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PROTECTION OF NEURONS AND MICROGLIA AGAINST ETHANOL IN A MOUSE MODEL OF FETAL ALCOHOL SPECTRUM DISORDERS BY PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-γ AGONISTS

Cynthia J.M. Kane, **Kevin D. Phelan**, **Lihong Han**, **Renea R. Smith**, **Jin Xie**, **James C. Douglas**, and **Paul D. Drew**
Department of Neurobiology and Developmental Sciences, College of Medicine, University of Arkansas for Medical Sciences

INTRODUCTION

Fetal Alcohol Spectrum Disorders (FASD) and related disorders occur in approximately 1% of live births in the United States and represent the most common cause of mental retardation (May et al., 2009). FASD is caused by ethanol induced neurodegeneration in the developing central nervous system (CNS). Pathology in the cerebellum, hippocampus, cerebrum, corpus callosum, basal forebrain, and other brain regions persists throughout life and underlies the dysfunction associated with this disorder (Guerri et al., 2009). FASD is commonly associated with significant lifetime disability, and thus the prevention and treatment of this syndrome is greatly needed (Stratton, 1996).

The effects of ethanol on the developing cerebellum have been extensively investigated. Brain imaging of children exposed to prenatal ethanol reveals defects throughout the cerebellum (O'Hare et al., 2005). Cerebellar maturation that occurs during the third trimester of human development has been well modeled in the neonatal rat with developmental equivalence during the first 2 postnatal weeks (Clancy et al., 2001; Cudd, 2005). In the neonatal rat, the cerebellum has been studied as a prototype for ethanol effects on neuronal development. The two major neuronal populations in the cerebellum, Purkinje cells and granule cells, are susceptible to ethanol with high vulnerability on postnatal days 4–6 (Goodlett and Eilers, 1997; Napper and West, 1995; Pierce et al., 1999), with deficits in these neuronal populations persisting in the adult animal (Goodlett et al., 1991; Green et al., 2002). Ethanol toxicity in cerebellar granule cells has been particularly well studied in culture. Granule cells require trophic support which can be provided by neurotrophins or NMDA receptor activation in culture. Ethanol interferes with normal neurotrophic support resulting in death of granule cells (Bhave et al., 1999; Bonthius et al., 2003; Snell et al., 2001).

CORRESPONDING AUTHOR: Cynthia J.M. Kane, Ph.D., Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, 4301 W. Markham, #846, Little Rock, AR 72205, kanecynthiaj@uams.edu, 501-626-9995 telephone, 501-526-6756 fax.

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Under normal physiological conditions, microglia promote neuronal development and survival by secreting factors including neurotrophins and protective cytokines, as well as by sequestering neurotransmitters (Nakajima et al., 2001; Streit et al., 2008). However, upon CNS insult, microglia can become pathologically activated leading to neuropathology, neuroinflammation, and/or neurodegeneration [reviewed in (Kaur et al., 2010; Lucin and Wyss-Coray, 2009; Lynch, 2009; Rivest, 2009)]. Studies in adult animal models of alcohol abuse also provide clues to the effects of ethanol on microglia [reviewed in (Crews and Nixon, 2009)]. In these models, ethanol administration induces an activated morphological phenotype and stimulates production of inflammatory and neurotoxic molecules. Collectively, these studies suggest that ethanol may stimulate neuron cell death, at least in part, through stimulation of neuroinflammatory and neurodegenerative processes in the CNS.

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear receptor family of proteins. The role of PPAR-γ in glucose metabolism and adipogenesis is well established (Bajaj et al., 2007). More recently, PPAR-γ has been demonstrated to modulate inflammatory responses, including those in the CNS (Bernardo et al., 2000; Diab et al., 2002; Diab et al., 2004; Drew et al., 2008; Heneka et al., 2001; Niino et al., 2001; Petrova et al., 1999; Xu and Drew, 2007). PPAR-γ is expressed on cells of the central nervous system including microglia, astrocytes, oligodendrocytes, and neurons (Bernardo and Minghetti, 2008). The cyclopentenone prostaglandin 15-deoxy-Δ12,15 prostaglandin J2 (PGJ) is an endogenous ligand of PPAR-γ, while synthetic ligands include thiazolidinediones such as pioglitazone (PIO) and rosiglitazone, which are commonly prescribed for the treatment of type II diabetes (Stumvoll and Haring, 2002; Wagstaff and Goa, 2002). Interestingly, PPAR-γ agonists inhibit the activation of primary microglia and suppress the production of pro-inflammatory cytokines and chemokines by these cells (Bernardo et al., 2000; Diab et al., 2002; Petrova et al., 1999). We and others have demonstrated that PPAR-γ agonists, including the thiazolidinediones (Heneka et al., 2001; Niino et al., 2001) and PGJ (Diab et al., 2002), inhibit the development of experimental autoimmune encephalomyelitis, an animal model of the CNS inflammatory disease multiple sclerosis. This suggests that PPAR-γ agonists may be used therapeutically to limit pathology in neuroinflammatory and neurodegenerative disease, including that resulting from ethanol exposure.

The proposed studies were designed to test the general hypotheses that ethanol induces death of microglia and neurons in vivo and in primary cultures, and that PPAR-γ agonists protect against ethanol mediated cytotoxicity. Accumulating evidence suggests that ethanol induced inflammation contributes to ethanol induced neurodegeneration. The current studies demonstrate that PPAR-γ agonists protect primary microglia and cerebellar granule cell neurons from ethanol induced toxicity. Furthermore, these studies demonstrate that PPAR-γ agonists also protect microglia and cerebellar Purkinje cell neurons from the toxic effects of ethanol using a novel in vivo mouse model of FASD. Collectively, these studies suggest that PPAR-γ agonists may be effective in protecting against the development of FASD in humans.

