

# Research Article PPAR Agonist Rescue of Ethanol-Impaired Brain Insulin Signaling: Cerebellar Slice Culture Model

Tran Le,<sup>1</sup> Ming Tong,<sup>1</sup> VanAnh Nguyen,<sup>1</sup> and Suzanne M. de la Monte<sup>2</sup>

<sup>1</sup>Department of Medicine, Rhode Island Hospital, Alpert Medical School of Brown University, Providence, RI 02903, USA <sup>2</sup>Departments of Pathology (Neuropathology), Neurology, Neurosurgery, and Medicine, Rhode Island Hospital,

Alpert Medical School of Brown University, Providence, RI 02903, USA

Address correspondence to Suzanne M. de la Monte, suzanne\_delamonte\_md@brown.edu

Received 2 November 2012; Revised 10 November 2012; Accepted 20 November 2012

Copyright © 2013 Tran Le et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract Cerebellar abnormalities in fetal alcohol spectrum disorder (FASD) have been linked to brain insulin resistance and oxidative stress. Peroxisome proliferator-activated receptor (PPAR) agonists, which have insulin sensitizer and antioxidant effects, could potentially be used to treat neurodevelopmental abnormalities in FASD. Slice cultures generated with cerebella from control or ethanol-exposed rat pups were treated with vehicle, or a PPAR- $\alpha$ , PPAR- $\delta$ , or PPAR- $\gamma$  agonist to examine effects on cytotoxicity, histopathology, insulin signaling, and neuronal/glial protein expression. PPAR agonists reduced ethanol-mediated cytotoxicity; restored cerebellar architecture; increased expression or activation of insulin/IGF-1 receptors, IRS-1, Akt, and PRAS40; and inhibited GSK-3*β*. PPAR agonists increased the expression of Hu, myelin-associated glycoprotein-1, and choline acetyltransferase, and decreased the oxidative stress in ethanol-exposed cultures. The PPAR- $\delta$  and PPAR- $\gamma$ agonists were more effective than the PPAR- $\alpha$  agonist in abrogating the adverse effects of ethanol. Therefore, early treatment with PPAR- $\delta$  or PPAR- $\gamma$  agonists may help prevent long-term consequences of FASD.

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

#### **1** Introduction

Alcohol abuse during pregnancy causes fetal alcohol spectrum disorder (FASD), which results in long-term deficits in cognitive and motor functions [29,35]. Ethanol mediates its adverse effects on the immature brain by inhibiting insulin and insulin-like growth factor (IGF) signaling [14]. Adverse effects of ethanol occur at multiple levels in the insulin/IGF-1 cascades, from the most proximal step (i.e., receptor binding) to distal steps, including those that regulate growth, survival, metabolism, neuronal migration, and plasticity [2,9,22,44]. Consequently, ethanol (1) inhibits insulin and IGF-1 receptor tyrosine kinases (RTKs) and their downstream effector molecules, including insulin receptor substrate (IRS) proteins [42], (2) inhibits phosphotidyl-inositol-3-kinase (PI3K) and Akt, (3) activates glycogen synthase kinase  $3\beta$  (GSK- $3\beta$ ) [14,23,38,42], and (4) increases phosphatases that negatively regulate RTKs

(PTP-1 $\beta$ ) and PI3K (PTEN) [23,42]. Akt promotes cell survival, migration, energy metabolism, and neuronal plasticity; and it inhibits GSK-3 $\beta$ , which increases cellular stress and apoptosis [18]. Therefore, ethanol inhibition of insulin/IGF signaling could account for a number of neurodevelopmental abnormalities in FASD, including microcephaly, mental retardation, motor impairments, and attention/learning deficits.

Ethanol exposures cause insulin/IGF-1 resistance in the brain. Mechanistically, the receptors fail to adequately transmit intracellular signals that mediate growth, metabolic, and homeostatic functions. However, ethanol also causes neurotoxic injury and thereby promotes oxidative stress, DNA damage, and mitochondrial dysfunction, and it activates pro-inflammatory and pro-apoptotic cascades [4, 6, 12, 14]. Together, insulin/IGF resistance and neurotoxic injury cause cell loss and compromise functions needed for brain development. Preventing long-term FASD effects requires both arms of the equation to be addressed. This concept led us to consider the therapeutic use of peroxisome proliferator activated receptor (PPAR) agonists in FASD.

PPARs are nuclear hormone receptors that function as transcription factors and regulate gene expression [19,24, 25]. PPAR- $\alpha$  is abundantly expressed in liver, adipose tissue, muscle, and kidney. PPAR- $\beta/\delta$  is highly expressed in brain, and PPAR- $\gamma$  is widely expressed throughout the body. Ligand binding causes PPARs to heterodimerize with retinoid X receptors [34,36]. The resulting complex regulates target genes by binding to peroxisome proliferator hormone response elements of DNA promoters [34,36]. PPAR signaling regulates cellular differentiation and energy metabolism. In addition, PPAR agonists have anti-inflammatory and antioxidant effects [26,31,34,41]. Therefore, PPARs are attractive as potential therapeutic targets in FASD. Herein, we characterize the therapeutic effects of PPAR- $\alpha$ , PPAR- $\delta$ , and

PPAR- $\gamma$  agonists on ethanol-impaired cerebellar insulin signaling in an ex vivo precision slice culture model.

