Protection against alcohol-induced neuronal and cognitive damage by the PPARγ receptor agonist pioglitazone

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Abstract
Binge alcohol drinking has emerged as a typical phenomenon in young people. This pattern of drinking, repeatedly leading to extremely high blood and brain alcohol levels and intoxication is associated with severe risks of neurodegeneration and cognitive damage. Mechanisms involved in excitotoxicity and neuroinflammation are pivotal elements in alcohol-induced neurotoxicity. Evidence has demonstrated that PPARγ receptor activation shows anti-inflammatory and neuroprotective properties. Here we examine whether treatment with the PPARγ agonist pioglitazone is beneficial in counteracting neurodegeneration, neuroinflammation and cognitive damage produced by binge alcohol intoxication. Adult Wistar rats were subjected to a 4-day binge intoxication procedure, which is commonly used to model excessive alcohol consumption in humans. Across the 4-day period, pioglitazone (0, 30, 60 mg/kg) was administered orally twice daily at 12-h intervals. Degenerative cells were detected by fluoro-jade B (FJ-B) immunostaining in brain regions where expression of pro-inflammatory cytokines was also determined. The effects of pioglitazone on cognitive function were assessed in an operant reversal learning task and the Morris water maze task. Binge alcohol exposure produced selective neuronal degeneration in the hippocampal dentate gyrus and the adjacent entorhinal cortex. Pioglitazone reduced FJ-B positive cells in both regions and prevented alcohol-induced expression of pro-inflammatory cytokines. Pioglitazone also rescued alcohol-impaired reversal learning in the operant task and spatial learning deficits in the Morris water maze. These findings demonstrate that activation of PPARγ protects against neuronal and cognitive degeneration elicited by binge alcohol exposure. The protective effect of PPARγ agonist appears to be linked to inhibition of pro-inflammatory cytokines.

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1. Introduction
Chronic alcohol intoxication resulting from binge drinking results in significant activation of neurodegenerative processes (Vetreno and Crews, 2015; Vetreno et al., 2016). The alcoholic brain shows reduction in brain volume and weight, enlargement of ventricles and gray as well as white matter shrinkage in cortical and subcortical structures (Pfefferbaum et al., 1992; Vetreno and Crews, 2015). Alcohol-induced neuropathological alterations in brain structure and function are often correlated with impairments in cognitive processes (Bowden and McCarter, 1993; White, 2003). In laboratory animals, it is well established that large doses of alcohol (11–15 g/kg/day) administered over a short period of time (4 days) reliably produce neurotoxicity in various cortical limbic areas including the hippocampal dentate gyrus (DG) and the entorhinal cortex (EC, (Collins et al., 1996; Crews et al., 2004; Obernier et al., 2002a)). The binge model of human alcoholic neurdogeneration, which mimics a single cycle of binge intoxication in human alcoholics (Braconi et al., 2010), is further validated by the fact that during alcohol intoxication animals reach sustained, high blood alcohol levels (BALs), commonly observed among alcoholics. Furthermore, neuronal deficits in animals treated with this
Binge alcohol model are associated with significant cognitive dysfunctions, such as learning and memory impairment as well as behavioral deficits including maladaptive perseverant behavior (Cippitelli et al., 2010a,b; Obernitz et al., 2002b).

It has been proposed that alcohol-induced brain damage may result from imbalance in expression and activation of transcription factors that regulate anti-inflammatory /pro-survival versus pro-inflammatory/pro-death processes (Crews and Nixon, 2009). Indeed, both in vitro and in vivo evidence have shown that alcohol shifts this balance toward neuroinflammation by decreasing CAMP responsive element-binding protein (CREB)-mediated signaling (Zou and Crews, 2006) or increasing toll-like receptor (TLR) 4 and downstream nuclear factor κB (NF-κB) signaling (Alfonso-Laeches et al., 2010; Crews et al., 2006; Davis and Syapin, 2004; Fernandez-Lizarbe et al., 2013).

 Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone receptor superfamily. They are involved in the transcriptional control of activated transcription factors of the nuclear hormone receptor superfamily. They are involved in the transcriptional control of genes regulating various physiological processes such as lipidhomeostasis, glucose metabolism, inflammation, and cellular differentiation and proliferation (Desvergne and Wahli, 1999). Agonists of the isoform PPARγ such as pioglitazone or rosiglitazone are commonly used medications in the treatment of type II diabetes. Beside their effect on metabolic disorders, PPARγ agonists also modulate inflammatory responses, including immune activity in the central nervous system (Kapadia et al., 2008). This is consistent with the observation that PPARγ was found in several brain regions both in neuronal and non-neuronal cells (Moreno et al., 2004) although a recent study indicates that PPARγ is constitutively expressed at higher levels in neurons than non-neuronal cells (Warden et al., 2016). Thus, PPARγ may be an important therapeutic target for neurodegenerative diseases. Accordingly, neuroprotective potential of PPARγ agonists has been demonstrated in animal models of acute CNS insults [i.e., spinal cord injury (Park et al., 2007)] and models of chronic CNS injuries including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Diab et al., 2002; Kapadia et al., 2008; Kaundal and Sharma, 2010; Schinto et al., 2009). Efficacy of PPARγ agonists was also shown in a mouse model of fetal alcohol spectrum disorders (Drew et al., 2015; Kane et al., 2011).