METHODS

Animals

C57BL/6 strain mice were obtained from Harlan Laboratories (Indianapolis, IN) and housed in the federally approved campus Division of Laboratory Animal Medicine. Adult mice were bred to produce pups for either cell cultures or experimental treatment. All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee.

Culture of microglia and neurons

Primary mouse microglia cultures were prepared as described (Drew and Chavis, 2001) with modifications. Briefly, meninges were removed from the cerebral hemispheres of 1- to 4-day-old C57BL/6 mice. The cortical tissue was minced and cells were separated by trypsinization, followed by trituration. Debris was removed by filtering the cell suspension through a 70 μm cell strainer. Cells were centrifuged at 153 ×*g* for 5 min at 4°C and then resuspended in DMEM medium containing 10% fetal bovine serum (Hyclone; Logan, UT), 1.4 mm L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, OPI medium supplement, and 0.5 ng/ml mouse GM-CSF (BD Pharmingen; San Diego, CA). Except where noted otherwise, tissue culture medium and reagents were obtained from BioWhittaker (Walkersville, MD). The cells were plated into tissue culture flasks and cultured at 37°C/5% CO_2 until confluent, generally 7–10 days following plating of cells. Flasks were then shaken overnight (200 rpm, 37°C) to release microglia and oligodendrocytes from the more adherent astrocytes. The suspended cells were replated for 2–3 h and flasks were shaken gently to separate oligodendrocytes from the more adherent microglia. Purified microglia were plated at 1.25×10^{5}/ cm^2 in 96-well plates and cultured in standard medium modified to contain 1% fetal bovine serum and treated with vehicle or 110 mM ethanol for 4 days. In a subset of cultures, 50 μM PIO or 2.5 μM PGJ was added. Culture medium served as vehicle control. During the period of ethanol treatment, ethanol treated cultures were incubated at 37°C in closed chambers equilibrated with 5% CO_2 and 110 mM ethanol in water to stably maintain the concentration of ethanol in the medium (experimentally determined to be 105–110 mM). Control cultures that were not treated with ethanol were incubated at 37°C in closed chambers equilibrated with 5% CO_2 and water.

Cerebellar granule cells were prepared from 4-day-old mice as we have described previously (Kane et al., 1996) with minor modification. Cells were cultured at 2.5×10^5 cells/cm^2 in poly-L-lysine coated 24-well plates in DMEM:Ham's F12 (90:10) with 10% Hyclone calf serum, 5 mM KCl, 100 μM NMDA, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Ara-C was added to the cultures at 3 μM from day 2–6 in order to remove contaminating proliferating cells. Cultures were returned to standard medium for 2 days. Granule cells were treated with 50 μM PIO (Cayman Chemical; Ann Arbor, MI) or 5 μM PGJ (Cayman Chemical; Ann Arbor, MI) for 24 h, which continued as cells were subsequently treated with vehicle or 110 mM ethanol for an additional 48 h. Fresh culture medium served as vehicle control. Closed, equilibrated chambers were used for both vehicle and ethanol treated granule cell cultures as described for microglial cultures above.

The purity of microglia and neuron cultures was evaluated by immunohistochemical staining. Microglia were selectively stained with isolectin B4 (Sigma; St. Louis, MO). Neurons were specifically identified with anti-MAP2 antibody (Sigma; St. Louis, MO). Astrocytes were specifically identified with glial fibrillary acidic protein antibody (Dako; Carpinteria, CA). Microglial cultures were determined to be >96% pure and granule cell cultures were determined to be >98% pure by quantification of stained cells (data not shown).

Cell viability of cultured cell populations

Cell viability of cultured microglia were determined by MTT reduction assay, as described previously (Xu and Drew, 2007). Optical density was determined using a Spectromax 190 microplate reader (Molecular Devices) at 570 nm. Results were reported as percentage of viability relative to untreated control cultures.

Cell viability of cultured cerebellar granule cell neurons were determined by unbiased counting of surviving MAP2 positive neurons. At the conclusion of experimental treatment,

cells were fixed with methanol at room temperature. Cells were stained with mouse anti-MAP2 primary antibody at 1:500 dilution in PBS + 0.2% Tween-20 overnight at 4°C and Alexa 488 labeled anti-mouse secondary antibody. Cell counts were performed in photomicrographs taken with a Nikon E600 microscope. Four photomicrographs were obtained from each of 4 replicate wells in each treatment condition. Cell counts were calculated as the mean ± standard deviation. Three independent experiments were performed and the mean ± standard error of these experiments was calculated.