## 2 Methods

# 2.1 Organotypic cerebellar slice cultures

Long-Evans rat pups were administered intraperitoneal (IP) injections (50  $\mu$ L) of saline or 2.5 g/kg ethanol (in saline) on postnatal days (P) 3, 5, 7, and 9 [16, 37]. On P10, the rats were sacrificed and cerebella were harvested. After a brief rinse in Hank's balanced salt solution (HBSS), the cerebella were chilled in Petri dishes containing ice-cold HBSS. Individually, cerebella were positioned in a Mcllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, UK) for slicing at 250  $\mu$ m intervals in the sagittal plane, following the manufacturer's protocol. The slices were gently separated under a dissecting microscope. Two or three slices were placed (non-overlapping) on the bottoms of BD Falcon  $8\,\mu\text{m}$  pore culture inserts that were seated into 12-well Nunc plates. Each well contained  $300 \,\mu\text{L}$  of pre-warmed (37 °C) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 25 mM KCl, 9 g/L glucose, 10 mM non-essential amino acids, 4 mM glutamine, 120 IU/mL penicillin, and 120  $\mu$ g/mL streptomycin.

Cultures were maintained at 37 °C in sealed humidified chambers equilibrated with 5% CO<sub>2</sub>, 20% O<sub>2</sub>, and 75% N2. Control cultures generated with cerebella from salineinjected rat pups, were incubated in chambers in which water was vaporized from the reservoir tray. Cultures from ethanol-exposed pups were incubated in chambers with 50 mM ethanol supplied in the reservoir tray to permit continuous ethanol exposure [1]. The reservoir fluid and culture media supplemented with vehicle, or a PPAR- $\alpha$ (GW7647, 10 nM), PPAR-δ (L-165,041, 10 nM), or PPAR- $\gamma$  (Fmoc-Leu, 20  $\mu$ M) agonist (Calbiochem, San Diego, CA, USA) were changed daily. GW7647 is a urea-substituted thioisobutyric acid and PPAR- $\alpha$  agonist that has lipidlowering activity in rats. L-165,041 is a PPAR- $\delta$  agonist and a derivative of phenoxyacetic acid. Fmoc-Leu is an activator of PPAR- $\gamma$  and has potent insulin-sensitizing activity. The concentrations used were based on exploratory studies and  $IC_{50}$  data provided by the manufacturer.

After 72 and 96 h in culture, cytotoxicity was gauged using the Vybrant Cytotoxicity Assay Kit (Molecular Probes-Invitrogen, Carlsbad, CA, USA), which measures glucose-6-phosphate-dehydrogenase (G6PD) release into the medium. Cultures were harvested at the 96 h time point to examine histopathology, insulin signaling through insulin and IGF-1 receptors, IRS-1, and Akt pathways by multiplex enzyme-linked immunosorbant assays (ELISAs), and immunoreactivity to neuronal and glial cell proteins and indices of oxidative stress by duplex direct binding ELISAs. For histological studies, representative samples from each culture were fixed in 10% neutral buffered formalin and embedded in paraffin. Histological sections  $(5 \,\mu\text{m} \text{ thick})$  were stained with hematoxylin and eosin (H&E). Tissues from the same wells were snap-frozen in dry ice-chilled methanol and stored at  $-80 \,^{\circ}\text{C}$  for later analysis by multiplex or duplex ELISA.

## 2.2 Cytotoxicity assay

Cytotoxicity in the cultures was measured using the Vybrant Cytotoxicity Assay Kit (Molecular Probes, Eugene, OR, USA), which measures the release of G6PD into the culture medium. The assays were performed according the manufacturer's protocol. In brief,  $50 \,\mu$ L of culture supernatant were transferred to a white OptiPlate (PerkinElmer, Waltham, MA, USA) and incubated for 30 min at 37 °C with a reaction mixture containing 4 mM resazurin. Fluorescence intensity was measured in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA; Ex/Em: 530/590 nm). Results were normalized to tissue protein concentration in the wells.

#### 2.3 Multiplex ELISA

Bead-based multiplex ELISAs were used to assess the integrity of signaling through the insulin and IGF-1 receptors, insulin receptor substrate, type 1 (IRS-1), and downstream through Akt-related pathways using the Akt Total and Phospho 7-Plex Panels (Bio-Rad, Hercules, CA, USA). The Akt Total 7-Plex panel measured immunoreactivity to insulin receptor (IN-R), IGF-1 receptor (IGF-1R), IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K), and glycogen synthase kinase  $3\beta$  (GSK- $3\beta$ ). The Akt Phospho 7-Plex panel measured immunoreactivity to pypY1162/1163-IN-R, <sup>pYpY1135/1136</sup>-IGF-1R, <sup>pS312</sup>-IRS-1, <sup>pS473</sup>-Akt, <sup>pT246</sup>-PRAS40, <sup>pTpS421/424</sup>-p70S6K, and <sup>pS9</sup>-GSK3 $\beta$ . Samples containing 200 mg protein were incubated with the beads according to the manufacturer's protocol. Captured antigens were reacted with biotinylated second epitope antibodies and phycoerythrin-conjugated streptavidin. Immunoreactivity was detected and quantified in a Bio-Plex 200 apparatus (Bio-Rad). Data are expressed as fluorescence light units (FLU) corrected for protein concentration [28].

