Journal of Behavioral and Brain Science, 2026, 16(3), 95-126
https://www.scirp.org/journal/jbbs
ISSN Online: 2160-5874
ISSN Print: 2160-5866

Dose-Effects of Dietary Soy on Ethanol-Impaired Molecular Pathways Mediating Cerebellar Neurodevelopment and Function in Experimental Fetal Alcohol Spectrum Disorder

Jason Ziplow[1], Ming Tong[2,3], Suzanne M. de la Monte[3,4,5*]

[1]Departments of Neuroscience and Biology, Brown University, Providence, RI, USA
[2]Department of Medicine, Rhode Island Hospital, Brown University Health, Providence, RI, USA
[3]Alpert Medical School of Brown University, Providence, RI, USA
[4]Departments of Pathology and Laboratory Medicine, Neurosurgery, and Neurology, Rhode Island Hospital, Brown University Health, Providence, RI, USA
[5]Women & Infants Hospital, Brown University Health, Providence, RI, USA
Email: jlziplow@chop.org, mtong216@gmail.com, *Suzanne_DeLaMonte_MD2Brown.edu

How to cite this paper: Ziplow, J., Tong, M. and de la Monte, S.M. (2026) Dose-Effects of Dietary Soy on Ethanol-Impaired Molecular Pathways Mediating Cerebellar Neurodevelopment and Function in Experimental Fetal Alcohol Spectrum Disorder. *Journal of Behavioral and Brain Science*, **16**, 95-126.
https://doi.org/10.4236/jbbs.2026.163004

Received: February 10, 2026
Accepted: March 28, 2026
Published: March 31, 2026

Abstract

Fetal alcohol spectrum disorder (FASD) is associated with sustained impairments in motor function mediated by neurotoxic injury and dysregulated intracellular signaling through insulin and insulin-like growth factor (IGF) networks that support neuronal growth, survival, energy metabolism, and plasticity. Although neurodevelopmental abnormalities in FASD can be reduced by supplying pregnant dams with 100% soy as the sole protein source, modified approaches are needed for feasible human translation. The research goals were to characterize the effects of soy dosing on cerebellar structure and function, and the expression of insulin/IGF signaling mediators, neuronal-glial molecules, and neurotrophins in an FASD model. Pregnant Long Evans rats were fed isocaloric liquid diets containing 0% or 26% ethanol (caloric) with 0%, 10%, 30%, 50%, or 100% soy as the protein source. Ethanol and soy were withdrawn on postnatal day 1 (P1). Rotarod (RR) testing of cerebellar function was performed on P15. On P35, the offspring were sacrificed, and cerebella were harvested for image analysis and protein expression. Prenatal alcohol exposure significantly impaired RR performance, reduced brain size, and caused cerebellar hypoplasia with reduced Tau and increased glial fibrillary acidic protein (GFAP) immunoreactivity. There were no consistent residual effects of ethanol on upstream insulin/IGF signaling molecules 5 weeks after its post-

natal withdrawal. Soy doses of 30% or higher nearly normalized brain weight, as well as the densities of cerebellar Purkinje and granule cell neurons in the ethanol group. Soy dose-dependent increases in RR performance were observed in controls but not in the ethanol group. Although the ethanol-mediated reductions in Tau and increases in GFAP were not prevented by dietary soy, the control and ethanol cerebella exhibited similar dose-dependent increases in neurotrophin expression. In conclusion, maternal soy intake, at doses as low as 10%, can prevent or reduce cerebellar structural and functional abnormalities associated with FASD and support the expression of neurotrophins involved in plasticity. Sustained deficits after ethanol withdrawal were associated with reduced Tau and increased GFAP, which likely compromised neuronal cytoskeletal function and contributed to persistent neuroinflammatory/oxidative stress-related injury.

Keywords

Fetal Alcohol Spectrum Disorder, Cerebellum, Dietary Soy, Insulin Signaling, Neuronal Proteins, Glial Proteins

1. Introduction

Fetal Alcohol Spectrum Disorder (FASD) is unquestionably the most preventable neurodevelopmental disorder worldwide, as it is caused by heavy chronic or binge ethanol exposure in the prenatal period [1]-[4]. FASD encompasses a broad spectrum of neurodevelopmental abnormalities, ranging from subtle to severe [5]-[8]. Mild forms frequently go undetected and therefore untreated, compromising the ability to achieve successful education, employment, and societal status. Severe forms, designated as fetal alcohol syndrome (FAS), exhibit characteristic craniofacial dysmorphic features [5]-[8] and lead to significant and often debilitating long-term neurocognitive, behavioral, and motor deficits. The severity of FASD is proportional to the amount of alcohol consumed by the mother during pregnancy [2]. Public health preventive measures and mechanistically based therapeutic interventions are needed to prevent or reduce the adverse effects of prenatal alcohol exposure, particularly with respect to the long-term consequences in the offspring [8].

In previous publications, we showed that FASD-related cognitive neurobehavioral deficits were associated with broadly compromised intracellular signaling through insulin and insulin-like growth factor (IGF) networks [9]. Given the critical roles of these pathways in brain development and the considerable array of functional deficits that result from their impairments, including downstream alterations in gene expression [10]-[13], novel approaches are needed to mitigate the adverse effects of early developmental alcohol exposure [12]-[15]. In addition, more information is needed on the spectrum of molecular and biochemical pathologies in major central nervous system (CNS) cell types, including neurons, oligodendrocytes, and astrocytes, to advance the design of therapeutic interven-

tions. To this end, we have already conducted a series of experiments demonstrating the therapeutic or preventive effects of dietary soy and its bioactive constituents in FASD models [9] [16]-[18]. Correspondingly, independent studies have shown that dietary soy has preventive effects on insulin resistance-related and metabolic diseases across various organs and tissues [19]-[27]. Furthermore, a recent meta-analysis of the health benefits and safety of dietary soy corroborates the notion that it is suitable for nutritional support in women across the lifespan [28].

In previous studies, dietary interventions included 100% soy isolate as the protein source [9] [16] [29] [30]. Despite significant neurodevelopmental benefits, human translational feasibility remains low. Therefore, the present study was designed to characterize soy's dose effects on brain insulin/IGF signaling and neuronal/glial functions to determine the threshold amounts of dietary soy that can significantly reduce the adverse effects of prenatal ethanol on brain development. The research focused on the cerebellum because: 1) it is a major CNS target of ethanol-mediated neurotoxicity in FASD [31] [32]; 2) ethanol inhibits insulin/IGF signaling in the developing cerebellum [33] [34]; and 3) insulin sensitizer agents have been shown to enhance neurodevelopment in models of brain insulin/IGF resistance, including those caused by ethanol exposure [35] [36].

2. Materials and Methods

2.1. Materials

Critical reagents and instruments used in this study are listed in **Table A1**. The bicinchoninic acid (BCA) kit, MaxiSorp 96-well plates, horseradish peroxidase (HRP)-conjugated secondary antibodies, and Superblock (TBS) were purchased from Thermo-Fisher Scientific (Bedford, MA, USA). The soluble fluorophores, Amplex UltraRed and 4-Methylumbelliferyl phosphate (4-MUP) were from Life Technologies (Carlsbad, CA, USA). Vector Laboratories Inc. (Newark, CA, USA) was the source of the Proton Biotin Protein Labeling Kit and Alkaline Phosphatase-conjugated Streptavidin. The rat Akt Pathway Total and Phospho Multiplex panels were from MilliporeSigma (Burlington, MA, USA) (**Table A2**). Primary antibodies used for direct binding duplex enzyme-linked immunosorbent assays (ELISAs) are listed in **Table A3**. Fine chemicals were purchased from CalBiochem (Carlsbad, CA, USA) or Sigma-Aldrich (St Louis, MO, USA).

2.2. Experimental Model

The FASD model was generated in Long Evans rats in accordance with our protocol, which was approved by the Brown University Health Institutional Animal Care and Use Committee (IACUC) of Rhode Island Hospital [16]. Females purchased from Charles River Laboratories (Willmington, MA, USA) were mated, and 6 days after detecting sperm in the vaginal canal, isocaloric control (0% ethanol) or ethanol-containing (26% caloric pharmaceutical grade) liquid diets (BioServ, Frenchtown, NJ, USA) were initiated. To study the dose effects of dietary soy, both the control and ethanol diets were supplied with protein consisting of

0% soy/100% casein, 10% soy/90% casein, 30% soy/70% casein, 50% soy/50% casein, or 100% soy/0% casein. The liquid diets were initiated on gestation day 6 to minimize fetal demise and failed implantation, which frequently accompany earlier gestation ethanol feeding [37]. The liquid diets continued until postnatal day 1 (P1), after which the dams were maintained on standard chow without further ethanol exposure. Upon weaning, the pups were fed chow and provided free access to water. Apart from fetal demise marked by fetal resorption [9], prenatal alcohol exposure does not reduce longevity into adulthood.

2.3. Rotarod Testing

On P16, cerebellar motor function was assessed by subjecting the offspring to 10 consecutive trials of incrementally increased rod rotation speed from 1.5 to 6.0 rpm using a Rotamex 5 instrument (Columbus Instruments, Columbus, OH, USA) [15]. The latencies to fall were automatically recorded with photocells positioned over the rod. To prevent exercise fatigue, all trials were halted after 45 seconds of work, and the rats were allowed 10 minutes of rest between each trial. The data were analyzed using two-way ANOVA with the Tukey post hoc test.

2.4. Cerebellar Tissue Processing for Image Analysis and Protein Studies

P35 was the experimental endpoint. The rats were sacrificed with terminal isoflurane anesthesia. Fresh cerebella were hemisected through the vermis. One hemisphere was fixed in 10% neutral buffered formalin, paraffin-embedded, and sectioned for staining with hematoxylin and eosin. Image analysis with Stereologer software (Stereology Resources, Inc., Chester, MD, USA, available at https://srcbiosciences.com/stereologer-software, accessed on 24 February 2025) was used for unbiased stereological quantification of granule and Purkinje cell densities, as described [17]. Gray matter volume was measured with the volume probe, and granule and Purkinje cell abundances were assessed using dissector probes. Cortical volumes were measured using the area point-count method at a 4x magnification. Purkinje and granule cells were counted, respectively, using frame sizes of 40% and 0.5% of the screen height, whereas granule cells were counted using a frame size that was 0.5% of the screen height at 40x magnification.

The non-fixed cerebellar hemisphere was snap-frozen on dry ice and stored at −80˚C for biochemical studies. For protein assays, 100 mg tissue samples were homogenized in 5 volumes of buffer (150 mM NaCl, 50 mM Tris-Base pH 7.5, 0.1% Triton X-100, 5 mM EDTA pH 8.0, 10 mM EDTA, 50 mM NaF) supplemented with protease (1 mM PMSF, 0.1 mM TPCK, 2 µg/ml aprotinin, 2 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 mM NaF, 1 mM $Na_4P_2O_7$) and phosphatase (2 mM Na_3VO_4) inhibitors [16]. The samples were homoginzed using a TissueLyser II instrument (Qiagen, Germantown, MD, USA) and 5-mm diameter stainless steel beads. Supernatant fractions obtained by centrifuging the samples at 14,000xg for 10 minutes at 4˚C were used in immunoassays. Protein concentrations were meas-

ured with the BCA assay.

2.5. Multiplex Enzyme-Linked Immunosorbent Assay (ELISA)

Magnetic multiplex bead-based platform kits (Invitrogen, Carlsbad, CA, USA) were used to measure total and phosphorylated protein levels in the insulin/IGF/IRS-Akt pathway (**Table A2**). In brief, 100 µg aliquots of cerebellar protein homogenate were incubated with antibody-coated beads. The captured antigens were detected with biotinylated secondary antibodies and phycoerythrin-conjugated Streptavidin. Immunoreactivity was measured in a Luminex MAGPIX instrument (Diasorin, Austin, TX, USA) with xPONENT software. MAGPIX calibration and verification standards were used throughout, and standard curves were generated for each analyte. Data are expressed in arbitrary fluorescence light units (FLU) calculated from the standard curves.

2.6. Duplex ELISAs

Duplex ELISAs: Direct binding duplex ELISAs measured immunoreactivity to neuronal and glial proteins [38]. The antibodies, their sources, characteristics, validations, and final concentrations are listed in **Table A3**. Triplicate samples containing 50 ng protein in 50 µL bicarbonate-binding buffer were first added to 96-well MaxiSorp plates for overnight adsorption at 4˚C. Non-specific binding sites were masked with Superblock TBS. The samples were then incubated with primary antibodies (0.2 - 5.0 µg/mL) overnight at 4˚C. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and the Amplex UltraRed soluble fluorophore. Fluorescence intensity was measured (Ex 530 nm/Em 590 nm) in a Spectra-Max M5 Multimode Plate Reader (Molecular Devices, Sunnyvale, CA, USA). The results were normalized to large acidic ribosomal protein (RPLPO) (Proteintech Group Inc., Chicago, IL) as a control for sample loading [39] [40]. The second phase of the duplex ELISA was accomplished by incubating the samples with biotin-conjugated anti-RPLPO, followed by streptavidin-conjugated alkaline phosphatase. RPLPO immunoreactivity was detected with 4-Methylumbelliferyl phosphate (4-MUP), and fluorescence was measured in a SpectraMax M5 (Ex 360 nm/Em 450 nm).

2.7. Data Analysis

Statistical analysis was performed, and graphs were generated using GraphPad Prism 10.6 (GraphPad Software Inc., Boston, MA). Inter-group comparisons were made using one-way or two-way analysis of variance (ANOVA) with Tukey post hoc multiple comparisons. Software-generated statistically significant ($P \leq 0.05$) or trendwise ($0.05 < P < 0.10$) differences are included in the Tables and Graphs.

3. Results

3.1. Experimental Group Characteristics

The 175 offspring, comprised of 90 males and 85 females, were divided among 5

control and 5 ethanol diet groups. The diets contained 0%, 10%, 30%, 50% or 100% soy as the protein source, with casein added to achieve 100% of the protein requirements. Within each group, there were no significant differences in the proportions of males and females that were gestationally exposed to the control or ethanol diet, as assessed by chi-square analysis (Table 1).