Animal treatment

Mice were used as a neonatal model of FASD paralleling the previously described neonatal rat model (Kane et al., 1997; Light et al., 1998; Sonderegger et al., 1982). Pups (2 days old) were separated randomly into the following treatment groups: handled control, vehicle control, ethanol treated, and agonist + ethanol treated. Ethanol treated animals were administered 3.5 g/kg/day ethanol in 15% w/v solution of vehicle by gavage as we have previously described (Kane et al., 1997; Light et al., 2002b; Light et al., 1998; Pierce et al., 1997). Blood alcohol concentrations were determined to be 250–325 mg/dl. Gavage controls were given vehicle containing isocaloric dextrose. PPAR-γ agonists were dissolved in saline and were administered by i.p. injection at the following concentrations: PIO (0.5 mg/kg/day) (Victor et al., 2006) or PGJ (0.5 mg/kg/day) (Diab et al., 2002; Natarajan and Bright, 2002). Vehicle control animals were administered the same volume of saline as the agonists and the same volume of vehicle (isocaloric dextrose) as ethanol. Handled control animals were removed from the dam for the same period as the other animals and handled only for weighing. Preliminary analysis indicated that the cell counts for the handled control and isocaloric vehicle groups were not significantly different and the isocaloric vehicle group is reported as vehicle control.

Tissue preparation and staining

Animals were anesthetized with halothane, and perfused with saline followed by 4% periodate-lysine-paraformaldehyde fixative (Whiteland et al., 1995). Serial sagittal sections of the cerebellar vermis with attached brainstem were prepared at 50 μm thickness. Immunohistochemistry was performed with calbindin D28k antibody (Light et al., 2002a) to permit identification and counting of Purkinje cells, which are the only cells to stain with anti-calbindin D28K antibody in the cerebellar vermis. Tissue sections were incubated with primary antibody at 1:500 dilution for 4 h at room temperature followed by detection with biotin-labeled anti-mouse secondary antibody and the Vectastain ABC kit streptavidin-horeseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Isolectin B4 histochemistry was performed to permit identification and counting of total microglia. Tissue sections were incubated with peroxidase labeled isolectin B4 reagent at 1:50 dilution for 1 h at room temperature then 24 h at 4°C and processed with diaminobenzidine for peroxidase reaction. Every other serial section of cerebellum was processed for detection of either Purkinje cells or microglia. Each stain was performed on 9–10 sections sampled in an even distribution across the tissue, providing appropriate sampling to apply the stereological cell count method.

Cell counts with stereology

Stained cells were counted in lobule IX of the cerebellar vermis by unbiased stereology (West et al., 1991) using the Bioquant Nova® Stereology software program (Bioquant Image Analysis Corporation; Nashville, TN,) and a Nikon E600 microscope, ASI motorized stage with XYZ encoder, and Sony video camera. The limits of lobule IX were defined in two dimensions by the pial surface and a straight line defined by the nadir of the sulcus between lobule VIII and IX and the nadir of the sulcus between lobule IX and X. The counting frame was specific for each cell type based on our previous experience: microglia

(75 μm × 75 μm), and Purkinje cells (120 μm × 120 μm). The dissector height was 20–30 μm and 15–20 dissectors were positioned randomly by the stereology program over the defined tissue area. Cell counts were performed by an experienced individual who was blinded to the experimental conditions. Data were tabulated in the Bioquant® database. The estimated total number of cells was calculated using the reference volume, according to the Cavalieri Principle, and the numerical cell density determined with the optical fractionator within the Bioquant® program. The coefficient of error approximated 0.08 and the nugget percent variance approximated 0.2 across measurements.

Statistical analysis of data

Statistical analysis was performed with Statview® (SAS Institute Inc., Cary, NC) software. Data derived from in vitro culture studies were analyzed by one-way ANOVA followed by the Bonferroni/Dunn post-hoc test. Data derived from in vivo animal studies were analyzed by one-way ANOVA followed by the Fisher PLSD post-hoc test.

RESULTS

The effect of ethanol on viability and survival of microglial cells from the developing brain was modeled in cell culture. Compared to vehicle treated cultures, survival in ethanol treated cultures was 67.2 ± 3.4% (Figure 1). The surviving cells often exhibited smaller cell bodies and shorter, broader processes compared to vehicle treated control cultures (Figure 2). To determine if PPAR-γ agonists have the ability to protect against ethanol cytotoxicity, selected agonists were added to microglial cultures. In control studies, in the absence of ethanol, PPAR-γ agonists did not alter microglial cell viability or morphology relative to vehicle treated cultures. However, importantly, the thiazolidinedione PIO and the natural endogenous agonist PGJ protected microglia against ethanol mediated cell death. Collectively, these studies demonstrate that ethanol decreases viability of microglia and PPAR-γ agonists protect against ethanol mediated microglial cell death.

The impact of ethanol on viability and survival of granule cells from the developing cerebellum was also modeled in cell culture. Neuron cultures were treated for a shorter period of time (2 days vs. 4 days) than microglial cultures because these neurons are more sensitive to the toxic effects of ethanol under these culture conditions. Neuron survival was determined by counting cells stained by immunocytochemistry with anti-MAP2 antibody. This allowed specific positive identification and counting of granule neurons, which was particularly important since ethanol was demonstrated to be toxic to microglia (Figure 1) and microglia are known to be present, although not abundantly, in primary cerebellar granule cell cultures. Compared to vehicle treated cultures, survival in ethanol treated cultures was 25.6 ± 4.3% (Figure 3). The surviving cells often exhibited beaded processes compared to cells in vehicle treated control cultures (Figure 4). The ability of PPAR-γ agonists to protect against ethanol toxicity was also determined in the neuron cultures. In control cultures in the absence of ethanol, there was no significant difference in cell survival or morphology in the presence of either PPAR-γ agonist. However, addition of PPAR-γ agonists to cultures provided a significant level of protection against ethanol induced cell death. These studies demonstrate that ethanol decreases viability of neurons and that PPAR-γ agonists protect against ethanol mediated granule cell death.

