

Effects of a novel beta lactam compound, MC-100093, on the expression of glutamate transporters/receptors and ethanol drinking behavior of alcohol preferring rats

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List of abbreviations:

AUD	Alcohol use disorder
BLA	Basal lateral amygdala
GLT-1	Glutamate transporter 1
GPCRs	G-protein coupled receptors
MCL	Mesocorticolimbic system
mGluR	Metabotropic glutamate receptor
MPEP	Methyl-2-(phenylethynyl) pyridine
NAc	Nucleus accumbens
PFC	prefrontal cortex
PPAR	Peroxisome proliferator-activated receptors
SUDs	Substance use disorders
xCT	Cystine/glutamate exchanger transporter

Abstract

Chronic ethanol exposure affects the glutamatergic system in several brain reward regions such as the nucleus accumbens (NAc). Our laboratory has shown that chronic exposure to ethanol reduced the expression of glutamate transporter 1 (GLT-1) and cystine/glutamate exchanger transporter (xCT) and, as a result, increased extracellular glutamate concentrations in the NAc of alcohol preferring (P) rats. Moreover, previous study from our laboratory reported that chronic ethanol intake altered the expression of certain metabotropic glutamate receptors in the brain. In addition to central effects, chronic ethanol consumption induced liver injury, which is associated with steatohepatitis. In the present study, we investigated the effects of chronic ethanol consumption in the brain and liver. Male P rats had access to free choice of ethanol and water bottles for five weeks. Chronic ethanol consumption reduced GLT-1 and xCT expression in the NAc shell but not in the NAc core. Furthermore, chronic ethanol consumption increased fat droplet content as well as peroxisome proliferator-activated receptor alpha (PPAR- α) and GLT-1 expression in the liver. Importantly, treatment with the novel beta-lactam compound, MC-100093, reduced ethanol drinking behavior and normalized the levels of GLT-1 and xCT expression in the NAc shell as well as normalized GLT-1 and PPAR- α expression in the liver. In addition, MC100093 attenuated ethanol-induced increases in fat droplet content in the liver. These findings suggest that MC-100093 might be a potential lead compound to attenuate ethanol-induced dysfunction in glutamatergic system and liver injury.

Keywords: Ethanol dependence, nucleus accumbens, glutamate, GLT-1, xCT, PPARs, MC-100093

Significance statement

This study identified a novel beta-lactam, MC100093, that has demonstrated upregulatory effects on GLT-1. MC-100093, reduced ethanol drinking behavior and normalized levels of GLT-1 and xCT expression in the NAc shell as well as normalizing GLT-1 and PPAR- α expression in the liver. In addition, MC100093 attenuated ethanol-induced increases in fat droplet content in the liver.

Introduction

Alcohol use disorder (AUD) is a complex chronic relapsing disorder that leads to long-lasting neuroadaptations of neurotransmitter systems. Changes in the glutamatergic system are implicated in the development and maintenance of ethanol dependence (Kalivas 2009). Dysregulation of the glutamatergic system in key brain reward regions of the mesocorticolimbic (MCL) system such as the nucleus accumbens (NAc), basal lateral amygdala (BLA), prefrontal cortex (PFC), and hippocampus are commonly observed consequences of chronic ethanol exposure, including altered expression of glutamate receptors and transporters in key reward brain regions (Goodwani et al 2017). Among other brain regions, the NAc has been extensively studied for its crucial role in the development of substance use disorders (SUDs), including ethanol (Heinze et al 2009, Müller et al 2016, Neasta et al 2011). Chronic ethanol exposure reduced the expression of glutamate transporter 1 (GLT-1) and cystine/glutamate exchanger transporter (xCT), and consequently increased extracellular glutamate concentration in the NAc of alcohol preferring (P) rats (Alhaddad et al 2014b, Bridges et al 2012, Das et al 2015). These effects were suggested to be mediated through reduced synaptic glutamate clearance and diminished metabotropic glutamate receptor 2 (mGluR2) associated with inhibition of synaptic release. xCT is co-localized with GLT-1 in astrocytes to regulate extracellular glutamate concentrations. Reduction in xCT expression may cause a decrease in extracellular glutamate concentration in the brain, which can lead to loss of glutamatergic tone on presynaptic mGluR2/3, and consequently increasing synaptic glutamate release (Javitt et al 2011, Moran et al 2005). We suggest that GLT-1, xCT and mGluR2/3 are important to regulate simultaneously extracellular glutamate concentrations in the brain. Moreover, treatment with GLT-1 modulators (e.g. Ceftriaxone and MS-153, Figure 1) upregulated GLT-1 and xCT expression in the NAc

while attenuating ethanol drinking behaviors (Alhaddad et al 2014a, Alhaddad et al 2014b). These studies suggest that glutamate transporters might be potential targets for the treatment of AUDs. Neuroadaptive changes involved in AUDs include downregulation of metabotropic glutamate receptor 5 (mGluR5) and its signaling pathway (Carroll 2008, Leurquin-Sterk et al 2018). mGluR5 has also been shown to be involved in relapse to ethanol intake as shown in mGluR5 knockout mouse model (Parkitna et al 2013). Additionally, 2-methyl-6-(phenylethynyl) pyridine (MPEP) antagonized mGluR5 action and reduced ethanol consumption in mice (Olive et al 2005). Moreover, mGluR1 signaling within the NAc shell has crucial role in maintaining ethanol consumption under limited access in mice (Lum et al 2014). The expression of mGluR1 is increased in animals exposed to ethanol (Obara et al 2009). Furthermore, mGluR1 antagonism was associated with attenuation of ethanol drinking behavior (Besheer et al 2008a, Lum et al 2014). These latter findings provide further support for the hypothesis that manipulation of glutamate transporters and/or receptors may serve as effective pharmacotherapeutics to treat AUDs.

In addition to its effects in the brain, ethanol exposure causes chronic liver injury, particularly steatohepatitis (De la Monte et al 2009, Zeng et al 2019). Peroxisome proliferator-activated receptors (PPAR- α and PPAR γ) play critical roles in adipose expansion and in the control of its functions (Anghel & Wahli 2007, You & Crabb 2004). Ethanol-induced fatty liver has been linked to the blocking of PPAR- α activity both in vitro (Fischer et al 2003) and in vivo (Hong et al 2004, Nanji et al 2004). Moreover, PPAR agonists reduce ethanol drinking behavior (Stopponi et al 2011), neurodegeneration (Mandrekar-Colucci et al 2013), and ethanol-induced liver injury (Enomoto et al 2003). It is noteworthy that ethanol, in addition to its direct action on the brain, may impair neurotransmitter function required for certain aspects of neuroplasticity,

learning, and memory mediated through biochemical feedback in the liver-brain axis (De la Monte et al 2009). Accordingly, these studies suggest a role for the liver-brain axis in ethanol dependence.

