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Pioglitazone Blocks Ethanol Induction of Microglial Activation and Immune Responses in the Hippocampus, Cerebellum, and Cerebral Cortex in a Mouse Model of Fetal Alcohol Spectrum Disorders

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Abstract

Background—Fetal alcohol spectrum disorders (FASD) result from fetal exposure to alcohol and are the leading cause of mental retardation in the United States. There is currently no effective treatment that targets the causes of these disorders. Thus, novel therapies are critically needed to limit the neurodevelopmental and neurodegenerative pathologies associated with FASD.

Methods—A neonatal mouse FASD model was used to examine the role of the neuroimmune system in ethanol-induced neuropathology. Neonatal C57BL/6 mice were treated with ethanol, with or without pioglitazone, on postnatal days 4 through 9 and tissue was harvested one day post-treatment. Pioglitazone is a peroxisome proliferator-activated receptor (PPAR)- γ agonist that exhibits anti-inflammatory activity and is neuroprotective. We compared the effects of ethanol with or without pioglitazone on cytokine and chemokine expression and microglial morphology in the hippocampus, cerebellum, and cerebral cortex.

Results—In ethanol-treated animals compared to controls, cytokines IL-1 β and TNF- α mRNA levels were increased significantly in the hippocampus, cerebellum, and cerebral cortex. Chemokine CCL2 mRNA was increased significantly in the hippocampus and cerebellum. Pioglitazone effectively blocked the ethanol-induced increase in the cytokines and chemokine in all tissues to the level expressed in handled-only and vehicle-treated control animals. Ethanol also produced a change in microglial morphology in all brain regions that was indicative of microglial activation, and pioglitazone blocked this ethanol-induced morphological change.

Conclusions—These studies indicate that ethanol activates microglia to a pro-inflammatory stage and also increases the expression of neuroinflammatory cytokines and chemokines in diverse regions of the developing brain. Further, the anti-inflammatory and neuroprotective PPAR- γ agonist pioglitazone blocked these effects. It is proposed that microglial activation and inflammatory molecules expressed as a result of ethanol treatment during brain development

contribute to the sequelae associated with FASD. Thus, pioglitazone, and anti-inflammatory pharmaceuticals more broadly, have potential as novel therapeutics for FASD.

Keywords

Ethanol; Brain; Fetal alcohol spectrum disorder; Neuroinflammation; Microglia

INTRODUCTION

Despite public health warnings, 12% of pregnant women drink alcohol (Floyd et al., 2009). Consequently, fetal alcohol spectrum disorders (FASD) occur in 1% of births and are the leading cause of mental retardation in the United States (May et al., 2009). Alcohol has profound effects on the developing central nervous system (CNS) resulting in widespread neuropathology and associated disabilities which persist throughout life (Mattson et al., 2011, Riley et al., 2011).

Ethanol causes significant neuropathology throughout the brain including in the hippocampus, cerebellum, and cerebral cortex in both humans and animal models. In rodent models of FASD, pathology in these regions can result from exposure at any stage of gestation and the early postnatal period. This later period in the rodent corresponds to the stage of brain development that occurs in the late second to third trimester of human gestation (Clancy et al., 2001). Studies in postnatal rodents have revealed that ethanol exposure at various times during postnatal days 3 through 16 (P3–16) produces impaired brain growth, inhibition of neurogenesis, aberrant neuronal migration, neuronal loss, neuronal dysmorphology including disruption of dendritic development, electrophysiological disruption, and neurochemical disturbances. Ethanol exposure of the hippocampus and cerebral cortex of the human and the rodent leads to deficits in learning, memory, executive function, and emotion (Mattson et al., 2011, Riley et al., 2011). These deficits are attributable to pathology in these brain regions (Sowell et al., 2002, Slawecki et al., 2004, Gil-Mohapel et al., 2010, Puglia et al., 2010, Wilson et al., 2011, Zink et al., 2011, Banuelos et al., 2012, Everett et al., 2012, El Shawa et al., 2013, Leigland et al., 2013, Joseph et al., 2014, Wagner et al., 2014). Ethanol exposure of the developing cerebellum of humans and rodents leads to deficits in neuron cell number, electrophysiological characteristics, motor function, and cerebellar dependent learning (Hamre et al., 1993, Klintsova et al., 1998, Autti-Ramo et al., 2002, Brown et al., 2007, Servais et al., 2007, Mattson et al., 2011, Riley et al., 2011).