Neonatal rat models of FASD have been employed for some time and have productively established the current understanding of the mechanisms underlying the neuroanatomical and cellular pathology that is associated with alcohol consumption during the last trimester of brain development. Further advancement would be facilitated by development of a corresponding mouse model in order to probe and manipulate molecular signaling mechanisms during ethanol exposure. For this purpose, neonatal mice were treated in

parallel to the method published for neonatal rats of the same age (Kane et al., 1997; Light et al., 1998; Sonderegger et al., 1982) in order to reproduce the developmental neuropathology produced by ethanol in the cerebellum (Goodlett and Eilers, 1997; Light et al., 2002a; Napper and West, 1995; Pierce et al., 1999).

Interactions between microglia and neurons are critical to neuron development and survival. However, the impact of ethanol on microglia in the developing brain is unknown. Ethanol is cytotoxic to immature neurons in the developing cerebellum causing significant apoptosis of both Purkinje cells and granule cells when ethanol exposure occurs during the early postnatal period. To investigate whether ethanol is toxic to developing microglial cells, neonatal mice were administered ethanol by gavage. The microglial population in vehicle treated control animals was estimated by stereology to be 1.11 (± 0.13) × 10^5 cells in lobule IX of the vermis (Figure 5). In ethanol treated animals, the number of microglia was 0.77 (± 0.12) × 10^5 cells. This reflects a significant 30.4% loss of microglia due to ethanol exposure during postnatal days 3–5. The surviving microglia exhibited an altered morphology with shorter, thicker, and less elaborately branched processes compared to the thin, highly branched appearance of cell processes in control animals administered vehicle (Figure 6).

In the culture studies of primary microglia, ethanol cytotoxicity was blocked by treatment with the PPAR-γ agonists, PIO or PGJ. These agents were tested for their effectiveness to also protect microglia against ethanol in the animal model. In control studies, treatment of animals with agonist alone, in the absence of ethanol, did not alter the size of the microglial population or microglial morphology. Importantly, PPAR-γ agonists abrogated ethanol induced loss of microglia and morphological change in the developing cerebellum. The cerebellar vermes of animals treated with ethanol in conjunction with PIO contained 1.14 (± 0.36) × 10^5 microglia. Similarly, the cerebellar vermes of animals treated with ethanol in conjunction with PGJ contained 1.13 (± 0.12) × 10^5 microglia. These studies demonstrate that PPAR-γ agonists provide meaningful protection of microglia against ethanol mediated cell loss in the developing cerebellum. These studies correlate with the in vitro studies indicating that PPAR-γ agonists protect cerebral microglia from the toxic effects of ethanol. It should be noted that cerebral microglia were used in these studies instead of cerebellar microglia due to our extensive experience in characterization of cerebral microglia and due to the fact that few laboratories have successfully prepared and characterized primary cerebellar microglia.

We investigated whether ethanol is toxic to developing Purkinje cells in the mouse model as we and others have previously shown in the rat model. Purkinje neurons are more sensitive to ethanol in this exposure paradigm than other cerebellar neurons and ethanol induced apoptosis of neurons is particularly well characterized in this population. The Purkinje cell population in vehicle treated control animals was estimated by stereology to be 1.15 (± 0.12) × 10^4 cells in lobule IX of the vermis (Figure 7). In ethanol treated animals, the number of Purkinje cells was 0.81 (± 0.07) × 10^4. This reflects a significant 29.1% loss of neurons due to ethanol exposure during postnatal days 3–5. There was reduced calbindin staining in the soma and dendrites of the surviving Purkinje cells in ethanol treated animals compared to control animals administered vehicle (Figure 8).

In the culture studies of cerebellar granule cells, the PPAR-γ agonists protected against ethanol induced neurotoxicity. The animal model was then utilitized to investigate whether neuronal vulnerability to ethanol could be blocked by these agonists in vivo. In control studies, treatment of animals with agonist alone, in the absence of ethanol, did not alter the size of the Purkinje cell population in lobule IX of the cerebellar vermis. Importantly, PPAR-γ agonists significantly reduced ethanol mediated loss of these cells in the developing cerebellum. The cerebellar vermes of animals treated with ethanol in conjunction with PIO

contained 1.07 (± 0.82) × 10^4 Purkinje cells. Similarly, the cerebellar vermes of animals treated with ethanol in conjunction with PGJ contained 1.09 (± 0.92) × 10^4 Purkinje cells. These studies demonstrate that PPAR-γ agonists provide meaningful protection of neurons against ethanol mediated cell loss in the developing cerebellum. Future studies will be performed to determine if PPAR-γ agonists protect other cerebellar neurons including granule cells.

