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Research Article

PPAR-δ Agonist Rescue of Brain Insulin/IGF Signaling Impairments Following Developmental Exposure to Alcohol

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Abstract *Background.* Neurodevelopmental abnormalities in fetal alcohol spectrum disorder (FASD) are associated with impaired insulin and IGF signaling, which is needed for neuronal growth, survival, and plasticity. We characterized effects of the L-165,041 PPAR-δ agonist insulin sensitizer on cerebellar structure and function in relation to insulin/IGF and neurotrophin signaling in an FASD model. *Methods.* On postnatal days (P) 2, 4, 6, and 8, rat pups were administered (i.p.) 2 g/kg of ethanol or saline; and on P5, P7, P9, and P11, they were treated with saline or L-165,041. Rotarod tests assessed motor function. Cerebella were studied biochemically. *Results.* Ethanol-impaired motor function and signaling through the insulin receptor and Akt were abrogated by PPAR-δ agonist treatments, whereas neurotrophin expression was unaffected. Conclusions: PPAR-δ agonists may help normalize cerebellar function in FASD by supporting insulin signaling through cell survival pathways, but additional approaches are needed to restore neurotrophin expression for neuronal plasticity.

Keywords insulin resistance; fetal alcohol spectrum disorder; insulin sensitizers; PPAR agonists; neurotrophins; cerebellum

1. Introduction

Alcohol abuse during pregnancy causes fetal alcohol spectrum disorder (FASD), which results in long-term deficits in cognitive and motor functions [48,49,63]. Ethanol mediates its adverse effects on the immature brain, in part, by inhibiting insulin and insulin-like growth factor (IGF) signaling [17]. These defects occur at multiple levels in the signaling cascades, beginning with receptor binding, which is critical for activating the intrinsic receptor tyrosine kinases (RTKs), and extending to distal regulators of growth, survival, metabolism, neuronal migration, and plasticity [4,9,10,28,62,79]. With regard to the signaling cascades, ethanol (1) inhibits phosphorylation and activation of insulin and IGF-1 RTKs, and their immediate downstream effector molecules, including insulin receptor substrate (IRS) proteins [51,77]; (2) inhibits phosphotidyl-inositol-3-kinase (PI3K) and Akt, and activates glycogen synthase kinase 3β (GSK-3β) [17,29,51,73,77]; and (3) increases phosphatases that negatively regulate RTKs (PTP-1b) and PI3K (PTEN) [29,77,78]. Akt promotes cell survival, migration, energy metabolism, and neuronal plasticity; and it inhibits GSK-3β, which increases cellular stress and apoptosis [23]. Therefore, ethanol inhibition of insulin/IGF signaling could account for many of the neurodevelopmental abnormalities in FASD, including microcephaly, mental retardation, motor impairments, and attention/learning deficits.

Ethanol-mediated impairments in insulin and IGF-1 signaling are tantamount to the insulin and/or IGF resistance that occurs in type 2 diabetes mellitus and non-alcoholic fatty liver disease. Besides insulin/IGF resistance, ethanol exerts neurotoxic effects, either directly or through acetaldehyde formation [76]. Neurotoxic effects of ethanol lead to increased oxidative stress, DNA damage, mitochondrial dysfunction, neuro-inflammation, and apoptosis [6,7,15,17]. Therefore, in brains with FASD, cell loss and impaired functions are likely mediated by the combined effects of insulin/IGF resistance and neurotoxic injury. Correspondingly, prevention of FASD's long-term effects will require both arms of the equation to be addressed. This concept led us to consider using peroxisome proliferator activated receptor (PPAR) agonists to rescue the functional deficits in FASD.

PPARs are nuclear hormone receptors that function as transcription factors to regulate gene transcription [26,36,39]. The three major PPAR isoforms include PPAR-α, β/δ, and γ. PPAR-α is abundantly expressed in liver, adipose tissue, muscle, and kidney. PPAR-β/δ is highly expressed in brain, and PPAR-γ is widely expressed throughout the body. Agonist binding causes PPARs to heterodimerize with retinoid x receptors [26,36,39]. The resulting complex regulates target genes by binding to peroxisome proliferator hormone response elements in DNA promoters [26,36,39]. Physiological effects of PPAR signaling include

cellular differentiation and energy metabolism. Enhanced insulin sensitivity imparted by PPAR-γ agonists led to their use in diabetes mellitus [66] and non-alcoholic fatty liver disease [61]. Since PPAR agonists also have anti-inflammatory and antioxidant effects [17,21,25,27,72], these compounds could be exploited to restore insulin responsiveness and also reduce neurotoxic injury caused by ethanol exposure, including in FASD.

Previously, we showed that PPAR-α, PPAR-δ, and PPAR-γ agonists could help restore liver structure and function in experimental alcohol-induced steatohepatitis; however, the PPAR-δ and PPAR-γ agonists were more effective than PPAR-α [11]. Herein, we characterize the therapeutic effects of the L-165,041 PPAR-δ agonist in relation to motor deficits and cerebellar insulin/IGF resistance caused by early postnatal binge ethanol exposures (2 g/kg, i.p. on postnatal days 2, 4, 6, and 8). We also examined the effects of L-165,041 on ethanol-impaired neurotrophin expression, because neurotrophin signaling mediates neuronal plasticity during development [31,42, 47,57]. This study focuses on the therapeutic effects of a PPAR-δ agonist because PPAR-δ is the most abundantly expressed PPAR isoform in brain [17], and the L-165,041, PPAR-δ agonist was previously shown to be effective for restoring brain and liver structure and function in other experimental models of insulin resistance [14,38,58].