## 2.4 Duplex ELISA

We used duplex direct-binding ELISAs to examine effects of ethanol and PPAR agonists on neuronal (Hu), astrocytic (glial fibrillary acidic protein; GFAP), oligodendrocyte (Olig-1 and myelin associated glycoprotein-1; MAG-1), choline acetyltransferase (ChAT),  $\beta$ -actin, 4-hydroxy-2nonenal (HNE), and 3-nitrotyrosine (NTyR). Slice culture homogenates were prepared in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.1% Triton X-100, and protease (1 mM PMSF, 0.1 mM TPCK, 1 mg/mL aprotinin, 1 mg/mL pepstatin A, 0.5 mg/mL leupeptin, 1 mM NaF, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>), and phosphatase  $(2 \text{ mM Na}_3 \text{VO}_4)$  inhibitors. Direct binding ELISAs were performed in 96-well MaxiSorp plates. Protein samples diluted in bicarbonate buffer (100 ng/50  $\mu$ L) were adsorbed to well bottoms by overnight incubation at 4°C, and then blocked for 3 h with 2% bovine serum albumin (BSA) in TRIS-buffered saline (TBS). After washing, the samples were incubated with primary antibody (0.1-0.4 µg/mL) for 1 h at 37 °C. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody and the Amplex UltraRed soluble fluorophore. Fluorescence intensity was measured (Ex 565 nm/Em 595 nm) in a SpectraMax M5 microplate reader (Molecular Devices). Subsequently, the samples were incubated with biotin-conjugated antibodies to large ribosomal protein (RPLPO), and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000; Vector, Burlingame, CA, USA) and the 4methylumbelliferyl phosphate (4-MUP) fluorophore (Invitrogen-Molecular Probes, Eugene, OR, USA). Fluorescence (Ex 360/Em 450) intensity was measured in a SpectraMax M5. Binding specificity was determined from parallel negative control incubations in which the primary or secondary antibody was omitted. The ratios of immunoreactivity corresponding to specific protein/RPLPO were calculated and used for inter-group statistical comparisons [28].

#### 2.5 Statistics

All assays were performed with 10–12 samples per group. Inter-group comparisons were made using two-way ANOVA tests and Bonferroni multiple comparisons post-tests (GraphPad Prism 5, San Diego, CA, USA).

#### **3 Results**

## 3.1 Cytotoxicity studies

At the 72-hour time point, control cultures treated with vehicle, the PPAR- $\alpha$ , or PPAR- $\delta$  agonist had similar levels of G6PD release; whereas those treated with the PPAR- $\gamma$  agonist had somewhat elevated levels of G6PD release although the difference to the other sub-groups did not reach significance (Figure 1(a)). At the 96-hour time point, culture supernatant G6PD levels were generally reduced relative to the 72-hour time point, and were similar among the four control groups (Figure 1(b)). Ethanol+vehicle-treated cultures had significantly higher levels of G6PD release relative to control at both the 72- and 96-hour time points. PPAR agonist treatments had no significant effect on G6PD release at the 72-hour time point, but after 96 h, ethanol-associated G6PD release was suppressed and rendered similar to control (Figure 1(b)).

#### 3.2 Histopathology

Formalin-fixed, paraffin-embedded histological sections of control slice cultures harvested at the 96-hour time



**Figure 1:** PPAR agonist inhibition of ethanol-induced neurotoxicity. Organotypic slice cultures were generated from postnatal day (P) 10 control or ethanol-exposed rat pups. Cultures were treated with vehicle, or a PPAR- $\alpha$  (GW7647), PPAR- $\delta$  (L-165,041), or PPAR- $\gamma$  (Fmoc-Leu) agonist. After 72 h incubation (a) and 96 h (b) in culture, G6PD release was measured in media. G6PD activity was normalized to protein content in the slice cultures.

point exhibited a relative preservation of the cerebellar cortical architecture with well-delineated external and internal granule cell, Purkinje cell, and molecular layers (Figure 2(a)). PPAR agonist treatments produced subtle alterations in cell density and thickness of the granule and molecular layers, while having minimal effect on the Purkinje layer (Figures 2(b), 2(c), and 2(d)). The PPAR- $\alpha$  agonist increased cell density in the external granule layer, reduced cell density in the internal granule layer, and narrowed the molecular layer (Figure 2(b)). The PPAR- $\delta$  agonist reduced the external granule cell population (Figure 2(c)). Cultures treated with the PPAR- $\gamma$  agonist closely resembled those in the control+vehicle group, except for modest reductions in the internal granule cell layer (Figure 2(d)).

Ethanol+vehicle cultures had conspicuously reduced cell densities in the external and internal granule cell



**Figure 2:** Histological features of cerebellar organotypic slice cultures. Organotypic slice cultures generated from P10 (a)– (d) control or (e)–(h) ethanol-exposed rat pups were treated with (a) and (e) vehicle, or a (b) and (f) PPAR- $\alpha$  (GW7647), (c) and (g) PPAR- $\delta$  (L-165,041), or (d) and (h) PPAR- $\gamma$  (Fmoc-Leu) agonist. Ninety-six-hour old cultures were fixed in formalin, and embedded in paraffin. Histological sections were stained with hematoxylin and eosin. Panels depict representative areas of cerebellar cortex with the external granule cell (egc), molecular (m), Purkinje (arrows), and internal granule cell (igc) layers.

layers, expansion of the molecular layer, and cell loss with gaps in the Purkinje layer (Figure 2(e)). PPAR- $\alpha$  agonist treatments caused further loss of Purkinje cells and thinning of the internal granule cell layer relative to ethanol+vehicle (Figure 2(f)). In contrast, the PPAR- $\delta$  and PPAR- $\gamma$  agonist treatments nearly abolished the adverse effects of ethanol by restoring the cerebellar cortical architecture and cellularity within the external granule, molecular, and Purkinje cell layers (Figures 2(g) and 2(h)). The PPAR- $\gamma$  agonist was most effective in preventing adverse effects of ethanol on cerebellar structure since the resulting histological features were similar to control+vehicle (Figures 2(h) and 2(a)).