 Immune or brain pro-inflammatory signaling is not only relevant for neurodegeneration. Recent evidence supports the influence of the neuroimmune system on learning and memory and neuropaesthetic (Williamson and Bilbo, 2013). Thus, actions of alcohol on neuroimmune function may be important for the development of aspects of alcohol dependence such as escalation of consumption, craving, tolerance, and withdrawal (Crews et al., 2011; Robinson et al., 2014). PPARγ agonists have been recently shown to modulate excessive alcohol consumption, alcohol withdrawal symptoms and stress but not cue-induced reinstatement of alcohol seeking in rats (Stopponi et al., 2013, 2011), thus providing promising preclinical evidence for novel and effective alcohol addiction medications.

 Stemming from the initial work, which supports a role of pioglitazone in reducing alcohol addiction and its emerging protective effects in various neurological dysfunctions, here we examine whether treatment with pioglitazone is beneficial in attenuating neurodegeneration elicited by excessive alcohol exposure. First we characterized fluoroujade B (FJ-B) immunohistochemistry in the DG and the EC, two brain regions known to be sensitive to alcohol-induced neurotoxicity. Subsequently, using operant reversal learning and spatial orientation strategy-shift tasks, we monitored the effect of PPARγ activation on re-learning and cognitive flexibility. Finally, we examined the ability of pioglitazone to modulate expression of pro-inflammatory cytokines as a possible mechanism for its neuroprotective actions following binge alcohol exposure.

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River, Calco, Italy), weighing 300 to 350 g (8–10 weeks old) at the beginning of the experiments, were pair-housed with free access to tap water and food pellets (4RF18, Settimo Milanese, Italy) except where specified. The animals were maintained in a temperature- and humidity-controlled vivarium on a reverse 12-h light/dark cycle (lights off at 8:30 AM). Behavioral experiments were conducted during the dark phase of the cycle. Animals were handled three times before each experiment. All procedures followed the EU Directive for Care and Use of Laboratory Animals and were approved by the Ethical Committee of the University of Camerino, Italy.

2.2. Drugs

Pioglitazone was prepared from the pharmaceutical form Actos (30 mg tablets, Takeda). Tablets were suspended in distilled water to reach dosages of 30 mg/kg and 60 mg/kg. Pioglitazone was administered orally (by gavage) in a 1 ml/kg volume. Alcohol solution (20% vol/vol) was prepared by diluting 95% alcohol with water and made available orally.

2.3. Binge alcohol treatment

All rats used in the present study (N = 157) were subjected to a 4-day binge intoxication (or to a control) procedure in which alcohol (20% vol/vol) was administered orally every 8 h to reach doses of 11–15 g/kg/day as previously described (Cippitelli et al., 2014; Majchrowicz, 1975). Alcohol was administered in a vehicle made up with water, 6% sucrose and 14.7% milk powder (Mellin, Milan, Italy). Alcohol treated animals were given a priming dose of 5 g/kg of body weight. Additional alcohol was administered every 8 h for 4 consecutive days at 8:00 AM, 4:00 PM, and 12:00 AM based on the animals’ estimated BAL, as determined using a six-point intoxication scale (Majchrowicz, 1975). Control (CON) rats received equal volumes of the vehicle. Four batches of rats were used. One batch (N = 29) was employed for histochemical analysis of FJ-B. These rats were assigned to two groups of 17 alcohol exposed, in turn divided into three groups of 5–6 receiving vehicle, pioglitazone (PIO) 30 mg/kg, or PIO 60 mg/kg twice daily at 12-h intervals across the 4-day binge period (7:00 AM and 11:00 PM), and 12 control exposed divided into three groups of 4 rats receiving vehicle, PIO 30 mg/kg, or PIO 60 mg/kg. A second group (N = 30; 8 non-alcohol exposed vehicle treated, 8 non-alcohol exposed treated with PIO 60 mg/kg, 7 alcohol exposed vehicle treated and 7 alcohol exposed receiving PIO 60 mg/kg) was employed for gene expression analysis. The third (N = 53) and the fourth (N = 43) batch of rats were divided into 4 groups as described above and used to examine operant reversal learning and to determine BALs and reversal learning in the Morris Water Maze (MWM), respectively (Supplemental Table 1).

2.4. BALs

Twenty-four blood samples (150–200 μl) consisting of 6 non-alcohol exposed vehicle treated, 6 non-alcohol exposed treated with PIO 60 mg/kg, 6 alcohol exposed vehicle treated and 6 alcohol exposed receiving PIO 60 mg/kg were taken from the rat tail vein 60 min after the last administration of the 4-day binge alcohol pro-
was then assayed from 5 μl plasma aliquots using an AnLab instrument (Lunenburg, MA). Single point calibrations from 25–400 mg% were done for each set of samples with reagents provided by AnLab (Gilpin et al., 2009; Richardson et al., 2008). Instrumentation background determined from non-alcohol treated samples was subtracted to calculate BAL of alcohol-treated animals.

