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Alcohol hangover induces nitric oxide metabolism changes by impairing NMDA receptor-PSD95-nNOS pathway

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ABSTRACT

Alcohol hangover is defined as the combination of mental and physical symptoms experienced the day after a single episode of heavy drinking, starting when blood alcohol concentration approaches zero. We previously evidenced increments in free radical generation and an imbalance in antioxidant defences in non-synaptic mitochondria and synaptosomes during hangover. It is widely known that acute alcohol exposure induces changes in nitric oxide (NO) production and blocks the binding of glutamate to NMDAR in central nervous system. Our aim was to evaluate the residual effect of acute ethanol exposure (hangover) on NO metabolism and the role of NMDA receptor-PSD95-nNOS pathway in non-synaptic mitochondria and synaptosomes from mouse brain cortex. Results obtained for the synaptosomes fraction showed a 37% decrease in NO total content, a 36% decrease in NOS activity and a 19% decrease in nNOS protein expression. The *in vitro* addition of glutamate to synaptosomes produced a concentration-dependent enhancement of NO production which was significantly lower in samples from hangover mice than in controls for all the glutamate concentrations tested. A similar patter of response was observed for nNOS activity being decreased both in basal conditions and after glutamate addition. In addition, synaptosomes exhibited a 64% and 15% reduction in NMDA receptor subunit GluN2B and PSD-95 protein expression, respectively. Together with this, glutamate-induced calcium entry was significant decreased in synaptosomes from alcohol-treated mice. On the other hand, in non-synaptic mitochondria, no significant differences were observed in NO content, NOS activity or nNOS protein expression. The expression of iNOS remained unaltered in synaptosomes and non-synaptic mitochondria. Here we demonstrated that hangover effects on NO metabolism are strongly evidenced in synaptosomes probably due to a disruption in NMDAR/PSD-95/nNOS pathway.

1. Introduction

The abuse of alcohol consumption induces adverse effects on multi-organs, including the liver, pancreas, heart, and central nervous system. Alcohol hangover constitutes the main negative consequence after binge drinking. This state is defined as the combination of mental and physical symptoms experienced the day after a single episode of heavy drinking, starting when blood alcohol concentration approaches zero [1]. We previously established that alcohol hangover induced substantial negative changes in motor and affective behavior during at least 20 h since hangover onset [2,3]. Since synaptosomes constitute a suitable approach to study bioenergetics and mitochondrial function in the synapses [4], we previously demonstrated that oxidative stress is generated at the onset of alcohol hangover. The increment in free radicals generation and the imbalance in antioxidant defenses were mostly observed in the synaptosomal fration revealing that mitochondria at the synapse were deeply affected [5].

Other authors evidenced that adolescent binge alcohol exposure affects brain function through mitochondrial impairment [6,7] and we recently verified that mitochondrial dysfunction triggered by acute ethanol exposure could be persistent and evidenced at the onset of the hangover state mainly at synaptic terminals [8]. In addition, it is known

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[1] Note of authors: AK and SLA regret to communicate that unfortunately our dear colleague Juanita Bustamante passed away by the time we were finishing the manuscript. We will never forget her huge enthusiasm for scientific research and wish to express our profound recognition and admiration for her work.

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that acute alcohol exposure can lead to changes in nitric oxide (NO) production, which is associated with different cognitive impairments such as memory and learning deficiencies [9].

Interestingly, we previously observed that ethanol hangover does not only impair mitochondrial function but also NO metabolism in crude mitochondrial fractions from brain cortex and cerebellum [10,11].

The activation of N-methyl-D-aspartate NMDA receptors (NMDARs) plays a central role in essential physiological processes [12]. It is widely known that ethanol blocks the binding of glutamate to NMDAR among other effects that compromise central nervous system [13]. NMDAR physiological activation results in the opening of its ion channel allowing calcium influx to the cell. Neuronal nitric oxide synthase (nNOS) is activated by calcium entry resulting in an increased NO production [14]; this activation pathway seems to be critically dependent on the postsynaptic density protein-95 (PSD-95), a scaffolding protein which binds simultaneously to the NMDAR and nNOS via its two N-terminal PDZ (PSD-95/Discs large/zona occludens-1) domains [15]. Thus, PSD-95 mediates a specific association between NMDAR activation, Ca^{2+} influx and NO production [15]. It was reported that PSD-95 changes could be associated with alcohol-induced behaviour changes, being also this protein postulated as a key mediator of the effects of multiple abuse drugs. For instance, it was demonstrated that PSD-95 knockout mice presented an exacerbated sensibility to acute ethanol exposure compared with wild-type animals [16]. Moreover, it was established that PSD95-nNOS interaction is critical for synaptic connections [17], and therefore, prolonged suppression of PSD95-nNOS signaling may lead to unknown risks [18].

There are no previous reports focusing on the study of NO metabolism associated to NMDAR-PSD-95 impairment due to alcohol aftereffects (hangover). In line with this, and considering our previous research, it was of interest to study the mechanisms involved in the changes in NO production at the hangover state and to analyze if NO production alterations at the synapses could be associated to NMDAR-PSD-95 impairment.

Therefore, the aim of the work was to study alcohol residual effects on NO metabolism by detecting NO total content and NOS activity and expression in non-synaptic mitochondria and synaptosomes from mouse brain cortex. Moreover, the role of NMDA activation on NO content and production together with NMDAR GluN2B subunit and PSD-95 protein expression were evaluated in the same experimental model of alcohol hangover.

2. Materials and methods

2.1. Materials

$CaCl_2$, catalase, p-coumaric acid, dithiothreitol (DTT), EDTA, EGTA, FFA-BSA, Folin reagent, glutamic acid, glutathione, Hepes, H_2O_2, HRP, KH_2PO_4, KCl, K_2HPO_4, KCN, L-arginine, luminol, malic acid, mannitol, $MgCl_2$, NADPH, NaH_2PO_4, Na_2HPO_4, NaN_3, NO_2, Nω-Nitro-L-arginine (L-NNA), oxyhemoglobin, sodium dodecilsulphate (SDS), superoxide dismutase (SOD), succinate, sucrose, Trizma base, Tween were obtained from Sigma Chemical Co. (Saint Louis, Missouri, United States). Acrylamide, APS, 2-mercaptoethanol, bisacrylamide, Laemmli buffer, and tetramethylethylenediamine (TEMED) were acquired from Bio-Rad California, USA. The probes DAF-2 and DAF-2 DA were purchased from Calbiochem, San Diego, CA, USA. Goat polyclonal antibody anti-PSD-95 (RRID:AB_298846), rabbit anti-NMDA receptor GluN2B subunit polyclonal antibody (RRID:AB_2247794), rabbit polyclonal antibody anti-inducible nitric oxide synthase (iNOS) (RRID:AB_2152861), mouse monoclonal antibody for β-actin (RRID: AB_626632) and mouse monoclonal antibody for β-tubuline (RRID: AB_628408) were acquired from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA or from AbcamCambridge, CB2 0AX, UK. Goat voltage-dependent anion channel antibody (VDAC, RRID: AB_793935) was obtained from Molecular Probes Inc., Eugene, Oregon, United States. All other reagents were of analytical grade.

2.2. Animals

Male Swiss mice (*Mus musculus*) (RRID: MGI_5906796) weighing 30 g were used. Animals were from Harlan Laboratories, Indianapolis, Indiana, United States, without genetic modifications. Mice were bred in the Animal Housing of Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires) (ID 13208537, CrlFfyb:SW). Animals were housed in a soundproof room, under controlled temperature (22 ± 2 °C) and humidity, with a 12:12-h light:dark cycle photoperiod (lights on at 7:00 a.m.), and received standard rat chow and tap water *ad libitum*. Animal handling and treatment, as well as all experimental procedures were reviewed in accordance with the guidelines of the National Institute of Health (USA), and with the 6344/96 regulation of the Argentinean National Drug Food and Medical Technology Administration (ANMAT). Additionally, the present study had the legal ethical accreditation from the Ethics Committee for Laboratory Animal Handling of Facultad de Farmacia y Bioquímica from Universidad de Buenos Aires where the protocol was performed (Res. 1019–2019). All efforts were made to minimize suffering and reduce the number of animals used.

