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Adolescent Binge Alcohol Exposure Affects the Brain Function Through Mitochondrial Impairment

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Abstract In the young population, binge drinking is a pattern of problematic alcohol consumption, characterized by a short period of heavy drinking followed by abstinence which is frequently repeated over time. This drinking pattern is associated with mental problems, use of other drugs, and an increased risk of excessive alcohol intake during adulthood. However, little is known about the effects of binge drinking on brain function in adolescents and its neurobiological impact during the adulthood. In the present study, we evaluated the effects of alcohol on hippocampal memory, synaptic plasticity, and mitochondrial function in adolescent rats after a binge drinking episode in vivo. These effects were analyzed at 1, 3, or 7 weeks post alcohol exposure. Our results showed that binge-like ethanol pre-treated (BEP) rats exhibited early alterations in learning and memory tests accompanied by an impairment of synaptic plasticity that was total and partially compensated, respectively. These changes could be attributed to a rapid increase in oxidative damage and a late inflammatory response induced by post ethanol exposure. Additionally, BEP alters the regulation of mitochondrial dynamics and modifies the expression of mitochondrial permeability transition pore (mPTP) components, such as cyclophilin D (Cyp-D) and the voltage-dependent anion channel (VDAC). These mitochondrial structural changes result in the impairment of mitochondrial bioenergetics, decreasing ATP production progressively until adulthood. These results strongly suggest that teenage alcohol binge drinking impairs the function of the adult hippocampus including memory and synaptic plasticity as a consequence of the mitochondrial damage induced by alcohol and that the recovery of hippocampal function could implicate the activation of alternative pathways that fail to reestablish mitochondrial function.

Keywords Alcohol · Binge drinking · Mitochondria · Oxidative stress · Synaptic dysfunction

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Introduction

Alcohol is the most commonly used licit substance, and its excessive consumption is the third cause of death worldwide [1]. Recent data indicate that global alcohol consumption has reached up to 6.2 l of pure alcohol per person from 15 years old and over, which corresponds to 13.5 g of pure alcohol per day (GISAH data). Among alcohol consumers, the biggest problem is the young population from the ages 15–24 years, who drink on average 112 g of alcohol per day [2, 3]. This group presents a troubling pattern of alcohol consumption known as "binge drinking." This drinking pattern is characterized by a heavy alcohol use in a short period followed by a

period of abstinence which can continue with additional intermittent alcohol intake [4]. Accumulative evidence shows that adolescents consume more than twice drinks per occasion than adults [4]. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) indicates that binge drinking produces a blood alcohol concentration (BAC) of 0.08 g/dL or above which corresponds to consuming five or more drinks in males or, four or more drinks in females, over a period of 2 hours [5].

In adolescents, binge drinking has many negative short- and long-term effects, which includes depression [6] and social rejection [7], and it can serve as a "gateway" for the use of other drugs such as tobacco or marijuana [3]. Also, evidence suggests that there is a close relationship between early alcohol consumption during adolescence and an increased risk of excessive alcohol intake during adulthood [8]. Alcohol consumption before 14 years of age is associated with a fourfold increase in the risk of alcohol dependence in adulthood [2, 9], but the mechanisms that links this early drinking with prospective alcohol dependence is still unknown [10].

During adolescence, the brain undergoes a maturation process that requires changes in neurotransmission and synaptic plasticity, which are accompanied by structural modifications in regions such as the hippocampus, prefrontal cortex, and limbic system [11]. Therefore, it is probable that adolescent brain could be more vulnerable to the harmful effects of alcohol binge drinking than the mature adult brain [12]. Alcohol intoxication results in brain damage and neuronal death [13, 14], being oxidative stress and inflammation in some part responsible for this phenomenon [13]. Interestingly, cognitive defects, oxidative stress, and inflammation could be related to alterations of mitochondrial function [15–18]. Mitochondria play a major role in synaptic plasticity [19], and they are the primary source of reactive oxygen species (ROS) production [18]. The mitochondrial injury also promotes an inflammatory response in the brain affecting synaptic function and memory loss [15, 16]. Also, chronic alcohol consumption alters the mitochondrial membrane properties and leads to mitochondrial dysfunction and neurotoxicity in the cerebral cortex [20]. However, it is not clear how mitochondrial failure contributes to neurotoxicity induced by alcohol binge drinking during adolescence, and whether these effects are associated with changes in adult brain function.

In the present work, we studied the effects of alcohol binge drinking on the brain in adolescent rats and if these changes persisted into adulthood. Rats were subjected to cognitive tests at 1, 3, or 7 weeks post exposure and later their hippocampus were analyzed through histochemical and biochemical assays. Our results showed that binge-like ethanol pretreated (BEP) rats exhibited alterations in learning and memory post alcohol treatment, accompanied by defects in synaptic plasticity and oxidative damage. These effects were compensated by a mechanism that may involve the activation of the Nrf-2 pathway. Complementary studies showed the appearance of a late inflammatory response and variations in the expression of proteins that control mitochondrial dynamics and the mitochondrial permeability transition pore (mPTP). Finally, these changes resulted in mitochondrial bioenergetics defects that persist until 7 weeks post alcohol administration.

Altogether, these results indicate that teenage binge-like alcohol consumption generates harmful effects on the brain which remain over time or appear belatedly post ethanol exposure, affecting the hippocampal function through mitochondrial impairment. These observations suggest that mitochondrial dysfunction is a significant contributor to neurotoxic damage induced by alcohol binge drinking during adolescence.

Materials and Methods

Animals Male Sprague–Dawley rat pups, postnatal day 25 (PND25), were housed in groups of four rats per cage and maintained to 22 °C on a 12:12 h light–dark cycle, with food and water ad libitum previous to alcohol binge drinking administration. The animals were treated and handled according to the National Institutes of Health guidelines (NIH, Baltimore, MD). The experimental procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile and Universidad Autónoma de Chile.

Reagents and Antibodies Ethanol was obtained from Merck Millipore (catalog number 107017). The primary antibodies used here were mouse anti-β-actin (sc-47778, Santa Cruz Biotechnology, Inc.), rabbit anti-β-actin (sc-722, Santa Cruz Biotechnology, Inc.), rabbit anti-Opa1 (PA1-16991, Thermo Scientific), rabbit anti-Cyclophilin D (PA3–022, Thermo Scientific), rabbit anti-Fis1 (sc-98,900, Santa Cruz Biotechnology, Inc.), rabbit anti-Mfn1 (H-65) (sc-50330, Santa Cruz Biotechnology, Inc.), rabbit anti-Mfn2 (H-68) (sc-50331, Santa Cruz Biotechnology, Inc.), mouse anti-VDAC1 (B-6) (sc-390996, Santa Cruz Biotechnology, Inc.), rabbit anti-phospho-DRP1 (Ser616) (4494, Cell Signaling), mouse anti-DRP1 (C-5) (sc-271583, Santa Cruz Biotechnology, Inc.), rabbit anti-iNOS (PA3-030A, Thermo Scientific), anti-NF-κB p65 (C22B4, Cell Signaling), mouse anti-Glial Fibrillary Acidic Protein (GFAP) (G3893 Sigma-Aldrich), goat anti-AIF1/IBA1 (Isoform 3) (SAB2500042 Sigma-Aldrich), rabbit anti-nitrotyrosine (n-tyr) (US Biological Life Sciences), and anti-8-hydroxyguanine (8OHdG) (Santa Cruz Biotechnology).

Binge-Like Ethanol Protocol in Rats Doses of ethanol (3.0 g/kg, 25% *w/v* mixed in isotonic saline) or saline solution were administrated in an intraperitoneal (i.p.) injection beginning on PND25 as previously described [21]. A second dose was given on PND26, followed by two consecutive days with

gaps of 2 days without injections, during 2 weeks (PND 25, 26, 29, 30, 33, 34, 37, and 38). The injected i.p. volumes were dependent on the weight of each animal. According to these variations in the time, amounts administered were 1–3 ml. After a single dose of ethanol in the binge drinking protocol, the maximum blood ethanol concentrations (BEC) reach 210 ± 11 mg⁄dL at 30 min post-injection, followed by a gradual decline to 540 min later. This same binge drinking protocol has been carried out previously, and the BEC also have been reported [21].

Behavioral Testing

The behavioral tests were performed at 1, 3, or 7 weeks post alcohol exposure. After the cognitive tasks, biochemical and histological analysis were immediately performed.

Memory Flexibility Test

The Morris water maze test was performed in a modified manner as previously described [22]. Briefly, rats were trained in a 160-cm diameter circular water maze (opaque water, 50 cm deep, 22–23 °C, with an 11-cm platform at 1 cm below water). Every trial lasts 90 s as a maximum, 10 s on the platform at the end of trials with an inter-trial interval of 15 min as a minimum. Each animal was trained for one pseudo-random location of the platform per day for 5 days (using day 1 as training and the rest of 4 days as testing), with a new platform location each day. Up to 15 trials were performed per day, until the criterion of three consecutive trials with an escape latency <20 s was reached. Upon testing completion, the rats were gently removed from the maze and returned to its cage. The animals were tested for the next location on the following day. Data were collected using a video tracking system coupled to Honestech TVR 2.5 program and analyzed off-line in ANY-MAZE software.

Novel Object Recognition Test

At different time points after ethanol injection protocol, rats were habituated in three chamber box (35 × 40 × 45 cm each room) during 3 days, 10 min per day. The fourth day performed familiarization phase, where two identical objects were presented to rats, one in each lateral chamber while rat started this phase on the central chamber. Both objects were placed in the center of respective chamber at the same distance from the wall of the box. Rats were allowed to explore both objects freely during 10 min. The box was cleaned using ethanol 70% between subjects. Four hours post start of training phase was performed in testing phase where one object was replaced by a novel object. Newly, rats began the testing phase in the central chamber and were allowed to explored freely during 10 min. Data were collected using a video tracking system coupled to Honestech TVR 2.5 program and analyzed off-line in ANY-MAZE software.

Social Interaction Test

At different time points after ethanol injection protocol, rats were habituated in three chamber box (35 × 40 × 45 cm each chamber) during 10 min. Then, one object (cage) and one rat (previously unknown to testing rat) inside a cage were presented to rats, one in each lateral chamber at the same distance from the wall. Rats started at the center chamber and were allowed to explore freely during 10 min. Finally, the empty cage was replaced by another rat (new rat, also unknown to testing rat). The testing rat was allowed to explored freely during 10 min. Data were collected using a video tracking system coupled to Honestech TVR 2.5 program and analyzed off-line in ANY-MAZE software.

