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4-Phenylbutyric Acid Protects Ethanol-induced Damage in the Developing Mouse Brain

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Abstract

Background: Ethanol exposure during pregnancy may result in fetal alcohol spectrum disorders (FASD). One of the most deleterious consequences of ethanol exposure is neuronal loss in the developing brain. Previously we have shown ethanol exposure induced neuroapoptosis in the brain of postnatal day 4 (PD4) mice but not PD12 mice. This differential susceptibility may result from insufficient cellular stress response system such as unfolded protein response (UPR, also known as ER stress) in PD4 mice. In this study, we compared the effect of ethanol on endoplasmic reticulum (ER) stress in PD4 and PD12 mice and determined whether the inhibition of ER stress could protect the developing brain from ethanol-damage.

Methods: We used a third trimester equivalent mouse model of FASD. PD4 and PD12 C57BL/6 mice were subcutaneously injected with saline (control), ethanol, ethanol plus 4-phenylbutyric acid (4-PBA), a chemical chaperone known as ER stress inhibitor, and 4-PBA alone. The expression of apoptosis marker, ER stress markers, and markers for glial cell activation were examined in the cerebral cortex.

Results: Ethanol induced neuroapoptosis and increased the expression ER stress markers, such as ATF6, GRP78, IRE1 α , MANF and caspase-12 in PD4 but not PD12 mice. Ethanol exposure also activated microglia and astrocytes. Interestingly, treatment of 4-PBA attenuated ethanol-induced neuroapoptosis. Moreover, 4-PBA inhibited the expression of aforementioned ER stress markers and ethanol-induced glial activation in PD4 mice.

Conclusions: ER stress plays an important role in ethanol-induced damage to the developing brain. Inhibition of ER stress is neuroprotective and may provide a new therapeutic strategy to treat FASD.

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Conflict of interest

The authors declare that they have no competing interests

Keywords

Development; fetal alcohol syndrome; neurotoxicity; unfolded protein response

Introduction

Fetal alcohol exposure may cause damage to the developing fetus which leads to fetal alcohol spectrum disorders (FASD). The developing central nervous system (CNS) is particularly vulnerable to ethanol exposure. Neuronal loss in the developing CNS is one of most devastating consequences of ethanol exposure which may account for many symptoms associated with FASD patients. The mechanisms underlying ethanol-induced neuronal loss, however, are complex and remain unclear. Animal models for FASD have demonstrated a temporal window of neuron susceptibility to ethanol toxicity (Maier et al., 1999, Siler-Marsiglio et al., 2006).

The endoplasmic reticulum (ER) regulates the folding, maturation and export of most secreted and transmembrane proteins. Under some cellular stress conditions, unfolded or misfolded proteins accumulate in the ER and trigger intracellular signal transduction pathways cumulatively known as ER stress response or unfolded protein response (UPR) (Ron, 2002, Xu et al., 2005). The ER stress response or UPR is mediated by three transmembrane ER sensor proteins: PERK, IRE1 α , and ATF6. These sensors are occupied by GRP78 which prevents their activation. Accumulation of unfolded/misfolded proteins in the ER results in the disassociation of ER sensors with GRP78 and the subsequent activation of the sensors. PERK is a Ser/Thr protein kinase; when activated, it phosphorylates eIF2 α , resulting in the inhibition of translation. IRE1 α is a bifunctional kinase and endoribonuclease. Oligomerization of IRE1 α excises one intron of its target mRNA XBP1. Spliced XBP1 mRNA codes for the active form of the transcription factor XBP1s. XBP1s regulates genes involved in protein folding and ER-associated protein degradation (ERAD). Upon ER-stress, ATF6 translocates from ER to the Golgi apparatus where it is cleaved and acts as a transcription factor to regulate UPR genes. The activation of these transducers results in protective adaptive responses to improve protein folding and lowering protein synthesis (Ron, 2002). However, sustained ER stress ultimately leads to irreversible damage and cell death (Xu et al., 2005, Rasheva and Domingos, 2009). We previously demonstrated that ethanol exposure increased a number of ER stress in multiple brain regions of early postnatal mice (Ke et al., 2011). Interestingly, in an effort to compare the expression profile of genes involved in apoptosis and ER stress in developing brains, we discovered that pro-apoptotic effectors are expressed at higher levels, whereas genes involved in UPR are expressed at lower levels in the brain of postnatal day 4 (PD4) mice (alcohol-sensitive period) compared to PD12 (alcohol-resistant period) (Alimov et al., 2013). Therefore, we hypothesized that ER stress plays an important role in neuroapoptosis and the vulnerability of developing brains may result from inadequate UPR systems to couple with ER stress.