2. Methods

2.1. Early postnatal binge ethanol exposure model

Long-Evans rat pups were administered intraperitoneal (i.p.) injections (50 μL) of sterile saline or 2 g/kg ethanol (in saline) on postnatal days (P) 2, 4, 6, and 8 [20,41, 71]. Blood alcohol levels 30 min after injection ranged from 152 to 305 g/dL, which was within the range we regularly observe in adult rats administered ethanol by the same dose and route. Pups were further treated with i.p. vehicle (saline) or the L-165,041 PPAR-δ agonist (2 μg/Kg) on P5, P7, P9, and P11. The PPAR dose, route of administration, and treatment frequency were based on prior studies demonstrating the effectiveness of this approach for restoring insulin responsiveness following ethanol exposure [11,58]. The delay in initiating treatment was intended to produce a treatment rather than prophylaxis model. The alternate day treatments kept the total number of i.p. injections to once daily. The i.p. rather than oral route of drug delivery ensured that the treatments were the same in all rats. Injections were made between 12:00 p.m. and 2:00 p.m. Rats were weighed weekly and monitored daily. The analyses were focused on cerebellar function and gene expression because the cerebellum is a major target of ethanol-induced neurotoxicity and teratogenesis [63]. The early postnatal exposure model was used because the rodent cerebellum develops postnatal, and the experimental conditions mimic the effects of binge drinking in the third trimester of human pregnancy [42,60].

2.2. Neurobehavioral assessments

Rotarod testing with a fixed incremental speed protocol was used to assess motor function [52,64]. On P20, rats were subjected to rotarod testing on a Rotamex-5 apparatus (Columbus Instruments). Ten rats per group were individually administered 10 incremental speed trials up to 10 rpm (rod rotation), with 10-min rest between trials. A fixed rather than accelerated speed protocol was used, and limits were set on the maximum rod rotation speed due to concerns that rats impaired by ethanol exposure might have been severely challenged by more robust testing of motor skills. Latency to fall was detected with photocells over the rod [52]. Trials were stopped after 30 seconds to avoid fatigue. Data from trials 1–3 (2–5 rpm), 4–7 (5–7 rpm), and 8–10 (8–10 rpm) were grouped and analyzed using the Mann-Whitney test. On P30, rats were sacrificed. Cerebella were divided in the mid-sagittal plane; one hemisphere was snap-frozen and stored at $-80\,^{\circ}$C; the other was preserved in Histofix (Amresco, Ohio) and embedded in paraffin with the mid-sagittal cut surface facing down. Routine histological sections were stained with hematoxylin and eosin. The Lifespan-Rhode Island Hospital IACUC committee approved these experiments.

2.3. Multiplex enzyme-linked immunosorbent assay (ELISA)

We used bead-based multiplex ELISAs to examine insulin and IGF-1 signaling by measuring immunoreactivity to the insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, GSK-3β, $^{\mathrm{pYpY1162/1163}}$-IR, $^{\mathrm{pYpY1135/1136}}$-IGF-1R, $^{\mathrm{pS312}}$-IRS-1, $^{\mathrm{pS473}}$-Akt, and $^{\mathrm{pS9}}$-GSK-3β [43]. 200 μg of protein was incubated with antibody-bound beads. Captured antigens were detected with biotinylated secondary antibody and phycoerythrin-conjugated streptavidin. Plates were read in a Bio-Plex 200 (Bio-Rad, Hercules, CA, USA). Data are expressed as fluorescence light units (FLU) corrected for protein concentration.

2.4. Duplex ELISA

We used duplex direct-binding ELISAs to examine effects of ethanol and PPAR-δ agonist treatments on neurotrophins (glial cell line-derived neurotrophic factor-GDNF; brain derived neurotrophic factor-BDNF; nerve growth factor-NGF; NT3; and NT4/5), choline acetyltransferase (ChAT), tau, phospho-tau, and glial fibrillary acidic protein (GFAP) expression. Immunoreactivity was normalized to large ribosomal protein (RPLPO) [43]. Fluorescence was measured in a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). Binding specificity was determined from negative control incubations with primary or secondary antibody

