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Different brain oxidative and neuroinflammation status in rats during prolonged abstinence depending on their ethanol relapse-like drinking behavior: Effects of ethanol reintroduction

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ABSTRACT

Rationale: Accumulating evidence suggests that chronic alcohol consumption is associated with excessive oxidative damage and neuroinflammatory processes and these events have been associated to early alcohol withdrawal. In the present research we wonder if brain oxidative stress and neuroinflammation remains altered during prolonged withdrawal situations and whether these alterations can be correlated with relapse behavior in alcohol consumption. The effects of alcohol reintroduction were also evaluated

Methods: We have used a model based on the alcohol deprivation effect (ADE) within a cohort of wild-type male Wistar rats. Two subpopulations were identified according to the alcohol relapse-like drinking behavior displayed (ADE and NO-ADE subpopulations). Oxidized and reduced glutathione content was determined within the hippocampus and the amygdala using a mass spectrometry method. The levels of mRNA of seven different inflammatory mediators in the prefrontal cortex of rats were quantified. All the analyses were performed in two different conditions: after 21-day alcohol deprivation (prolonged abstinence) and after 24 h of ethanol reintroduction in both subpopulations.

Results: ADE and NO-ADE rats showed different endophenotypes. ADE rats always displayed a significant lower alcohol intake rate and ethanol preference than NO-ADE rats. The results also demonstrated the existence of altered brain redox and neuroinflammation status after prolonged abstinence exclusively in ADE rats. Moreover, when ethanol was reintroduced in the ADE subpopulation, altered oxidative stress and neuroinflammatory markers were restored.

Conclusions: Present findings provide new mechanisms underlying the neurobiology of relapse behavior and suggest the development of new pharmacological approaches to treat alcohol-induced relapse.

1. Introduction

Alcohol Use Disorders (AUDs), formerly called alcohol dependence or alcohol abuse, are complex, chronic disorders with a high relapse rate (Koob and Volkow, 2016; Rehm, 2011). Thus, even after successful detoxification and abstinence treatment, an alcohol-dependent patient remains at risk of relapse. Drug craving may even incubate over time, leading to a relapse risk several months after detoxification (Pickens et al., 2011). Literature shows that 60–80% of abstinent alcoholics will relapse during their lifetime (Barrick and Connors, 2002; Weiss et al., 2001). All in all, drug seeking and relapse are the main clinical problems related to AUDs. Thus, deepen the understanding of the underlying neurobiology of relapse behavior could be essential for improving available treatments to reduce the relapse rate or, to a lesser extent, reduce alcohol intake (Cannella et al., 2019; Reilly et al., 2014; Spanagel and Vengeliene, 2013).

Accumulating evidence from preclinical and clinical studies suggests that chronic alcohol consumption is associated with excessive oxidative

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damage and reduced levels of endogenous antioxidants, leading to excessive reactive oxygen species (ROS) production (Das et al., 2007; Jung and Metzger, 2016; Peng et al., 2005). These alterations in the oxidative stress status are not only restricted to the period of consumption itself but also have been linked to the early withdrawal phase. Thus, Huang et al. (2009) demonstrated the existence of alterations in the oxidative stress status only during early, but not prolonged, alcohol withdrawal symptoms in alcoholic patients. In experimental animals, Jung et al. (2008) clearly showed that oxidative stress is even more intense during early withdrawal than during previous ethanol exposure. At present, it is not known if these changes in oxidative brain status are also evident in prolonged withdrawal.

On the other hand, it has been suggested that inflammatory factors also play a key role in the development of alcohol-related behavioral and mood disorders (Kelley and Dantzer, 2011; Leclercq et al., 2014). In the case of AUDs, a number of animal and human studies have demonstrated the role of neuroinflammation in the pathophysiology of the disease (Robinson et al., 2014). The neuroinflammatory response has been observed at the mRNA and/or protein level when different cells or tissues are directly exposed to ethanol (Alfonso-Loeches et al., 2010, 2009, 2014). Systemic administration of ethanol to experimental animals also induces neuroinflammation. Thus, binge intoxication induces an inflammatory response in the brain of rats (Crews et al., 2006; Pascual et al., 2007) or mice (Crews et al., 2013; Kane et al., 2014; Montesinos et al., 2016), as does chronic exposure to ethanol in mice (Alfonso--Loeches et al., 2010; Whitman et al., 2013; Lippai et al., 2013). The inflammatory response has also been described after 24-hour ethanol withdrawal and a 15-day period of alcohol exposure. Curiously, this response was dependent on the cytokine and the brain region considered (Knapp et al., 2016).

A very important aspect that, according to our available information, has not been explored so far is whether the phenomena of alteration of the oxidative stress state of the brain and neuroinflammation, appear in late withdrawal situations and whether these alterations can be correlated with relapse behavior in alcohol consumption.

It is well-known that the alcoholic population is heterogeneous in nature (Epstein et al., 1995; Lesch and Walter, 1996; Windle and Scheidt, 2004). In fact, AUDs can be considered the result of a complex interplay between polygenic, environmental, and neurobiological components leading to very heterogeneous patient populations (Cannella et al., 2019). Identifying specific behavioral and genetic traits that could predispose individuals to develop drug abuse is a major goal in the field of Neurobiology of Addiction (Reilly et al., 2014; Spanagel and Vengeliene, 2013). The phenomenon of relapse in alcoholics is another behavioral trait of AUDs that clearly shows a great heterogeneity in patient populations. The neurobiology of alcohol relapse has tried to be studied with some success using different animal models. Among the repertoire of animal models of relapse presently available, the model based on the alcohol deprivation effect (ADE) is probably one of the most commonly used preclinical approach to study the ethanol relapse-like drinking behavior. This model is considered an excellent model in its face, predictive and ecological validity (Bell et al., 2012; Spanagel, 2017). The use of the ADE model reveals the existence of an enormous heterogeneity in the relapse behaviour in non-selected rat lines that has been linked by many authors to greater variability in the expression of several genes that may have a great importance in the functioning of critical brain areas (Vengeliene et al., 2014). To date, it is not known whether this heterogeneity in relapse behavior is related to a different neuroinflammatory response, with a differentiated alteration in the oxidative stress control mechanisms, or with both phenomena at the same time.

Therefore, the main aim of the current study was to investigate the existence of brain oxidative stress and to evaluate the neuroinflammation status in the late ethanol withdrawal in two subpopulations of male Wistar rats with a long experience in voluntary alcohol consumption. These subpopulations were selected according to the alcohol relapse-like drinking behavior displayed after experiencing several deprivation and reintroduction episodes. This research focuses particularly on the exploration of a crucial stage of the disease: the abstinence period which leads to the relapse phenomenon, and more specifically to the long-lasting abstinence period (21 days). We also examined the effect of the alcohol reintroduction in brain oxidative stress as well as in the expression of different neuroinflammation factors. We have focused our present research on the hippocampus, amygdala and prefrontal cortex (PFC) as these brain areas are highly affected by ethanol consumption and abstinence-induced damage (Chefer et al., 2011; Elibol-Can et al., 2011; Roberto et al., 2004).

2. Materials and methods

2.1. Animals

In this study, 58 male Wistar rats purchased from ENVIGO (Barcelona, Spain) were used. All animals, weighing 356 ± 3.2 g at the beginning of the experiment, were housed in individual cages in a temperature- and humidity-controlled room with a 12-hour inverted light/dark cycle (on 22:00, off 10:00). All the procedures were performed in accordance with Directive 2010/63/EU, Spanish laws (RD 53/2013) and animal protection policies. The Animal Care Committee of University of Valencia and the regional government (Conselleria de Agricultura, Medio Ambiente y Cambio Climático) approved and authorized all experiments.

2.2. Experimental design: long-term voluntary alcohol drinking procedure

As can be seen in Fig. 1, the 58 animals were divided into two experimental groups. Animals from the first experimental group (Experimental group 1; n = 43) were subjected to a long-term voluntary alcohol drinking procedure (total duration: 32 weeks) with repeated deprivation phases. This procedure has previously been used and validated under our experimental conditions (Orrico et al., 2013, 2014; Cano-Cebrián, 2021). During the procedure, animals were given continuous free access to tap water and 5%, 10% and 20% (v/v) ethanol solutions in their home cages. Alcohol drinking solutions were prepared from 96% v/v (Scharlau S.A., Spain) and then diluted with tap water to the different concentrations. Animals were subjected to three random deprivation periods (DPs). The duration of the drinking and DPs was irregular in order to prevent behavioural adaptations (Vengeliene et al., 2005). Moreover, when bottles were removed to quantify alcohol consumption, the position of the four bottles was always changed to avoid location preferences.

Animals from the second group of rats (Experimental group 2; n = 15) were distributed into two experimental subgroups, which were given continuous ad libitum access to tap water ("Water" subgroup; n = 9) or tap water and 5%, 10% and 20% alcohol dilutions ("Ethanol" subgroup; n = 6) during 32 weeks without any deprivation phase. After this period, these rats were euthanized to remove their brains.