Table 1. Rat treatment groups.

Diet	Treatment	Male	Female	Total	Chi-Square	P-Value
0% Soy	Control	3 (33%)	6 (67%)	9	0.926	N.S.
0% Soy	Ethanol	13 (52%)	12 (48%)	25		
10% Soy	Control	4 (36%)	7 (64%)	11	3.074	N.S.
10% Soy	Ethanol	10 (71%)	4 (29%)	14		
30% Soy	Control	12 (48%)	13 (52%)	25	0.383	N.S.
30% Soy	Ethanol	9 (39%)	14 (61%)	23		
50% Soy	Control	13 (65%)	7 (35%)	20	1.146	N.S.
50% Soy	Ethanol	6 (46%)	7 (54%)	13		
100% Soy	Control	7 (70%)	3 (30%)	10	0.945	N.S.
100% Soy	Ethanol	13 (52%)	12 (48%)	25		
Total		90	85	175	0.017	N.S.

Long-Evans pregnant rat dams were maintained on isocaloric control or ethanol-containing (26% caloric) liquid diets from gestation day 6 through delivery. The sources of dietary protein included from 0% to 100% soy with casein added to achieve 100% of the required protein composition. The number and percentage of male and female pups in each group are listed. Chi-square tests show no significant differences in sex distribution across groups.

3.2. Cerebellar Motor Function and Structure

Rotarod performance was assessed by comparing mean latency-to-fall across 10 trials at progressively higher rod rotation speeds. The two-way ANOVA tests demonstrated significant dietary soy effects in all 10 trials and significant ethanol effects in all trials except Trial #1 (Table 2). Significant interactive effects of significant dietary protein source (soy dose) x ethanol were detected in Trials #4-#6 and #9-#10. In addition, statistical trend effects of dietary protein x ethanol interactions on performance were observed for Trials #3 and #8.

Table 2. Two-way ANOVA for trial-based rotarod performance-dietary protein source and ethanol factors.

	Protein Source		Ethanol Factor		Dietary Protein x Ethanol	
Rotarod Trial	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
1	**5.079**	**0.0008**	0.734	N.S.	0.155	N.S.
2	**5.097**	**0.0008**	**10.27**	**0.0018**	0.8059	N.S.
3	**6.368**	**0.0001**	**12.58**	**0.0006**	*2.090*	*0.086*
4	**5.573**	**0.0001**	**27.11**	**<0.0001**	**5.429**	**0.0004**
5	**4.366**	**0.0024**	**34.31**	**<0.0001**	**5.29**	**0.0006**
6	**4.092**	**0.0037**	**35.85**	**<0.0001**	**4.325**	**0.0026**
7	**3.883**	**0.005**	**30.05**	**<0.0001**	1.404	N.S.

Continued

8	**6.707**	**<0.0001**	**16.60**	**<0.0001**	*2.341*	*0.0586*
9	**7.085**	**<0.0001**	**16.59**	**<0.0001**	**2.579**	**0.041**
10	**11.78**	**<0.0001**	**25.40**	**<0.0001**	**3.847**	**0.0055**

Long Evans rat offspring of pregnant dams that were maintained on isocaloric control or ethanol-containing (26% caloric) liquid diets from gestation day 6 through delivery. Maternal sources of dietary protein included from 0% to 100% soy, with casein added to achieve 100% of the required protein composition. On postnatal day 15, the offspring were subjected to 10 consecutive progressively challenging rotarod performance tests to evaluate cerebellar motor function. Results were analyzed by two-way ANOVA with post hoc Tukey tests. Significant ($P \leq 0.05$) differences are highlighted with bold font. Statistical trend effects ($0.05 < P < 0.10$) are italicized. The graphs and significant post hoc Tukey test results are shown in Figure 1 and Figure 2.

To clearly illustrate the within-group and between-group exposure effects, graphs were generated to show the clustered and overall performance of the control and ethanol groups (Figure 1) or to present direct comparisons for each trial (Figure 2). The most striking observation was that among controls, dietary soy dose impacted performance such that exposures between 30% and 100% soy significantly increased the latencies to fall relative to the effects of 0% or 10% soy (Figure 1). In contrast, the effects of dietary soy dose were modest and limited to the least challenging trials, *i.e.*, #1-#3. The control-versus-ethanol paired comparisons by soy dose demonstrated that inter-group differences were mainly observed at 50% and 100% dietary soy, and largely attributable to the positive effects in controls versus minimal effects in the ethanol group (Figures 2(B)-(J)). Inter-group differences were not detected for Trial #1 (Figure 2(A)) or for most of the more challenging trials in rats exposed to 30% or lower levels of dietary soy.

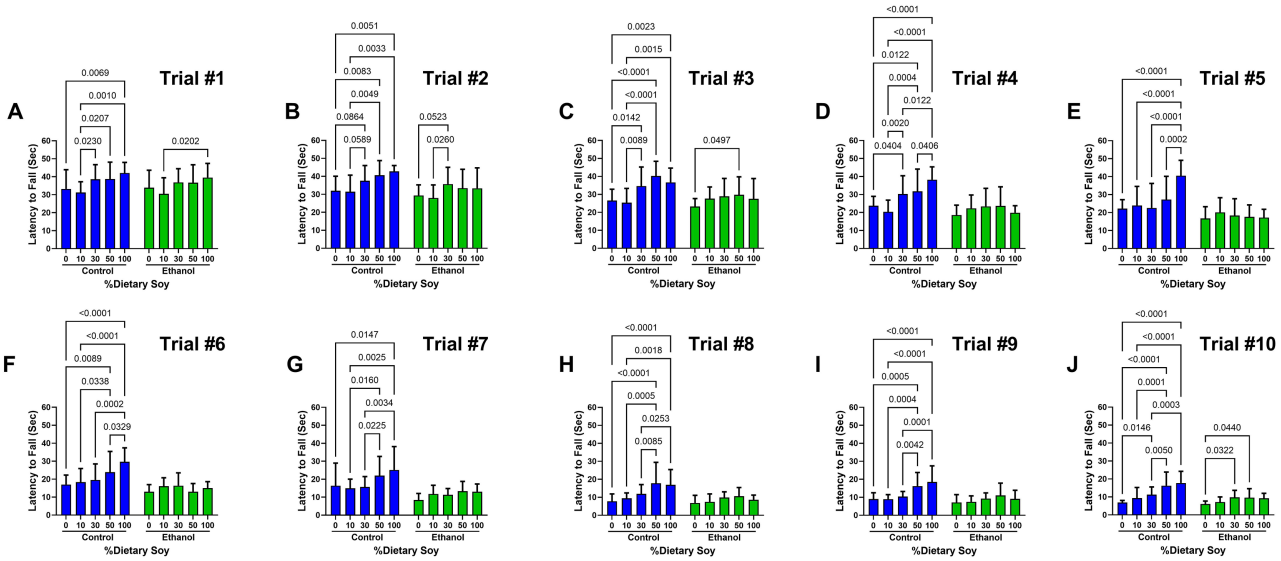

Figure 1. Rotarod Performance-Soy dose effects on performance in control and ethanol-exposed offspring. (A)-(J) Rotarod performance was measured over 10 consecutive trials of increasing rod rotation speed (See Table 2). The latencies to fall off the rod (seconds) were optically recorded. Graphs depict the mean ± S.D. of latencies in rats exposed to 0% (control) or 26% caloric ethanol in liquid diets containing 0%, 10%, 30%, 50%, or 100% soy as the protein source, with casein added to meet 100% of the dietary protein requirement. Results were analyzed by trial using ANOVA and Tukey's test for within-group comparisons. Significant differences are displayed.

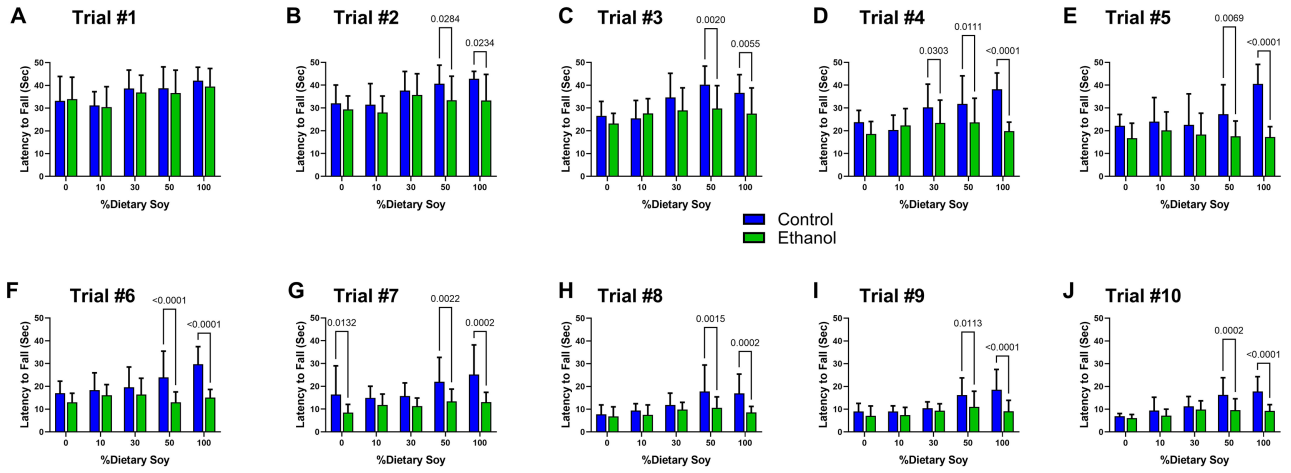

Figure 2. Rotarod Performance-Ethanol exposure effects on performance with increasing dietary soy dose. (A)-(J) Rotarod performance was measured over 10 consecutive trials of increasing rod rotation speed (See Table 2). The latencies to fall off the rod (seconds) were optically recorded. Graphs depict the mean ± S.D. of latencies in rats exposed to 0% (control) or 26% caloric ethanol in liquid diets containing 0%, 10%, 30%, 50%, or 100% soy as the protein source, with casein added to meet 100% of the dietary protein requirement. Results were analyzed by trial using ANOVA and Tukey's test for between-group comparisons at each soy dose. Significant differences are displayed.

3.3. Dietary Soy Effects on the Offspring's Experimental Endpoint Body Weight and Brain Weight

The experimental endpoint was P35. Two-way ANOVA detected significant soy dose, ethanol, and soy dose x ethanol interactions with respect to body weight (Table 3). However, regarding brain weight, significant soy dose and soy dose x ethanol interactive effects were observed, whereas ethanol itself was not a significant factor (Table 3).

Table 3. Two-way ANOVA table for body and brain weights.

	Soy Dose		Ethanol Factor		Soy x Ethanol	
Offspring	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
Body Weight (P35)	**12.67**	**<0.0001**	**9.529**	**0.0024**	**4.766**	**0.0012**
Brain Weight (P35)	**8.525**	**<0.0001**	2.559	N.S.	**5.058**	**0.0008**

Long Evans rat offspring of pregnant dams that were maintained on isocaloric control or ethanol-containing (26% caloric) liquid diets from gestation day 6 through delivery. Soy comprised 0%, 10%, 30%, 50%, or 100% of the protein source, and with casein added to provide 100% of the required protein. Postnatal day 35 (P35), experimental endpoint body and brain weight comparisons were made using two-way ANOVA. Significant results are represented with bold font. The corresponding graphs with significant ($P < 0.05$) post hoc Tukey test results are shown in Figure 3.

Graphs were generated to optimally illustrate the within- and between-group effects of exposure on body weight (Figure 3(A), Figure 3(B)), brain weight (Figure 3(C), Figure 3(D)), **and the brain weight/body weight ratios (Figure 3(E), Figure 3(F)).** Body weight distributions varied within each group, but the main effect was that rats exposed to 100% soy in utero were significantly heavier than the other groups (Figure 3(A)). The paired comparisons demonstrated that

within the 10% and 30% soy groups, ethanol-exposed rats were significantly heavier than controls, whereas the opposite was observed in the 50% dietary soy group (Figure 3(B)). The effects of soy dose and ethanol were clearer for brain weight. Among controls, the lowest mean brain weight was observed in the 10% soy group, and rats exposed to 100% soy protein had significantly higher brain weights than rats exposed to 10% or 30% soy (Figure 3(C)). In the ethanol group, the lowest mean brain weight was in the 0% soy group. Exposure to 10% soy or higher significantly increased brain weight. The largest effect occurred with 100% soy, such that the mean brain weight was significantly higher than in all the other ethanol groups (Figure 3(C)). The paired control versus ethanol paired comparisons demonstrated significantly lower mean brain weight in ethanol+ 0% soy and ethanol + 50% soy groups (Figure 3(D)).