2.5. FJ-B staining

FJ-B was purchased from Histochem, Inc., (Jefferson, AR) and used as a marker of degenerating neurons (Schmued and Hopkins, 2000). Three hours after the last alcohol gavage, rats were perfused with 4% paraformaldehyde under isoflurane anesthesia. Horizontal 20-μm cryosections were obtained, allowing visualization in the same section of both ventral hippocampi containing the DG and the EC. Sections were mounted directly on gelatin-coated slides and stained for FJ-B according to the manufacturer’s protocol. Dry slides were cleared in xylene and cover-slipped with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI). For cell density analysis an Olympus microscope (Olympus Corporation, Japan) equipped with a Fitch filter was used. Six horizontal sections containing the bilateral hippocampi and the respective EC regions were analyzed for degenerating cells between 5.6 to 6.6 mm ventral from bregma. Cell counting was conducted as previously described (Cippitelli et al., 2010a,b; Cippitelli et al., 2014). Degenerating granule cells of the entire DG were measured using the program Image J (Schneider et al., 2012). Results for EC degeneration are depicted as counts per square mm by dividing the total number of degenerating cells found in 48 examined microscope fields, equivalent to 16.8 square millimeters (single field area was 0.35 square millimeters × 4 fields per side × 2 sides per section × 6 sections per animal, for a total of 16.8 square millimeters) with a 20× microscope objective. Data for EC and DG degenerating cells are presented as number per square millimeter.

2.6. RNA isolation, cDNA synthesis and real time polymerase chain reaction

Rats were decapitated three hours after the last alcohol gavage, the same time point used to harvest samples for histochemical analysis of FJ-B. Brains were quickly removed, areas of interest were dissected and snap frozen in −80 °C isopentane, and stored at −80 °C until use. RNA isolation and cDNA synthesis was executed as previously described (Drew et al., 2015). Briefly, tissue was homogenized using a BBX24B Bullet Blender Blue homogenizer with 0.5 mm RNase-free beads for approximately 6 min at speed 8 (Next Advance, Averill Park, NY). RNA was isolated from tissue homogenate using the RNeasy Lipid Tissue Mini Kit and optional DNase set according to the manufacturer’s instructions (Qiagen, Valencia, CA). RNA concentration and integrity were evaluated using an Agilent 2100 Bioanalyzer with its associated RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). The iScript cDNA synthesis kit was used to prepare cDNA as described by the manufacturer (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using TaqMan® primers (assays # Rn00580432_m1 for IL-1β, Rn01410330_m1 for IL-6, Rn00667689_m1 for β-actin; ThermoFisher Scientific, Waltham, MA) and SsoAdvanced™ Universal Probes Supermix (Bio-Rad). Data were calculated as the mean ΔCt relative to the housekeeping gene β-actin. The ΔΔCt method was employed to generate fold expression variance of ethanol and drug treated groups compared to control.

2.7. Operant reversal learning task

This procedure was conducted in operant conditioning chambers (Med Associates, Inc., St. Albans, VT) enclosed in lit, sound-attenuating and ventilated cubicles and equipped with two retractable levers located in the front panel, laterally (on the right and left side) to a food pellet magazine. The pellet dispenser was positioned behind the front panel of the chambers. Chambers were also equipped with visual stimuli located above the levers (right and left cue lights) and near the top of the chamber on the back panel (house light). A microcomputer controlled the delivery of the reinforcer, presentation of visual stimuli and recording of the behavioral data. Operant training and testing methods used are described elsewhere (Abdul-Monim et al., 2003).

2.7.1. Training

Rats maintained at food restriction regimen (16–18 g per day) were initially trained for 4 days to press the lever for delivery of one 45-mg food pellet (Test Diet, 5-TUM, Richmond, IN) in 20-min daily sessions under a fixed ratio 1 (FR-1) reinforcement schedule using two active levers. Subsequently, rats were trained to press either the right or the left lever for food delivery under FR-1 for an additional 4 days. The active lever was varied from day to day to avoid lever bias. Rats were then trained to respond for food in presence and absence of a visual cue for 20 days. In the former case reward contingencies were right lever-right cue (RR) and left-lever left cue (LL), in the latter case, reward contingencies were right lever-left cue (RL) or left lever-right cue (LR). Thus, half of animals that were initially rewarded following RR or LL schedule for 10 days (varied day to day) were switched to RL or LR for other 10 days and the other half did the opposite. The experimental session began with the house light being illuminated. After 3 s, the levers, together with the stimulus cue, were presented. Following a lever press, the levers were retracted and the house light was extinguished for a 3-s time out period. A correct response on the active lever resulted in delivery of a food pellet and an incorrect response resulted in no food delivery. The house light was then turned on again and the cycle repeated. The experimental session was terminated following 128 lever presses (the total number including active and inactive lever presses) or 40 min (cut-off). At the end of this training period all rats were subjected to the 4-day binge alcohol or control treatment in which rats were pre-treated with PIO 60 mg/kg dose or its vehicle as described above.

