick) were stained with (h) and (e). (a), (e), (i) Vehicle treated control cerebellar cortex showing (a) complex foliation (folding), (e) uniform external granule cell (E), molecular (M), and internal granule (I) layers with (i) distinct cellular cores of white matter (W). Note small and medium size indentations corresponding to secondary sulcation along the lengths of folia ((a); box). (b), (f), (j) NDEA exposed cerebellar cortex with hypotrophic and straight simplified folia ((b); box), (f) irregular thickness of the external granule and molecular layers, and (j) thinning of white matter cores relative to control. (c), (g), (k) Binge ethanol exposures resulted in (c), (g) pronounced hypotrophy of the cerebellar cortex with irregular blunting of the folia ((c); box), and (k) reduced fiber density in white matter. (d), (h), (l) Ethanol *NDEA resulted in simplification and broadening of the folia* ((d); box), (h) narrowing of the external granule cell layer, and (l) cortical thinning as well as extreme reductions in white matter fibers with gaps in tissue integrity. Original magnifications, (a)–(d), 20×; (e)–(l) 100×.

Table 1: Effects of early postnatal binge ethanol and NDEA exposures on cerebellar neuronal and glial protein expression: two-way ANOVA summary.

Protein	Ethanol effect		NDEA effect		Ethanol×NDEA effect	
	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
Hu	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
MAG-1	11.53	.0015	16.87	.0002	0.067	N.S.
GFAP	29.31	$< .0001$	7.127	.0106	4.067	.049
Tau	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
pTau	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
pTau/Tau	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
ChAT	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
AChE	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
HNE	9.136	.0042	0.881	N.S.	4.625	.037

Immunoreactivity in cerebella of P10 rats exposed to ethanol, NDEA, or both during development was measured by duplex ELISAs with RPLPO as an internal control. Corresponding results are displayed in Figure 2. Data were analyzed by two-way ANOVA with the post-hoc Tukey significance test. Columns show significant F-ratios and P-values for ethanol NDEA, and interactions between ethanol and NDEA exposures.

