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Oleoylethanolamide prevents neuroimmune HMGB1/TLR4/NF-kB danger signaling in rat frontal cortex and depressive-like behavior induced by ethanol binge administration

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ABSTRACT

Alcohol abuse is frequently characterized by a specific pattern of intake in binge drinking episodes, inducing neuroinflammation and brain damage. Here, we characterized the temporal profile of neuroinflammation in rats exposed to intragastric binge ethanol administrations (3 times/day × 4 days) and tested the anti-inflammatory/neuroprotective properties of the satiety factor oleoylethanolamide (OEA). Pre-treatment with OEA (5 mg/kg, i.p.) previous each alcohol gavage blocked the expression of high mobility group box 1 (HMGB1) danger signal and the innate immunity Toll-like receptors 4 (TLR4) in frontal cortex, and inhibited the nuclear factor-kappa B (NF-kB) proinflammatory cascade induced by alcohol binge administration. OEA reduced the levels of interleukin-1beta (IL-1β), the monocyte chemoattractant protein-1 (MCP-1), and the enzymes cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in ethanol binged animals. Elevations in plasma tumor necrosis factor alpha (TNF-α) and IL-1β after ethanol were also inhibited by OEA. OEA also prevented ethanol-induced lipid peroxidation, caspase-8 and pro-apoptotic caspase-3 activation in frontal cortex. Additionally, OEA blocked the rise in blood corticosterone levels after ethanol with no alteration in blood ethanol levels and may affect ethanol-induced gut permeability for endotoxin. Finally, OEA, administered as a pre-treatment during the ethanol binge, exerted antidepressant-like effects during acute withdrawal. Altogether, results highlight a beneficial profile of OEA as a potent anti-inflammatory, antioxidant, neuroprotective and antidepressant-like compound to treat alcohol abuse.

Keywords Alcohol binge, HMGB1, neuroinflammation, neuroprotection, oleoylethanolamide, TLR4.

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INTRODUCTION

Binge heavy alcohol drinking is a pattern of alcohol consumption common among some alcoholics. Alcohol-induced brain damage and neurodegeneration have been proposed to occur as a result of binge drinking episodes (Hunt 1993; Agartz *et al.* 1999; Obernier, Bouldin & Crews 2002a), and compelling evidence indicates that alcohol binge-induced neuroinflammation may contribute to the neurotoxic effects of the drug (Hamelink *et al.* 2005; Crews *et al.* 2006; Tajuddin *et al.* 2014).

A 4-day binge ethanol model (12 intragastric doses), the so-called Majchrowicz binge intoxication model (Majchrowicz 1975), and slight modifications of it, have been used extensively to describe the neuroinflammatory and neurodegenerative properties of alcohol binge drinking. For example, ethanol binge causes microglia activation, increases the nuclear factor-*kappa* B (NF-κB)-DNA

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binding activity, upregulates the expression of cyclooxygenase-2 (COX-2) (Knapp & Crews 1999; Obernier *et al.* 2002a; Crews *et al.* 2006) and induces brain injury and neurodegeneration in cerebral cortex and hippocampus (Crews *et al.* 2000; Hamelink *et al.* 2005; Tajuddin *et al.* 2014) associated with cognitive deficits (Obernier *et al.* 2002b). Additionally, the above cited binge ethanol intoxicating model induces sustained high blood ethanol levels (BEL) similar to those documented in chronic alcoholics (Adachi *et al.* 1991; Snyder *et al.* 1992).

Chronic administrations of ethanol enhance NF-κB-related inflammatory mediators and activate Toll-like receptors (TLR)4 and TLR3 signaling pathways, inducing apoptosis, brain injury and neurodegeneration (Valles *et al.* 2004; Alfonso-Loeches *et al.* 2010; Qin & Crews 2012). The neuroimmune activation induced by chronic alcohol may involve the danger signaling molecule high mobility group box 1 (HMGB1), which binds Toll-like receptors 4 (TLR4) (Crews *et al.* 2013; Zou & Crews 2014). HMGB1 expression was increased in postmortem alcoholic orbitofrontal cortex and its levels correlated with lifetime alcohol consumption (Crews *et al.* 2013). Also in humans, increases in peripheral proinflammatoy cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β), activation of microglia and enhanced brain expression of the chemoattractant protein 1 (MCP-1) have been documented in alcohol-dependent subjects (McClain & Cohen 1989; McClain *et al.* 2004; He & Crews 2008). Inflammation was also correlated to depressive symptoms and alcohol craving (Leclercq *et al.* 2012).

The endocannabinoid system has been studied for years for its anti-inflammatory and homeostatic properties. A structural analog of the endocannabinoid anandamide belonging to the *N*-acylethanolamine family, the lipid mediator oleoylethanolamide (OEA), has emerged as an interesting bioactive molecule with anti-inflammatory and neuroprotectant actions in the brain. OEA was first discovered as a satiety factor with no activity at traditional cannabinoid receptors (Rodriguez de Fonseca *et al.* 2001; Fu *et al.* 2003), and growing evidence indicates that it mediates a variety of different actions through activation of peroxisome proliferator-activated receptor-alpha (PPAR-α). Current studies in animal models indicate that OEA may have putative neuroprotective properties against CNS disorders such as stroke (Sun *et al.* 2007), Parkinson's disease (Gonzalez-Aparicio *et al.* 2014), depression (Jin *et al.* 2015) or addiction (Melis *et al.* 2008; Bilbao *et al.* 2013, 2015).

Recently, we have documented potent anti-inflammatory and anti-anhedonic actions of OEA in a LPS model of neuroinflammation (Sayd *et al.* 2014). Interestingly, a main role for OEA in alcoholism has been also reported: OEA regulates alcohol self-administration and relapse, reduces the withdrawal symptoms of alcohol, and it is released in blood and different tissues following exposure to alcohol, presumably as a homeostatic protective mechanism (Bilbao *et al.* 2015). To further investigate the role of OEA in alcohol pathology we tested here the effects of OEA in the neuroimmune HMGB1/TLR4/NF-kB pro-inflammatory and danger signaling pathway in an animal model of alcohol binge drinking and tested its effects modulating depressive-like symptoms and anxiety associated with ethanol withdrawal.

MATERIALS AND METHODS

Animals

Eighty-seven male Wistar rats (from Harlan, Spain) weighing 250–300 g were used. Animals were housed in groups ($n = 4$–6) on a reverse 12-h light/dark cycle under standard conditions of temperature and humidity. Standard food (A04 SAFE, Scientific Animal Food and Engineering, Augy, France) and tap water were available *ad libitum* in the home cage. All animals were maintained under constant conditions for 10 days before experiments.

All experimental protocols were adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense of Madrid following European legislation (2010/63/UE).

Binge ethanol treatment

Animals were treated with intragastric (i.g.) ethanol 3 times per day using i.g. cannulae (16-G needle, Fisher Scientific, Waltham, MA, USA), following a slight modification of a standard paradigm of 4-day binge alcohol intoxication protocol (Obernier *et al.* 2002a,2002b) (Fig. 1a). Ethanol doses were administered every 8 h during a total time of 4 days. Ethanol-treated rats received an initial *loading* dose of 5 g/kg of ethanol in a solution of 30 percent (w/v) and then a maximum of 3 g/kg for additional doses, which were determined in base of the animals' BEL. This repeated binge-pattern ethanol paradigm maintained relatively constant intoxicating BEL in a range of sedation/ataxia (19\0–430 mg/dl, Table 1), according to the six-point behavioral ethanol intoxication scale (Majchrowicz 1975). The average dose of ethanol/rat/day (days 1–4) was 7.06 g/kg, similar to other studies (Obernier *et al.* 2002a,2002b). See Supporting Information for more details.

Blood ethanol level determination

To verify intoxication, BEL were ascertained in blood samples taken from tail 120 min before and after the 15:00 h ethanol i.g. administration by using electrochemical

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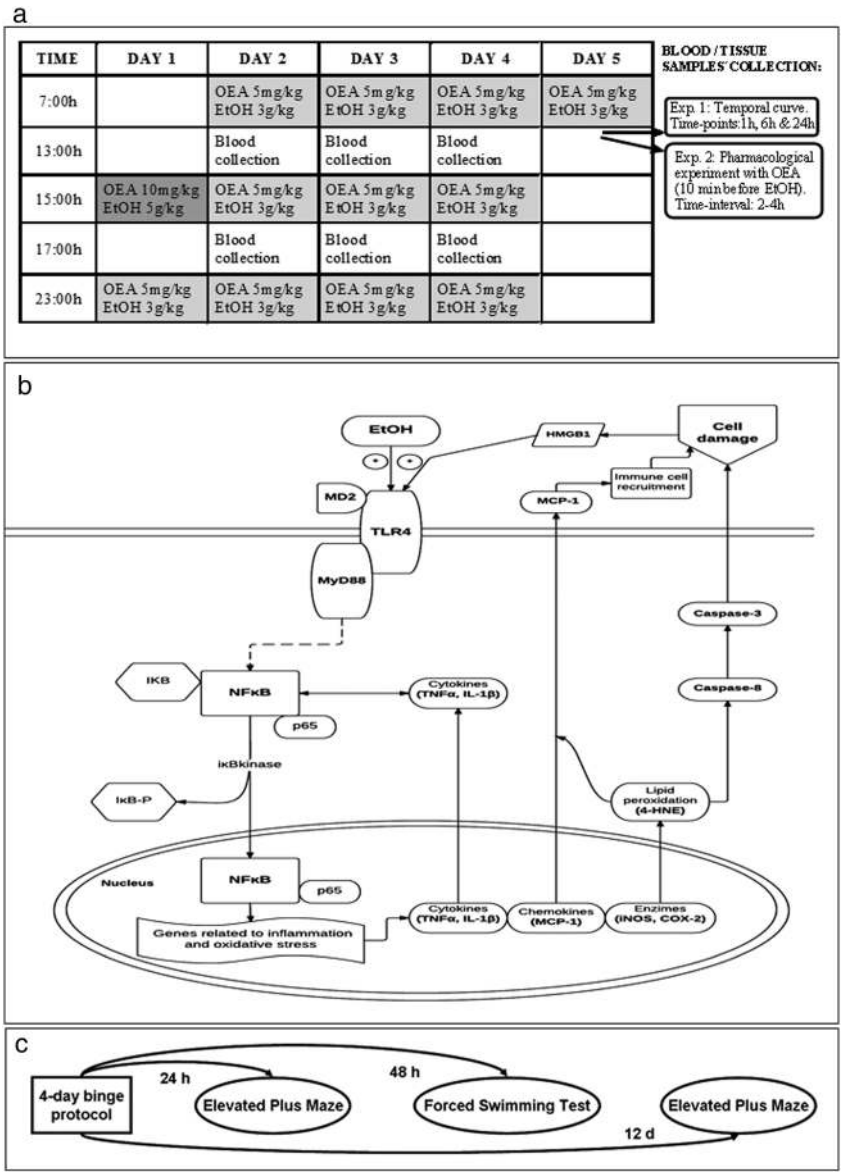