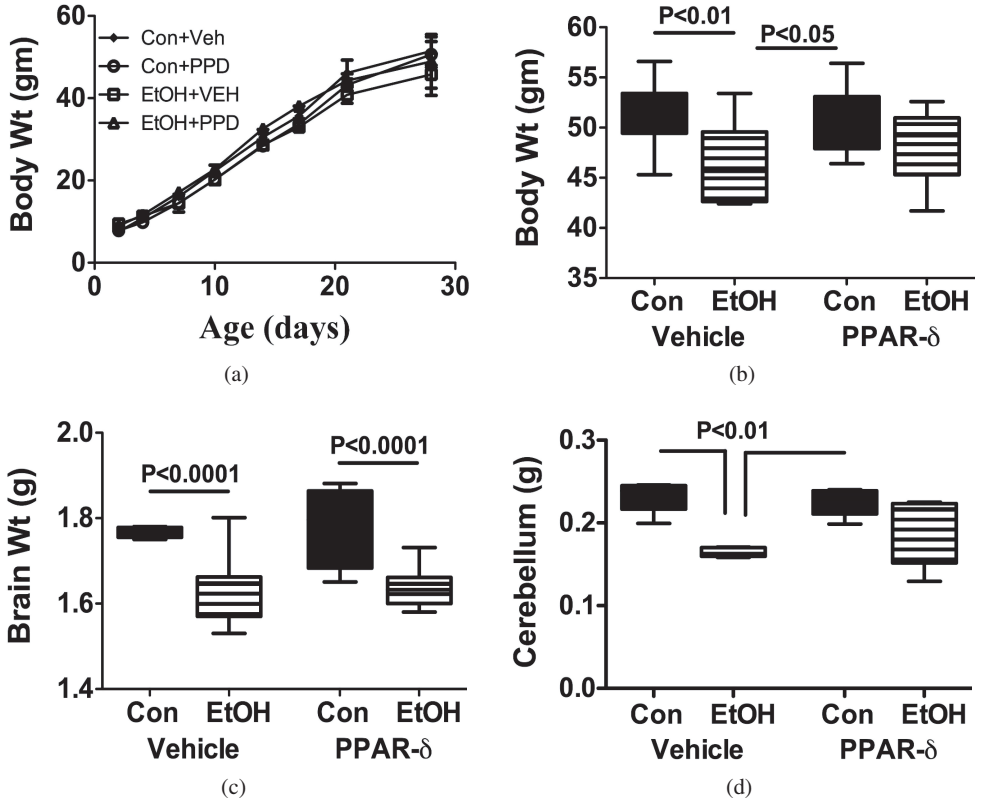

Figure 1: Effect of early postnatal binge ethanol exposures and PPAR-δ agonist treatments on (a) body growth, (b) body weight, (c) brain weight, and (d) cerebellar weight. Long Evans rats were given i.p. injections of ethanol (2 g/kg) or saline on postnatal days (P) 2, 4, 6, and 8, and i.p. injections of the L-165-041 PPAR-δ agonist (2 μg/kg) on P5, P7, P9, and P11. (a) Growth curves reflected by changes in body weight over the course of the experiment. (b) Mean body weights at the time of sacrifice. (c) Mean brain weights at the time of sacrifice. (d) Mean cerebellar weights at the time of sacrifice. Data in panel (a) depict body weight means $\pm$SD values measured over time. Box plots in panels (b)–(d) reflect median values (horizontal bars), 95% confidence interval limits (upper and lower edges of boxes), and range (stems). Inter-group statistical comparisons were made by two-way ANOVA using the post-hoc Bonferroni test for significance. Abbreviations: Con = control; Veh = vehicle; EtOH = ethanol treated; PPD = PPAR-δ agonist treated.

omitted. Calculated ratios of specific protein/RPLPO immunoreactivity were used for inter-group comparisons.

2.5. Statistics

All assays were performed with 10–12 individual brains per group, using two to three rat brains from each of four litters. Box plots depict medians (horizontal bars), 95% confidence limit intervals (upper and lower limits of boxes), and range (upper and lower stems). Inter-group comparisons were made using two-way ANOVA tests and Bonferroni multiple comparisons post-hoc tests (GraphPad Prism 5, San Diego, CA, USA).

3. Results

3.1. Effects of ethanol and PPAR-δ agonist treatments on motor performance

Body growth was continuous in all groups (Figure 1(a)), although at sacrifice, ethanol-exposed rats had reduced mean body weights relative to control (Figure 1(b)). However, inter-group differences were significant only for the ethanol+vehicle group. The mean whole brain weights were significantly lower in ethanol-exposed relative to control rats, irrespective of PPAR-δ agonist treatments (Figure 1(c)). In contrast, the mean cerebellar weight was significantly lower in ethanol+vehicle relative to either control group (Figure 1(d)). Although mean cerebellar weight was also somewhat lower in the ethanol+PPAR-δ agonist-treated group, the differences from control were not statistically significant (Figure 1(d)).

Rotarod performance differed significantly among the groups due to ethanol exposures ($F = 4.754$, $P = .031$) and interactive effects of ethanol and PPAR-δ agonist treatments ($F = 4.98$, $P = .028$). For trials 1–3, performance was similar among controls ($\pm$ PPAR-δ agonist) and ethanol+vehicle treated rats, and all were significantly worse than in the ethanol+PPAR-δ agonist group (Figure 2(a)). For

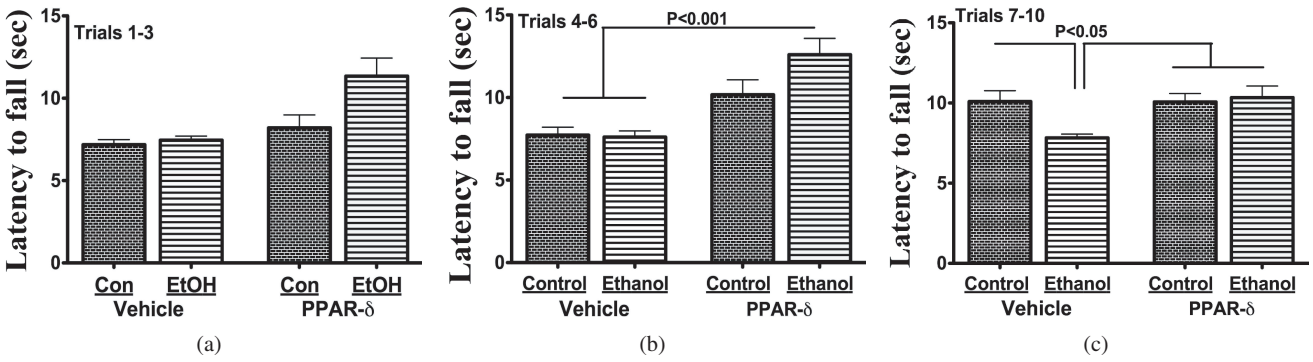

Figure 2: Effects of early postnatal ethanol exposures and PPAR-δ agonist treatments on rotarod motor performance. Long-Evans rats were given i.p. injections of ethanol (2 g/kg) or saline on postnatal days (P) 2, 4, 6, and 8, and i.p. injections of the L-165-041 PPAR-δ agonist (2 μg/kg) on P5, P7, P9, and P11. On P20, the rats were subjected to Rotarod testing over 10 incremental speed trials. Latency to fall (seconds) from the rotating rod was recorded automatically from a photocell above the rod. Results reflect mean $\pm$SEM for each group. Inter-group comparisons using results obtained from trials (a) 1–3, (b) 4–7, and (c) 8–10 were analyzed with the Mann-Whitney test.