β -lactam compounds have shown promise as pharmacotherapeutics to treat SUDs, including AUDs. Ceftriaxone is a prototypical β -lactam antibiotic that has been shown to reduce ethanol intake, while up-regulating GLT-1 and xCT levels in the MCL (Rao & Sari 2012). However, because ceftriaxone is an antibiotic, efforts have been made to construct β -lactam products that lack antibiotic activity. For instance, structural modification of the cephalosporin ring system and side chains of ceftriaxone resulted in the creation of a novel beta-lactam compound, MC-100093 (Figure 1), which has enhanced GLT-1 up-regulatory properties compared to ceftriaxone (Abou-Gharbia et al 2017, Childers et al 2020b). *In vitro* pharmacokinetics and pharmacology and *in vivo* pharmacokinetic data for MC-100093 have been previously reported (Knackstedt et al 2021). MC-100093 enhanced glutamate uptake in an astrocyte-neuron co-culture model by 23.5% over that of control values with an IC_{50} value of 0.1 μ M. Ceftriaxone displayed similar efficacy but was significantly less potent (IC_{50} approx. 3 μ M). Unlike ceftriaxone, MC-100093 showed no antimicrobial effects against select gram-positive and gram-negative bacteria at concentrations up to 256 μ g/mL. MC-100093 displayed no affinity for 43 G-protein coupled receptors (GPCRs) and transporters at a concentration of 10 μ M as assessed through the NIMH Psychoactive Drug Screening Program (Besnard et al 2012). *In vitro*, MC-100093 demonstrated high aqueous solubility (> 10 mM in 2% DMSO/phosphate buffered saline), high stability in rodent and human liver microsomes, low binding to plasma protein (35% bound), and low partitioning into lipid membranes (83% free, unbound). *In vivo* pharmacokinetic studies found

that MC-100093 displayed reasonable bioavailability following oral administration ($F\% = 28\%$). The lack of significant oxidative metabolism suggests that bioavailability following intraperitoneal administration should be similar to that found with oral dosing. The brain-to-plasma (B/P) ratio for MC-100093 was 0.28 (28%). In vivo half-life was found to be moderate (4.2 hours i.p., 5.26 hours p.o.) and was likely the result of renal elimination, given the lack of oxidative metabolism found in liver microsomes and the long half-life found with MC-100093 in plasma ($t_{1/2} \gg 240$ minutes). These results contrast with those revealed with ceftriaxone, which displays essentially no significant oral bioavailability and very low brain penetration; B/P = 1% (Granero et al 1995).

Given the above, we hypothesized that treatment with MC-100093 would upregulate GLT-1 in key MCL regions while attenuating ethanol intake. Using P rat as an established animal model of chronic ethanol drinking and AUD (Bell et al 2017), we investigated the effects of chronic ethanol exposure and MC-100093 treatment on glutamatergic receptor and transporter levels as well as that of PPARs in the liver and brain.

Materials and Methods

Animals

Male P rats were received from Indiana University School of Medicine (Indianapolis, IN, USA), and were housed in a 21°C vivarium on a standard 12/12 h light/dark cycle. Rats had free access to water and food throughout the experiment. Institutional Animal Care and Use Committee (IACUC) of The University of Toledo approved all experimental procedures, in accordance with

the guidelines governing the use of animals in research of the National Institutes of Health as described in the Guide for the Care and Use of Laboratory Animals.

Ethanol drinking protocol

We used an ethanol drinking procedure as described previously (Alhaddad et al 2014b). Briefly, animals started the experimental drinking procedure at age of 90 days. Rats had five weeks of free-choice access to three-bottle choice drinking, 0%, 15% and 30%, v/v ethanol. Daily measurement of ethanol intake was performed (g of ethanol intake/kg of body weight/day). We measured the baseline ethanol drinking by averaging the intake during the last three weeks. Three groups of P rats (n = 8-9/group) were exposed to ethanol for five weeks. At Week 6, rats received either MC-100093 (50 mg/kg, i.p.) (Ethanol-MC group), ceftriaxone (200 mg/kg, i.p.) (Ethanol-CEF group, which served as a positive control), or equivolume of saline vehicle (Ethanol group) for five days. Following criteria for the development of ethanol dependence, rats whose average ethanol intake ≤ 4 g/kg/day were excluded from the study (Bell et al 2012, Li et al 1987, Sari & Sreemantula 2012). Beside the ethanol exposed groups, another group of P rats (n = 8), was exposed to only water and food throughout the exposure procedure (control group). This procedure results in pharmacologically relevant blood ethanol concentration (50–200 mg%) (Bell et al 2006).

Brain and liver tissue dissection

At the completion of pharmacological challenges while the rats had free-choice access to ethanol, P rats were removed from their home-cage and rapidly euthanized by CO₂ inhalation

followed by decapitation with the guillotine the day following the fifth and final injection. Brains were isolated and immediately frozen on dry ice and stored at -80°C . The stereotaxic coordinates provided by Paxinos and colleague's rat brain stereotaxic atlas (Paxinos et al 2007) were used to isolate NAc-core and NAc-shell using a cryostat apparatus (-20°C), and samples were returned to -80°C for subsequent Western blot analyses. We used a micro-punch procedure under a laboratory microscope (10x) to dissect the NAc-core and NAc-shell. Liver samples were extracted after decapitation, with the anterior-ventral lobe removed and stored at -20°C for subsequent analyses.

Western Blot analyses

Western blot assays were performed to measure the expression of GLT-1, xCT, mGluR1, mGluR5, PPAR- α , PPAR γ , GAPDH, and β -tubulin in the NAc-core and NAc-shell as has been done in previous studies from our laboratory (Alhaddad et al 2014a, Alhaddad et al 2014b). Liver, NAc-core, and NAc-shell tissues were lysed using a lysis buffer containing protease and phosphatase inhibitors. A detergent compatible protein assay (Bio-Rad, Hercules, CA, USA) was used to quantify the amount of protein for each sample. Samples were mixed with laemmli dye and loaded with equal amounts on polyacrylamide gels (10%) for protein separation using an electrophoresis apparatus. Polyvinylidene difluoride (PVDF) membranes were further used to transfer proteins electrophoretically from the gels. Membranes were incubated in 5% fat-free milk in Tris-buffered saline with Tween-20 (TBST) for 30-60 minutes at room temperature. Membranes were then incubated with appropriate primary antibodies overnight at 4°C : rabbit anti-GLT-1 (1:5000, Abcam, ab41621), rabbit anti-xCT (1:2000, Abcam, ab175186), rabbit anti-mGluR1 (1:1000, Abcam, ab82211), rabbit anti-mGluR5 (1:1000, Abcam, ab76316), rabbit anti-

PPAR- α (1:1000, Abcam, ab24509), and rabbit anti-PPAR γ (1:1000, Abcam, ab209350). Mouse anti- β -tubulin (1:1000; BioLegend) was used as a loading control antibody for the brain tissue and rabbit anti-GAPDH (1:5000; cell signaling) was used as a loading control antibody for the liver tissues. Membranes were washed five times on the next day with TBST followed by incubation with appropriate secondary antibody (1:5000) for 90 minutes at room temperature. Chemiluminescent reagents (Super Signal West Pico, Pierce Inc.) were used to detect proteins using a GeneSys imaging system, which digitized the blot images. Water-control group data was represented as 100% and all other values were expressed relative to this control group for detection of changes in the expression of all targeted proteins in the brain and liver samples as previously described (Alhaddad et al 2020a).

Liver Oil Red O staining

Fat content was measured in formalin-fixed liver sections (Frozen liver sections placed in 4% formalin, 10 μ m thick) using Oil Red O (Sigma, CAS Number 1320-06-5) staining. Liver sections were placed on slides and stained with freshly prepared Oil Red O solution for 15 minutes and then rinsed with 60% isopropanol. Slides were then rinsed with distilled water and mounted in aqueous mounting media and prepared for imaging. The magnitude of Oil Red O staining was determined using a color video camera attached to an Olympus VS120 slide scanning microscope at 20 \times magnification. Olympus OlyVIA software was used to analyze images. Lipid droplets were quantified using Image J pro (NIH). Data are presented as the mean \pm SEM of the Oil Red O staining for each group.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism software, with p-value of 0.05 or less considered statistically significant. We performed a two-way (mixed model) ANOVA followed by Bonferroni multiple comparison post-hoc test to analyze daily ethanol and water consumption, as well as body weight changes. For the Western blot and Oil Red O staining analysis, we performed one-way ANOVA with Newman-Keuls post hoc tests to measure differences between groups as a percentage (relative to control values).