2.3. Experimental procedures

2.3.1. Alcohol hangover model

Ethanol (15% w/v) was prepared by diluting a 95% stock solution of ethanol with 0.9% saline solution (SS). Animals received an injection (i.p.) of ethanol (3.8 g/kg body weight) or saline (8:00 a.m.). Standard rat chow and tap water were available *ad libitum* and there was no fasting period before or after ethanol injection. The choice for ethanol dose was based on previous studies [19–21]. The selection of i.p. administration and the criterion for hangover onset at 6 h after ethanol injection were based on our previous work. Moreover, we previously demonstrated by using this experimental model, that alcohol hangover provoked significant motor and affective behavior impairment, which was also associated with oxidative stress and mitochondrial dysfunction in non-synaptic mitochondria and synaptosomes from brain cortex [3,5,8,10,11,22]. For the purpose of the present work, observations of animals' behavior (locomotion, self-grooming, signs of aggression or hyperactivity, etc.) in their home cages were conducted to keep the experiment similar to other studies.

2.3.2. Isolation of subcellular fractions

Six hours after injection, at the onset of alcohol hangover, animals were sacrificed by cervical dislocation in accordance with the directive systems of protection of vertebrate animals for scientific research. Brains were weighed, and the brain cortices were dissected and homogenized at a ratio of 1 g cortex/5 mL in a medium consisting of 230 mM mannitol, 70 mM sucrose, and 5 mM Hepes (pH 7.4), supplemented with 1 mM EDTA (MSHE). Homogenates were centrifuged at 600×*g* for 10 min at 4 °C to discard nuclei and cell debris. The supernatant was decanted and centrifuged again at 8000×*g* for 10 min; the resulting pellet was washed and resuspended in MSH buffer and the last supernatant obtained was designated as the cytosolic fraction.

Further mitochondrial purification and synaptosomal fraction separation were performed by Ficoll gradient [23] with modifications. The crude mitochondrial fraction was resuspended in MSHE buffer and layered on Ficoll gradients containing steps of 13%, 8%, and 3% Ficoll [24]. The gradients were centrifuged at 11500×*g* for 30 min. After centrifugation, the original sample was separated into two fractions: a pellet at the bottom of the tube, corresponding to a fraction of heavy mitochondria that are mainly non-synaptic, and the fraction occurring at the 8% layer that contained synaptosomes [25]. This subcellular fraction was washed in MSH buffer (0.23 M mannitol, 0.07 M sucrose, and 5 mM HEPES; pH = 7.4) by centrifugation at 11500×*g* for 10 min. Finally, synaptosomes were resuspended in MSH buffer. The whole procedure

was carried out at 0–2 °C. Submitochondrial membranes were obtained from mitochondria by twice freezing, thawing, and homogenizing by passing the suspension through a 15/10 hypodermic needle [26]. Protein concentration was determined by the Folin phenol reagent, using bovine serum albumin as the standard, according to Lowry (1951) [27]; this process was used to normalize the results obtained for each subcellular fraction.

Mitochondrial yield of subcellular preparations was estimated as previously described by our group by the determination of the activity of monoamine oxidase (MAO), both in total homogenates and in mitochondrial or synaptosomal fractions from control animals; the estimate was 30–45 mg mitochondrial protein/g brain tissue, both for non-synaptic mitochondria and synaptosomal fractions. Non-synaptic mitochondria were less than 5% contaminated with synaptosomal components, according to acetylcholinesterase activity determinations [28]. In addition, using a detailed flow cytometry analysis, we recently demonstrated that both fractions contained NAO-positive particles, indicating the presence of mitochondria either free or inside the synaptosomes [25].

2.3.3. Nitric oxide content detection by flow cytometry

Nitric oxide total content was determined in freshly prepared synaptosomes and non-synaptic mitochondria fractions by a cytometric method based on the use of potentiometric probe DAF-2 DA for synaptosomes or DAF-2 for non-synaptic mitochondria [29]. The probe DAF-2 DA penetrates through the synaptosome membrane, and acetate esters are hydrolyzed by cytosolic esterases yielding DAF-2, which can react with NO forming the highly fluorescing triazolofluorescein (DAF-2T; λ_{exc} = 495 nm/λ_{em} = 515 nm). Synaptosomes or non-synaptic mitochondria (25 μg protein/mL) were incubated at 37 °C for 30 min in the presence of 10 μM DAF-2-DA (synaptosomes) or DAF-2 (non-synaptic mitochondria) in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, 2 mM phosphate, 1 mM $MgCl_2$. Sample incubation medium for synaptosomes also contained 1.3 mM $CaCl_2$. Samples were protected from light until acquired by the FACSCalibur flow cytometer (Becton--Dickinson, USA). Auto-fluorescence was evaluated in samples without the probe. In addition, 5 mM GSNO and 1 mM L-NNA were added as positive and negative controls, respectively. Furthermore, the role of NMDAR activation on NO content was analyzed in synaptosomes by preincubating with glutamate in different concentrations (0–2.5 mM) before flow cytometer adquisition.

DAF-2T fluorescence was analyzed using the median value of the distribution of fluorescence events for each treatment. A common marker (M1) was fixed on control median value representing approximately 50% of the fluorescent events. Histogram differences in DAF-2T fluorescence were quantified as the number of events which drop under the median value of the relative fluorescence distribution corresponding to M1. A higher number of DAF-2T relative fluorescence events under M1 would reflect decreased NO levels. Quantification of results was shown as bar graphs in which data were expressed as the percentage of control DAF-2T relative fluorescence intensity taking control as 100%.

2.3.4. Nitric oxide synthase activity by spectrophotometry

Nitric oxide production was measured in brain cortex synaptosomal membranes and non-synaptic mitochondria (0.1–0.5 mg protein/mL) by using a double-beam dual wavelength spectrophotometer, following the oxidation of oxyhemoglobin to methemoglobin at 577–591 nm ($\varepsilon_{577\text{-}591}$ = 11.2 mM^{-1}. cm^{-1}) at 37 °C [30,31]. The reaction medium contained 50 mM phosphate buffer (pH = 7.4 for synaptosomal membranes and pH = 5.8 for non-synaptic mitochondria), 50 μM L-arginine, 100 μM NADPH, 10 μM DTT, 1 mM $CaCl_2$ and 25 μM oxyhemoglobin (expressed per heme group). In order to avoid the presence of $O_2^{\cdot-}$ and H_2O_2, 4 μM Cu–Zn superoxide dismutase and 0.1 μM catalase were also added to the reaction medium.

It was previously demonstrated that NO production, detected by this technique, was 73% reduced by the competitive NOS inhibitor L-NNA [26,30].

Additionally, nNOS activity dependent on NMDAR activity was analyzed in synaptosomes by adding glutamate. Thus, synaptosomal membranes were incubated 2 min with increasing glutamate concentrations (0–2.5 mM) and nNOS enzymatic activity was determined as described above. Results were expressed as nmol NO produced per minute per milligram protein.