trials 4–6, vehicle-treated control and ethanol-exposed rats performed similarly, and both were significantly worse than the ethanol+PPAR-δ agonist group (Figure 2(b)). For the most challenging trials (#7–#10), ethanol+vehicle-treated rats had a significantly shorter mean latency to fall than all other groups (Figure 2(c)). In addition, (1) controls exhibited steady improvements with each trial, despite increasing difficulty; (2) ethanol-treated rats had similar performance levels irrespective of rod rotation speed, suggesting that motor learning was limited; and (3) PPAR-δ agonist treatments enhanced (trials 4–6) or normalized (trials 7–10) motor performance in ethanol-exposed rats. Enhanced performance could have been due to improved cerebellar function due to increased insulin responsiveness, but other factors possibly related to improved skeletal muscle function must be considered [19, 24].

Cerebella from ethanol-exposed rats exhibited irregular thinning of the molecular and granule cell layers and white matter cores, and numerous gaps corresponding to loss of Purkinje cells (Figure 3(b)) relative to control (Figure 3(a)). PPAR-δ agonist treatments reduced the severity of ethanol-mediated cerebellar injury (Figure 3(d)), although it did not completely restore cerebellar architecture relative to either control group (Figures 3(a) and 3(c)).

3.2. Effects of ethanol and PPAR-δ agonist treatments on insulin/IGF-1 signaling through IRS-1, Akt, and GSK-3β

Multiplex ELISAs measured total and phosphorylated insulin receptor, IGF-1 receptor, IRS-1, Akt, and GSK-3β. In addition, we calculated the ratios of phosphorylated/total protein to assess relative phosphorylation. Two-way ANOVA tests demonstrated significant inter-group differences or trends with respect to expression of the insulin receptor, IGF-1 receptor, and IRS-1 (Table 1). Bonferroni post tests revealed that early postnatal ethanol exposure led to significant increases in insulin receptor, IGF-1 receptor, and IRS-1 expression (Table 1). These ethanol-associated abnormalities reflect insulin/IGF-1/IRS-1 resistance in juvenile cerebella. The ethanol-associated increase in IGF-1 receptor expression could reflect a compensatory response to insulin/IRS-1 resistance. PPAR-δ agonist treatments resolved ethanol's long-term effects on insulin receptor, IGF-1 receptor, and IRS-1; and they significantly increased Akt expression in ethanol-exposed relative to control cerebella (Table 1).

Two-way ANOVAs detected inter-group differences in variance with respect to ethanol's effects on expression levels of $^{pYpY1162/1163}$-insulin receptor (decreased-statistical trend), $^{pYpY1135/1136}$-IGF-1 receptor (increased), and ^{pS473}-Akt (decreased). The PPAR-δ agonist treatments rendered the mean levels of $^{pYpY1162/1163}$-insulin receptor, $^{pYpY1135/1136}$-IGF-1 receptor, and ^{pS312}-IRS-1 similar in the control and ethanol-exposed groups, and significantly increased the levels of ^{pS473}-Akt and ^{pS9}-GSK-3β in ethanol-exposed relative control cerebella (Table 2).

With regard to relative phosphorylation, $^{pYpY1162/1163}$-insulin receptor/total insulin receptor, ^{pS312}-IRS-1/total IRS-1, and ^{pS473}-Akt/total Akt were significantly reduced by early postnatal ethanol exposure (Table 2). Note that although Ser phosphorylation of IRS-1 can be inhibitory, ^{S312}-IRS-1 is not [33, 35], indicating that reduced ^{S312}-IRS-1 reflects impaired IRS-1 signaling. In contrast, the mean levels of $^{pYpY1135/1136}$-IGF-1 receptor/total IGF-1 receptor and ^{pS9}-GSK-3β/total GSK-3β were similar in control and ethanol-exposed cerebella, irrespective of PPAR-δ agonist treatments (Table 2). Importantly, the results demonstrate that the long-term adverse effects of developmental ethanol