Slice Preparation and Electrophysiology Transverse slices (400 μm) from the dorsal hippocampus were cut under cold artificial cerebrospinal fluid (ACSF; in mM: 124 NaCl, 2.6 NaHCO3, 10 D-glucose, 2.69 KCl, 1.25 KH2PO4 2.5 CaCl2, 1.3 MgSO4, y 2.60 NaHPO4) using a Vibratome (BSK microslicer DTK-1500E) and incubated in ACSF for 1 h at room temperature. In all experiments, 10 μM PTX was added to suppress inhibitory GABA transmission. Slices were transferred to an experimental chamber (2 ml), superfused (3 ml/min, at room temperature) with gassed ACSF and visualized by transillumination with a binocular microscope (Amscope). To evoke field excitatory postsynaptic potentials (fEPSPs), we stimulated with bipolar concentric electrodes (Tungsten, 125 μm OD diameter, Microprobes) and connected to an isolation unit (Isoflex, AMPI, Jerusalem, Israel). The stimulation was in the Stratum Radiatum within 100–200 μm from the recording site. Recordings were filtered at 2.0–3.0 kHz, sampled at 4.0 kHz using an A/D converter, and stored with WinLTP program. The basal excitatory synaptic transmission was measured using an input/output curve protocol, which consisted of eight stimuli ranging from 200 to 900 μA (the interval between stimuli was 10 s). To generate LTP, we used high-frequency stimulation (HFS), which consisted of 3 or 4 trains of 100 pulses at 100 Hz of stimuli with an inter-train interval of 20 s. Data were analyzed off-line with pClamp 10 (Molecular Devices).

Immunoblotting The hippocampus of the treated or control rats were dissected on ice and immediately processed as previously described [22, 23]. The number of control or alcohol-treated rats analyzed were $n = 3$, $n = 4$, and $n = 5$ at 1, 3, and 7 weeks post treatment, respectively. Briefly, the hippocampal tissue was homogenized in RIPA buffer (10 mM Tris-Cl, pH 7.4, EDTA 5 mM, 1% NP-40, 1% sodium deoxycholate, and

1% SDS) supplemented with a protease inhibitor mixture and phosphatase inhibitors (25 mM NaF, 100 mM Na_3VO_4, and 30 μM $Na_4P_2O_7$) using a homogenizer and then sequentially passed through syringes of different calibers. The protein samples were centrifuged twice at 14,000 rpm for 20 min at 4 °C. The protein concentrations were determined using the BCA Protein Assay Kit (Pierce). The samples were resolved by SDS-PAGE, followed by immunoblotting on PVDF membranes. The membranes were incubated with the primary antibodies and anti-mouse, anti-goat, or anti-rabbit IgG peroxidase-conjugated antibodies (Pierce, Rockford, IL) and developed using an ECL kit (Luminata Forte Western HRP substrate, Millipore).

Immunofluorescence Rats were anesthetized using ketamine and xylazine and then intracardially perfused with paraformaldehyde 4% in PBS. Washes and immune reagent dilutions were performed using PBS with 0.2% Triton X-100 (PBS-T) throughout all of the immunofluorescence experiments, with three washes per antibody incubation and after glycine-NaBH4 incubation. Tissue sections were first permeabilized with PBS-T for 30 min. To diminished endogenous fluorescence, sections were incubated in NaBH4 10 mg/ml and 0.15 M glycine for 30 min. Sections were incubated with 3% bovine serum albumin (BSA) at room temperature for 1 h. Primary antibodies were incubated overnight at 4 °C in BSA solution, and secondary antibodies were applied for 2 h at room temperature in BSA solution. During last washes, Hoechst was added to nuclei staining. After immunofluorescence, sections were mounted on gelatin-coated slices, air-dried, and coverslipped using a fluorescent mounting medium (Dako North America, Inc.).

Image Analysis Stained brain sections were photographed using an Olympus BX51 microscope coupled to MicroPublisher 3.3 RTV camera (QImaging, Surrey, Canada). The images were loaded into ImageJ software (NIH) for analysis, adjusting the threshold manually in every picture.

Measurement of ATP Concentration ATP concentration was measured in the hippocampal lysate using a luciferin/luciferase bioluminescence assay kit (ATP determination kit no. A22066, Molecular Probes, Invitrogen). The amount of ATP in each sample was calculated from standard curves and normalized to the total protein concentration.

Statistical Analysis The results are presented as graphs depicting the mean ± standard deviation. Statistical significance was determined using one-way ANOVA with Bonferroni's post-test. p values >0.05 and ≤0.05 were regarded, respectively, as not statistically significant and as statistically significant. In the figures, p values between 0.01 and 0.05 are marked with one asterisk, p values between 0.001 and 0.01 with two asterisks, and p values less than 0.001 are shown with three asterisks. All statistical analyses were performed using Prism software (GraphPad Software, Inc.).

Results

Binge-Like Ethanol Treatment Affects Cognitive Performance in Adolescent Rats

Binge-like ethanol treatment induced damage to the cortical olfactory regions in brain adolescent rats [24], and chronic ethanol consumption causes cognitive impairment especially in young humans and rodents [13]. Clinical reports showed hippocampal volume and white matter reduction along with a deficit in hippocampal function in adolescents after severe alcohol consumption [25]. In the present study, we performed three different behavioral tasks at three different time points after ethanol or saline injections: 1, 3, and 7 weeks (Fig. 1). We tested a modified spatial memory paradigm task associated with an assay to evaluate episodic memory (memory flexibility) in saline (SP) and BEP rats (Fig. 1a). This water maze protocol requires of up 15 trials of the rodents to learn to escape onto the hidden platform at one location, and then, the platform is move to a new point. Different areas are used successively during 4 days. As previously described, in such day earlier positions of the platform are encoded in long-term memory, potentially causing interference in the next day. Memory retrieval must be selective for the most recently encoded location, evaluating the hippocampal formation in episodic-like memory [26]. Analysis of average needed to reach criterion indicated non-differences comparing all experimental groups submitted to these tests (Fig. 1b). Similar trials to reach criterion were required in control and BEP rats at 1, 3, and 7 weeks post ethanol treatment (Fig. 1). These results indicate no impairment in episodic memory after ethanol administration at any time measured. Additionally, we evaluated alcohol-treated animals using recognition memory test (novel object recognition (NOR)) (Fig. 1c). We analyzed saline pretreatment (SP) group that showed a preference for novel object compared to the old object. On the other hand, rats in BEP group showed no preference for the new object and they spent equivalent times with novel and older objects (Fig. 1d). Complementary analysis of alcohol-treated animals (second and third period) and SP showed a preference for novel object compared to the old object (Fig. 1d), showing alteration only 1 week after ethanol exposure.

Finally, we evaluated rats in a social affiliation and social memory paradigm (social interaction (SI) test) (Fig. 1e). During social affiliation phase of the task, rats in all groups spent more time exploring with the rat compared with time exploring the object (empty cage) (Fig. 1f). Preference index

was similar between all experimental groups (Fig. 1f). In social novelty/preference phase of the task, rats previously treated with ethanol and measured 1 week after the treatment did not show preference for novel rat, exploring similar times with both rats (the known and the novel) indicated by a similar preference index (Fig. 1g). Rats in all others time evaluated showed a preference for novel rat compared with known rat (Fig. 1g). To eliminate the possibility of treatment-induced alterations of locomotor activity, we analyzed the swimming rate in the MWM, the average speed in the NOR and the average travel speed in the SI and no significant differences were observed between the experimental groups (Supplementary Table 1). These results indicate that young animals submitted to ethanol treatment showed an early impairment in recognition memory tasks, event that is reverted over time and that was not apparent at long-term.