In recent years, it has become clear that chronic alcohol abuse results in immune activation in the CNS, which is believed to contribute to neurodegeneration (Crews et al., 2011). The effect of ethanol on neuroinflammation in adolescent, adult, and aged animals has been extensively documented (Crews et al., 2011, McClain et al., 2011, Kane et al., 2013, Kane et al., 2014). However, the effect of ethanol on immune activation in the developing CNS has just begun to be investigated (Saito et al., 2010, Kane et al., 2011, Tiwari et al., 2011). We demonstrated recently that ethanol causes death of Purkinje neurons in mice treated on P3–5 (Kane et al., 2011), as has been well documented in a variety of FASD models (Hamre et al., 1993, Pierce et al., 1999, Dikranian et al., 2005). We also documented that ethanol was toxic

to cerebellar granule cell neurons in culture as previously shown (Pantazis et al., 1993, Kane et al., 2011). In that study, we discovered that ethanol caused loss of microglial cells in vivo in the cerebellum and was toxic to microglia in culture. Interestingly, in the context of the current report, microglia that survived the toxic effects of ethanol had the phenotype of activated pro-inflammatory cells (Kane et al., 2011). Similarly, Saito et al. (2010) have suggested microglial activation of phagocytic cells in the cingulate cortex of an FASD model.

Microglia play important roles in the developing CNS such as phagocytosis of debris from cells eliminated during normal development (Bilbo et al., 2012) and synaptic pruning critical to the maturation of synapses (Nayak et al., 2014). Microglia also help to maintain the homeostasis of the normal CNS by producing neurotrophic factors and sequestering neurotransmitters (Nakajima et al., 2001, Streit et al., 2008). However, in response to CNS insult, microglia become activated and chronic microglial activation can contribute to neuropathology (Rivest, 2009). Thus, investigating the effects of ethanol insult on microglia in the developing CNS is particularly meaningful.

Peroxisome proliferator activated receptor (PPAR)- γ is a member of the nuclear receptor family of proteins. PPAR- γ agonists including thiazolidinediones such as pioglitazone were classically defined by their ability to modulate glucose and lipid homeostasis and they are commonly used in the treatment of type II diabetes (Cariou et al., 2012). More recently, PPAR- γ has been shown to modulate inflammatory responses, including immune activity in the CNS. We and others have demonstrated that PPAR- γ agonists suppress the activation of microglia (Diab et al., 2002) and are effective in suppressing inflammation in animal models of neuroinflammation and neurodegeneration (Heneka et al., 2001, Diab et al., 2002, Tureyen et al., 2007). Importantly, in the neonatal mouse model of FASD, we discovered that pioglitazone and other PPAR- γ agonists prevent ethanol-induced neuron and microglia loss and microglial activation in the developing cerebellum (Kane et al., 2011).

The current study was designed to evaluate the effects of ethanol on cytokine and chemokine expression and microglial pro-inflammatory activation in diverse brain regions in the third-trimester neonatal mouse model of FASD. The hippocampus, cerebellum, and cerebral cortex were evaluated because ethanol exposure during development is known to cause significant damage to these regions resulting in long term impairment of motor function, synaptic plasticity, learning and memory, as well as alterations in emotion and addiction. We demonstrate for the first time that ethanol induces neuroinflammation with the expression of pro-inflammatory molecules and microglial activation in all of these brain regions. Furthermore, we demonstrate that the PPAR- γ agonist pioglitazone suppresses ethanol-induced neuroinflammation. These studies suggest that pioglitazone, and perhaps other anti-inflammatory therapeutics, may be effective in the treatment of FASD.

MATERIALS AND METHODS

Animal treatment and blood ethanol concentration assay

Mice (C57BL/6) were purchased from Jackson Laboratories. A breeding colony was established in the institution's Division of Laboratory Animal Medicine facility to produce