2.3. Experiments

Three experiments were designed to achieve the planned aims.

2.3.1. Experiment 1: identification of two subpopulations of rats according to their alcohol relapse-like drinking behavior

In this experiment, we studied the manifestation of the ADE in the cohort of 43 rats of Experimental Group 1. Ethanol intake (expressed as g/kg/day), total fluid intake (expressed in mL), total ethanol preference (expressed in percentage as the quotient of mL of ethanol consumption and total fluid intake) and ethanol preference from every alcohol dilution (mL of 5, 10 or 20% ethanol consumption and total alcohol intake, expressed in percentage) were calculated individually. Basal values in each animal were determined by averaging the obtained values during

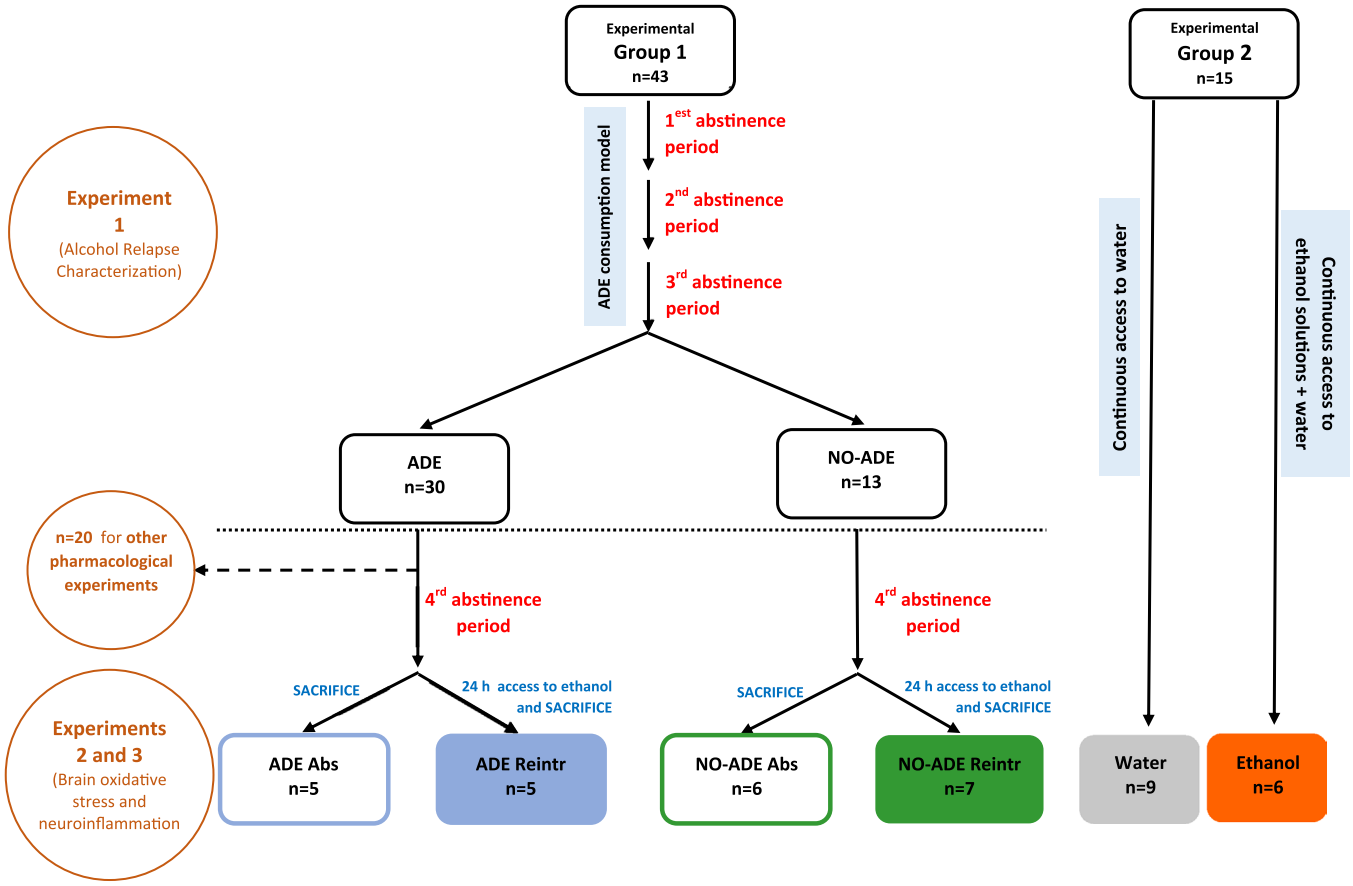

Fig. 1. Experimental protocol used in Experiment 1, 2 and 3. Animals from Experimental group 1 (n = 43) were categorised in two different subpopulations, ADE and NO-ADE, based on the manifestation of the Alcohol Deprivation Effect (ADE) (Experiment 1). For the development of experiments 2 and 3, 23 rats were selected. In a random-manner, 10 rats from the ADE and 13 rats from the NO-ADE subgroup were assigned to the ADE Abstinence (represented in framed blue; n = 5), ADE Reintroduction (solid blue; n = 5), NO-ADE Abstinence (framed green; n = 6) and NO-ADE Reintroduction (solid green; n = 7) subgroups. "Abstinence" animals were sacrificed after 21-day ethanol abstinence, while "Reintroduction" animals were sacrificed after 24-hours ethanol reintroduction. Animals from the second group of rats (Experimental group 2; n = 15) were distributed into two experimental subgroups, which were given continuous ad libitum access to tap water ("Water" subgroup; n = 9) or tap water and 5%, 10% and 20% alcohol dilutions ("Ethanol" subgroup; n = 6) during 32 weeks without any deprivation phase. After this period, all animals were sacrificed in order to obtain their brains. The same color legend has been applied in the other Figures. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

the last three pre-abstinence days. The measurements for the three days following the reintroduction of the ethanol bottles (post-abstinence days) were also calculated in order to determine the manifestation of the ADE phenomenon. N.B. the ADE is a marked and transient increase in the alcohol intake over basal values following a period of deprivation which is correlated with the loss of control associated with the alcohol relapse-like drinking behavior (Spanagel, 2017). All animals were subjected to three random abstinence periods. A positive ADE was considered when alcohol consumption increased by more than 50% with respect to the basal consumption determined before the deprivation period (Sinclair et al., 1973). Animals that, during the experiment, displayed two or three positive ADEs were assigned to the so-called "ADE" subgroup, while rats that expressed only one or no ADE episodes were assigned to the "NO-ADE" subgroup. After this first adscription, we proceeded in experiment 1 to characterize the drinking phenotype of both subgroups by analysing several variables such as: total ethanol intake (g/kg/day), total fluid intake (mL/day), weight (kg), total ethanol preference (%), and particular ethanol dilution preferences (%). For this characterization, data from the entire cohort of rats (n = 43) was used.

To perform Experiments 2 and 3 (see below), twenty-three of the forty-three rats in Experimental Group 1 above were forced to undergo a fourth period of abstinence. Of these twenty-three rats, eleven rats from the ADE (n = 5) and NO-ADE (n = 6) subgroups were sacrificed after 21-day of abstinence (hereinafter referred to as "ADE Abs" and "NO-ADE Abs" subgroups) and their brains were extracted. The twelve remaining rats were not only subjected to the fourth abstinence period but also, at the end of this period, they were allowed free access to alcohol for 24 h, after which they were euthanized and their brains processed as well. Hereinafter, we will refer to these 12 animals as "ADE Reintr" (n = 5) and "NO-ADE Reintr" (n = 7) subgroups. The remaining twenty of the forty-three rats in Experimental Group 1 were extracted from the present research and were used for other pharmacological experiments (see Fig. 1). Animals of the "Water" (n = 9) and "Ethanol" (n = 6) subgroups, belonging to Experimental Group 2, were used as control groups for experiments 2 and 3.

2.3.2. *Experiment 2: determination of brain oxidation levels*

Drug seeking and relapse are under the control of specific brain nuclei including the hippocampus, amygdala, PFC, insula and dorsal striatum (Koob and Volkow, 2016), so that experiment 2 and 3 focused on some of them. Probably, it would have been interesting to determine both oxidative and neuroinflammatory response in the same nuclei, however, the amount of biological material available led us to perform our study in different brain regions. For the measurement of reduced Glutathione (GSH) and oxidized Glutathione (GSSG) levels, the hippocampus and amygdala were dissected. Tissues were homogenized in phosphate buffered saline (PBS) and 10 mmol/L N-ethylmaleimide

(NEM) (Sigma-Aldrich, St. Louis, MO, USA) (pH 7.0), with a tissue-buffer ratio of 1:4. Then, perchloric acid solution was added to obtain a final concentration of 4% and samples were centrifuged at 11.000 rpm for 15 min at 4 °C. Supernatants were injected in the chromatographic system (UPLC-MS/MS).