Results

Effect of MC-100093 and ceftriaxone treatment on ethanol consumption

Treatment with MC-100093 or ceftriaxone significantly reduced ethanol consumption compared to saline treatment. Statistical analysis of ethanol drinking data revealed a significant Drug by Day interaction ($F_{10,138} = 17.49$, $p < 0.0001$). Tukey's multiple comparison tests showed a significant decrease in ethanol consumption from treatment Day 2 through treatment Day 5 in the MC-100093 and ceftriaxone groups compared to the Ethanol-Saline group (Fig.2 A). However, ethanol consumption was significantly reduced in ceftriaxone group compared to MC-100093 group in Day 2 through Day 5.

Effects of MC-100093 and ceftriaxone treatment on water intake

Water consumption was significantly increased in MC-100093- and ceftriaxone-treated groups compared to the saline treated group. Statistical analysis of water consumption data revealed a significant Drug by Day interaction ($F_{10,138} = 6.987$, $p < 0.0001$). Tukey's multiple comparison tests showed a significant increase in water consumption from treatment Day 2 through treatment Day 5 in the ceftriaxone group and at Treatment Day 5 in the MC-100093 group compared to the Ethanol-Saline group (Fig.2 B). Noteworthy, there was no significant difference in the total fluid intake across all groups (data not shown).

Effects of MC-100093 and ceftriaxone treatment on body weight

Statistical analysis of body weight data revealed a non-significant Drug by Day interaction ($F_{10,138} = 0.0045$, $p > 0.999$). Neither MC-100093 nor ceftriaxone treatment had a significant effect on animal body weights (Fig.2 C).

Effect of MC-100093 and ceftriaxone on the expression of GLT-1 in the NAc-core and NAc-shell

We investigated the effects of MC-100093 or ceftriaxone on GLT-1 expression in P rats exposed to a chronic ethanol drinking protocol. One-way ANOVA revealed no significant difference in GLT-1 expression in the NAc-core, among all tested groups ($F_{3, 28} = 0.734$, $p > 0.05$, $n = 8/$ group), Fig 3A. However, there was a significant difference in GLT-1 expression among the four groups in the NAc-shell ($F_{3, 28} = 4.85$, $p < 0.01$, $n = 8/$ group). Newman-Keuls post-hoc analyses revealed a significant decrease in GLT-1 expression in the NAc-shell of the Ethanol group compared to the Water control group ($p < 0.05$), while its expression was significantly

increased in groups treated with MC-100093 ($p < 0.05$) or ceftriaxone ($p < 0.01$) in the NAc-shell as compared to the Ethanol-saline group. No significant changes were detected between the Water control, Ethanol-MC-100093, and Ethanol-CEF groups as shown in Fig 3B.

Effect of MC-100093 and ceftriaxone on the expression of xCT in the NAc-core and NAc-shell

One-way ANOVA revealed no significant difference in xCT expression in the NAc-core, among all tested groups ($F_{3, 28} = 0.265$, $p > 0.05$, $n = 8/$ group), Fig 3C. However, there was a significant difference in protein expression of xCT among the four groups in the NAc-shell ($F_{3, 28} = 4.48$, $p < 0.05$, $n = 8-9/$ group). Newman-Keuls post-hoc analyses showed a significant decrease in xCT expression in NAc-shell of the Ethanol group compared to the Water control group ($p < 0.05$). The analysis also showed that MC-100093 ($p < 0.01$) and ceftriaxone ($p < 0.05$) significantly increased xCT expression in the NAc-shell as compared to the Ethanol-Saline group. No significant changes were detected between the Water control, Ethanol-MC-100093, and Ethanol-CEF groups as shown in Fig 3D.

Effect of MC-100093 and ceftriaxone on the expression of metabotropic glutamate receptors in the NAc-core and NAc-shell

We next investigated the effects of MC-100093 or ceftriaxone on protein expression of mGluRs in the NAc. One-way ANOVA revealed no significant difference in mGluR1 expression among all tested groups in the NAc-core ($F_{3, 27} = 0.802$, $p > 0.05$, $n = 7-8/$ group), and NAc-shell ($F_{3, 28} = 0.890$, $p > 0.05$, $n = 8/$ group), Fig. 4A and Fig. 4B, respectively. There was no significant difference in mGluR5 expression among all groups in the NAc-core ($F_{3, 27} = 1.084$, $p > 0.05$, $n =$

7-8/ group), Fig 4C. However, mGluR5 expression was significantly down-regulated in the ethanol group compared to the Water control, Ethanol-MC-100093, and Ethanol-CEF groups in the NAc-shell ($F_{3, 28} = 2.707$, $p < 0.05$, $n = 8/$ group). No significant changes in mGluR5 expression were detected between the Water control, Ethanol-MC-100093, and Ethanol-CEF groups, Fig 4D.

Effect of MC-100093 and ceftriaxone on the expression of PPAR- α in the NAc-core and NAc-shell

There were no significant changes in PPAR- α expression between all tested groups in the NAc-core ($F_{3, 28} = 0.922$, $p > 0.05$, $n = 8/$ group) and NAc-shell ($F_{3, 20} = 1.609$, $p > 0.05$, $n = 6/$ group) as shown in Fig. 4E and Fig. 4F, respectively.

Effect of chronic ethanol exposure, MC-100093, and ceftriaxone on fat deposition in the liver

Oil Red O staining was used to detect the liver fat content for each group. One-way ANOVA revealed a significant change in fat content between groups ($F_{3, 15} = 5.543$, $p < 0.01$, $n = 4-5/$ group). Newman-Keuls post-hoc analyses revealed higher fat droplet content in the ethanol group ($p < 0.05$) compared to the Water control, ethanol-MC or ethanol-CEF groups. However, no significant differences were observed between the Water control, ethanol-MC or ethanol-CEF groups (Fig. 5A).

Effects of MC-100093 and ceftriaxone on the protein expression of PPARs in the liver

We also investigated the effects of MC-100093 or ceftriaxone on protein expression of PPAR- α in the liver of P rats exposed to a chronic ethanol drinking protocol. One-way ANOVA revealed a significant change in PPAR- α expression ($F_{3, 28} = 4.223$, $p < 0.05$, $n = 8/$ group), Fig 6A. Newman-Keuls post-hoc analyses showed that PPAR- α expression was significantly up-regulated in the ethanol group compared to the Water control group ($p < 0.01$) and that treatment with MC-100093 or ceftriaxone normalized PPAR- α expression. However, PPAR- γ expression was not changed significantly between all treatment groups ($F_{3, 28} = 0.747$, $p > 0.05$, $n = 8/$ group), Fig 6B.

Effects of MC-100093 and ceftriaxone on GLT-1 and xCT expression in the liver

There was a significant increase in GLT-1 protein expression in the liver of the ethanol group ($F_{3, 28} = 3.042$, $p < 0.05$, $n = 8/$ group), Fig 6C. Newman-Keuls post-hoc analyses showed that GLT-1 expression was significantly up-regulated in the ethanol group compared to the Water control group ($p < 0.05$), and that treatment with MC-100093 or ceftriaxone normalized GLT-1 expression. However, one-way ANOVA revealed no significant change in xCT expression between all treatment groups ($F_{3, 28} = 2.613$, $p > 0.05$, $n = 8/$ group), Fig 6D.