#### 3.3 Insulin/IGF-1/IRS-1 signaling

Using a multiplex bead-based ELISA, we observed consistently lower mean levels of insulin receptor (IN-R), IGF-1 receptor (IGF-1R), IRS-1, <sup>pYpY1162/1163</sup>-IR, <sup>pYpY1135/1135</sup>-IGF-1R, and <sup>S312</sup>-IRS-1 in vehicle-treated ethanol-exposed relative to control cultures (Figure 3). However, the inter-group differences were only statistically significant for <sup>pYpY1162/1163</sup>-IR, <sup>pYpY1135/1135</sup>-IGF-1R, and <sup>S312</sup>-IRS-1, indicating that the main effects of ethanol were to impair tyrosine phosphorylation of the insulin and IGF-1 receptors, and S312 phosphorylation of IRS-1. Although ethanol-associated reductions in IN-R, IGF-1R, and IRS-1 expressions were prominent, the inter-group differences did not achieve statistical significance due to the large variances and standard deviations.

In control cultures, the total and phosphorylated levels of IN-R, IGF-1R, and IRS-1 tended to not vary with respect to PPAR-agonist treatment. The main exceptions occurred with respect to the PPAR- $\gamma$  agonist, which reduced expression of IN-R, IRS-1, <sup>pYpY1162/1163</sup>-IN-R, and <sup>S312</sup>-IRS-1 relative to vehicle-, the PPAR- $\alpha$  agonist, and/or the PPAR- $\delta$  agonist treatments. In ethanol-exposed cultures, the effects of PPAR agonists varied with sub-type. PPAR- $\alpha$  agonist treatments sharply increased IN-R, IGF-1R, and IRS-1 expressions, and modestly increased pYpY1135/1135-IGF-1R and S312-IRS-1 expressions. The PPAR- $\delta$  agonist treatments produced small to moderate increases in IN-R, IGF-1R, IRS-1, pYpY1162/1163-IN-R, pYpY1135/1135-IGF-1R, and S312-IRS-1 expressions relative to ethanol+vehicle. The PPAR- $\gamma$ agonist had little or no effect on total or phosphorylated IN-R, IGF-1R, and IRS-1 levels in ethanol-exposed cultures. However, because the PPAR- $\gamma$  agonist tended to reduce expression of these same proteins and phospho-proteins in control cultures, the inter-group differences observed in ethanol+vehicle cultures were either abolished or markedly curtailed by PPAR agonist treatments (Figure 3).

## 3.4 Akt pathway signaling

Analysis of the downstream pathways through Akt demonstrated consistently lower mean levels of both total and phosphorylated Akt, GSK-3 $\beta$ , p70S6K, and PRAS40 in ethanol+vehicle relative to control+vehicle cultures (Figure 4). The inter-group differences were statistically



**Figure 3:** Multiplex ELISA data characterizing effects of ethanol and PPAR agonist treatments on upstream mediators of insulin/IGF-1 signaling in 96-hour cerebellar slice cultures. Cerebellar culture homogenates were used in bead-based multiplex ELISAs to measure immunoreactivity to the (a) insulin receptor (InR), (b) IGF-1 receptor (IGF-1R), (c) IRS-1, (d) <sup>pYpY1162/1163</sup>-InR, (e) <sup>pYpY1135/1136</sup>-IGF-1R, and (f) <sup>pS312</sup>-IRS-1. Inter-group comparisons were made using 2-way ANOVA tests with Bonferroni post hoc tests for significance.

significant for  $p^{S473}$ -Akt,  $p^{S9}$ -GSK- $3\beta$ ,  $p^{TpS421/424}$ -p70S6K, and  $p^{T246}$ -PRAS40, indicating that the main effect of the ethanol exposures was to impair Akt signaling through cell growth, survival, and metabolic pathways. Despite prominent ethanol-associated reductions in total Akt, GSK- $3\beta$ , and p70S6K, the differences relative to control did not achieve statistical significance due to large variances and standard deviations.

In control cultures, PPAR- $\alpha$  agonist treatments increased expression of  ${}^{pTpS421/424}$ -p70S6K and  ${}^{pT246}$ -PRAS40, PPAR- $\delta$  agonist treatments increased the mean levels of  ${}^{pT246}$ -PRAS40, and PPAR- $\gamma$  agonist treatments reduced the levels of  ${}^{pS473}$ -Akt and  ${}^{pTpS421/424}$ -p70S6K relative to vehicle (Figure 4). In contrast, the mean total and phosphorylated levels of Akt, GSK- $3\beta$ , p70S6K, PRAS40, and  ${}^{pS9}$ -GSK- $3\beta$  were relatively unaffected by the PPAR-agonist treatments. In the ethanol-exposed cultures, PPAR- $\alpha$  agonist treatments increased Akt, p70S6K,  ${}^{pS9}$ -GSK- $3\beta$ , and  ${}^{pT246}$ -PRAS40; and PPAR- $\delta$  agonist treatments increased the levels of p70S6K,  ${}^{pS9}$ -GSK- $3\beta$  and  ${}^{pT246}$ -PRAS40; and PPAR- $\delta$  agonist treatments increased the levels of p70S6K,  ${}^{pS473}$ -Akt,  ${}^{pS9}$ -GSK- $3\beta$  and  ${}^{pT246}$ -PRAS40 relative to vehicle. In contrast, PPAR- $\gamma$  agonist treatments had minimal or no effect on the total