The chromatographic system consisted of a Micromass QuatroTM triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Zspray electrospray ionization source operating in the positive ion mode with a LC-10A Shimadzu (Shimadzu, Kyoto, Japan) coupled to the MassLynx 4.1 software for data acquisition and processing. Samples were analyzed by reversed-phase UPLC with a C18 Mediterranea SEA column (Teknokroma, Barcelona, Spain) (5.060.21 cm) with 3 mm particle size. In all cases, 20 µl of the supernatant were injected onto the analytical column. The mobile phase consisted of the following gradient system (min/%A/%B) (A, 0.5% formic acid; B, isopropanol/acetonitrile 50/50; 0,5% formic acid): 5/100/0, 10/0/100, 15/0/100, 15.10/100/0, and 60/100/0. The flow rate was set at 0.2 mL/min. Positive ion electrospray tandem mass spectra were recorded with the electrospray capillary set at 3 kV and a source block temperature of 120 °C. Nitrogen was used as drying and nebulizing gas at flow rates of 500 and 30 L/h, respectively. Argon at 1.5610–3 mbar was used as collision gas for collision-induced dissociation. An assay based on UPLC-MS/MS with multiple reaction monitoring was developed using the transitions *m/z*, cone energy (V), collision energy (eV) and retention time (min) for each compound that represents favorable fragmentation pathways for these protonated molecules (Table 1). This procedure was previously validated under our experimental conditions (Rius-Pérez et al., 2020). Calibration curves were obtained using twelve-point (0.01–100 mmol/l) standards (purchased from Sigma-Aldrich, St Louis, USA) for each compound. The concentrations of metabolites were expressed as nmol/mg of protein.

2.3.3. Experiment 3: determination of neuroinflammation

The gene expression levels of different inflammatory mediators, such as TNF-α, IL-6, IL-1β, iNOS, Nfκβ, HMGB1 and NLRP3, were determined in brain prefrontal cortex (PFC). RNA was extracted using Trizol according to the manufacturer's instructions (Sigma). RNA was measured in a NanoDrop ND-1000 Spectrophotometer (260/280 nm ratio). First-strand cDNA synthesis was performed with the NZY First-Strand cDNA Synthesis Flexible PAck (NZYtech) using 1000 ng of total RNA according to the manufacturer's instructions. The RT-PCR reactions contained LightCycler 480 SYBR Green I Master (2 ×; Roche Applied Science), 5 µM forward and reverse primers, and 1 µl of cDNA. RT-PCR was performed in a LightCycler® 480 System (Roche). The relative expression ratio of a target/reference gene was calculated according to the Pfaffl equation (Pfaffl, 2001). Housekeeping cyclophilin A (PPIA) was used as an internal control. The sequences of primers used in this study are: *PPIA-F* 5′ TGTGCCAGGGTGGTGACTTT 3′, *PPIA-R* 5′ CGTTTGTGTTTGGTCCAGCAT 3′; *IL1β-F* 5′ CAGCAGCATCTCGACAAGAG 3′, *IL1β-R* 5′ CATCATCCCACGAGTCACAG 3′; *IL6-F* 5′ TGTGCAATGGCAATTCTGAT 3′, *IL6-R* 5′ CGGAACTCCAGAAGACCAGAG 3′; *TNFα-F* 5′ GGTGGGCTGGGTAACAAGTA 3′, *TNFα-R* 5′ AGGGACAAACCACAATATAGGAAAA 3′; *HMGB1-F* 5′ ATCTAAATACGGATTGCTCAGGAA 3′, *HMGB1-R* 5′ AGGGACAAACCACAATATAGGAAAA 3′; *iNOS-F* 5′ ACCAGCACCTACCAGCTCAA 3′, *iNOS*-R 5′ CCCTTTGTTGGTGGCATACT 3′; *Nfκβ-F* 5′ CAAGAGTGACGACAGGGAGAT 3′, *Nfκβ-R* 5′ GCCAGCAGCATCTTCACAT 3′. Fluorescence was recorded in the annealing/elongation step in each cycle. To check the specificity of the primers, a melting curve analysis was performed at the end of each PCR. All these procedures were previously validated in our laboratory (Alfonso-Loeches et al., 2014; Ureña-Peralta et al., 2020; Vallés et al., 2004).

Table 1.-
Transitions and retention times for analytes determined by LC–MS/MS.

Analyte	Cone (V)	Collision (eV)	Transition (*m/Z*)	Retention time (min)
GS-NEM	30	15	433 > 304	4.32
GSSG	30	25	613 > 355	1.46

2.4. Statistical analysis

To analyze, after each deprivation period (DP), the relapse-like drinking behavior in experiment 1, two statistical analyses were performed: first, a two-way repeated measures ANOVA with *time* being the within-groups factor and *subpopulation* the between-groups factor. Alcohol intake or preference along 6 days (three days before and after each DP were used. Secondly, the ethanol consumption or preference of the 3 days before and after the considered DP were collapsed and compared by using a paired Student's t-test. Moreover, ethanol intake and ethanol preference between the ADE and NO-ADE groups along the four consumption periods experienced were analyzed using a mixed two-way ANOVA, with *consumption period* being the within-groups factor and *subpopulation* the between-groups factor. For this comparison, data from individual rats were collapsed at each consumption period considered (Figs. 2D and 3D). In experiment 1, particular ethanol-dilution preferences between the ADE and NO-ADE groups along months were analyzed through a 3-way ANOVA with *months* being the within-groups factor and *ethanol dilution* and subpopulation the between-groups factors (Fig. 4A). Moreover, after collapsing data along time, a 2-way ANOVA was performed with *ethanol dilution* and *subpopulation* being the studied factors (Fig. 5B). Whenever significant differences were found, post-hoc adjusted Bonferroni tests were performed.

In experiments 2 and 3, a power analysis was performed that revealed a sample size of N = 5/group was determined necessary to detect the key variables at an α level of $p < 0.05\%$ and 80% power. In both experiments, levels of GSH, GSSG, GSH/GSSG and mRNA expression were analyzed using one-way analysis of variance (ANOVA). Additionally, a two-way ANOVA was also performed with *subpopulation* and *alcohol reintroduction* being the factors analyzed. Whenever significant differences were found, a post-hoc Tukey test was performed. All data are presented as mean ± standard error (SE). Analyses were carried out using GraphPad Prism v.8.0.1. and IBM SPSS Statistics v.26.

3. Results

3.1. Experiment 1: identification of two subpopulations of rats depending on their alcohol relapse-like drinking behavior

Based on the manifestation or not of the ADE phenomenon, experiment 1 allowed us to categorize our rats according to their relapse-like drinking behavior. According to the obtained results, 30 animals fitted the ADE group conditions, while the other 13 rats were assigned to the NO-ADE group. Fig. 2A shows the average alcohol intake, expressed as grams per kilogram per day, and the time course in both experimental groups. Data included in each framed-line rectangle (see Fig. 2A), were used to perform a two-way ANOVA for repeated measures at each DP. The significant effects on alcohol intake detected were: *time* [$F(5, 205) = 8.930$; $p < 0.001$], *subpopulation* [$F(1, 41) = 7.150$; $p = 0.011$] and *subpopulation x time* interaction [$F(5, 205) = 6.108$; $p < 0.001$] in DP-1; *time* [$F(5, 205) = 10.628$; $p < 0.001$] and *subpopulation x time* interaction [$F(5, 205) = 2.839$; $p = 0.017$] in DP-2; *subpopulation* [$F(1, 41) = 5.855$; $p = 0.020$] and *subpopulation x time* interaction [$F(5, 205) = 2.542$; $p = 0.029$] in DP-3. Data extracted and collapsed from Fig. 2A have been used to perform additional statistical analysis depicted in Fig. 2B, C and D.

As can be observed in Fig. 2B (ADE group) and according to paired t-tests, average ethanol intake increased significantly, with respect to the basal value, along the three consecutive deprivation periods assayed: [$T(29) = -7.521$; $p < 0.0001$], [$T(29) = -5.046$; $p < 0.0001$], and [$T(29) = -2.605$; $p = 0.014$], respectively, thus confirming the deprivation effect in this group of animals. On the other hand, animals assigned