Sodium 4-phenylbutyrate acid (4-PBA) is often considered an “ER stress inhibitor” primarily due to its properties as a chemical chaperone to improve protein folding and reduce ER stress in multiple disorders (Mimori et al., 2012, Mizukami et al., 2010, Qi et al.,

2004). 4-PBA has been approved for clinical use to treat protein folding diseases (Burrows et al., 2000, Rubenstein and Zeitlin, 2000, Perlmutter, 2002). Despite the fact that ethanol exposure can cause ER stress, it is unclear whether 4-PBA provides protection against ethanol-induced neurotoxicity. In this study, we aimed to determine whether 4-PBA protects neurons against ethanol-induced damage in the cerebral cortex and the underlying mechanisms. Our results showed the treatment of 4-PBA alleviated ethanol-induced neuroapoptosis, glial activation and ER stress responses, suggesting 4-PBA can be a potential therapeutic agent for FASD.

MATERIALS AND METHODS

Materials

4-PBA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-GRP78, anti-eIF2 α , and anti-ATF6 antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA). Anti-cleaved caspase-3, anti-phospho-eIF2 α (Ser51), and anti-IRE1 α antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-phospho-IRE1 α (Ser724), Anti-MANF, and Anti-caspase-12 antibodies were obtained from Abcam (Cambridge, MA). Anti-Iba-1 antibody was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). Anti-glial fibrillary acidic protein (GFAP) antibody was obtained from Sigma Chemical Co. (St. Louis, MO). Ketamine and xylazine were obtained from Henry Schein Animal Health (Dublin, OH).

Animals and treatment

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained at the Animal Facility of the University of Kentucky Medical Center. All procedures were performed in accordance with the guidelines set by the NIH and the Animal Care and Use Committee of the University of Kentucky. A well-established mouse model of postnatal ethanol exposure was employed (Alimov et al., 2013, Ke et al., 2011, Liu et al., 2009, Olney et al., 2002, Young et al., 2008). Postnatal 4-day-old (PD4) or 12-day-old (PD12) mouse pups were weighed and randomly assigned to 4 groups: control (saline), ethanol, ethanol plus 4-PBA and 4-PBA alone. Each pup in control or ethanol group was injected subcutaneously with saline or ethanol (2.5 g/kg, 20% solution in saline) twice at time 0 and 2 hours. Eight hours after the first ethanol injection the brains were removed and processed for immunoblotting or immunohistochemical analysis. For chemical inhibition of ER stress, pups received 4-PBA diluted in Phosphate Buffered Saline (PBS) at a dose of 100 mg/kg 1 day and 0.5 hour prior to ethanol exposure by injection. The concentration was selected based on previous studies of 4-PBA injection in neonatal or adult animals (Jeanne et al., 2015, Carlisle et al., 2014, Shimizu et al., 2014). Pups of both genders were used in this study and we did not analyze male and female animals separately.

Preparation of protein extracts and Immunoblotting

The procedure for preparation of protein extracts from brain tissues has been previously described (Ke et al., 2011, Alimov et al., 2013). Briefly, mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg), and the brain was immediately dissected. The tissues were frozen in liquid nitrogen and stored at -80°C . The

tissues were homogenized using an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5% NP-40, 0.25% SDS, 5 µg/ml leupeptin, and 5 µg/ml aprotinin. Homogenates were centrifuged at 20,000 × g for 30 min at 4°C and the supernatant fraction was collected.