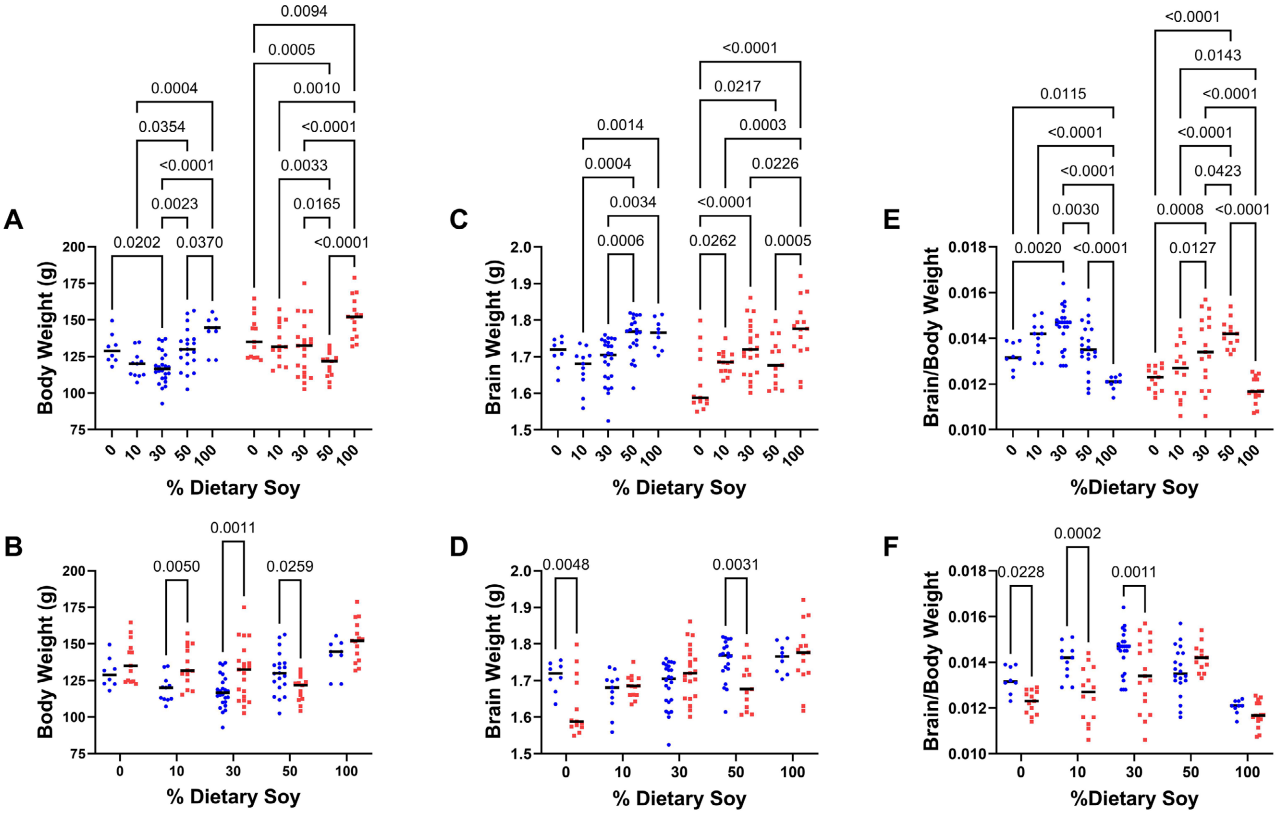

Figure 3. Offspring Metrics. Effects of dietary soy dose and ethanol on body and brain weights. Pregnant Long Evans dams were maintained on isocaloric liquid diets containing 0% (control) or 26% caloric ethanol and 0%, 10%, 30%, 50%, or 100% soy as the protein source, with casein added to meet 100% of the dietary protein requirement. Ethanol and soy were withdrawn on postnatal day 1 (P1). P35 was the experimental endpoint. Scatter plots depict within-group effects of dietary soy dose on (A) body weight, (C) brain weight, and (E) brain/body weight ratios, and between-group differences in the effects of soy dose on (B) body weight, (D) brain weight, and (F) brain/body weight ratios in P35 offspring. Data were analyzed by two-way ANOVA (Table 3) and the Tukey post hoc test.

Otherwise, the control and ethanol groups exposed to 10%, 30%, or 100% dietary soy had comparable mean brain weights. The data for the brain weight/body weight ratios show progressively increasing values from 0% to 30% soy in the con-

trol and ethanol groups, but sharp reductions in the 100% soy groups, reflecting the effects of increased body weight (Figure 3(E)). Inter-group comparisons demonstrated lower mean brain weight-to-body weight ratios associated with ethanol and with dietary protein ranging from 0% to 30% soy. The combined effects of soy-mediated increases in brain and body weight rendered the brain-to-body weight ratios similar when the diets contained 50% or 100% soy as the protein source (Figure 3(F)).

3.4. Soy and Ethanol Effects on Cerebellar Neuronal Populations

Image analysis with unbiased stereology was used to quantify the densities of Purkinje and granule cell neurons in the cerebellar cortex. The control within-group one-way ANOVA tests demonstrated significant dietary soy dose effects on Purkinje cell density and a statistical trend effect on granule cell density (Table 4). The ethanol within-group one-way ANOVA test demonstrated a significant dietary soy dose effect on granule cell but not Purkinje cell density (Table 4).

Table 4. Impact of dietary soy dose on cerebellar structure within control and ethanol groups.

Group	Purkinje Cell Density		Granule Cell Density	
	F-Ratio	P-Value	F-Ratio	P-Value
Control	**4.459**	**0.0078**	2.640	*0.057*
Ethanol	1.039	N.S.	**4.071**	**0.012**

Image analysis of the cerebellar cortex with unbiased stereology assessed the effects of prenatal ethanol and dietary soy dose on Purkinje and granule cell densities. Cells were counted at high magnification using an optical dissector method and the results were normalized to tissue volume based on Cavalieri point grid method. The data were analyzed by one-way ANOVA to determine the effect of soy dose on neuronal cell density within the control and ethanol groups. $P \leq 0.05$ was considered as statistically significant. Statistical trend effects ($0.05 < P < 0.10$) are italicized. NS = not significant. See the corresponding graphs in Figure 4.

Two-way ANOVA revealed significant ethanol effects on Purkinje cell density, and significant soy dose, ethanol, and soy dose x ethanol interactions on granule cell density (Table 5). The corresponding graphs of paired comparisons between the control and ethanol groups by soy dose, with post hoc Tukey test results, showed significantly lower Purkinje and granule cell neuron densities in the ethanol + 0% dietary soy samples (Figure 4(A) and Figure 4(B)). In both groups, the

Table 5. Two-Way ANOVA tests of soy dose and ethanol exposure effects on cerebellar structure.

	Soy Dose Factor		Ethanol Factor		Soy Dose x Ethanol	
Cell Type	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
Purkinje	1.619	N.S.	**5.224**	**0.027**	1.801	N.S.
Granule	**3.418**	**0.0156**	**6.463**	**0.0144**	**3.511**	**0.0137**

Histological sections of cerebellar cortex were evaluated using stereology-based image analysis to assess the effects of prenatal ethanol and dietary soy dose on Purkinje and granule cell densities. Cells were counted at high magnification using an optical dissector method and the results were normalized to tissue volume based on Cavalieri point grid method. The results were analyzed by two-way ANOVA with post hoc Tukey tests. $P \leq 0.05$ was considered as statistically significant. See the corresponding graphs in Figure 4.

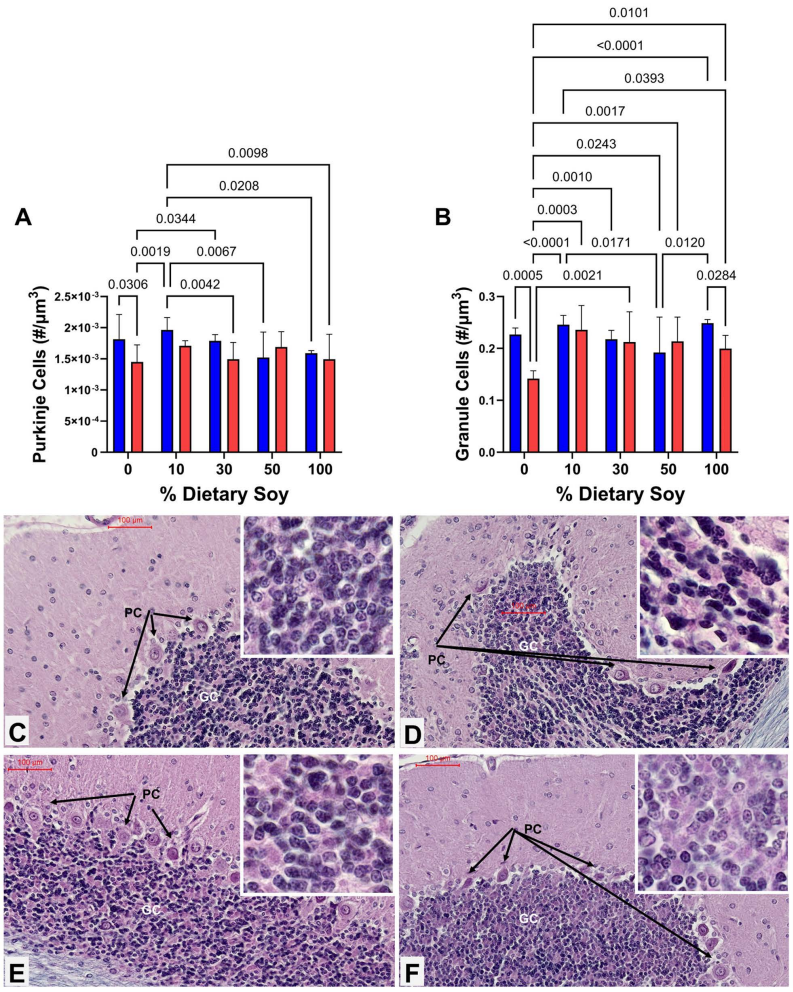

Figure 4. Cerebellar Morphometrics. Histological sections of cerebellar cortex (vermis) from control and ethanol-exposed Long Evans rat P35 offspring were subjected to image analysis to evaluate the effects of prenatal alcohol exposure and dietary soy dose (0%, 10%, 30%, 50%, or 100% of the protein source) on Purkinje and granule cell densities. Numerical densities of (A) Purkinje and (B) granule cells were quantified per cubic micron of cortical gray matter present on the slide with the aid of an Olympus BX60 light microscope (Olympus America Inc., Center Valley, PA) with an attached MS-2000 XYZ Inverted Stage (Applied Scientific Instrumentation, Eugene, OR) and Stereologer software (Stereology Resource Center, Inc., Chester, MD). Unbiased counting frames were applied under software control. Cells were counted at high magnification (40x) using an optical dissector method and normalized to volume using the Cavalieri point grid method. The results were analyzed by two-way ANOVA (Table 4 and Table 5) and Tukey post hoc tests. Significant ($P \leq 0.05$) inter-group differences for each soy dose are displayed in the panels. (C-F) Representative histological images of cerebellar cortex from the (C) Control + 0% soy, (D) Ethanol + 0% soy, (E) Control + 100% soy, and (F) Ethanol + 100% soy groups. H&E-stained slides were photographed at 200x. Insets show images of Granule cells (GC) photographed at 600x. PC = Purkinje cells. Note smaller area (volume) of the GC zone and loss of PC in Panel D versus Panel C. Ethanol-reduced GC density is highlighted by the relatively sparse populations of small dark nuclei shown in the inset in D versus C. Dietary soy had minimal effect on the PC and GC populations in control cerebella (compare E to C), but it conspicuously increased the abundance of PC and GC in the ethanol +100% soy (F) compared with ethanol + 0% soy (D). 100-µm scale bars are shown in each panel for size/magnification reference.

modest dietary soy-related increases in Purkinje cell densities abolished their inter-group statistical differences. A similar phenomenon occurred with respect to the granule cells, except at the 100% soy dose, which was associated with a significantly lower density in the ethanol group (**Figure 4(B)**).

Representative photographs of H&E-stained cerebellar cortex sections demonstrate the effects of ethanol and dietary soy on the Purkinje and Granule cell populations (**Figures 4(C)-(F)**). Control cerebella from the offspring of dams fed with liquid diets that contained no alcohol and had 0% Soy/100% casein as the protein source, exhibited abundant populations of Purkinje and granule cells (**Figure 4(C)**). In contrast, gestational ethanol exposure coupled with diets containing 0% soy/100% casein resulted in reduced cerebellar volumes with loss of both Purkinje and granule cells (**Figure 4(D)**). Large gaps in the Purkinje cell layer reflect neuronal loss. The reduced density of dark round nuclei in the granule cell layer reflects combined effects of cell loss and impaired proliferation. Control cerebella from offspring of dams fed with diets that contained 100% soy/0% casein as the protein source (**Figure 4(E)**) were similar to controls in the 0% soy/100% casein group. In contrast, in ethanol-exposed offspring, 100% dietary soy increased the population of Purkinje cells, resulting in fewer gaps, and increased the density of cells in the granule cell layer (**Figure 4(F)**). The histological features of cerebella from the ethanol-exposed, 100% Soy/0% Casein group were similar to those of the corresponding control group (**Figure 4(E)**), corresponding with the results obtained by image analysis.

3.5. Multiplex Immunoassays of Insulin Receptor/IGF-1 Receptor/IRS-1 Pathway

Previous studies showed that chronic ethanol exposures impair insulin and IGF-1 signaling through IRS and Akt pathways in the brain, and correlated those effects with alcohol-related neurobehavioral dysfunctions [12] [14] [15] [41]-[43]. To examine the extent to which dietary soy's effects on cerebellar structure were mediated by restoration of these signaling networks, the samples were analyzed with multiplex total and phosphoprotein ELISAs (**Table A2**). In addition, the calculated relative levels of protein phosphorylation (p/T) were assessed. Two-way ANOVA detected significant soy dose effects on all signaling, phosphorylated signaling, and calculated relative levels of protein phosphorylation (**Table 6**). In addition, significant ethanol effects were detected for IRS1 and $^{pYpY1135/1136}$-IGF-1R, and significant soy dose x ethanol interactive effects were observed for $^{pYpY1135/1136}$-IGF-1R, ^{pS636}-IRS-1, ^{pS473}-Akt, and the relative levels of IGF-1R and Akt phosphorylation.

Table 6. Two-way ANOVA table for insulin, IGF, Akt pathway molecules.

	Soy Dose Factor-		Ethanol Factor		Soy Dose x Ethanol	
Biomarker	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
Insulin-R	**86.28**	**<0.0001**	0.0329	N.S.	0.074	N.S.