2.7.2. Testing

Following 5-day recovery from alcohol intoxication the operant training on all reward contingencies was briefly recalled (2 sessions for each randomly scheduled reward contingency). On testing day, rats were first given access to the same reward contingency of the training session used on the previous day for a maximum period of 5 min or 25 lever presses (including both active and inactive lever presses, initial task). Then, after 2 min time-out signalled by the house light being turned off in which animals remained in the operant chamber, the reward contingency was reversed so that animals rewarded in the presence of the visual cue (RR or LR) were rewarded for responses made on the lever not signalled by the cue (LR or RL, respectively) and vice versa (reversal task). Similar to the initial task, the reversal task was carried out for a maximum period of 5 min or following a total number of 25 lever presses including correct and incorrect responses.

2.8. MWM task

Spatial acquisition and cognitive flexibility were assessed following methods described previously (Cippitelli et al., 2010a;...
The apparatus consisted of a circular pool (55 cm diameter) and a squared platform (16 cm²) submerged below the water surface. Behavior was recorded and analyzed using a computerized video-tracking system (EthoVision 4, Noldus Information Technology, Wageningen, The Netherlands). The pool was placed in a room with distal cues represented by objects present in the text room (i.e., doors, black rectangle drawn in a wall).

2.8.1. Reference memory

Beginning 5 days after the final gavage treatment, in which rats were pre-treated with PIO 60 mg/kg or its vehicle and treated with alcohol or control, acquisition of reference memory was assessed on four daily trials over 6 days. Four random entry points were used. If a rat did not escape to the platform within 60 s, it was guided to the platform and allowed to remain on the platform for 10 s. The rat was then removed and placed into a holding cage for 50 s before the next trial, so the total intertrial interval was 60 s. The platform remained in the same location throughout the 6 days of acquisition training. Results are means of four daily trials for latency to reach the submerged platform. Swimming speed was an index of locomotor activity.

2.8.2. Learning of a new platform position

Following reference memory testing, which provided 6 days of acquisition training and was 11 days after the last alcohol dose, the platform was moved diagonally across from its initial position, and the new learning was assessed. Each animal performed four trials separated by 60-s intertrial intervals, starting from four random entry points. The rest of the procedure was as described above. Time swum to reach the new platform location, distance to reach the new location, entries into the quadrant of the previous platform location as well as time swum in the previous platform location indicated learning of the new condition. For each re-learning variable, differences between trials (“savings”) served as an index of re-learning. Swimming speed was an index of locomotor activity.

2.9. Statistical analysis

Histological data from alcohol-induced neurodegeneration were analyzed separately for DG and EC using the non-parametric multiple independent samples Kruskal-Wallis test (Ethovision 4, Noldus Information Technology, Wageningen, the Netherlands). The pool was placed in a room with distal cues represented by objects present in the text room (i.e., doors, black rectangle drawn in a wall).

3. Results

3.1. Pioglitazone treatment prevents alcohol-induced neurodegeneration in the hippocampal DG and the EC

Because very low numbers of FJ-B positive cells were present in the groups that were not alcohol exposed, these groups had low variance compared with groups that did receive alcohol. The analysis was therefore conducted using non-parametric approaches (see Statistical Analysis). These analyses demonstrated an overall significant effect of alcohol treatment in inducing neuronal cell death in the DG ($p < 0.05$) as well as the EC ($p < 0.05$). Specifically, the most intense damage was found in the granule cells of the DG and the layer III pyramidal cells of the lateral EC (Supplemental Fig. S1). In the DG, neurotoxicity was reduced by daily treatment with pioglitazone. Pairwise comparisons showed that alcohol exposed animals treated with the 60 mg/kg dose of pioglitazone had significantly reduced levels of neurodegeneration as compared to rats treated with only alcohol ($p < 0.05$, Fig. 1A–E). Similar to the DG findings, treatment with 60 mg/kg of pioglitazone prevented alcohol-induced neurotoxicity in the EC ($p < 0.05$, Fig. 1F–J). Pioglitazone 30 mg/kg did not block neurodegeneration in the DG or EC, but a non-significant statistically reduction was observed. In areas other than the hippocampal DG granule cells and the EC layer III pyramidal cells, FJ-B positive cells were too few to be reliably counted. Thus, although alcohol-induced neurotoxicity was also observed in brain regions such as the piriform cortex, neurodegeneration was not quantified.

3.2. Binge treatment leads to high, sustained BALs that are not influenced by pioglitazone

BALs were 393.1 ± 24.2 and 401.5 ± 20.0 in alcohol treated animals without or with pioglitazone treatment, respectively.