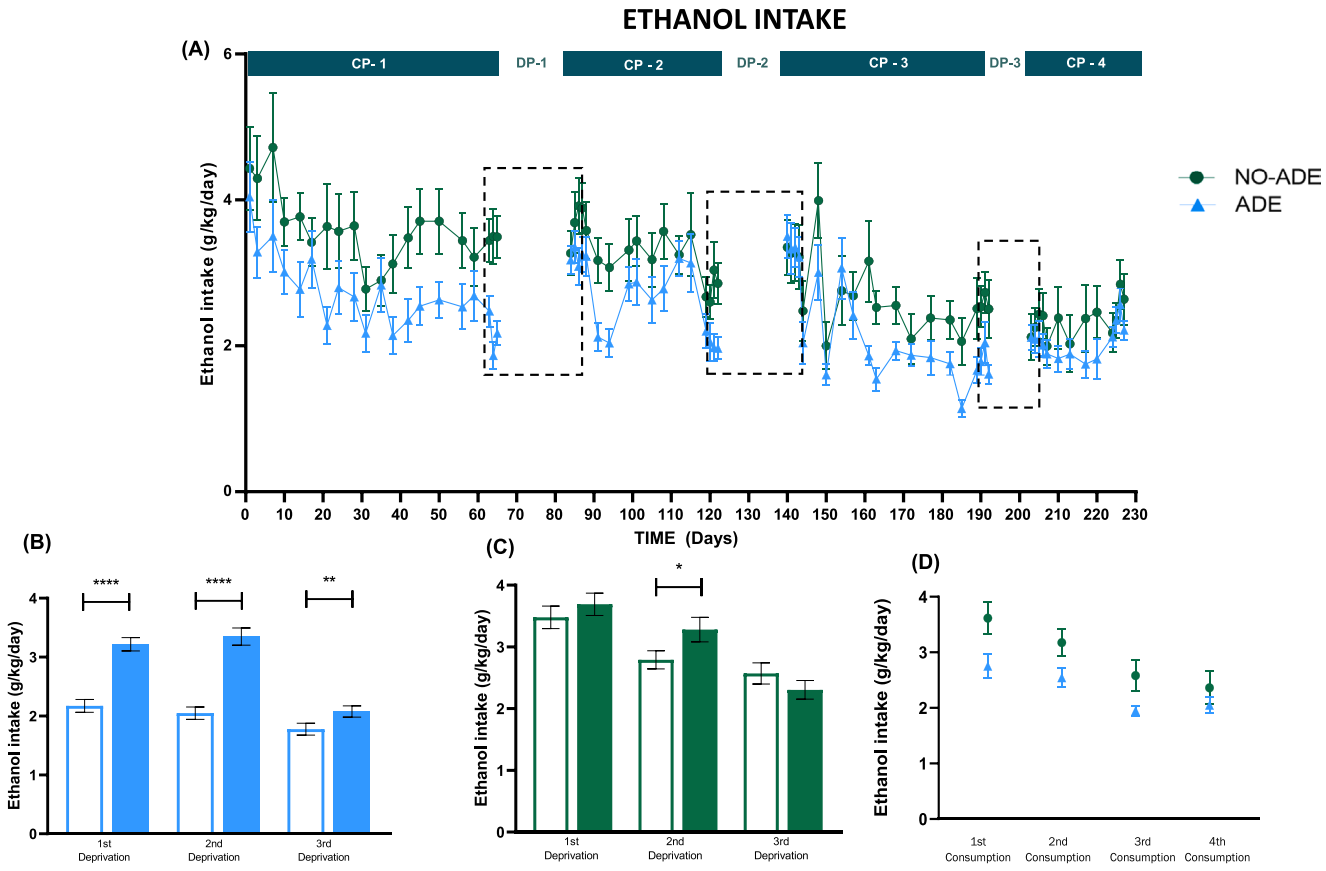

Fig. 2. Average voluntary ethanol intake, expressed in g/kg/day, displayed by rats categorized in the ADE (blue triangle, n = 30) or NO-ADE (green circle, n = 13) subgroups. (A) time course in both experimental groups along the entire experiment. As depicted, animals experienced 4 consumption (CP) and 3 deprivation periods (DP). (B and C) Manifestation of the ADE phenomenon in two different subpopulations. Bars represent the collapsed values of alcohol intake determined during the 3 days before and after each deprivation period in the ADE (B) and NO-ADE (C) subgroup. (D) Compared average voluntary ethanol intake between ADE and NO-ADE rats along the four ethanol-consumption periods assayed, excluding the 3 days post-abstinence in each period. Asterisks denote significant differences relative to the pre- and post-abstinence period (panel B and C) or between ADE and NO-ADE groups (panel D). [* $p < 0.05$; * * $p < 0.01$; * ** * $p < 0.0001$]. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

to the NO-ADE subgroup (see Fig. 2C) clearly showed a different relapse-like drinking behavior pattern. Specifically, neither in the first [$T(12) = -0.592$; $p = 0.564$] nor in the third [$T(12) = 1.391$; $p = 0.189$] deprivation period did animals display a significant increase in average ethanol intake. Moreover, differences detected in the second deprivation period were not very intense [$T(12) = -2.254$; $p = 0.044$], confirming that these animals tend not to show relapse behavior. Another differential behavioral trait between these two subpopulations of rats is the voluntary alcohol intake displayed along time. As can be observed in Fig. 2A, from the huge amount of data collected, on most days, ADE rats displayed a lower alcohol consumption than NO-ADE animals. The two-way ANOVA for repeated measures confirmed these observations. ANOVA revealed a significant *subpopulation* effect [$F(1, 41) = 5.485$; $p = 0.024$] and *consumption period* effect [$F(1, 41) = 5.485$; $p = 0.024$] on alcohol intake (Fig. 2D).

When alcohol preference was analyzed between the ADE and NO-ADE groups, the results obtained were even clearer. Fig. 3A shows the time course of average alcohol preference in both experimental groups (ADE and no ADE). Data included in each framed-line rectangle (see Fig. 3A), were used to perform a two-way ANOVA for repeated measures at each DP. The significant effects detected on alcohol preference were: *time* [$F(5, 205) = 8.976$; $p < 0.001$], *subpopulation* [$F(1, 41) = 10.621$; $p = 0.002$] and *subpopulation x time* interaction [$F(5205) = 3.383$; $p = 0.006$] on alcohol preference in DP-1; *time* [$F(5, 205) = 3.148$; $p = 0.009$], *subpopulation* [$F(1, 41) = 6.185$; $p = 0.017$] and *subpopulation x time* interaction [$F(5, 205) = 3.515$; $p = 0.005$] in DP-2; *time* [$F(5, 205) = 2.664$; $p = 0.023$] and *subpopulation* [$F(1, 41) = 13.489$; $p = 0.001$] in DP-3. As can be observed in Fig. 3B, average ethanol preference significantly increased from approximately 40% to nearly 60%, with respect to the basal value, along the three consecutive deprivation periods assayed: [$T(29) = -7.596$; $p < 0.0001$], [$T(29) = -5.407$; $p < 0.0001$], and [$T(29) = -3.084$; $p = 0.004$] in the ADE subgroup, thus re-confirming the expression of the ADE phenomenon in this subpopulation of rats. Conversely, animals assigned to the NO-ADE subgroup (Fig. 3C) did not show significant changes in their alcohol preference after experiencing a DP. In Fig. 3C, statistical analysis results were: $T(12) = -1.259$; $p = 0.231$], [$T(29) = -0.054$; $p = 0.957$] and [$T(12) = 1.064$; $p = 0.308$] for the 1st, 2nd and 3rd DP, respectively. Curiously, as can be observed in Fig. 3D, ADE rats displayed a lower ethanol preference than NO-ADE rats throughout the experiment. The two-way ANOVA for repeated measures confirmed these observations. ANOVA revealed a significant *subpopulation* effect [$F(1, 41) = 10.841$; $p = 0.002$] and *consumption period* effect [$F(1, 41) = 5.485$; $p = 0.024$] on alcohol preference (Fig. 3D).

Particular preferences for each ethanol dilution were also explored in both subpopulation of rats along time. As can be observed in Fig. 4, ADE and NO-ADE rats displayed, once again, differential behaviors. The three-way ANOVA for repeated measures confirmed these observations. ANOVA revealed a significant *ethanol dilution* effect [$F(2, 123) = 185.771$; $p < 0.001$] and *ethanol dilution* x subpopulation interaction effect [$F(2, 123) = 3.944$; $p = 0.022$] on particular alcohol preference (Fig. 4A). As the factor *months* revealed no statistical differences, an