Discussion

Chronic ethanol consumption was associated with dysregulation of the glutamatergic system in both the brain and liver of P rats. More specifically, the expression of GLT-1, xCT, and mGluR5

were downregulated in the NAc-shell, while the liver exhibited upregulation in GLT-1 expression. Moreover, the steatotic liver in alcohol dependent rats showed higher protein expression of PPAR- α and GLT-1. More importantly, the novel beta lactam compound, MC-100093, attenuated ethanol drinking behavior, and this effect was associated with normalized changes in several glutamatergic and PPAR protein products associated with chronic ethanol drinking. It is noteworthy that MC-100093 is orally bioavailable with essentially no antibiotic activity, and is found to be a potent GLT-1 up-regulator, which has the potential to attenuate cocaine reinstatement and reverses a neurodegenerative phenotype in an animal model of cerebral palsy (Childers et al 2020a). Our results demonstrate that MC-100093 may be a potential pharmacotherapeutic to treat AUDs with preferable characteristics (i.e. high oral bioavailability and no antibiotic actions) compared to ceftriaxone. Nevertheless, ceftriaxone was able to attenuate ethanol drinking behavior to a greater extent than MC-100093. This is probably due to the use of one low dose of MC-100093 (50 mg/kg), which represents one of the limitations in this study. Further studies are warranted to explore the effect of higher doses. MC-100093 (50 mg/kg, i.p.) attenuated ethanol intake and this effect was associated with normalized GLT-1 and xCT levels in the NAc-shell. This is in line with our previous finding that demonstrated chronic ethanol drinking induces down-regulation of glutamate transporters in the NAc-shell and treatment with GLT-1 up-regulators attenuated ethanol drinking behaviors (Alasmari et al 2020, Hasan et al 2020). Furthermore, we previously found that chronic ethanol consumption induced glucocorticoid resistance and a neuroinflammatory response in the NAc-shell but not in the NAc core (Alasmari et al 2020, Alhaddad et al 2020b), which supports the involvement neuroimmune signaling of the NAc-shell in the pathology of AUDs, as well as putatively SUDs. Likewise, chronic ethanol consumption reduced the expression of mGluR5 in

the NAc-shell, and its normalization was associated with attenuation of drinking behavior and neuroimmune signaling (Alasmari et al 2020). It is noteworthy that mGluR5 signaling is strongly implicated in the development and maintenance of ethanol dependence in animal models. For instance, blockade of mGluR5's action resulted in reduction in ethanol consumption by mice (Hodge et al 2006), as well as reductions in both consumption and ethanol self-administration under a progressive ratio (PR) schedule of reinforcement by P rats (Besheer et al 2008b, Schroeder et al 2005). Moreover, activation of mGluR5 resulted in anti-inflammatory effects by inhibiting microglial activation and the induction of neuroimmune signaling (Byrnes et al 2009, Loane et al 2009). In this study, we further confirmed the role of mGluR5 in ethanol drinking behavior by demonstrating that chronic ethanol consumption induced downregulation of mGluR5 expression in the NAc-shell. Additionally, MC-100093 or ceftriaxone treatment was associated with normalizing levels of mGluR5 expression in alcohol dependent animals, which may contribute to previously observed attenuation of ethanol drinking behavior by Type I mGluR antagonists. Together, the previous studies along with the current findings provide strong support that the NAc-shell mediates continuous ethanol consumption and/or self-administration. In this regard, chronic ethanol exposure induced dysregulation of glutamate receptors and/or transporters in the NAc-shell provides a potential pharmacotherapeutic target to treat AUDs, and possibly SUDs.

The role of PPARs in ethanol dependence is well documented. Several studies have shown that PPAR agonists reduce ethanol drinking behavior in animal models. For instance, Gemfibrozil, a PPAR- α agonist, reduced ethanol drinking behavior in outbred rats (Barson et al 2009); pioglitazone and rosiglitazone reduced ethanol drinking and stress-induced relapse in selected

Mardinian sP rats (Stopponi et al 2013, Stopponi et al 2011). The mechanism underlying PPAR-associated reduction in ethanol consumption is possibly mediated through modulation of the neuroimmune system centrally (Blednov et al 2017). However, PPAR- α activation in the liver also stimulates hepatic catalase and hydrogen peroxide that leads to ethanol aversion via conversion of ethanol into acetaldehyde, which represents another possible mechanism for its reduction of drinking behavior (Karahanian et al 2015). To assess this hypothesis, we used our chronic ethanol drinking model and measured the expression of PPARs in the liver and brain. The expression of PPAR- α in the NAc-core and NAc-shell were not significantly changed. The present experimental conditions prevented the detection of PPAR- γ in the brain, probably due to lower expression levels compared to other organs (Cullingford et al 1998, Moreno et al 2004). In the liver, although the PPAR- γ expression was not changed, we found that chronic ethanol consumption significantly increased PPAR- α level. In line with findings from a previous study (Fischer et al 2003), we found that our ethanol drinking paradigm was associated with the development of fatty liver. Previously, exposure to ethanol for four weeks did not alter the expression of PPAR- α but was associated with reduced PPAR/RXR binding to its consensus sequence in mice (Fischer et al 2003). Our results suggest that increased PPAR- α expression in P rats exposed to ethanol for six weeks increased liver fat contents. Of note, it has been suggested that PPAR- α agonists reduced ethanol consumption through activation of PPAR- α in the liver (Karahanian et al 2015). Interestingly, our results showed that the expression of GLT-1 was up-regulated in the liver, with no change in xCT levels. Previous work indicated that PPAR- γ activation increased the expression of GLT-1 at the transcriptional level (Romera et al 2007). However, little is known about the involvement of PPAR- α in GLT-1 function. Nevertheless, it has been shown that PPAR- α signaling promotes GLT-1 endocytosis in astrocytes (Huang et al

2017). The present findings provide further support for a connection between PPAR- α and GLT-1 activity. Further, we showed that treatment with MC-100093 or ceftriaxone alleviated liver steatosis and normalized PPAR- α as well as GLT-1 levels in the liver. However, whether these effects are directly related to MC-100093 and ceftriaxone action on the liver is not investigated in the present study. Further studies are warranted to explore the effect of MC-100093 treatment on PPAR- α and GLT-1 levels in the liver as well as other glutamate receptors (e.g. NMDA receptors) in the brain. Thus, this study revealed the efficacy of both MC-100093 and ceftriaxone in mitigating chronic ethanol intake and its consequences in both the brain and liver.

In summary, we demonstrated here that chronic ethanol drinking induced dysregulation of the glutamatergic system in both the brain and liver. In addition to liver steatosis, chronic ethanol consumption was associated with increased PPAR- α expression in the liver. Treatment with MC-100093 or ceftriaxone attenuated ethanol drinking behavior, which was associated with normalization of glutamatergic-associated proteins in both the brain and liver as well as attenuating fatty deposition in the liver. This study presents a new potential candidate, MC-100093, pharmacotherapeutic for the treatment of ethanol dependence and liver injury caused by chronic ethanol consumption.

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Authorship Contributions

Participated in research design: H.A. and Y.S.

Conducted experiments: H.A. and W.W.

Contributed new reagents or analytic tools: M. A.G., W.C., R.B., E.M, and Y.S.

Performed data analysis: H.A. and W.W.

Contributed to the writing of the manuscript: All authors

All authors revised the manuscript and approved the final version.

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Conflict of interest

The authors declare no conflict of interest.

Legends to Figures

Figure 1. Chemical structure of GLT-1 uptake modulators: MS-153, Ceftriaxone and MC-100093. MC-100093 synthesized by structural modification of the cephalosporin ring system and side chains of ceftriaxone.