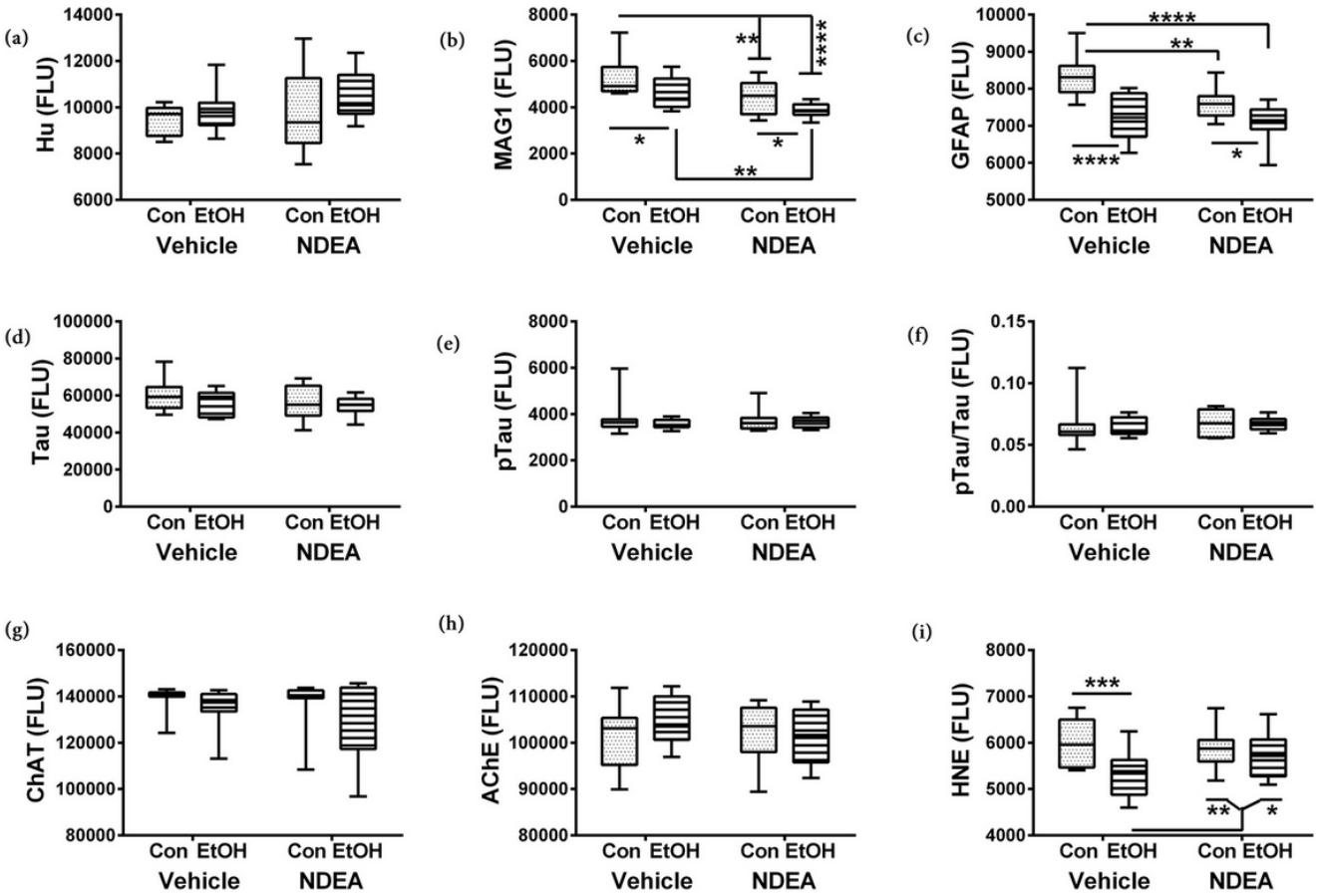

Figure 2: *Effects of ethanol and NDEA exposures on neuronal and glial cell protein expression.* Protein homogenates were prepared from fresh frozen cerebella harvested from P10 pups exposed to vehicle, ethanol, NDEA, or ethanol and NDEA in the early postnatal period. Immunoreactivity to (a) Hu, (b) myelin-associated glycoprotein 1 (MAG-1), (c) glial fibrillary acidic protein (GFAP), (d) Tau, (e) pTau, (g) choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and (i) 4-hydroxy-2-nonenal (HNE) was measured by duplex ELISA with results normalized to RPLPO (see Section 2). In addition, relative tau phosphorylation was assessed by the calculated ratio of pTau/Tau (f). Results were analyzed by two-way ANOVA (Table 1). Post-hoc significance tests determined the specific intergroup differences as shown in the panels.

Table 2: Effects of early postnatal binge ethanol and NDEA exposures on cerebellar ER stress gene expression: two-way ANOVA summary.

Protein	Ethanol effect		NDEA effect		Ethanol×NDEA effect	
	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
GRP78	1.190	N.S.	25.49	$< .0001$	0.407	N.S.
EDEM-1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
$p58^{IPK}$	5.311	.030	16.64	.0004	0.007	N.S.
ATF4	13.04	.001	0.051	N.S.	3.266	.083
CHOP	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
PDI	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
BAX	5.256	.031	0.005	N.S.	1.164	N.S.
WARS	2.600	.119	N.S.	N.S.	N.S.	N.S.
HERPUD	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

ER stress pathway gene expression in cerebella of P10 rats that were developmentally exposed to ethanol, NDEA, or both was measured by probe-based duplex qRT-PCR analysis with Actin as the internal control. Corresponding results are displayed in Figure 3. Data were analyzed by two-way ANOVA with the post-hoc Tukey significance test. Columns show significant F-ratios and P-values for ethanol NDEA, and interactions between ethanol and NDEA exposures.