2.3.5. Western blot assays

Protein expression of nNOS and iNOS in synaptosomal membranes and non-synaptic mitochondria was analized by Western blot assays. In addition, in synaptosomal membranes, NMDA receptor GluN2B subunit and PSD-95 protein expression was determined. For this purpose, sample fractions (80 μg protein) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (ranging from 7.5% to 12%, depending on the proteins), blotted onto a nitrocellulose membrane (Bio-Rad, München, Germany) and probed primarily with primary antibodies (dilution 1:500), as follows: rabbit polyclonal antibodies against neuronal and inducible nitric oxide synthase (nNOS & iNOS), rabbit polyclonal antibodies for NMDA GluN2B subunit, goat polyclonal antibodies for PSD-95. As loading controls, mouse monoclonal antibodies for β-tubulin were used in a dilution 1:1000. After that, the nitrocellulose membrane was incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:5000), as follows: anti-goat for PSD-95, anti-rabbit for Glu N2B subunit of NMDA receptor, nNOS and iNOS, and anti-mouse for β-tubulin, followed by chemiluminescence reaction of 0.2 mM coumaric acid and 1.25 mM luminol in the presence of 1.76 mM H_2O_2 for 2 min and revealed on X-ray films using a Hypercassette™ from Amersham Life Science. Densitometric analysis of control and treated bands were evaluated through NIH Image J 1.47b software.

2.3.6. Calcium influx by glutamate

Evaluation of calcium uptake was carried out in freshly isolated synaptosomes from control and alcohol-treated mice by flow cytometry. Cytometric assay was based on the use of the acetoxymethyl ester derivative of the green fluorescent indicator Fluo-4 acetoxymethyl ester (FLUO-4AM; λ_{exc} = 494 nm/λ_{em} = 506 nm). Synaptosomes (25 μg protein/mL) were incubated at 37 °C for 20 min in the presence of 230 nM FLUO-4AM in buffer containing 120 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM Na_2SO_4, 0.015 M D-glucose, 0.01 M pyruvate, 10 mM Hepes, 0.4% (w/v) FFA-BSA and 1.3 mM $CaCl_2$. To assess the possible role of calcium on the impairment of NO production after NMDAR activation at the onset of alcohol hangover, fluorometric signal was detected before and after 1 mM glutamate stimulus. Samples were protected from light until acquired by the FACSCalibur flow cytometer (Becton-Dickinson, USA). Auto-fluorescence was evaluated in samples without the probe. In addition, 5 μM ionomycin and 1 mM EGTA-EDTA were added as positive and negative controls, respectively [32].

FLUO-4AM fluorescence was analyzed using the median value of the distribution of fluorescence events for each treatment. A common marker (M1) was fixed on control histogram fluorescence events. Differences in FLUO-4AM relative fluorescence were quantified as the number of events which drop under M1. A higher number of FLUO-4AM relative fluorescence events under M1 would reflect an increase in Ca^{2+} influx. Quantification of data was shown as a bar graph showing the percentage of FLUO-4AM relative fluorescence intensity taking control at basal condition as 100%.

2.4. Statistical analysis

Data were checked for normality before each analysis. For this, all data were evaluated by the Kolmogorov-Smirnov test to follow a posterior parametric or nonparametric statistical analysis. Since data followed a parametric distribution, results were presented as mean ± SEM. The analysis of the results was performed using unpaired Student *t*-test or ANOVA to analyze the significance of differences between groups.

IBM SPSS Statistics (22.0 version) software was used and a difference was statistically significant when $p < 0.05$.

3. Results

3.1. Nitric oxide content

Nitric oxide total content was detected by flow cytometry using DAF-2DA and DAF-2 probes in synaptosomes and non-synaptic mitochondria respectively. Results of synaptosomes NO content are shown in Fig. 1A while the same for non-synaptic mitochondria are shown in Fig. 1B.

Dot plots of Forward Scatter-Height (FSC–H) versus Side Scatter-Height (SSC–H) indicating the gated synaptosomes or non-synaptic mitochondria populations from control samples are shown in Fig. 1A (i) and 1B (i), respectively. Histograms showing DAF relative intensity fluorescence for a typical experiment are shown for control and alcohol conditions for each subcellular fraction (Fig. 1A ii, iii and Fig. 1B ii, iii). Overlaid histograms were included to simplify the qualitative visualization of the results (Fig. 1A iv and Fig. 1B iv). Bar graphs show percentage of control DAF-2T relative fluorescence intensity taking control as 100% (Fig. 1A v and Fig. 1B v).

Synaptosomes showed a 37% decrease in NO total content ($p < 0.05$; Fig. 1A v). In this subcellular fraction, the preincubation with L-NNA as negative control induced a 58% decrease in NO content ($p < 0.05$, Fig. 1A v) while the addition of a NO donor, GSNO, increased DAF-2T fluorescence by 96% ($p < 0.05$, Fig. 1A v).

No significant differences were observed in NO content in non-synaptic mitochondria (Fig. 1B v). As expected, DAF-2T relative fluorescence intensity decreased 38% after L-NNA pre-incubation ($p < 0.05$; Fig. 1B v) and increased 97% after the addition of GSNO (Fig. 1B v).

3.2. Nitric oxide synthase activity

In order to verify if differences in NO total content in fresh subcellular fractions could be explained by differences in enzyme activity, NO production was determined in synaptosomal and non-synaptic mitochondrial membranes at the onset of alcohol hangover. Enzyme activity resulted to be 36% decreased by alcohol hangover in synaptosomes as compared with controls ($p < 0.05$, Fig. 2A). On the other hand, no significant differences were observed between alcohol-treated mice and controls in non-synaptic mitochondria (Fig. 2B).