3.3. Neuroprotection by pioglitazone is associated with inhibition of alcohol-induced increase in cytokine expression in hippocampus and EC

Analysis of gene expression revealed 1.8-fold induction of interleukin 6 (IL-6) mRNA in the hippocampus of rats treated with alcohol [main alcohol effect: ($F_{(1,24)} = 21.2, p < 0.001$)]. This effect was not accompanied by a main pioglitazone effect ($F_{(1,24)} = 0.8, NS$) or interaction between alcohol and pioglitazone ($F_{(1,24)} = 3.1, NS$). Administration of pioglitazone attenuated alcohol induction of IL-6 expression in the hippocampus (Fig. 2A). Analysis of gene expression also revealed robust 2.5-fold induction of IL-6 mRNA in the EC of rats treated with alcohol ($F_{(1,26)} = 36.1, p < 0.001$). Administration of pioglitazone blocked alcohol induction of IL-6 expression in the EC to levels observed in vehicle treated controls or treatment with pioglitazone alone (main pioglitazone effect ($F_{(1,26)} = 28.1, p < 0.001$); interaction “pioglitazone × alcohol” ($F_{(1,26)} = 5.4, p = 0.05$), Fig. 2B).

Interleukin 1 beta (IL-1β) mRNA expression in the hippocampus was not significantly altered by treatment with alcohol or pioglitazone, or co-administration ($F_{(1,24)} = 1.7, NS$, ($F_{(1,24)} = 0.6, NS$), and ($F_{(1,24)} = 0.8, NS$, respectively). However, there was a trend to increased IL-1β expression with alcohol treatment, and suppression of the alcohol effect by pioglitazone (Fig. 2C). In contrast, IL-1β mRNA expression in the EC was increased 1.8-fold by alcohol treatment ($F_{(1,24)} = 7.5, p < 0.05$) and blocked by co-
Fig. 1. Binge alcohol-induced neurotoxicity in the dentate gyrus (DG) and the entorhinal cortex (EC), and its prevention by pioglitazone (0, 30, 60 mg/kg). Sections were stained by Fluoro-Jade B (FJ-B) to visualize neurodegeneration. Panels (A), (B), (C), and (D) show representative sections (~6.1 mm from bregma, 20X magnification) visualizing labeled DG neurons in animals non-alcohol exposed treated with vehicle (PIO 0-CON), alcohol exposed treated with vehicle (PIO 0-ALC), pioglitazone 30 (PIO 30-ALC) and 60 mg/kg (PIO 60-ALC), respectively. Panels (F), (G), (H), (I) show representative sections (~6.1 mm from bregma, 20X magnification) visualizing labeled EC neurons in PIO 0-CON, PIO 0-ALC, PIO 30-ALC and PIO 60-ALC treated rats, respectively. Panels (E) and (J) show quantification of the histological data demonstrating alcohol-induced neurodegeneration and its prevention by pioglitazone in the DG and the EC, respectively. Data are the mean number of FJ-B positive cells/mm² ± SEM (N = 4–6 per group). #p < 0.05 vs. PIO 0-CON group; *p < 0.05 vs. PIO 0-ALC group. For detailed statistics, see “Results”.

Fig. 2. Effect of alcohol (ALC) and pioglitazone (PIO) on expression of interleukin 6 (IL-6) and interleukin 1β (IL-1β) genes in the hippocampus (A and C) and entorhinal cortex (EC, B and D). Pioglitazone (60 mg/kg) blocked alcohol-induced neuroinflammation in the hippocampus and EC. Brain structures were dissected, RNA prepared, cDNA synthesized, and mRNA levels evaluated by real-time polymerase chain reaction. β-actin expression served as normalization control. Results are expressed as fold change relative to vehicle treated controls (PIO 0-CON). Values are mean ± SEM. N = 6–8 per treatment group. ***p < 0.001, **p < 0.01, *p < 0.05. For detailed statistics see “Results”.

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administration of pioglitazone \((F_{1,24} = 11.1, p < 0.01)\), but no interaction between the alcohol and pioglitazone was detected \((F_{1,24} = 1.7, \text{NS})\). Pioglitazone blocked alcohol induction of IL-1\(\beta\) expression to the level in vehicle treated control or treatment with pioglitazone alone \((\text{Fig. 2D})\).

3.4. Pioglitazone prevents alcohol-impaired reversal learning ability in the operant task

The sequence of the reward contingencies did not alter the correct performance of the task during training \([F(1,49) = 0.0, \text{NS})\) and \((F_{1,49} = 0.1, \text{NS})\), Supplemental Figs. S2A and S2B, respectively). Post-intoxication training was not altered either by alcohol, pioglitazone or the combined treatment \([F(1,49) = 0.6, \text{NS}), \text{Supplemental Fig. S3}\). Also, these manipulations did not affect the performance in the initial task \([F(1,49) = 3.1, \text{NS}), \text{Fig. 3A}\]. In contrast, overall performance in the reversal task was significantly modified. ANOVA showed “alcohol effect” × “pioglitazone treatment” interaction \((F_{1,49} = 5.1, p < 0.05)\) with pairwise comparisons showing reduced ability of rats treated with alcohol only to perform the correct response \((p < 0.05)\) while pioglitazone reversed the cognitive deficit induced by alcohol intoxication \((p < 0.05, \text{Fig. 3B})\).