DISCUSSION

FASD is caused by exposure of the developing fetus to ethanol. It is associated with a variety of neurodevelopmental pathologies that persist throughout life resulting in devastating consequences both at an individual and a societal level. Throughout gestation, ethanol can cause dramatic effects on neuron viability and function with different neuronal populations exhibiting vulnerability at different times. For example, ethanol exposure during the later phases of brain development causes specific consequences in the cerebellum, including neuronal apoptosis (Light et al., 2002a), disrupted formation of dendrites and synapses, and dysregulated expression of neurotransmitters and their receptors, and neurotrophins and their receptors (Karacay et al., 2008; Light et al., 2002b; Light et al., 1989a, b; Pierce et al., 2010). These discoveries were possible because of the development of neonatal rat models of FASD which mimic the third trimester of brain development in the human. In the course of the present investigation, the paradigm we established previously in development of the neonatal rat model (Kane et al., 1997; Light et al., 1998) was used to establish a parallel mouse model. Accordingly, herein, we validate that the mouse model described reproduced the loss of neurons that has been reported in the rat. This was quantified by stereological cell counts of Purkinje cells in lobule IX of the vermis, which represents one of the most ethanol vulnerable regions of the brain in the neonatal rodent (Pierce et al., 1989; Pierce et al., 1999). Because the effects of ethanol on glia in the developing nervous system are less well characterized, the mouse model was then utilized to probe the impact of ethanol on microglial cells under the same in vivo treatment conditions that generated neuron cell death.

Microglia are resident immune cells in the CNS and function in innate immune responses. It is well understood that microglia have dual roles in the CNS (Kaur et al., 2010; Lucin and Wyss-Coray, 2009; Lynch, 2009; Rivest, 2009). Under normal conditions they provide trophic support to neurons. However, in response to CNS infection or injury, they undergo a progressing gradient of activation and play an important role in ridding the CNS of pathogens or removing debris resulting from injury. The effects of ethanol in stimulating an immune response by microglia is complex and appears to be determined by a variety of factors including age of the experimental organism, as well as the paradigm used in the treatment and withdrawal of ethanol (Crews and Nixon, 2009). For example, a single short term binge exposure to ethanol did not induce expression of pro-inflammatory cytokines in the CNS (Zahr et al., 2010). In contrast, intermittent binge exposure to alcohol induced an activated microglial morphological phenotype and stimulated production of inflammatory and neurotoxic molecules including NO, COX2, cytokines such as TNFα and IL1β, and chemokines including MCP1 and MIP1α and β (Alfonso-Loeches et al., 2010; He and Crews, 2008; Pascual et al., 2009; Qin et al., 2008). In other studies, ethanol induction of pro-inflammatory cytokine and chemokine expression in the CNS was cooperatively enhanced by subsequent exposure to the bacterial surface protein lipopolysaccharide, suggesting that ethanol may prime microglia to convert to an activated phenotype (Qin et al., 2008). Although molecules including NO, TNFα, and IL1β play an important role in immune responses against pathogens, these molecules can also be toxic to host cells including neurons. Collectively, these studies suggest that agents that limit ethanol induced

immune responses by microglia may be effective in limiting ethanol induced neurodegeneration.

PPARs are members of the nuclear receptor family originally characterized as modulators of glucose and lipid metabolism (Bajaj et al., 2007; Desvergne and Wahli, 1999). In addition, the role of these receptors in altering immune responses has more recently become appreciated. PPAR-γ agonists have been demonstrated to suppress the development of experimental autoimmune encephalitis, an animal model of multiple sclerosis, which is characterized by CNS inflammation and associated demyelination (Diab et al., 2002; Heneka et al., 2001; Niino et al., 2001). These agonists have also been demonstrated to potently suppress the production of pro-inflammatory molecules by microglia, suggesting that agonist suppression of experimental autoimmune encephalitis occurs, at least in part, through suppression of glial cell activation (Bernardo et al., 2000; Diab et al., 2002; Diab et al., 2004; Drew et al., 2008; Heneka et al., 2001; Niino et al., 2001; Petrova et al., 1999; Xu and Drew, 2007). Currently, we demonstrate that PPAR-γ agonists protect neurons and microglia in vitro and in vivo from the toxic effects of ethanol. Since ethanol has been demonstrated to induce immune activity in the CNS, this suggests that PPAR-γ agonists may limit ethanol induced neuroinflammation and associated neurodegeneration.

Mechanistically, PPAR-γ is believed to regulate genes that encode proteins important in lipid and glucose metabolism by binding to peroxisome proliferator-activated response elements found in the promoters of these genes. In contrast, PPAR-γ agonists principally limit inflammation by suppressing the expression of genes encoding pro-inflammatory cytokines and chemokines through a process termed receptor-dependent transrepression. This process is believed to involve physical interaction between PPAR-γ and other transcription factors which normally activate genes encoding pro-inflammatory molecules resulting in suppression of the activity of these genes (Kamei et al., 1996; Kerppola et al., 1993). Examples of transcription factors that associate with PPAR-γ to suppress activation of genes encoding pro-inflammatory molecules by receptor dependent transrepression include NF-κB, AP-1, and STAT-1 (Li et al., 2000). In addition to acting through receptor-dependent mechanisms, PPAR-γ agonists have been demonstrated to suppress inflammation through receptor independent mechanisms. For example, these agonists can suppress specific steps in NF-κB signaling pathways through receptor independent mechanisms (Castrillo et al., 2000; Rossi et al., 2000; Straus et al., 2000). In the future, it will be important to determine the mechanisms by which PPAR-γ agonists protect neurons and microglia from the toxic effects of ethanol.