Continued

IGF-1R	**20.48**	**<0.0001**	0.055	N.S.	0.069	N.S.
IRS1	**169.2**	**<0.0001**	**10.02**	**0.0026**	0.76	N.S.
Akt	**11.21**	**<0.0001**	1.235	N.S.	1.304	N.S.
GSK-3β	**9.908**	**<0.0001**	0.598	N.S.	0.289	N.S.
pY-Insulin-R	**15.13**	**<0.0001**	2.064	N.S.	1.039	N.S.
pY-IGF-1R	**15.99**	**<0.0001**	**4.226**	**0.045**	**11.21**	**<0.0001**
pS-IRS-1	**16.27**	**<0.0001**	0.391	N.S.	**3.319**	**0.017**
pS-Akt	**11.12**	**<0.0001**	*2.914*	*0.094*	**4.339**	**0.0043**
pS-GSK-3β	**5.80**	**0.0006**	0.418	N.S.	0.810	N.S.
pY/T-Insulin R	**39.87**	**<0.0001**	0.338	N.S.	1.125	N.S.
pY/T-IGF-1R	**27.95**	**<0.0001**	0.003	N.S.	**2.529**	**0.05**
pS/T-IRS-1	**145.9**	**<0.0001**	1.049	N.S.	0.696	N.S.
pS/T-Akt	**35.27**	**<0.0001**	2.136	N.S.	**9.707**	**<0.0001**
pS/T-GSK-3β	**4.304**	**0.0045**	0.072	N.S.	1.029	N.S.

Immunoreactivity was measured in 100 µg cerebellar protein using commercial Total Akt and Phospho-Akt Magnetic Bead-Based ELISA panels and a MAGPIX instrument. The calculated levels of relative protein phosphorylation (p/T) were also compared. Inter-group comparisons were made using mixed-model ANOVA and post-hoc Tukey tests with correction for multiple comparisons. F-Ratios correspond to measured soy dose, ethanol, and soy dose x ethanol interactive effects. Significant differences (bold font) have P-values < 0.05. Statistical trend effects (0.05 < P < 0.10) are italicized. NS = not significant. R = receptor; IGF-1 = insulin like growth factor type 1; IRS-1 = insulin receptor substrate, type 1; GSK-3β = glycogen synthase kinase 3β; pY = phosphotyrosine; pS = phosphoserine. See Table A2 for details of protein phosphorylation sites.

Box and whisker plots display the effects of ethanol and dietary soy on insulin/IGF-1/IRS-1-Akt pathway proteins, phosphoproteins, and the relative levels of protein phosphorylation (Figure 5). The main overall effects observed were that, with few exceptions, the levels of immunoreactivity at each soy dose were similar for the control and ethanol samples, and the most prominent shifts in immunoreactivity were associated with the 50% and 100% soy protein diets. To simplify the graphic presentation, the significant Tukey comparisons displayed were focused on effects relative to the 0% soy diet groups. Insulin R (Figure 5(A)) and IGF-1R (Figure 5(B)) were similarly expressed at relatively low levels in cerebellar tissue from control and ethanol-exposed rats in the 0% to 50% soy groups, and both were significantly elevated with the 100% soy diet (compared with all other groups. In contrast, IRS1 (Figure 5(C)), Akt (Figure 5(D)), and GSK-3β (Figure 5(E)) immunoreactivities exhibited downward trends from 0% to 50% soy, followed by a further significant decline in IRS-1, and moderate increases in Akt and GSK-3β in the 100% soy diet groups.

The levels of $^{pYpY1162/1163}$-Insulin R (Figure 5(F)), $^{pYpY1135/1136}$-IGF-1R (Figure 5(G)), ^{pS636}-IRS-1 (Figure 5(H)), and ^{pS473}-Akt (Figure 5(I)) trended upward from the 0% to 30% or 50% soy diets, then declined to levels below those in the 0% soy groups. In addition, for each of these phosphoproteins, significant ethanol-inhibitory effects were observed in the 50% soy groups. The ^{pS9}-GSK3β levels were relatively uniform over the full soy dose range except for a significant reduction in

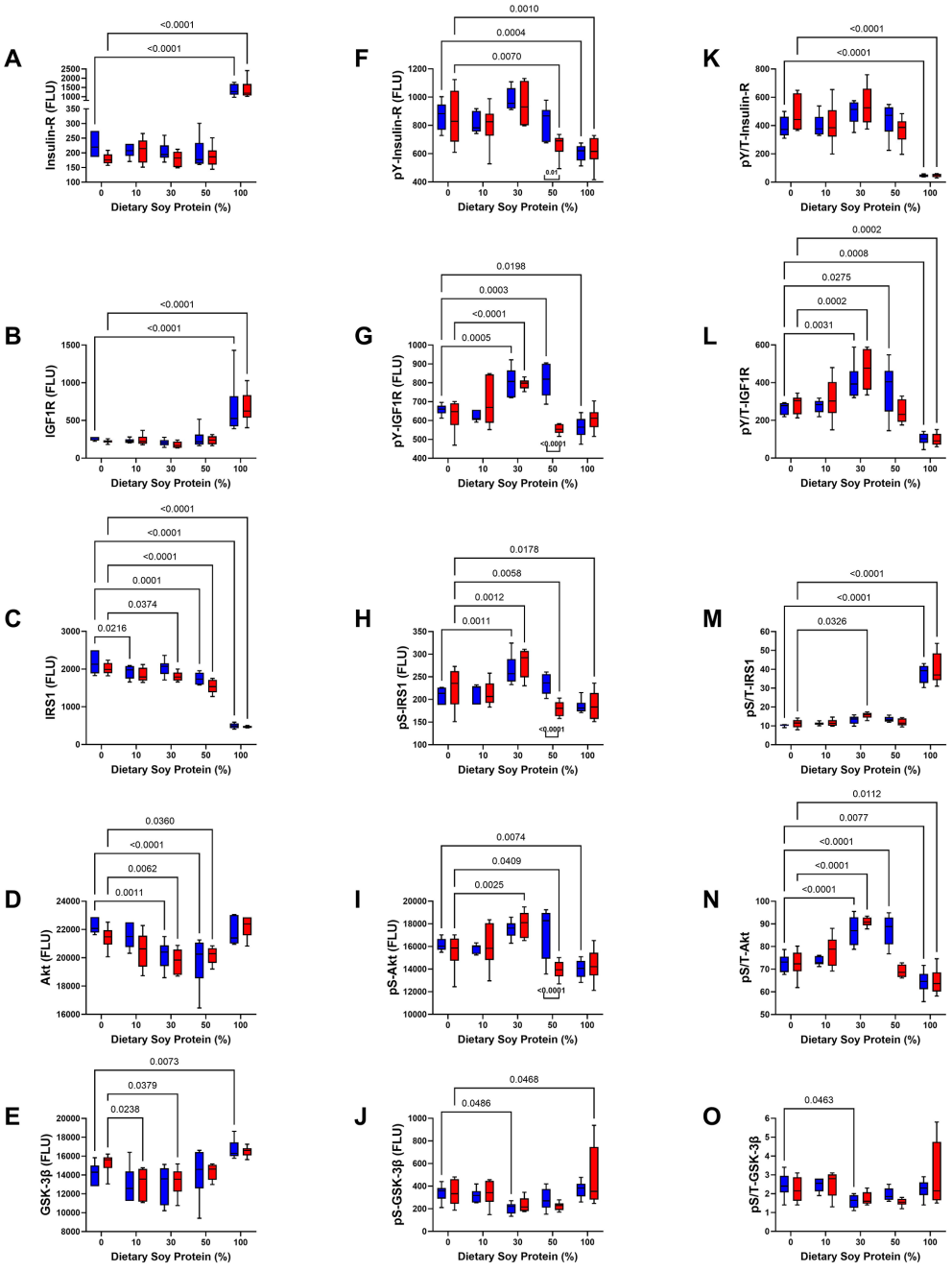

Figure 5. Insulin/IGF Signaling Network. Prenatal ethanol and dietary soy dose effects on (A)-(E) protein, (F-J) phosphoprotein, and (K)-(O) the relative levels of signaling protein phosphorylation in cerebellar tissue homogenates from the offspring of dams fed with isocaloric liquid diets containing 0% or 26% caloric ethanol and 0%, 10%, 30%, 50%, or 100% soy as the protein source, with casein added to meet 100% of the dietary protein requirement. Commercial multiplex bead-based immunoassay kits (Table A2) were used to measure immunoreactivity to signaling proteins (A) insulin receptor (Insulin-R), (B) IGF-1R, (C) IRS-1, (D) Akt, (E) GSK-3β, phorphorylated (pY or pS) signaling molecules (F) pYpY1162/1163-Insulin-R, (G) pYpY1135/1136-IGF-1R, (H) pS312-IRS-1, (I) pS473-Akt, and (J) pS9-GSK-3β, and the calculated relative levels (pY/T or pS/T) of signaling protein phosphorylation (K) pY/T-Insulin R, (L) pY/T-IGF-1R, (M) pS/T-IRS-1, (N) pS/T-Akt, and (O) pS/T-GSK-3β. The data were analyzed by two-way ANOVA (Table 6) and the Tukey post hoc test. Significant ($P \leq 0.05$) results are displayed within the panels.

the 30% soy control group and a significant increase in the 100% soy ethanol group relative to corresponding 0% soy samples (Figure 5(J)). The profiles observed for soy dose-related shifts in the relative phosphorylation levels of Insulin R (Figure 5(K)), IGF-1R (Figure 5(L)), Akt (Figure 5(N)), and GSK-3β (Figure 5(O)) were nearly identical to those observed for the corresponding tyrosine- or serine-phosphorylation. In contrast, the calculated relative levels of pS-IRS1 phosphorylation were uniformly low in the 0% to 50% soy groups but significantly elevated in the 100% soy compared with all other groups (Figure 5(M)).

3.6. Neuroglial Studies

Neuronal and glial protein expression was measured by duplex ELISA with results normalized to acidic ribonuclear protein (RPLPO). Two-way ANOVA detected significant soy dose effects on Tau, pTau, ubiquitin, and choline acetyltransferase (ChAT) (Table 7) and significant ethanol effects on Tau, ChAT, and glial fibrillary acidic protein (GFAP). Significant soy dose x ethanol interactive effects were observed for Tau, ubiquitin, ChAT, and GFAP. Box and whisker plots were configured to clearly display soy dose effects in control and ethanol-exposed cerebella (Figures 6(A)-(E)) or the comparative effects of ethanol relative to control at each dietary soy dose (Figure 6(F)-(J)), with the significant post hoc Tukey results.

Table 7. Two-way ANOVA table for neuroglial proteins.

	Soy Dose Effect		Ethanol Effect		Soy Dose x Ethanol Interactive Effect	
Biomarker	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
Tau	**3.118**	**0.0179**	**12.54**	**0.0006**	**2.638**	**0.0377**
pTau	**38.89**	**<0.0001**	0.147	N.S.	1.539	N.S.
Ubiquitin	**62.04**	**<0.0001**	1.69	N.S.	**4.604**	**0.0018**
ChAT	**17.5**	**<0.0001**	**12.92**	**0.0005**	**6.868**	**<0.0001**
GFAP	0.763	N.S.	**28.16**	**<0.0001**	**5.922**	**0.0002**

Immunoreactivity was measured by direct-binding ELISA in 50 ng samples of cerebellar protein homogenates. Immunoreactivity was measured with primary antibodies targeting Tau, phospho-Tau (pTau), ubiquitin, choline acetyltransferase (ChAT), or glial fibrillary acidic protein (GFAP), horseradish peroxidase-conjugated secondary antibody, and Amplex UltraRed soluble fluorophore. Fluorescence intensity (Ex 560 nm/Em 590 nm) was measured in a SpectraMax M5. Results were normalized to the internal control ribosomal protein, RPLPO. The data was analyzed using mixed-model ANOVA and post-hoc Tukey tests (See Figure 6). $P \leq 0.05$ was considered statistically significant (bold font). NS = not significant.

Tau immunoreactivity was significantly elevated in the 100% soy relative to most lower soy doses, while in the ethanol group, Tau was increased in the 10% soy diet relative to most other doses (Figure 6(A)). pTau (Figure 6(B)) and ubiquitin (Figure 6(C)) immunoreactivities were lowest in the 0% soy control and ethanol groups and significantly increased across the full range of dietary soy exposure. However, the peak levels were not correlated with the maximum soy dose. ChAT immunoreactivity was significantly reduced from 0% to 10% soy in control

samples, but significantly reduced by 10%, 30% or 50% dietary soy in the ethanol group (Figure 6(D)). The levels of GFAP immunoreactivity were relatively consistent across the dietary soy dose range in both control and ethanol samples, although significant differences were detected due to an isolated increase in the 30% soy control group, and inhibitory effects of 10% and 30% soy in the ethanol group (Figure 6(E)).