Alcohol Binge Drinking Impairs Synaptic Transmission in Adolescent Rats

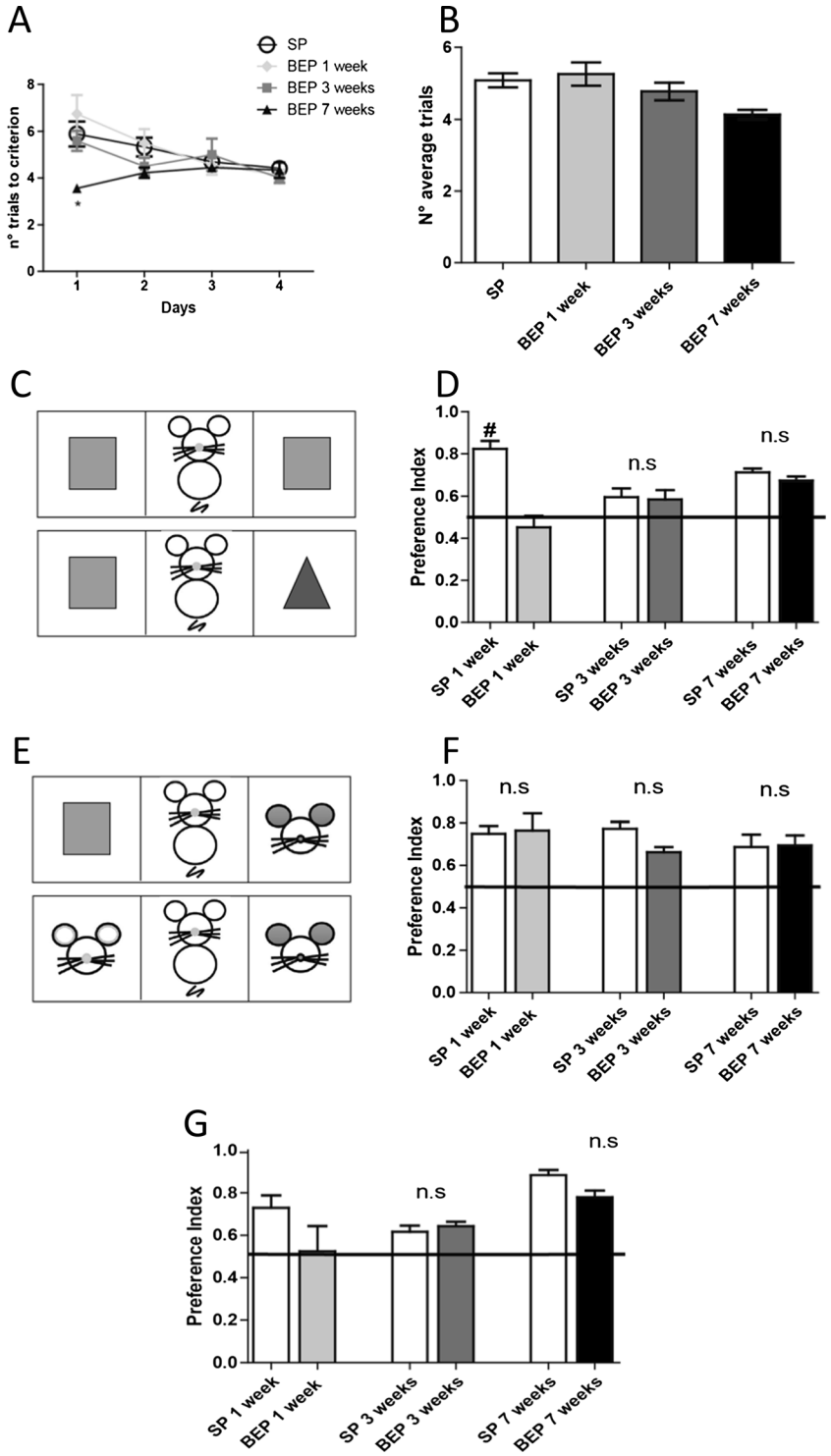
Alcoholism affects diverse signaling molecules involved in synaptic plasticity in the hippocampus such as glutamate and gamma-aminobutyric acid (GABA) both neurotransmitters which are responsible for excitatory and inhibitory responses, respectively [27]. Some reports have shown that alcohol treatment affects neuronal activity and generates excitotoxicity in hippocampal neurons [28]. However, cellular changes associated with ethanol-induced cognitive impairment during binge drinking are poorly understood. Therefore, we evaluated the synaptic plasticity in SP and BEP animals by studying the long-term potentiation (LTP) magnitude in the hippocampal CA3–CA1 transmission [28]. We observed a partial LTP response at 1 week after BEP (Fig. 2a(a), d), and a loss of LTP signal 7 weeks after BEP (Fig. 2a(c), d). In contrast, group animals of 3 weeks after BEP showed no differences in LTP induction (Fig. 2Ab and D) compared with SP rats. Moreover, we performed input–output experiments analyzing the synaptic strength of field excitatory postsynaptic potential (fEPSP) slopes obtained from different stimulation intensities in the same experimental groups. We observed a decrease in the synaptic strength only in rats 1 week after BEP (Fig. 2b(a), b), whereas the groups with 3 and 7 weeks after BEP presented similar levels to that of the control group (Fig. 2b). These results suggest that BEP alters the synaptic function in rats 1 week after ethanol exposure with consequences in synaptic plasticity (LTP) 7 weeks after treatment. Complementary studies were made to evaluate if BEP alters presynaptic function using the paired pulse facilitation assay. We found that a weak facilitation in rats 1 week after BEP (Fig. 2c(a), b), whereas in rats 3 and 7 weeks after BEP, the paired pulse ratio was similar to control group. These data suggest that BEP alters the release of neurotransmitters and decreases postsynaptic responsiveness in animals analyzed 1 week after BEP protocol. In summary, young BEP rats showed alterations in presynaptic (facilitation index) and postsynaptic activity (input/output curve), principally at 1 week after BEP, and these changes in initial times of exposition produce consequences in synaptic plasticity (LTP) at 7 weeks after ethanol exposure.

Adolescent Binge-Like Ethanol Exposure Induces Brain Inflammation in Adult Rats

Evidence indicates that alcohol consumption activates the immune response in the brain contributing to neurodegeneration associated with ethanol intoxication [6, 8, 29, 30]. Exposure to high alcohol concentrations increases the expression of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 and several chemokines in astrocytes in vitro [31]. Interestingly, application of alcohol binge drinking protocol increased cyclooxygenase-2 (COX-2) levels in adolescent rats suggesting a significant inflammatory response [21]. Brain samples from animals submitted to BEP protocol were collected for biochemical analysis 1, 3, and 7 weeks after treatment. We evaluated nuclear factor-κB (NF-κB) (pro-inflammatory factor) [32] (Fig. 3a) and ionized calcium-binding adapter molecule 1 (Iba1) (microglia activator) [33] (Fig. 3b) and glial fibrillary acidic protein (GFAP) [34] (Fig. 3c). Our results showed a significant decrease in NF-κB levels both at 1 and 3 weeks post ethanol consumption. However, animals with 7 weeks after treatment presented increased levels of NF-κB (Fig. 3b). When we evaluated the Iba1 expression, similar levels were observed in control and rats exposed to alcohol, at 1 and 3 weeks post administration, meanwhile at 7 weeks a drastic increase was observed (Fig. 3c, d). Finally, analysis of GFAP levels showed significant changes over time (Fig. 3e, f). BEP animals showed no differences compared to SP at 1 week, while at 3 weeks, GFAP expression was decreased and at 7 weeks, levels were significantly increased (Fig. 3f). Overall, these results indicate that BEP induces a delayed inflammatory response, which is evident only 7 weeks post ethanol consumption.

Adolescence Binge-Like Ethanol Consumption Affects the Regulation of the Mitochondrial Fusion/Fission Cycle in the Rat Brain

Mitochondria contribute to cellular bioenergetics producing ATP [35] and play a significant role in alcohol metabolism [36]. Interestingly, impaired mitochondrial function and energy metabolism was described following an acute and chronic ethanol exposure in different cell types, i.e., hepatocytes [37]. Mitochondria are dynamic organelles that continuously undergo fission and fusion events, a process known as mitochondrial dynamics [37–39]. Impaired mitochondrial dynamics is related to neurotoxicity and loss of neuroplasticity and memory [17, 40, 41]. We evaluated the levels of the main proteins



that control mitochondrial dynamics such as dynamin-related GTPases, mitofusins (Mfn1 and Mfn2), and optic atrophy 1 (OPA1), which are responsible for fusion of outer and inner mitochondrial membranes, respectively [38, 42]. Figure 4 shows that animals subjected to BEP had significantly decreased Mfn1 and Mfn2 levels compared to SP rats 1-week post treatment (Fig. 4a, d). Animals with 3 weeks post alcohol consumption, Mfn1 levels increased reaching similar levels to control rats, meanwhile Mfn2 levels remained lower (Fig. 4b, d), and at 7 weeks, although Mfn1 expression was restored in the BEP group, Mfn2 remained decreased compared to the SP group (Fig. 4c, d). Also, when we evaluated Opa1 expression levels we also observed abnormalities (Fig. 5). Different Opa1 isoforms are expressed as a result of alternative splicing and

◀ **Fig. 1** Effect of ethanol binge-like administration on hippocampal-dependent tasks in adolescent rats. **a** Memory flexibility task measure at three different time points, 1 week after injections (n = 8), 3 weeks after injections (n = 10), and 7 weeks after injections (n = 9). SP values considering three time points (no differences each other, n = 27). **b** Memory flexibility test average number of trials considering 4 days in the same bar. There were no differences between experimental groups. **c**, **d** Novel object recognition test. **c** Representative scheme of testing. **d** Preference index calculated as time spent with novel object divided by total exploration time. Same three different time points were measured (1 week after injections (n = 6): 3 weeks after injections (n = 7, SP group and n = 6 BEP group) and 7 weeks after injections (n = 7, SP group and n = 6 BEP group). **e**–**g** Social interaction test. **e** Representative scheme of testing. **f** Social affiliation phase of the task in where same three previous time points were measured (1 week after injections n = 6, 3 weeks after injections n = 7, 7 weeks after injections n = 7, SP group and n = 6 BEP group). *Upper bars* correspond preference index calculated to object (time spent with object divided for total exploration time) and *down bars* correspond preference index calculated respect to each rat. *Asterisks* indicate the difference between both preference indexes in the same experimental group. There were no changes between groups. **g** Social novelty/preference phase of the task. *Upper bars* correspond to preference index calculated to known rat and *down bars* correspond preference index calculated respect to novel rat. *Asterisks* indicate a difference between both preference indexes in the same experimental group. Ethanol treatment showed no differences between preference indexes 1 week after ethanol exposure. *$p < 0.05$, *ns* not significant, two-way ANOVA (**a**, **e**, **f**), t test (**c**)

are commonly identified as Long-Opa1 (L-Opa1) [43]. L-Opa1 is also proteolytically processed; favoring the loss of its transmembrane domain and triggering the formation of short Opa1 forms (S-Opa1). In normal conditions both L- and S-Opa1 forms, which are necessary for mitochondrial fusion, can be detected. However, in the presence of cellular stress conditions, L-Opa1 is mainly cleaved into S-Opa1, inhibiting mitochondrial fusion [44]. Figure 5 showed that animal samples at 1 week (Fig. 5a) BEP had increased L-Opa1 levels (Fig. 5d), without affecting the levels of S-Opa1 forms (Fig. 5e). In contrast, rats at 3 weeks after BEP had reduced L-Opa1 levels, maintaining S-Opa1 levels similar to the SP group (Fig. 5b, d, and e). Finally, brain samples from 7 weeks BEP animals presented low L-Opa1 levels, accompanied by a significant increase in form 1 and form 2 S-Opa1 levels (Fig. 5c–e). These results indicate that ethanol binge-like consumption affects mitochondrial fusion event that persists over time.

Also, we measured the levels of proteins that participate in mitochondrial fission processes. Dynamin-related protein 1 (Drp1) is recruited to the outer mitochondrial membrane to constrict mitochondria, inducing its division [44]. Phosphorylation at Ser616 of Drp1 protein stimulates this process [45]. Drp1 also interacts with other mitochondrial receptor proteins such as Fission 1 (Fis1) [38, 42, 46]. Interestingly, we observed that brain samples of 1 week after the BEP protocol had normal levels of total Drp1, but a drastic increase in Fis1 expression was observed (Fig. 6a, g). However, when we analyzed phospho-Drp1 levels, a significant increase was observed (Fig. 6d, h). At 3 weeks post ethanol treatment, the total Drp1 levels were decreased compared to control rats (Fig. 6b, g), whereas the phosphorylated form of Drp1 was drastically increased (Fig. 6e, h) and Fis1 expression also remained increased (Fig. 6b, g). Finally, total Drp1 and Fis1 levels at 7 weeks post alcohol consumption were significantly decreased compared to control rats (Fig. 6c, g), while the levels of phosphorylated Drp1 were similar to the control group (Fig. 6f, h). In summary, these results suggest that binge-like ethanol consumption promotes a fast mitochondrial fission. Figure 6i schematizes the alterations in mitochondrial dynamics in where alcohol treatment increased fission events at 1 and 3 weeks after dosage, whereas at 7 weeks post treatment, the balance is restored. Interestingly, animals with 7 weeks after ethanol treatment showed reduced both mitochondrial fusion and fission processes compared to saline treated animals (Fig. 6i).