neonatal mice from multiparous dams. All animal protocols were approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Ethanol in Intralipid 20% (Fresenius Kabi, Uppsala, Sweden) nutritional vehicle or Intralipid 20% alone was administered daily by intra-esophageal gavage at 4 g/kg/day on P4–9. Mice were also administered either pioglitazone (12.5 mg/kg/day as used in our previous studies (Diab et al., 2004); Cayman, Ann Arbor, MI) or water vehicle by gavage on P4–9. On P4, drug or water vehicle was given two hr before ethanol treatment and on P5–9 it was given one hr before ethanol treatment. Animals in the ethanol, pioglitazone plus ethanol, and vehicle treatment groups were distributed in multiple litters across a total of 11 litters for histology and 10 litters for RNA analysis. Additional litters of handled-only control animals were not gavaged but were handled only for marking and weighing. A group of pioglitazone plus vehicle animals was not included in this study as we have previously published the lack of an effect of pioglitazone alone in a related FASD model (Kane et al., 2011). Both sexes of animals were analyzed and no significant difference in the treatment response was identified between the sexes. Tissue was harvested one day after the final ethanol treatment. In separate animals, blood ethanol concentrations were evaluated on P9 at several intervals between 30–360 minutes following ethanol treatment using an Analox AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

RNA isolation and cDNA synthesis

Animals were anesthetized with isoflurane and perfused transcardially with phosphate buffered saline (PBS) containing heparin. The brain was harvested, the hippocampus, cerebellum, and cerebral cortex were dissected and frozen in liquid nitrogen. A BBX24B Bullet Blender Blue homogenizer (Next Advance, Averill Park, NY) was used to homogenize the tissues. The RNeasy Mini Kit (Qiagen, Valencia, CA) was used to isolate RNA and DNA was removed with DNaseI (Qiagen). A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) was used to determine the concentration and quality of RNA. cDNA was prepared using the iScript™ system (Bio-Rad, Hercules, CA).

Real-time polymerase chain reaction

Real-time polymerase chain reaction (rtPCR) was used to quantify IL-1 β , TNF- α , and CCL2 mRNA levels using a CFX96 Real-time PCR Detection system (Bio-Rad, Hercules, CA). rtPCR analyses were performed in duplicate using TaqMan® primers (Applied Biosystems, Foster City, CA) and SsoFast™ Probes Supermix (Bio-Rad). Data were calculated as the mean Ct relative to β -actin. The Ct method was used to generate fold expression differences between experimental groups and vehicle-treated control. Statistical comparison was performed using ANOVA and post-hoc t-tests with Bonferroni correction for multiple comparisons (GraphPad Prism®, San Diego, CA).

Tissue preparation, immunostaining, and morphometry

Animals were anesthetized with isoflurane and perfused transcardially with a brief heparinized PBS flush followed by phosphate buffered 4% paraformaldehyde fixative. Parasagittal sections of the cerebellum and coronal sections of the remaining brain were cut at 50 μ m using a Leica VT1200S vibrating blade microtome and serial sections were collected in phosphate buffer (PB) containing 0.05% sodium azide. Every sixth section was

stained for Iba-1 using peroxidase immunohistochemistry and the VectaStain ABC Elite kit (Vector Laboratories, Burlingame, CA). Sections were treated with endogenous peroxidase blocking solution (1:1 solution of methanol:6% hydrogen peroxide) for 17 minutes followed by rinses in PB. Sections were incubated in PB containing 0.3% Triton-X (PBT) and 9% normal goat serum for 30 minutes for blocking. Sections were then incubated in rabbit anti-Iba-1 antibody (Wako Chemicals, Richmond, VA) diluted 1:1000 in PBT containing 3% normal goat serum for 23 hours at room temperature. Sections were rinsed in PB and incubated 1 hour with biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) diluted 1:100 in PBT containing 1.5% normal goat serum. Sections were rinsed in PB, incubated in VectaStain reagent A (1:50) and B (1:50) in PB for 1 hour at room temperature, and rinsed in PB. Peroxidase reaction was performed for 5 minutes with 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and 0.004% hydrogen peroxide in PB followed by rinses in PB. Photomicrographs were obtained with a CoolSNAP^{cf} digital camera (Photometrics, Tucson, AZ) using an Olympus BX51 microscope. MetaMorph[®] imaging software (Molecular Devices, Sunnyvale, CA) was utilized to capture images from the CA1 region of the hippocampus, lobule V of the cerebellar cortex, and the parietal region of the cerebral cortex. Quantitative morphometric analysis of Iba-1 immunostained tissue was performed in real time with a CoolSNAP^{es} digital camera, an Olympus BX51 microscope, and MetaMorph[®] software. Three sections containing the region of interest were quantified from each of three animals in each treatment group. The relative cell area was assessed in the hippocampal CA1 region and parietal region of the cerebral cortex by image thresholding to represent the stained cell profiles and region measurement of the % thresholded area of the region. The relative cell area was assessed in lobule V of the cerebellar cortex by image thresholding of stained cell profiles and integrated morphometric analysis of the area of single cells. The territory occupied by individual cells and their processes was assessed by defining a perimeter at the tips of the cell processes and estimation of the area within the perimeter. Results were calculated as a ratio relative to the value observed in vehicle-treated control animals. Statistical comparison was performed using ANOVA and post-hoc t-tests with Bonferroni correction for multiple comparisons (GraphPad Prism[®]).

