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Aspirin and N-acetylcysteine co-administration markedly inhibit chronic ethanol intake and block relapse binge-drinking: Role of neuroinflammation-oxidative stress self-perpetuation.

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Abstract:	Chronic alcohol intake leads to neuroinflammation and cell injury, proposed to result in alterations that perpetuate alcohol intake and cued-relapse. Studies show that brain oxidative stress is consistently associated with alcohol-induced neuroinflammation, and literature implies that oxidative stress and neuroinflammation perpetuate each other. In line with a self-perpetuating mechanism, it is hypothesized that inhibition of either oxidative stress or neuroinflammation could reduce chronic alcohol intake and relapse. The present study conducted on alcohol-preferring rats shows that chronic ethanol intake was inhibited by 50-55% by the oral administration of low doses of either the antioxidant N-acetyl cysteine (40 mg/kg/day) or the anti-inflammatory aspirin (ASA; 15 mg/kg/day), while the co-administration of both dugs led to a 70-75% ($p<0.001$) inhibition of chronic alcohol intake. Following chronic alcohol intake, a prolonged alcohol deprivation and subsequent alcohol re-access, relapse-drinking resulted in blood-alcohol levels of 95-100 mg/dl in 60 minutes which were reduced by 60% by

	either N-acetyl cysteine or aspirin, and by 85% by the co-administration of both drugs (blood-alcohol: 10-15 mg/dl; $p<0.001$). Alcohol intake either on the chronic phase or following deprivation and re-access led to a 50% reduction of cortical glutamate transporter GLT-1 levels, while aspirin administration fully returned GLT-1 to normal levels. N-acetyl cysteine administration did not alter GLT-1 levels, while N-acetyl cysteine may activate the cystine/glutamate transport xCT, presynaptically inhibiting relapse. Overall, the study suggests that a neuroinflammation/oxidative stress self-perpetuation cycle maintains chronic alcohol intake and relapse drinking. The co-administration of anti-inflammatory and antioxidant agents may have translational value in alcohol-use-disorders.

Aspirin and N-acetylcysteine co-administration markedly inhibit chronic ethanol intake and block relapse binge-drinking: Role of neuroinflammation-oxidative stress self-perpetuation.

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Running Title: Alcohol, oxidative stress-neuroinflammation.

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ABSTRACT

Chronic alcohol intake leads to neuroinflammation and cell injury, proposed to result in alterations that perpetuate alcohol intake and cued-relapse. Studies show that brain oxidative stress is consistently associated with alcohol-induced neuroinflammation, and literature implies that oxidative stress and neuroinflammation perpetuate each other. In line with a self-perpetuating mechanism, it is hypothesized that inhibition of either oxidative stress or neuroinflammation could reduce chronic alcohol intake and relapse. The present study conducted on alcohol-preferring rats shows that chronic ethanol intake was inhibited by 50-55% by the oral administration of low doses of either the antioxidant N-acetyl cysteine (40 mg/kg/day) or the anti-inflammatory aspirin (ASA; 15 mg/kg/day), while the co-administration of both dugs led to a 70-75% ($p<0.001$) inhibition of chronic alcohol intake. Following chronic alcohol intake, a prolonged alcohol deprivation and subsequent alcohol re-access, relapse-drinking resulted in blood-alcohol levels of 95-100 mg/dl in 60 minutes which were reduced by 60% by either N-acetyl cysteine or aspirin, and by 85% by the co-administration of both drugs (blood-alcohol: 10-15 mg/dl; $p<0.001$). Alcohol intake either on the chronic phase or following deprivation and re-access led to a 50% reduction of cortical glutamate transporter GLT-1 levels, while aspirin administration fully returned GLT-1 to normal levels. N-acetyl cysteine administration did not alter GLT-1 levels, while N-acetyl cysteine may activate the cystine/glutamate transport xCT, presynaptically inhibiting relapse. Overall, the study suggests that a neuroinflammation/oxidative stress self-perpetuation cycle maintains chronic alcohol intake and relapse drinking. The co-administration of anti-inflammatory and antioxidant agents may have translational value in alcohol-use-disorders.