3.5. Pioglitazone improves acquisition of spatial memory and prevents alcohol-induced deficits in spatial learning in the MWM

Patterns of acquisition of spatial memory in the MWM were modified by the treatments with both alcohol and pioglitazone \((60 \text{mg/kg})\). ANOVA revealed a main effect of alcohol intoxication \((F_{1,39} = 5.0, p < 0.05)\) accompanied by a main effect of pioglitazone \((F_{1,39} = 6.0, p < 0.05)\) indicating that alcohol impaired reference memory abilities while pioglitazone improved them. Interestingly, the pioglitazone-induced enhancement of spatial memory acquisition occurred regardless of the contingent alcohol treatment, as demonstrated by the absence of “alcohol effect” × “pioglitazone effect” interaction. However, navigation latency to reach the hidden platform decreased over the 6 days of training [main time effect: \((F_{5,395} = 37.6, p < 0.001)\)] and did so in all groups, as shown by a lack of “alcohol effect” × “pioglitazone effect” × “day” interaction \([F(5,395) = 0.5, \text{NS}), \text{Fig. 4A}\]. Swim speed \((\text{Fig. 4B})\), a measure of locomotor activity, was not altered by alcohol treatment (main effect: \(F_{1,39} = 0.0, \text{NS})\) or pioglitazone (main effect: \(F_{1,39} = 2.3, \text{NS})\). Speed decreased across the 6 training days \((F_{5,393} = 16.4, p < 0.001)\) in all treatment groups examined \((F_{5,393} = 1.3, \text{NS})\).

Following 6 days of acquisition trials, animals were tested for a new spatial learning task, in which the platform was moved to the opposite quadrant of the pool. Learning of the new platform location differed between groups for distance traveled to reach the new platform location \([F(3,39) = 3.3, p < 0.05)\) and time swum to reach this new location \([F(3,39) = 4.2, p < 0.05)\] while the time course of this learning was different in all variables examined \([“group” × “trial” interaction for distance traveled to reach the new platform location \((F_{9,117} = 3.3, p < 0.01)\), time to reach this new location \((F_{9,117} = 2.5, p < 0.05)\), entries into the original training quadrant \((F_{9,117} = 2.8, p < 0.01)\) and time swum in the original platform quadrant \([F_{9,117} = 2.2, p < 0.05), \text{Supplemental Fig. S4})\].

The differential course of re-learning reflected differences in savings (improvement of performance). When analyzing savings between trial 1 and trial 2, when most of the learning normally occurs, savings for time spent swimming to reach the new platform location \((\text{Fig. 4C})\) differed among treatments. ANOVA showed a main effect of alcohol \((F_{1,39} = 5.2, p < 0.05)\) accompanied by a main effect of pioglitazone \((F_{1,39} = 5.8, p < 0.05)\) and interaction of the two factors \((F_{1,39} = 6.8, p < 0.05)\) with post hoc comparisons indicating decreased savings of alcohol exposed rats as compared to non-exposed controls \((p < 0.01)\), an effect prevented by pioglitazone \((p < 0.01 \text{ vs alcohol exposed group})\). A similar pattern was found for total distance swum to reach the new location \((\text{Fig. 4D})\): main effect of alcohol: \((F_{1,39} = 3.9, p < 0.05)\); main effect of pioglitazone: \((F_{1,39} = 11.6, p < 0.01)\); interaction: \((F_{1,39} = 7.3, p < 0.05)\) with post hoc analysis showing alcohol-induced decrease in this variable \((p < 0.01)\) reversed by pioglitazone \((p < 0.001 \text{ vs alcohol exposed group})\). Savings for entries into the previously trained quadrant \((\text{Fig. 4E})\) also differed \([“alcohol” × “pioglitazone” interaction \((F_{1,39} = 4.8, p < 0.05)\)] with post hoc analysis showing difference between alcohol exposed and control exposed rats \((p < 0.05)\) as well as prevention by pioglitazone \((p < 0.05)\). Significant treatment interaction was also observed for time spent swimming in the original platform quadrant \((F_{1,39} = 4.1, p < 0.05, \text{Fig. 4F})\). Conversely, swim speed did not differ between the groups during the new learning task [main alcohol effect: \((F_{1,39} = 0.2, \text{NS})\]; main pioglitazone effect: \((F_{1,39} = 0.0, \text{NS})\); interaction \((F_{1,39} = 0.0, \text{NS})\)]

4. Discussion

We show here that binge-like alcohol exposure produces neuronal death in the EC and hippocampal DG and that this is associated with alcohol-induced immune responses. Pioglitazone prevents neurodegeneration and neuroinflammation. In parallel, pioglitazone prevents alcohol-impaired re-learning performance both in an operant task and in a spatial navigation task.