The immunoblotting procedure has been previously described (Ke et al., 2011, Alimov et al., 2013). In brief, aliquots of the protein samples (30 µg) were separated on an SDS-polyacrylamide gel by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% bovine serum albumin (BSA) or 5% nonfat milk in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 (TBST) at room temperature for 1 hour. Subsequently, the membranes were probed with primary antibodies directed against target proteins overnight at 4°C. The final dilutions for primary antibodies were: anti-cleaved-caspase-3 antibody, 1:1000; anti-p-eIF2α (Ser51) antibody 1:1000; anti-eIF2α antibody 1:1000; anti-ATF6 antibody 1:1000; anti-GRP78 antibody 1:1000; anti-p-IRE1α(S724) antibody 1:1000; anti-IRE1α antibody 1:1000; anti-caspase-12 antibody 1:1000; anti-MANF antibody 1:1000; anti-Iba-1 antibody 1:1000; anti-GFAP antibody 1:1000 and anti-actin antibody, 1:5000. After 3 quick washes in TBST, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Hts, IL). The immune complexes were detected by the enhanced chemiluminescence method (Amersham). The density of immunoblotting was quantified with the software of Quantity One (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry (IHC)

After treatments, the mice were anesthetized with ketamine/xylazine, and then perfused with saline followed by 4% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.2). The brains were removed and post-fixed in 4% paraformaldehyde for an additional 24 hours and then transferred to 30% sucrose. The brain was sectioned at 40 µm with a sliding microtome (Leica Microsystems, Wetzlar, Germany).

The procedure for IHC staining has been previously described (Ke et al., 2011, Alimov et al., 2013). In brief, the mounted sections were incubated in 0.3% H₂O₂ in methanol for 30 min at room temperature and then treated with 0.1% TritonX-100 for 10 min in PBS. The sections were washed with PBS three times, and then blocked with 1% BSA and 0.01% TritonX-100 for 1 hour at room temperature. The sections were incubated with an anti-cleaved-caspase-3 antibody (1:1000), anti-p-eIF2α antibody (1:500), anti-caspase-12 antibody (1:1000), anti-MANF antibody (1:1000), anti-Iba-1 antibody (1:1000), or anti-GFAP antibody (1:1000) overnight at 4°C. Negative controls were performed by omitting the primary antibody. After rinsing in PBS, sections were incubated with a biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA; 1:200) for 1 hour at room temperature. The sections were washed 3 times with PBS, then incubated in avidin–biotin–peroxidase complex (Vector Laboratories Inc. 1:100 in PBS) for 1 h and developed in 0.05% 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich) containing 0.003% H₂O₂ in PBS. The images were recorded using an Olympus microscope (BX61) equipped with a DP70 digital camera.

Statistical Analysis

All analyses were performed using the SPSS software (SPSS, Chicago, IL, USA). The data were expressed as mean $\pm$ SEM. Differences among experimental groups were analyzed by one-way Analysis of Variance (ANOVA). Differences in which the p value was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific post hoc comparisons between treatment groups were examined with the Tukey test.

Results

4-PBA decreases ethanol-induced caspase-3 activation in the brain of PD4 mice

Consistent with previous findings, we observed that ethanol increased the expression of cleaved-caspase-3 (c-caspase-3), an apoptosis marker in the cerebral cortex of PD4 mice ($F_{(3,8)} = 15.7$, $p = 0.001$) but not PD12 mice by immunoblotting (Fig. 1A and 1B). This result was further confirmed by the increase of IHC staining of c-caspase-3 in the cerebral cortex of PD4 mice ($F_{(3,12)} = 11.8$, $p = 0.001$) (Fig. 1C) but not in PD12 mice (data not shown). To investigate whether 4-PBA can protect brains from ethanol-induced damage, we administered 4-PBA (100 mg/kg) 1 day and 0.5 hour prior to ethanol exposure by subcutaneous injection on PD4 or PD12. The concentration was selected based on previous studies of 4-PBA injections in neonatal or adult animals (Jeanne et al., 2015, Carlisle et al., 2014, Shimizu et al., 2014). 4-PBA significantly reduced ethanol-induced expression of c-caspase-3 in the cerebral cortex as shown by immunoblot ($p = 0.001$) and IHC ($p = 0.004$) (Fig. 1A, 1B and 1C). The treatment of 4-PBA alone, however, does not have a significant effect on the expression of c-caspase-3.