Keywords: acetyl salicylic acid, ASA, astrocytosis, glutamate, microglia, oxidative stress

A number of studies have shown that chronic alcohol intake or its administration leads to the synthesis of pro-inflammatory cytokines, astrocytosis and microglial activation; hallmarks of neuroinflammation[1-7]. The mechanism of alcohol-induced neuroinflammation is in part mediated by the influx of gut lipopolysaccharide (LPS) into the systemic circulation. Acetaldehyde, generated from ethanol metabolism, increases the permeability of gut endothelial tight-junctions allowing the influx of bacterial LPS into the portal circulation[8], further releasing tumor necrosis factor-alpha (TNF-α) by hepatic macrophages, which through the systemic circulation reaches the brain via TNF-α receptors present in the blood brain barrier [9, 10].

A factor that potentiates neuroinflammation is brain oxidative stress generated upon chronic ethanol intake or its systemic administration [5, 6, 11-13]. Brain oxidative stress is generated by most drugs of abuse [14], and is seen after a single dose of cocaine [15]. For alcohol, increases in reactive oxygen species follow the oxidation of dopamine released during the stimulation of mesolimbic dopamine system [16-18], while dopamine released is metabolized by monoamine oxidase generating hydrogen peroxide [14]. Further, at the extracellular physiological pH the auto-oxidation of catechol groups of dopamine leads to superoxide-ion formation [19]. Additionally, chronic ethanol intake or its administration leads to increases in brain cytochrome CYP2E1 levels[20], which generate superoxide ion radicals and hydrogen peroxide[21]. In turn, reactive oxygen species induce neuroinflammation via the NF-kB pathway by inactivation of the NF-kB inhibitor IkB, leading to the generation of pro-inflammatory cytokines such as TNF-α[22]. TNF-α further activates NADPH oxidase [23, 24], which generates hydrogen peroxide. Additionally, TNF-α increases the generation of mitochondrial superoxide ions [25]. Thus, the literature strongly implies the existence of an oxidative stress-neuroinflammation vicious-cycle perpetuating both oxidative stress and neuroinflammation.

The alcohol and drug addiction field has paid special attention to neuroinflammation and cell injury as the main factors perpetuating the use of ethanol and other addictive drugs [26]. Which of either, oxidative stress or neuroinflammation, initiates the self-perpetuating cycle is not clear, although for several drugs of abuse oxidative stress likely precedes neuroinflammation - since for other drugs increases in systemic LPS have not been reported as for alcohol [14, 15].

The relationship between neuroinflammation and alcohol self-administration was demonstrated following the single administration of LPS to mice [27]. Lipopolysaccharide likely acting via Toll-like receptor-4 (TLR-4) increases alcohol self-administration [28]. Montesinos et al [29] showed that the increased ethanol self-administration that follows chronic ethanol administration is inhibited by nalmefene, an inhibitor of TLR-4. Increases in voluntary ethanol intake were also shown in mice following neuroinflammation induced by the administration of dsRNA poly(I:C), which acts via a different pathway; namely the TLR-3 route [30]. Noteworthy, in detoxified alcoholics a strong relationship is seen between alcohol craving, plasma LPS and pro-inflammatory cytokines[10].

The existence of a neuroinflammation-oxidative stress self-perpetuating cycle suggests that the administration of an *antioxidant* may inhibit neuroinflammation as well as reducing chronic alcohol intake and relapse drinking. Recent studies [31, 32] showed that administration of the antioxidant (and glutathione precursor) N-acetyl cysteine to rats chronically self-administering ethanol solutions fully reversed alcohol-induced brain oxidative stress, abolished neuroinflammation, markedly inhibited chronic ethanol intake and partially prevented relapse-binge drinking. Such a marked inhibitory effect of N-acetyl cysteine was confirmed in alcohol cued- seeking operant studies in rats [33,34]. Operant studies by Gass et al [35]showed that following a period of chronic ethanol self-administration and deprivation, alcohol-associated cues markedly

elevate glutamate levels in the nucleus accumbens of rats, in line with the hypothesis that cued-induced increases in glutamatergic state lead to drug seeking and relapse in animals that have learned to self-administer the drug [36]. It has been proposed that the likely mechanisms involved in the reduction of ethanol intake induced by N-acetyl cysteine is an increase in cystine-glutamate exchange via the astrocyte xCT transporter, activating inhibitory presynaptic (or peri-synaptic, *vide-infra*) mGlu2/3 receptors, thus reducing cued-released glutamate levels[34, 37].