Binge-like Alcohol Treatment Induced Oxidative Stress in Adolescent Rat Brains

One of the most important mechanisms that describe ethanol-induced brain damage is the loss of intracellular redox balance [24]. Chronic alcohol ingestion leads to an increase in the production of ROS and also affects several antioxidant pathways in numerous tissues such as heart, liver, and brain [29]. Overproduction of ROS may trigger morphological and functional alterations in neurons which could lead to neurotoxicity in the brain [30]. Mitochondria are the primary source of ROS; therefore, it is possible that mitochondrial injury, produced by alterations of proteins involved in mitochondrial dynamics, may induce excessive ROS production and further oxidative damage (Fig. 6). To test this hypothesis, we evaluated the oxidative stress damage of hippocampal tissue using immunofluorescence with two different protein oxidation markers: nytro-tyrosine (proteins with the NO_2 group on tyrosine residues) and 8-hydroxyguanine (DNA damage) in BEP and control animals (Fig. 7 and S1). We evaluated oxidative damage at three different locations on the hippocampus, dentate gyrus (DG), cornusammonis 1 (CA1), and cornusammonis 3 (CA3). Representative images of CA3 marked with nytro-tyrosine are shown in Fig. 7a. Nytro-tyrosine marker showed an increase in CA3 region of the hippocampus 1 week after ethanol treatment, while this effect decreased to control group levels at 3 weeks after ethanol treatment. Brain tissue analyzed at 7 weeks after ethanol protocol showed no apparent differences between saline and ethanol-treated groups (Fig. 7b). However, ethanol-treated group showed a decrease in nytro-tyrosine immunoreactivity at this time measured (Fig. 7b). Also, in CA1 region of the hippocampus, there was a similar pattern of immunoreactivity than in CA3. One week after BEP, ethanol group showed an increase in nytro-tyrosine immunoreactivity, while at 3 weeks after alcohol treatment, there were no differences. At 7 weeks after alcohol treatment, nytro-tyrosine

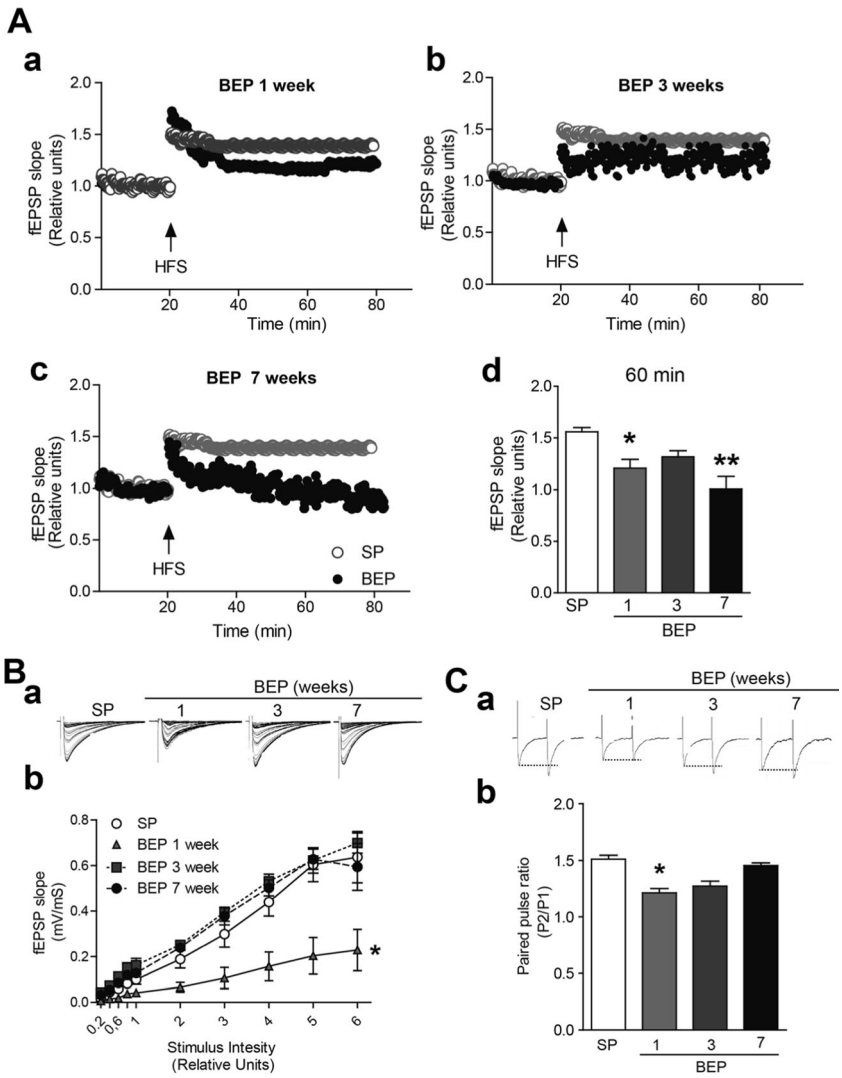


Fig. 2 Ethanol binge-like treatment alters the synaptic transmission in adult rats. **a** LTP was generated in hippocampal slices from SP and BEP animals, 1 (*a*), 3 (*b*), and 7 (*c*) weeks after treatment. *The arrow* indicates LTP induction by HFS and the plots show the fEPSP slope at different times. **a**(*d*) Quantification of synaptic response was evaluated at 60 min post-induction of LTP. **b**(*a*) Representative fEPSP at different stimulus intensities for SP and BEP animals at several times after treatment. **b**(*b*) Input-output curves are showing the relationship between fEPSP slope and stimulus intensity. **c**(*a*) Representative fEPSP traces at interstimulus intervals of 50 ms. **c**(*b*) Paired pulse facilitation (PPF) of the fEPSP of hippocampal slices from SP and BEP animals 1, 3, and 7 weeks after treatment. *The dots and bars* represent the mean ± SE from seven different slices. Three animals were used per experimental group. Statistical significant differences were calculated by one-way ANOVA, followed by Bonferroni's post hoc test (*$p < 0.05$; **$p < 0.01$)

immunoreactivity was significantly lower in ethanol-treated group compared to SP (data no shown). When we measured 8-hydroxiguanine immunoreactivity, we observed a different pattern. Only at 1 week after alcohol treatment, immunoreactivity of 8-hydroxiguanine in BEP group was lower in the three regions of the hippocampus, instead of higher levels seen with a nytro-tyrosine marker, while at any other time measured, immunoreactivity of SP and BEP groups were similar (Supplementary Fig. 1). To discard differences in the density of cells in the hippocampus, we also evaluated Nissl stain. No significant differences were observed in both experimental groups at 1, 3, and 7 weeks (Supplementary Fig. 3). We also evaluated the effects of binge drinking on the levels of oxidative stress-mediated damage in the hippocampus of control and alcohol-exposed rats using the anti-nitrotyrosine (n-tyr) antibody (Fig. 7c). Adolescent rats exposed to binge drinking had elevated levels of peroxynitrite-modified proteins at 1-week post treatment (Fig. 7c); however, at 3 and 7 weeks post alcohol consumption, the levels of oxidative stress were restored to normal levels. These results indicate that ethanol induces an early oxidative response that is reverted in adult animals.

Ethanol Binge-Like Treatment Affects Mitochondrial Permeability Transition Pore in Adolescent Rats

Mitochondrial permeability transition pore (mPTP) is a protein complex formed in the inner membrane of the mitochondria during mitochondrial calcium overload allowing permeability to molecules less than 1.5 kDa [47]. The opening of mPTP triggers uncoupling of oxidative phosphorylation and ATP depletion and can finally lead to mitochondrial swelling and cell death [47]. mPTP is formed by the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), and cyclophilin D (Cyp-D); however, Cyp-D is considered the promoter of mPTP opening in different cell types and tissues [48]. We measured the expression of mPTP components in SP and BEP groups. Figure 8a show that Cyp-D