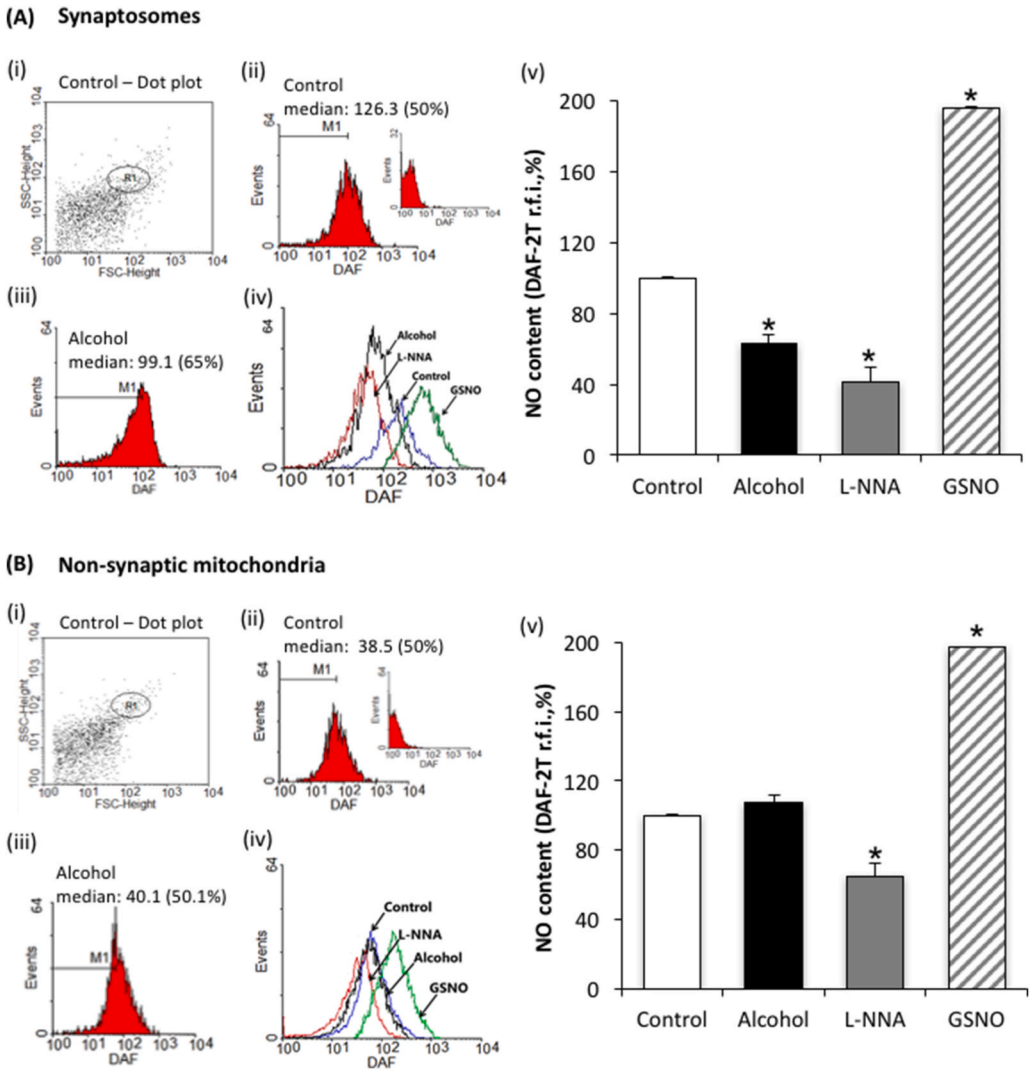

Fig. 1. Effect of alcohol hangover on nitric oxide (NO) content in synaptosomes and non-synaptic mitochondria isolated from mouse brain cortex. Brain cortex subcellular fractions were loaded with the probe DAF-2-DA and DAF-2 for synaptosomes (A) and non-synaptic mitochondria (B) respectively and direct measurements of NO were obtained by flow cytometry. Typical dot plot of FSC-H versus SSC-H indicating a gated mitochondrial population (R1) for control mice are shown in panels (i) for both subcellular fractions. Typical histograms of gated events versus relative fluorescence intensity (FL-1) are shown for control- or alcohol-treated mice in panel (ii) and (iii) respectively for both subcellular fractions. Samples without probe used for autofluorescence are presented as insets. Negative and positive controls were carried out in the presence of 1 mM L-NNA and 5 mM S-nitrosoglutathione (S-nitrosylating agent) (GSNO) respectively. Typical overlapped histograms showing control, alcohol, L-NNA and GSNO experimental conditions are shown in panel (iv) for both subcellular fractions. Bar graph quantification of DAF-2T relative fluorescence events is shown in panel (v). Fluorescence events were quantified as the number of events which drop under a common marker M_1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. Bars represent the mean ± SEM. The cytometric analysis was performed three times for each treatment (control- and alcohol-treated mice). Experiments were performed in triplicate ($n = 9$, corresponds to the number of total assays for each experimental condition). $p < 0.05$ compared with control; ANOVA, Bonferroni's test.

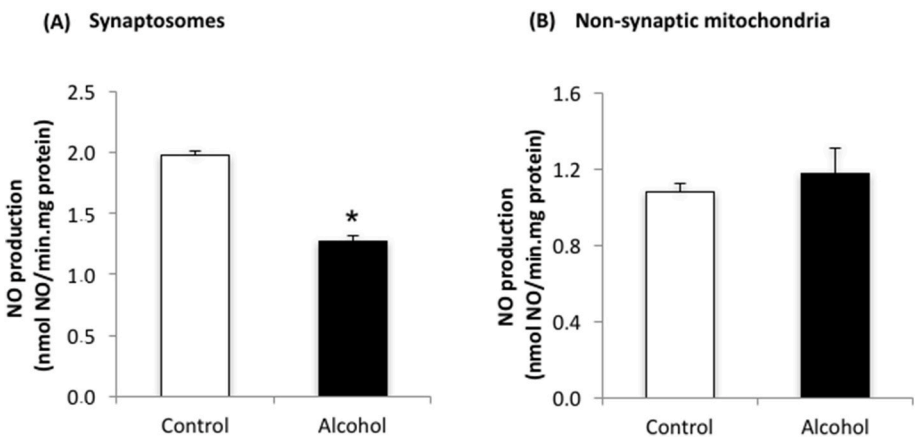

Fig. 2. Effect of alcohol hangover on nitric oxide synthase activity in synaptosomes and non-synaptic mitochondria isolated from mouse brain cortex. Nitric oxide synthase activity was determined in synaptosomal and non-synaptic mitochondrial membranes in both control- and alcohol-treated mice following the oxidation of oxyhemoglobin to methemoglobin at 577–591 nm. Results are expressed as specific enzyme activity. The whole procedure for mice treatment and subcellular fractionation was replicated three times and NOS activity assays were performed in triplicate (n = 9, corresponds to the number of total assays for each experimental condition). Data are shown as mean ± SEM. *p < 0.05, compared with control; Student's *t*-Test for independent sample comparisons.

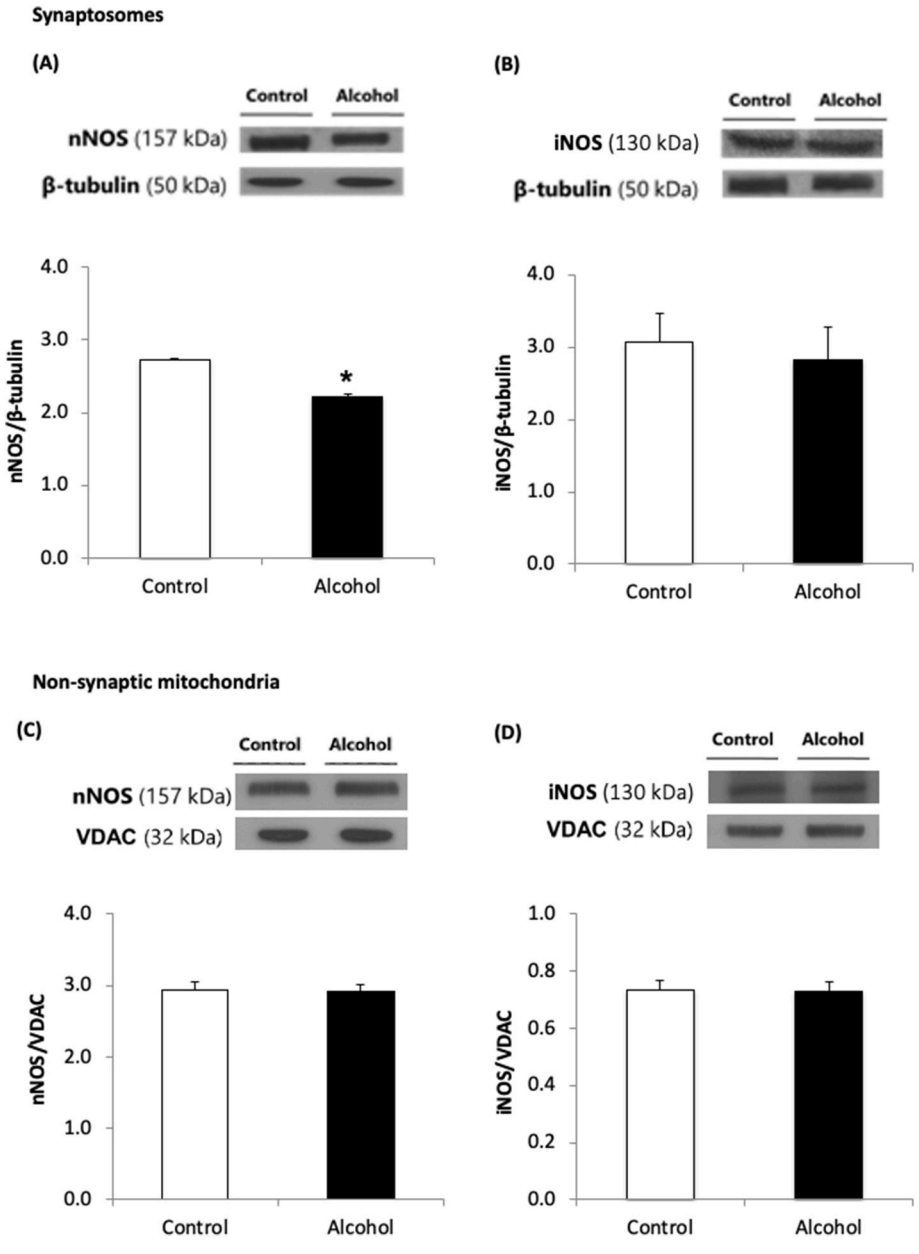

Fig. 3. Effect of alcohol hangover on nNOS and iNOS protein expression in synaptosomal and non-synaptic mitochondrial membranes. Western blots of nNOS and iNOS were carried out in both subcellular fractions for control- and alcohol-treated mice. Results show immunoblots from a single representative Western blot assay and the ratio between the expression of nNOS or iNOS versus that of β-tubulin (for synaptosomes) or VDAC (for non-synaptic mitochondria). The whole procedure for mice treatment and subcellular fractionation was replicated three times and membrane samples were processed in quadrupled for Western blot assays (n = 12, corresponds to the number of total assays for each experimental condition). Data are expressed as mean ± SEM. *p < 0.05, compared with control; Student's *t*-Test for independent sample comparisons.