RESULTS

Our prior studies in a neonatal mouse model of FASD demonstrated that ethanol treatment caused significant depletion of the microglial population (Kane et al., 2011). Further, the surviving microglia exhibited morphology that was suggestive of activated, pro-inflammatory microglia found in neurodegenerative and neuroinflammatory conditions. Here, we have investigated this further with both molecular and cellular markers to determine if microglia are functionally activated to a pro-inflammatory stage and if neuroinflammation is present in diverse regions of the brain that are vulnerable to ethanol exposure in FASD.

Neonatal mice were administered ethanol in nutritional vehicle or vehicle alone as control. Some animals that received ethanol also received pioglitazone. The peak blood ethanol concentration (BEC) was determined at intervals between 30 and 360 minutes after ethanol

treatment. The peak BEC was 401 ± 16 (mean $\pm$ SD) mg/dl in ethanol-treated animals and 397 ± 11 in pioglitazone plus ethanol-treated animals 90 minutes after ethanol treatment.

The levels of mRNA encoding chemokines and cytokines were quantified in the brain parenchyma one day after the last dose of ethanol. Expression of IL-1 β , TNF- α , and CCL2 mRNA was increased in the brain following ethanol exposure compared to vehicle-treated controls. The levels of all three cytokines and chemokines were elevated in the hippocampus in ethanol-treated animals compared to vehicle-treated controls (Fig. 1): 3.9 ± 0.60 -fold ($p < 0.001$, $F(3,22) = 14.2$) for IL-1 β , 3.2 ± 0.70 -fold ($p < 0.01$, $F(3,23) = 6.10$) for TNF- α , and 2.1 ± 0.39 -fold ($p < 0.05$, $F(3,23) = 4.97$) for CCL2. The levels of all three cytokines and chemokines were elevated in the cerebellum in ethanol-treated animals compared to vehicle-treated controls (Fig. 2): 3.6 ± 0.36 -fold ($p < 0.001$, $F(3,23) = 24.0$) for IL-1 β , 2.0 ± 0.20 -fold ($p < 0.001$, $F(3,23) = 10.2$) for TNF- α , and 1.5 ± 0.11 -fold ($p < 0.05$, $F(3,21) = 5.04$) for CCL2. The levels of IL-1 β and TNF- α were elevated in the cerebral cortex in ethanol-treated animals compared to vehicle-treated controls (Fig. 3): 2.1 ± 0.26 -fold ($p < 0.001$, $F(3,22) = 10.9$) for IL-1 β and 1.6 ± 0.17 -fold ($p < 0.05$, $F(3,23) = 4.44$) for TNF- α . Expression of CCL2 was not significantly changed in the cerebral cortex ($F(3,22) = 1.29$, $p = 0.30$). For all genes in all tissues, there was no significant difference in the levels of chemokine or cytokine expression in handled-only control animals compared to vehicle-treated control animals (Figs. 1–3).

We investigated the ability of the PPAR- γ agonist pioglitazone to block the effects of ethanol on cytokine and chemokine expression by treating animals with or without pioglitazone during the period of ethanol treatment. Pioglitazone treatment prevented the ethanol-induced increase in cytokines and chemokines (Figs. 1–3). In all three tissues, animals that received pioglitazone in addition to ethanol exhibited IL-1 β , TNF- α , and CCL2 mRNA levels that were equivalent to handled-only and vehicle-treated control animals. Ethanol-treated animals expressed higher levels of all three mRNAs in all three tissues compared to animals treated with ethanol plus pioglitazone, except for CCL2 in the cerebellum and TNF- α and CCL2 in the cerebral cortex. Co-treatment with pioglitazone indicates that the induction of these molecules can be blocked with this anti-inflammatory pharmaceutical.