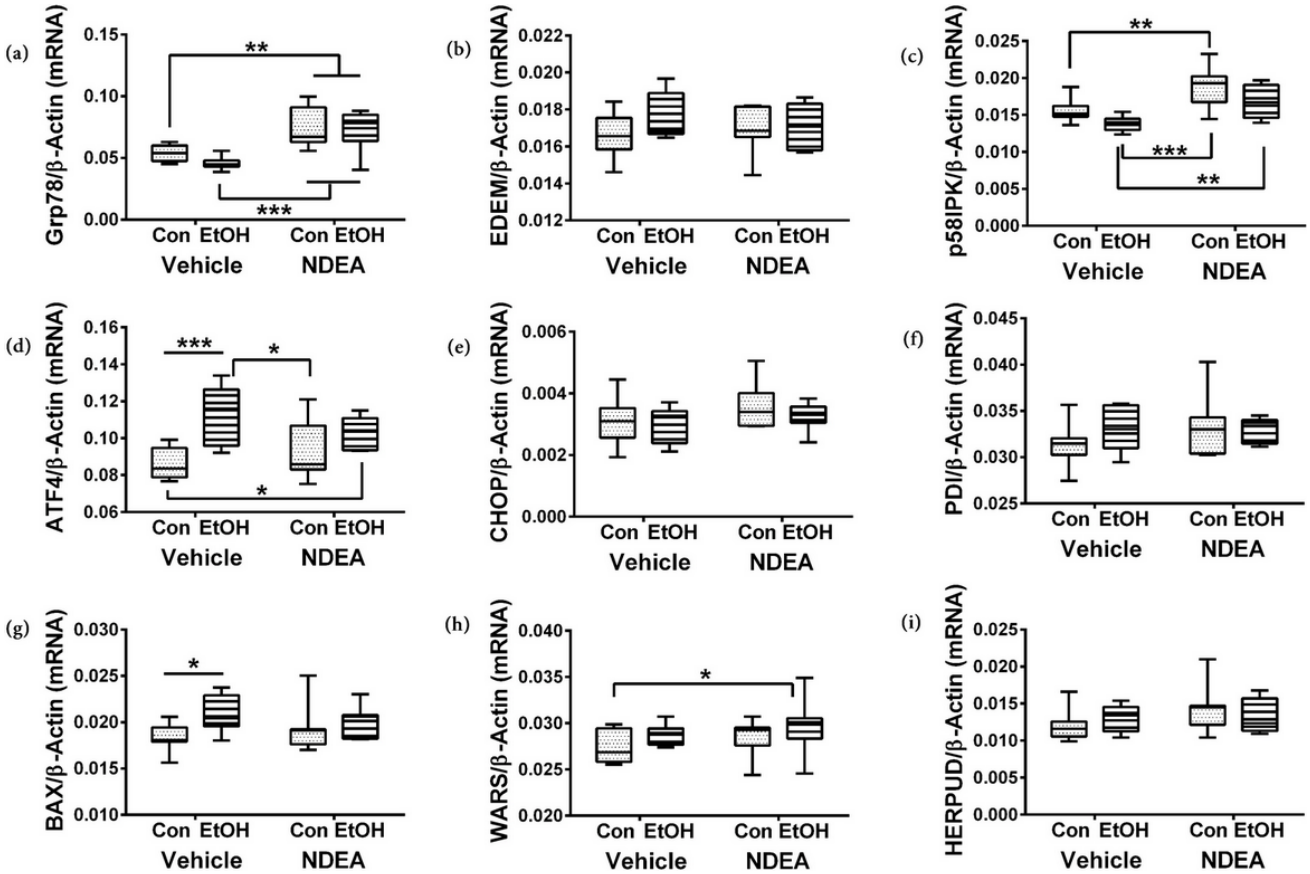

Figure 3: *Effects of ethanol and NDEA exposures on ER stress gene activation.* RNA extracted from cerebella was reverse transcribed, and the cDNAs were used to measure gene expression by probe hydrolysis-based qRT-PCR analysis with results normalized to β-actin. Graphs depict relative levels of gene expression for (a) glucose regulated protein 78 (GRP78), (b) ER degradation-enhancing α-mannosidase-like protein (EDEM), (c) inhibitor of interferon-induced and double stranded RNA activated kinase ($p58^{IPK}$), (d) activating transcription factor-4 (ATF-4), (e) C/EBP homologous protein (CHOP), (f) protein disulphur isomerase (PDI), (g) Bcl2-associated X protein (BAX), (h) tryptophanyl-tRNA synthetase (WARS), and (i) homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 (HERPUD). Results were analyzed by two-way ANOVA (Table 1). Post-hoc significance tests determined the specific intergroup differences as shown in the panels.