Figure 2. Effects of five consecutive days of MC-100093 (50 mg/kg, i.p.) or ceftriaxone (200 mg/kg, i.p.) treatment on (A) Ethanol consumption (g/kg of average body weight/24hr), (B) Water intake (mL/day), and (C) Body weight (grams). Statistical analyses revealed that treatment with MC-100093 or ceftriaxone significantly reduced ethanol consumption from D2 through Day 5, with a concomitant significant increase in water consumption from Day 2 through Day 5 by the Ethanol-ceftriaxone group and on Day 5 by the Ethanol-MC-100093 group as compared to the Ethanol-Saline group. However, ethanol consumption was significantly lower

and water consumption was significantly higher in the Ethanol-ceftriaxone group as compared to the Ethanol-MC-100093 group on Day 2 through Day 5. There were no significant effects of MC-100093 or ceftriaxone treatment on body weight. The values are expressed as mean \pm SEM ($n = 8$ /group for Ethanol-Saline group and $n = 9$ /group for Ethanol-MC and Ethanol-ceftriaxone groups), (* $p < 0.05$ and ** $p < 0.01$, and # $p < 0.0001$).

Figure 3. Effects of five consecutive days of MC-100093 or ceftriaxone treatment on: (A) GLT-1 expression in the NAc-core. Upper panel: Representative immunoblot of GLT-1 and β -tubulin in the NAc-core. Lower panel: Quantitative analysis revealed a non-significant difference in GLT-1 expression among Water Control, Ethanol, Ethanol-MC-100093, and Ethanol-CEF groups in the NAc-core. (B) GLT-1 expression in the NAc-shell. Upper panel: Representative immunoblot of GLT-1 and β -tubulin in the NAc-shell. Lower panel: Quantitative analysis revealed a significant downregulation of GLT-1 expression in Ethanol group compared to Water control group. However, Ethanol-MC-100093, and Ethanol-CEF groups showed significantly higher levels of GLT-1 expression compared to the ethanol group, with no significant change between the Water control, Ethanol-MC-100093, and Ethanol-CEF groups, in the NAc-shell. (C) xCT expression in NAc-core. Upper panel: Representative immunoblot of xCT and β -tubulin in the NAc-core. Lower panel: Quantitative analysis revealed a non-significant difference in xCT expression among the Water Control, Ethanol, Ethanol-MC-100093, and Ethanol-CEF groups in NAc-core. (D) xCT expression in the NAc-shell. Upper panel: Representative immunoblot of xCT and β -tubulin in the NAc-shell. Lower panel: Quantitative analysis revealed a significant down-regulation of xCT expression in the Ethanol group compared to the Water control group. However, Ethanol-MC-100093, and Ethanol-CEF groups showed significantly

higher level of xCT compared to the ethanol group, with no significant change between the Water control, Ethanol-MC-100093, and Ethanol-CEF groups, in the NAc-shell. Control group data were represented as 100% (i.e. relative to water-control). The values are expressed as mean \pm SEM (n = 8/group), (* p < 0.05 and ** p < 0.01).

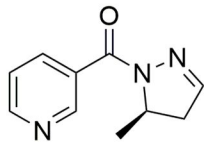
Figure 4. Effects of five consecutive days of MC-100093 or ceftriaxone treatment on: (A) Protein expression of mGluR1 in the NAc-core. Upper panel: Representative immunoblot of mGluR1 and β -tubulin in the NAc-core. Lower panel: Quantitative analysis revealed a non-significant difference in mGluR1 expression among Water Control, Ethanol, Ethanol-MC-100093, and Ethanol-CEF groups in the NAc-core. (B) mGluR1 expression in the NAc-shell. Upper panel: Representative immunoblot of mGluR1 and β -tubulin in the NAc-shell. Lower panel: Quantitative analysis revealed non-significant differences in mGluR1 expression among the Water Control, Ethanol, Ethanol-MC-100093, and Ethanol-CEF groups in NAc-shell. (C) mGluR5 expression in the NAc-core. Upper panel: Representative immunoblot of mGluR5 and β -tubulin in the NAc-core. Lower panel: Quantitative analysis revealed a non-significant difference in mGluR5 expression among the Water Control, Ethanol, Ethanol-MC-100093, and Ethanol-CEF groups in the NAc-core. (D) mGluR5 expression in NAc-shell. Upper panel: Representative immunoblot of mGluR5 and β -tubulin in the NAc-shell. Lower panel: Quantitative analysis revealed a significant down-regulation in mGluR5 expression in the Ethanol group compared to the Water Control group in the NAc-shell. However, there was no significant change in mGluR5 expression in the Ethanol-MC-100093, and Ethanol-CEF groups compared to the Water control group. (E) PPAR- α expression in the NAc-core. Upper panel: Representative immunoblot of PPAR- α and β -tubulin in the NAc-core. Lower panel:

Quantitative analysis revealed non-significant differences in PPAR- α expression among the Water Control, Ethanol, Ethanol-MC-100093, and Ethanol-CEF groups in the NAc-core. (F) PPAR- α expression in the NAc-shell. Upper panel: Representative immunoblot of PPAR- α and β -tubulin in the NAc-shell. Lower panel: Quantitative analysis revealed non-significant differences in PPAR- α expression among the Water Control, Ethanol, Ethanol-MC-100093, and Ethanol-CEF groups in the NAc-shell. Control group data were represented as 100% (i.e. relative to water-control). The values are expressed as mean \pm SEM (n = 7-8/group), (* p < 0.05 and ** p < 0.01).

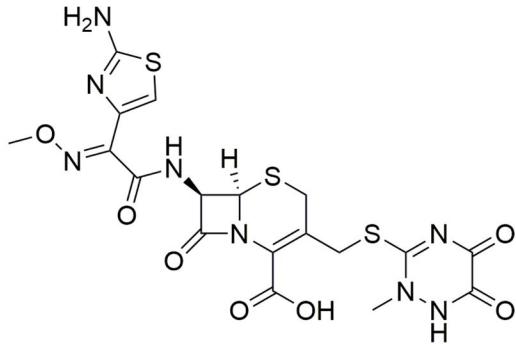
Figure 5. Fat droplets in Oil Red O-stained liver sections. (A) Quantitative analysis revealed a significant increase in liver fat content (high fat deposition) in the ethanol group compared to the Water control group. There was no significant difference in fat content between the Water control, ethanol-MC-100093, and ethanol-CEF groups. (B) Representative Oil Red O-stained liver sections of the Water control, ethanol, ethanol-MC and ethanol-CEF groups. Higher fat content was observed in liver sections of the ethanol group as compared to the Water control, ethanol-MC-100093, or ethanol-CEF groups. The values are expressed as mean \pm SEM (n = 4-5/group), (* p < 0.05).

Figure 6. Effects of five consecutive days of MC-100093 or ceftriaxone on: (A) PPAR- α expression in the liver. Upper panel: Representative immunoblot of PPAR- α and GAPDH in the liver. Lower panel: Quantitative analysis revealed a significant increase in PPAR- α expression in the ethanol group compared to control the Water control group in the liver. However, there

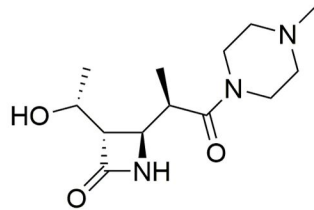
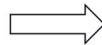
was no significant change in PPAR- α expression in the Ethanol-MC-100093, and Ethanol-CEF groups compared to the Water control group. (B) PPAR- γ expression in the liver. Upper panel: Representative immunoblot of PPAR- γ and GAPDH in the liver. Lower panel: Quantitative analysis revealed no significant difference in PPAR- γ expression among all groups. (C) GLT-1 expression in the liver. Upper panel: Representative immunoblot of GLT-1 and GAPDH in the liver. Lower panel: Quantitative analysis revealed a significant increase in GLT-1 expression in the ethanol group compared to the Water control group in the liver. However, there was no significant change in GLT-1 expression in the Ethanol-MC-100093, and Ethanol-CEF groups compared to the Water control group. (D) xCT expression in the liver. Upper panel: Representative immunoblot of xCT and GAPDH in the liver. Lower panel: Quantitative analysis revealed no significant difference in xCT expression among all groups. Control group data were represented as 100% (i.e. relative to water-control). The values are expressed as mean \pm SEM (n = 8/group), (* p < 0.05 and ** p < 0.01).