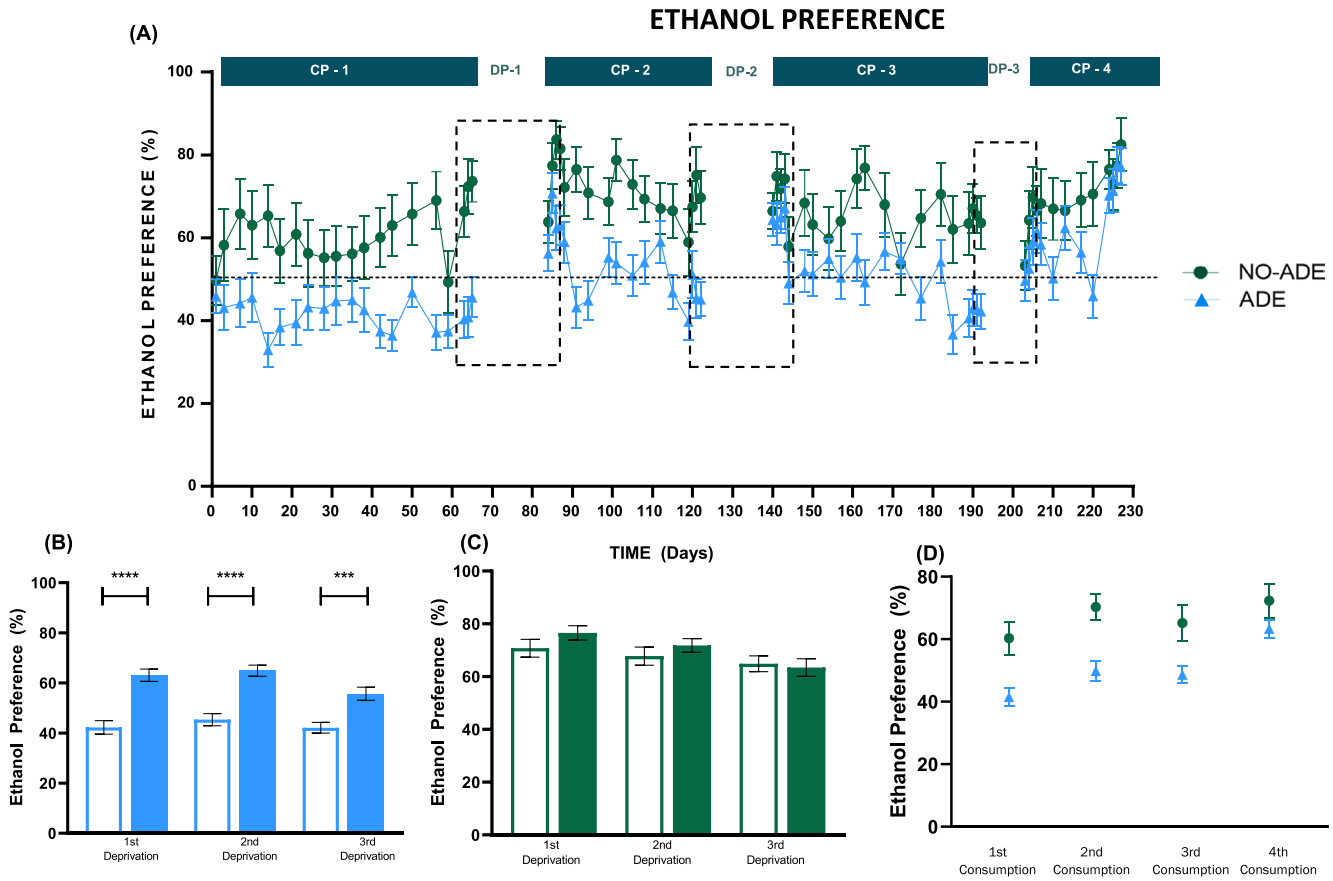

Fig. 3. Average ethanol preference, expressed in % of total fluid intake, displayed by ADE (blue triangle, n = 30) and NO-ADE (green circle, n = 13) rats. (A) time course in both experimental groups along the entire experiment. As depicted, animals experienced 4 consumption (CP) and 3 deprivation periods (DP). (B and C) Manifestation of the relapse-like drinking behavior in ADE and NO-ADE rats. Bars represent the collapsed values of alcohol preference determined during the 3 days before and after each deprivation period in the ADE (B) and NO-ADE (C) subgroup. (D) Compared average ethanol preference between ADE and NO-ADE rats along the four ethanol-consumption periods assayed, excluding the 3 days post-abstinence in each period. Asterisks denote significant differences relative to the pre- and post-abstinence period in ADE (panel B) and NO-ADE (panel C) subgroups. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

additional 2-way ANOVA, collapsing data, was performed. As depicted in Fig. 4B, animals depending on the subpopulation considered displayed a different alcohol consumption pattern, as the interaction between *subpopulation* x *ethanol dilution* ($F_{2,30} = 27{,}75$; $p < 0.001$) was statistically significant. As can be observed in this Figure, ADE animals showed a significant lower preference for 5% ethanol dilution in comparison with NO-ADE animals ($p < 0.001$), however a significant higher preference for 20% ethanol dilution was detected in ADE rats with respect to NO-ADE rats ($p < 0.001$).

The total fluid intake, i.e. the consumed volume of water and ethanol per day was also analysed. No statistical differences between the two experimental groups were found. ADE and NO-ADE rats consumed an average volume of 38.62 ± 0.71 and 35.56 ± 0.55 mL/day, respectively. Finally, the body weight of the animals was checked throughout the experiment. Their growth-curves did not differ from previous ones obtained in our laboratory (data not shown) (Orrico et al., 2013; Cano-Cebrián et al., 2021). At the end of the experiment, ADE and NO-ADE rats weighed 612.4 ± 9.4 and 581.7 ± 11.6 g, respectively. No significant differences between the two experimental groups were detected.

3.2. Experiment 2: determination of brain oxidation levels

To assess the possible oxidative stress by ethanol intake, the levels of GSSG and GSH were determined in two different brain regions: hippocampus (Fig. 5A, B) and amygdala (Fig. 6A, B). As the GSSG/GSH ratio is one of most common index of oxidative stress, it was also calculated (Figs. 5C, and 6C). As can be observed in Figs. 5C and 6C, under our experimental conditions, animals with long-term exposure to voluntary ethanol consumption did not show statistical differences with respect to control animals when the GSSG/GSH ratio in either the hippocampus or amygdala was analyzed. However, the most remarkable results were obtained within the hippocampus, where a rather large difference between the ADE and NO-ADE groups in the GSSG/GSH ratio was detected when animals were subjected to a 21-day deprivation period. In particular, as can be observed in Fig. 5C, rats of the ADE group suffered a great increase in oxidative stress levels during abstinence (4.147 ± 0.557) with respect to the water (1.378 ± 0.227; $p < 0.0001$) or ethanol exposed rats (2.005 ± 0.468; $p = 0.005$). However, the GSSG/GSH ratio in NO-ADE rats (0.917 ± 0.382) remained invariable with respect to water and ethanol groups during the abstinence period. Additionally, Tuckey's post-hoc test also detected statistical differences between the ADE Abs and NO-ADE Abs groups ($p < 0.0001$). Another interesting aspect that emerges from the results obtained is the role of alcohol when it is reintroduced. In particular, whereas high oxidative stress levels, as revealed by GSSG/GSH levels, were detected in ADE rats during abstinence, these levels were normalized after 24 h of ethanol reintroduction, decreasing from 4.147 ± 0.557–0.649 ± 0.272. These statistical results were confirmed through an additional analysis. Concretely, 2-way ANOVA revealed a significant subpopulation [$F(1, 14) = 28.730$; $p < 0.001$], alcohol reintroduction [$F(1, 14) = 38.670$; $p < 0.001$] and alcohol reintroduction x subpopulation interaction effect [$F(1, 14) = 25.210$; $p < 0.001$] on hippocampal GSSG/GSH ratio. It

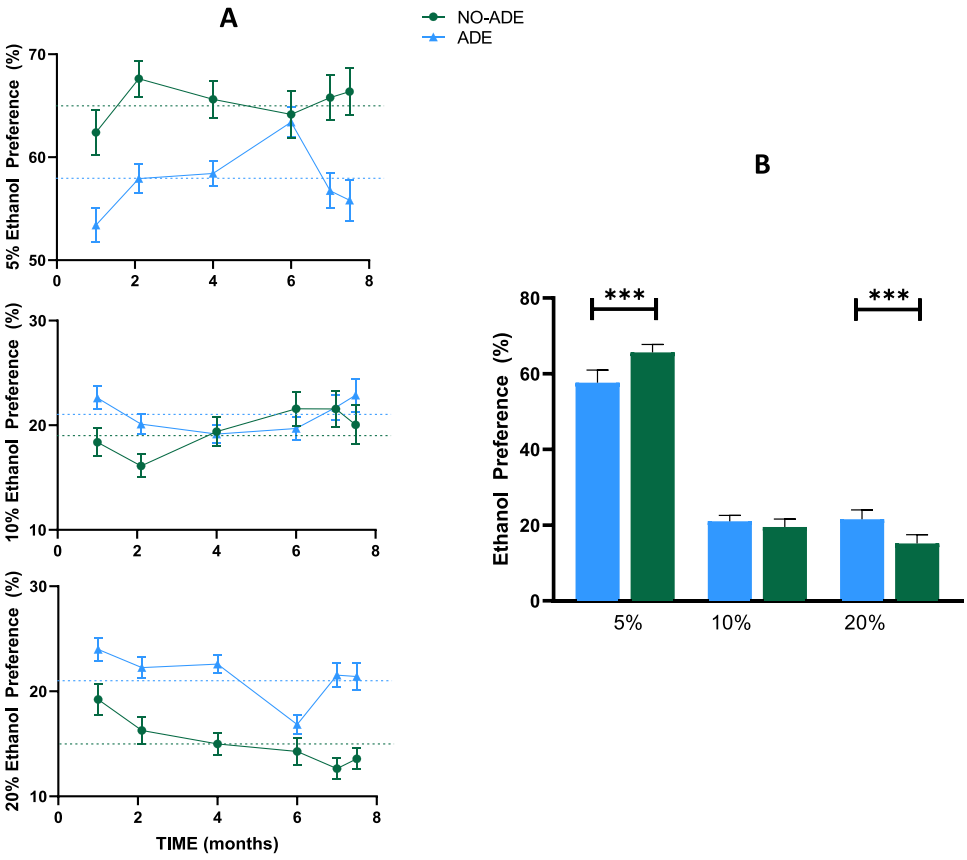

Fig. 4. (A) 5%, 10% and 20% ethanol preference, expressed in % of each solution with respect of the total volume of alcohol consumed, displayed by ADE (blue triangle, n = 30) and NO-ADE (green circle, n = 13) rats along time. As the three-way ANOVA for repeated measures did not reveal a significant time effect, an additional 2-way ANOVA using collapsed data was performed. These data are depicted in panel (B). Asterisks indicate significant differences between ADE and NO-ADE groups with respect to the preference displayed by rats depending on the different ethanol dilutions available [* ** $p < 0.001$]. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