Figure 1 Experimental design of the study. a) Binge alcohol drinking experimental protocol. Rats received an initial loading dose of 5 g/kg of 30 percent ethanol (*w/v*) via oral gavage and a maximum of 3 g/kg of ethanol in each subsequent dose every 8 h. Blood was collected from the tail vein 2 h before and 2 h after of the 15:00 h ethanol gavage administration on days 2 to 4 in order to determine blood ethanol levels (BEL), and the ethanol doses were titrated to maintain relatively constant intoxicating BEL (see Table 1). The average doses of ethanol/rat were 8 g/kg, 7.5 g/kg, 7.9 g/kg and 4.9 g/kg, for days 1 to 4, respectively, and the average dose of ethanol/rat/day (1–4) was 7.06 g/kg. In a first experiment we checked the time-course of neuroinflammation 1 h, 6 h and 24 h after last ethanol gavage. In the second experiment, OEA (5 mg/kg, i.p., loading dose 10 mg/kg) was injected as pre-treatment 10 min before each ethanol gavage, and blood/brain tissue samples were collected 2–4 h after last ethanol administration. b) Proposed HMGB1-mediated neuroimmune TLR4/MyD88/NF-kB signaling pathway activated by alcohol, leading to oxidative stress, caspase-3 activation and cell damage. HMGB1 is a danger cytokine recruited by alcohol that activates immune MyD88-dependent TLR4 signaling pathway, which induces the NF-kB p65 subunit translocation to the nucleus and increase in its transcriptional activity. The NF-kB-mediated pro-inflammatory cascade involves the release of cytokines, such as TNF-α and IL-1β, that induce more NF-kB activation and further neuroinflammation, and the chemokine MCP-1 (that is also induced by impaired oxidative stress), which mediates microglia recruitment and exacerbates neurodegeneration. NF-kB transcriptional activity induces the expression of other pro-inflammatory markers, such as COX-2 and iNOS, leading to oxidative and nitrosative stress. Lipid peroxidation, measured by 4-HNE accumulation, induces a cellular redox status related with caspase-8-mediated activation of caspase-3 and apoptotic cell death. c) Experimental design for behavioral studies during acute ethanol withdrawal. Animals were tested in The Elevated Plus Maze 24 h and 12 days after the alcohol binge protocol, and the Forced Swimming test was performed 48 h after the last ethanol administration. [HMGB1: High mobility group box 1; TLR4: Toll-like receptors 4; MD2: myeloid differentiation protein-2; MyD88: myeloid differentiation factor 88; NF-kB: nuclear factor-kappa B transcription factor (subunit p65); IkB: IkappaB inhibitory protein; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin-1beta; MCP-1: monocyte chemoattractant protein-1; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; 4-HNE: 4-hydroxynonenal]

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Table 1 Blood ethanol levels.

Treatment	*Time of blood collection*	*Day 2*	*Day 3*	*Day 4*	
		BEL (g/dl)	*BEL (g/dl)*	*BEL (g/dl)*	
Vehicle + EtOH	13:00	193.29 ± 38.22	341.73 ± 32.48**	303.32 ± 37.17*	$N = 10$
	17:00	274.96 ± 27.17	422.35 ± 18.00***	430.31 ± 19.72***	$N = 10$
OEA + EtOH	13:00	217.14 ± 41.58	342.57 ± 40.25*	320.68 ± 36.63	$N = 9$
	17:00	321.88 ± 34.08	404.17 ± 27.65	429.76 ± 29.25*	$N = 9$

Data represent BEL 2 h before and 2 h after the 15:00 h alcohol administration in vehicle or OEA pre-treated animals. There were differences in the BEL between the day 2 and days 3 or 4 of treatment in both groups. Pretreatment with OEA did not modify the BEL achieved during days 2–4 of alcohol binge treatment neither before nor after ethanol gavage. Results are expressed as means ± S.E.M. Different from day 2 in the same treatment group: *$p < 0.05$. **$p < 0.001$. BEL: blood ethanol levels; EtOH: ethanol; OEA: oleoylethanolamide.

detection of an enzymatic reaction with an AM1 Alcohol Analyzer (Analox Instruments, London, UK).

Experimental design, drug administration and tissue/plasma collection

Time-course of the main neuroinflammatory changes induced by the ethanol binge protocol (Fig. 1a)

Animals ($n = 22$) subjected to the 4-day ethanol binge treatment by gavage (see Fig. 1a) were sacrificed 1 h, 6 h and 24 h after the last ethanol administration by using a lethal dose of sodium pentobarbital (300 mg/kg, i.p., Dolethal®, Spain). Brains were isolated from the skull, and meninges and blood vessels were carefully discarded. The frontal cortex was excised and frozen at −80 °C until assayed. Blood was collected by cardiac puncture using trisodium citrate (3.15 percent *w*/*v*) as anticoagulant. Plasma was obtained by blood centrifugation (2000 g) 15 min at 4 °C and stored at −20 °C until determinations.

This first experiment allowed us to observe the timing of expression of the main inflammatory markers. According to this, we designed a second experiment in which we collected the brain tissue samples in a range between 2 and 4 h after last ethanol gavage. We chose this interval between 1 h and 6 h to detect, both, early changes in inflammatory parameters, i.e. TNF-α upregulation, as well as the activation of posterior markers, such as COX-2 expression (Fig. 2).

Effects of repeated OEA administration in the alterations in neuroinflammatory markers induced by ethanol binge exposure (Fig. 1a)

In this second experiment ($n = 40$ animals) we aimed to test the anti-inflammatory/neuroprotectant effects of OEA in the TLR4 signaling cascade (Fig. 1b) in a model of binge drinking alcohol abuse.

OEA (5 mg/kg, i.p., except first loading dose at 10 mg/kg i.p.; synthesized as described in Giuffrida *et al.* 2000) was dissolved in vehicle (5 percent Tween-80 in saline) and injected 10 min before each ethanol gavage administration. We previously reported anti-inflammatory actions of OEA at 10 mg/kg i.p. in frontal cortex prior a single injection of LPS (Sayd *et al.* 2014). In this study we injected OEA repeatedly as a pre-treatment of each ethanol gavage. The doses of OEA chosen in this study are in the range of doses proven to be pharmacologically active (5–20 mg/kg, i.p.) (Bilbao *et al.* 2015).

We also performed a time-course ($n = 18$) of OEA (3 h, 6 h and 24 h) to discard any effect in control animals (Supporting Information, page S8).

Effects of repeated OEA administration in the depressive-like behavior and anxiety during early ethanol withdrawal (Fig. 1c)

In this third experiment ($n = 25$) we repeated the binge ethanol treatment and OEA pre-treatment explained for experiment 2 and tested the animals in the Elevated Plus Maze 24 h and 12 days after the last ethanol gavage and in the Forced Swimming Test 48 h after the ethanol binge protocol.

Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic protein extracts were obtained as previously published (Sayd *et al.* 2014). See Supporting Information for details.

Western blot analysis

Brain frontal cortices were homogenized by sonication in 400 µl of PBS (pH = 7.4) mixed with a protease inhibitor cocktail (Complete, Roche®, Madrid, Spain) followed by centrifugation at 12 000 *g* for 10 min at 4 °C. Homogenates with adjusted protein levels were mixed with Laemmli sample buffer (BioRad®, CA, USA) containing β-mercaptoethanol (50 µl/ml of

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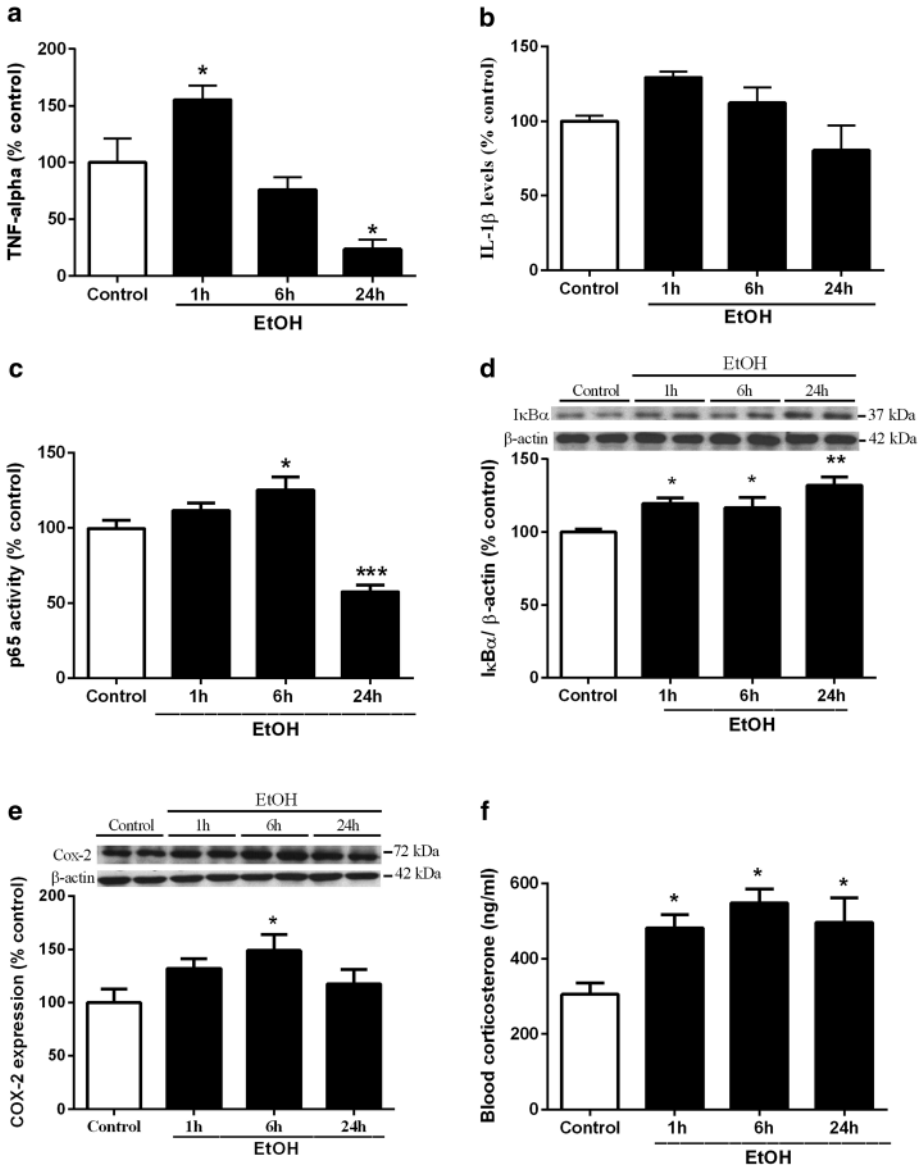


Figure 2 Time-course of ethanol binge drinking-induced rise in frontal cortex proinflammatory mediators and blood corticosterone. Parameters were measured 1 h, 6 h and 24 h after last ethanol administration. a) TNF-α levels in frontal cortex were upregulated 6 h after binge treatment and downregulated 24 h post-treatment; b) IL-1β levels after ethanol binge treatment showed a tendency to increase but were not significantly elevated; c) the activity of p65 subunit of NFkB was increased and decreased in frontal cortex nuclear extracts 6 h and 24 h after ethanol binge, respectively; d) the levels of IkBα were elevated at all times tested after ethanol binge treatment; e) the enzyme COX-2 was upregulated 6 h after ethanol exposure; f) ethanol binge treatment increased blood corticosterone levels 1 h, 6 h and 24 h after last ethanol gavage. Data represents the mean ± S.E.M. ($n = 3–6$). Different from control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Laemmli), and 1 mg/ml was loaded into an electrophoresis gel. Proteins were blotted onto a nitrocellulose membrane (Amersham Ibérica®, Spain) with a semi-dry transfer system (Bio-Rad®, CA, USA), incubated with specific primary and secondary antibodies (see Supporting Information) and revealed by ECL™-kit (Amersham Ibérica®, Spain). Autoradiographs were quantified by densitometry (NIH ImageJ® software, National Biosciences, Lincoln, Nebraska USA) and expressed as optical density (O.D.). In all Western blot analyses, the housekeeping β-actin protein was used as loading control.

Real-time polymerase chain reaction (RT-PCR) analysis

Total cytoplasmic RNA was prepared from samples of frontal cortex using TRIZOL® reagent (Invitrogen, Grand Island, NY, USA). Aliquots were converted to cDNA using random hexamer primers. Quantitative changes in mRNA levels were estimated by RT-PCR,

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carried out in the presence of SYBR green probe using a 20-l reaction in a Rotor-Gene (Corbett Research®, Mortlake, NSW, Australia). See Supporting Information for additional description and sequence details of the primers used (Table 1S).