MS-153



Ceftriaxone



MC-100093

Figure 1

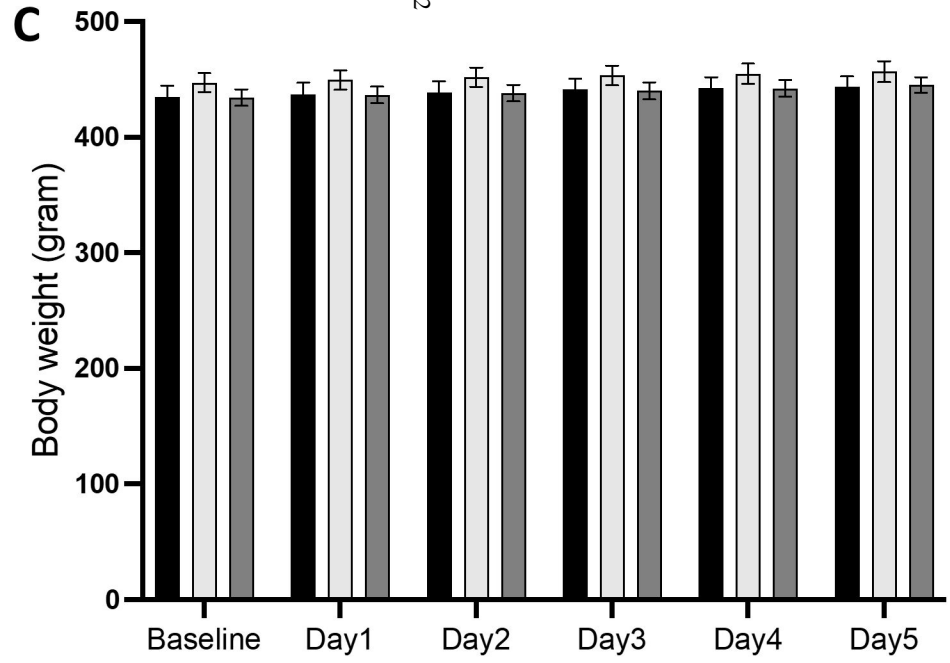
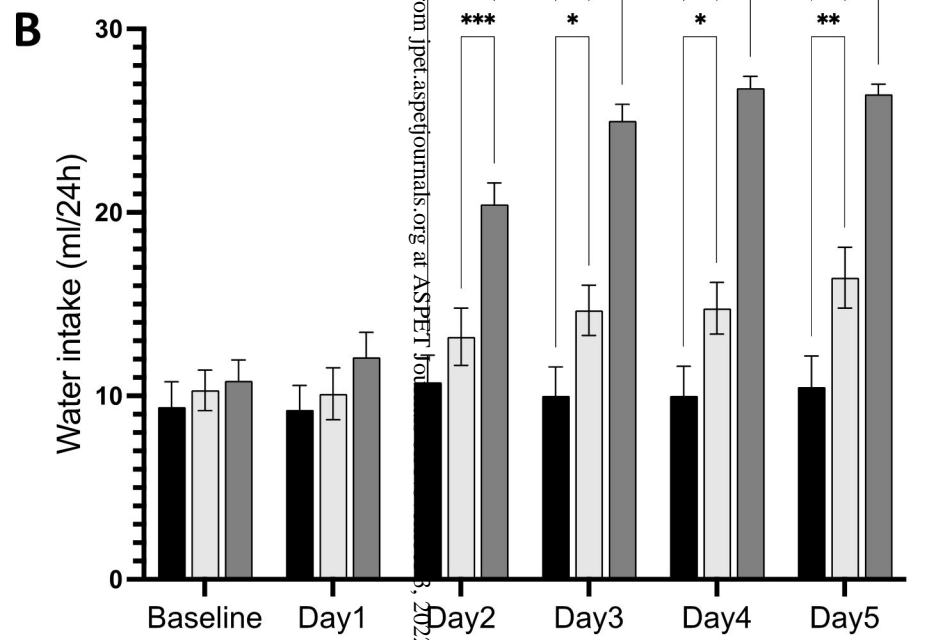
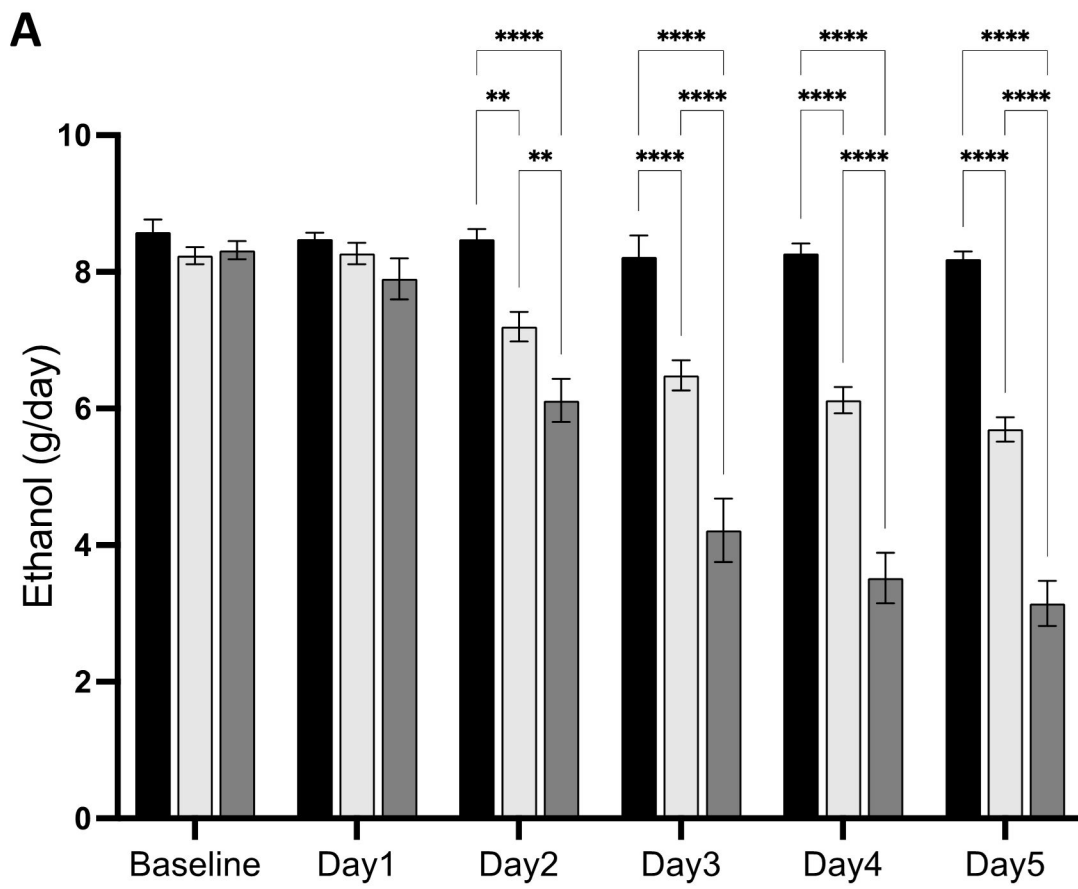
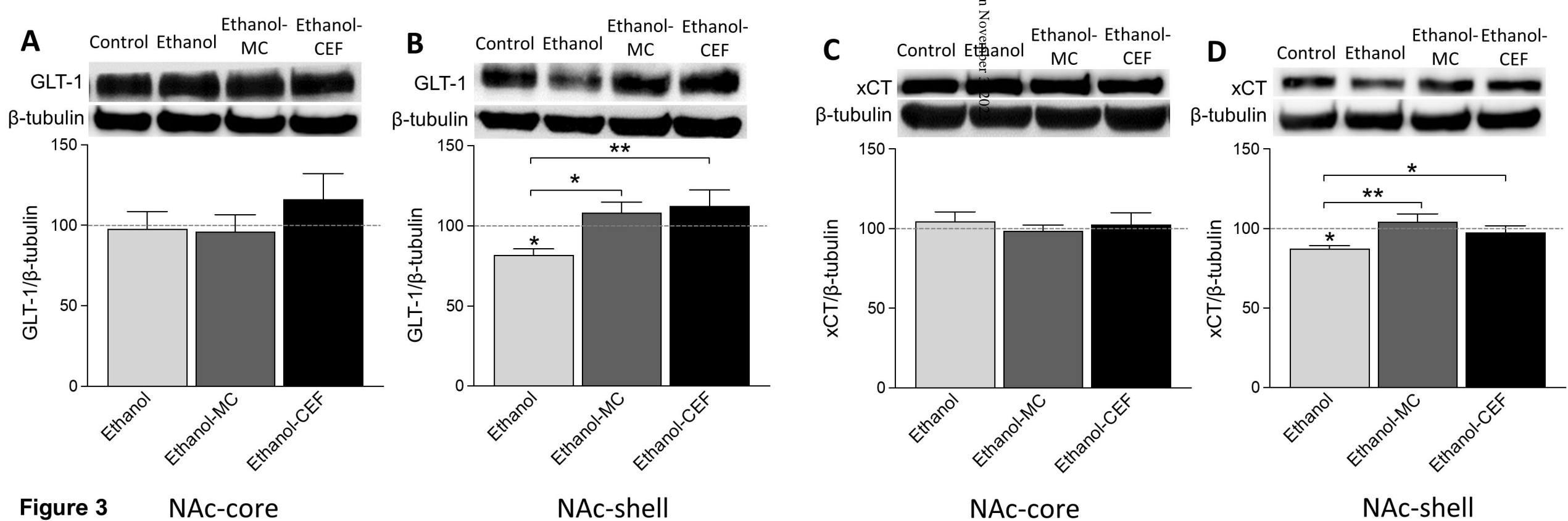