Previous studies have demonstrated that PPAR-γ agonists can alter the viability of neurons. This receptor is expressed by neurons suggesting that these agonists may regulate neuron viability through receptor dependent mechanisms (Inestrosa et al., 2005; Smith et al., 2003). These agonists can protect neurons from the toxic effects of agents including NMDA (Zhao et al., 2006) and apolipoprotein E4 (Brodbeck et al., 2008). The mechanisms by which PPAR-γ agonists alter neuron viability have not been completely elucidated. However, studies indicate that these agonists may modulate apoptosis of neurons through altering the expression of the anti-apoptotic factor Bcl-2. The mechanisms by which PPAR-γ induction of Bcl-2 protects neurons is not completely clear but may result from maintaining mitochondrial function and limiting the toxic effects of reactive oxygen species on these cells (Fuenzalida et al., 2007). PPAR-γ agonists could also protect neurons through production of neurotrophic factors. This possibility has not been extensively investigated and there is some indication that PPAR-γ agonists may actually reduce the expression of neurotrophic factors (Lee et al., 2010). However, ethanol has been demonstrated to increase translocation of the pro-apoptotic protein bax to the mitochondrial membrane, which is associated with neuron cell death. Interestingly, the neurotrophic factor brain derived

neurotrophic factor blocks translocation of Bax and protects neurons from the toxic effects of ethanol (Heaton et al., 2006; Heaton et al., 2011). In addition to directly altering neuron cell viability, PPAR-γ agonists are capable of modulating neuron viability indirectly by suppressing glial cell activation (Combs et al., 2000; Kim et al., 2002; Zhao et al., 2006). Interestingly, in addition to altering neuron viability, PPAR-γ agonists are also capable of modulating the differentiation and proliferation of neural stem cells (Morales-Garcia et al., 2011; Wada et al., 2006). Future studies are needed to determine the mechanisms by which PPAR-γ agonists protect neurons in animal models of FASD.

The current studies tested the hypothesis that ethanol induces death of microglia and neurons in vivo and in vitro and that the ethanol neuropathology could be blocked by agonists to PPAR-γ. We determined that ethanol causes loss of microglia as well as neurons in the cerebellum in a mouse model of FASD. Further, we established in the mouse model that either synthetic or endogenous PPAR-γ agonists limit this ethanol mediated cell loss. The phenomena of ethanol induced cell death and protection by PPAR-γ agonists was reproduced in primary cultures of microglia and neurons. This suggests that these agonists may be effective therapeutically in limiting the neuropathology associated with FASD. Importantly, PPAR-γ agonists including the thiazolidinediones PIO and rosiglitazone are commonly used for the treatment of type II diabetes and have good safety profiles. This should facilitate evaluation of the therapeutic efficacy of PPAR-γ agonists in the treatment of neuroinflammatory and neurodegenerative disorders, perhaps including ethanol mediated disease. To this end, additional basic research is needed to better determine the mechanisms by which PPAR-γ agonists protect neuronal cells from the toxic effects of ethanol, which would facilitate a rational approach to treatment of ethanol induced neuropathology.

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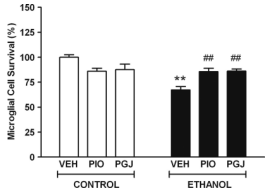

Figure 1. Microglial cell survival in vitro

Primary cultures of purified microglial cells were treated with vehicle (VEH) or 110 mM ethanol (E) for 4 days, without or with 50 μM PIO or 2.5 μM PGJ. Cell survival was determined using the MTT viability assay. Data was normalized to 100% survival in vehicle treated control cultures. Results shown are the mean ± SE of three independent experiments with six replicates in each experiment. Statistical analysis was performed with one-way ANOVA and the Bonferroni/Dunn post hoc test. [$F(5,12) = 8.887$, $p = 0.001$]. (** $p<0.005$ compared to each of the three control conditions; ## $p<0.005$ compared to the VEH + ethanol condition).

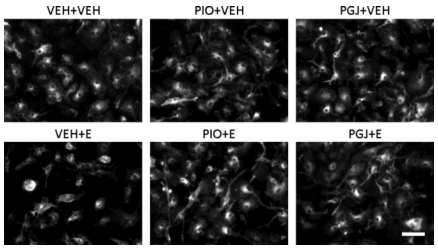

Figure 2. Morphology of microglial cells in vitro

Representative images of isolectin-stained cultures of microglial cells in primary culture. Cells were treated as described in Figure 1. Scale bar = 50 microns.