HMGB1 levels, proinflammatory cytokines (TNF-α, IL-1β) and chemokine MCP-1 levels

Levels of soluble HGMB1, TNF-α, IL-1β and MCP-1 were determined by commercially available enzyme-linked immunosorbent assays (ELISA) kits (Elabscience Biotechnology Co., Ltd., China, for HMGB-1; RayBiotech®, GA, USA, for TNF-α, IL-1β and MCP-1). HMGB1 nuclear/cytosolic fraction was determined by western blot analyses. See Supporting Information for details.

NF-κB transcription factor assay

Nuclear extracts were used for determination of NF-κB transcription factor activity by using an ELISA-based kit (Cayman Chemicals©, Tallin, Estonia). Frontal cortex nuclear extracts were incubated with specific NF-κB p65 subunit response element probes, and p65 bounded to its response element probe was detected using a specific antibody against this subunit. Horseradish peroxidase-labeled secondary antibody was added and the binding was detected spectrophotometrically at 450 nm. Measurement was performed according to the manufacturer's instructions. This assay is specific for p65 activation, and it does not cross-react with other NF-κB subunits, such as p50. Data were normalized by the amount of total protein.

Lipid peroxidation

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as 4-hydroxynonenal (4-HNE), a natural bi-product of lipid peroxidation. The levels of HNE protein adducts in lysates from cerebral cortex were measured using a 96-well OxiSelect™ HNE Adduct Competitive ELISA Kit (Cell Biolabs®, San Diego, CA, USA) (see Supporting Information).

Measurement of caspase-3 activity

Caspase-3 activity was determined using a fluorimetric assay kit (Abnova®, Taiwan) according to the manufacturer's protocol. This commercial kit allows the measurement of the DEVD-dependent caspase activity (see Supporting Information for details).

Plasma corticosterone and LPS levels

Corticosterone and LPS were measured in plasma by using commercially available kits by radioimmunoassay (R.I.A.) (Coat-a-Count®, Siemens, LA, USA) or color-based enzymatic reaction (Hycult Biotech, Uden, The Netherlands), respectively (see Supporting Information for details).

Protein assay

Protein levels were measured using the Bradford's method based on the principle of protein-dye binding (Bradford 1976).

Behavioral tests

Rats were tested in the Forced Swimming Test 2 days after the last ethanol gavage (see Fig. 1c for experimental design), and specific behaviors were recorded with a digital camcorder for a period of 5 min. Same rats were also tested in an infrared photo beams-equipped Elevated Plus Maze (Panlab, Barcelona, Spain) 1 day and 12 days after the last ethanol gavage, allowing the animals to explore the maze freely for 5 min. The tests were performed 3 h after of the beginning of the dark phase. See Supporting Information for details of both tests.

Statistical analyses

Data in text and figures are expressed as mean ± S.E.M. Data were analyzed by two-way analysis of variance (ANOVA), comparing the factors [alcohol/water] versus [OEA/vehicle], followed by a Bonferroni *post hoc* test, and by one-way ANOVA followed by Newman–Keuls *post hoc* test when appropriate. The BEL data were analyzed by repeated-measures two-way ANOVA followed by a Bonferroni *post hoc* test (treatment × daily determination) for both the pre-gavage and post-gavage BEL determinations. Data from behavioral tests were examined for normality with the Kolmogorov–Smirnov test and then analyzed by parametric two/one-way ANOVA followed by Bonferroni/Newman–Keuls *post hoc* tests (forced swimming test) or by the non-parametric Kruskal–Wallis ANOVA followed by Dunn's *post hoc* test for multiple comparisons (elevated plus maze) when appropriate. A P value ≤ 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Time-course of alcohol binge-induced pro-inflammatory markers in frontal cortex and rise in plasma corticosterone levels

Frontal cortex TNF-α was up-regulated 1 h after ethanol administration [55 percent of increase over control

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values (80.29 ± 17 pg/mg protein)] and the cytokine content decreased 24 h after treatment (Fig. 2a; $F_{(3,11)} = 9.45$; $P = 0.0002$). IL-1β showed a tendency to be increased 1 h after ethanol exposure [29 percent of increase over control values (75.5 ± 6.6 pg/mg protein)], but data did not reach statistical significance (Fig. 2b). The expression of p65 subunit of NF-kB increased in nuclear extracts of frontal cortex 6 h after ethanol treatment, and decreased 24 h after the ethanol binge, when compared with the control group (Fig. 2c; $F_{(3,11)} = 20.48$; $P < 0.0001$). Levels of the NF-kB inhibitory protein I*kappa*B-alpha (IκBα) remained elevated 1 h, 6 h and 24 h after alcohol treatment in frontal cortex cytosolic extracts (Fig. 2d; $F_{(3,13)} = 6.73$; $P = 0.0056$) and the enzyme COX-2 showed an upregulation of its expression 6 h after the ethanol administration (Fig. 2e; $F_{(3,11)} = 3.69$; $P = 0.04$). Finally, ethanol binge exposure elevated plasma corticosterone levels 1 h, 6 h and 24 h after last administration (Fig. 2f; $F_{(3,15)} = 3.91$; $P = 0.03$).

Based on this pattern of expression of main pro-inflammatory markers between 1 h and 6 h after last ethanol gavage, we chose to extract brain/plasma samples in a time interval of 2–4 h after the binge protocol to study the pharmacological effects of OEA.

Blood ethanol levels

BEL achieved during the experiment were in the range reported by others (Knapp & Crews 1999; Obernier *et al.* 2002a,2002b; Crews *et al.* 2006). We studied whether OEA may modify BEL during days 2–4 of the binge treatment (Table 1). A repeated-measures two-way ANOVA detected differences between BEL across the days of the binge protocol ($F_{(2,\ 17)} = 10.17$; $P = 0.0003$; $F_{(2,\ 17)} = 15.41$; $P < 0.0001$) and showed that OEA pre-treatment did not alter the BEL achieved during days 2–4 of ethanol binge treatment neither in the pre-gavage ($F_{(1,\ 17)} = 0.13$; $P = 0.72$, n.s.) nor in the post-gavage ($F_{(1,\ 17)} = 0.18$; $P = 0.67$, n.s.) BEL determination. BEL were also determined immediately before tissue extraction (day 5), being 431.98 ± 34.90 g/dl for the vehicle + ethanol group and 445.82 ± 32.70 g/dl for the OEA + ethanol group (Student's *t*-test, $P = 0.78$, n.s.).

Ethanol binge alterations in HMGB1/TLR-4 signaling pathway and effect of OEA pre-treatment

In order to explore whether the ethanol-induced pro-inflammatory mediators such as cytokines or NF-κB were discharged by activation of innate immunity receptors, we checked the influence of ethanol binge in the expression of TLR4 and its endogenous activator, the danger signaling cytokine HGMB1. Additionally, we checked the TLR4 co-receptor myeloid differentiation protein-2 (MD2), and the expression of the myeloid differentiation factor 88 (MyD88), which is an adapter protein of the TLR4 intracellular signal transduction cascade.

We observed main effects of ethanol exposure and OEA pretreatment in HMGB1 expression. The increase in HMGB1 induced by ethanol binge ($F_{(1,13)} = 19.14$, $P = 0.0008$) was prevented by pre-treatment with OEA (Fig. 3a; $F_{(1,\ 13)} = 4.58$, $P = 0.05$). Analyses of the HMGB1 nuclear/cytoplasmic ratio (Fig. 3b) revealed that the total increase in HMGB1 expression (Fig. 3a) in ethanol exposed animals may be because of an increase of the cytokine in both nucleus and cytoplasm, maintaining ethanol-treated rats higher levels in the nucleus versus the cytoplasm (n/c ratio = 1.21). OEA pretreatment decreased ethanol-induced HMGB1 expression more efficiently in the cytosolic fraction (n/c ratio = 1.48) indicative of an activity at the HMGB1 release from nucleus to the cytoplasm rather than at the synthesis level.

There was a main effect of ethanol binge treatment in both TLR4 mRNA (Fig. 3c; $F_{(1,\ 17)} = 10.08$, $P = 0.005$) and protein expression (Fig. 3d; $F_{(1,\ 24)} = 10.04$, $P = 0.004$), a main effect of OEA in TLR4 mRNA ($F_{(1,\ 17)} = 6.08$, $P = 0.0246$) and an interaction between ethanol exposure and OEA ($F_{(1,\ 24)} = 9.67$, $P = 0.0048$) in TLR4 protein levels. The pre-treatment with OEA was able to reduce the ethanol-induced upregulation in TLR-4 mRNA (Fig. 3c; $F_{(3,\ 17)} = 5.86$, $P = 0.0061$) and protein expression (Fig. 3d; $F_{(3,24)} = 8.15$, $P = 0.0006$).

Ethanol exposure in a binge pattern increases the protein expression of MD2 (Fig. 3e; $F_{(1,\ 27)} = 6.83$, $P = 0.0145$), but data of mRNA expression did not reach statistical significance (data not shown; $F_{(1,\ 31)} = 3.79$, $P = 0.06$, n.s.). Main effect of OEA pretreatment ($F_{(1,\ 27)} = 4.24$, $P = 0.04$) and an interaction (ethanol exposure × pre-treatment) ($F_{(1,\ 17)} = 4.57$, $P = 0.0418$) were also found in MD2 protein levels. OEA pre-treatment inhibited the ethanol-induced increase in MD2 (Fig. 3e; $F_{(3,30)} = 5.52$, $P = 0.044$).

Ethanol binge induced a main effect in MyD88 mRNA ($F_{(1,\ 15)} = 10.34$, $P = 0.0058$; data not shown) and protein expression (Fig. 3f; $F_{(1,\ 30)} = 9.55$, $P = 0.0043$) and an interaction (ethanol exposure × pre-treatment) was also found in MyD88 protein levels ($F_{(1,\ 30)} = 4640$, $P = 0.0394$). Pre-treatment with OEA inhibited the ethanol-induced increase in MyD88 protein expression (Fig. 3f; $F_{(3,\ 32)} = 5.46$, $P = 0.042$).

Effects of OEA in frontal cortex and plasma proinflammatory cytokines TNF-α and IL-1β and in the chemokine MCP-1

Ethanol binge treatment increased the expression of both TNF-α and IL-1β mRNA levels in frontal cortex, although OEA pre-treatment did not prevent this effect (Supporting