4-PBA alleviates ethanol-induced ER stress in the brain of PD4 mice

ER stress has been proposed to play a vital role in ethanol-induced brain damage (Ke et al., 2011). We have previously demonstrated that the brain develops more effective defensive systems, such as UPR, as it matures shown by the higher expression levels of UPR genes in the ethanol-resistant PD12 mice than the ethanol-sensitive PD4 mice (Alimov et al., 2013). To determine whether ER stress may account for the differential effects of ethanol exposure to PD4 and PD12 brains, we examined the expression of a group of ER stress markers in the cerebral cortex by immunoblot. In PD4 mice, ethanol significantly increased the expression of ATF6 ($F_{(3,8)} = 14.2$, $p = 0.001$), GRP78 ($F_{(3,8)} = 12.4$, $p = 0.002$), MANF ($F_{(3,8)} = 36.5$, $p = 0.001$), p-IRE1 α ($F_{(3,8)} = 13.3$, $p = 0.002$), phosphorylated eIF2 α (p-eIF2 α) ($F_{(3,8)} = 16.2$, $p = 0.001$) and caspase-12 ($F_{(3,8)} = 5.0$, $p = 0.031$) (Fig. 2A). While 4-PBA alone had little effect on the basal level of the selected ER stress markers, it abolished the ethanol-induced increase of these proteins. Compared with the ethanol-treated group, 4-PBA significantly decreased ethanol-induced expression of ATF6 ($p = 0.003$), GRP78 ($p = 0.037$), MANF ($p = 0.007$), p-IRE1 α ($p = 0.010$), p-eIF2 α ($p = 0.023$), and caspase-12 ($p = 0.027$). In contrast, neither ethanol exposure nor treatment of 4-PBA has an effect on the expression of these ER stress markers in PD12 mice (Fig. 2B).

Additional IHC staining was performed on MANF, p-eIF2 α and caspase-12 in PD4 brains and the results were consistent with that of immunoblotting. Ethanol significantly increased

the expression of caspase-12 ($F_{(3,12)} = 9.6$, $p = 0.002$), p-eIF2 α ($F_{(3,12)} = 11.7$, $p = 0.001$), and MANF ($F_{(3,12)} = 7.7$, $p = 0.004$) in PD4 cerebral cortex. While 4-PBA alone had little effect on these proteins, it significantly decreased ethanol-induced expression of caspase-12 ($p = 0.038$), p-eIF2 α ($p = 0.04$), and MANF ($p = 0.042$).

4-PBA inhibits ethanol-induced glial activation in the brain of PD4 mice

Astrocytes and microglia are the two main types of glial cells in the central nervous system that normally provide support and protect neurons. They can be activated under pathological conditions to either release proinflammatory factors or phagocytose apoptotic neurons and release anti-inflammatory mediators (Saito et al., 2016). To address whether astrocytes and microglia are activated by ethanol exposure, we examined the expression of Iba-1 for microglia and GFAP for astrocytes in the cerebral cortex of PD4 and PD12 brains. As shown by Figure 3A and 3B, ethanol increased the expression of Iba-1 ($F_{(3,8)} = 6.5$, $p = 0.016$) and GFAP ($F_{(3,8)} = 13.0$, $p = 0.002$) in PD4 mice but not in PD12 mice. Interestingly, 4-PBA restored the expression of Iba-1 ($p = 0.048$) and GFAP ($p = 0.029$) in PD4 brains, compared with the ethanol-treated group (Fig. 3A and 3C). The immunoblot results were consistent with that of IHC staining (Fig. 3C). Ethanol significantly increased the expression Iba-1 ($F_{(3,12)} = 7.9$, $p = 0.003$) and GFAP ($F_{(3,12)} = 6.4$, $p = 0.008$) in PD4 cerebral cortex, while 4-PBA blocked ethanol-induced expression of Iba-1 ($p = 0.016$) and GFAP ($p = 0.017$). 4-PBA alone had little effect on Iba-1 or GFAP.