Figure 2

- Saline
- MC-100093 50mg/kg
- Ceftriaxone 100 mg/kg

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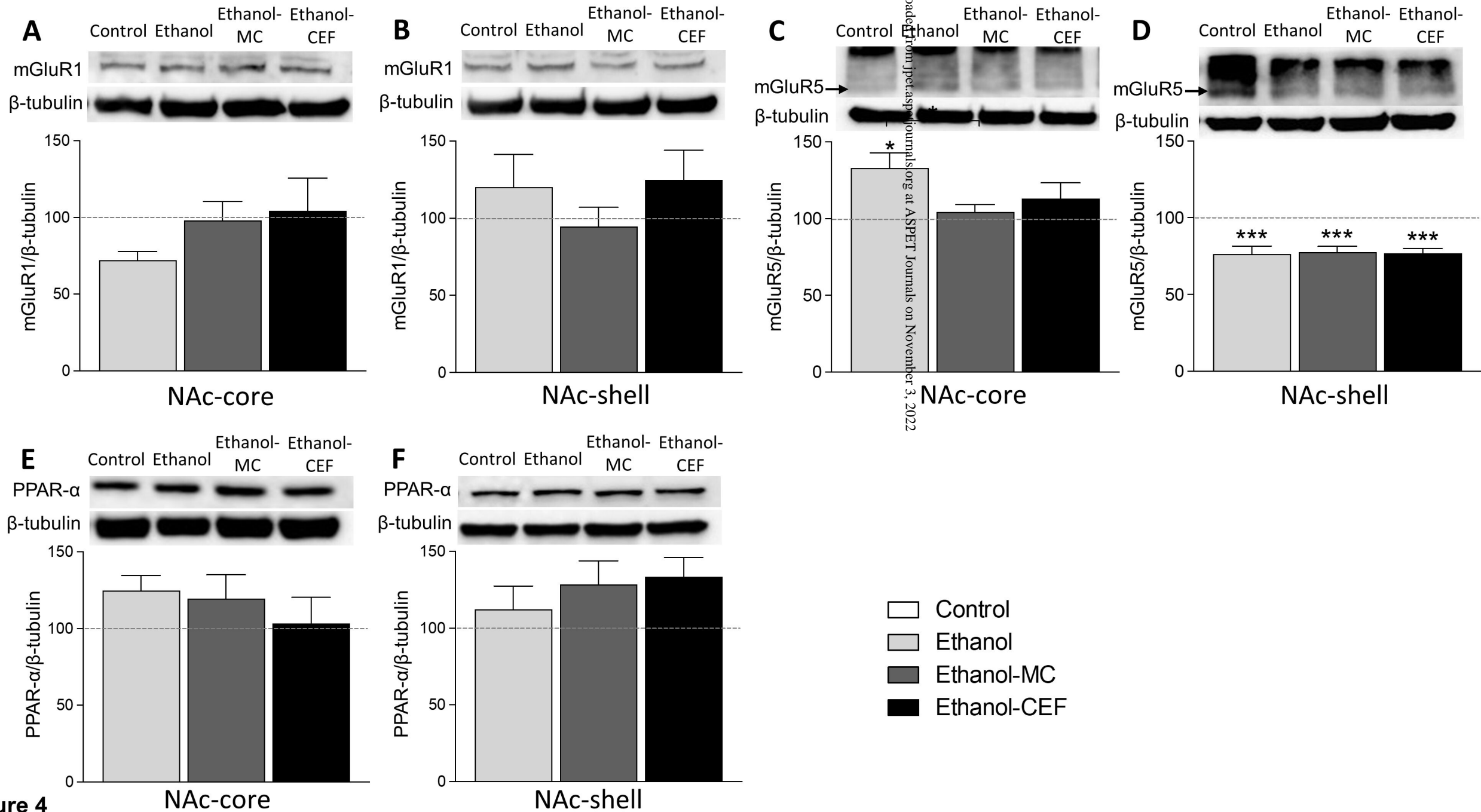
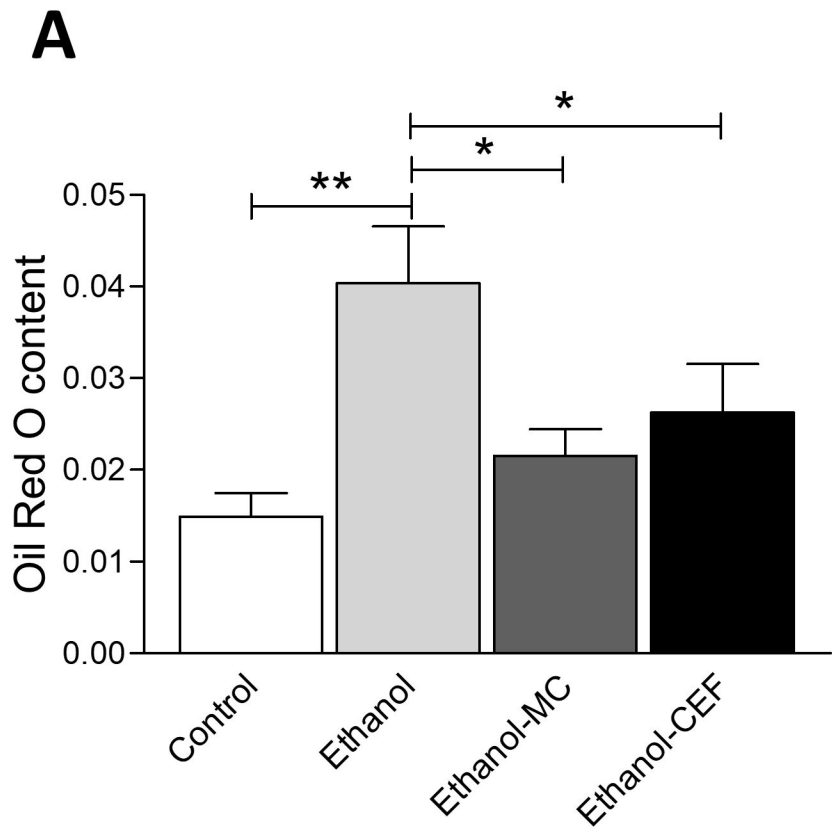