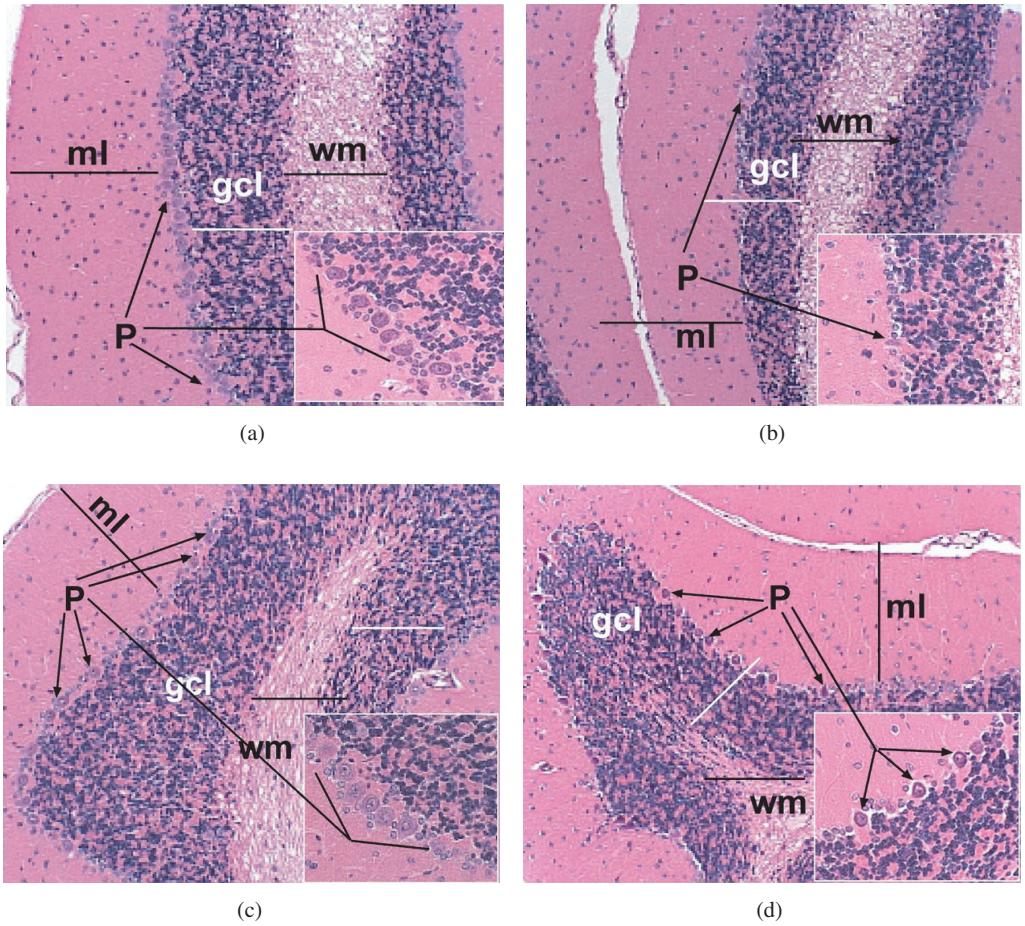

Figure 3: Effects of early postnatal ethanol exposures and PPAR-δ agonist treatments on cerebellar structure. Cerebella from control and ethanol exposed rats that were treated with vehicle or a PPAR-δ agonist were harvested on P30. (a) H&E stained histological sections depict cerebellar vermis of (a) control+vehicle, (b) ethanol+vehicle, (c) control+PPAR-δ agonist, and (d) ethanol+PPAR-δ agonist-treated rats. Note narrowing of the molecular layer (ml), granule cell layer (gcl), and white matter cores (wm), and low cell density in the Purkinje layer (P) of ethanol+vehicle cerebella (b). There is expansion of the ml and gcl with increased Purkinje cell density in cerebella of ethanol+PPAR-δ agonist-treated rats (d). PPAR-δ agonist treatments did not fully preserve the Purkinje layer or white matter cores in ethanol-exposed rats. Insets show representative neuronal densities in the Purkinje layer. Larger panel images were photographed at 200$\times$ and the insets at 400$\times$. Bars corresponding to ml, gcl, and wm are the same length in all panels.

exposure are not mediated by activation of GSK-3β as occurs in the perinatal period [73,77].

The long-term adverse effects of ethanol on phosphorylation of the insulin receptor, IRS-1, and Akt were resolved by the PPAR-δ agonist treatments. Moreover, the PPAR-δ agonist treatments significantly enhanced Akt phosphorylation/activation above control levels. However, scrutiny of those responses revealed two distinct effects of the PPAR-δ agonist treatments on Akt signaling: (1) they reduced levels of Akt, ^{pS473}-Akt, and ^{pS473}-Akt/total Akt in control cerebella; and (2) they strikingly increased ^{pS473}-Akt and ^{pS473}-Akt/total Akt in the ethanol-exposed cerebella (Table 2). These opposing effects accounted for the higher levels of Akt, ^{pS473}-Akt, and ^{pS473}-Akt/total Akt in the ethanol-exposed relative to control PPAR-δ agonist-treated cerebella.

3.3. Effects of ethanol and PPAR-δ agonist on neuronal and glial proteins

We used duplex ELISAs to assess effects of early postnatal ethanol exposures on neuronal and glial proteins and to determine if ethanol-induced abnormalities could be prevented by PPAR-δ agonist treatments. We focused our investigations on neuronal (Tau) and astrocyte (GFAP) cytoskeletal proteins, neurotrophins (NGF, BDNF, GDNF, NT3, NT4/5), and regulators of acetylcholine production (ChAT) because of their roles in cerebellar development and function (Table 3). Two-way ANOVA tests demonstrated

Table 1: Effects of early postnatal binge ethanol exposures and PPAR-δ agonist treatments on cerebellar insulin/IGF-1 signaling.

	Con±Veh	EtOH±Veh	Con±PPAR-δ	EtOH±PPAR-δ	EtOH factor	PPAR-δ factor	EtOH×PPAR-δ interaction	Bonferroni P-values
Protein								
In-R	286.7 ± 16.3	384.3 ± 36.98	298.8 ± 30.1	308 ± 35.7	0.049		0.091	Veh*
IGF-1R	473.1 ± 45.6	730.7 ± 61.35	499.4 ± 48.7	495.6 ± 37.9	0.018	0.046	0.015	Veh**
IRS-1	$1,412 \pm 197.1$	$2,061 \pm 81.84$	$1,860 \pm 130.8$	$1,851 \pm 141.2$	0.038		0.033	Veh**
Akt	$14,586 \pm 1,066$	$12,316 \pm 1,193$	$10,312 \pm 829.8$	$13,832 \pm 1,300$		0.098	0.011	PPAR-δ^{*}
GSK-3β	$6,450 \pm 853.9$	$6,063 \pm 430.2$	$5,284 \pm 124.1$	$5,258 \pm 245$				
Phospho-Protein								
pIn-R	229.5 ± 22.5	197.3 ± 16.9	200.7 ± 13.8	175.6 ± 8.2	0.092			
pIGF-1R	79.33 ± 6.9	154.5 ± 20.9	99.1 ± 13.9	106.2 ± 19.5	0.020		0.049	Veh**
pIRS-1	33.42 ± 2.9	35.2 ± 3.9	32.3 ± 2.2	30.58 ± 1.2				
pAkt	$5,398 \pm 1,047$	$2,028 \pm 281.4$	$1,941 \pm 182.3$	$5,194 \pm 749.6$			< 0.0001	Veh**; PPAR-δ^{**}
pGSK-3β	204.8 ± 17.9	163.9 ± 12.9	168.2 ± 12.9	191.6 ± 15.2			0.043	
Phospho/Total Protein								
p/T In-R	0.177 ± 0.03	0.096 ± 0.009	0.136 ± 0.03	0.098 ± 0.01	0.011			Veh*
p/T IGF1R	0.276 ± 0.018	0.39 ± 0.082	0.359 ± 0.08	0.355 ± 0.06				
p/T IRS1	0.079 ± 0.011	0.048 ± 0.003	0.075 ± 0.01	0.066 ± 0.01	0.014			Veh*
p/T Akt	0.847 ± 0.13	0.343 ± 0.048	0.431 ± 0.09	1.012 ± 0.18			0.0002	Veh*; PPAR-δ^{**}
p/T GSK-3β	0.014 ± 0.001	0.014 ± 0.002	0.017 ± 0.003	0.015 ± 0.002				