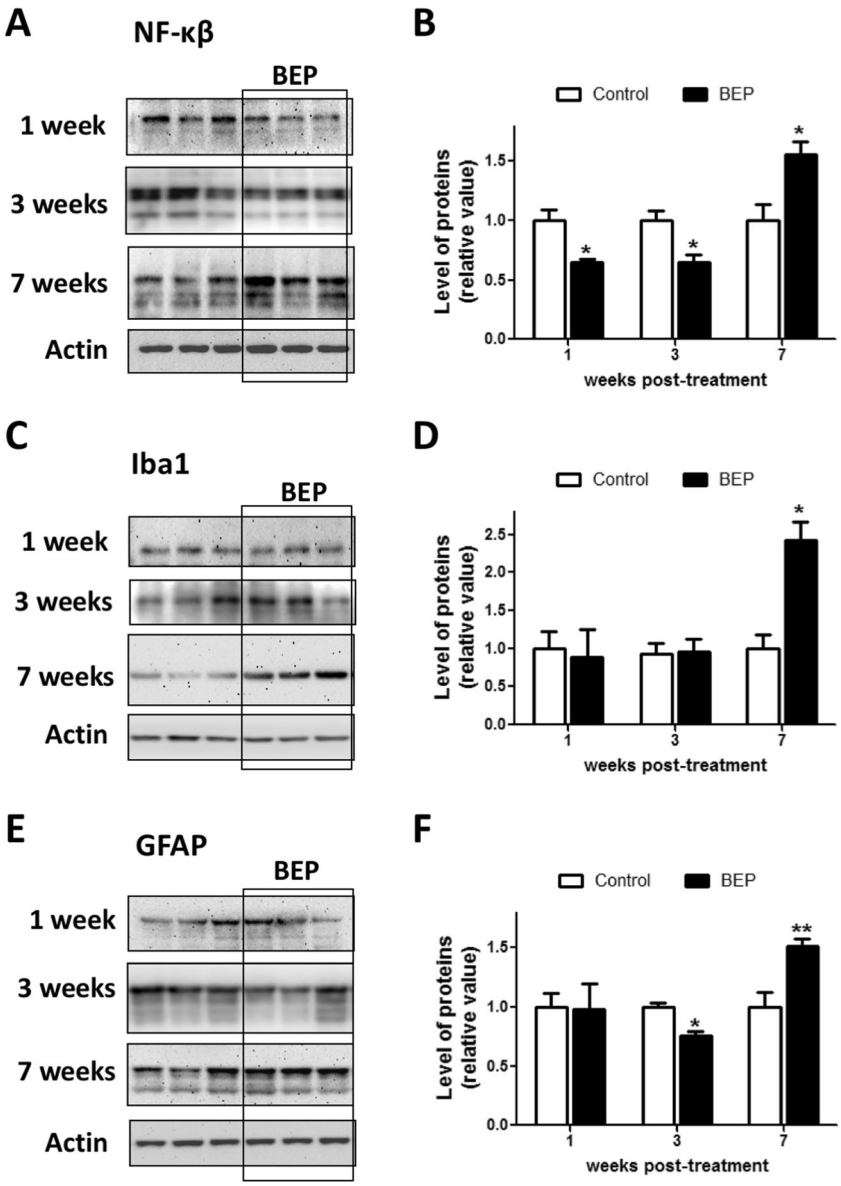


Fig. 3 Adolescent binge-like ethanol treatment induces a delayed inflammatory response. Representative images of Western blot analysis of hippocampal samples from control (SP) and ethanol-treated rats (BEP). The levels of **a** NF-κβ, **c** Iba1, and **e** GFAP proteins were measured at 1, 3, and 7 weeks after alcohol treatment, as indicated by the densitometric analysis (**b**, **d**, and **f**, respectively). Protein levels are expressed as the relative value of control animals, and the *dotted line* indicated control levels. *The graph* represents a whole analysis of $n = 3$, $n = 4$, and $n = 5$ different animals at 1, 3, and 7 weeks, respectively. Bars represent the mean ± SEM. $*p < 0.05$; $**p < 0.01$, $\#p < 0.001$

levels were significantly decreased in BEP animals at 1, 3, and 7 week post treatment. Also, Fig. 8b showed that VDAC expression was significantly decreased at 3 and 7 weeks post treatment. Finally, ANT protein expression levels were similar in all experimental groups, as presented in Fig. 8c, at all time points analyzed.

Ethanol Binge-Like Consumption Affects ATP Production in Adolescent Rat Brains

Our results suggest that the application of BEP protocol to rats induced several compensatory responses to restore oxidative damage produced by alcohol intake (Fig. 7). Acute treatment with high concentrations of ethanol produces a significant increase in the levels and transcriptional activity of nuclear factor erythroid-2 related factor 2 (Nrf2) in primary neurons [49]. Nrf2, under stress conditions, regulates the expression of several antioxidants, anti-inflammatory, and detoxification enzymes, as well as proteins that participate in the reduction of oxidative damage through the improvement of mitochondrial function [50]. Nevertheless, it is unknown whether these or other compensatory responses can restore oxidative homeostasis after binge-like ethanol exposition in vivo. To evaluate this possibility, we measured Nrf2 expression levels in both SP and BEP groups at different times. Our results indicated that at 1-week Nrf2 levels increase after ethanol binge-like protocol, an effect that was significant after 3 and 7 weeks post alcohol treatment (Fig. 9a, b) suggesting that Nrf2 signaling could be responsible for restoring oxidative damage observed in BEP rats.

Brain cells use high amounts of ATP for normal activity and cell signaling [51]. Disruption of mitochondrial dynamics

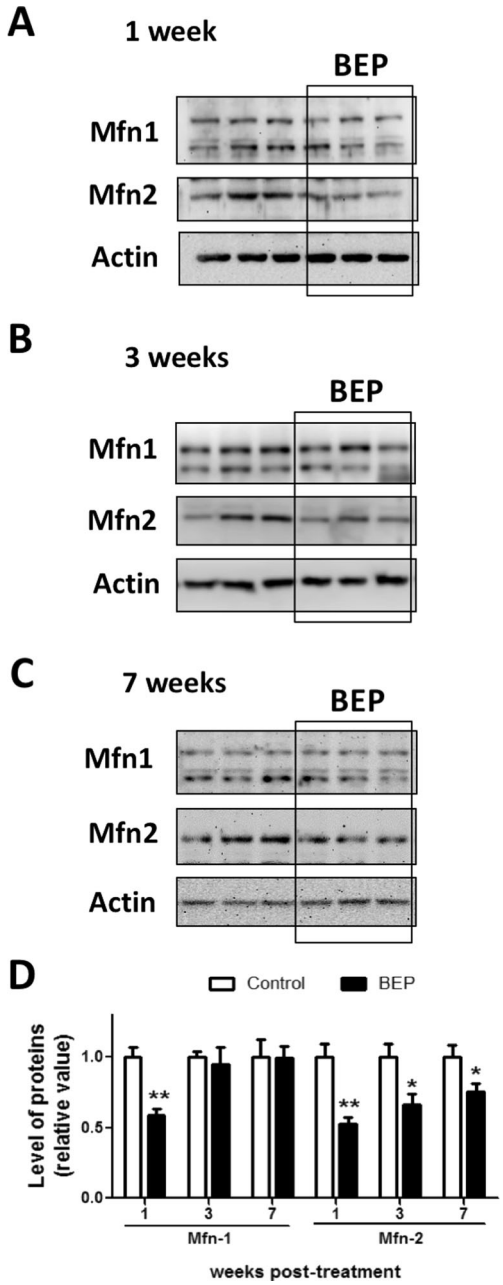


Fig. 4 Ethanol binge-like drinking affects mitochondrial dynamics regulation in adolescent rats. Representative Western blot of hippocampal lysates from control and ethanol-treated (BEP) rats. Expression levels of Mfn1 and Mfn2 were measured using specific isoform antibodies, in both control and binge alcoholized rats at **a** 1 week, **b** 3 weeks, and **c** 7 weeks post treatment. Densitometric analysis of both proteins is shown in (**d**). Protein levels are expressed as the relative value of control animals, and control levels are indicated by the *dotted line*. *The graph* represents a whole analysis of $n = 3$, $n = 4$, and $n = 5$ different animals at 1, 3, and 7 weeks, respectively. *Bars* represent the mean ± SEM. $*p < 0.05$; $**p < 0.01$, $\#p < 0.001$

may result in mitochondrial membrane potential loss and reduced ATP production [52]. In our studies, we observed severe alterations in the major proteins necessary for mitochondrial fission/fusion regulation (Figs. 4, 5, and 6). In this context, we measured ATP production, using an ATP measuring kit where bioluminescence is proportional to quantitative ATP concentration, as shown in Fig. 9c. We measured ATP produced in the hippocampus from both SP and BEP groups at 1, 3, and 7 weeks. Our results indicate that at 1-week post treatment animals exposed to alcohol had decreased ATP levels, an effect that was significant at 3 and 7 weeks post ethanol exposure (Fig. 9d). These observations suggest a significant impairment of mitochondrial bioenergetics in rats exposed to binge-like ethanol treatment.

Discussion

In the present study, we showed that adolescence binge-like alcohol consumption triggers detrimental effects in hippocampal function; several effects persisted until adulthood, whereas others were compensated overtime. We observed alterations in learning and memory accompanied by disrupted synaptic transmission, which was partially compensated, a delayed inflammatory response, an imbalance of mitochondrial dynamics, and decreased expression of mPTP components. We also found increased Nrf2 levels which possibly correspond to the antioxidant response against alcohol treatment; however, this mechanism is not enough to maintain an efficient mitochondrial function, because insufficient ATP production in the hippocampus is evident for up to 7 weeks post alcohol treatment (Fig. 9). In summary, these results indicate that adolescent binge-like alcohol results in severe structural and functional alterations in hippocampal cells, specifically in the mitochondria, which remain in time and affect adult brain function.

Several studies have shown that adolescent brains are more vulnerable that adult brains to ethanol toxicity, even when this is ingested in the pattern of binge drinking [13, 24]. Bouts of binge drinking produce necrotic neurodegeneration in rat brains, which could explain the loss of volume and white matter detected in the brains of adolescents that drank alcohol in the binge drinking pattern [53, 54]. Adolescent rats exposed to a binge drinking exhibited behavioral deficits associated with hippocampal function just 24 h after the last ethanol administration (PND 39) and 3 weeks after the last administration (PND 60), using only conditional discrimination learning test in a wooden Y-maze [21]. In our studies, we apply three different cognitive tests to evaluate the effect of binge drinking on learning and memory in adolescent rats submitted to binge drinking. Memory flexibility, a variant of water maze, and novel object recognition, both are tests which mainly measure the hippocampal functionality [55, 56]. We were able to observe the differences between experimental groups only 1 week after the alcohol treatment and only in NOR test, which could indicate that effect of ethanol is not only dependent of hippocampus and is highly sensitive to the test used. By that reason, we applied the social interaction test which is