3.3. Nitric oxide synthase protein expression

In order to explore whether NOS activity changes involved an alteration in enzyme expression, nNOS and iNOS protein expression was assessed by Western blot in both synaptosomal and non-synaptic mitochondria membranes at the onset of alcohol hangover. In each Western blot assay, membrane samples isolated from tissue of alcohol-treated mice were run in parallel with control samples. Results are presented in Fig. 3 showing immunoblots from a single representative experiment and the quantification of optical density expressed as the ratio between target protein (nNOS or iNOS) and loading control (β-tubulin for synaptosomes and VDAC for non-synaptic mitochondria).

In synaptosomes, nNOS expression was 19% decreased compared with control ($p < 0.05$, Fig. 3A) while no significant changes were

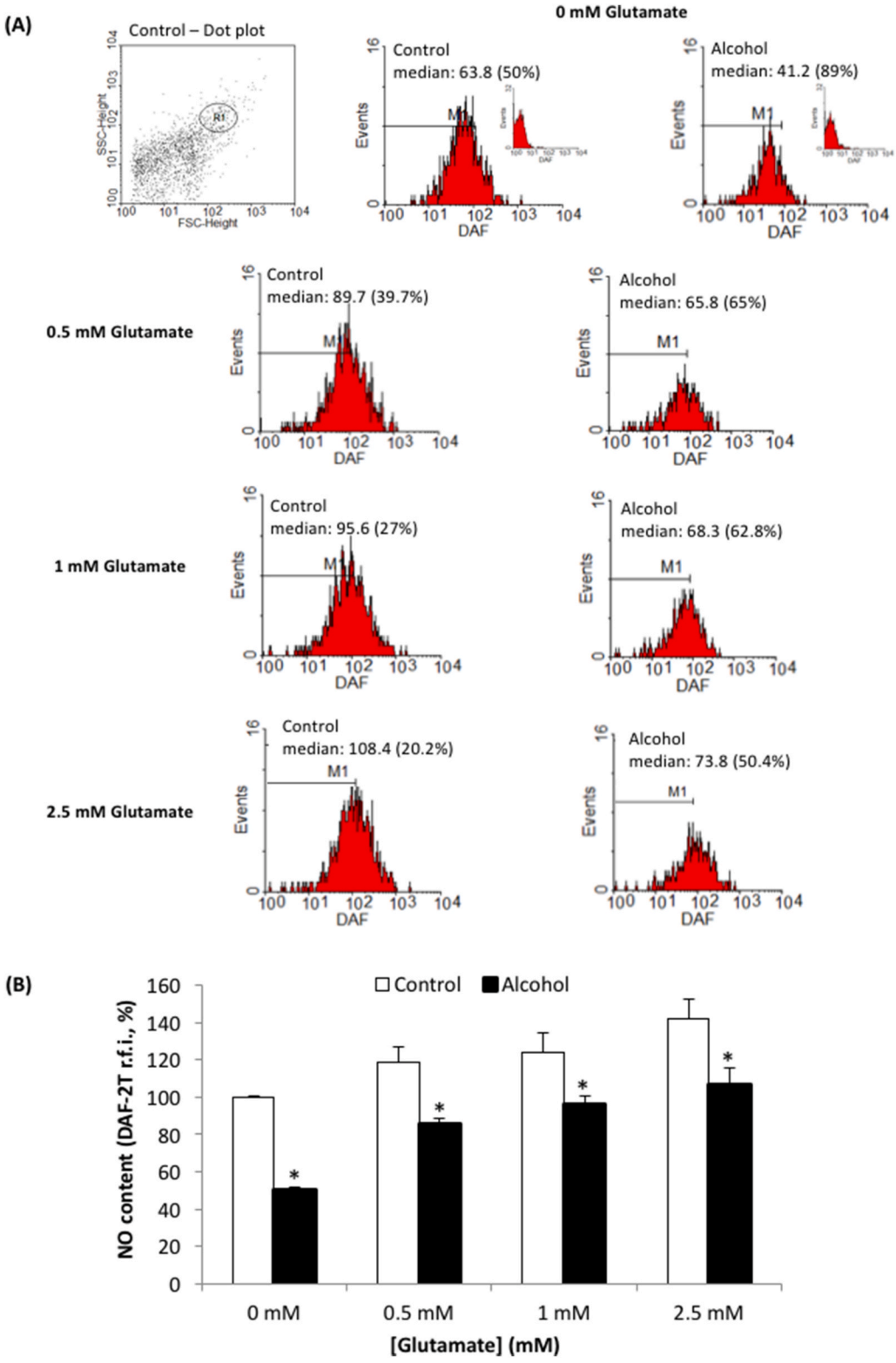

Fig. 4. Effect of alcohol hangover on nitric oxide (NO) content in synaptosomes isolated from mouse brain cortex measured after NMDAR activation. Brain cortex synaptosomes were loaded with the probe DAF-2-DA and direct measurements of NO were obtained by flow cytometry. Typical dot plot of FSC-H versus SSC-H indicating a gated synaptosomes population (R1) for control mice is shown (A). The effect of NMDAR activation on NO content was analyzed by adding increasing glutamate concentrations (0–2.5 mM) on control and alcohol-treated groups right before flow cytometer adquisition. Typical histograms of gated events versus relative fluorescence intensity (FL-1) are shown for control- or alcohol-treated mice in response to the different glutamate concentrations. Particularly, autofluorescence histogram for samples without probe are presented as insets for 0 mM glutamate in both control and alcohol synaptosomes. DAF-2T fluorescence was analyzed using the median value of the distribution of fluorescence events from each treatment. A common marker (M1) was fixed on control median value representing approximately 50% of the fluorescent events (for 0 mM glutamate). Histogram differences in DAF-2T fluorescence were quantified as the number of events which drop under the median value of the relative fluorescence distribution corresponding to M1. A higher number of DAF-2T relative fluorescence events under M1 would reflect decreased NO levels (for comparisons between control and alcohol). Bar graph quantification of DAF-2T relative fluorescence events is shown in panel (B). Fluorescence events were quantified as the number of events which drop under a common marker M_1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. Bars represent the mean ± SEM. Analysis was performed three times for each treatment (control- and alcohol-treated mice). Experiments were performed in triplicate ($n = 9$, corresponds to the number of total assays for each experimental group and glutamate concentration). *$p < 0.05$ compared with control; ANOVA, Bonferroni's test.

observed for iNOS protein expression (Fig. 3B). In non-synaptic mitochondria no significant changes were found either for nNOS or iNOS protein expression (Fig. 3 C, D).