and insulin/IGF signaling through survival and metabolic pathways [8,35], similar to effects of ethanol [9,17,25, 27]. Herein, we utilized an early postnatal binge ethanol exposure model because during the interval studied, the adverse effects of ethanol are robust and produce substantial deficits in cerebellar development and function [34].

4.2. Cerebellar pathology

Histological studies demonstrated that both ethanol and NDEA impaired cerebellar development, and that their effects were manifested within a relatively short interval after treatment. These observations correspond with the evidence that the first 9–10 postnatal days of life represent a highly vulnerable period during which the teratogenic effects of alcohol are prominent. The findings also suggest that the same window of vulnerability exists for NDEA neurotoxicity. The morphologic effects of binge ethanol exposures on cerebellar development were striking and representative of previous observations using this model [11]. In earlier studies, we showed that cerebellar hypoplasia and hypotrophy in FASD were mediated impairments in cell survival, migration, proliferation, and differentiation [6,30]. In addition, the dysmorphic blunting of folia corresponds to ethanol-mediated impairments in cerebellar morphogenesis [6].

NDEA treatment also produced structural abnormalities in the cerebellum, but the main effect was to render the folia thin and simplified relative to control. Combined ethanol+NDEA exposures produced blunting and simplification of folia with marked irregularities in the thickness of cortical layers. Importantly, NDEA, ethanol, and NDEA+ethanol exposures lead to white matter hypotrophy and/or hypoplasia (glial cell loss or failed proliferation). This is a point of interest because white matter hypotrophy is a major and consistent feature of FASD [32]. The somewhat distinct dysmorphic effects of ethanol versus NDEA on cerebellar development could reflect differences in the treatment schedules (P3, P5, P7, and P9 for ethanol versus P7 only for NDEA) and therefore aggregate dose. Alternatively, the mechanisms by which CNS injury occurs may differ for ethanol and NDEA, despite similarities in outcomes.

4.3. Ethanol and NDEA impair glial protein expression

ELISAs were used to evaluate cellular profiles and related gene expression following ethanol and/or NDEA exposures. Despite striking morphological effects of these treatments, we detected no significant alterations in Hu, Tau, pTau (neuronal), or enzymes that regulate cholinergic function (ChAT and AChE). In contrast, ethanol and NDEA exposures reduced both MAG-1 and GFAP, corresponding to the white matter hypotrophy and/or hyperplasia observed in the cerebella. These findings suggest that glial cells are selective and immediately vulnerable targets of ethanol and NDEA exposures in the early postnatal period. Deficiencies in MAG-1 indicate that the myelin-generating and maintenances function of oligodendrocytes [13] were impaired, and that subsequent myelination of the developing and maturing brain was at risk. Correspondingly, one of the major abnormalities in FASD is white matter hypotrophy with reduced white matter thickness [21] caused by deficits in myelin production and/or maintenance [21,23].

Previous studies linked ethanol-mediated impairments in myelin protein expression to inhibition of insulin/IGF signaling [20] and loss of myelin producing cells [19, 40]. The finding that NDEA also inhibits MAG-1 protein expression is novel but consistent with previous results showing that NDEA impairs insulin/IGF signaling in the brain [36]. Importantly, the findings herein suggest that NDEA and ethanol share mechanisms of white matter hypotrophy during development, and support our hypothesis that developmental exposures to nitrosamines can contribute to FASD-associated neurodevelopmental abnormalities in the brain. The reductions in HNE expression in ethanol-exposed cerebella is discordant with previous observations, but may pertain to the time period of analysis. However, the significantly elevated levels of HNE in NDEA±ethanol versus ethanol treatment alone suggest that NDEA produces oxidative injury leading to lipid peroxidation. Reductions in MAG-1 protein could have been mediated in part by increased degradation of myelin [21,22,32].