Discussion

Our previous studies suggest that ER stress may be involved in ethanol-induced neuroapoptosis and vulnerability of immature brain to ethanol exposure could result from a deficiency in the stress response system such as UPR (Alimov et al., 2013). To test this hypothesis, we used a well-established mouse model for FASD to investigate ethanol-induced apoptosis, ER stress, and glia activation in the brain. Consistent with previous findings, we demonstrated c-caspase-3 is expressed at a higher level on PD4 than PD12 whereas UPR mediators including ATF6 and GRP78 are expressed at a lower level on PD4 than PD12. The ethanol exposure regimen, two doses of 2.5 g/kg per dose with 2 hours apart, induced the expression of c-caspase-3 on PD4 but not PD12. It also increased a number of ER stress markers, including ATF6, GRP78, MANF, p-eIF2 α , p-IRE1 α , and caspase-12 as well as glial cell markers such as Iba-1 and GFAP on PD4 but not PD12. Strikingly, treatment of 4-PBA, a known ER stress inhibitor, not only suppresses the expression of the aforementioned ER stress markers, but also blocked the expression of c-caspase-3, suggesting ER stress contributes to ethanol neurotoxicity.

ER stress is an evolutionarily conserved cellular stress response that is initiated when sensor proteins at the ER membrane sense accumulation of unfolded/misfolded proteins (Hetz and Saxena, 2017, Ron and Walter, 2007, Walter and Ron, 2011). Depending on the nature of the ER stress, downstream transcription or translation programs are triggered to either alleviate stress or result in cell death. In this study, we showed that IRE1 α , one of the most evolutionarily conserved sensor proteins, is induced by ethanol exposure on PD4. IRE1 α is a transmembrane ER-resident protein that has dual protein kinase and RNase activities

(Iwawaki et al., 2001). Once activated, IRE1 α can autophosphorylate and regulate the splicing of XBP1 mRNA (Walter and Ron, 2011). Spliced XBP1 encodes a transcription factor XBP1s that modulate many genes involved in protein folding and secretion and ER-associated degradation to maintain ER homeostasis (Hetz et al., 2011, Acosta-Alvear et al., 2007, Lee et al., 2003). However, recent studies have indicated XBP1s can also target sequence-specific mRNAs for degradation through a process known as regulated IRE1 α -dependent decay (RIDD) leading to cell death (Hetz et al., 2015, Maurel et al., 2014). ATF6 is another ER stress sensor that is induced by ethanol in this study. Upon ER-stress, ATF6 translocates to the Golgi apparatus and is cleaved by proteases. The N-terminal fragment of ATF6 acts as a transcription factor that moves into the nucleus and regulates the expression of XBP1 (Walter and Ron, 2011) and other UPR mediators such as GRP78 (Jørgensen et al., 2000), leading to an increase in ER protein folding capacity and ER homeostasis.

ER stress can lead to cell death, though the molecular mechanisms remain poorly understood (Hetz and Saxena, 2017). Caspase-12 is an ER residing caspase and can be activated by ER stress (Yoneda et al., 2001, Szegezdi et al., 2003). Activation of caspase-12 has been reported in animal models and cell culture models for a number of neurodegenerative diseases and is believed to act on some downstream effectors including caspase-3 leading to cell death (Siman et al., 2001, Nakagawa et al., 2000, Chun et al., 2001, Oubrahim et al., 2002). Caspase-12-deficient mice are resistant to ER-directed pro-apoptotic signals but their cells still undergo other apoptotic pathways, suggesting a central role of caspase-12 in ER-stress induced apoptosis (Nakagawa et al., 2000). In this study, caspase-12 exhibited an expression pattern similar to c-caspase-3 upon ethanol exposure and treatment of ER stress inhibitor, suggesting caspase-12 is involved in ethanol-induced neuroapoptosis. However, future experiments using caspase-12 deficient mice or caspase-12 inhibitors are necessary to confirm the role of caspase-12 in ethanol neurotoxicity.