Imunoreactivity was measured by multiplex ELISA. Data reflect mean ±SEM of fluorescence light units (immunoreactivity) normalized to protein content in the samples ($N = 10$ samples/group). Results were analyzed by two-way ANOVA and Bonferroni post tests. *Abbreviations and symbols:* In = insulin; R = receptor; p = phospho; p/T = relative levels of phosphorylated/total protein; EtOH = ethanol; Con = control; Veh = vehicle (saline); EtOH factor = ethanol exposure as the source of variation; PPAR factor = PPAR-δ agonist treatment as the source of variation; EtOH × PPAR = interaction between ethanol exposure and PPAR-δ agonist treatment as the source of variation; P-value = P values generated by Bonferroni multiple comparisons post tests demonstrating significant ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$) differences between control and EtOH groups treated with vehicle (Veh) or between control and EtOH groups treated with the PPAR-δ agonist (PPAR-δ).

Table 2: Effects of early postnatal binge ethanol exposures and PPAR-δ agonist treatments on cerebellar neuro-glial and neurotrophin proteins.

Gene	Con±Veh	EtOH±Veh	Con±PPAR-δ	EtOH±PPAR-δ	EtOH factor	PPAR-δ factor	EtOH×PPAR-δ interaction	Bonferroni P-values
Tau	20.32 ± 0.78	19.6 ± 0.59	31.79 ± 1.62	20.84 ± 0.96	< 0.00001	< 0.0001	< 0.0001	PPAR-δ^{****}
pTau	3.78 ± 0.41	3.27 ± 0.28	4.58 ± 0.22	3.13 ± 0.28	0.002			PPAR-δ^{**}
GFAP	10.85 ± 0.45	13.93 ± 0.55	22.89 ± 1.03	15.40 ± 0.79		0.005	< 0.0001	Veh*; PPAR-δ^{****}
ChAT	179.3 ± 19.1	185.2 ± 9.4	369.8 ± 16.9	203.2 ± 14.2	< 0.0001	< 0.0001	< 0.0001	Veh*; PPAR-δ^{****}
GDNF	8.18 ± 0.46	5.29 ± 0.62	9.83 ± 0.41	6.82 ± 0.37	< 0.0001	0.0018		Veh***; PPAR-δ^{***}
BDNF	9.20 ± 0.83	11.83 ± 1.30	10.34 ± 1.19	9.57 ± 0.31				
NGF	7.82 ± 0.32	7.67 ± 0.28	9.31 ± 0.23	7.25 ± 0.27	0.0003	0.059	0.0013	PPAR-δ^{***}
NT3	19.02 ± 1.4	15.99 ± 0.48	21.82 ± 0.62	16.08 ± 0.56	< 0.0001	0.096	0.117	Veh*; PPAR-δ^{****}
NT4/5	10.74 ± 0.52	10.77 ± 0.30	11.42 ± 0.54	10.87 ± 0.38				

Immunoreactivity was measured by a duplex ELISA with levels normalized to ribonuclear protein measured in the same wells. Data reflect mean ±SEM of 12 samples per group. Results were analyzed by two-way ANOVA and the Bonferroni multiple comparisons posttest. Significant P-values reflecting significant variance with respect to column (Col; Control vs Ethanol), row (Vehicle (Veh) vs PPAR-δ agonist treatment), and interactions between column and row are indicated. *Abbreviations and symbols:* GFAP = glial fibrillary acidic protein; ChAT = choline acetyltransferase; GDNF = glial derived neurotrophic factor; BDNF = brain derived neurotrophic factor; NGF = nerve growth factor; NT = neurotrophin; EtOH = ethanol; Con = control; Veh = vehicle (saline); EtOH factor = ethanol exposure as the source of variation; PPAR factor = PPAR-δ agonist treatment as the source of variation; EtOH×PPAR = interaction between ethanol exposure and PPAR-δ agonist treatment as the source of variation; P-Value = P values generated by Bonferroni multiple comparisons post tests demonstrating significant ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$) differences between control and EtOH groups treated with vehicle (Veh) or between control and EtOH groups treated with the PPAR-δ agonist (PPAR-δ).

Table 3: Functions of neuronal and glial proteins studied.