3.4. Nitric oxide content due to NMDAR activation

Since nNOS enzyme activity depends on NMDA receptor activity, NO total content due to NMDAR activation was evaluated in fresh synaptosomes by flow cytometry using DAF-2-DA (see Materials and Methods, section 2.3.3). Fresh synaptosomes were exposed to increasing glutamate concentrations (0–2.5 mM) in order to stimulate NMDAR. Thus, synaptic nNOS coupled to NMDAR was capable of producing NO by NMDAR-dependent calcium influx. Results are shown in Fig. 4. The gated synaptosomes population is shown in a dot plot of Forward Scatter- Height (FSC–H) versus Side Scatter-Height (SSC–H) (Fig. 4A). Typical histograms for DAF-2T relative fluorescence intensity are shown for each glutamate concentration for both control and alcohol conditions.

As expected, it can be observed that DAF-2T relative fluorescence intensity increased in the control group as the glutamate concentration increased. This is clearly represented by the changes in the median values of DAF-2T fluorescence.

Quantification of relative fluorescence intensity events is shown in a bar graph in Fig. 4B in which control values were taken as 100%. As previously observed, median values for DAF-2T relative fluoresce intensity clearly were decreased by 50% in synaptosomal samples from hangover mice at the baseline condition (0 mM glutamate), as compared with control samples ($p < 0.05$, Fig. 4B). The addition of 0.5–2.5 mM glutamate produced a concentration-dependent enhancement of NO production, as observed by an increase in DAF-2T relative fluorescence intensity in both control and alcohol synaptosomes. However, values observed for alcohol-treated mice were significantly lower than controls for all the glutamate concentrations tested, being: 118% vs. 85% for 0.5 mM, 124% vs. 96% for 1 mM and 142% vs. 106% for 2.5 mM control vs. alcohol, respectively ($p < 0.05$, Fig. 4B).

3.5. Nitric oxide synthase activity-dependent on NMDAR

With the purpose of verifying the effects of hangover on the NMDAR-dependent activation of nNOS, NOS activity was determined in synaptosomal membranes exposed to increasing glutamate concentrations (0–2.5 mM). Results are shown in Fig. 5. As it was observed for NO total content, NO production increased depending on glutamate concentration in both control and alcohol samples. Nevertheless, NO production in alcohol-treated mice remained lower than controls for all the glutamate concentrations assayed, being decreased by 54% (for basal conditions without glutamate), 30% (0.5 mM glutamate), 48% (1 mM glutamate) and 52% (2.5 mM glutamate) ($p < 0.05$, Fig. 5).

3.6. NMDAR and PSD-95 protein expression

Since it resulted to be interesting to deeply analyze if the detrimental effect of alcohol hangover on NO metabolism could be associated with NMDAR dysfunction, protein expression of GluN2B subunit and PSD-95 was evaluated by Western blot. Results are shown in Fig. 6. Synaptosomes from alcohol-treated mice showed a significant 64% reduction in GluN2B expression ($p < 0.05$, Fig. 6A). In addition, PSD-95 protein expression was 15% decreased by alcohol hangover as compared with control group ($p < 0.05$, Fig. 6B).

3.7. Calcium influx by glutamate

In order to assess the role of NMDAR activity on NO production at the onset of alcohol hangover, Ca^{2+} influx in synaptosomes by glutamate stimulus was evaluated by flow cytometry. Fig. 7 includes the dot plot for control group and representative histograms showing relative fluorescence events for control synaptosomes together with positive and negative assay controls. Results obtained indicate that as expected, calcium influx was markedly increased by glutamate in both control and alcohol groups as compared with basal condition ($p < 0.05$). However, glutamate-induced calcium entry was 38% decreased in synaptosomes from alcohol-treated mice as compared with control ($p < 0.05$). As expected, ionomycin induced a 50% increase in calcium influx and EGTA-EDTA reduced calcium entry by almost 90%.

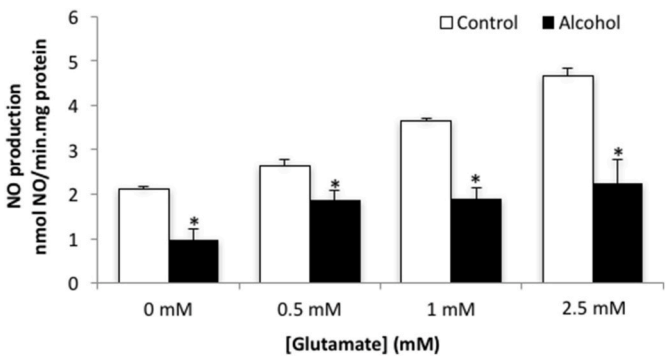

Fig. 5. Effect of alcohol hangover on neuronal nitric oxide synthase (nNOS) activity in synaptosomes isolated from mouse brain cortex dependent on NMDAR activation. Nitric oxide production by nNOS due to NMDAR activation was determined in synaptosomal and non-synaptic mitochondrial membranes in both control- and alcohol-treated mice following the oxidation of oxyhemoglobin to methemoglobin at 577–591 nm. The effect of NMDAR activation on NO production by nNOS was analyzed by adding increasing glutamate concentrations (0–2.5 mM) on control and alcohol samples. Results are expressed as specific enzyme activity. The whole procedure for mice treatment and subcellular fractionation was replicated three times and NOS activity assays were performed in triplicate ($n = 9$, corresponds to the number of total assays for each experimental condition). Data are shown as mean ± SEM. *$p < 0.05$, compared with control; ANOVA, Bonferroni's test.

4. Discussion

Alcohol hangover represents the condition that comprises the residual effects of acute alcohol exposure. Bae and colleagues (2005) have proposed that ethanol damage in CNS is provoked due to its site of action within the cell membrane at synapses [33]. We previously demonstrated the occurrence of mitochondrial dysfunction and oxidative stress mainly affecting mitochondria present at the synapse at the onset of alcohol hangover [5,8]. As we also reported alterations in NO metabolism in total mitochondrial fractions [5,10], it was interesting to investigate hangover effects on NO metabolism in synaptosomes and non-synaptic mitochondria and the possible role of NMDAR-PSD-95 at the synapses in brain cortex.

Nitric oxide is considered, a modulator and neuroprotector of neuronal functioning at physiological levels [34]. Specifically, NO is produced postsinaptically mainly associated to NMDAR activation and can diffuse to the presynaptic sites and act as a retrograde neurotransmitter. In addition, NO may also be released by the presynaptic ending in peripheral nitrergic nerves acting as anterograde neurotransmitter or neuromodulator [35]. The present study shows that NO total content and production were significantly decreased in synaptosomes by alcohol hangover probably associated with a reduction of synaptic proteins PSD-95 and NMDA receptor.

The effects of ethanol in brain NOS activity have been deeply studied in both animals and humans. It is known that EtOH selectively affects nNOS activity in different brain cells, which may be related to behavioural alcohol-dependent patterns and to the changes due to EtOH itself on cells or tissue functions [36]. In addition, chronic exposure of cortical neurons to EtOH appears to increase nNOS expression thus increasing NO levels due to enhancement of NMDA-stimulated NO synthesis [9,