One of the molecular targets of ER stress transducers is MANF, which expression was also increased in ethanol-exposed PD4 brains in this study. Two ER stress response elements (ERSE I and II) are located in the promoter region of MANF, which can be recognized by UPR mediators: cleaved ATF6 and spliced XBP1 (Mizobuchi et al., 2007, Oh-Hashi et al., 2013, Wang et al., 2018a). MANF is up-regulated upon ER-stress and it can alleviate ER-stress induced neuronal cell death (Apostolou et al., 2008, Yu et al., 2010). As a neurotrophic factor, MANF is required for the development and survival of neurons in normal or pathological conditions (Voutilainen et al., 2009, Palgi et al., 2009, Chen et al., 2012, Petrova et al., 2003). It protects cortical neurons from ER-stress induced neurodegeneration caused by tunicamycin or thapsigargin treatment and cerebral ischemia (Apostolou et al., 2008, Airavaara et al., 2010, Airavaara et al., 2009, Huang et al., 2016). It also attenuates neuronal damage in 6-hydroxydopamine (6-OHDA)-induced rat model of Parkinson's disease (Voutilainen et al., 2009, Voutilainen et al., 2011). Recently, MANF was also reported to be required for retinal development in *Drosophila* (Walkowicz et al., 2017) and protected the degeneration of rod and cone photoreceptors in transgenic rats (Lu et al., 2018). In this study, upregulation of MANF may be a protective action in response ethanol-induced ER stress.

Microglia and astrocytes are the two main types of glial cells that play important roles in many homeostatic processes in the CNS. Under pathological conditions, they can be activated to either release pro-inflammatory cytokines or phagocytose apoptotic neurons and release anti-inflammatory mediators; the imbalance between these functions can result in neuroinflammation and neurodegeneration (Jäkel and Dimou, 2017, Temburni and Jacob, 2001). There are many studies showing that ethanol can activate microglia or astrocytes in experimental models for FASD and produce proinflammatory cytokines and reactive oxygen species (ROS) that may contribute to the long-lasting cellular, physiological and neurobehavioral deficits (Chastain and Sarkar, 2014, Kane and Drew, 2016, Saito et al., 2016, Wilhelm and Guizzetti, 2015, Wang et al., 2018b, Li et al., 2015). However, glial activation following ethanol exposure may also have a neuroprotective role as activated glial cells can either produce anti-inflammatory cytokines such as IL-10 and TGF- β or phagocytose apoptotic neurons in response to neuronal damage (Chastain and Sarkar, 2014, Saito et al., 2016). In this study, the expression of microglia and astrocytes are increased by ethanol exposure and reduced by 4-PBA, a pattern similar to c-caspase-3 expression. However, it is difficult to distinguish whether glial activation is the result of secondary response to neuronal damage or a direct effect of ethanol exposure. Future experiments are necessary to address the role of glial activation in ethanol neurotoxicity.

4-PBA is a small fatty acid that has been approved by the FDA to treat urea cycle disorders (Lichter-Konecki et al., 2011). Recent studies have significantly expanded the application of 4-PBA as a therapeutic molecule to a variety of diseases like cancer, cystic fibrosis, Alzheimer's disease and Parkinson's disease (Camacho et al., 2007, Zeitlin et al., 2002, Zhou et al., 2011, Cuadrado-Tejedor et al., 2011). The therapeutic effects of 4-PBA in these diseases are believed to involve two primary mechanisms: a chemical chaperone and histone deacetylase inhibitor (HDACi)(Miller et al., 2011, Basseri et al., 2009, Xiao et al., 2011, Yam et al., 2007). In this study, 4-PBA alone did not exhibit toxic effects on developing brains, but reduced ethanol-induced expression of ER stress markers. This may result from the HDAC inhibitory property of 4-PBA as it has been shown that deacetylation can regulate the stability and transcriptional activity of XBP1s and other UPR proteins (Wang et al., 2011, Karanam et al., 2007, Lassot et al., 2005, Bobrovnikova-Marjon et al., 2008). 4-PBA inhibition of ethanol-induced ER stress in the developing brain may also result from its property as a chemical chaperone that facilitates protein folding and reduces ER stress (Kolb et al., 2015, Cortez and Sim, 2014). More importantly, 4-PBA significantly reduced the ethanol-induced cell death, implying a potential therapeutic value of 4-PBA in treating FASD.