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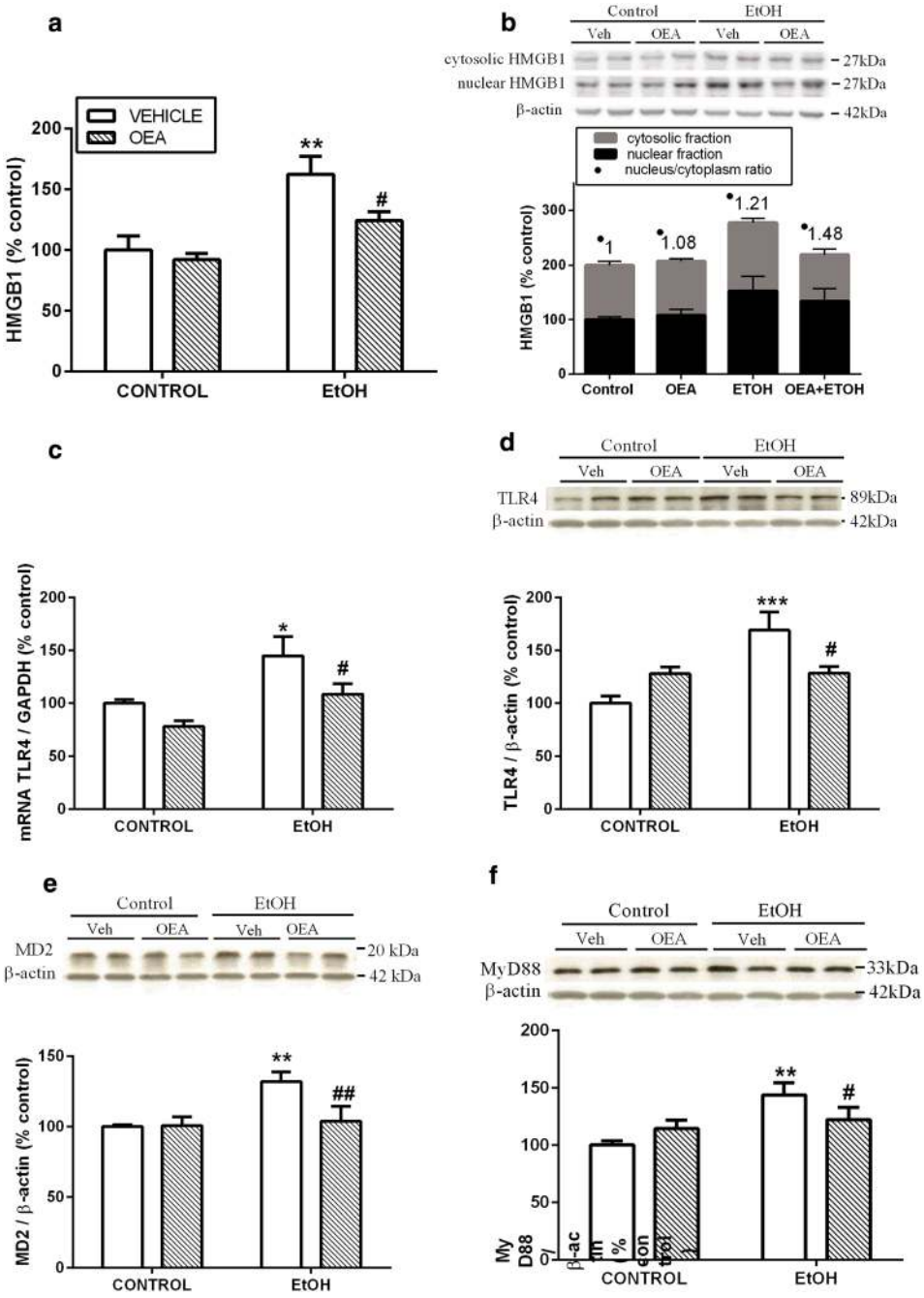


Figure 3 Effects of pretreatment with OEA in the HMGB1/TLR4 signaling pathway activated by ethanol binge exposure in frontal cortex. a) HMGB1 levels measured by enzyme-linked immunosorbent assays; b) HMGB1 nuclear/cytoplasmic ratio by western blot analyses; data are expressed as a percentage of controls in each fraction. The expression of HMGB1 in control animals was higher in nucleus than in cytoplasm (n/c ratio = 1.3); c) mRNA relative levels of TLR4; d) protein TLR4 levels; e) protein levels of MD2; f) protein levels of MyD88. Parameters measured 2–4 h after last ethanol administration. Data represents the mean ± S.E.M. (n = 4–10). mRNA levels were normalized by GAPDH and densitometric data in the western blot analyses of bands of interest were normalized by β-actin (lower band). Different from control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Different from ethanol-treated animals: #$P < 0.05$; ##$P < 0.01$

Information, Fig. 1S). TNF-α protein levels were increased in frontal cortex of rats under the binge ethanol treatment ($F_{(1, 16)} = 2.56$, $P = 0.1291$) [24 percent of increase over control values (95.2 ± 3.1 pg/mg protein)], and were not prevented by OEA (Fig. 4a; $F_{(3,16)} = 0.02$, $P = 0.89$, n.s.). In plasma, we observed a robust increase in TNF-α levels in ethanol-treated animals ($F_{(1,14)} = 44.64$, $P < 0.0001$) [141 percent of increase over control values (113.0 ± 5.1 pg/mg protein)] and an interaction between ethanol and OEA pre-treatment ($F_{(1,14)} = 4.85$, $P = 0.045$). One-way ANOVA indicated that OEA pre-treatment partially prevented the increase in

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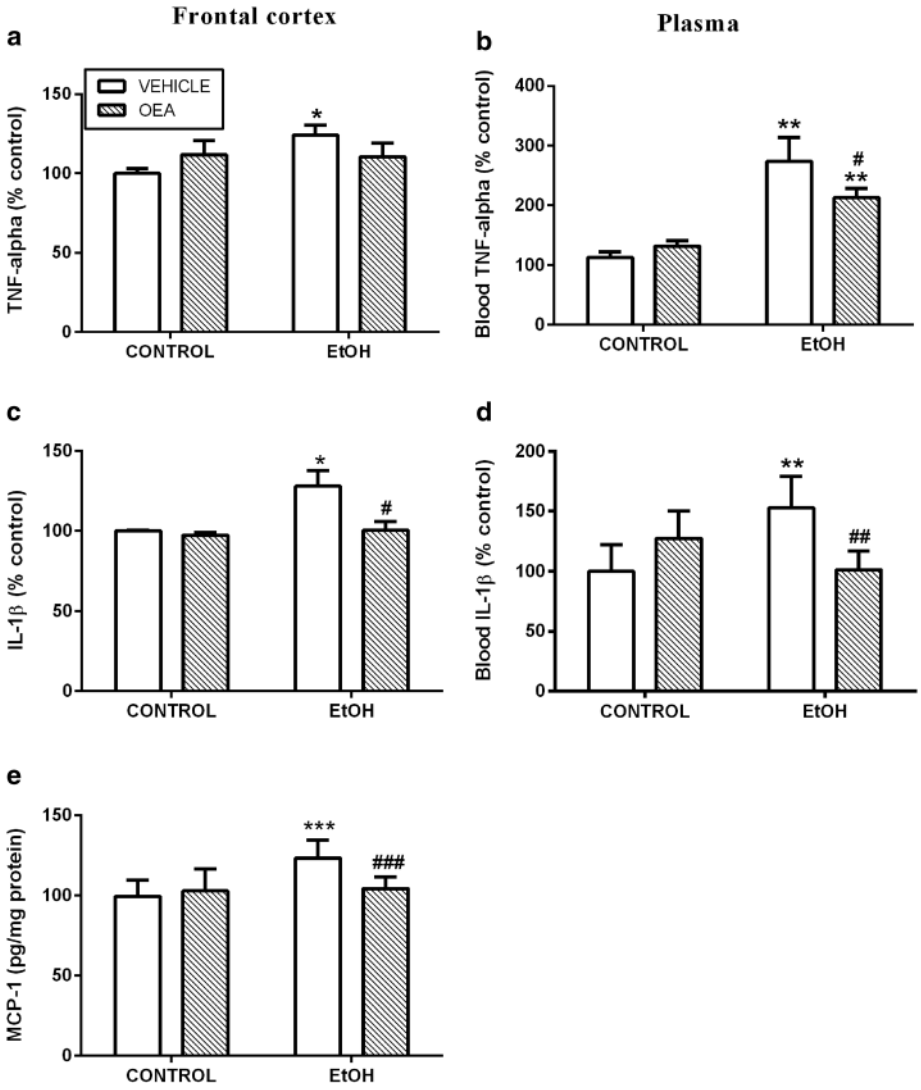


Figure 4 Release of TNF-α, IL-1β and MCP-1 in frontal cortex and/or plasma after pharmacological treatments. Enzyme-linked immunosorbent assay (ELISA) data of frontal cortex levels (left panel) of TNF-α (a), IL-β (c) and MCP-1 (e). ELISA-detected plasma levels (right panel) of TNF-α (b) and IL-β (d). Parameters measured 2–4 h after last ethanol administration. Data represents the mean ± S.E.M. ($n = 3$–6). Different from control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Different from ethanol-treated animals: #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$

plasma TNF-α observed after ethanol binge (Fig. 4b; $F_{(3,17)} = 15.59$, $P < 0.0001$).

Ethanol-treated animals showed an increase in frontal cortex IL-1β protein levels ($F_{(1,16)} = 4.497$, $P = 0.0499$) [27 percent of increase over control values (79.31 ± 0.29 pg/mg protein)], which was prevented by OEA pretreatment (Fig. 4c; $F_{(1,16)} = 4.22$, $P = 0.05$). In plasma, an interaction between pre-treatment with OEA and ethanol administration was observed ($F_{(1,20)} = 18.15$, $P = 0.0004$). Ethanol binge increased plasma IL-1β levels [53 percent of increase over control values (85.29 ± 7.8 pg/mg protein)], an effect that was prevented by OEA (Fig. 4d; $F_{(3,20)} = 8.20$, $P = 0.0009$).

Regarding MCP-1 levels, there was an interaction between ethanol administration and OEA pretreatment ($F_{(1,33)} = 10.06$, $P = 0.0033$) and main effects of ethanol ($F_{(1,33)} = 4.69$, $P = 0.0377$) and OEA pretreatment ($F_{(1,33)} = 12.88$, $P = 0.0011$). Ethanol binge increased MCP-1 levels in frontal cortex and OEA pre-treatment prevented this effect (Fig. 4e; $F_{(3,33)} = 9.92$, $P < 0.0001$).

Effect of OEA in NF-κB expression and activity after ethanol binge exposure

We explored the canonical NF-κB pro-inflammatory pathway in nuclear and cytosolic extracts of frontal cortex after the pharmacological treatments. There was a main effect of ethanol binge treatment in p65 mRNA levels (Fig. 5a; $F_{(1,33)} = 44.14$, $P < 0.0001$) and an interaction between ethanol treatment and OEA pretreatment ($F_{(1,33)} = 9.10$, $P = 0.0049$). One-way ANOVA revealed that pre-administration of OEA partially prevented the

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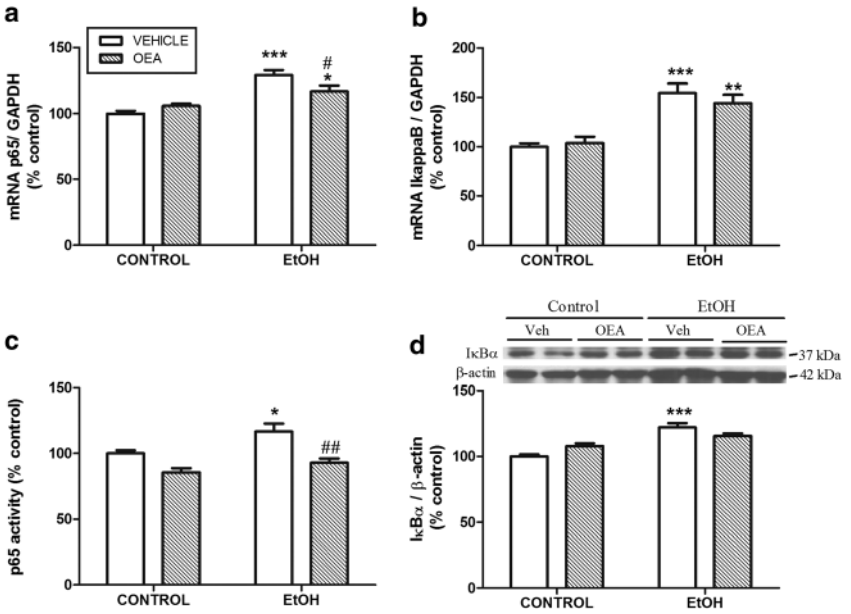


Figure 5 Activation of frontal cortex NFκB induced by ethanol binge exposure and effects of OEA pre-treatment. a) mRNA relative levels of nuclear p65 subunit of NFκB; b) mRNA levels of cytosolic NFκB inhibitory protein IκBα; c) p65 nuclear activity measured by an ELISA-based kit; d) protein levels of IκBα in cytosolic extracts. Parameters measured 2–4 h after last ethanol administration. Data represents the mean ± S.E.M. ($n = 4$–10). Densitometric data of IκBα were normalized by β-actin (lower band). Different from control group: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Different from ethanol-treated animals: $\#P < 0.05$; $\#\#P < 0.01$

ethanol-induced increase in p65 mRNA levels (Fig. 5a; $F_{(3,36)} = 19.87$, $P < 0.0001$). In the activity assay, there were main effects of alcohol exposure ($F_{(1,16)} = 6.55$, $P = 0.021$) and OEA pretreatment ($F_{(1,16)} = 16.66$, $P = 0.0009$), being the increase in p65 nuclear activity induced by ethanol counteracted by OEA pre-treatment (Fig. 5c).