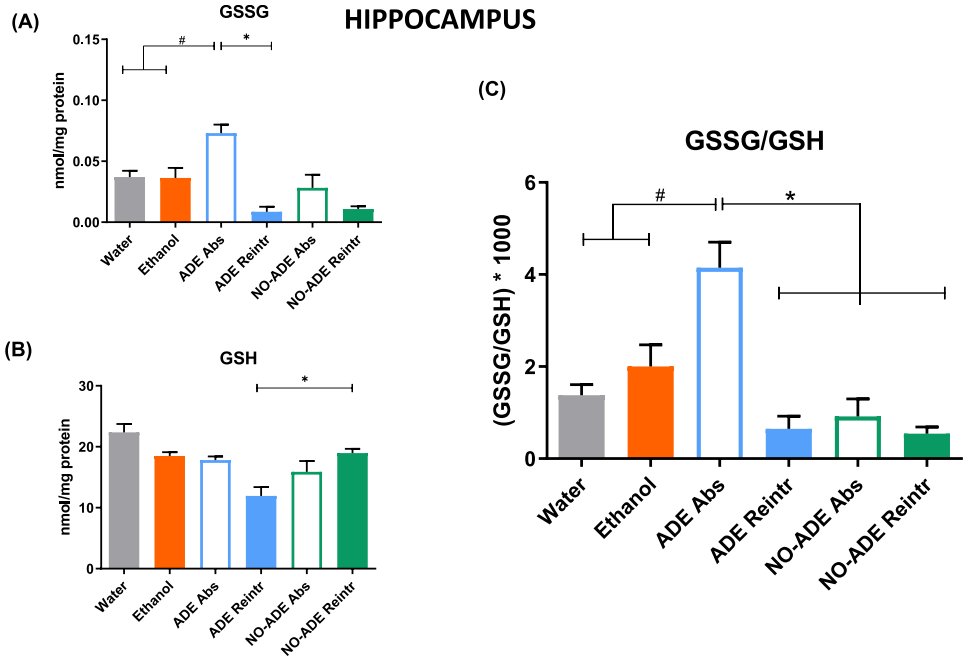

Fig. 5. (A) GSSG and (B) GSH levels, both expressed as nmol/mg protein, and (C) GSSH/GSH ratio determined in the **rat Hippocampus** under the different experimental conditions described in Fig. 1. The color legend is the same as the one described in Fig. 1. One-way ANOVA results were: (A) $F(5,28) = 8.523$; $p < 0.001$; (B) $F(5,27) = 8.320$; $p < 0.001$; (C) $F(5,24) = 13.770$; $p < 0.001$. Asterisk (*) indicates significant differences among groups, and the hash symbol (#) indicates significant differences with respect to "water" or "ethanol" groups.

is important to note that, in a general, GSSG levels displayed similar tends than that observed in the GSSG/GSH ratio. Thus, during abstinence a marked and significant increase in GSSG is noted only in ADE rats (0.073 ± 0.007 nmol/mg protein), when compared to either the Water (0.037 ± 0.005 nmol/mg protein; $p = 0.022$) or Ethanol groups (0.036 ± 0.008 nmol/mg protein; $p = 0.043$). Curiously, the reintroduction of ethanol in abstinent ADE rats rapidly alleviated this rise and restored GSSG levels (0.008 ± 0.004 nmol/ mg protein; $p < 0.0001$). As previously, 2-way ANOVA also detected a significant *subpopulation* [$F(1, 15) = 9.821$; $p = 0.007$], *alcohol reintroduction* [$F(1, 15) = 36.240$; $p < 0.001$] and *alcohol reintroduction* x *subpopulation* interaction [$F(1, 15) = 11.990$; $p = 0.003$] effects on hippocampal GSSG

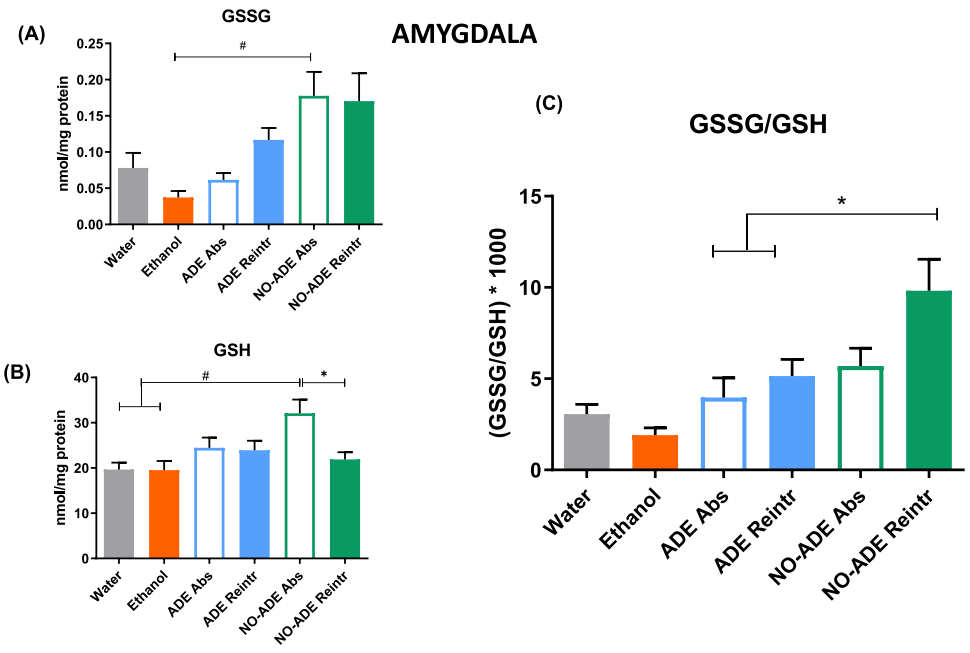

Fig. 6. (A) GSSG and (B) GSH levels, both expressed as nmol/mg protein, and (C) GSSH/GSH ratio determined in the **rat Amygdala** under the different experimental conditions described in Fig. 1. The color legend is the same as the one described in Fig. 1. One-way ANOVA results were: (A) $F(5,26) = 4.764$; $p = 0.032$ (B) $F(5,26) = 4.997$; $p = 0.0024$ (C) $F(5,22) = 8.156$; $p = 0.0002$. Asterisk (*) indicates significant differences among groups, and hash symbol (#) indicates significant differences with respect to the "water" or "ethanol" groups.

values.

Concerning data obtained in the amygdala, one-way ANOVA detected statistical differences when GSSG, GSH or GSSG/GSH ratio were analyzed. It is especially remarkable that during abstinence, GSSG levels in NO-ADE rats (0.178 ± 0.033 nmol/mg protein) were significantly higher ($p = 0.014$) when compared with those obtained in EtOH rats (0.037 ± 0.009 nmol/mg protein). Strikingly, this effect was not detected in ADE rats. Additionally, 2-way ANOVA revealed a significant subpopulation [$F(1, 15) = 7.033$; $p = 0.018$] effect on GSSG levels determined in the amygdala. Similar results were obtained when GSH levels were analyzed, suggesting the existence of biochemical differences between both subpopulations. On the other hand, GSSG/GSH values showed that NO-ADE Reint subgroup showed clear differences with respect to the other experimental conditions, an aspect that, from our present knowledge we are not able to reasonably interpret. Again, the existence of significant differences in GSH/GSSG ratio between the ADE Reint and NO-ADE Reint subgroups ($p = 0.036$) could be indicative of differences between the two subpopulations. The comparison