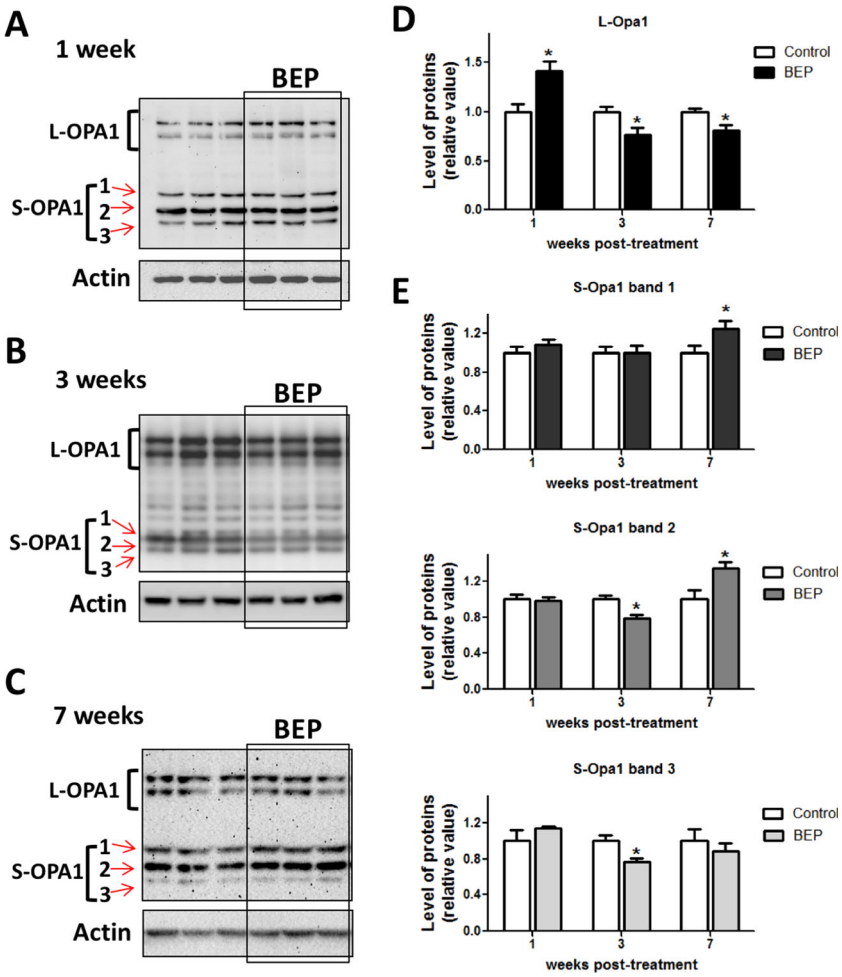


Fig. 5 Adolescent binge-like ethanol treatment affects Opa1 expression. Representative Western blot of hippocampus obtained from saline and alcoholized (BEP) rats at different times post treatment. Expression levels of long-Opa1 (L-Opa1) and short-Opa1 (S-Opa1) forms measured at **a** 1 week, **b** 3 weeks, and **c** 7 weeks post ethanol exposure. Densitometric analysis of **d** L-Opa1 and **e** the three S-Opa1 forms. Protein levels are expressed as the relative value of control animals and the *dotted line* indicates the control levels. *The graph* represents a whole analysis of $n = 3$, $n = 4$, and $n = 5$ different animals at 1, 3, and 7 weeks, respectively. *Bars* represent the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$, $\#p < 0.001$

related to hippocampal function, but other brain structures that are also regulating this performance in this test. In this case, we observe, similar to NOR, that ethanol binge drinking exposure affected rat performance just after 1 week. The significant differences both in the recognition memory and in social interaction were compensated overtime. There is a possibility that the cognitive impairment observed at 1-week post-BEP treatment may be attributed exclusively to the last dose administrated. However, it is possible that these compensatory effects are related to some adverse effects to a cellular level that was reverted, including the synaptic strength, presynaptic neurotransmitter release, and oxidative stress, in addition to the activation of the Nrf-2 signaling pathway. Posterior studies could resolve this question, evaluating the synaptic and cognitive performance after a single binge.

Despite the evidence presented above, the cellular mechanisms involved in the cognitive impairment caused by ethanol consumption are still unknown, and it has been suggested that this may be due to excitotoxicity [57]. Animals submitted to chronic alcohol treatment had increased glutamate levels and alterations in the expression of both glutamate receptor and transporter [58]. Alcoholic patients also present higher glutamate levels in their cerebrospinal fluid (CSF) [59]. Changes in the expression of two ionotropic glutamatergic receptors, *N*-methyl-D-aspartate (NMDA) [60, 61] and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) have been reported [62, 63]. In our studies, we showed that BEP induced alterations in the synaptic transmission, using electrophysiological tools in the field recording configuration of hippocampal slices. We observed that after 1 week of ethanol exposure, the synaptic strength was decreased, but after 3 and 7 weeks, this effect in the CA3–CA1 circuit is compensated. Similar results are observed in the presynaptic neurotransmitter release using paired pulse facilitation (PPF), in where the decreased in PPF index after 1 week of ethanol exposure indicated that presynaptic mechanism is affected under BEP protocol, and this effect similar to what we observed in synaptic strength is compensated for 3 and 7 weeks post-BEP. Using LTP protocol to analyze synaptic plasticity, we can obtain differences in plasticity between SP and BEP groups 1 and 7 weeks after we finish the ethanol protocol, but not at 3 weeks after BEP. These surprising observations could be explicated by the mechanisms underlying synaptic plasticity in LTP process [64]. Synaptic strength and PPF index are an indicator of

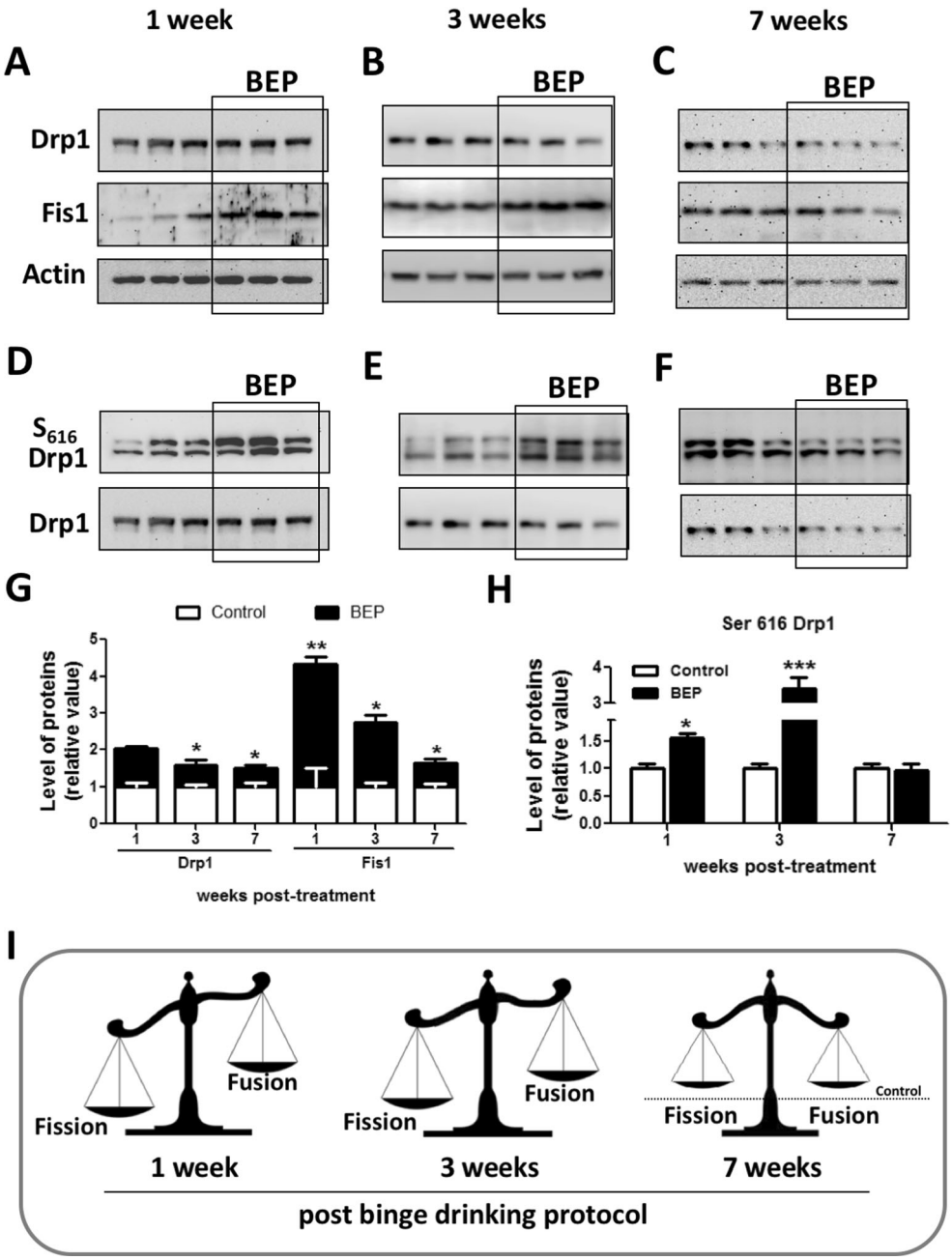


Fig. 6 Ethanol binge-like consumption affects mitochondrial fission in adolescent brain rats. Representative images of a Western blot of hippocampal samples from control and ethanol-treated (BEP) rats. Analysis of the expression of Drp1 and Fis1 proteins at **a** 1 week, **b** 3 weeks, and **c** 7 weeks post binge-like ethanol protocol. The levels of phosphorylated Drp1 at Ser616 (S_{616} Drp1) were evaluated in both control and alcohol-treated groups at **d** 1 week, **e** 3 weeks, and **f** 7 weeks post exposure. *Graphs* indicate the densitometry of **g** total Drp1 and Fis 1 and **h** phospho-Drp1 at all analyzed times. Protein levels are expressed as the relative value of control animals, and the *dotted line* indicates the control levels. **i** Schematic summary of alterations observed in the mitochondrial fission/fusion balance after ethanol binge-like consumption. *Graph* represents the analysis of $n = 3$, $n = 4$, and $n = 5$ different animals at 1, 3, and 7 weeks, respectively. *Bars* represent the mean ± SEM. $*p < 0.05$; $**p < 0.01$, $\#p < 0.001$

the basal transmission including the placed of ionic channels and structural spine configuration at the synapsis [65]. Also, LTP is an indicator of a dynamic process such as synaptic plasticity which could be affected by conditions in where basal transmission is not altered. Considering the complex changes that BEP protocol induce is possible to observe this behavior in glutamatergic synapses which are in constant dynamics changes to counteract the effect generated by ethanol exposure. It has been reported that acute ethanol intoxication partially inhibits NMDAR blocking LTP induction. However, in choric ethanol conditions, there are persistent changes in both glutamate- and GABA-mediated transmission through variations in the synapses structure at long-time [66]. In agreement with our findings at 7 weeks, studies in hippocampal slices treated with ethanol showed a decrease in the generation of LTP even when ethanol is withdrawn for 1 month or more time [66]. These studies suggest that the impairment in synaptic plasticity at 7 weeks post-BEP could be the consequence of repeat alcohol exposure more than that related to the last dose. However, more studies are necessary to validate this possibility, evaluating the synaptic plasticity overtime after a single binge drinking administration.