Ethanol binge treatment also increased the mRNA and protein expression of IκBα (Fig. 5b and d; $F_{(1,35)} = 50.64$, $P < 0.0001$; $F_{(1,33)} = 37.69$, $P < 0.0001$) and interacted with OEA pretreatment ($F_{(1,33)} = 8.81$, $P = 0.0055$). Pre-treatment with OEA did not counteract the increase in IκBα mRNA levels and protein expression induced by ethanol (Fig. 5b and d; $F_{(1,35)} = 0.17$, $P = 0.6848$ n.s.; $F_{(1,33)} = 0.17$, $P = 0.067$, $P = 0.7970$, n.s., respectively).

Effect of OEA pre-treatment in iNOS and COX-2 mRNA expression and lipid peroxidation after ethanol binge exposure

The activation of NF-κB culminates in the production of inflammatory mediators such as the inducible enzymes iNOS and COX-2. The model of ethanol binge used in this study induced a robust increase in the mRNA expression of iNOS ($F_{(1,17)} = 11.87$, $P = 0.0031$) that was completely prevented by pre-treatment with OEA (Fig. 6a; $F_{(1,17)} = 8.71$, $P = 0.0089$). The maximal expression of COX-2 observed after ethanol treatment in the time-course experiment took place 6 h after the end of the binge protocol (see back Fig. 2f), which might explain the absence of a significant effect of ethanol in the regulation of the COX-2 mRNA in the second experiment, in which brain samples were collected between 2 and 4 h after the end of the last ethanol administration (Fig. 6b; $F_{(1,32)} = 0.07$, $P = 0.7975$, n.s.). However, the two-way ANOVA detected a main effect of OEA pre-treatment ($F_{(1,32)} = 7.17$, $P = 0.0011$) and an interaction (ethanol × OEA) ($F_{(1,32)} = 6.94$, $P = 0.0129$). One-way ANOVA analyses revealed that OEA decreased mRNA COX-2 levels in ethanol but not vehicle-treated animals (Fig. 6b; $F_{(3,32)} = 4.26$, $P = 0.0123$).

As a result of an overactivation of pro-inflammatory pathways, the mechanisms for oxidative cellular stress may be activated inducing the formation of lipid peroxides that will affect the cell viability. We checked in our experiments the formation of the HNE adduct, a reactive compound formed by decomposition of lipid peroxides. We observed an increase in the HNE formation in frontal cortex of animals exposed to the ethanol binge treatment (Fig. 6c; $F_{(1,15)} = 14.56$, $P = 0.0017$) and an effect of OEA pretreatment ($F_{(1,15)} = 7.21$, $P = 0.017$). OEA decreased the formation of HNE, being the effect significant only in ethanol-treated animals (Fig. 6c; $F_{(3,18)} = 7.03$, $P = 0.0036$).

Effects of OEA in caspase-8 and pro-apoptotic caspase-3 after ethanol binge treatment

We studied whether the induction of the TLR4/NF-κB signaling cascade by ethanol culminated in neuronal

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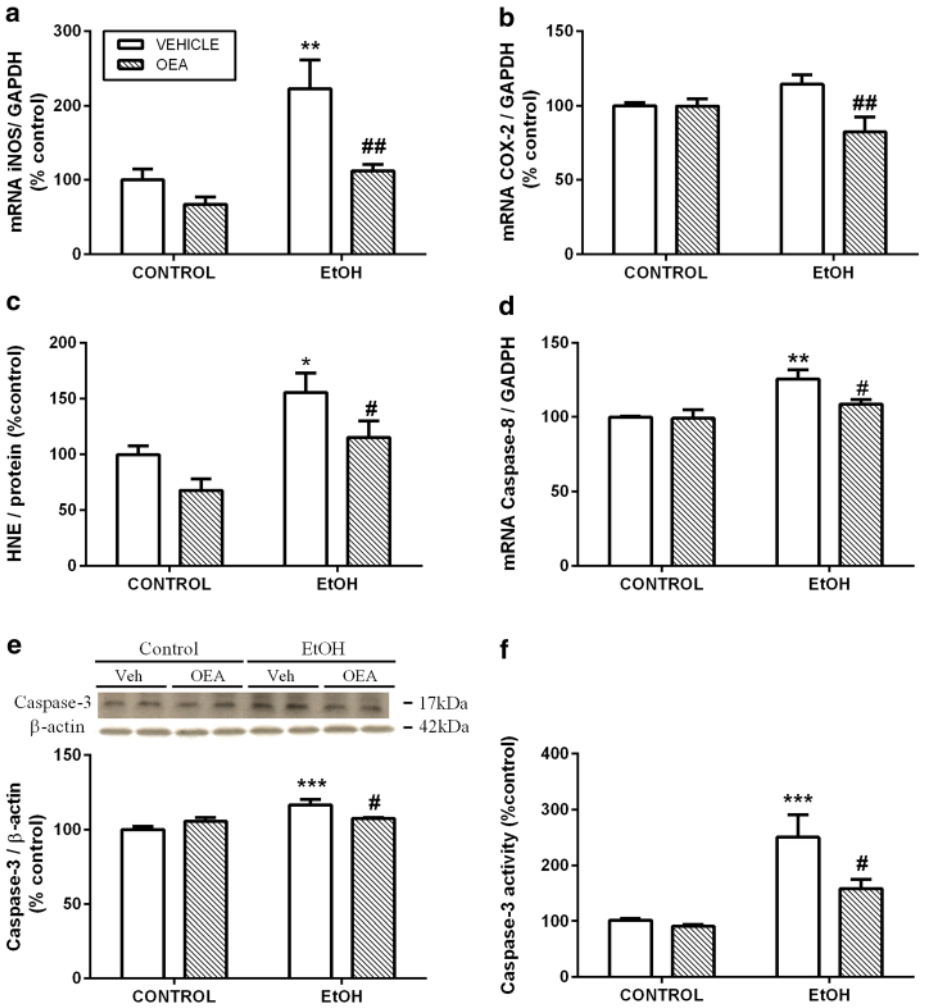


Figure 6 Role of OEA in ethanol-induced upregulation of iNOS and COX-2, lipid peroxidation, caspase 8 and caspase-3 expression and activity in frontal cortex. a) mRNA relative levels of iNOS; b) mRNA relative levels of COX-2; c) relative levels of 4-hydroxynonenal (4-HNE), a natural product of lipid peroxidation; d) mRNA relative levels of caspase-8, a signaling intermediate of caspase-3 activation; e) caspase-3 protein levels; f) caspase-3 activity measured by fluorimetric assay. Parameters measured 2–4 h after last ethanol administration. Data represents the mean ± S.E.M. ($n = 4$–10). Different from control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Different from ethanol-treated animals: #$P < 0.05$; ##$P < 0.01$

damage. We checked the expression and activity of the pro-apoptotic enzyme caspase-3 in frontal cortices of animals under the different pharmacological treatments, and the upregulation of caspase-8, which acts as an intermediate caspase in the TLR4-MyD88/caspase-8/caspase-3 signaling pathway (Liu *et al.* 2013).

Caspase-8 mRNA was upregulated by ethanol binge ($F_{(1,15)} = 13.37$, $P = 0023$), and OEA pretreatment reverted this effect (Fig. 6d; $F_{(3,18)} = 6.95$, $P = 0037$). Caspase-3 mRNA was upregulated by alcohol, but OEA was not able to prevent this effect (Fig. S2, Supporting Information). Regarding caspase-3 protein levels (Fig. 6e), we observed an interaction between alcohol exposure and OEA pre-treatment ($F_{(1,15)} = 7.95$, $P = 0.0129$) and a main effect of ethanol ($F_{(1,15)} = 12.30$, $P = 0.0032$). One-way ANOVA analyses indicated that pretreatment with OEA prevented the overexpression of caspase-3 induced by ethanol (Fig. 6e; $F_{(3,18)} = 7.29$, $P = 0.0031$). Additionally, the ethanol binge treatment induced an increase in the activity of caspase-3 in frontal cortex ($F_{(1,32)} = 20.98$, $P < 0.0001$), which was reverted by pretreatment with OEA (Fig. 6f; $F_{(1,32)} = 4.744$, $P = 0.0369$).

Effects of OEA in plasma corticosterone and LPS levels

To check the effects on hypothalamic–pituitary–adrenal (HPA) axis, we tested the influence of OEA in ethanol-induced rise in corticosterone levels (Fig. 7a). Data revealed a significant effect of treatment ($F_{(1,29)} = 5.44$, $P = 0.00268$) and an interaction between ethanol binge and OEA pretreatment ($F_{(1,29)} = 4.77$, $P = 0.0373$). A rise in corticosterone levels was observed in animals exposed

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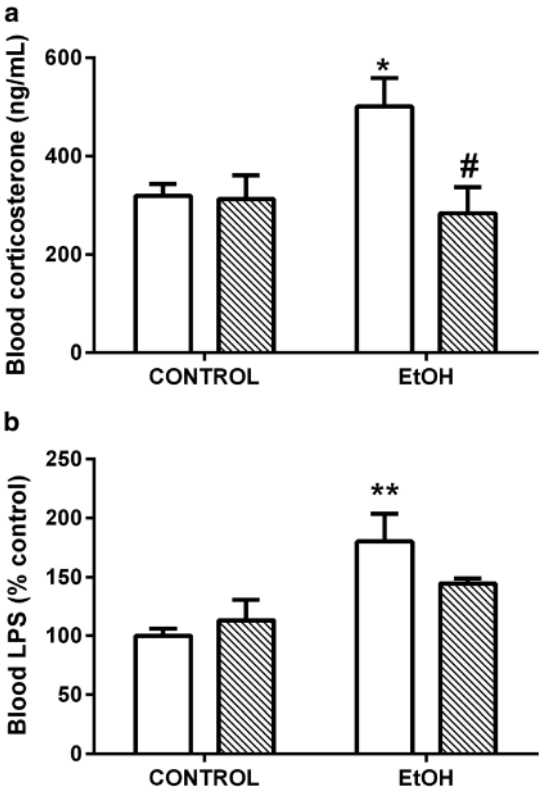


Figure 7 Role of OEA in ethanol-induced upregulation of plasma corticosterone levels and gut permeability for LPS. a) Plasma corticosterone levels detected by radioimmunoassay (R.I.A.); b) plasma LPS levels detected by enzymatic assay. Parameters measured 2–4 h after last ethanol administration. Data represents the mean ± S.E.M. (n = 6–9). Different from control group: *$P < 0.05$; **$P < 0.01$. Different from ethanol-treated animals: #$P < 0.05$

to the ethanol binge treatment, which was totally prevented by pre-treatment with OEA ($F_{(3,29)} = 4.21$, $P = 0.0136$). OEA did not modify plasma corticosterone levels in control animals (Fig. 7a).

Additionally, we studied the effect of OEA pre-treatment in the gut permeability for endotoxins by checking the levels of LPS in plasma after treatments. Ethanol binge treatment increases significantly the levels of LPS in plasma about an 80 percent versus controls ($F_{(1,27)} = 12.40$, $P = 0.0015$). Animals pre-treated with OEA and receiving ethanol showed reduced levels of plasmatic LPS (44 percent of increase over controls), a value that did not differ neither with control group nor with the ethanol group. Two-way ANOVA indicated no significant effect of pretreatment ($F_{(1,27)} = 0.50$, $P = 0.4872$) and no interaction between ethanol exposure and OEA pretreatment ($F_{(1,27)} = 2.376$, $P = 0.01348$).