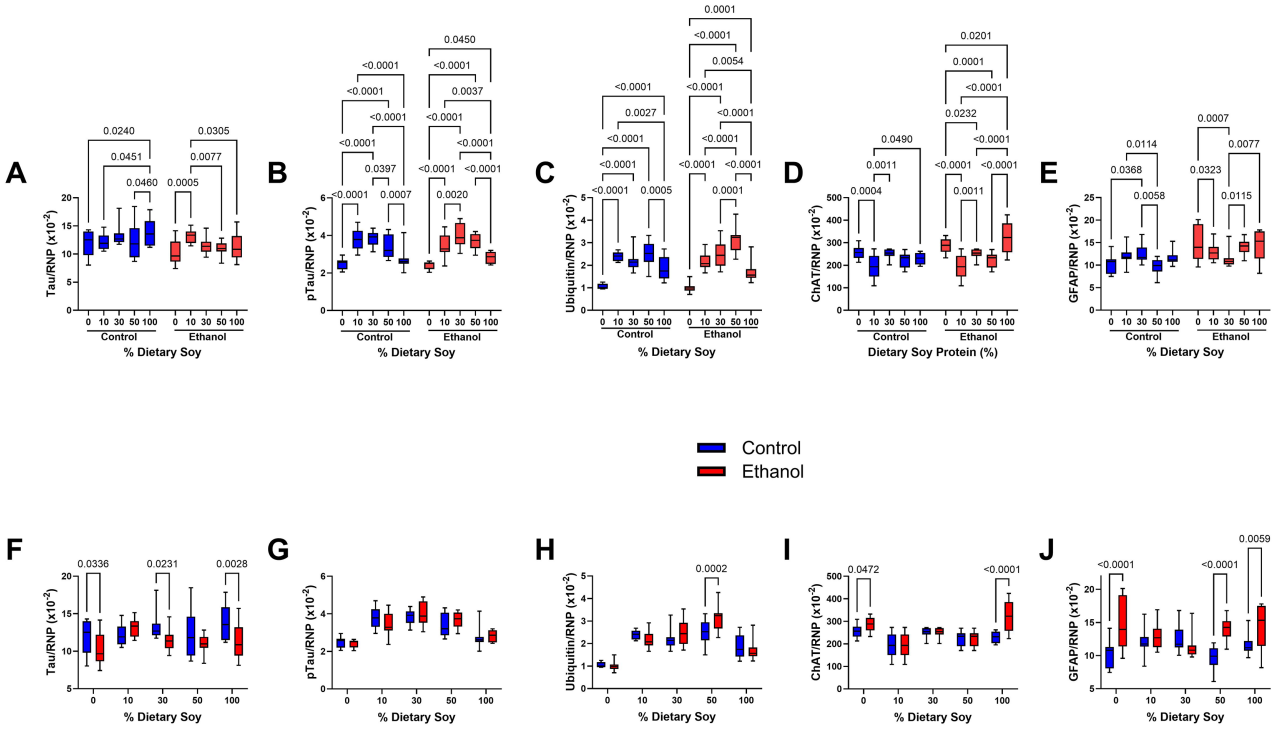

Figure 6. Dietary Soy Dose and Ethanol Effects on Cerebellar Neuroglial Protein Expression. Cerebellar tissue from the offspring of dams fed with isocaloric liquid diets containing 0% or 26% caloric ethanol and 0%, 10%, 30%, 50%, or 100% soy as the protein source, with casein added to meet 100% of the dietary protein requirement was used to measure immunoreactivity to (A, F) Tau (BDNF), (B, G) pS396-Tau (pTau), (C, H) ubiquitin, (D, I) choline acetyltransferase (ChAT), and (E, J) glial fibrillary acidic protein (GFAP) by duplex ELISA. Results were normalized to acidic ribosomal phosphoprotein PO (RPLPO) housekeeping molecule. Graphs (A)-(E) show within-group (control or ethanol) effects of soy dose on immunoreactivity levels, and (F)-(J) show the between-group differences in responses to increasing soy dose. Data were analyzed by two-way ANOVA (Table 7) and the Tukey post hoc test. Significant differences ($P < 0.05$) are shown within the panels.

The graphs designed to compare the effects of ethanol revealed significantly reduced Tau (Figure 6(F)) and increased GFAP (Figure 6(J)) at 0%, 100% and either the 30% or 50% soy dose. ChAT immunoreactivity was also significantly elevated at 0% and 100% soy doses in the ethanol group (Figure 6(I)). Ubiquitin was significantly increased by ethanol only in the 50% dietary soy group (Figure 6(H)). No significant inter-group differences were observed for pTau across the soy dose range (Figure 6(G)).

3.7. Neurotrophin Studies

Neurotrophin protein expression was measured by duplex ELISA with results

normalized to acidic ribonuclear protein (RPLPO). Two-way ANOVA detected significant soy dose effects on brain-derived neurotrophic factor (BDNF), nerve growth factor beta (NGFβ), glial-derived neurotrophic factor (GDNF), Neurotrophin 3 (NT3), and NT4, but significant ethanol effect restricted to GDNF, and significant soy dose x ethanol interactive effects for BDNF (Table 8). Box-and-whisker plots were configured to display soy dose effects in control and ethanol-exposed cerebella (Figures 7(A)-(E)) or to compare ethanol relative to control at each dietary soy dose (Figures 7(F)-(J)), with the significant post hoc Tukey results shown.

Table 8. Two-way ANOVA table for neurotrophins.

	Soy Dose Factor		Ethanol Factor		Soy x Ethanol	
Biomarker	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
BDNF	**3.536**	**0.0094**	1.891	N.S.	**2.747**	**0.0319**
NGFβ	**17.57**	**<0.0001**	0.940	N.S.	1.228	N.S.
GDNF	**47.93**	**<0.0001**	**7.773**	**0.0062**	1.406	N.S.
NT3	**40.28**	**<0.0001**	0.5873	N.S.	1.328	N.S.
NT4	**22.58**	**<0.0001**	0.524	N.S.	0.830	N.S.

Immunoreactivity was measured by direct-binding ELISA in 50 ng samples of cerebellar protein homogenates. Immunoreactivity was measured with primary antibodies targeting brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), Neurotrophin 3 (NT3), or Neurotrophin 4 (NT4), horseradish peroxidase-conjugated secondary antibody, and Amplex UltraRed soluble fluorophore. Fluorescence intensity (Ex 560 nm/Em 590 nm) was measured in a SpectraMax M5. Results were normalized to the internal control ribosomal protein, RPLPO. The data was analyzed using mixed-model ANOVA and post-hoc Tukey tests (See Figure 7). $P < 0.05$ was considered statistically significant (bold font). NS = not significant.

In contrast to the neuroglial proteins, the neurotrophins were concordantly modulated with dietary soy dose in control and ethanol samples (Figures 7(A)-(E)). For BDNF, the levels of immunoreactivity were relatively consistent across the dietary soy dose range in both control and ethanol samples, except for the significantly reduced level observed in the 50% soy + ethanol group (Figure 7(A)). For NGFβ, cerebellar immunoreactivity was similar from 0% to 50% soy but significantly increased in both control and ethanol groups exposed to 100% soy (Figure 7(B)). In addition, a modest dose-dependent downward trend in NGFβ was detected in ethanol samples from the 10%, 30%, and 50% soy models. The profiles observed for GDNF (Figure 7(C)), NT3 (Figure 7(D)), and NT4 (Figure 7(E)) were similarly arched, with generally dose-related increases in immunoreactivity from 0% to 30% or 50% soy, followed by a tapered response to 100% dietary soy. Graphs comparing dietary soy dose effects in ethanol versus control samples (Figures 7(F)-(J)) showed relatively few significant differences. Those differences were restricted to ethanol-associated significant reductions in BDNF in samples from the 10% soy model (Figure 7(F)), and GDNF from the 10% and 100% soy models (Figure 7(H)).

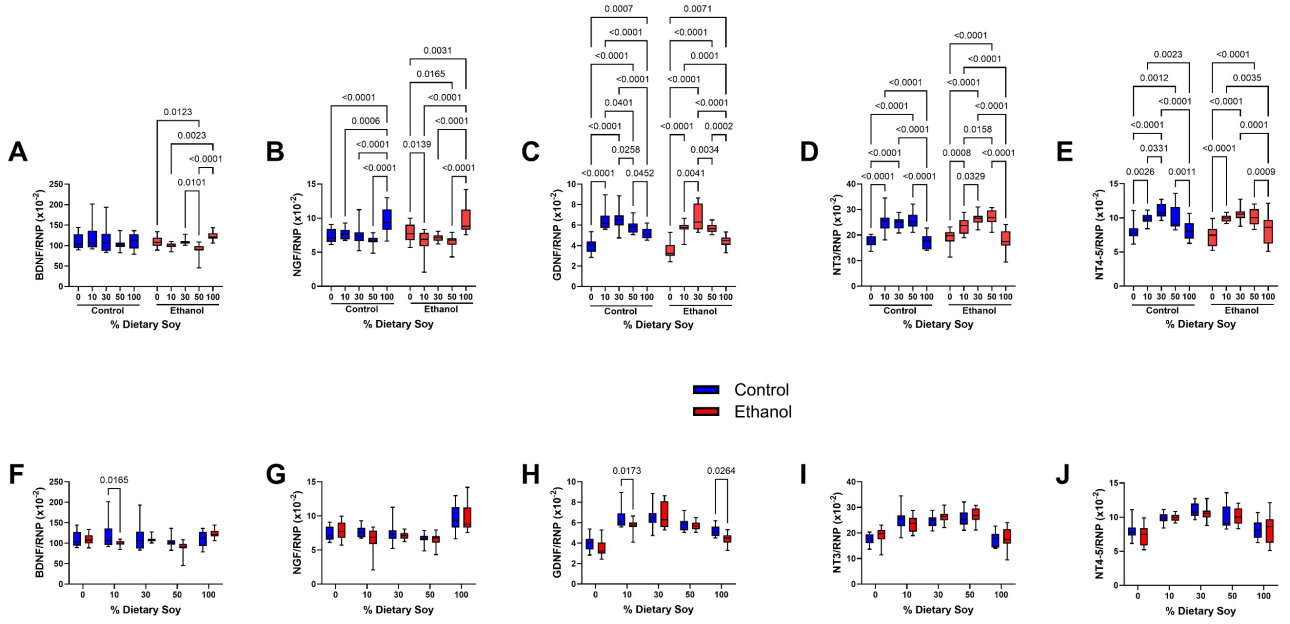

Figure 7. Dietary Soy Dose and Ethanol Effects on Cerebellar Neurotrophin Expression. Cerebellar tissue from the offspring of dams fed with isocaloric liquid diets containing 0% or 26% caloric ethanol and 0%, 10%, 30%, 50%, or 100% soy as the protein source, with casein added to meet 100% of the dietary protein requirement was used to measure immunoreactivity to (A, F) brain-derived neurotrophic factor (BDNF), (B, G) Nerve growth factor beta (NGF-β), (C, H) glial-derived neurotrophic factor (GDNF), (D, I) Neurotrophin 3 (NT3), and (E, J) Neurotrophin 4 (NT4) by duplex ELISA. Results were normalized to acidic ribosomal phosphoprotein PO (RPLPO) housekeeping molecule. Graphs (A)-(E) show within-group (control or ethanol) effects of soy dose on immunoreactivity levels, and (F)-(J) show the between-group differences in responses to increasing soy dose. Data were analyzed by two-way ANOVA (**Table 8**) and the Tukey post hoc test. Significant differences ($P < 0.05$) are shown within the panels.

4. Discussion

This manuscript reports the effects of graded gestational dietary soy doses on postnatal cerebellar function, structure, and expression of neuroglial and neurotrophin molecules in an experimental model of FASD. In previous reports using related models, we demonstrated phenotypic craniofacial features of FASD in the late fetal period [9], ethanol dose-dependent increases in severity of cerebellar hypotrophy and hypocellularity [12] [33], and sustained cognitive and motor impairments in early adolescence [15] [17] [43] [44]. Remarkably, the co-administration of gestational dietary soy comprising 100% of the required protein in the dam's diet significantly attenuated FASD features in the offspring, including neurodevelopmental abnormalities [9]. In addition, we demonstrated that 100% gestational dietary soy was protective through adolescence, resulting in normalization of brain weight and performance on spatial learning and memory tasks [16] [18]. Known benefits of dietary soy include its insulin-sensitizing and antioxidant/anti-inflammatory effects [19] [21]-[27] [45]. The rationale for testing the preventive or therapeutic effects of soy in FASD models is that several critical neurodevelopmental abnormalities caused by prenatal alcohol exposure are mediated by impairments in signaling through insulin and insulin-like growth factor (IGF) pathways, increased neuroinflammation, and oxidative injury [13] [17] [33] [46]

[47]. However, since complete replacement of dietary protein with soy would be unrealistic and therefore have limited translational application for most pregnant women, our next goal was to determine if lower dietary soy doses were also neuroprotective, and to characterize mechanistic effects in relation to insulin and IGF networks and the expression of neuroglial and neurotrophin molecules utilized for developmental brain maturation and plasticity. This study focused on the cerebellum because of its prominent vulnerability to heavy ethanol exposure across the lifespan. Ethanol and dietary soy interventions were implemented prenatally, during rodents' rudimentary cerebellar development, and when Purkinje cells are mainly formed [35]. The experimental design enabled analysis of postnatal cerebellar development, beyond the window of gestational ethanol and soy exposures, mimicking aspects of the human condition associated with maternal alcohol consumption in pregnancy.

In rodents, cerebellar development is robust from birth through P12, *i.e.*, the third-trimester equivalent of human fetal development [48], coinciding with rapid granule cell proliferation [49]. Prenatal alcohol exposures, including those leading to FASD, damage both Purkinje and granule cells [31] [32] [50], which are the major cerebellar cortical neuron populations mediating output and regulation of motor functions [51]. Correspondingly, in the present study, we detected significant impairments in cerebellar motor function associated with prenatal alcohol exposure using rotarod testing. Image analysis of cerebellar cortical tissue revealed proportionally greater ethanol-associated reductions in granule cells compared with Purkinje cells. Additional studies showed that prenatal ethanol exposure did not impair body growth, indicating no evidence of failure to thrive. Instead, the significantly reduced brain weight reinforced the concept that prenatal ethanol exposures cause sustained and relatively selective neurodevelopmental impairments.

The protective effects of dietary soy were evident at the lowest dose (10%). One interesting effect was the significant increase in body weight observed in the ethanol group exposed to 10% or 30% dietary soy, illustrating positive effects on growth. However, even more striking was that, at all soy doses, brain weight in the ethanol group was significantly increased relative to 0% soy; the effects were largely dose-dependent, and at most soy doses, brain weight was normalized relative to control. Concerning the cerebellar cortex, the lowest soy dose (10%) effectively increased and normalized the densities of Purkinje and granule cells in the ethanol group. However, unlike the whole brain, the supportive effects were not dose-dependent, and they varied across the soy dose range. Nonetheless, the findings show that dietary soy has significant protective effects on brain growth and cerebellar cortical neuron populations in a moderately severe prenatal ethanol exposure model of FASD.

Rotarod testing of cerebellar function was more informative concerning the effects of ethanol and the interventional benefits of dietary soy. Ethanol-impaired performance was documented, consistent with previous reports [13] [15] [44].

Dietary soy, particularly at doses of 30% or higher, enhanced the performance of both control and ethanol-exposed rats. However, deficits in the ethanol group persisted and worsened as the task challenges increased, despite cerebellar neuron densities being normalized or nearly normalized. In contrast, among controls, performance was significantly improved with the 50% or 100% soy protein diets compared with lower doses, even at the most challenging rotarod rotation speed. Therefore, discrepancies between neuronal structural and functional integrities existed and required further analysis. Potential mediators include ethanol's adverse effects on neuronal functions related to energy metabolism and plasticity. We extended our investigations along these lines using molecular and biochemical studies to measure cerebellar total and phosphoproteins that mediate insulin and IGF signaling, neuronal-glial structural proteins, and neurotrophins.