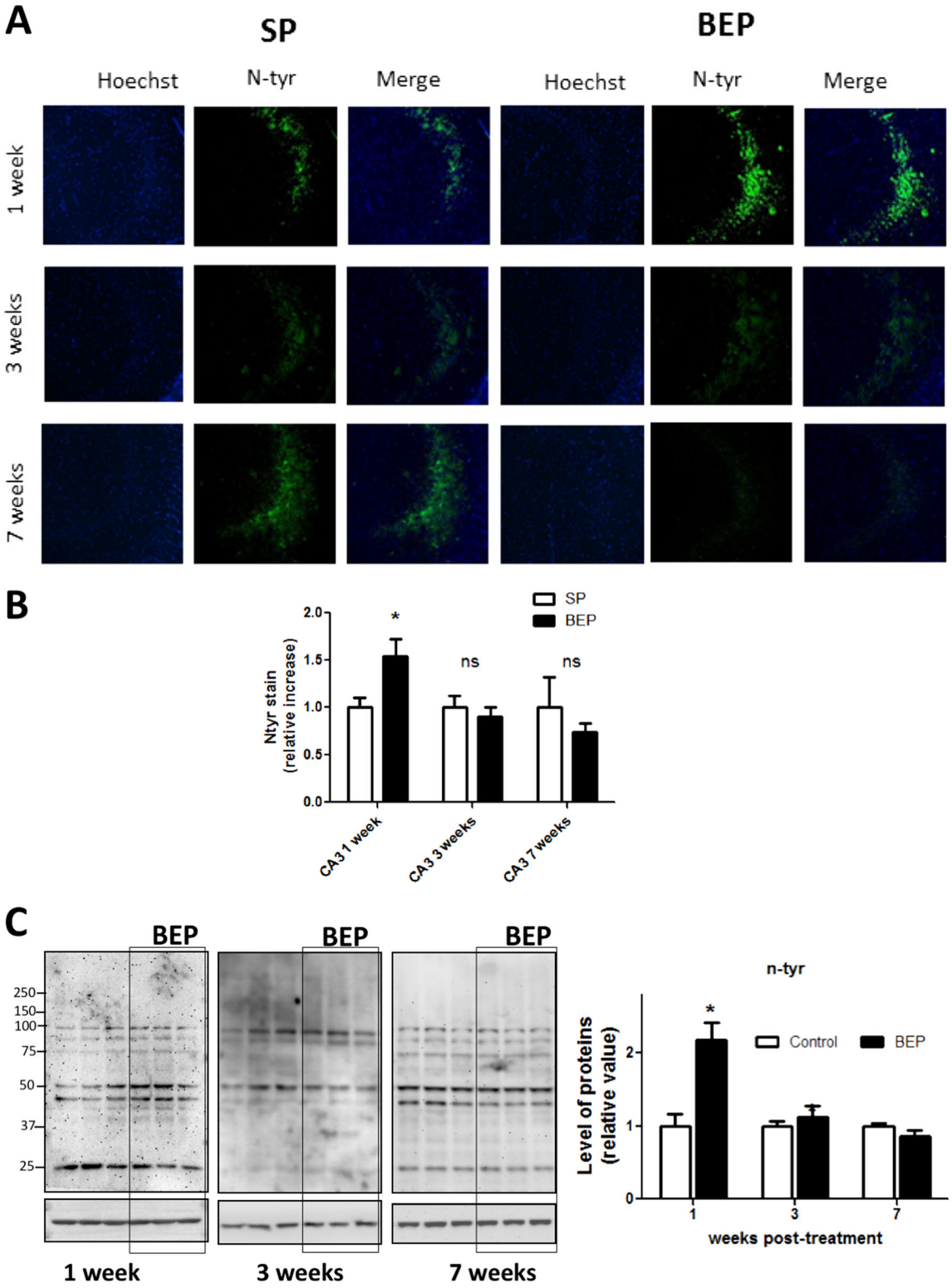
The recovery of cognitive abilities and partial compensation of synaptic functions at 3 and 7 weeks post binge drinking ethanol exposure is interesting and reflect the plasticity of the neuronal circuits. It has been described that alcohol

consumption depresses brain function by altering the balance between inhibitory and excitatory neurotransmission. Short-term alcohol exposure enhances the inhibitory function and decreases the excitatory function, inhibiting LTP induction and triggering memory loss [13]. Instead, after long-term alcohol exposure has been suggested that the brain attempts to restore equilibrium, decreasing inhibitory neurotransmission and enhancing excitatory neurotransmission [66, 67]. On another hand, during alcohol withdrawal, the synaptic system is again imbalanced, and LTP is deficiently induced until 1 month posterior to alcohol exposure [66, 67]. These antecedents could explicate, almost in part, the compensatory effects observed at long-time in the cognitive and social tasks and the deficient LTP seen at 7 weeks post alcohol treatment.

Among the several alterations reported by alcohol consumption, brain inflammation could contribute to neuronal dysfunction and finally to neurodegeneration [30]. The alcohol-induced inflammatory process involves the activation of pro-inflammatory transcriptional factors and the production of chemokines, cytokines, and enzymes participating in the immune response [31]. Our results indicate that alcohol increases the levels of inflammatory markers in both microglia and astrocytes. Nevertheless, this increase is observed only 7 weeks post-BEP. Other studies propose that inflammation could further activate the transcription factor NF-κB [30], which can indirectly potentiate glutamate neurotoxicity [68]. Complementary to these observations, we observed an increased in NF-κB levels, simultaneously with Iba1 and GFAP 7 weeks after BEP. In this scenario, analyzing the whole hippocampus through Western blot assays, and in the absence of in situ studies, we are incapable to discriminate that cellular type is the main responsible of the inflammatory response. Independent of the source of inflammation, up-regulation of inflammatory molecules also plays a major role in neuronal loss and neurodegenerative diseases or brain insults [69]; therefore, the inflammation induced by adolescent BEP observed in our study could affect the cellular function.

Mitochondria are organelles that contribute to cellular bioenergetics, participate in redox balance and mediate cell death [18]. Mitochondria are dynamic organelles which continuously undergo fission and fusion events [52]. The balance between fission and fusion regulates both mitochondrial distribution and energy production [46]. Therefore, alterations in this process could affect mitochondrial function. Impaired mitochondria were observed following ethanol exposure in hepatocytes and cerebellar cells [37, 70, 71]. Hepatocytes isolated from chronically ethanol-fed rats showed defects in mitochondrial volume and dynamics [37]. Because of their high metabolic demands, neurons are particularly sensitive to mitochondrial dysfunction [72]. Hence, alterations in mitochondria can result in synaptic impairment and memory loss [17]. Our study is the first to show the key role of mitochondria in alcohol binge drinking in vivo. We found severe perturbations in mitochondria that could partially explain the defects observed in synaptic transmission and memory processes. We observed altered expression of proteins involved in fusion and fission events (Figs. 4, 5, 6). Normal mitochondrial fission in neurons allows mitochondrial renewal, redistribution, and proliferation, whereas fusion enables its communication, facilitating mitochondrial movement and distribution into the synapses [73]. An imbalance between fusion and fission is also associated with neurodegeneration and brain insults [74], and changes in the regulation of mitochondrial fission have been considered as an early event in cell death [75]. We showed an increase of mitochondrial fission proteins accompanied by a decreased expression of fusion proteins at 1 and 3 weeks post-BEP. These results suggest that BEP could induce degenerative processes in the brain through mitochondrial failure. At 7 weeks, we observed that the balance of mitochondrial dynamics is restored; however, the levels of both fission and fusion proteins remained decreased compared with control animals. Interestingly, a lower rate of mitochondrial dynamics has been linked to mitochondrial dysfunction [65]. A double mutant with a reduced expression of Drp1 and Opa1 proteins showed deficient mitochondria respiration, and a diminish response to stress, which could lead to decreased mitophagy [76]. Therefore, it is possible that a similar consequence may occur in our rats exposed to ethanol and that this could contribute to functional alterations of the brain.

The main effectors involved in alcohol intoxication are oxidative stress and the inflammatory response [77], which together may contribute to cognitive impairment [53]. We observed an early oxidative damage at 1-week post-BEP. Overproduction of ROS can induce neuronal damage, including disruption of intracellular Ca2+ homeostasis, which is considered the basis of excitotoxicity in the brain [78]. Then, the oxidative stress observed in hippocampal tissue of neurons exposed to alcohol could contribute to the changes seen in synaptic transmission, mainly at 1-week post alcohol treatment. Nevertheless, the redox imbalance produced after BEP is rapidly restored, since at 3 and 7 weeks the levels of oxidative markers were similar to control levels. These observations suggest that alcohol binge-like consumption does not affect antioxidants enzymes but alcohol intoxication produce the activation of compensatory pathways to restore the redox balance. Studies that have evaluated the effects of alcohol consumption in the brain have found several potential sources of oxidative stress molecules as the oxide nitric synthase enzyme (NOS) [21, 79] or cytochrome P450 2E1 (CYP2E1) [80, 81]. In our case, during the alcohol binge drinking protocol, the mechanism by which oxidative species are generated is not clear. Mitochondria are the main source of ROS in cells; therefore, oxidative damage induced by alcohol may be caused by mitochondrial injury. In fact, here, we showed negative changes in mitochondrial dynamic proteins post-BEP, and it has been suggested that altered mitochondrial dynamics could



trigger negative changes in mitochondrial bioenergetics [17, 46, 74]. Therefore, we propose that adolescent ethanol exposure affects mitochondrial dynamics which could ultimately impair its function, resulting in increased ROS production and further oxidative damage at 1-week post treatment. Interestingly at 3 and 7 weeks, the 4-HNE protein adducts and protein nitration was restored to normal levels, suggesting the activation of antioxidant pathways including Nrf-2 signaling to compensate the toxic effects of binge drinking exposure.