Effects of OEA in control animals

The time-course of OEA in control animals (Supporting Information, Table S2) indicates that OEA did not significantly modify parameters in controls. However, because some tendencies are observed versus the control levels at 3 h after the injection (i.e. TLR4 and p65), we cannot discard completely that OEA may affect control animals at early time points.

Effects of OEA in depressive-like symptoms and anxiety

In order to determine the effects of OEA in ethanol-related behaviors during withdrawal, we performed the Forced Swimming test and the Elevated Plus Maze test to check depressive-like symptoms and anxiety, respectively. Our results showed that ethanol binge drinking induced reductions in the swimming and climbing times (Fig. 8a and b; $F_{(1,21)} = 3.281$, $P = 0.05$; $F_{(1,21)} = 7.326$, $P = 0.0132$, respectively) and increases in the immobility and latency (Fig. 8c and d; $F_{(1,21)} = 9.53$, $P = 0.0056$; $F_{(1,21)} = 9.53$, $P = 0.006$, respectively) in the Forced Swimming test. An interaction between OEA treatment and ethanol binge was also observed in the immobility and latency parameters ($F_{(1,21)} = 4.43$, $P = 0.0475$; $F_{(1,21)} = 9.53$, $P = 0.006$, respectively). The pre-treatment with OEA was able to prevent the alcohol-induced depressive-like symptoms, normalizing the swimming, immobility and latency measures (Fig. 8a, c and d; $F_{(3,21)} = 2.935$, $P = 0.05$; $F_{(3,21)} = 5.77$, $P = 0.048$; $F_{(3,21)} = 4.17$, $P = 0.018$, respectively).

Regarding anxiety-like behaviors, the Kruskal–Wallis ANOVA indicated that there was a general effect of treatments in the percentage of entries (Fig. 8e; $H = 8.074$, $P = 0.05$) and time (Fig. 8f; $H = 7.702$, $P = 0.0526$) spent in open arms of the elevated plus maze 24 h after the ethanol binge. Although there was a clear tendency showing an apparent anxiolytic effect of OEA in ethanol-treated and control animals, the Dunn's *post hoc* test did not found significant effects in multiple comparisons. The effect of treatments in the same measures of anxiety, percentage of entries (Fig. 8g) and time (Fig. 8h) spent in open arms, was not significant 12 days after the ethanol binge ($H = 6.377$, $P = 0.09$ and $H = 5.619$, $P = 0.13$,n.s., respectively).

DISCUSSION

In this study we described the time-course of neuroinflammation induced by ethanol binge intoxication and we provided evidence of the anti-inflammatory and neuroprotectant effects induced by OEA. Our results indicate that OEA interfered with the neuroimmune HMGB1/TLR4/MyD88 danger signaling associated with NFkB-mediated pro-inflammatory cascade and protected against the overactivity of the pro-apoptotic enzyme caspase-3 in rat frontal cortex, all elicited by ethanol binge intoxication. Additionally, OEA inhibited the HPA

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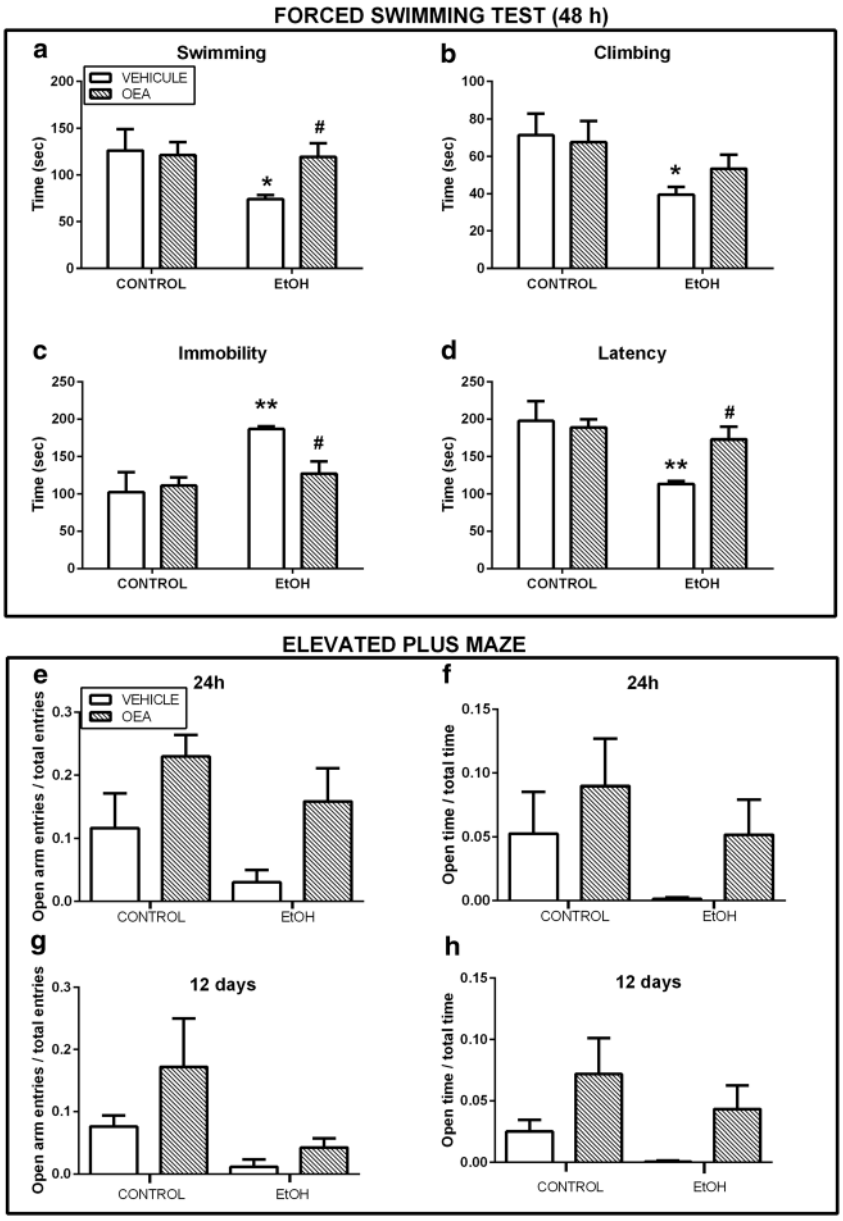


Figure 8 Antidepressant-like and anxiolytic effects of OEA in ethanol binge acute withdrawal. OEA was administered as a pretreatment before each ethanol gavage and animals were tested in the Forced Swimming Test (upper panel) and the Elevated Plus Maze (bottom panel) 48 h and 24 h/12 days, respectively, after the alcohol binge protocol. In the Forced Swimming Test, decreases in swimming (a), climbing (b) and latency (d) times and increases in immobility (c) are indexes of depressive-like behavior. In the Elevated Plus Maze, a decrease in the percentage of entries in the open arms (e, g) and in the percentage of time spent in the open arms (f, h) are considered measures of anxiety. Data represents the mean ± S.E.M. ($n = 5$–8). Different from control group: $^{*}P < 0.05$; $^{**}P < 0.01$. Different from ethanol-treated animals: $\#P < 0.05$

axis activation under alcohol binge exposure with no alteration in the ethanol metabolism.

One of the main findings of the present study was that OEA pretreatment affected the expression and signaling of innate immune receptors TLR4 under alcohol intoxicating conditions. Recent evidences indicate that alcohol recruits and activates TLR4 in cerebral cortex (Alfonso-Loeches *et al.* 2010; Crews *et al.* 2013) including the prefrontal cortex (Vetreno & Crews 2012; Pascual *et al.* 2014), specifically in glial cells (Blanco *et al.* 2005; Fernandez-Lizarbe, Montesinos & Guerri 2013). Ethanol-induced TLR4 signaling triggers the induction of a cascade of pro-inflammatory mediators that affect myelination (Alfonso-Loeches *et al.* 2012; Montesinos *et al.* 2015), induce brain damage (Alfonso-Loeches *et al.* 2010, Fernandez-Lizarbe *et al.* 2013), cognitive impairments (Pascual *et al.* 2011, 2014; Montesinos *et al.* 2015) and anxiety-related behaviors (Pascual *et al.* 2015) associated with the drug abuse. Increased expression of TLR2, TLR3 and TLR4 has been observed in

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human brains of alcoholics and in mice treated chronically with ethanol (Crews *et al.* 2013). The mechanisms underlying ethanol activation of TLR4 may include, apart from the direct activation, the recruitment of the nuclear cytokine HMGB1 that is released from neurons to the extracellular space inducing stimulation of both glial and neuronal TLR4. The binding of HMGB1 to TLR4 in glial cells activates a cascade of pro-inflammatory mediators and induce the expression and release of more HGMB1, which magnifies the inflammatory response (Crews *et al.* 2013; Zou & Crews 2014). It has been described that HMGB1/TLR4 signaling increased brain neuroimmune activation in alcohol dependence (Crews *et al.* 2013) and has been associated with cognitive deficits (Mazarati *et al.* 2011). Here, we proved that one of the mechanisms by which OEA may exert its anti-inflammatory and protective actions in the brain is by blocking the activation of the innate immune system, specifically through HMGB1/TLR4 signaling cascade inhibition associated with cell damage. HMGB1 is hyperacetylated and translocated from the nucleus to the cytoplasm under damage conditions, including alcohol exposure, where it exerts its anti-inflammatory properties (Zou & Crews 2014). Here, we showed that ethanol increased total HMGB1 as a consequence of the increase in nuclear and cytosolic fractions. OEA inhibited the total expression of HMGB1, appearing to affect more the HMGB1 cytosolic fraction, which may indicate that OEA exerted its anti-inflammatory actions by inhibiting the release of HGMB1 from the nucleus to the cytosol in the ethanol-stimulated condition rather than inhibiting the synthesis. To further confirm this hypothesis, the effect of OEA in the acetylation of HMGB1 should be performed.

Another mechanism for ethanol activation of TLR4 may involve an increase in the intestinal permeability to endotoxins, because elevated levels of blood LPS (which is a TLR4 ligand) and leaky gut have been documented in alcohol-dependent animals and alcohol-dependent non-cirrhotic humans (Adachi *et al.* 1995; Leclercq *et al.* 2012). Indeed, LPS administration greatly increased the neuroinflammatory effects of ethanol in brain and blood (Qin *et al.* 2008) and sensitized ethanol-induced anxiety during withdrawal (Breese *et al.* 2008). We have previously showed that OEA blocks the frontal cortex NF-κB-dependent neuroinflammatory pathway induced by LPS and counteracts the anhedonic-like behavior associated with neuroinflammation (Sayd *et al.* 2014). In the present study, we tested the plasma levels of LPS and found that animals treated with ethanol had increased levels of circulating endotoxin. Pre-treatment with OEA reduced plasma LPS in ethanol-treated animals to levels that not significant differ neither from controls nor from ethanol group. These data suggest that OEA may weakly affect the gut permeability induced by ethanol, although this hypothesis needs to be further confirmed, and has special relevancy because of the fact that OEA is formed in the gut in a diet-dependent manner. Whether OEA may alter the gut permeability for other endotoxins reducing peripheral inflammation after ethanol abuse requires more specific studies by 16S rRNA sequencing to be determined in future investigations.