Our studies demonstrate that cytokines and chemokines are significantly increased in the brain following ethanol exposure suggesting that ethanol exposure results in glial expression of pro-inflammatory molecules as a result of glial activation. To assess activation of the microglia, immunohistochemical staining with Iba-1 was performed to determine the morphological phenotype of the cells. The normal morphology of microglia in the gray matter of the CA1 region of the hippocampus, lobule V of the cerebellar cortex, and the parietal region of the cerebral cortex is distinctive due to the architecture of each neuroanatomical region, as seen in handled-only and vehicle-treated control animals (Fig. 4A–F). Although the cellular morphology varies between regions, a majority of the microglia in normal brain at this age exhibit small somas and long, thin, and highly branched processes. Ethanol treatment altered the normal microglial morphology such that many of the cells in ethanol-treated animals exhibited hypertrophy of the soma and exhibited processes that were shorter, broader, and less highly branched (Fig. 4G–I). The altered

microglial morphology in ethanol-treated animals suggests microglial activation (Raivich et al., 1999). Because pioglitazone blocked ethanol induction of pro-inflammatory cytokine and chemokine expression (Figs. 1–3), we investigated its ability to prevent the morphological change. Animals were treated with or without pioglitazone during the period of ethanol treatment. Pioglitazone treatment suppressed the ethanol-induced change in microglial morphology (Fig. 4J–L) such that the cells generally had the morphology of those in handled-only and vehicle-treated control animals (Fig. 4A–F).

To more precisely address the change in microglial morphology, quantitative morphometry of Iba-1 stained microglia was performed. In the hippocampus CA1, the cell area was quantified by analysis of the percent area of the region occupied by microglia. The area occupied by microglia was increased in ethanol-treated animals compared to handled-only and vehicle-treated controls (Fig. 5A, $F(3,7) = 207$, $p < 0.0001$). Administration of pioglitazone to ethanol-treated animals blocked the ethanol-induced change in cell area. The territory of tissue occupied by individual microglial cell bodies and processes was analyzed. The cell territory was similar in handled-only, vehicle-treated, and pioglitazone plus ethanol-treated animals (Fig. 5D). However, the cell territory in ethanol-treated animals was reduced in the hippocampus ($F(3,8) = 9.23$, $p < 0.01$). In cerebellar lobule V, the microglial cell area was increased in ethanol-treated animals compared to handled-only and vehicle-treated controls (Fig. 5B, $F(3,8) = 24.1$, $p < 0.001$). The territory occupied by the cells was decreased by ethanol treatment compared to the other three treatment groups (Fig. 5E, $F(3,8) = 34.9$, $p < 0.0001$). Administration of pioglitazone to ethanol-treated animals blocked the ethanol-induced change in cell area and territory in the cerebellum. In the parietal region of the cerebral cortex, analysis revealed that the area occupied by stained microglial profiles was increased in ethanol-treated animals (Fig. 5C, $F(3,7) = 35.2$, $p < 0.0001$). The territory occupied by the cells was significantly decreased by ethanol treatment compared to the other three treatment groups (Fig. 5F, $F(3,8) = 18.0$, $p < 0.001$). Administration of pioglitazone to ethanol-treated animals blocked the ethanol-induced change in cell area and territory in the parietal cortex. Together with the qualitative observations illustrated in Fig. 4, these results reflect ethanol-induced change in microglial morphology evidenced by cellular hypertrophy and retraction of processes. Together with cytokine and chemokine induction, the morphological change in the microglia and suppression of both the molecular and cellular changes by treatment with pioglitazone suggests that ethanol treatment may generate a pro-inflammatory stage of microglial activation that is associated with neuroinflammatory and neurodegenerative events.

DISCUSSION

It is well established that maternal consumption of alcohol during pregnancy can produce FASD with deficits in CNS development (Mattson et al., 2011, Riley et al., 2011). Human functional and structural imaging studies and investigations in animal models of FASD demonstrate that the CNS disabilities are caused by ethanol-induced neuropathology throughout the brain. Despite the knowledge that has been gained, there are no effective treatments that target the neuropathological causes of FASD. This study investigated the role of the neuroimmune system in FASD neuropathology, uncovering a potentially pathogenic role for microglial cells and neuroinflammatory events.