Abbreviation	Full name	Function
NGF	Nerve growth factor	*Secreted neurotrophin.* Regulates neuronal growth, regeneration, and plasticity, and myelin repair. Expressed late in post-mitotic neurons.
		Cerebellum: Inhibits neurotoxic effects of ethanol [2,32,46,55] and stimulates neuronal growth via Erk MAPK [74]; ethanol inhibits TrkA and p75 NGF receptor expression on Purkinje cells during development [22] and NGF secretion [30].
BDNF	Brain-derived neurotrophic factor	*Secreted neurotrophin.* Promotes neurogenesis, and neuronal survival, growth, plasticity, and differentiation. Signals by activating receptor tyrosine kinase (TrkB) in the cerebellum.
		Cerebellum: Supports neuronal survival [55,56], activity-dependent inhibitory synaptogenesis [68], and has anti-apoptotic effect on ethanol-exposed cerebellar granule neurons [1]. BDNF expression inhibited by ethanol [57].
GDNF	Glial-derived neurotrophic factor	Promotes survival and differentiation of dopaminergic and motor neurons, and inhibits axotomy-induced apoptosis.
		Cerebellum: Promotes survival and differentiation of Purkinje cells [54] and attenuates ethanol-induced Purkinje cell loss [50].
GFAP	Glial fibrillary acidic protein	Astrocyte cytoskeletal protein. Expressed in astrocytes and Bergmann glia.
		Cerebellum: Ethanol delays development of Bergmann glia, correlating with disturbances in neuronal migration [65,69].
NT-3[a]	Neurotrophin 3	Neurotrophin with high-level binding and activation of TrkC neurotrophin receptor. Regulates neurogenesis.
		Cerebellum: Expressed in early post-mitotic neurons; supports neuronal survival; induces neuronal differentiation and migration in external granule cell layer; synaptogenesis in Purkinje cells [55]. Ethanol inhibits NT3 secretion [30] and expression of TrkB and TrkC neurotrophin receptors, impairing actions of NT-3 [42].
NT-4/5	Neurotrophin 4	Neurotrophin that signals through TrkB and mediates cerebellar granule cell neurogenesis.
		Cerebellum: Promotes activity-dependent inhibitory synaptogenesis. Ethanol inhibits expression of TrkB and TrkC neurotrophin receptors, impairing actions of NT-4/5 [42,68].
ChAT	Choline acetyltransferase	Key enzyme for acetylcholine synthesis. Acetylcholine is major neurotransmitter for cerebellarmotor function.
		Cerebellum: ChAT inhibited by chronic ethanol exposure [73,75].
Tau	Tau	A type II microtubule-associated protein localized mainly in axons; binds to tubulin subunits, stabilizing microtubule structure; involved in axonal outgrowth needed to maintain synaptic connections [37].
		Cerebellum: Ethanol inhibits tau expression [73].
pTau	Phospho-tau	Phosphorylated tau increases with insulin resistance [40,67].
		Cerebellum: Ethanol increases tau phosphorylation via insulin resistance-mediated increases in GSK-3β activity and oxidative stress [73].

significant inter-group differences with respect to: (1) binge ethanol effects on Tau, pTau, GDNF, GFAP, ChAT, NGF, and NT3; (2) PPAR-δ agonist effects on Tau, GDNF, GFAP, ChAT, and NGF; (3) and interactions between ethanol and PPAR-δ agonists on Tau, GFAP, ChAT, and NGF (Table 2). Bonferroni post tests demonstrated that the mean levels of GDNF and NT3 were significantly reduced, while GFAP was significantly increased (Table 2) in ethanol+vehicle relative to control. In contrast, Tau, pTau, ChAT, BDNF, NGF, and NT4/5 were similar in the control and ethanol-exposed, vehicle-treated cerebella. Furthermore, Bonferroni post tests demonstrated that PPAR-δ agonist treatments increased expression of several neuro-glial proteins in control brains, including Tau, pTau, GFAP, and ChAT, but produced no significant responses in ethanol-exposed cerebella. The combined effects of increased protein expression in controls and virtually absent responses in the ethanol group rendered the mean levels of cerebellar Tau, pTau, GFAP, ChAT, GDNF, NGF, and NT3 significantly higher in the control+PPAR-δ agonist treated relative to the corresponding ethanol group.

4. Discussion

4.1. Effects of PPAR-δ agonist treatments on ethanol-impaired cerebellar function and structure

This study utilized an early postnatal binge ethanol exposure model in which rat pups were exposed to ethanol during the period of cerebellar development. Importantly, this model corresponds to binge alcohol drinking in third trimester of human pregnancy [42,60]. The results demonstrated that early postnatal binge ethanol exposures impair juvenile

cerebellar function as shown by the shorter latencies to fall measured with the most challenging rotarod trials. Correspondingly, cerebellar weight and neuronal populations were reduced by ethanol exposure. PPAR-δ agonist treatments normalized function, increased weight, and partly restored the histological architecture of the cerebellum in ethanol-exposed rats. Since the PPAR-δ agonist treatments were initiated on P5, several days after the ethanol treatments were started (P2), most likely, the rescue effects were mediated by enhancement of cell survival/reduced cell death and increased neurogenesis. This interpretation fits with the finding that the PPAR-δ agonist treatments increased signaling through Akt, which promotes cell survival, growth, and metabolism. These findings highlight the importance of insulin/IGF-1 signaling networks in relation to brain development.

Ethanol-induced reductions in granule cells were probably due to increased cell death and reduced proliferation [3, 8,9,10,70] following inhibition of insulin signaling [18]. Immature granule cells utilize insulin and IGF-1 for growth, survival, migration, and metabolism, and ethanol impairs insulin/IGF signaling in the developing brain [13,16,18,28, 79]. Rescue effects of the PPAR-δ agonist were likely mediated by enhanced signaling through the insulin receptor and Akt. Previous reports demonstrated roles for insulin-PI3K-Akt in survival, growth, and maintenance of cerebellar granule cells [31,42].