**Figure 4:** Multiplex ELISA data characterizing effects of ethanol and PPAR agonist treatments on Akt pathways networks in 96-hour cerebellar slice cultures. Cerebellar slice culture protein homogenates were used in bead-based multiplex ELISAs to measure immunoreactivity to (a) Akt, (b) GSK-3 $\beta$ , (c) p70S6K, (d) PRAS40, (e) <sup>pS473</sup>-Akt, and (f) <sup>pS9</sup>-GSK-3 $\beta$ , (g) <sup>pTpS421/424</sup>-p70S6K, and (h) <sup>pT246</sup>-PRAS40. Inter-group comparisons were made using two-way ANOVA tests with Bonferroni post hoc tests for significance.

or phosphorylated levels of Akt, GSK-3 $\beta$ , p70S6K, and PRAS40 (Figure 4).

## 3.5 Neuronal and glial protein expression

Duplex ELISAs were used to measure Hu (neuronal marker), glial fibrillary acidic protein (GFAP; astrocyte marker), myelin-associated glycoprotein-1 (MAG-1; mature oligodendrocyte marker), Olig-1 (oligodendrocyte transcription factor), choline acetyltransferase (ChAT),  $\beta$ -actin, 4-hydroxy-nonenal (HNE; lipid peroxidation marker), and 3-nitrotyrosine (N-TYR; oxidative stress marker). Immunoreactivity was normalized to large ribosomal protein (RPLPO) measured in the same wells. Ethanol+vehicle treatments significantly inhibited cerebellar expression of Hu, MAG-1, ChAT, and  $\beta$ -actin, and increased expression



**Figure 5:** Characterization of ethanol and PPAR agonist treatment effects on neuronal and glial proteins and indices of oxidative stress in cerebellar slice cultures. Direct binding duplex ELISAs were used to measure (a) Hu, (b) GFAP, (c) MAG-1, (d) Olig-1, (e) ChAT, (f)  $\beta$ -Actin, (g) HNE, and (h) N-Tyr. Immunoreactivity was normalized to large ribosomal protein (RPLPO) measured in the same wells. Inter-group comparisons were made by two-way ANOVA using with Bonferroni post hoc tests.

of GFAP and N-TYR relative to the control+vehicle cultures (Figure 5). The overall effects of PPAR agonist treatments were relatively modest, as they resulted in few meaningful changes in the relative levels of the proteins examined. However, it is noteworthy that in the ethanol-exposed cultures, treatment with a PPAR- $\delta$  or PPAR- $\gamma$  agonist increased MAG-1 expression, narrowing the differences relative to control (Figure 5(c)), and the PPAR- $\delta$  agonist reduced expression of both HNE and NTyR, resulting in significantly lower levels of the latter in the ethanol group (Figure 5(h)). The other PPAR-agonist associated effects that resulted in similar mean levels of Hu, GFAP, ChAT, and  $\beta$ -actin in control and ethanol-exposed cultures were consequences of modest declines in protein expression in the control groups and/or slight shifts in protein expression in the ethanol-exposed groups.

# 4 Discussion

FASD is the most common preventable and costly cause of mental retardation and attention-deficit hyperactivity disorders in the United States. FASD is caused by chronic or binge prenatal alcohol exposures. In addition to its teratogenic effects in the brain, prenatal alcohol exposures lead to low birth weight, pre-term delivery, fetal demise, and cognitive-motor deficits. Our previous studies linked cerebellar hypoplasia and neuronal migration disorders to neuronal insulin resistance and oxidative stress in experimental FASD [3,6,13,14]. Besides the impairments in growth, survival, and neuronal migration, ethanolimpaired brain insulin signaling inhibits mitochondrial function and neurotransmitter gene expression. Ethanolassociated increases in oxidative stress are mediated by mitochondrial dysfunction, impairments signaling through metabolic pathways, and increased acetaldehyde generation. The present study was designed to determine if PPAR agonists could preserve cerebellar structure, reduce neurotoxic injury, and enhance insulin/IGF-1 signaling mechanisms impaired by developmental exposure to ethanol. The underlying hypothesis was driven by the earlier findings that PPAR agonists (1) provide neuroprotection in other models of brain insulin resistance, (2) can prevent experimental alcohol-induced liver disease, and (3) are therapeutically effective in human insulin resistance-associated diseases, including diabetes mellitus, non-alcoholic steatohepatitis, and neurodegeneration.

We used cerebellar slice cultures to examine the therapeutic/protective effects of PPAR agonists in an experimental model of FASD that produces in long-term structural, functional, and gene expression abnormalities in the cerebellum [20]. Slice cultures are more advantageous than isolated neuronal cultures as an ex vivo model because gene expression and signal transduction can be measured in tissue that retains its normal 3-dimensional architecture and has all cell types represented. We compared responses to PPAR- $\alpha$ , PPAR- $\delta$ , and PPAR- $\gamma$  agonists because all three receptor subtypes are expressed in the brain, although PPAR- $\delta$ is most abundant. In addition, previous studies showed that cellular and tissue responses to different PPAR agonist subtypes can vary with tissue type and disease model [5, 10, 11, 26, 33, 39, 40].