How neuroinflammation may play a role in cued-relapse alcohol intake is not clear. However, the cued-released glutamate at the tripartite synapse is potentiated by a low glutamate removal due to the deficient astrocyte glutamate transporter GLT-1 induced by oxidative stress, which accompanies neuroinflammation, leading to the inactivation of the transporter sulfhydryl moieties by oxidation[38]. The relevance of the GLT-1 transporter is shown by the administration of the antibiotic ceftriaxone which increases GLT-1 levels in several brain areas, including the prefrontal cortex, where it is associated with a marked inhibition of chronic ethanol intake [39] and also reducing the intake of other drugs of abuse [40, 41].

Mesenchymal stem cells and the products secreted by these cells, which display (i) strong antioxidant and (ii) anti-inflammatory properties, were recently shown to increase GLT-1 levels and to greatly reduce chronic alcohol intake and relapse-drinking[42,43]. Further, both ethanol-induced oxidative stress and neuroinflammation were fully abolished by mesenchymal stem cells. Indeed, mesenchymal stem cells have been termed “guardians of inflammation” [44]. Pre-incubation of mesenchymal stem cells with aspirin has been shown to increase their anti-inflammatory action [45]. Since the action of mesenchymal stem cells results from a multi-target effect, elucidating which of the several anti-inflammatory and antioxidant factors secreted exert

their anti-alcohol action will require further studies. Thus, other options are conceivable where the effect on chronic ethanol intake and relapse are investigated following the administration of known anti-inflammatory and antioxidant drugs.

A number of studies have shown that the anti-inflammatory drug aspirin (acetyl salicylic acid or ASA) activates the synthesis of peroxisome proliferator-activated receptor-gamma (PPARγ) known to have strong anti-inflammatory effects [46,47]. A wealth of data also has shown that moderate doses of acetyl salicylic acid (ASA) by acetylating cyclooxygenases I and II promote the synthesis of potent anti-inflammatory fatty acid metabolites (Lipoxins)[48,49]. Aspirin may additionally inhibit the NF-kB pathway [50]. Presently, an ongoing multicenter clinical trial (Australia, U.S.) investigates the use of Aspirin versus placebo in a severe inflammatory condition such as sepsis[51]. Further a recently clinical trial, in its double-blind phase, showed the effectiveness of the combination of aspirin plus N-acetylcysteine in the treatment of bipolar depression [52] a condition in which a major neuroinflammatory component exists.

Given the above, in the present studies we determined whether the: (i) *anti-inflammatory aspirin* (ASA) and the (ii) *antioxidant N-acetyl cysteine* (NAC) would be effective and synergize each other in reducing both chronic ethanol intake and relapse like-binge drinking in a rat model of high ethanol intake. The study tested the effect of aspirin at doses that do not induce gastric damage [53] and of N-acetyl cysteine within the doses previously used in humans [54, 55].

MATERIALS AND METHODS

Animals

Adult female rats, Wistar-derived, bred for over 90 generations as alcohol consumers (University of Chile Bibulous; UChB) [56, 57] were used in the experiments. Animals were maintained on a 12 h light/dark cycle (lights off a 7:00 PM) and regularly fed a soy-protein rodent diet (Cisternas, Santiago Chile). Experimental procedures were approved by the Ethics Committee for Experiments with Laboratory Animals at the Medical Faculty (Protocol CBA# 0994 FMUCH) and by the Chilean Council for Science and Technology Research (CONICYT).

Drugs and treatments

Alcohol solutions for drinking experiments were prepared from absolute alcohol (Merck, Darmstadt Germany) diluted to 10 or 20% alcohol (v/v) in tap water. Initially, N-acetylcysteine (Sigma, St. Louis,MO) for i.p. administration was dissolved in saline, adjusted with NaOH to pH 7.2 and injected in volume of 5.0 mL/kg at a dose of 70 mg/kg/day for two days, while subsequently N-acetylcysteine (NAC) for oral administration was dissolved in water, adjusted with NaOH to pH 7.2, and administered in 5.0 mL/kg by gavage at a dose of 40 mg/kg/day. Acetylsalicylic acid (ASA), (St. Louis, MO, USA) also known as Aspirin, was dissolved in water, adjusted with NaOH to pH 7.2, and given in volume of 5.0 mL/kg by oral gavage at a dose of 15 mg/kg/day to avoid gastric damage [53]. When the combination of N-acetylcysteine + acetylsalicylic acid was administered, both were dissolved in water, adjusted with NaOH to pH 7.2 and given in a volume of 5 mL/kg by oral gavage at a dose of 40 mg/kg of N-acetylcysteine plus 15 mg/kg of acetylsalicylic acid. In initial studies, ethanol access was continued while NAC, ASA or NAC+ASA were administered. In subsequent studies NAC, ASA or NAC+ASA were administered only during the alcohol deprivation period and discontinued 24 hours prior to allowing alcohol re-access.