Since Purkinje cells developed in utero, prior to the period of binge ethanol administration, ethanol-associated reductions in Purkinje cells were likely due to decreased survival. Correspondingly, the rescue effects of PPAR-δ agonist treatments were likely mediated by enhanced survival and metabolic function with increased signaling through the insulin receptor and Akt. Protective effects of the PPAR-δ agonist also could have been due to its anti-oxidant properties. A third consideration pertains to neurotrophin signaling which is critical to Purkinje cell function and survival [42,55,68]. Although ethanol-associated reductions in GDNF and NT3 could have contributed to the loss of cerebellar neurons, failure of the PPAR-δ agonist to enhance neurotrophin expression vis-à-vis improvements in cerebellar structure and function suggests that insulin/IGF-1 signaling have dominant roles in cerebellar development.

4.2. PPAR-δ agonist rescue of ethanol-impaired insulin receptor signaling

Chronic prenatal ethanol exposure impairs insulin and IGF-1 signaling through IRS-1, PI3K, Akt, and Erk MAPK, and increases GSK-3β activation [73,77]. Short-term ethanol exposures produce similar effects on cultured cerebellar neurons [18,28,77]. New data generated by these studies include the findings that: (1) ethanol-mediated impairments in insulin signaling persist in the brain well beyond the period of exposure; (2) later effects of early postnatal binge ethanol exposures are less striking and more limited in scope than the abnormalities produced by in utero (earlier) ethanol exposures, i.e. P2; (3) ethanol-mediated inhibition of insulin signaling in the brain can be abrogated by treatment with a PPAR-δ agonist; and (4) PPAR-δ agonist associated improvements in cerebellar function and structure correlate with rescue effects on insulin receptor and Akt signaling. The most parsimonious explanation of these findings is that the PPAR-δ agonist enhances insulin responsive genes to increase survival and metabolic function, and decreases oxidative stress. The net result was to increase "normalize" cerebellar development and motor function in the juvenile/early adolescent period.

Factors that may have been responsible for the limited preservation of cerebellar structure and function in ethanol+vehicle-exposed rats include, significantly increased levels of IGF-1 receptor, tyrosine phosphorylated IGF-1 receptor, and IRS-1 expression. Since insulin and IGF-1 have similar signaling networks that flow through IRS proteins and lead to activation of PI3K-Akt and inhibition of GSK-3β, enhanced signaling through IGF-1 receptors and IRS-1 could have compensated for deficits in insulin receptor signaling in ethanol-exposed brains. Previous studies demonstrated that insulin receptors are co-expressed with IGF-1 or IGF-2 receptors in sub-populations of immature cerebellar neurons [12]. Conceivably, immature neurons that have dual or triple insulin/IGF receptor expression may be more resistant to the inhibitory effects of ethanol on insulin receptor function due to availability of alternative and redundant mechanisms for activating downstream survival and metabolic pathways.

Although it is not apparent why the impairments in brain insulin signaling persist beyond the period of ethanol exposure, the consequences could include ongoing cell loss due to reduced survival mechanisms and increased oxidative stress. The finding of impaired Akt signaling in juvenile cerebella suggests that insulin signaling has a more dominant role than IGF-1 in activating Akt pathways. Based on the PPAR-δ agonist rescue effects on cerebellar structure, function, and Akt signaling, it appears that the inhibition of Akt signaling contributes greatly to the neuronal loss in ethanol-exposed cerebella [16,58,77,78].

In contrast to the effects of chronic prenatal ethanol exposure [5,13,16,44], GSK-3β activity (increased GSK-3β protein and/or reduced ^{pS9}-GSK-3β) was not increased in ethanol-exposed juvenile cerebella. GSK-3β promotes oxidative stress, DNA damage, mitochondrial dysfunction, apoptosis, and disordered neuronal migration [16,34,45,53, 59]. Normalization of GSK-3β activity in ethanol-exposed juvenile cerebella suggests compensatory mechanisms down-regulated GSK-3β activity and thereby helped

maintain cerebellar structure. The increased levels of total and tyrosine phosphorylated IGF-1 receptor could have mediated these effects in the ethanol+vehicle group. On the other hand, the cerebellar hypotrophy and reductions in cerebellar neuron populations suggest that the activation of GSK-3β prior to the PPAR-δ agonist intervention, caused permanent neuronal loss, whereas concomitant treatment reduced further neuronal loss due to enhanced insulin receptor and Akt signaling and reduced oxidative stress.

4.3. Adverse effects of ethanol and rescue effects of PPAR-δ agonist treatments on neurotrophin signaling mechanisms

Although neurotrophins are regarded as critical for cerebellar function, we only detected significant reductions in GDNF and NT3 expression in the ethanol+vehicle group. Moreover, the structural and functional rescues afforded by the PPAR-δ agonist were not associated with significant increases in neurotrophin expression. These findings suggest that insulin and IGF-1 actions play a more prominent role than neurotrophin signaling in relation to cerebellar development and function in the juvenile period.

Acetylcholine modulates cerebellar function and is produced by ChAT, which is regulated by insulin and IGF-1 and inhibited by ethanol [73]. Tau expression and phosphorylation are also regulated by insulin and IGF-1. The finding of intact ChAT, Tau, and pTau expression in the ethanol+vehicle group could have been due to up-regulation of IGF-1 signaling. The finding that PPAR-δ agonist treatments increased expression of ChAT and cytoskeletal proteins in control cerebella suggests that with intact insulin signaling, PPAR-δ agonists enhance neuronal structure and function. This suggests new ways in which mature brain function could be stimulated. The main conclusions of this study are that: (1) structural abnormalities in the juvenile cerebellum caused by early postnatal binge ethanol exposures can be prevented or reduced by treatment with a PPAR-δ agonist; and (2) all aspects of neuronal function that are modulated by insulin signaling, e.g., neurotrophin signaling and damaged by developmental ethanol exposures, are not accessible to PPAR-δ agonist treatments; and therefore, additional approaches are needed to fully restore brain function in FASD.

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