Previous studies showed that ethanol impairs insulin/IGF/Akt pathway signaling in developing, including adolescent brains [12] [13] [16] [52] [53]. Insulin/IGF-Akt signaling supports a broad range of brain functions, including neuronal growth, survival, development, energy metabolism, migration, and synaptic plasticity [10] [53] [54]. Previous studies have shown that in FASD models, ethanol impairs signaling through the insulin and IGF-1 receptors, IRS, and Akt via alterations in protein expression or protein phosphorylation [15] [33] [43]. However, the severity or extent of these abnormalities is greater with higher levels of ongoing ethanol exposure compared with delayed effects associated with several weeks of ethanol withdrawal in the postnatal period [16] [33] [43]. In the present study, the FASD models were generated using liquid diets containing a moderate (26%) rather than high (37%) caloric ethanol level, and the brains were harvested for study on P35, 5 weeks after ethanol withdrawal. Therefore, the abnormalities detected reflect sustained pathologies resulting from earlier exposures and not reversed by the cessation of ethanol feeding. In the present study, there were no ethanol-specific effects on the expression of signaling proteins, phosphoprotein expression, or the relative levels of signaling protein phosphorylation. In essence, the inhibitory effects of prenatal alcohol exposure on upstream (insulin receptor, IGF-1 receptor) and intermediate (IRS1, Akt, GSK-3β) signaling molecules appeared to have been abrogated by the ethanol withdrawal. Since dietary soy, for the most part, similarly modulated the expression of signaling proteins and phosphoproteins in the control and ethanol groups, it is unlikely that the interventional effects on cerebellar structure and function were mediated via these mechanisms. However, further downstream signaling molecules such as mTOR [55]-[57] and other interconnected pathways, such as Notch [44] [58] and Wnt [13] [18], that were not evaluated, may not have been normalized by ethanol withdrawal or dietary soy, and thereby contributed to the ethanol-related sustained deficits in cerebellar motor function.

The effects of ethanol and soy on the expression of neuronal and glial molecules have not been extensively studied. The developing cerebellum utilizes neuronal and glial structural proteins to support growth, maturation, neuronal plasticity,

myelination, and blood-brain barrier functions. The findings in this study are discussed in relation to the interactive effects of insulin/IGF signaling and oxidative stress since dietary soy targets their related pathologies [27] [45] [59]. Tau, which is abundantly expressed in cerebellar neurons, is bidirectionally regulated with insulin signaling such that insulin signaling regulates the Tau gene [60], and tau regulates insulin signaling [61]. For example, deletion of tau impairs hippocampal response to insulin via the insulin receptor and PTEN [62]. In the present study, ethanol exposure, with or without dietary soy, had significant sustained inhibitory effects on Tau protein expression. Therefore, this long-term effect of prenatal alcohol exposure in the cerebellum was not ameliorated by the insulin-sensitizing effects of dietary soy. Potential consequences include disruption of the neuronal cytoskeletal architecture needed to establish and maintain intercellular connectivity. In contrast, pTau similarly increased with soy dose in both control and ethanol-exposed cerebella. Since the responses were similar in both the control and disease states, the effects of soy were most likely physiological rather than pathological and were mediated by the actions of one or more kinases activated in insulin/IGF signaling networks. Insulin signaling promotes Tau phosphorylation, which is critical to neuronal cytoskeletal assembly and stability [63]. Although tau hyperphosphorylation by GSK-3β can be a feature of brain insulin resistance in various disease states [64] including chronic ethanol exposure [65] [66], the model utilized in these studies had alcohol-off, and the multiplex Akt Pathway ELISA failed to detect aberrantly increased GSK-3 activity. Therefore, the results likely reflect physiological responses to dietary soy enhancement of insulin signaling.

Choline acetyltransferase is utilized to generate acetylcholine, one of the most abundant cerebellar neurotransmitters mediating motor functions. ChAT activity was previously shown to be stimulated by insulin/IGF networks [67] [68] and inhibited by acute or subacute ethanol exposure [12] [69]. However, in this study, the ethanol and dietary soy exposures were limited to the prenatal period. There were no significant residual effects of either ethanol or soy, except at the 100% soy dose, which significantly increased ChAT in the ethanol group. This effect may reflect a supra-normal insulin-sensitizing effect of soy, but further studies are needed to account for that result.

The ubiquitin proteasome system (UPS) targets proteins for proteasomal degradation, including regulatory factors involved in insulin signaling [70]. Mechanistically, the UPS works through inducible ubiquitylation pathways that regulate internalization of insulin/IGF1 receptors and downstream signaling targets. Consequently, ubiquitin has critical roles in regulating insulin secretion and influencing insulin/IGF1 pathways that regulate cell growth, tissue development, and metabolism. On the other hand, insulin sensitizers, including peroxisome-proliferator activated receptor (PPAR) agonists are known to up-regulate the UPS and facilitate intracellular trash removal and minimize protein aggregation [71]. No specific links between soy-mediated increases in ubiquitin immunoreactivity and other molecular or biochemical cerebellar pathologies were identified.

GFAP, a marker of mature, activated astrocytes, was significantly increased by ethanol exposure, corresponding with previous reports [29] [72]. GFAP is stimulated by ciliary neurotrophic factor via the JAK-STAT pathway [73]. Astrocyte activation causes cellular and tissue injury via the elaboration and secretion of proinflammatory cytokines and reactive oxygen species [74]. The persistently elevated GFAP expression in the cerebellum beyond the ethanol exposure period suggests that chronic astrocyte activation contributed to the sustained cerebellar dysfunction, as evidenced by impaired rotarod performance. The failure of dietary soy to consistently suppress ethanol-induced GFAP suggests that GFAP is not responsive to insulin/IGF stimulation.

The developing cerebellum expresses neurotrophins and their receptors. BDNF and NGF interact with tropomyosin receptor kinase (Trk). The binding of BDNF to its TrkB receptor activates signaling important for synaptogenesis in that it stimulates cerebellar neuron axonal and dendritic arborization [75] and survival of differentiated granule cells [76]. BDNF/TrkB signals through PI3K/Akt and MAPK to promote neuronal functions and survival [75], paralleling the effects of insulin/IGF stimulation. NGF functions by binding to TrkA and $p75^{NTR}$, inducing neuronal-type differentiation. In addition, NGF augments glucose-dependent insulin secretion in pancreatic beta cells and islets [77], and glucose stimulates NGF expression. Except for isolated incidences, neither BDNF nor NGF was significantly modulated by dietary soy or ethanol, indicating that long-term adverse effects of prenatal ethanol exposure on cerebellar motor dysfunction are not mediated by impaired expression of these neurotrophins. Further studies are needed to determine whether TrkA or TrkB receptor expression and function were adversely impacted, contributing to the sustained impairments in cerebellar structure and function.

NT3 supports the survival of neurons and neuroblast/neuroprogenitor cells and is upregulated by neuroregulin, PDGF, and CNTF but not IGF1 or NGF [78]. NT4/5 produces structural changes in cortical neurons [79], and like BDNF, it stimulates cerebellar neuron neurite extension and survival of differentiated granule cells [76]. GDNF, a member of transforming growth factor-beta superfamily, cooperates with insulin to enhance cellular proliferation [80], and works through PI3k-Akt. During development, GDNF promotes neural progenitor cell proliferation, migration, differentiation, and survival, and it contributes to synaptic plasticity. Besides neuronal expression during development, GDNF can be upregulated in astrocytes and microglia by injury and inflammation [81]. All three neurotrophins were expressed at the lowest levels in the absence of dietary soy, irrespective of ethanol exposure. Dietary soy caused dose-dependent increases in NT3, NT4/5, and GDNF expression with minimal inhibitory effects of ethanol. These findings highlight the positive effects of soy's insulin-sensitizing, anti-inflammatory, anti-oxidant effects on neurotrophins that have key roles in neuroplasticity, neuronal survival, and neuronal maturation, and suggest that these responses contribute to enhanced neurobehavioral functions, such as performance

on the Morris water maze [16] [18] [82]. Mechanistically, the crosstalk between neurotrophins and insulin/IGF networks likely mediated the stimulatory effects of dietary soy. Altogether, these findings suggest that dietary soy supports cognitive-behavioral-motor functions during development by enhancing neurotrophin expression and function, even after prenatal ethanol exposures that can cause FASD.

5. Conclusions

- This study examined the effects of maternal dietary soy on postnatal cerebellar development in an FASD Long Evans rat model. Alcohol exposure and soy administration were limited to the period of gestation.
- Dietary soy prevented alcohol-related reductions in brain weight and significantly increased cerebellar granule neuron density in ethanol-exposed offspring.
- Despite improvements in cerebellar structure, motor performance was equally impaired in the ethanol-exposed offspring, irrespective of dietary protein dose.
- Postnatal cessation of alcohol exposure abolished many of the abnormalities in insulin/IGF-1/IRS1 pathway signaling that were previously associated with acute or subacute ethanol exposures. The 100% dietary soy diet similarly increased cerebellar expression of insulin and IGF-1 receptors in both the control and ethanol groups, thereby enhancing growth and metabolic signaling, irrespective of ethanol exposure.
- Therapeutic effects of dietary soy on brain structure and function are broad and mediated through enhanced neurotrophin expression. NGF, GDNF, NT3 and NT4 were increased by dietary soy at various dietary concentrations, suggesting their importance as a widely available means of bolstering cognitive and motor performance during development, including in FASD.
- Although Tau and pTau increased with various dietary soy doses irrespective of ethanol exposure, prenatal ethanol exposure significantly muted these effects with respect to Tau, thereby contributing to the long-term inhibitory effects on neuronal architectural integrity. On the other hand, the increased ChAT expression in the ethanol+ 100% dietary soy cerebella marks rescue of cholinergic neurotransmission utilized for motor function. The persistently elevated level GFAP likely reflects sustained astrocytic activation and neuroinflammation as a contributing factor mediating chronic cerebellar hypocellularity and motor dysfunction in FASD.

Author Contributions

Conceptualization, SMdlM; Methodology, SMdlM, MT, JZ, Software, JZ, SMdlM; Validation, MT; Formal Analysis, SMdlM, MT, JZ, Investigation, MT, JZ; Resources, SMdlM; Data Curation, MT, JZ; Writing original/initial draft, SMdlM, JZ.; Writing Final version, Review & Editing, SMdlM; Visualization, SMdlM; Supervision, MT, SMdlM; Project Administration, SMdlM, MT; Funding Acquisi-

tion, SMdlM. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health, grants R01AA011431, R01AA028408, and R01AA032106. The APC was funded by R01AA011431.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee (IACUC) at Lifespan/Brown University Health (Protocol #500221, initially approved 03/15/2021). The protocol adheres to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Data Availability Statement

The data underlying this article will be shared at reasonable request to the corresponding author.

Acknowledgements

The authors have reviewed and edited the output and take full responsibility for the content of this publication. The authors thank Donna Palange and Rolf Carlson for administrative support throughout this research.

Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

[1] Nayak, R.B. and Murthy, P. (2008) Fetal Alcohol Spectrum Disorder. *Indian Journal of Pediatrics*, **45**, 977-983.

[2] Osborn, J.A., Harris, S.R. and Weinberg, J. (1993) Fetal Alcohol Syndrome: Review of the Literature with Implications for Physical Therapists. *Physical Therapy*, **73**, 599-607. https://doi.org/10.1093/ptj/73.9.599

[3] Gomez, D.A. and Abdul-Rahman, O.A. (2021) Fetal Alcohol Spectrum Disorders: Current State of Diagnosis and Treatment. *Current Opinion in Pediatrics*, **33**, 570-575. https://doi.org/10.1097/mop.0000000000001071

[4] Riley, E.P. and McGee, C.L. (2005) Fetal Alcohol Spectrum Disorders: An Overview with Emphasis on Changes in Brain and Behavior. *Experimental Biology and Medicine*, **230**, 357-365. https://doi.org/10.1177/15353702-0323006-03

[5] Blanck-Lubarsch, M., Dirksen, D., Feldmann, R. and Hohoff, A. (2023) A Systematic Review: Facial, Dental and Orthodontic Findings and Orofacial Diagnostics in Patients with FASD. *Frontiers in Pediatrics*, **11**, Article ID: 1169570.

https://doi.org/10.3389/fped.2023.1169570

[6] Wozniak, J.R., Riley, E.P. and Charness, M.E. (2019) Clinical Presentation, Diagnosis, and Management of Fetal Alcohol Spectrum Disorder. *The Lancet Neurology*, **18**, 760-770. https://doi.org/10.1016/s1474-4422(19)30150-4

[7] Janczewska, I., Wierzba, J., Cichon-Kotek, M. and Janczewska, A. (2019) Fetal Alcohol Spectrum Disorders-Diagnostic Difficulties in the Neonatal Period and New Diagnostic Approaches. *Developmental Period Medicine*, **23**, 60-66.