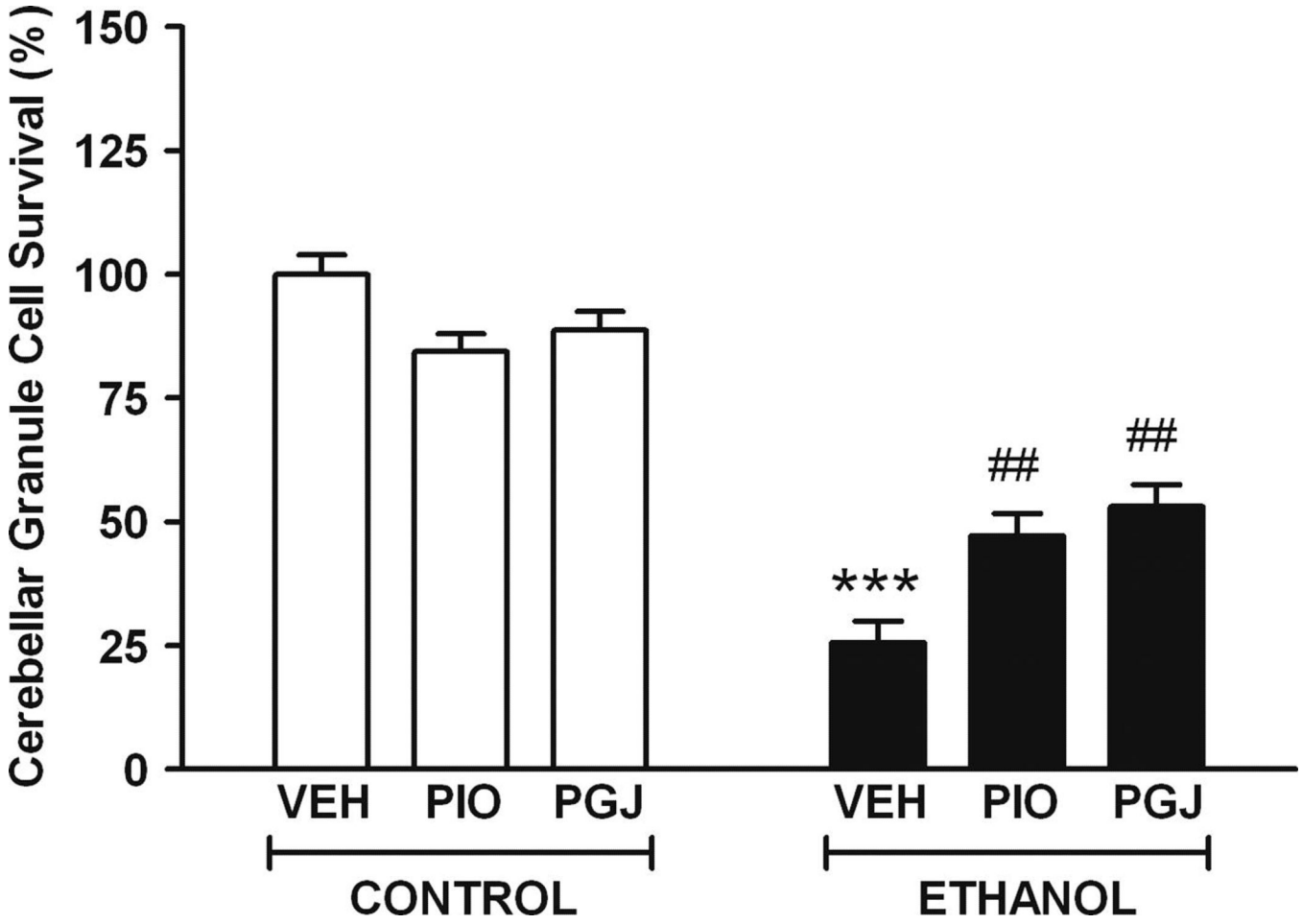

Figure 3. Cerebellar granule cell survival in vitro

Primary cultures of granule cells isolated from the cerebellum were treated without or with 50 μM PIO or 5 μM PGJ for 24 h followed by addition of vehicle (VEH) or 110 mM ethanol (E) for 48 h. Cell counts of MAP2-stained neurons were performed from 4 replicate photomicrographs obtained from each of 4 replicate wells per treatment condition. Data was normalized to 100% survival in vehicle treated control cultures. Results shown are the mean ± SE of three independent experiments. Statistical analysis was performed with one-way ANOVA and the Bonferroni/Dunn post hoc test. [$F(5,12) = 49.450$, $p < 0.0001$]. (*** $p<0.001$ compared to each of the three control conditions; ## $p<0.005$ compared to the VEH + ethanol condition).

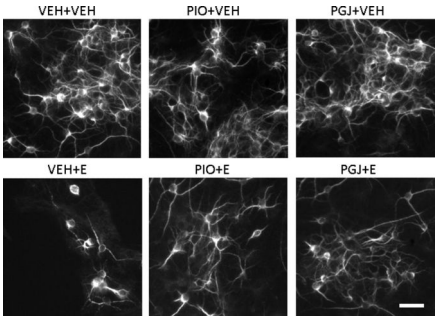

Figure 4. Morphology of cerebellar granule cells in vitro

Representative images of MAP2-stained cultures of cerebellar granule cells. Cells were treated as described in Figure 3. Scale bar = 30 microns.

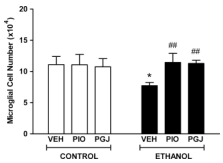

Figure 5. Protection of microglial cells in the postnatal mouse cerebellum in vivo

Mouse pups were administered PIO or PGJ on postnatal days 2–5. Animals were given ethanol at 3.5 g/kg on postnatal days 3–5 and sacrificed on postnatal day 6. Control animals were administered vehicle (VEH) in lieu of agonist or ethanol (E). Sagittal tissue sections were stained with isolectin B4 and the number of microglial cells in the cerebellar vermis was quantified using stereological methods. Results shown are the mean ± SE of six animals in each treatment group. Statistical analysis was performed with one-way ANOVA and the Fisher PLSD post hoc test. [$F(5,30) = 3.261$, $p = 0.0181$]. (* $p<0.01$ compared to each of the three control conditions; ## $p<0.005$ compared to the VEH + ethanol condition).

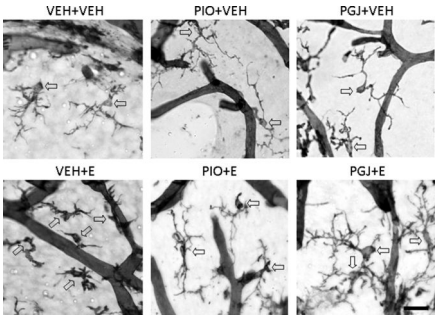

Figure 6. Morphology of microglial cells in the postnatal mouse cerebellum in vivo

Representative micrographs of isolectin B4 stained microglial cells in the cerebellum. Animals were treated as described in Figure 5. Representative microglial cells are indicated with arrows; the isolectin B4 positive tubular structures are blood vessels. Scale bar = 25 microns.