Mechanisms by which ethanol injures the brain may include inflammation-derived oxidative stress and corticosterone production. Oxidative stress has been proposed as a major factor for alcohol-associated neurobiological effects (Hamelink *et al.* 2005; Haorah *et al.* 2008; Collins *et al.* 2014). Because of the suggested role of inflammation and oxidative stress in alcohol-induced brain damage, several antioxidants have been tested as neuroprotectant drugs for alcohol abuse. Interestingly, only some of them have been proven to be effective preventing alcohol-induced damage. Butylated hydroxytoluene (BTH) inhibited NF-κB nuclear activity and prevented the brain damage induced by ethanol in the 4-day binge intoxicating model (Crews *et al.* 2006). The diuretic furosemide and the non psychoactive cannabinoid Cannabidiol protected against ethanol brain damage by an antioxidant effect (Collins *et al.* 1998; Hamelink *et al.* 2005). The docosahexaenoic acid reduced neuroinflammation, oxidative stress and ethanol-induced neurodegeneration in the same ethanol binge model used in the present study (Tajuddin *et al.* 2014). However, the antioxidant ebselen and a blueberry extract failed to afford neuroprotection against ethanol binge-induced brain damage (Crews *et al.* 2006), and there are contradictory results for the effect of α-tocofecol (Hamelink *et al.* 2005; Crews *et al.* 2006). Chronic alcohol-induced cognitive deficits were abrogated by antioxidants (Tiwari & Chopra 2013). In our ethanol binge study, OEA showed a similar antioxidant profile, reducing oxidative and nitrosative stress. The anti-oxidant actions of OEA in the brain under stimulating conditions have been shown previously by us and other authors (Sayd *et al.* 2014; Jin *et al.* 2015), which also confirm that, despite a little tendency observed here, OEA appears to not significantly affect antioxidant capacity in control animals (Sayd *et al.* 2014; Jin *et al.* 2015). Additionally, we showed that OEA decreased the ethanol-induced rise in blood corticosterone levels. Altogether, these results suggest a predominant role for oxidative stress and HPA axis activation in the neuronal damage induced by alcohol binge. The inhibition of blood corticosterone levels induced by OEA may be protective for the neurodegeneration induced by ethanol, because it has been shown that elevated circulating corticosterone partially mediates ethanol binge-induced neurotoxicity in the brain (Cippitelli *et al.* 2014). Although it is to note that prolonged increases in glucocorticoids may exert an anti-inflammatory effect. In line with our results, OEA

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normalized the hyperactivity of the HPA axis after chronic unpredictable mild stress (Jin *et al.* 2015).

We described that ethanol binge intoxication induced the release of proinflammatory cytokines in brain and blood. The effects of OEA in cytokine reduction were observed both at central and peripheral levels. The fact that the study with OEA was done in an interval in which brain TNF-α levels after ethanol binge were modest (because they were measured at a time-point in the descending curve of the cytokine expression) may explain the absence of OEA effect in cortical TNF-α levels, while the peak of blood TNF-α levels was substantial to appreciate the inhibitory effect of OEA. Nevertheless, OEA appears to only partially decrease plasma TNF-α levels in ethanol-treated rats compared with a more robust effect in the inhibition of plasma IL-1β. The ethanol-induced increase in plasma TNF-α levels is much more pronounced (141 percent) that the increase in IL-1β (53 percent), which may indicate that higher doses of OEA will be required to completely prevent the upregulation of TNF-α levels in plasma. We reported previously that OEA reduced both plasma and frontal cortex TNF-α under neuroinflammatory experimental conditions (Sayd *et al.* 2014) and it has been proven that OEA crosses the blood brain barrier after i.p. administration (Gonzalez-Aparicio *et al.* 2014). Nevertheless, plasma cytokine alterations are known to induce brain cytokine responses. Thus, peripheral cytokines can enter the brain by volume diffusion in the circumventricular organs and/or through saturable cytokine transporters at the blood-brain barrier. In addition, IL-1 receptors located in perivascular macrophages and brain endothelial cells results in local production of inflammatory prostaglandins. Ultimately, the engagement of immune-to-brain communications leads to the production of pro-inflammatory cytokines by microglial cells (revised in Dantzer *et al.* 2008). Disregarding the sources of production, increases in the pro-inflammatory cytokines IL-1β and TNF-α have been linked to brain HMGB1/TLR4 activity altered by ethanol (Crews *et al.* 2013; Zou & Crews 2014).

OEA is an analog of anandamide with no activity at traditional cannabinoid receptors that binds the nuclear PPAR-α. The anti-inflammatory actions of OEA reported here could be related to PPAR-α targeting, because the activation of this nuclear receptor has been shown to inhibit transcriptional activity related with inflammation by repressing NF-kB and the activator protein-1 (AP-1) signaling (reviewed in Stahel *et al.* 2008). We observed that OEA blocked the expression and activity of the p65 subunit of NF-kB, which mediates most of the transcriptional activity of this nuclear factor, with no significant effect in the NF-kB inhibitory protein IkBα. The translocation of NF-kB p65 subunit to the nucleus induces the transcription of more pro-inflammatory cytokines, which are release to the cytoplasm and activate NF-kB again in an amplified inflammatory loop. It is to note that prolonged increases in IkBα may contribute to the loss of proinflammatory gene induction at later time points, because IkBα sequestrates NF-kB in the cytosol. NF-kB transcriptional activity produces MCP-1, which induce immune cells migration to damage cells. We observed that OEA reduces, not only IL-1β and TNF-α, but also MCP-1 production. This finding may reveal unknown properties of OEA for the treatment of Wernicke's encephalopathy, a neuropsychiatric condition associated with alcoholism, because thiamine (vitamin B1) deficiency, a core element for the disorder, has been correlated with brain levels of MCP-1 (Zahr *et al.* 2014), which caused microglia recruitment/activation and exacerbated neurodegeneration (Yang *et al.* 2011).

Ethanol-induced NF-kB activity increased also the expression of the enzymes COX-2 and iNOS, and the production of the oxidative stress marker 4-HNE, which were blocked by OEA pretreatment. According to our results, other studies showed increased COX-2 immunostaining in frontal cortex of rats exposed to the binge ethanol intoxicating model (Knapp & Crews 1999) and increased 4-HNE adduct formation following the 4-day intoxicating ethanol binge protocol (Tajuddin *et al.* 2014). This model of alcohol binge intoxication and its slight modifications have shown to activate phospholipase A2 (PLA2) and mobilize arachidonic acid (AA), resulting in ROS production and oxidative stress, neuroinflammation and neurodegeneration (Collins *et al.* 2014). The increase in frontal cortex COX-2 levels reported here and by other authors (Knapp & Crews 1999) after ethanol binge intoxication may be partially explained by the increase in AA, which is the COX-2 substrate in the production of prostaglandins.

Another relevant finding of our study is the potential ability of OEA to suppress the overactivity of the pro-apoptotic enzyme caspase-3 under stimulated conditions. OEA inhibited also the alcohol-induced increase in caspase-8 mRNA, an upstream intermediate in the caspases' cascade for apoptosis cell death (Liu *et al.* 2013; Viceconte *et al.* 2015), which is dependent of HNE-linked redox status (Liu *et al.* 2000) and has been related with NF-κB activation in response to TLR4 activation (Lemmers *et al.* 2007). More remarkable, ethanol binge induced an upregulation of caspase-3 expression and activity in frontal cortex, which were both inhibited by the pre-treatment with OEA. This finding reveals an up to date undescribed mechanism that OEA may use to protect the brain. Precisely, NFκB-mediated caspase-3 apoptotic signaling has been related with the cognitive deficits associated with ethanol exposure (Tiwari & Chopra 2012; Tiwari & Chopra 2013).

Of great interest are the data provided by behavioral experiments, indicating that OEA may modulate negative

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aspects associated with early ethanol withdrawal at a behavioral level. Thus, OEA showed antidepressive-like properties in a forced swimming test 48 h after alcohol abuse and tendency to counteract alcohol-induced anxiety 24 h after the binge. The pattern of modulation of anxiety was less pronounced 12 days after the treatments. Despite the absence of significant effects in anxiety, we cannot discard that OEA may reduce anxiety during acute ethanol binge withdrawal, and it is very intriguing the possible anxiolytic effect of OEA in control (non anxious) animals. The explanation of this phenomenon remains elusive and requires further studies on the action of OEA in non-treated animals. On the contrary, the antidepressant-like properties of OEA shown here are robust and in agreement with other authors (Jin *et al.* 2015). In a previous study, we also showed that OEA modulates motivational behavior by decreasing the anhedonia in a saccharin preference test (Sayd *et al.* 2014). Interestingly, endogenous *N*-acylethanolamines are altered with antidepressant treatments. OEA levels are increased in rat frontal cortex or hippocampus after pharmacological treatment with the antidepressants N-acetylcysteine and imipramine, suggesting that, the *N*-acylethanolamines, including OEA, may play a prominent role in the mechanism of action of antidepressant drugs (Smaga *et al.* 2014). The antidepressant effects of OEA may be related to regulation of brain-derived neurotropic factor (BDNF) in hippocampus and prefrontal cortex (Jin *et al.* 2015), the regulation of HPA axis and/or its antiinflamatory/antioxidant profile (Jin *et al.* 2015, and this paper).

Recent studies indicate that OEA may act as a homeostatic signal that regulates multiple physiological adaptations after brain insults. Indeed, increased OEA levels have been documented after several CNS pathologies, including ethanol addiction (Bilbao *et al.* 2015). Thus, an exogenous administration of OEA (Sayd *et al.* 2014) or an increased bioviability of it (Ribeiro *et al.* 2015) might serve as a potent strategy to suppress inflammation and as a neuroprotective pharmacologic maneuver. The neuroinflammation induced by ethanol may play a role in the pathology of ethanol addiction, because activation of inflammatory signaling by LPS increased ethanol drinking (Blednov *et al.* 2011). OEA regulates multiple physiological adaptations after ethanol abuse, including reduction of ethanol self-administration and relapse (Bilbao *et al.* 2015), ethanol-induced neuroinflammation and brain damage (this study) or attenuation of the withdrawal symptoms after ethanol intake (Bilbao *et al.* 2015). Collectively, these results highlight the beneficial profile of OEA as a pharmacotherapy for different aspects of ethanol abuse. From a clinical perspective, it remains to be determined in future investigations whether OEA will be also effective as an antidote against alcohol intoxication when administered after and not during ethanol abuse.

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Authors Contribution

MA performed all biochemical studies; MA, FA and RGH participated in the behavioral studies, tissue/blood extractions and BEL determinations; AS and FJP synthesized the OEA; BGB participates in the study design, acquisition of data and revision of the manuscript; JCJ and FRF provided methodological support and supervised the manuscript for intellectual content; LO designed the study, performed experiments and statistical analysis, and wrote the manuscript.

References

Adachi J, Mizoi Y, Fukunaga T, Ogawa Y, Ueno Y, Imamichi H (1991) Degrees of alcohol intoxication in 117 hospitalized cases. J Stud Alcohol 52:448–453.

Adachi Y, Moore LE, Bradford BU, Gao W, Thurman RG (1995) Antibiotics prevent liver injury in rats following long-term exposure to ethanol. Gastroenterology 108:218–228.

Agartz I, Momenan R, Rawlings RR, Kerich MJ, Hommer DW (1999) Hippocampal volume in patients with alcohol dependence. Arch Gen Psychiatry 56:356–363.

Alfonso-Loeches S, Pascual-Lucas M, Blanco AM, Sanchez-Vera I, Guerri C (2010) Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. J Neurosci 30:8285–8295.

Alfonso-Loeches S, Pascual M, Gómez-Pinedo U, Pascual-Lucas M, Renau-Piqueras J, Guerri C (2012) Toll-like receptor 4 participates in the myelin disruptions associated with chronic alcohol abuse. Glia 60:948–964.

Bilbao A, Blanco E, Luque-Rojas MJ, Suarez J, Palomino A, Vida M, Araos P, Bermudez-Silva FJ, Fernandez-Espejo E, Spanagel R, Rodriguez de Fonseca F (2013) Oleoylethanolamide dose-dependently attenuates cocaine-induced behaviours through a PPARalpha receptor-independent mechanism. Addict Biol 18:78–87.

Bilbao A, Serrano A, Cippitelli A, Pavón FJ, Giuffrida A, Suárez J, García-Marchena N, Baixeiras E, Gómez de Heras R, Orio L, Alén F, Ciccocioppo R, Cravatt BF, Parsons LH, Piomelli D, Rodríguez de Fonseca F (2015) Role of the satiety factor oleoylethanolamide in alcoholism. Addict Biol(in press). doi: 10.1111/adb.