In adults, it is well known that alcohol has profound, yet often dichotomous effects on the peripheral immune system. For example, alcoholics are often immunocompromised but exhibit elevated levels of pro-inflammatory molecules including cytokines and chemokines (Achur et al., 2010). Studies have evaluated the effects of alcohol on the neuroimmune system in adult rodents and humans. There, it is clear that ethanol increases the expression of pro-inflammatory cytokines and chemokines as well as other immune molecules such as nitric oxide and cyclooxygenase-2 in the brain (He et al., 2008, Qin et al., 2008, Alfonso-Loeches et al., 2010, Kane et al., 2013, Kane et al., 2014). It is notable that the amount of ethanol-induced neuroinflammation varies depending on several factors including the route of ethanol administration, peak BEC, duration of ethanol exposure, period of withdrawal, and age of the animal. Compared to adults, little is known concerning the effects of ethanol on immune responses in the developing CNS.

Recently, we demonstrated in the cerebellum that mice exposed to ethanol from P3–5 exhibited a significant loss of Purkinje cell neurons and microglia on P6, and that the remaining microglia exhibited an activated morphological phenotype (Kane et al., 2011). These studies also demonstrated that ethanol was toxic to cultured cerebellar granule cell neurons and microglia. Our current studies indicate that ethanol increases the expression of the pro-inflammatory cytokines IL-1 β and TNF- α in the hippocampus, cerebellum, and cerebral cortex of mice treated from P4–9 with analysis on P10. Expression of the pro-inflammatory chemokine CCL2 was increased in the hippocampus and cerebellum, but not in the cerebral cortex. Perhaps CCL2 was increased in specific regions within the cerebral cortex however this would not have been detected in this study since the entire cerebral cortex was analyzed. The current study quantified changes in RNA levels and it might be expected that protein levels would reflect the changes in RNA; this will be important to ascertain in further studies. It is difficult to ascribe the changes in gene expression to exclusively microglia because astrocytes and to a more limited extent neurons also express these cytokines and chemokines. But, meaningfully, we further demonstrate both qualitatively and quantitatively that ethanol induces microglia in the hippocampus, cerebellum, and cerebral cortex to exhibit a morphological change in which the cellular processes become shorter, broader, and less branched and the somas undergo hypertrophy, indicative of microglial activation. Together, our current and previous findings indicate that ethanol induces microglial activation and expression of neuroinflammatory molecules in the neonatal mouse model of FASD. In another study, rats treated from P7–9 expressed increased IL-1 β and TNF- α levels in the cerebral cortex and hippocampus when measured at P28. This suggests that inflammation persists in the CNS for a significant period of time following cessation of ethanol exposure (Tiwari et al., 2011). Although in vitro studies describe the direct effect of ethanol on microglial activation and survival (Fernandez-Lizarbe et al., 2009, Kane et al., 2011, Fernandez-Lizarbe et al., 2013), it has not been established in vivo whether the neuroinflammatory response that accompanies ethanol exposure is a direct effect of ethanol on glia or whether it is a consequence of the ethanol effect on neurons. However, collectively, current and previous studies suggest that if the microglial response and neuroinflammation is detrimental as in other neurodegenerative disorders in humans and animals, anti-inflammatory therapies may be effective throughout an extended timeframe in infants with FASD.