Figure 4



B

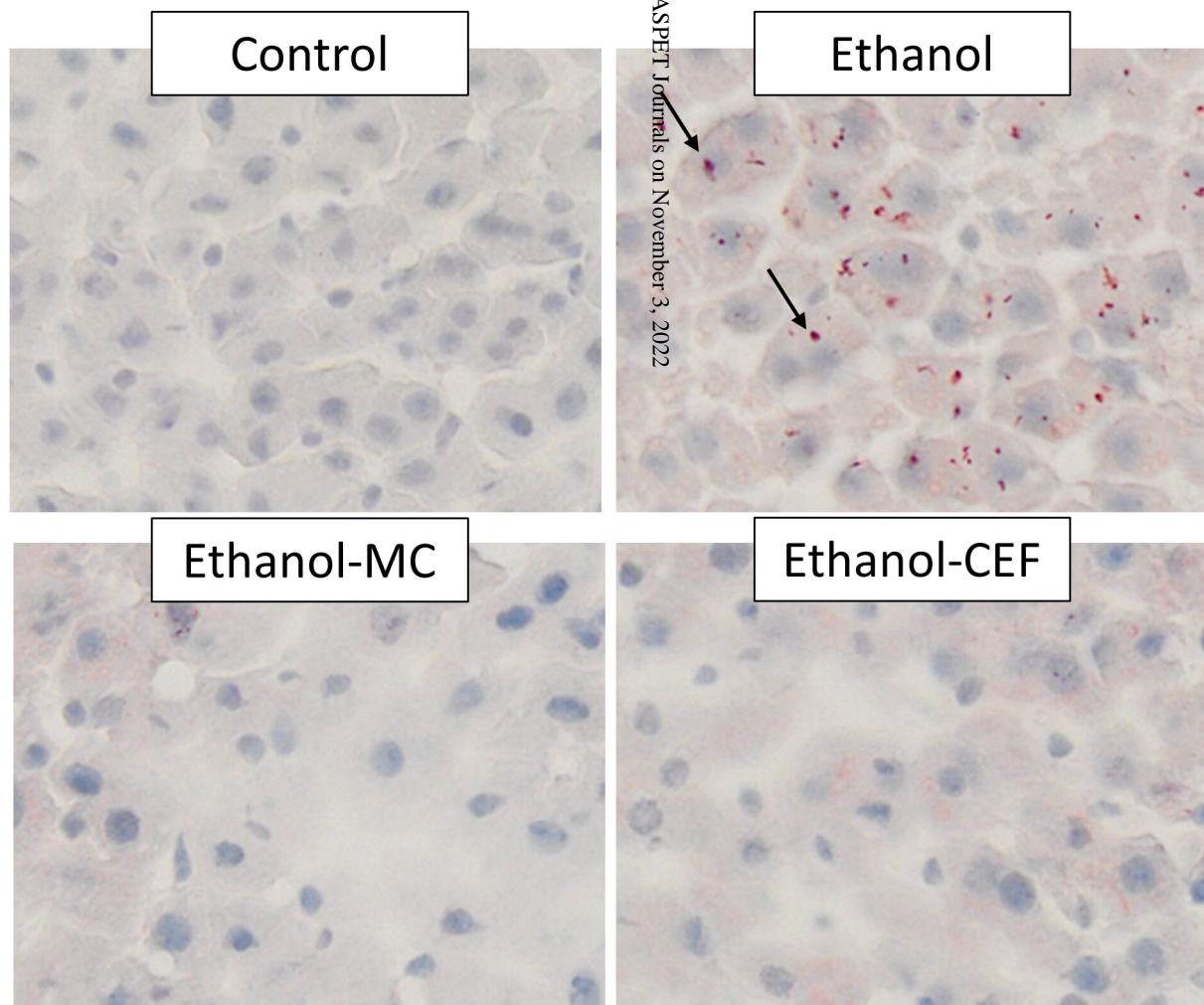


Figure 5

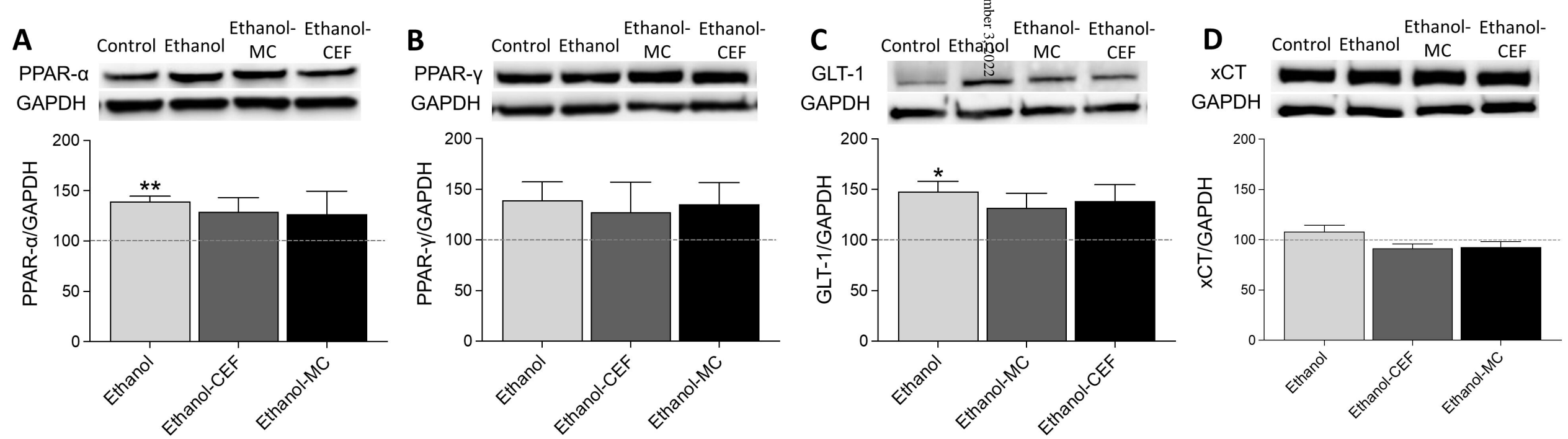


Figure 6