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Blanco AM, Vallés SL, Pascual M, Guerri C (2005) Involvement of TLR4/type I IL-1 receptor signaling in the induction of inflammatory mediators and cell death induced by ethanol in cultured astrocytes.

Blednov YA, Benavidez JM, Geil C, Perra S, Morikawa H, Harris RA (2011) Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. Brain Behav Immun 25 Suppl 1:S92–S105.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Breese GR, Knapp DJ, Overstreet DH, Navarro M, Wills TA, Angel RA (2008) Repeated lipopolysaccharide (LPS) or cytokine treatments sensitize ethanol withdrawal-induced anxiety-like behavior. Neuropsychopharmacology 33:867–876.

Cippitelli A, Damadzic R, Hamelink C, Brunnquell M, Thorsell A, Heilig M, Eskay RL (2014) Binge-like ethanol consumption increases corticosterone levels and neurodegeneration whereas occupancy of type II glucocorticoid receptors with mifepristone is neuroprotective. Addict Biol 19:27–36.

Collins MA, Tajuddin N, Moon KH, Kim HY, Nixon K, Neafsey EJ (2014) Alcohol, phospholipase A2-associated neuroinflammation, and omega3 docosahexaenoic acid protection. Mol Neurobiol 50:239–245.

Collins MA, Zou JY, Neafsey EJ (1998) Brain damage due to episodic alcohol exposure in vivo and in vitro: furosemide neuroprotection implicates edema-based mechanism. FASEB J 12:221–230.

Crews F, Nixon K, Kim D, Joseph J, Shukitt-Hale B, Qin L, Zou J (2006) BHT blocks NF-kappaB activation and ethanol-induced brain damage. Alcohol Clin Exp Res 30:1938–1949.

Crews FT, Braun CJ, Hoplight B, Switzer RC 3rd, Knapp DJ (2000) Binge ethanol consumption causes differential brain damage in young adolescent rats compared with adult rats. Alcohol Clin Exp Res 24:1712–1723.

Crews FT, Qin L, Sheedy D, Vetreno RP, Zou J (2013) High mobility group box 1/Toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence. Biol Psychiatry 73:602–612.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9:46–56.

Fernandez-Lizarbe S, Montesinos J, Guerri C (2013) Ethanol induces TLR4/TLR2 association, triggering an inflammatory response in microglial cells. J Neurochem 126:261–273.

Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, Piomelli D (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 425:90–93.

Giuffrida A, Rodriguez de Fonseca F, Piomelli D (2000) Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. Anal Biochem 280:87–93.

Gonzalez-Aparicio R, Blanco E, Serrano A, Pavon FJ, Parsons LH, Maldonado R, Robledo P, Fernandez-Espejo E, de Fonseca FR (2014) The systemic administration of oleoylethanolamide exerts neuroprotection of the nigrostriatal system in experimental Parkinsonism. Int J Neuropsychopharmacol 17:455–468.

Hamelink C, Hampson A, Wink DA, Eiden LE, Eskay RL (2005) Comparison of cannabidiol, antioxidants, and diuretics in reversing binge ethanol-induced neurotoxicity. J Pharmacol Exp Ther 314:780–788.

Haorah J, Ramirez SH, Floreani N, Gorantla S, Morsey B, Persidsky Y (2008) Mechanism of alcohol-induced oxidative stress and neuronal injury. Free Radic Biol Med 45:1542–1550.

He J, Crews FT (2008) Increased MCP-1 and microglia in various regions of the human alcoholic brain. Exp Neurol 210:349–358.

Hunt WA (1993) Are binge drinkers more at risk of developing brain damage? Alcohol 10:559–561.

Jin P, Yu HL, Tian L, Zhang F, Quan ZS (2015) Antidepressant-like effects of oleoylethanolamide in a mouse model of chronic unpredictable mild stress. Pharmacol Biochem Behav 133:146–154.

Knapp DJ, Crews FT (1999) Induction of cyclooxygenase-2 in brain during acute and chronic ethanol treatment and ethanol withdrawal. Alcohol Clin Exp Res 23:633–643.

Leclercq S, Cani PD, Neyrinck AM, Starkel P, Jamar F, Mikolajczak M, Delzenne NM, de Timary P (2012) Role of intestinal permeability and inflammation in the biological and behavioral control of alcohol-dependent subjects. Brain Behav Immun 26:911–918.

Lemmers B, Salmena L, Bidère N, Su H, Matysiak-Zablocki E, Murakami K, Ohashi PS, Jurisicova A, Lenardo M, Hakem R, Hakem A (2007) Essential role for caspase-8 in toll-like receptors and NfκB signaling. J Biol Chem 282:7416–7423.

Liu W, Kato M, Akhand AA, Hayakawa A, Suzuki H, Miyata T, Kurokawa K, Hotta Y, Ishikawa N, Nakashima I (2000) 4-Hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. J Cell Sci 113:635–641.

Liu JW, Zhu HT, Chen KL, Qiu C, Tang KF, Niu XL (2013) Selenium attenuates high glucose-induced ROS/TLR4 involved apoptosis of rat cardiomyocyte. Biol Trace Elem Res 156:262–270.

Majchrowicz E (1975) Induction of physical dependence upon ethanol and the associated behavioral changes in rats. Psychopharmacologia 43:245–254.

Mazarati A1, Maroso M, Iori V, Vezzani A, Carli M (2011) High-mobility group box-1 impairs memory in mice through both toll-like receptor 4 and Receptor for Advanced Glycation End Products. Exp Neurol 232:143–148.

McClain CJ, Cohen DA (1989) Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. Hepatology 9:349–351.

McClain CJ, Song Z, Barve SS, Hill DB, Deaciuc I (2004) Recent advances in alcoholic liver disease. IV. Dysregulated cytokine metabolism in alcoholic liver disease. Am J Physiol Gastrointest Liver Physiol 287:G497–G502.

Melis M, Pillolla G, Luchicchi A, Muntoni AL, Yasar S, Goldberg SR, Pistis M (2008) Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. J Neurosci 28:13985–13994.

Montesinos J, Pascual M, Pla A, Maldonado C, Rodriguez-Arias M, Minarro J, Guerri C (2015) TLR4 elimination prevents synaptic and myelin alterations and long-term cognitive dysfunctions in adolescent mice with intermittent ethanol treatment. Brain Behav Immun 45:233–244.

Obernier JA, Bouldin TW, Crews FT (2002a) Binge ethanol exposure in adult rats causes necrotic cell death. Alcohol Clin Exp Res 26:547–557.

Obernier JA, White AM, Swartzwelder HS, Crews FT (2002b) Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. Pharmacol Biochem Behav 72:521–532.

Pascual M, Balino P, Alfonso-Loeches S, Aragon CM, Guerri C (2011) Impact of TLR4 on behavioral and cognitive dysfunctions

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associated with alcohol-induced neuroinflammatory damage. Brain Behav Immun 25 Suppl 1:S80–S91.

Pascual M, Balino P, Aragon CM, Guerri C (2015) Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: role of TLR4 and TLR2. Neuropharmacology 89:352–359.

Pascual M, Pla A, Minarro J, Guerri C (2014) Neuroimmune activation and myelin changes in adolescent rats exposed to high-dose alcohol and associated cognitive dysfunction: a review with reference to human adolescent drinking. Alcohol Alcohol 49:187–192.

Qin L, Crews FT (2012) NADPH oxidase and reactive oxygen species contribute to alcohol-induced microglial activation and neurodegeneration. J Neuroinflammation 9:5.

Qin L, He J, Hanes RN, Pluzarev O, Hong JS, Crews FT (2008) Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. J Neuroinflammation 5:10.

Ribeiro A, Pontis S, Mengatto L, Armirotti A, Chiurchiu V, Capurro V, Fiasella A, Nuzzi A, Romeo E, Moreno-Sanz G, Maccarrone M, Reggiani A, Tarzia G, Mor M, Bertozzi F, Bandiera T, Piomelli D (2015) A potent systemically active N-acylethanolamine acid amidase inhibitor that suppresses inflammation and human macrophage activation. ACS Chem Biol 10:1838–1846.

Rodriguez de Fonseca F, Navarro M, Gomez R, Escuredo L, Nava F, Fu J, Murillo-Rodriguez E, Giuffrida A, LoVerme J, Gaetani S, Kathuria S, Gall C, Piomelli D (2001) An anorexic lipid mediator regulated by feeding. Nature 414:209–212.

Sayd A, Anton M, Alen F, Caso JR, Pavon J, Leza JC, Rodriguez de Fonseca F, Garcia-Bueno B, Orio L (2014) Systemic administration of oleoylethanolamide protects from neuroinflammation and anhedonia induced by LPS in rats. Int J Neuropsychopharmacol 18. doi: 10.1093/ijnp/pyu111

Smaga I, Bystrowska B, Gawkinski D, Pomierny B, Stankowicz P, Filip M (2014) Antidepressants and changes in concentration of endocannabinoids and N-acylethanolamides in rat brain structures. Neurotox Res 26:190–206.

Snyder H, Williams D, Zink B, Reilly K (1992) Accuracy of blood ethanol determination using serum osmolality. J Emerg Med 10:129–133.

Stahel P, Smith WR, Bruchis J, Rabb CH (2008) Peroxisome proliferator-activated receptors: "key" regulators of neuroinflammation after traumatic brain injury. PPAR Res 2008:538141.

Sun Y, Alexander SP, Garle MJ, Gibson CL, Hewitt K, Murphy SP, Kendall DA, Bennett AJ (2007) Cannabinoid activation of PPAR alpha; a novel neuroprotective mechanism. Br J Pharmacol 152:734–743.

Tajuddin N, Moon KH, Marshall SA, Nixon K, Neafsey EJ, Kim HY, Collins MA (2014) Neuroinflammation and neurodegeneration in adult rat brain from binge ethanol exposure: abrogation by docosahexaenoic acid. PLoS One 9: e101223.

Tiwari V, Chopra K (2012) Attenuation of oxidative stress, neuroinflammation, and apoptosis by curcumin prevents cognitive deficits in rats postnatally exposed to ethanol. Psychopharmacology (Berl) 224:519–535.

Tiwari V, Chopra K (2013) Resveratrol abrogates alcohol-induced cognitive deficits by attenuating oxidative–nitrosative stress and inflammatory cascade in the adult rat brain. Neurochem Int 62:861–869.

Valles SL, Blanco AM, Pascual M, Guerri C (2004) Chronic ethanol treatment enhances inflammatory mediators and cell death in the brain and in astrocytes. Brain Pathol 14:365–371.

Vetreno RP, Crews FT (2012) Adolescent binge drinking increases expression of the danger signal receptor agonist HMGB1 and Toll-like receptors in the adult prefrontal cortex. Neuroscience 226:475–488.

Viceconte N, Burguillos MA, Herrera AJ, De Pablos RM, Joseph B, Venero JL (2015) Neuromelanin activates proinflammatory microglia through a caspase-8-dependent mechanism. J Neuroinflammation 12:5.

Yang G, Meng Y, Li W, Yong Y, Fan Z, Ding H, Wei Y, Luo J, Ke ZJ (2011) Neuronal MCP-1 mediates microglia recruitment and neurodegeneration induced by the mild impairment of oxidative metabolism. Brain Pathol 21:279–297.

Zahr NM, Alt C, Mayer D, Rohlfing T, Manning-Bog A, Luong R, Sullivan EV, Pfefferbaum A (2014) Associations between in vivo neuroimaging and postmortem brain cytokine markers in a rodent model of Wernicke's encephalopathy. Exp Neurol 261:109–119.

Zou JY, Crews FT (2014) Release of neuronal HMGB1 by ethanol through decreased HDAC activity activates brain neuroimmune signaling. PLoS One 9: e87915.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 RT-PCR analyses of TNF-α (left) and IL-β (right).
Figure S2 RT-PCR analyses of Caspase-3.
Table S1 RT-PCR primers´sequence details.
Table S2 Time-course of OEA in TLR4, p65 and iNOS mRNA and protein in control animals.

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