Taken together, this is the first study to show that 4-PBA offers protection against ethanol neurotoxicity in the developing brain. Although the mechanisms underlying the therapeutic effects of 4-PBA require further investigation, our study suggests that targeting ER stress may provide an effective therapeutic approach for FASD.

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Abbreviations:

ATF6	activating transcription factor 6
CNS	central nervous system
eIF2α	eukaryotic translation initiation factor 2 α
FASD	fetal alcohol spectrum disorders
GFAP	glial fibrillary acidic protein
GRP78	78-kDa glucose-regulated protein
Iba-1	ionized calcium binding adaptor molecule 1
IRE1α	inositol-requiring enzyme 1 α
MANF	mesencephalic astrocyte derived neurotrophic factor
PERK	pancreatic endoplasmic reticulum kinase
UPR	unfolded protein response
XBP1s	x-box binding protein 1

Reference

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Figure 1: Effect of ethanol and 4-PBA on the expression of c-caspase-3 during development
 C57BL6 mice of PD4 or PD12 were exposed to ethanol for 8 h with or without pre-treatment of 4-PBA (100 mg/kg), then sacrificed and cerebral cortices were processed. **A:** The expression of cleaved caspase-3 (c-caspase-3) on PD4 was evaluated by immunoblotting as described in the Materials and Methods. Relative levels of c-caspase-3 were determined by densitometry and normalized to actin. **B:** The expression of c-caspase-3 on PD12 was evaluated by immunoblotting and quantified. **C:** C57BL6 mice of PD4 were exposed to ethanol for 8 h with or without pre-treatment of 4-PBA (100 mg/kg), then sacrificed and cerebral cortices were processed for immunohistochemistry (IHC). The number of c-caspase-3-positive cells in the cerebral cortex was determined in twenty randomly selected sections using the software Image Lab 5.2. Four-five animals were analyzed for each group. Bar = 200 μ m in the upper panel and 50 μ m in the lower panel. * denotes significant difference ($p < 0.05$).

Figure 2: Effect of ethanol and 4-PBA on the expression of ER stress markers during development

C57BL6 mice of PD4 or PD12 were exposed to ethanol for 8 h with or without pre-treatment of 4-PBA (100 mg/kg), then sacrificed and cerebral cortices were processed. **A:** The expression of ER stress markers, ATF6, GRP78, p-IRE1α, p-eIF2α, MANF and caspase-12 was evaluated by immunoblotting as described in the Materials and Methods. Relative levels of these ER stress markers were determined by densitometry and normalized to actin. **B:** The expression of the selected ER stress markers on PD12 was evaluated by immunoblotting and quantified. **C:** C57BL6 mice of PD4 were exposed to ethanol for 8 h with or without pre-treatment of 4-PBA (100 mg/kg), then sacrificed and cerebral cortices were processed for IHC. The number of MANF-, caspase-12- or p-eIF2α-positive cells in the cerebral cortex was determined in twenty randomly selected sections using software of

Image Lab 5.2. Four-five animals were analyzed for each group. Bar = 200 μm in the upper panel and 50 μm in the lower panel. * denotes significant difference ($p < 0.05$).

Figure 3: Effect of ethanol and 4-PBA on glial activation during development

C57BL6 mice of PD4 or PD12 were exposed to ethanol for 8 h with or without pre-treatment of 4-PBA (100 mg/kg), then sacrificed and cerebral cortices were processed. **A:** The expression of Iba1 and GFAP was evaluated by immunoblotting as described in the Materials and Methods part. Relative levels of the Iba1 and GFAP were determined by densitometry and normalized to actin. **B:** The expression of Iba1 and GFAP on PD12 was evaluated by immunoblotting and quantified and normalized to actin. **C:** C57BL6 mice of PD4 were exposed to ethanol for 8h with or without pre-treatment of 4-PBA (100 mg/kg), then sacrificed and cerebral cortices were processed for IHC. The number of Iba1- and GFAP-positive cells in the cerebral cortex was determined in twenty randomly selected sections using software of Image Lab 5.2. Four-five animals were analyzed for each group.

Bar = 200 μm in the upper panel and 50 μm in the lower panel. * denotes significant difference ($p < 0.05$).