Extensive neurodegeneration was indicated by FJ-B staining in the DG of the hippocampus and in the adjacent EC. Sparse degenerative neurons were detected in other areas of the brain but their number was low compared to the DG and the EC. These findings are consistent with previous observations indicating that binge alcohol intoxication caused significant neuronal loss, particularly in these regions (Cippitelli et al., 2010a; Collins et al., 1996; Crews et al., 2004; Obernier et al., 2002a). We also found that the PPAR\(\gamma\) agonist pioglitazone prevented in a dose dependent manner neurotoxic consequences of the binge alcohol treatment by suppressing the number of FJ-B positive cells in both the hippocampal DG and the EC. The dose dependence of the effect suggests that, in order to evaluate the potential clinical implications, the plasma drug levels at which this response is achieved should be compared to the levels that are achieved in humans with FDA-approved doses. In agreement with these results, previous studies have shown that activation of PPAR\(\gamma\) exerts a marked protective response in various models of neurodegenerative diseases (Diab et al., 2002; Kapadia et al., 2008; Kaundal and Sharma, 2010). This effect appears to be linked to the ability of PPAR\(\gamma\) to attenuate neuronal inflammation and to reduce activation of inflammatory cytokines (Kapadia et al., 2008; Yi et al., 2008). Interestingly, the PPAR\(\alpha\) agonist oleylethanolamide protects against alcohol-induced inflammatory signaling and apoptosis in the cerebral cortex of adolescent rats with binge drinking (Anton et al., 2016). Here, for the first time, we provided evidence that PPAR\(\gamma\) activation is also neuroprotective against binge alcohol drinking, possibly through inhibition of the neuroimmune response (Drew et al., 2015; Kane et al., 2013, 2014, 2011).

To confirm this hypothesis, we examined IL-1\(\beta\) and IL-6 mRNA expression levels. These cytokines are involved in neuroinflammation initiated by excessive alcohol use (Alfonso-Loeches et al., 2010; Crews and Nixon, 2009; Drew et al., 2015; Kane et al., 2013, 2014). IL-1\(\beta\) and IL-6 are well characterized as pro-inflammatory molecules. In addition, it should be noted that IL-6 is suggested to possess anti-inflammatory activities under some circumstances (Schaper and Rose-John, 2015). We found that both the DG and the EC are sensitive to neuroinflammatory processes as dynamic changes were observed in the expression of these inflammatory cytokines in response to alcohol treatment. However, these
alcohol-induced changes were more prominent in the EC. There was robust alcohol-induced upregulation of IL-6 and IL-1β expression in the EC that was abolished by concurrent administration of pioglitazone. This suggests, on the one hand, these cytokines may contribute to alcohol-induced neurodegeneration and, on the other, the effect of pioglitazone may be related to its ability to...
The intimate mechanisms linking PPARγ function to immune system regulation has not been fully determined yet. However, it is known that PPARγ agonists suppress the production of pro-inflammatory molecules including cytokines and chemokines by CNS glial cells. PPARγ agonists are believed to principally suppress inflammation by repressing the transcription of genes encoding these pro-inflammatory molecules. This is believed to occur through a mechanism referred to as receptor-dependent trans-repression in which PPARγ physically interacts with transcription factors, blocking the activation of immune responsive genes (Daynes and Jones, 2002). PPARγ has been shown to act through receptor-dependent trans-repression to suppress the activity of a variety of transcription factors including NF-κB (Li et al., 2000). PPARγ can also alter transcription through receptor-independent mechanisms, including suppressing specific steps in NF-κB signaling pathways (Rossi et al., 2000; Straus et al., 2000). Interestingly, alcohol has been demonstrated to activate NF-κB (Blanco and Guerri, 2007; Ward et al., 1996). PPARγ can be expressed by glia in vivo, particularly under neuroinflammatory conditions (Diab et al., 2002; Warden et al., 2016). Thus, PPARγ suppression of immune activity by glia may indirectly protect neurons since pro-inflammatory molecules can be toxic to neurons. In addition, PPARγ agonists may directly alter the viability of neurons. Neurons have previously been demonstrated to express PPARγ suggesting that pioglitazone may regulate neuron viability directly through receptor-dependent mechanisms (Inestrosa et al., 2005). PPARγ agonists have been demonstrated to protect neurons from toxic insults by maintaining mitochondrial function and limiting reactive oxygen species formation in these cells (Fuenzalida et al., 2007). It will be important in future studies to further define the mechanisms by which PPARγ agonists protect hippocampal and EC neurons from the toxic effects of alcohol.