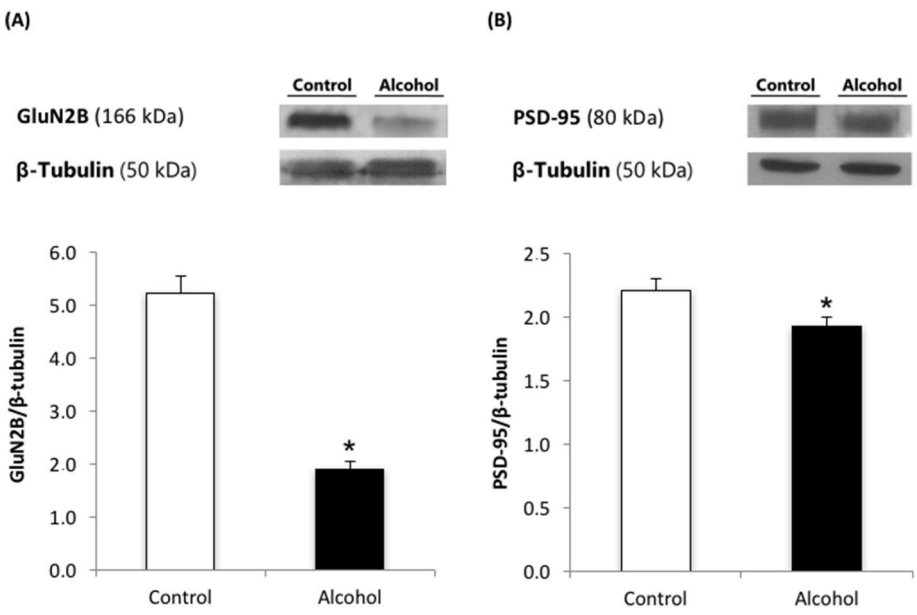

Fig. 6. Effect of alcohol hangover on NMDAR GluN2B subunit and PSD-95 proteins expression in synaptosomes isolated from mouse brain cortex. Western blots were carried out to analyze the expression of NMDAR GluN2B subunit (A) and PSD-95 (B) in synaptosomal membranes isolated from mice brain cortex at the onset of alcohol hangover. Results shown are immunoblots from a single representative and the ratio between the expression of NMDAR GluN2B subunit and PSD-95 protein versus that of β-tubulin protein. The whole procedure for mice treatment and subcellular fractionation was replicated three times and membrane samples were processed in quadrupled for Western blot assays (n = 12, corresponds to the number of total assays for each experimental condition). Data are expressed as mean ± SEM. *$p < 0.05$, compared with control; Student's *t*-Test for independent sample comparisons.

37]. In addition, nNOS activity was increased in the rat cerebellum due to its sensitivity to the oxidative insult triggered by ethanol [38]. On the other hand, acute exposure to EtOH may lead to inhibition of nNOS activity by decreasing NMDA-dependent NO synthesis [9] both in rat cortical neurons and in hippocampus slices [39]. The current finding showing low NO levels due to alcohol hangover is an interesting fact which indicates that acute EtOH effects are evidenced not only during the metabolizing period but also in the residual stage of exposure. In non-synaptic mitochondria no changes were detected in NO content or production by nNOS indicating a clear difference between results of both subcellular fractions in the response to the residual effect of acute ethanol exposure.

Related to nNOS, a previous study demonstrated an increased vulnerability to ethanol-induced neuronal loss in the neocortex and hippocampus in neonatal mice genetically deficient for nNOS, suggesting a neuroprotective role of NO in ethanol intoxication [40]. In this sense, our results showed that nNOS protein expression is reduced at the beginning of alcohol hangover specifically in synaptosomes but not in non-synaptic mitochondria.

Previously, other evidences indicated that acute and chronic exposure to alcohol impairs nNOS and eNOS in cerebral arterioles [41,42] and, even though the mechanism underlying this effect is still unknown, it seems to involve the formation of reactive oxygen species [43]. This could support the idea that the burden of reactive oxygen species generation triggered by the hangover state could alter nNOS expression and function. Moreover, global inhibition of nNOS enzyme itself is demonstrated to cause undesired systemic effects, such as deficits in motor functions and impairments in learning [44–46]; thus, the observed reduction in nNOS expression and function at the synapses could be related to our previous evidences showing negative long-lasting effects of hangover on motor and affective behavior [2,3].

We also assessed the protein expression of iNOS and no significant changes were observed due to the hangover state in both studied subcellular fractions. Regarding EtOH and iNOS activity, there are several evidences reporting counteractive effects depending on EtOH dose, exposure times and type of tissue [36]. For instance, low concentrations of EtOH (10 mM) promote inflammatory processes in brain and in glial cells by up-regulating cytokines and by increasing NO production due to iNOS activity enhancement [47]. On the other hand, administration of EtOH decreased corticosterone release through inhibition of iNOS activity in animals treated by repeated restraint stress [48]. Furthermore, it was established that different doses of EtOH affected iNOS expression in glial cells. For example, acute (6–24 h) exposure of activated human astrocytoma cells to 50 mM EtOH enhanced iNOS activity recovered from the cytosol, whereas 200 mM EtOH decreased it [49].

Regarding eNOS involvement in alcohol hangover effects, we conducted preliminary assays of this isoform expression. As we mentioned above, an impairment of eNOS protein expression was associated with acute and chronic exposure to alcohol in cerebral arterioles [41,42]. Interestingly, in our study, no detectable differences in eNOS protein expression due to residual effects of acute ethanol exposure were found (data not shown).

NMDA receptors are important regulators of synaptic signaling in the brain [50]. NMDARs activate several downstream signalling pathways one of which involves activation of nNOS, and the subsequent production of NO. Strong evidences support that activation of nNOS following NMDAR activation is a critical component of fear memory formation [51]. In the present work, as negative changes on NO content and production were observed in synaptosomes, a next step was to evaluate the role of NMDA activation on NO metabolism in the same experimental model. For this propose, synaptosomes were exposed to increasing glutamate concentrations and NO content and nNOS activity were analyzed. Data obtained showed that synaptosomes from both hangover or control mice exhibited increased levels of NO in response to increasing glutamate concentrations exposure. However, the response of NO generation was lower for synaptosomes from hangover mice than controls showing a clear after-effect of acute ethanol exposure. The same profile of response was evidenced both for NO content and production assays. A possible explanation for this effect could be that the NMDAR inhibition by ethanol persists long after ethanol metabolism [13]. Interestingly, we evidenced a strong reduction in GluN2B subunit protein expression at the onset of alcohol hangover. Thus, NO metabolism alterations could be due to the inhibitory effect of acute ethanol exposure on NMDAR expression and function.

Signal transmission by NMDAR relies on the interaction of this receptor with nNOS through the mediator protein PSD-95. Current findings showed a novel role for PSD-95 in mediating ethanol effects on behaviour. Indeed, functional deletion of PSD-95 produced hypersensitivity to ethanol intoxicating effects thus showing that PSD-95 could be a possible key mediator of the behavioral effects of ethanol [16]. Related to this, PSD-95 was postulated to play a role in the organization of glutamate receptors determining also the morphologic characteristics of synapse and affecting synaptic plasticity [52,53]. Additionally, it was suggested that, since PSD-95 contributes to the level of ethanol