The present study demonstrates specific changes in the neuroimmune system in the developing brain as a result of ethanol exposure. These neuroimmune changes have been best characterized in light of neurodegenerative and neuroinflammatory events, however, new understanding has broadened our perspective to include regenerative as well as degenerative roles for microglial activation and cytokines and chemokines. Equally as important, information is emerging that molecules classically defined as inflammatory including cytokines and chemokines and their receptors and other immune molecules are normally expressed in the developing CNS. For example, cytokines including IL-1 β and TNF- α play critical positive roles in brain development (Merrill, 1992). The major histocompatibility class I molecule, which is central to antigen presentation in the mature immune system, is crucial to activity-dependent synapse formation in the developing cortex (Corriveau et al., 1998). Importantly, activation of the neuroinflammatory system or changes in the microglial population, even transiently, in the developing brain leads to long-term detrimental consequences in the brain. As an example, early life infections and the associated inflammatory response increase susceptibility to cognitive and neuropsychiatric disorders later in life (Bilbo et al., 2012). Neonatal exposure to pathogens also results in an exaggerated neuroimmune response including microglial activation later in life, which is associated with cognitive impairment (Bilbo et al., 2012). Recent studies of the chemokine receptor CX3CR1 shed new light on the importance of the integrity of the neuroimmune system in early development. CX3CR1 is expressed by microglia and its ligand fractalkine is expressed by neurons. Their interaction is an essential component of microglial-neuron communication. Mice lacking CX3CR1 exhibit a transient reduction in microglia during early postnatal development with resulting deficiencies in synaptic pruning. As a result, later in life these mice exhibit weak synaptic transmission, decreased brain connectivity, and deficits in social interaction indicative of neuropsychiatric disorders (Zhan et al., 2014). Collectively, these studies suggest that even transient alterations in immune activity in the brain during critical developmental periods may contribute to the development of inflammatory, cognitive, and behavioral disorders in adults. Our current and previous studies indicating that ethanol produces microglial pro-inflammatory activation, neuroinflammatory events, and depletion of microglia (Kane et al., 2011) during brain development open the possibility of a link between ethanol-induced neuroinflammatory activity and the cognitive deficits and behaviors associated with FASD.

As discussed above, neuroinflammatory molecules can lead to neuronal death and dysfunction as well as amplify glial activation. We propose that neuroinflammation may contribute to the widespread FASD neuropathology in the developing hippocampus, cerebellum, and cerebral cortex. In the hippocampus, postnatal ethanol treatment causes reduction in pyramidal and granule cell populations and the neurochemistry and function of hippocampal neurons is impaired (Gil-Mohapel et al., 2010, Puglia et al., 2010, Everett et al., 2012). In the cerebral cortex, there is extensive neuronal loss, impaired neuronal maturation, aberrant development of neural circuitry, and disruption of neurochemical and electrophysiological function (Slawecki et al., 2004, Banuelos et al., 2012, El Shawa et al., 2013). Similarly in the cerebellum, there is extensive loss of Purkinje, granule cell, and deep cerebellar neurons with altered dendritic morphology and electrophysiological function (Smith et al., 1990, Hamre et al., 1993, Pierce et al., 1999, Dikranian et al., 2005, Servais et

al., 2007). Our findings in this study suggest that ethanol effects on microglia may specifically contribute to the pathology in all of these brain regions.

Several important areas remain to be explored. This study administered pioglitazone treatment one to two hr before the daily ethanol treatment. It will be important in future studies to investigate whether pioglitazone treatment at various times after ethanol treatment will provide protection against the long-term consequences of ethanol exposure in order to more closely mimic potential therapeutic application in humans. This study focused on the effect of ethanol and pioglitazone on microglial activation and expression of pro-inflammatory cytokines and chemokines. Because pro-inflammatory cytokines and chemokines are also expressed by astrocytes, it will be enlightening to evaluate whether astrogliosis also occurs in this model. Although we previously published a neuroprotective effect of pioglitazone against ethanol for cerebellar Purkinje cells *in vivo*, it will be important to analyze a potential neuroprotective effect of pioglitazone in each of these brain regions in future studies. This study analyzed microglia and neuroinflammation one day after the final ethanol treatment. It was not determined what period of ethanol treatment produced microglial activation and expression of pro-inflammatory molecules. Future studies may investigate a time course to determine whether it is the ethanol exposure itself or a period of withdrawal that generates the greatest neuroinflammatory response and also, importantly, investigate whether a single dose of ethanol produces these responses.

The specific changes in microglia and neuroimmune processes identified in our studies suggest concrete neuroimmune mechanisms that may contribute to the behavioral consequences in FASD. Ethanol-induced temporary or permanent loss of microglia during brain development might be predicted to lead to deficiency in synaptic pruning, electrophysiological abnormalities, and behavioral deficits in adulthood (Zhan et al., 2014). Ethanol-induced increases in microglial activation during brain development may be causative in long-term cognitive impairment (Bilbo et al., 2012). Even transient loss of microglia or changes in microglial activation may alter microglial interaction with synapses. In addition, activated microglia and pro-inflammatory cytokines are associated with neurodegeneration (Ransohoff et al., 2009, Saijo et al., 2011), which is widespread in the developing brain exposed to ethanol.