The EC and the hippocampus are closely interconnected regions and the circuitry from the EC to the hippocampal formation is considered to be critical for memory formation and spatial learning (Aggleton et al., 2000). Lesion studies have shown that damage of the hippocampal area mostly produces spatial reference deficits (Jarrard, 1993; Morris et al., 1982) whereas damage of the EC may also compromise reversal learning abilities (Eijkenboom et al., 2000; Hagan et al., 1992). However, cognitive impairment resulting from binge alcohol exposure includes reversal learning and object recognition deficits rather than deficits in acquisition of spatial reference memory in navigation or exploration tasks in rats (Cippitelli et al., 2010a,b; Obernier et al., 2002b). In these studies, re-learning deficits observed in binge alcohol treated animals have been closely associated with different navigation or exploratory patterns reflecting increased perseverative behavior as well as decreased cognitive flexibility. Here we tested the possibility that the observed neuroprotective role of pioglitazone was associated with prevention of alcohol-induced deficits in learning abilities. This hypothesis was verified by employing two behavioral strategy-shift tasks, both requiring intact cognitive ability and flexibility, though one devoid of a spatial component (i.e., operant reversal learning task) and the other heavily based on spatial orientation (i.e., learning of a new task in the MWM). In the non-spatial operant reversal learning task, we found a selective though weak deficit in cued reversal learning following alcohol treatment, which was reversed by concurrent administration of pioglitazone. These data confirm previous evidence that excessive alcohol treatment causes learning disabilities in non-spatial tasks (Cippitelli et al., 2010b; Garcia-Moreno and Cimadevilla, 2012; Takahashi et al., 2015) and demonstrate that pioglitazone ameliorates re-learning performance in a new cue-reward contingency. The learning of a new condition in the MWM is very sensitive to hippocampal as well as EC damage and, therefore, cognitive consequences of alcohol exposure were assumed to result in a more pronounced impairment. As expected, variables describing navigation strategies of alcohol treated rats were significantly changed from the control group. Since velocity of swimming was similar between groups, these data indicate alcohol-induced damage of new learning performance and reduced cognitive flexibility to engage the correct navigation strategy to reacquire the platform location, similar to what has been described previously (Cippitelli et al., 2010a; Obernier et al., 2002b). Pioglitazone successfully prevented this impairment. However, surprisingly, alcohol-induced cognitive impairment was not restricted to the re-learning performance, as a deficit in the acquisition of reference memory was also detected following binge alcohol treatment in the present study. This finding suggests that, under certain experimental conditions (i.e., different use of navigation cues or different experimental design), alcohol can produce damage to the hippocampal-mediated spatial reference memory. This evidence reconciles with previous observations obtained from lesion studies and supports the hypothesis that alcohol-induced impairments in spatial learning and memory parallel those induced by lesions (Matthews and Silvers, 2004). Even more surprisingly, we also observed that pioglitazone treatment was able to improve spatial reference memory for a fixed platform location compared to non-pioglitazone treated groups. Similarly, PPARγ agonist treatment was previously associated with improvements of hippocampal-dependent memory in mouse models for Alzheimer’s disease, indicating that PPARγ agonists may act as cognitive enhancers (Nenov et al., 2014). In addition, PPARγ activation has been shown to modulate alcohol intake and preference in two-bottle choice models of consumption but not in a limited access binge drinking model (Blednov et al., 2015). Investigation of potential anxiolytic or antidepressant activity of pioglitazone (Domí et al., 2016; Kemp et al., 2014) may suggest additional mechanisms of cognitive protection by pioglitazone following alcohol intoxication. Further study of the therapeutic potential of approved PPARγ agonists, including pioglitazone, in alcohol-induced neuropathology, and cognitive impairments, as well as alcohol consumption, may provide new strategies for intervention in alcohol use disorders.

In the present study, molecular events were assessed acutely and behavioral effects were assessed at later time points. As reported by Crews and Nixon (2009), neurodegeneration peaks shortly after the last dose of alcohol in this model, the time point that we used to measure neurodegeneration. We have previously demonstrated that spatial memory remains impaired 10 weeks in this alcohol model (Cippitelli et al., 2010b) and cognitive function was tested in the present study in a two week period following alcohol treatment. Alcohol treatment also inhibits neurogenesis, contributing to neuron loss and perhaps cognitive impairment (Nixon, 2006; Nixon and Crews, 2004). Given our finding that pioglitazone protects against alcohol-induced cognitive impairment, it is interesting that PPARγ activation has been shown to protect neurogenesis and cognitive function in disease models (Ormerod et al., 2013). The protective effects of pioglitazone appear to be long lasting as suggested by our data demonstrating pioglitazone protection against cognitive impairments two weeks after completion of alcohol and pioglitazone treatment.

5. Conclusions

Here we show a clear protective effect by the PPARγ agonist pioglitazone against neuronal damage caused by binge alcohol intoxication in brain regions underlying learning and memory processes. Consistently, pioglitazone confers a wide spectrum of protection against alcohol-induced impairment of spatial and non-
spatial learning abilities, and improves spatial reference memory. We also show that neuroinflammation importantly contributes to alcohol-induced neurotoxicity as dynamic changes in the expression of pro-inflammatory markers were detected following alcohol treatment. These changes were blocked by pioglitazone suggesting that anti-inflammatory mechanisms may be responsible for the protective effects of PPARγ agonists on alcohol-induced neuronal and cognitive damages. The present findings, together with our previous evidence describing the ability of PPARγ agonists to reduce excessive alcohol intake and vulnerability to relapse into alcohol seeking (Stopponi et al., 2011), provide a strong rationale for consideration of pioglitazone or its congeners as effective treatments for alcohol use disorders.

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Footnotes


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