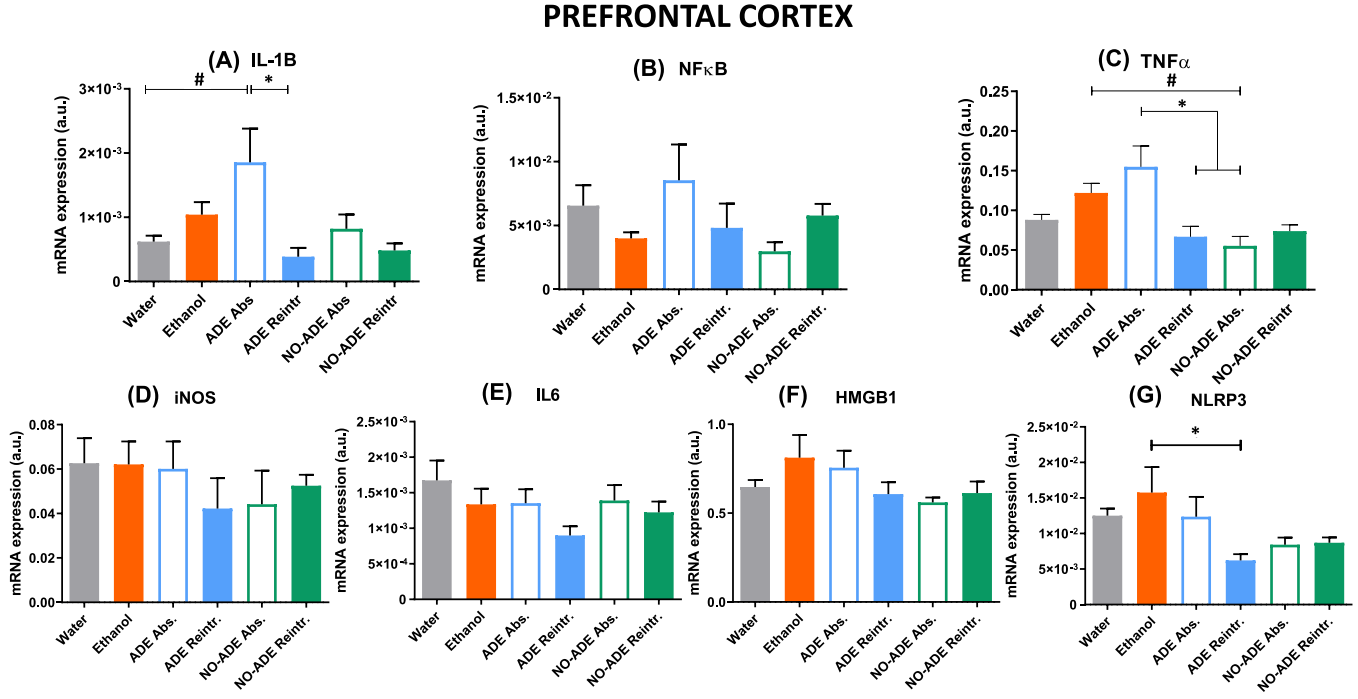

Fig. 7. Levels of (A) IL-1B, (B) NFκB, (C) TNFα, (D) iNOS, (E) IL6, (F) HMGB1 and (G) NLRP-3 mRNA expressed in arbitrary units in the **rat Prefrontal Cortex** under the experimental conditions described in Fig. 1. The color legend is the same as the one described in Fig. 1. One-way ANOVA results were: (A) $F(5,27) = 4.696$; $p = 0.003$ (B) $F(5,26) = 1.751$; $p = 0.158$ (C) $F(5,27) = 7.176$; $p = 0.0002$ (D) $F(5,25) = 0.643$; $p = 0.669$ (E) $F(5,28) = 1.443$; $p = 0.243$ (F) $F(5,27) = 1.449$; $p = 0.239$ (G) $F(5,25) = 3.027$; $p = 0.028$. Asterisk (*) indicates significant differences among groups, and the hash symbol (#) indicates significant differences with respect to the "water" or "ethanol" groups.

between the NO-ADE Abs and NO-ADE Reint group was almost significant ($p = 0.059$), suggesting the potential role of the reintroduction of alcohol in the GSSG/GSH values only in one of the identified subpopulations. Previous statistical results were confirmed through 2-way ANOVA, given that a significant *subpopulation* [$F(1, 13) = 7.117$; $p = 0.019$] and *alcohol reintroduction* [$F(1, 13) = 4.872$; $p = 0.045$] effect on the GSSG/GSH ratio were determined in the amygdala.

3.3. Experiment 3: analysis of the gene expression levels of neuroinflammatory mediators

The neuroinflammatory status was also analyzed in the ADE and NO-ADE rats. For this objective, under the same experimental conditions as in experiment 2, we evaluated the mRNA levels of different inflammatory modulators/mediators (IL-1B, IL6, TNFα, HMGB1, iNOS, NFκB and NLRP-3) in the PFC of the different groups of rats. The results obtained are depicted in Fig. 7. After three-week ethanol withdrawal, one-way ANOVA only detected statistical differences in the IL-1B, TNFα and NLRP3 mRNA expression. As can be observed in Fig. 7A, in the abstinence period, only ADE rats presented an increased IL-1B value of up to 200% with respect to the Water group. Tukey's test confirmed the existence of statistical differences between both experimental groups ($p = 0.014$). Conversely, after prolonged abstinence, NO-ADE rats showed no statistical differences with respect to rats from the Water ($p = 0.991$) or Ethanol group ($p = 0.987$). On the other hand, elevated IL-1B mRNA levels detected in ADE rats during abstinence rapidly normalized after 24 h ethanol reintroduction ($p = 0.0039$). This observation was confirmed by 2-way ANOVA given that a significant *alcohol reintroduction* [$F(1, 17) = 10.090$; $p = 0.006$] effect was determined. In the case of TNFα mRNA expression (Fig. 7C), during abstinence, ADE and NO-ADE showed statistical differences between them ($p = 0.0006$). The effects of alcohol reintroduction were analyzed as well. Again, it can be observed that the presence of ethanol, rapidly and significantly decreased TNFα mRNA expression in ADE rats ($p = 0.0024$). Similarly, in the case of TNFα mRNA expression, 2-way ANOVA confirmed a significant *subpopulation* [$F(1, 18) = 9.118$; $p = 0.007$], *alcohol reintroduction* [$F(1, 18) = 5.147$; $p = 0.036$] and *alcohol reintroduction* x *subpopulation* interaction [$F(1, 18) = 12.080$; $p = 0.003$] effects. Statistical analysis also detected differences in NLRP3 mRNA expression (Fig. 7E), but only between Ethanol and ADE Reintr conditions, a difficult comparison to interpret, given that in this case two aspects are involved: the abstinence experience and the alcohol reintroduction. A final observation should be noted: although statistical analysis could not confirm it, the rest of inflammatory modulators/mediators explored, always tended to decrease when alcohol was reintroduced in ADE subpopulation, i.e. when compared ADE Abs vs ADE Reintr.

4. Discussion

To the best of our knowledge, the present study provides the first evidence of both altered brain redox and neuroinflammation status after three-week ethanol abstinence in Wistar rats exposed to a long-term ethanol experience. In the current study, we demonstrate that oxidative and neuroinflammation status remain altered after long-term ethanol withdrawal. However, in our opinion, one of the most significant findings of the present study, is the fact that this effect was observed exclusively in rats that repeatedly displayed ethanol relapse-like drinking behavior. Moreover, according to our results, the reintroduction to alcohol consumption rapidly blunted these effects in this subpopulation of animals.

The understanding of the underlying neurobiology of relapse behavior could be crucial in the improvement of available treatments to reduce relapse, which is one of the main clinical problems related to AUDs. As the neurobiology of relapse behavior is difficult to study in patients, we have designed the present research applying the ADE model, which is, probably, one of the most commonly used preclinical approach to ethanol relapse-drinking behavior (Bell et al., 2012; Spanagel, 2017). This animal model encompassed the entire range of the addiction cycle, including acquisition and maintenance of drug taking, withdrawal and craving during periods of drug abstinence and ultimately relapse; processes that were repeated several times in this experimental model. All these facts support the model's high face and predictive validity (Leong et al., 2018; Bell et al., 2017). One of the few limitations of this animal model could be that the abstinence is not freely chosen by the rat. Thus, recently new rat models of relapse after voluntary abstinence (achieved either by introducing adverse consequences to drug taking or seeking or by providing mutually exclusive choices between the self-administered drug and nondrug rewards) are being developed. However, at present it is not known whether the use of these novel models will improve the predictive validity of classical relapse models because, to date, there are no published reports showing its postdictive validity using approved medications (Fredriksson et al., 2021).

Using a heterogeneous and non-selected cohort of rats as well as the aforementioned ADE model, we have been able to identify and categorize two different subpopulations of rats that showed specific behavioral endophenotypes related to their relapse-like drinking behavior. The obtained results also demonstrate that this behavioral trait correlates with their pattern of voluntary alcohol-drinking behavior. Specifically, animals with a high probability of displaying alcohol-relapse (called "ADE group") always showed lower alcohol intake rates than that observed in NO-ADE animals, as can be appreciated in Fig. 2D. Similar results were obtained when total ethanol preference was analyzed. Moreover, a great difference between both groups was detected when the particular pattern of ethanol consumption was analyzed (see Fig. 4). The present results also indicate that the applied criterion to categorize ADE/NO-ADE rats clearly allowed us to distinguish two subpopulations, of which ADE rats always displayed lower ethanol intake and lower ethanol preference, but a significantly higher preference for 20% ethanol dilution than NO-ADE rats. These results are consistent with previous observations extracted from the study of the ADE manifestation in various alcohol-preferring rat lines, which are widely used in alcohol research (Bell et al., 2017) since they were selected for their high ethanol preference or excessive alcohol drinking. After a single deprivation period, neither sP, nor HAD, nor AA lines show an ADE phenomenon (Agabio et al., 2000; Sinclair and Tiihonen, 1988; Vengeliene et al., 2003) and only alcohol-preferring P rats exhibit a robust ADE (McKinzie et al., 1998). In other words, these selective breeding rat lines emerged for the selection of animals characterized by their high ethanol preference or excessive alcohol drinking, and not because of their relapse-drinking behavior. Hence, according to the present results, it is plausible that the occurrence of the ADE is unlikely in these selected lines. It has been proposed that the high basal intake may explain the lack of ADE due to a ceiling effect (Vengeliene et al., 2014). In general, data obtained in the present paper demonstrate that male Wistar rats show specific behavioral endophenotypes related with their voluntary alcohol-drinking behavior (such as basal ethanol intake, total alcohol preference or particular preference for an alcohol dilution) that correlates with their relapse-like drinking behavior.