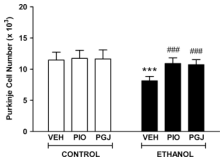

Figure 7. Protection of Purkinje cell neurons in the postnatal mouse cerebellum in vivo

Mouse pups were administered PIO or PGJ on postnatal days 2–5. Animals were given ethanol at 3.5 g/kg on postnatal days 3–5 and tissue was harvested on postnatal day 6. Control animals were administered vehicle (VEH) in lieu of agonist or ethanol (E). Sagittal tissue sections were stained with anti-calbindin D28K antibody to identify Purkinje cells. The number of these cells in lobule IX of the cerebellar vermis was quantified using stereological methods. Results shown are the mean ± SE of six animals in each treatment group. Statistical analysis was performed with one-way ANOVA and the Fisher PLSD post hoc test. [$F(5,30) = 8.932$, $p < 0.0001$]. (*** $p<0.001$ compared to each of the three control conditions; ### $p<0.001$ compared to the VEH + ethanol condition).

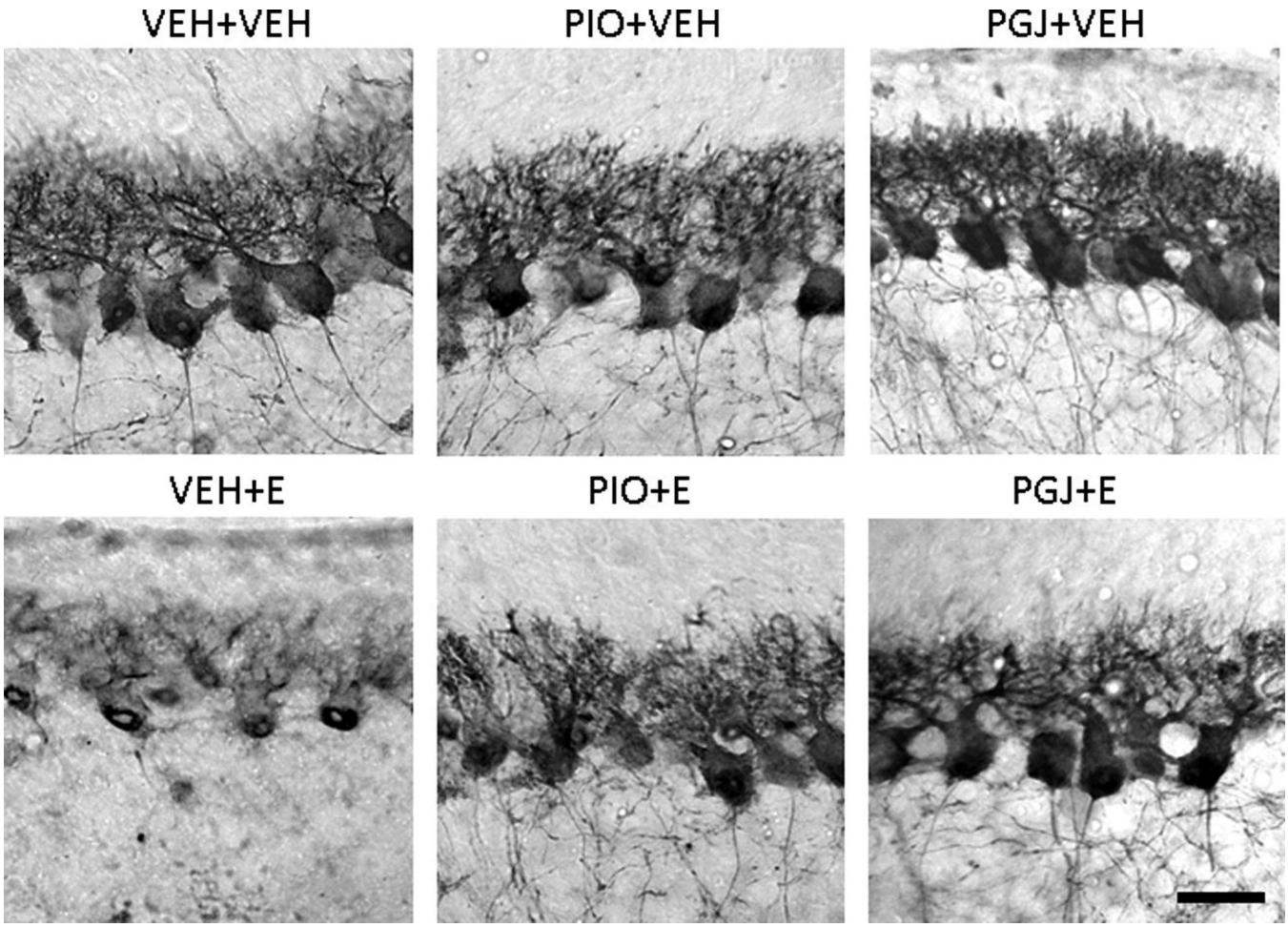

Figure 8. Morphology of Purkinje cells in the postnatal mouse cerebellum in vivo

Representative tissue sections stained with anti-calbindin D28K antibody to identify Purkinje cells in lobule IX. Animals were treated as described in Figure 7. Scale bar = 30 microns.