The increased cytotoxicity in ethanol-treated versus control cultures was expected based on previous reports [15, 30]. The PPAR agonist-mediated dampening of cytotoxicity in ethanol-exposed cultures corresponds with the antioxidant and pro-metabolic effects of these compounds. Histological studies revealed that PPAR- $\delta$  or PPAR- $\gamma$  agonist treatment of control cultures reduced cell density in the external granule layer; whereas the PPAR- $\alpha$  agonist increased cell density in the external granule layer, narrowed the molecular layer, and reduced cell density in the internal granule layer. Since maturation of the cerebellum is associated with depletion of the external granule layer due to migration of neuroblasts to the internal granule layer where they mature and extend neuritic processes, leading to increased neuropil volume and reduced cell body density, the findings suggest that PPAR- $\delta$  and PPAR- $\gamma$  agonists enhance normal cerebellar maturation and differentiation. In contrast, the PPAR- $\alpha$  agonist may compromise normal cerebellar maturation, as evidenced by the resulting poor cortical lamination with narrowing the molecular layer.

The ethanol-induced cell loss in granule and Purkinje layers of the cerebellum corresponds with the increased G6PD levels in culture supernatant (cytotoxic responses), and findings from our previous in vivo characterization of this model [20]. The PPAR- $\alpha$  agonist treatment provided no appreciable benefit relative to vehicle, and moreover resulted in further cell loss in the internal granule and Purkinje cell layers. These findings, together with those in control cultures, suggest that PPAR- $\alpha$  agonist treatments may be detrimental to brain development and maturation. In contrast, the structural restorations/preservations afforded by PPAR- $\delta$  or PPAR- $\gamma$  agonist treatments of ethanolexposed cultures correspond with the reductions in G6PD levels, and suggest that these agents have potential therapeutic application for supporting or enhancing cerebellar development in FASD. Moreover, the finding that control cerebellar maturation is also abetted by PPAR- $\delta$ or PPAR- $\gamma$  agonist treatments highlights the concept that insulin signaling is critical to normal development and that brain development may be supported by nutritional measures that optimize brain insulin signaling networks.

Examination of the upstream mediators insulin/IGF-1 signaling revealed that ethanol mainly impaired phosphorylation of the insulin and IGF-1 receptors and IRS-1, and that these effects were abrogated by treatment with PPAR agonists, particularly PPAR- $\delta$ . In addition, upstream insulin/IGF signaling could have been further enhanced by the PPAR- $\alpha$  or PPAR- $\delta$  agonist treatments due to associated increases in insulin and IGF-1 receptor expression. Mechanistically, increased receptor abundance could facilitate downstream signaling through both IRS-dependent and IRS-independent pathways.

Analysis of Akt pathway protein expression and phosphorylation revealed that ethanol exposure significantly reduced cerebellar Akt, GSK-3 $\beta$ , p70S6K, and PRAS40 phosphorylation. These effects could have contributed to the increased cytotoxicity in the cultures, as well as the structural pathology corresponding to cell loss and impaired neuronal migration (cortical lamination). The major effect of the PPAR agonists was to increase Akt, GSK-3 $\beta$ , and PRAS40 phosphorylation. Most likely, this response helped to restore survival and metabolic functions, with attendant relative preservation of the cerebellar cortical architecture. It is noteworthy that the PPAR- $\delta$  agonist was more effective than the PPAR- $\alpha$  or PPAR- $\gamma$  agonist for enhancing Akt pathway signaling and restoring cerebellar structure, consistent with earlier findings in models of brain and liver insulin resistance [10, 11, 32].

Ethanol reduced the expression of neuronal (Hu and ChAT) and mature oligodendrocye (MAG-1) proteins and increased expression of astrocyte (GFAP) and oxidative stress (N-TyR) molecules. These effects were prevented or reduced by the PPAR agonist treatments, particularly PPAR- $\delta$ , followed by PPAR- $\gamma$ , corresponding with the improvements in cerebellar histology and insulin signaling through the insulin receptor, Akt, and p70S6K, and reduced signaling through GSK-3 $\beta$ . Oligodendrocyte myelin synthesis and maintenance are regulated by insulin/IGF-1 [17,21,43], and white matter hypotrophy and atrophy are recognized consequences of ethanol-mediated neurotoxicity or neurodegeneration [7,8,27]. The finding that ethanolassociated inhibition of MAG-1 was only modestly reversed by the PPAR- $\delta$  or PPAR- $\gamma$  agonist treatments, suggests that other measures are needed to restore function and maturation of oligodendrocytes in FASD.

#### 5 Conclusions

In conclusion, the results suggest that PPAR agonists could be used prevent or substantially reduce the severity of FASD-associated structural and functional abnormalities in the cerebellum. However, it is also clear that each subtype of PPAR agonist provides a different level and quality of support for brain development in the context of FASD, and that no single subclass may provide adequate neuroprotection. Therefore, future therapeutic approaches should consider targeting 2 or 3 of the PPAR sub-types, perhaps through the use of hybrid agonists.

Acknowledgment Our research is supported by grants AA-11431, AA-12908, and AA-16126 from the National Institute for Alcohol Abuse and Alcoholism at the National Institutes of Health.