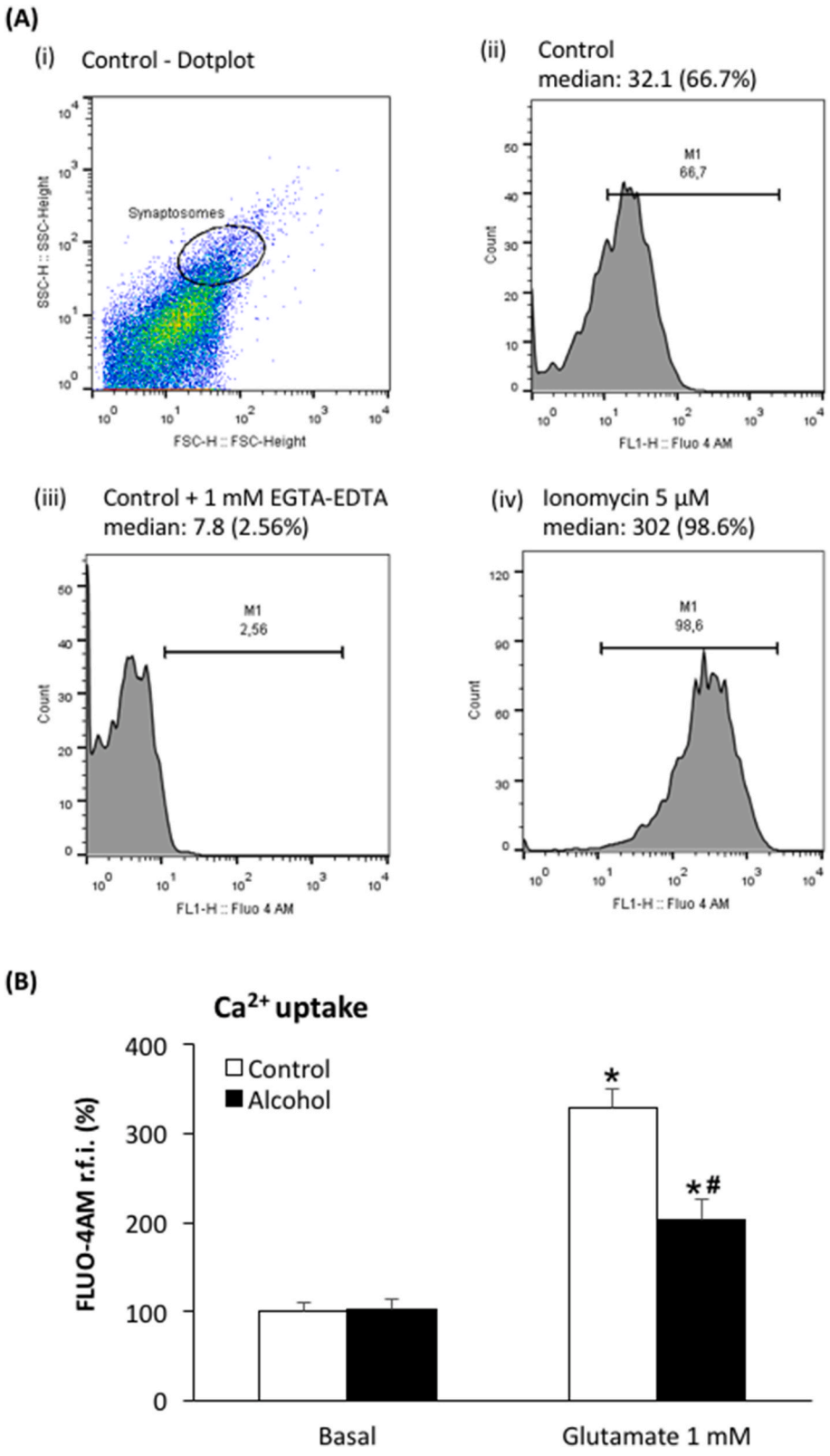

Fig. 7. Glutamate-induced calcium influx by brain cortex synaptosomes at the onset of alcohol hangover. Brain cortex synaptosomes were loaded with the probe FLUO-4AM and calcium entry was detected by flow cytometry. Calcium entry was detected before and 20 s after 1 mM glutamate stimulus. A: Typical dot plot of FSC-H versus SSC-H indicating the gated synaptosomes population (R1) (i) and histograms of gated events versus relative fluorescence intensity (FL-1) are shown for control synaptosomes (ii), negative (1 mM EGTA-EDTA) and positive (5 µM ionomycin) assay controls (iii, iv). FLUO-4AM fluorescence was analyzed using the median value of the distribution of fluorescence events for each treatment. A common marker (M1) was fixed on control histogram fluorescence events. Differences in FLUO-4AM relative fluorescence were quantified as the number of events which drop under M1. A higher number of FLUO-4AM relative fluorescence events under M1 would reflect a increase in Ca^{2+} influx. Bar graph quantification of FLUO-4AM relative fluorescence intensity (r.f.i.) is shown in panel (B) taking control at basal condition as 100%. Bars represent the mean ± SEM. Analysis was performed twice for each treatment (control- and alcohol-treated mice). Experiments were performed in triplicate (n = 6, corresponds to the number of total assays for each experimental group). *p < 0.05 compared with basal, #p < 0.05 compared with control; ANOVA, Bonferroni's test.

intoxication and influences ethanol intake, this protein could contribute to reward memory [54]. In our present work, we aimed at determining if PSD-95 could be altered at the beginning of alcohol hangover. Results showed that PSD-95 protein expression is significantly reduced in synaptosomes from ethanol-treated mice. This is an interesting fact since no previous studies had demonstrated that PSD-95 protein expression could be reduced at the residual stage of acute alcohol intoxication. Regarding the present facts, we observed a decrease in NO production associated to alteration of synaptic proteins of functional complex NMDAR-PSD-95-nNOS in another animal experimental model of treatment with levocabastine (Lores-Arnaiz et al., unpublished results).

In order to shed light upon the mechanism underlying alcohol

hangover effects on NO production reduction and the role of NMDAR impairment, we evaluated calcium influx in synaptosomes after glutamate stimulus by flow cytometry. Data showed that glutamate-induced calcium entry was significantly decreased as a result of residual effect of acute ethanol exposure. This supports our hypothesis that, at the beginning of alcohol hangover, the synthesis of NO is mainly reduced by the blockage of calcium entry due to the impairment of NMDA receptor.

At present, our results show strong evidence suggesting that ethanol could exert its negative after' effects on NO metabolism through impairing NMDAR-PSD-95-nNOS complex. Further studies could be carried out to elucidate if this pathway impairment is triggered only by ethanol or by its metabolism derived products such as acetaldehyde. Moreover, the alterations in NO synthesis might be a consequence of mitochondrial dysfunction and the exacerbated free radical production at the onset of alcohol hangover since an imbalance in bioenergetics and redox state could be involved in the loss of protein expression, assembly or function.

5. Conclusions

The present work demonstrated that ethanol induced impairments of NMDAR/PSD-95/nNOS pathway and NO synthesis observed in mouse brain cortex synaptosomes and non-synaptic mitochondria 6 h after acute ethanol exposure.

Results indicated that hangover induced a significant decrease in NO total content, nNOS activity and NO production due to NMDAR stimulation in synaptosomes. On the other hand, no changes were observed in NO content and nNOS activity in non-synaptic mitochondria. The expression of iNOS remained unaltered in synaptosomes and non-synaptic mitochondria. Particularly, GluN2B and PSD-95 protein expression was found reduced in synaptosomes at the onset of hangover. Moreover, glutamate-induced calcium entry was significant decreased in synaptosomes from alcohol-treated mice. Thus, it can be concluded that hangover effects on NO metabolism are strongly evidenced in synaptosomes probably due to a disruption in NMDAR/PSD-95/nNOS pathway. Furthermore, NO synthesis is mainly reduced by the blockage of calcium entry due to the impairment of NMDA receptor.

Declaration of competing interest

None.

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List of abbreviations

APS	ammonium persulfate
BAC	blood alcohol concentration
BSA	bovine serum albumin
FFA-BSA	free fatty acid BSA
CNS	central nervous system
DAF-2	4,5-Diaminofluorescein
DAF-2 DA	4,5-Diaminofluorescein diacetate
DAF-2T	4,5-Diaminofluorescein triazole
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EtOH	ethanol
FLUO-4AM	Fluo-4 acetoxymethyl ester
FSC-H:	forward scatter-height
GSNO	S-Nitrosoglutathione
iNOS	inducible nitric oxide synthase
L-NNA	Nω-Nitro-L-arginine
MAO	monoamine oxidase
NADH	nicotinamide adenine dinucleotide
NAO	10 N-nonylacridine orange
NMDAR	N-methyl-D-aspartate receptors
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PSD-95	postsynaptic density protein-95
SDS	sodium dodecilsulphate
SOD	superoxide dismutase
SS	saline solution
SSC-H:	side scatter-height
TEMED	tetramethylethylenediamine
VDAC	voltage-dependent anion channel

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