The inhibition of GFAP expression by ethanol or NDEA reflects impaired astrocyte function which could have been caused by injury or death of astrocytes. GFAP is the major component of astrocyte intermediate filaments [41]. Astrocytes have important and diverse roles in the CNS including neuronal support, maintenance of extracellular fluid integrity, guidance of neuronal migration, and formation of brain scaffolding [38]. Therefore, loss or compromise of astrocyte function could have negatively impacted the development and function of cerebellar neurons. For example, the dysmorphic effects of ethanol and NDEA on cerebellar cortical structure may have been caused by impairments in neuronal migration due to inhibition of insulin/IGF signaling [8], and inhibition of astrocyte function or survival [28].

While the findings with respect to ethanol corroborate previous evidence that ethanol inhibits glial-driven neuronal migration and reduces glial cell populations and myelination in FASD [10], the data regarding the effects of NDEA on oligodendrocyte and astrocyte protein expression are novel. Importantly, the results support the hypothesis that other toxic exposures during critical periods of development can mimic or possibly exacerbate effects of alcohol. The finding that combined exposures to ethanol and NDEA had less striking inhibitory effects on glial cell protein expression

was somewhat surprising, but perhaps explainable on the basis that ethanol and NDEA both utilize CyP2E1 and CyP2A6 enzymes for degradation [3,12]. Conceivably, in this model, competition for the same enzyme could have limited the generation of toxic metabolites of each, including acetaldehyde build-up. However, the long-term responses might differ due to lower levels of sustained oxidative injury. This question will be resolved in future studies.

4.4. Ethanol and NDEA exposures activate ER stress pathways in the FASD model

ER stress genes are transcribed to maintain equilibrium for homeostatic protein modification in the ER [26]. These genes are transiently expressed but their levels are altered by various stressors. ER stress pathway activation reflects decreased protein folding capacity and increased potential for cell damage. Failure to restore protein homeostasis leads to permanent cellular dysfunction followed by activation of proapoptotic mechanisms [26].

Although both ethanol and NDEA exposures activated ER stress genes, the specific genes that were significantly affected were not the same, suggesting that their mechanisms of action were distinct. While NDEA significantly increased expression of the GRP78 and $p58^{IPK}$, ethanol increased expression of ATF4 and BAX. In addition, a modest additive effect of NDEA+ethanol was observed with respect to WARS. The responses to NDEA (i.e., increased GRP78 which initiates UPR downstream signaling and prevents secretion of mal-folded proteins [14,39]) and $p58^{IPK}$, which is an ER stress target that functions through eIF2a to attenuate the UPR [37], suggest that NDEA may drive ER stress signaling and UPR activation. In contrast, effects of ethanol appear to be more complex and downstream leading to activation of proapoptotic mechanisms through Bax [31] and ATF4, promoting transcription of proapoptotic/antisurvival CHOP (see Supplementary Table 1).

4.5. Conclusions

Ethanol or NDEA exposures during early postnatal development have significant adverse effects on cerebellar structure, inhibit glial protein expression, and increase ER stress pathway activation. Consequences of impaired glial protein expression would include deficits in neuronal support and function, neuronal migration, myelination, and cortical morphogenesis. ER stress pathway activation reflects consequences of ethanol or NDEA mediated cellular injury, but the differences in activation of pathway components suggest that NDEA mainly initiates the UPR response, whereas ethanol activates downstream proapoptosis mechanisms. Therefore, the findings suggest that the adverse effects of ethanol and NDEA exposure during a highly vulnerable period of cerebellar development overlap and produce similar, but not identical, abnormalities in the brain. Moreover, although both activate ER stress pathways, the mechanisms of ethanol appear to be more responsible for cell loss (apoptosis), whereas NDEA exposures activate more upstream components of the pathway. The aggregate results suggest that NDEA exposures through diet could potentially mimic or modulate the phenotypic features of FASD with respect to brain development. Additional research is needed to better delineate long-term consequences of the independent and combined ethanol and NDEA exposures during development.

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