The present study demonstrates that the PPAR- γ agonist pioglitazone effectively blocks ethanol-induced neuroinflammatory events in the developing brain. PPAR- γ is important in glucose metabolism and adipogenesis as well as neuroprotection. We and others have previously demonstrated that PPAR- γ agonists suppress the production of pro-inflammatory molecules including cytokines, chemokines, and nitric oxide by microglia *in vitro* and *in vivo* in animal models of neuroinflammation and neurodegeneration (Heneka et al., 2001, Diab et al., 2002, Tureyen et al., 2007). It will be important to consider that, in addition to suppressing inflammation, PPAR- γ agonists also act through inflammation-independent mechanisms, particularly glucose and lipid homeostasis (Cariou et al., 2012). Further, one must consider that alterations in glucose metabolism may be linked to neuroinflammatory and neurodegenerative disorders, which is, for example, supported by the association between type II diabetes and an increased risk of Alzheimer's disease (Butterfield et al., 2014). We previously demonstrated that PPAR- γ agonists were capable of protecting

microglia and neurons in vivo in the cerebellum in a model of FASD (Kane et al., 2011). Here we demonstrate that pioglitazone blocks ethanol-induced microglial activation and production of the cytokines and chemokines IL-1 β , TNF- α , and CCL2 in the hippocampus, cerebellum, and cerebral cortex of neonatal mice. In the future, it will be important to determine the mechanisms by which PPAR- γ agonists protect neurons and microglia and block neuroinflammatory events in the developing CNS exposed to ethanol. Our current and previous studies open the possibility that PPAR- γ agonists or other anti-inflammatory pharmaceuticals may be effective in the treatment of FASD.

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Fig. 1.

Effect of ethanol and pioglitazone on IL-1 β (panel A), TNF- α (panel B), and CCL2 (panel C) expression in neonatal mouse hippocampus. Animals were given 4 g/kg/day ethanol (E) daily by gavage on P4–9 and mice were sacrificed 1 day after the final dose of ethanol. Handled-only (H) and vehicle-treated (V) mice were included as controls. Where indicated, animals were treated with ethanol plus 12.5 mg/kg/day pioglitazone (P+E). The hippocampus was dissected, RNA prepared, cDNA synthesized, and mRNA levels were evaluated by real-time PCR. Results were normalized against β -actin and are expressed as fold changes relative to vehicle-treated control mice. Values are mean $\pm$ SEM. PCR reactions were performed in duplicate on each sample. $n=6-8$ per treatment group. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Fig. 2. Effect of ethanol and pioglitazone on IL-1 β (panel A), TNF- α (panel B), and CCL2 (panel C) expression in neonatal mouse cerebellum. Animals were treated, cerebellum was dissected, and samples analyzed as described in Fig. 1. Results were normalized against β -actin and are expressed as fold changes relative to vehicle-treated control mice. Values are mean $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Fig. 3. Effect of ethanol and pioglitazone on IL-1 β (panel A), TNF- α (panel B), and CCL2 (panel C) expression in neonatal mouse cerebral cortex. Animals were treated, cerebral cortex was dissected, and samples analyzed as described in Fig. 1. Results were normalized against β -actin and are expressed as fold changes relative to vehicle-treated control mice. Values are mean $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Fig. 4.

Microglial morphology in neonatal mice treated with ethanol and pioglitazone. Animals were treated as described in Fig. 1. Photomicrographs illustrate the appearance of Iba-1 stained microglia in H, V, E, and P+E treated neonatal mice in the CA1 region of the hippocampus (panels A, D, G and J), lobule V of the cerebellar cortex (panels B, E, H and K), and the parietal region of the cerebral cortex (panels C, F, I and L). Handled animals = panels A–C. Vehicle animals = panels D–F. Ethanol-treated animals = panels G–I. P+E treated animals = panels J–L. Scale bar = 50 μ m.

Fig. 5. Quantitative morphometric analysis of microglial morphology in neonatal mice treated with ethanol and pioglitazone. Animals were treated as described in Fig. 1 and processed for Iba-1 immunohistochemistry as in Fig. 4. Iba-1 stained microglia from H, V, E, and P+E treated animals were compared for relative cell area (panels A–C) and relative cell territory (panels D–F). A and D: hippocampus CA1 region. B and E: cerebellum lobule V. C and F: parietal cortex. Results are expressed as fold changes relative to vehicle-treated control mice. Values are mean $\pm$ SEM. $n=3$ per treatment group. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.