## References

- E. D. Adickes, T. J. Mollner, and S. K. Lockwood, *Closed chamber system for delivery of ethanol to cell cultures*, Alcohol Alcohol, 23 (1988), 377–381.
- [2] M. C. Camp, R. D. Mayfield, M. McCracken, L. McCracken, and A. A. Alcantara, *Neuroadaptations of Cdk5 in cholinergic interneurons of the nucleus accumbens and prefrontal cortex of inbred alcohol-preferring rats following voluntary alcohol drinking*, Alcohol Clin Exp Res, 30 (2006), 1322–1335.
- [3] J. J. Carter, M. Tong, E. Silbermann, S. A. Lahousse, F. F. Ding, L. Longato, et al., *Ethanol impaired neuronal migration is* associated with reduced aspartyl-asparaginyl-beta-hydroxylase expression, Acta Neuropathol, 116 (2008), 303–315.
- [4] P. P. Cherian, S. Schenker, and G. I. Henderson, *Ethanol-mediated DNA damage and PARP-1 apoptotic responses in cultured fetal cortical neurons*, Alcohol Clin Exp Res, 32 (2008), 1884–1892.
- [5] W. Chokesuwattanaskul, Y. Yaqub, J. Suarez, J. Simoni, G. Simoni, K. Nugent, et al., *Effect of pioglitazone on platelet*

aggregation in a healthy cohort, Cardiology, 116 (2010), 253–256.

- [6] J. Chu, M. Tong, and S. M. de la Monte, Chronic ethanol exposure causes mitochondrial dysfunction and oxidative stress in immature central nervous system neurons, Acta Neuropathol, 113 (2007), 659–673.
- [7] S. M. de la Monte, *Disproportionate atrophy of cerebral white matter in chronic alcoholics*, Arch Neurol, 45 (1988), 990–992.
- [8] S. M. de la Monte, L. Longato, M. Tong, S. DeNucci, and J. R. Wands, *The liver-brain axis of alcohol-mediated neurodegeneration: role of toxic lipids*, Int J Environ Res Public Health, 6 (2009), 2055–2075.
- [9] S. M. de la Monte, T. Neely, J. Cannon, and J. Wands, *Ethanol impairs insulin-stimulated mitochondrial function in cerebellar granule neurons*, Cell Mol Life Sci, 58 (2001), 1950–1960.
- [10] S. M. de la Monte, M. Pang, R. Chaudhry, K. Duan, L. Longato, J. Carter, et al., *Peroxisome proliferator-activated receptor agonist treatment of alcohol-induced hepatic insulin resistance*, Hepatol Res, 41 (2011), 386–398.
- [11] S. M. de la Monte, M. Tong, N. Lester-Coll, M. Plater Jr., and J. R. Wands, *Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease*, J Alzheimers Dis, 10 (2006), 89–109.
- [12] S. M. de la Monte and J. R. Wands, *Mitochondrial dna damage and impaired mitochondrial function contribute to apoptosis of insulin-stimulated ethanol-exposed neuronal cells*, Alcohol Clin Exp Res, 25 (2001), 898–906.
- [13] S. M. de la Monte and J. R. Wands, Chronic gestational exposure to ethanol impairs insulin-stimulated survival and mitochondrial function in cerebellar neurons, Cell Mol Life Sci, 59 (2002), 882–893.
- [14] S. M. de la Monte and J. R. Wands, *Role of central nervous system insulin resistance in fetal alcohol spectrum disorders*, J Popul Ther Clin Pharmacol, 17 (2010), e390–e404.
- [15] S. M. de la Monte, X. J. Xu, and J. R. Wands, *Ethanol inhibits insulin expression and actions in the developing brain*, Cell Mol Life Sci, 62 (2005), 1131–1145.
- [16] H. K. de Licona, B. Karacay, J. Mahoney, E. McDonald, T. Luang, and D. J. Bonthius, A single exposure to alcohol during brain development induces microencephaly and neuronal losses in genetically susceptible mice, but not in wild type mice, Neurotoxicology, 30 (2009), 459–470.
- [17] A. J. D'Ercole, P. Ye, A. S. Calikoglu, and G. Gutierrez-Ospina, *The role of the insulin-like growth factors in the central nervous* system, Mol Neurobiol, 13 (1996), 227–255.
- [18] H. Dudek, S. Datta, T. Franke, M. Birnbaum, R. Yao, G. Cooper, et al., *Regulation of neuronal survival by the serine-threonine* protein kinase Akt, Science, 275 (1997), 661–665.
- [19] J. L. Evans, J. J. Lin, and I. D. Goldfine, Novel approach to treat insulin resistance, type 2 diabetes, and the metabolic syndrome: simultaneous activation of PPARα, PPARγ, and PPARδ, Curr Diabetes Rev, 1 (2005), 299–307.
- [20] A. Ewenczyk, J. Ziplow, M. Tong, T. Le, and S. M. de la Monte, Sustained impairments in brain insulin/IGF signaling in adolescent rats subjected to binge alcohol exposure during development, J Clin Exp Pathol, 2 (2012), 106.
- [21] S. Freude, U. Leeser, M. Müller, M. M. Hettich, M. Udelhoven, K. Schilbach, et al., *IRS-2 branch of IGF-1 receptor signaling is essential for appropriate timing of myelination*, J Neurochem, 107 (2008), 907–917.
- [22] H. Hallak, A. Seiler, J. Green, A. Henderson, B. Ross, and R. Rubin, *Inhibition of insulin-like growth factor-I signaling by ethanol in neuronal cells*, Alcohol Clin Exp Res, 25 (2001), 1058–1064.
- [23] J. He, S. de la Monte, and J. R. Wands, Acute ethanol exposure inhibits insulin signaling in the liver, Hepatology, 46 (2007), 1791–1800.