A great deal of evidence has been gathered demonstrating that alcohol triggers inflammatory responses and oxidative stress, especially after excessive consumption (Das and Vasudevan, 2007; Barcia et al., 2015; Quintanilla et al., 2018, 2007). The central nervous system is highly sensitive to oxidative stress, because of its high oxygen consumption and lipid content as well as its low antioxidant defense activity (Halliwell, 2006). Ethanol-induced hippocampal oxidative damage has also been well documented in rats (Almansa et al., 2013; Johnsen-Soriano et al., 2007; Quintanilla et al., 2018; Scolaro et al., 2012). However, under our experimental conditions GSH and GSSG levels were not modified by prolonged chronic ethanol consumption in the hippocampus nor amygdala. In previous papers in which significant effects were reported, rats were exposed to the Lieber-deCarli alcohol

liquid diet, that enhances the rate of alcohol consumed by the animal (Almansa et al., 2013; Johnsen-Soriano et al., 2007), or elevated ethanol doses (3 g/kg) were intraperitoneally administered (Scolaro et al., 2012). Nonetheless, when a lower ethanol dose was administered, for example by oral gavage (1.5 g/kg), no changes in GSH levels were observed in the frontal cortex, hippocampus or striatum of male Sprague–Dawley rats (Sommavilla et al., 2012). Similar conclusions can be reached when inflammatory responses are explored. Under our experimental conditions, prolonged chronic ethanol consumption did not alter any cytokine or inflammatory mediator. However, several studies have demonstrated that after chronic ethanol exposure, rats show significantly elevated cytokine expression in the hippocampus and cortex. Yet, in those experiments, once again, high ethanol doses were administered following a forced schedule (10 g/kg by oral gavage for 10 weeks), or animals were fed the Lieber-deCarli diet (5 months), respectively (Tiwari et al., 2009; Vallés et al., 2004). However, under the free-choice four-bottle paradigm used in our research, our male Wistar rats displayed a voluntary average ethanol intake of 2.21 ± 0.12 g/kg during 24 h, which is a low ethanol dosing rate compared to previous data reported. Our research is supported by the use of a high face, predictive and ecological validity model in the preclinical setting, the ADE model, in which alcohol consumption is voluntarily controlled by the animal and leads to a lower average alcohol dosage rate. According to obtained outcomes, this limited alcohol consumption was not enough to trigger significant oxidative/neuroinflammatory damage. However, and very interestingly, in ADE rats (animals that have displayed similar levels of ethanol intake but have experienced several abstinence periods) a robust brain redox disbalance after three-weeks abstinence was observed, suggesting the critical role that oxidative stress might play in triggering alcohol craving and relapse, as discussed in the next paragraph.

Once animals were separated in two clearly differentiated categories, i.e., rats that show relapse-like drinking behavior (ADE) or not (NO-ADE), the neurobiology of relapse behavior was explored after three-week alcohol withdrawal. Experiment 2 evidenced that after this prolonged abstinence period, a remarkable difference between the ADE and NO-ADE groups was detected in relation to brain oxidative status determined in the hippocampus, with the GSSG/GSH ratio being significantly higher in ADE than in NO-ADE rats. Our results correlate somewhat with clinical data according to which serum oxidative stress markers remain elevated after 1–2 weeks of alcohol detoxification (Huang et al., 2009). We additionally focused our studies on the amygdala as it is considered one possible locus for alcohol withdrawal-anxiety, a process related to craving and relapse behaviors (Harper et al., 2019). However, according to the results obtained, the quantification of the redox status during the abstinence period in the hippocampus has revealed more interesting results than in the amygdala. Similar conclusions were reached by Knapp et al. (2016) when several neuroimmune mRNAs in cortex, hippocampus, and amygdala were assessed in ethanol-withdrawn male rats. Strong increases in TNF-α, IL-1β and CCL-2 were detected both in the hippocampus and cortex. Nevertheless, no effect on any measure was detected in the amygdala.

With regard to neuroinflammation markers, although in the present research we analysed seven different neuroinflammation mediators in PFC, after a three-week ethanol abstinence period only the IL-1β and TNF-α mRNA expression remained altered and showed statistical differences between ADE and NO-ADE rats. Our results are in accordance with previous reports, using assimilable procedures to ours. Thus, Schneider et al. (2017) exposed male Wistar rats to 2 g/kg alcohol twice a day by oral gavage for 30 days. After a short alcohol cessation period (5 days), animals showed increased values of TNF-α, IL-1β, IL-6 and IL-18 in both, the hippocampus and frontal cortex. Knapp et al. (2016) reported that, rats that received a continuous 7% (w/v) ethanol diet followed by a 24-hour withdrawal period showed elevated cytokine levels in the cortex and hippocampus. In the clinical setting, Yen et al. (2017) reported that alcohol-dependent patients, during early withdrawal, demonstrated higher plasma cytokine levels than healthy controls ($p < 0.001$ for all cytokines analyzed: IL-2, IFN-γ, TNF-α, IL-4, IL-5 IL-6, IL-10, IL-1β, IL-8 and GM-GSF). However, after four weeks of alcohol abstinence, the levels of cytokine expression were significantly lower ($p < 0.001$; except for IL-1β and IL-5). In a study performed with 29 patients, after an average of 6 days of ethanol abstinence, increased serum levels of IL-6, IL-10 and IL-8 at the beginning of the abstinence period declined (González-Quintela et al., 2000). All in all, data evidence that the time course of each neuroinflammation mediator is different and depends on the brain nuclei considered. As can be observed, under our experimental conditions, only IL-1β and TNF-α remained altered after prolonged ethanol abstinence.

The effects of ethanol reintroduction in an ethanol-withdrawn population have also been explored in the present paper. Although alcohol is considered a prooxidant substance, our results evidence that it has different effects depending on the distinct endophenotypes displayed by the rats. In fact, when ethanol was reintroduced in ADE rats, it restored altered oxidative stress indicators as well as neuroinflammation markers such as IL-1β and TNF-α. This effect was not observed in NO-ADE rats. In our opinion, this result should be considered in future pharmacological research when an anti-relapse drug is being evaluated and the action mechanism is being explored, as not only the drug but also the alcohol could have an effect on the obtained results. For instance, when Quintanilla and colleagues (2008), evaluated N-Acetylcysteine (NAC) as a potential anti-relapse drug, it would have been very interesting to determine hippocampal GSSG and GSH levels before ethanol was reintroduced, and not only after a 60-minutes alcohol re-access, since the reduction in the GSSG/GSH ratio appreciated in the group treated with NAC, could be due to, at least in part, by ethanol reintroduction.

4.1. Conclusions

The main finding of this study is the demonstration that after a prolonged ethanol withdrawal, brain redox and neuroinflammation status remain altered, but only in animals that display ethanol-relapse behavior, possibly being a plausible key in the induction of the craving that will lead to the relapse process. According to the present results, the next step in our research should be the elucidation of the underlying mechanisms (precise causes), probably from the genetic point of view, that lead to the different redox/neuroinflammation status identified in the different individuals. These aspects could be considered a robust criterion for predicting the alcohol-addiction and non-addiction vulnerability of individuals. Additionally, our results gather mechanistic evidence concerning the use of antioxidant, antiinflammatories or combined therapies for preventing alcohol relapse, as currently being evaluated by different groups (Berríos-Cárcamo et al., 2020, Quintanilla et al., 2020). However, in future research, the effects induced by the ethanol reintroduction should be taken into consideration when the pharmacological mechanism of anti-relapse drugs are evaluated.

CRediT authorship contribution statement

All authors contributed substantially to this research, revised and approved the final version of this manuscript. **A. Polache:** Conceptualization, Writing – review & editing, Supervision. **S. Rius-Pérez:** Acquisition of data. **T. Zornoza:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision. **S. Fernández-Rodríguez:** Acquisition of data. **M.J. Cano-Cebrián:** Acquisition of data. **C. Guerri:** Methodology, Formal analysis. **S. Pérez:** Methodology, Formal analysis. **L. Granero:** Conceptualization, Writing – review & editing.

Conflict of interest

The Authors declare that there is no conflict of interest.

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