[8] Lange, S., Probst, C., Gmel, G., Rehm, J., Burd, L. and Popova, S. (2017) Global Prevalence of Fetal Alcohol Spectrum Disorder among Children and Youth. *JAMA Pediatrics*, **171**, 948-956. https://doi.org/10.1001/jamapediatrics.2017.1919

[9] Qi, W., Gundogan, F., Gilligan, J. and Monte, S.D.L. (2023) Dietary Soy Prevents Fetal Demise, Intrauterine Growth Restriction, Craniofacial Dysmorphic Features, and Impairments in Placentation Linked to Gestational Alcohol Exposure: Pivotal Role of Insulin and Insulin-Like Growth Factor Signaling Networks. *Alcohol*, **110**, 65-81. https://doi.org/10.1016/j.alcohol.2023.01.006

[10] Adamo, M., Raizada, M.K. and LeRoith, D. (1989) Insulin and Insulin-Like Growth Factor Receptors in the Nervous System. *Molecular Neurobiology*, **3**, 71-100. https://doi.org/10.1007/bf02935589

[11] Schubert, M., Brazil, D.P., Burks, D.J., Kushner, J.A., Ye, J., Flint, C.L., *et al*. (2003) Insulin Receptor Substrate-2 Deficiency Impairs Brain Growth and Promotes Tau Phosphorylation. *The Journal of Neuroscience*, **23**, 7084-7092. https://doi.org/10.1523/jneurosci.23-18-07084.2003

[12] Soscia, S.J., Tong, M., Xu, X.J., Cohen, A.C., Chu, J., Wands, J.R., *et al*. (2006) Chronic Gestational Exposure to Ethanol Causes Insulin and IGF Resistance and Impairs Acetylcholine Homeostasis in the Brain. *Cellular and Molecular Life Sciences CMLS*, **63**, 2039-2056. https://doi.org/10.1007/s00018-006-6208-2

[13] Tong, M., Ziplow, J., Chen, W.C., Nguyen, Q.G., Kim, C. and de la Monte, S.M. (2013) Motor Function Deficits Following Chronic Prenatal Ethanol Exposure Are Linked to Impairments in Insulin/IGF, Notch and Wnt Signaling in the Cerebellum. *Journal of Diabetes & Metabolism*, **4**, Article No. 238. https://doi.org/10.4172/2155-6156.1000238

[14] Andreani, T., Tong, M., Gundogan, F., Silbermann, E. and de la Monte, S.M. (2016) Differential Effects of 3rd Trimester-Equivalent Binge Ethanol and Tobacco-Specific Nitrosmine Ketone Exposures on Brain Insulin Signaling in Adolescence. *Journal of Diabetes Related Disorders*, **1**, 105-114.

[15] Ewenczyk, A., Ziplow, J., Tong, M. and de la Monte, S.M. (2012) Sustained Impairments in Brain Insulin/IGF Signaling in Adolescent Rats Subjected to Binge Alcohol Exposure during Development. *Journal of Clinical & Experimental Pathology*, **2**, Article No. 106. https://doi.org/10.4172/2161-0681.1000106

[16] Tong, M., Ziplow, J.L., Mark, P. and de la Monte, S.M. (2022) Dietary Soy Prevents Alcohol-Mediated Neurocognitive Dysfunction and Associated Impairments in Brain Insulin Pathway Signaling in an Adolescent Rat Model. *Biomolecules*, **12**, Article 676. https://doi.org/10.3390/biom12050676

[17] de la Monte, S.M., Tong, M., Ziplow, J., Mark, P., Van, S. and Nguyen, V.A. (2025) Impact of Prenatal Dietary Soy on Cerebellar Neurodevelopment and Function in Experimental Fetal Alcohol Spectrum Disorder. *Nutrients*, **17**, Article 812. https://doi.org/10.3390/nu17050812

[18] de la Monte, S.M., Tong, M., Ziplow, J., Mark, P., Van, S. and Nguyen, V.A. (2025) Dietary Soy Preserves Cognitive Function in Experimental Fetal Alcohol Spectrum

Disorder: Role of Increased Signaling through Notch and Gonadotropin Releasing Hormone Networks. *Journal of Behavioral and Brain Science*, **15**, 11-46. https://doi.org/10.4236/jbbs.2025.152002

[19] Khalil, D.A., Lucas, E.A., Juma, S., Smith, B.J., Payton, M.E. and Arjmandi, B.H. (2002) Soy Protein Supplementation Increases Serum Insulin-Like Growth Factor-I in Young and Old Men but Does Not Affect Markers of Bone Metabolism. *The Journal of Nutrition*, **132**, 2605-2608. https://doi.org/10.1093/jn/132.9.2605

[20] George, K.S., Muñoz, J., Akhavan, N.S., Foley, E.M., Siebert, S.C., Tenenbaum, G., *et al*. (2020) Is Soy Protein Effective in Reducing Cholesterol and Improving Bone Health? *Food & Function*, **11**, 544-551. https://doi.org/10.1039/c9fo01081e

[21] Mezei, O., Shay, N., Banz, W.J., Steger, R.W., Peluso, M.R. and Winters, T.A. (2003) Soy Isoflavones Exert Antidiabetic and Hypolipidemic Effects through the PPAR Pathways in Obese Zucker Rats and Murine RAW 264.7 Cells. *The Journal of Nutrition*, **133**, 1238-1243. https://doi.org/10.1093/jn/133.5.1238

[22] Wagner, J.D., Zhang, L., Shadoan, M.K., Kavanagh, K., Chen, H., Tresnasari, K., *et al*. (2008) Effects of Soy Protein and Isoflavones on Insulin Resistance and Adiponectin in Male Monkeys. *Metabolism*, **57**, S24-S31. https://doi.org/10.1016/j.metabol.2008.04.001

[23] Ronis, M.J., Chen, Y., Badeaux, J. and Badger, T.M. (2009) Dietary Soy Protein Isolate Attenuates Metabolic Syndrome in Rats via Effects on PPAR, LXR, and SREBP Signaling. *The Journal of Nutrition*, **139**, 1431-1438. https://doi.org/10.3945/jn.109.107029

[24] Bansal, N. and Parle, M. (2010) Soybean Supplementation Helps Reverse Age- and Scopolamine-Induced Memory Deficits in Mice. *Journal of Medicinal Food*, **13**, 1293-1300. https://doi.org/10.1089/jmf.2010.1132

[25] Hassan, S.M. (2013) Soybean, Nutrition and Health. In: El-Shemy, H.A., Ed., *Soybean—Bioactive Compounds*, IntechOpen. https://doi.org/10.5772/54545

[26] Lu, C., Wang, Y., Wang, D., Zhang, L., Lv, J., Jiang, N., *et al*. (2018) Neuroprotective Effects of Soy Isoflavones on Scopolamine-Induced Amnesia in Mice. *Nutrients*, **10**, Article 853. https://doi.org/10.3390/nu10070853

[27] Lu, C., Wei, Z., Jiang, N., Chen, Y., Wang, Y., Li, S., *et al*. (2022) Soy Isoflavones Protects against Cognitive Deficits Induced by Chronic Sleep Deprivation via Alleviating Oxidative Stress and Suppressing Neuroinflammation. *Phytotherapy Research*, **36**, 2072-2080. https://doi.org/10.1002/ptr.7354

[28] Li, W., Wei, X., Zhang, J., Wang, L., Chen, L., Li, F., *et al*. (2026) Soy Foods Consumption and Multiple Health Outcomes: An Umbrella Review of Meta-Analyses. *Molecular Nutrition & Food Research*, **70**, e70255. https://doi.org/10.1002/mnfr.70255

[29] de la Monte, S.M., Tong, M. and Delikkaya, B. (2023) Differential Early Mechanistic Frontal Lobe Responses to Choline Chloride and Soy Isoflavones in an Experimental Model of Fetal Alcohol Spectrum Disorder. *International Journal of Molecular Sciences*, **24**, Article 7595. https://doi.org/10.3390/ijms24087595

[30] de la Monte, S.M., Elgas, E., Tong, M., Delikkaya, B. and Yang, Y. (2023) Differential Rescue Effects of Choline Chloride and Soy Isolate on Metabolic Dysfunction in Immature Central Nervous System Neurons: Relevance to Fetal Alcohol Spectrum Disorder. *Diabetes Management*, **13**, 107-118.

[31] Lewis, P.D. (1985) Neuropathological Effects of Alcohol on the Developing Nervous System. *Alcohol and Alcoholism*, **20**, 195-200.

[32] Cealie, M.Y., Douglas, J.C., Swan, H.K., Vonkaenel, E.D., McCall, M.N., Drew, P.D.,

et al. (2024) Developmental Ethanol Exposure Impacts Purkinje Cells but Not Microglia in the Young Adult Cerebellum. *Cells*, **13**, Article 386. https://doi.org/10.3390/cells13050386

[33] de la Monte, S.M., Tong, M., Carlson, R.I., Carter, J.J., Longato, L., Silbermann, E., *et al*. (2009) Ethanol Inhibition of Aspartyl-Asparaginyl-β-Hydroxylase in Fetal Alcohol Spectrum Disorder: Potential Link to the Impairments in Central Nervous System Neuronal Migration. *Alcohol*, **43**, 225-240. https://doi.org/10.1016/j.alcohol.2008.09.009

[34] Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., *et al*. (1997) Regulation of Neuronal Survival by the Serine-Threonine Protein Kinase Akt. *Science*, **275**, 661-665. https://doi.org/10.1126/science.275.5300.661

[35] Vinters, H.V., Gatti, R.A. and Rakic, P. (1985) Sequence of Cellular Events in Cerebellar Ontogeny Relevant to Expression of Neuronal Abnormalities in Ataxia-Telangiectasia. *Kroc Foundation Series*, **19**, 233-255.

[36] Tong, M., Dominguez, C., Didsbury, J. and de la Monte, S.M. (2016) Targeting Alzheimer's Disease Neuro-Metabolic Dysfunction with a Small Molecule Nuclear Receptor Agonist (T3D-959) Reverses Dise Ase Pathologies. *Journal of Alzheimer's Disease & Parkinsonism*, **6**, Article No. 238. https://doi.org/10.4172/2161-0460.1000238

[37] Gundogan, F., Elwood, G., Longato, L., Tong, M., Feijoo, A., Carlson, R.I., *et al*. (2008) Impaired Placentation in Fetal Alcohol Syndrome. *Placenta*, **29**, 148-157. https://doi.org/10.1016/j.placenta.2007.10.002

[38] Tong, M., Gonzalez-Navarrete, H., Kirchberg, T., Gotama, B., Yalcin, E.B., Kay, J., *et al*. (2017) Ethanol-Induced White Matter Atrophy Is Associated with Impaired Expression of Aspartyl-Asparaginyl-β-Hydroxylase (ASPH) and Notch Signaling in an Experimental Rat Model. *Journal of Drug and Alcohol Research*, **6**, 1-12. https://doi.org/10.4303/jdar/236033

[39] Le, T., Tong, M., Nguyen, V. and de la Monte, S.M. (2013) PPAR Agonist Rescue of Ethanol-Impaired Brain Insulin Signaling: Cerebellar Slice Culture Model. *Journal of Drug and Alcohol Research*, **2**, 1-9. https://doi.org/10.4303/jdar/235611

[40] Stern-Straeter, J., Bonaterra, G.A., Hörmann, K., Kinscherf, R. and Goessler, U.R. (2009) Identification of Valid Reference Genes during the Differentiation of Human Myoblasts. *BMC Molecular Biology*, **10**, Article No. 66. https://doi.org/10.1186/1471-2199-10-66

[41] Cohen, A.C., Tong, M., Wands, J.R. and De La Monte, S.M. (2007) Insulin and Insulin-like Growth Factor Resistance with Neurodegeneration in an Adult Chronic Ethanol Exposure Model. *Alcoholism*: *Clinical and Experimental Research*, **31**, 1558-1573. https://doi.org/10.1111/j.1530-0277.2007.00450.x

[42] Xu, J., Yeon, J.E., Chang, H., Tison, G., Chen, G.J., Wands, J., *et al*. (2003) Ethanol Impairs Insulin-Stimulated Neuronal Survival in the Developing Brain. *Journal of Biological Chemistry*, **278**, 26929-26937. https://doi.org/10.1074/jbc.m300401200

[43] Zabala, V., Silbermann, E., Re, E., Andreani, T., Tong, M., Ramirez, T., *et al*. (2016) Potential Co-Factor Role of Tobacco Specific Nitrosamine Exposures in the Pathogenesis of Fetal Alcohol Spectrum Disorder. *Gynecology and Obstetrics Research - Open Journal*, **2**, 112-125. https://doi.org/10.17140/goroj-2-125

[44] Silbermann, E., Moskal, P., Bowling, N., Tong, M. and de la Monte, S.M. (2010) Role of Aspartyl-(Asparaginyl)-β-Hydroxylase Mediated Notch Signaling in Cerebellar Development and Function. *Behavioral and Brain Functions*, **6**, Article No. 68. https://doi.org/10.1186/1744-9081-6-68

[45] Clark, J., Taylor, C. and Zahradka, P. (2018) Rebelling against the (Insulin) Re-

sistance: A Review of the Proposed Insulin-Sensitizing Actions of Soybeans, Chickpeas, and Their Bioactive Compounds. *Nutrients*, **10**, Article 434. https://doi.org/10.3390/nu10040434

[46] Cantacorps, L., Montagud-Romero, S. and Valverde, O. (2020) Curcumin Treatment Attenuates Alcohol-Induced Alterations in a Mouse Model of Foetal Alcohol Spectrum Disorders. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, **100**, Article 109899. https://doi.org/10.1016/j.pnpbp.2020.109899

[47] Rafaiee, R., Mohseni, F., Khaksari, M., Garmabi, B., Masoudi, A., Jamali, Z., *et al*. (2025) Erythropoietin Improves Spatial and Nonspatial Memory Defects by Suppressing Oxidative Damage, Inflammation and Apoptosis against Ethanol Neurotoxicity in the Developing Male Rat Hippocampus. *Peptides*, **186**, Article 171368. https://doi.org/10.1016/j.peptides.2025.171368

[48] Rocamora, N., García-Ladona, F.J., Palacios, J.M. and Mengod, G. (1993) Differential Expression of Brain-Derived Neurotrophic Factor, Neurotrophin-3, and Low-Affinity Nerve Growth Factor Receptor during the Postnatal Development of the Rat Cerebellar System. *Molecular Brain Research*, **17**, 1-8. https://doi.org/10.1016/0169-328x(93)90065-w

[49] Kim, M., Jun, S., Park, H., Tanaka-Yamamoto, K. and Yamamoto, Y. (2023) Regulation of Cerebellar Network Development by Granule Cells and Their Molecules. *Frontiers in Molecular Neuroscience*, **16**, Article ID: 1236015. https://doi.org/10.3389/fnmol.2023.1236015

[50] West, J.R., Parnell, S.E., Chen, W.A. and Cudd, T.A. (2001) Alcohol-Mediated Purkinje Cell Loss in the Absence of Hypoxemia during the Third Trimester in an Ovine Model System. *Alcoholism: Clinical and Experimental Research*, **25**, 1051-1057. https://doi.org/10.1111/j.1530-0277.2001.tb02316.x