- [24] A. Jawerbaum and E. Capobianco, *Review: Effects of PPAR activation in the placenta and the fetus: implications in maternal diabetes*, Placenta, 32 (2011), S212–S217.
- [25] C. Lamers, M. Schubert-Zsilavecz, and D. Merk, *Therapeu*tic modulators of peroxisome proliferator-activated receptors (*PPAR*): a patent review (2008-present), Expert Opin Ther Pat, 22 (2012), 803–841.
- [26] G. Landreth, Therapeutic use of agonists of the nuclear receptor PPARγ in Alzheimer's disease, Curr Alzheimer Res, 4 (2007), 159–164.
- [27] P. J. Langlais and S. X. Zhang, Cortical and subcortical white matter damage without Wernicke's encephalopathy after recovery from thiamine deficiency in the rat, Alcohol Clin Exp Res, 21 (1997), 434–443.
- [28] L. Longato, K. Ripp, M. Setshedi, M. Dostalek, F. Akhlaghi, M. Branda, et al., *Insulin resistance, ceramide accumulation, and endoplasmic reticulum stress in human chronic alcohol-related liver disease*, Oxid Med Cell Longev, 2012 (2012), 479348.
- [29] S. N. Mattson, N. Crocker, and T. T. Nguyen, *Fetal alcohol spectrum disorders: neuropsychological and behavioral features*, Neuropsychol Rev, 21 (2011), 81–101.
- [30] S. M. Mooney and M. W. Miller, *Ethanol-induced neuronal death in organotypic cultures of rat cerebral cortex*, Brain Res Dev Brain Res, 147 (2003), 135–141.
- [31] L. Nagy, A. Szanto, I. Szatmari, and L. Széles, Nuclear hormone receptors enable macrophages and dendritic cells to sense their lipid environment and shape their immune response, Physiol Rev, 92 (2012), 739–789.
- [32] M. Pang, S. M. de la Monte, L. Longato, M. Tong, J. He, R. Chaudhry, et al., *PPARδ agonist attenuates alcohol-induced hepatic insulin resistance and improves liver injury and repair*, J Hepatol, 50 (2009), 1192–1201.
- [33] W. Pang, N. Li, D. Ai, X. L. Niu, Y. F. Guan, and Y. Zhu, Activation of peroxisome proliferator-activated receptor-γ downregulates soluble epoxide hydrolase in cardiomyocytes, Clin Exp Pharmacol Physiol, 38 (2011), 358–364.
- [34] J. Plutzky, The PPAR-RXR transcriptional complex in the vasculature: energy in the balance, Circ Res, 108 (2011), 1002–1016.
- [35] E. Riley, M. Infante, and K. Warren, *Fetal alcohol spectrum disorders: an overview*, Neuropsychol Rev, 21 (2011), 73–80.
- [36] M. V. Schmidt, B. Brüne, and A. von Knethen, *The nuclear hormone receptor PPARγ as a therapeutic target in major diseases*, ScientificWorldJournal, 10 (2010), 2181–2197.
- [37] J. M. Silvers, S. Tokunaga, G. Mittleman, T. O'Buckley, A. L. Morrow, and D. B. Matthews, *Chronic intermittent ethanol* exposure during adolescence reduces the effect of ethanol challenge on hippocampal allopregnanolone levels and Morris water maze task performance, Alcohol, 39 (2006), 151–158.
- [38] S. J. Soscia, M. Tong, X. J. Xu, A. C. Cohen, J. Chu, J. R. Wands, et al., *Chronic gestational exposure to ethanol causes insulin* and *IGF resistance and impairs acetylcholine homeostasis in the* brain, Cell Mol Life Sci, 63 (2006), 2039–2056.
- [39] M. Viana Abranches, F. C. Esteves de Oliveira, and J. Bressan, Peroxisome proliferator-activated receptor: effects on nutritional homeostasis, obesity and diabetes mellitus, Nutr Hosp, 26 (2011), 271–279.
- [40] Y. X. Wang, C. L. Zhang, R. T. Yu, H. K. Cho, M. C. Nelson, C. R. Bayuga-Ocampo, et al., *Regulation of muscle fiber type and running endurance by PPARδ*, PLoS Biol, 2 (2004), e294.
- [41] G. Wolf, Retinoic acid activation of peroxisome proliferationactivated receptor delta represses obesity and insulin resistance, Nutr Rev, 68 (2010), 67–70.
- [42] J. Xu, J. E. Yeon, H. Chang, G. Tison, G. J. Chen, J. Wands, et al., *Ethanol impairs insulin-stimulated neuronal survival in the developing brain: role of PTEN phosphatase*, J Biol Chem, 278 (2003), 26929–26937.

- [43] P. Ye, G. Kollias, and A. J. D'Ercole, *Insulin-like growth factor-I ameliorates demyelination induced by tumor necrosis factoralpha in transgenic mice*, J Neurosci Res, 85 (2007), 712–722.
- [44] F. X. Zhang, R. Rubin, and T. A. Rooney, *Ethanol induces apoptosis in cerebellar granule neurons by inhibiting insulin-like growth factor 1 signaling*, J Neurochem, 71 (1998), 196–204.