◀ **Fig. 7** Ethanol binge-like administration induces oxidative damage in the brain of adolescent rats. (**a**) Representative images from immunofluorescence of the CA3 region of the hippocampus obtained from animals at three different time points: 1 week after injections (n = 3), 3 weeks after injections (n = 3), and 7 weeks after injections (n = 3). Hoechst staining was used to identify the nuclei, nytro-tyrosine immunoreactivity and merge images are shown. **b** Quantification of images in (**a**) shows a comparison between the three different times after ethanol treatment. There was an increase in nytro-tyrosine immunoreactivity 1 week after ethanol injections, and there was no difference between the control and ethanol group at the other measured times. *p < 0.05; *ns* not significant. **c** Representative Western blot of hippocampal samples from control or ethanol-treated (BEP) rats at 1, 3, and 7 weeks, using the anti-n-tyr antibody and its densitometric analysis. Protein levels are expressed as the relative value of control animals and the dotted line indicates the control levels. Analysis of n = 3, n = 4, and n = 5 different animals at 1, 3, and 7 weeks, respectively. *Bars* represent the mean ± SEM. *p < 0.05; **p < 0.01, #p < 0.001

New roles for the mitochondria have been discovered over the last years. It has been proposed that perturbations in mitochondrial functions may activate inflammatory responses [15, 16, 82, 83]. A positive feedback loop could contribute to neuronal damage, since oxidative stress can trigger inflammation and this process produces more oxidative stress, inducing tissue damage and ultimately leading to chronic inflammation [16]. Studies using antioxidants support the idea that the alcohol-induced pro-inflammatory response is mediated by a primary oxidant effect [84]. Antioxidant treatment prevents ethanol binge drinking-induced damage [84], blocking the increase of COX-2 [77], and the use of antioxidants in vitro reduced NF-κB activation and neuronal death produced by ethanol [77]. Thus, these studies strongly suggest that the ethanol-induced oxidative stress triggers a pro-inflammatory response and altogether may cause long-term neurodegeneration.

Mitochondrial mPTP formation results in the loss of membrane potential and the release of apoptotic mediators that finally lead to cell death [85]. Interestingly, in our study, we observed that Cyp-D and VDAC levels are significantly decreased post-BEP. These changes suggest that despite the neurotoxicity induced by alcohol, several mechanisms prevent neuronal death which leads to decreasing mPTP components levels and therefore avoiding its formation. One mechanism that could explain almost in part the compensatory effects observed in our studies is related to activation of the Nrf-2 pathway. In this report, BEP produced an increment in Nrf-2 levels at 3 weeks, effect that is more clear at 7 weeks after BEP, indicating a continuous activation of this factor in response to alcohol treatment.

Finally, mitochondrial structure and dynamics are fundamental for their efficient function and ATP production [39,

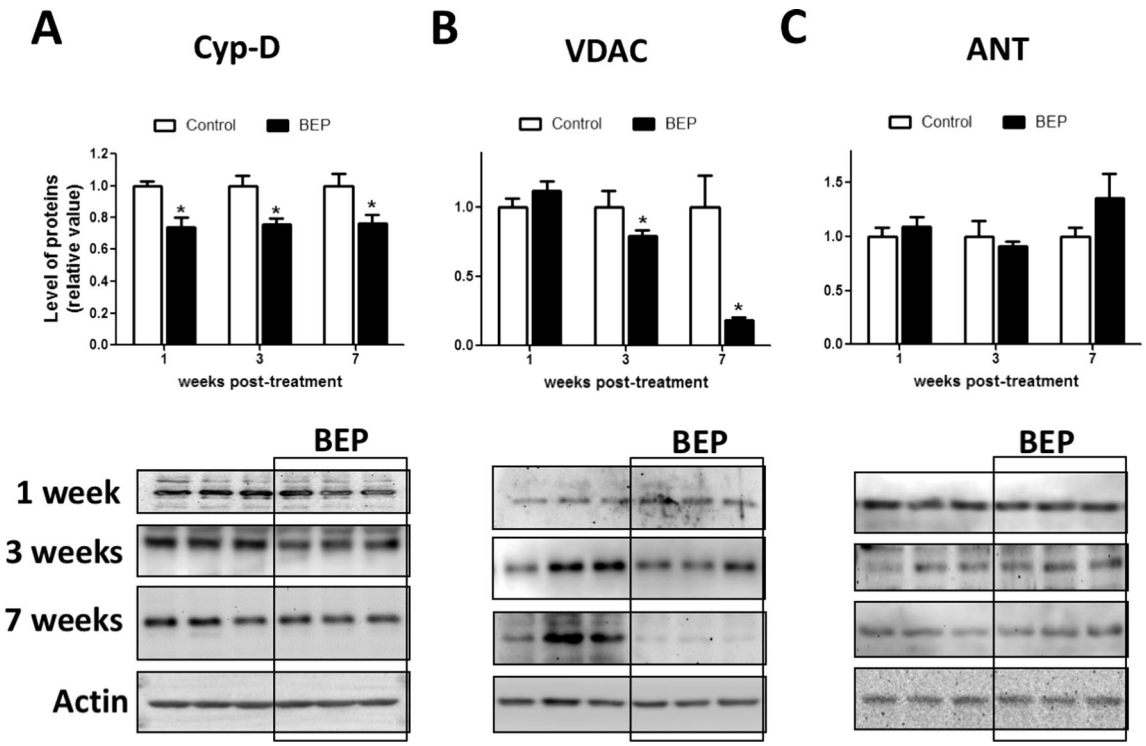


Fig. 8 Adolescent binge-like ethanol treatment modifies the expression of key components of the mitochondrial permeability transition pore (mPTP). Representative Western blot of hippocampal lysates from control and ethanol-treated (BEP) rats. Expression levels of **a** cyclophilin D (Cyp-D), **b** voltage-dependent anion channel (VDAC), and **c** the adenine nucleotide translocase (ANT) proteins in both saline and alcohol-treated groups at 1, 3, and 7 weeks post-injection protocol and its respective densitometry analysis. Protein levels are expressed as the relative value of control animals and control levels are indicated by the *dotted line*. Analysis of n = 3, n = 4, and n = 5 different animals at 1, 3, and 7 weeks, respectively. *Bars* represent the mean ± SEM. *p < 0.05; **p < 0.01, #p < 0.001

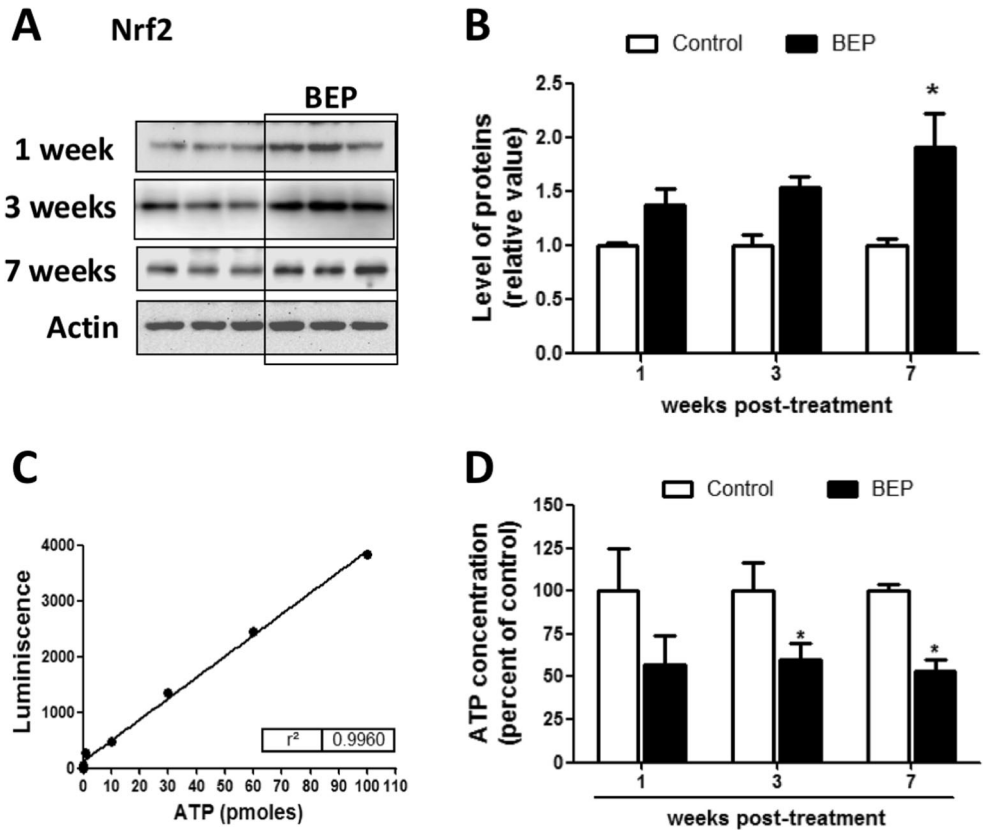


Fig. 9 Alcohol binge-like exposure increases Nrf2 levels and reduces ATP production in adolescent brain rats. **a** Representative Western blot of hippocampal samples from control and alcohol-treated (BEP) animals showing Nrf2 protein levels and its densitometric analysis (**b**). *Bars* represent the mean ± SEM. *$p < 0.05$; **$p < 0.01$. **c** ATP concentration was measured using an ATP detection kit (see methods). ATP concentration is directly proportional to the produced luminescence. **d** Graph indicating ATP concentration, expressed as a percent of the control, measured at 1, 3, and 7 weeks post saline or ethanol administration. *The graph* represents the analysis of $n = 3$, $n = 4$, and $n = 5$ different animals at 1, 3, and 7 weeks, respectively. Bars represent the mean ± SEM. *$p < 0.05$

46]. Previous reports have indicated that mitochondrial respiration is necessary for retention and consolidation of spatial memory in mice [86]. Mitochondria also play important roles in regulating synaptic function, modulation of calcium levels, and production of ROS [87]; therefore, alterations in mitochondrial bioenergetics or increased protein oxidization observed in our study could be responsible for synaptic impairment and finally the observed memory loss in animals exposed to alcohol. The decreased ATP production may be caused by changes in the number of mitochondria present in the hippocampus post treatment. To discard differences in the mitochondrial mass as consequence of increased mitophagy or decreased mitochondrial biogenesis, we used MitoTracker Green dye in coronal sections from unfixed brain tissue. Not significant differences were observed at 7 weeks post treatment in BEP rats in comparison with SP rats (Supplementary Figure 2). In the same sections, we analyzed the number of cells per field in the hippocampus, and similar results were observed in both control or treated rats (Supplementary Figure 2). Therefore, the decreased ATP production sustained over time could be the product of other factors. It is well-established that mitochondrial functions are affected when exposed to alcohol through increased levels of oxidative/nitrative stress [20, 77]. Damaged mitochondria may be responsible for the delayed inflammatory response observed at 7 weeks post treatment [16]. Consequently, the inflammatory process could contribute to the deficient mitochondrial function [88], such is suggested by decreased ATP production in the adulthood. In the following studies, the link between inflammation and mitochondrial dysfunction ethanol-mediated could be evaluated.

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Author Contributions CTR, RAQ, WC, JAO, and JML designed and coordinated the study. CTR, FJC, RMG, and CA conducted most of the experiments and performed the statistical analyses. CTR, WC, and RAQ wrote the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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