[51] Hull, C. and Regehr, W.G. (2022) The Cerebellar Cortex. *Annual Review of Neuroscience*, **45**, 151-175. https://doi.org/10.1146/annurev-neuro-091421-125115

[52] De La Monte, S.M., Yeon, J., Tong, M., Longato, L., Chaudhry, R., Pang, M., *et al*. (2008) Insulin Resistance in Experimental Alcohol-Induced Liver Disease. *Journal of Gastroenterology and Hepatology*, **23**, e477-e486. https://doi.org/10.1111/j.1440-1746.2008.05339.x

[53] de la Monte, S.M. and Wands, J.R. (2005) Review of Insulin and Insulin-Like Growth Factor Expression, Signaling, and Malfunction in the Central Nervous System: Relevance to Alzheimer's Disease. *Journal of Alzheimer's Disease*, **7**, 45-61. https://doi.org/10.3233/jad-2005-7106

[54] Kido, Y., Nakae, J. and Accili, D. (2001) The Insulin Receptor and Its Cellular Targets. *Journal of Clinical Endocrinology & Metabolism*, **86**, 972-979. https://doi.org/10.1210/jc.86.3.972

[55] Srivastava, V.K., Hiney, J.K. and Dees, W.L. (2018) Alcohol Delays the Onset of Puberty in the Female Rat by Altering Key Hypothalamic Events. *Alcoholism: Clinical and Experimental Research*, **42**, 1166-1176. https://doi.org/10.1111/acer.13762

[56] Yalcin, E.B., Tong, M., Delikkaya, B., Pelit, W., Yang, Y. and de la Monte, S.M. (2024) Differential Effects of Moderate Chronic Ethanol Consumption on Neurobehavior, White Matter Glial Protein Expression, and mTOR Pathway Signaling with Adolescent Brain Maturation. *The American Journal of Drug and Alcohol Abuse*, **50**, 492-516. https://doi.org/10.1080/00952990.2024.2355540

[57] Tong, M., Homans, C., Pelit, W., Delikkaya, B. and de la Monte, S.M. (2025) Progressive Alcohol-Related Brain Atrophy and White Matter Pathology Are Linked to Long-Term Inhibitory Effects on mTOR Signaling. *Biomolecules*, **15**, Article 413.

https://doi.org/10.3390/biom15030413

[58] Gundogan, F., Gilligan, J., Qi, W., Chen, E., Naram, R. and de la Monte, S.M. (2015) Dose Effect of Gestational Ethanol Exposure on Placentation and Fetal Growth. *Placenta*, **36**, 523-530. https://doi.org/10.1016/j.placenta.2015.02.010

[59] Ariyani, W. and Koibuchi, N. (2024) The Effect of Soy Isoflavones in Brain Development: The Emerging Role of Multiple Signaling Pathways and Future Perspectives. *Endocrine Journal*, **71**, 317-333. https://doi.org/10.1507/endocrj.ej23-0314

[60] Gonçalves, R.A., Wijesekara, N., Fraser, P.E. and De Felice, F.G. (2019) The Link between Tau and Insulin Signaling: Implications for Alzheimer's Disease and Other Tauopathies. *Frontiers in Cellular Neuroscience*, **13**, Article ID: 17. https://doi.org/10.3389/fncel.2019.00017

[61] Gratuze, M. and Planel, E. (2017) Regulation of Brain Insulin Signaling: A New Function for Tau. *Journal of Experimental Medicine*, **214**, 2171-2173. https://doi.org/10.1084/jem.20170979

[62] Marciniak, E., Leboucher, A., Caron, E., Ahmed, T., Tailleux, A., Dumont, J., *et al*. (2017) Tau Deletion Promotes Brain Insulin Resistance. *Journal of Experimental Medicine*, **214**, 2257-2269. https://doi.org/10.1084/jem.20161731

[63] Parra Bravo, C., Naguib, S.A. and Gan, L. (2024) Cellular and Pathological Functions of Tau. *Nature Reviews Molecular Cell Biology*, **25**, 845-864. https://doi.org/10.1038/s41580-024-00753-9

[64] Morales-Corraliza, J., Wong, H., Mazzella, M.J., Che, S., Lee, S.H., Petkova, E., *et al*. (2016) Brain-Wide Insulin Resistance, Tau Phosphorylation Changes, and Hippocampal Neprilysin and Amyloid-β Alterations in a Monkey Model of Type 1 Diabetes. *The Journal of Neuroscience*, **36**, 4248-4258. https://doi.org/10.1523/jneurosci.4640-14.2016

[65] Tong, M., Yu, R., Deochand, C. and de la Monte, S.M. (2015) Differential Contributions of Alcohol and the Nicotine-Derived Nitrosamine Ketone (NNK) to Insulin and Insulin-Like Growth Factor Resistance in the Adolescent Rat Brain. *Alcohol and Alcoholism*, **50**, 670-679. https://doi.org/10.1093/alcalc/agv101

[66] Tucker, A.E., Alicea Pauneto, C.D.M., Barnett, A.M. and Coleman, L.G. (2022) Chronic Ethanol Causes Persistent Increases in Alzheimer's Tau Pathology in Female 3xtg-Ad Mice: A Potential Role for Lysosomal Impairment. *Frontiers in Behavioral Neuroscience*, **16**, Article ID: 886634. https://doi.org/10.3389/fnbeh.2022.886634

[67] Kyriakis, J.M., Hausman, R.E. and Peterson, S.W. (1987) Insulin Stimulates Choline Acetyltransferase Activity in Cultured Embryonic Chicken Retina Neurons. *Proceedings of the National Academy of Sciences*, **84**, 7463-7467. https://doi.org/10.1073/pnas.84.21.7463

[68] Fishwick, K.J. and Rylett, R.J. (2015) Insulin Regulates the Activity of the High-Affinity Choline Transporter CHT. *PLOS ONE*, **10**, e0132934. https://doi.org/10.1371/journal.pone.0132934

[69] Re, E., Tong, M. and De la Monte, S.M. (2016) Tobacco Nitrosamine Exposures Contribute to Fetal Alcohol Spectrum Disorder Associated Cerebellar Dysgenesis. *International Journal of Biology*, **8**, 10-21. https://doi.org/10.5539/ijb.v8n3p10

[70] Balaji, V., Pokrzywa, W. and Hoppe, T. (2018) Ubiquitylation Pathways in Insulin Signaling and Organismal Homeostasis. *BioEssays*, **40**, e1700223. https://doi.org/10.1002/bies.201700223

[71] Schoonjans, K., Staels, B. and Auwerx, J. (1996) Role of the Peroxisome Proliferator-Activated Receptor (PPAR) in Mediating the Effects of Fibrates and Fatty Acids on

Gene Expression. *Journal of Lipid Research*, **37**, 907-925. https://doi.org/10.1016/s0022-2275(20)42003-6

[72] Dalçik, H., Yardimoglu, M., Fi˙li˙z, S., Gonca, S., Dalçik, C. and Erden, B.F. (2009) Chronic Ethanol-Induced Glial Fibrillary Acidic Protein (GFAP) Immunoreactivity: An Immunocytochemical Observation in Various Regions of Adult Rat Brain. *International Journal of Neuroscience*, **119**, 1303-1318. https://doi.org/10.1080/00207450802333672

[73] Wang, Y., Smith, S.B., Ogilvie, J.M., McCool, D.J. and Sarthy, V. (2002) Ciliary Neurotrophic Factor Induces Glial Fibrillary Acidic Protein in Retinal Müller Cells through the JAK/STAT Signal Transduction Pathway. *Current Eye Research*, **24**, 305-312. https://doi.org/10.1076/ceyr.24.4.305.8408

[74] Topper, L.A., Baculis, B.C. and Valenzuela, C.F. (2015) Exposure of Neonatal Rats to Alcohol Has Differential Effects on Neuroinflammation and Neuronal Survival in the Cerebellum and Hippocampus. *Journal of Neuroinflammation*, **12**, Article No. 160. https://doi.org/10.1186/s12974-015-0382-9

[75] Wang, C.S., Kavalali, E.T. and Monteggia, L.M. (2022) BDNF Signaling in Context: From Synaptic Regulation to Psychiatric Disorders. *Cell*, **185**, 62-76. https://doi.org/10.1016/j.cell.2021.12.003

[76] Gao, W., Zheng, J. and Karihaloo, M. (1995) Neurotrophin-4/5 (NT-4/5) and Brain-Derived Neurotrophic Factor (BDNF) Act at Later Stages of Cerebellar Granule Cell Differentiation. *The Journal of Neuroscience*, **15**, 2656-2667. https://doi.org/10.1523/jneurosci.15-04-02656.1995

[77] Houtz, J., Borden, P., Ceasrine, A., Minichiello, L. and Kuruvilla, R. (2016) Neurotrophin Signaling Is Required for Glucose-Induced Insulin Secretion. *Developmental Cell*, **39**, 329-345. https://doi.org/10.1016/j.devcel.2016.10.003

[78] Verdi, J.M., Groves, A.K., Fariñas, I., Jones, K., Marchionni, M.A., Reichardt, L.F., *et al*. (1996) A Reciprocal Cell-Cell Interaction Mediated by NT-3 and Neuregulins Controls the Early Survival and Development of Sympathetic Neuroblasts. *Neuron*, **16**, 515-527. https://doi.org/10.1016/s0896-6273(00)80071-9

[79] Antonini, A., Harris, S.L. and Stryker, M.P. (2023) Neurotrophin NT-4/5 Promotes Structural Changes in Neurons of the Developing Visual Cortex. bioRxiv 2023.

[80] Focke, P.J., Swetlik, A.R., Schilz, J.L. and Epstein, M.L. (2003) GDNF and Insulin Cooperate to Enhance the Proliferation and Differentiation of Enteric Crest-Derived Cells. *Journal of Neurobiology*, **55**, 151-164. https://doi.org/10.1002/neu.10204

[81] Duarte Azevedo, M., Sander, S. and Tenenbaum, L. (2020) GDNF, a Neuron-Derived Factor Upregulated in Glial Cells during Disease. *Journal of Clinical Medicine*, **9**, Article 456. https://doi.org/10.3390/jcm9020456

[82] Zeng, X., Wu, M., Cai, Y., Chen, H., *et al*. (2026) Prenatal Alcohol Exposure Impairs Offspring Cognition through Oxidative Stress Disrupting CREB/BDNF/TrkB Signaling and GABAergic Neuron Deficits. *Food and Chemical Toxicology*, **207**, Article 115820. https://doi.org/10.1016/j.fct.2025.115820

Appendix

Table A1. Critical reagents.

Material/Reagent/Equipment	Source	Company Location
Materials and Instruments		
Spectra-Max M5 Multimode Plate Reader	Molecular Devices	Sunnyvale, CA, USA
MaxiSorp 96-well plates	Thermo-Fisher Scientific	Bedford, MA, USA
Luminex MAGPIX Instrument	Diasorin	Austin, TX, USA
TissueLyser II Instrument	Qiagen	Germantown, MD, USA
Cellular and Immunoassay Reagents		
Bicinchoninic Acid Assay Reagents	Thermo-Fisher Scientific	Bedford, MA, USA
Superblock TBS	Thermo-Fisher Scientific	Bedford, MA, USA
Horseradish peroxidase-conjugated secondary antibodies	Thermo-Fisher Scientific	Bedford, MA, USA
Amplex UltraRed Soluble Fluorophore	Life Technologies	Carlsbad, CA, USA
Commercial Kits and Reagents for Multiplex Assays		
Total Akt Multiplex ELISA	MilliporeSigma	Burlington, MA, USA
Phospho-Akt Multiplex ELISA	MilliporeSigma	Burlington, MA, USA

Table A2. Multiplex Akt pathway (proteins and phosphoproteins).

Akt/Pathway Molecules	Protein Abbreviation	Phospho-protein
Insulin Receptor	Insulin-R	$^{pYpY1162/1163}$-Insulin R
Insulin-Like Growth Factor Receptor Type 1	IGF-1R	$^{pYpY1135/1136}$-IGF-1R
Insulin Receptor Substrate, Type 1	IRS1	^{pS636}-IRS-1
Akt (Protein Kinase B)	Akt	^{pS473}-Akt
Glycogen Synthase Kinase 3β	GSK-3β	^{pS9}-GSK3β

Table A3. Antibodies used in duplex and cellular ELISAs.

Antibody Targets*	Antibody Type	Final Concentration (µg/mL)	Commercial Source	Company Location	Catalogue#	RRID
Tau (Tubulin-associated unit)	Rabbit	3.1	Agilent/Dako, Santa Clara, CA	Santa Clara, CA, USA	REF A0024; Lot# 00033544	AB_10013724
pTau (PHF; S396) (Phosphorylated Tubulin associated unit)	Mouse	0.40	Cell Signaling	Danvers, MA, USA	#9632	AB_2266237
Ubiquitin	Rabbit	0.5	Abcam	Boston, MA, USA	ab7780	AB_306069
ChAT (choline acetyltransferase)	Rabbit	1:3000	Abcam	Boston, MA, USA	ab6168	AB_2244866
GFAP (glial fibrillary acidic protein)	Goat	0.5	Abcam	Boston, MA, USA	ab53554	AB_880202

Continued

BDNF (brain-derived neurotrophic factor)	Sheep	1.0	CHEMICON International	Temecula, CA, USA	AB1513P	AB_2064329
NGF β (nerve growth factor beta)	Sheep	1.0	CHEMICON International	Temecula, CA, USA	AB1528SP	AB_2893049
GDNF (glial-derived neurotrophic factor)	Chicken	1.0	Abcam	Boston, MA, USA	ab28956	AB_732566
NT3 (neurotrophin 3)	Sheep	1.0	CHEMICON International	Temecula, CA, USA	AB1517P	AB_2064366
NT4 (neurotrophin 4)	Sheep	1.0	CHEMICON International	Temecula, CA, USA	AB1519P	AB_2064371
RPLPO (acidic ribosomal phosphoprotein PO)	Rabbit	0.1	Prointech Group, Inc.	Chicago, IL, USA	sc-293260	RPL23 16086-1-AP

RRID = Research Resource Identifier.