Blood ethanol and glutathione determination.

Blood ethanol levels were determined in animals that had consumed alcohol chronically, were alcohol deprived for 9 days and offered ethanol re-access on day 10. Immediately, after 60 minutes of re-access to ethanol (water also available), 100 microliters of blood were collected from the tip of the tail under moderate acepromazine sedation (1 mg/kg i.p.). Samples were immediately mixed with 0.9ml of distilled water at 4°C in a glass vial sealed and analyzed by head space gas chromatography (Perkin Elmer SRI 8610) as previously described[58]

In studies in which the brain oxidative stress was determined, the ratio of oxidized (GSSG) and reduced (GSH) glutathione was determined in hippocampus as previously described [59]. Glutathione reductase (G3664), NADPH (N1630) and DTNB (5, 50-dithiobis-2-nitrobenzoic acid) (D-8130), used for the determination of glutathione, were purchased from Sigma-Aldrich.

Determination of astrocyte and microglia immunoreactivity.

Immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP) and the microglial marker ionized-calcium-binding adaptor molecule 1 (Iba-1) were evaluated in coronal cryo-sections of hippocampus (30um thick) as previously reported[59]. Nuclei were counterstained with DAPI. Microphotographs were taken from the lacunosum moleculare stratum of hippocampus using a confocal microscope (Olympus FV10i). The area analyzed for each stack was 0.04 mm^2 and the thickness (Z axis) was measured for each case. The total length and thickness of GFAP positive astrocyte primary processes and density of Iba-1 positive microglial cells were assessed using FIJI image analysis software (http://fiji.sc/Fiji) as previously reported [59].

Quantification of GLT-1 levels in prefrontal cortex.

Samples of prefrontal cortex of (i) N-acetylcysteine (NAC); (ii) acetyl salicylic acid (ASA), (iii) the combination NAC+ASA or (iv) vehicle-treated rats were procured and proteins were extracted using RIPA buffer (Thermo-Fisher) containing protease inhibitors. Western blot procedures to examine the levels of GLT-1 were performed using a guinea pig anti-GLT1 antibody (AB1783, Merck) as previously described[6]. The same membranes were also assessed for β-actin immunobloting as loading control (anti β-actin, sc-47778 Santa Cruz Biotechnology). Reactive bands were detected using the Odyssey Imaging System (LI-COR) and quantified using the Image Studio Lite 5.2 software.

Experiment 1: Chronic alcohol intake: Effect of oral N-acetylcysteine administration, Acetylsalicylic acid or the combination of N-acetylcysteine plus Acetylsalicylic acid.

Twenty four adult female UChB rats, weighing 190-210 g, were single-housed in individual cages at the age of 60 days and maintained on a 12 h light/dark cycle (lights off at 7:00 PM). Female animals were utilized in the present study due to the long-term nature of the experiments and the ability of maintaining stable body weights over time. Subsequently, rats received continuous concurrent free-choice access in the home cage to 10% (v/v) ethanol and water for 77 days. On day 78 rats were allowed to concurrent three-bottle choice access to 10% and 20% (v/v) ethanol and water for 24 additional days. On day 88, rats were divided in 4 groups (n=6/group) including: (1) vehicle group: rats were given saline the first two days by the intraperitoneal (i.p.) route, and by oral gavage the following eleven days, (2) N-acetylcysteine (NAC) group: on the first two days NAC was administered by the intraperitoneal (i.p.) route (70 mg/kg/day), and subsequently by oral gavage (40 mg/kg)for the following 11 days, (3) Acetylsalicylic acid (ASA) group: rats were given saline via i.p. on the first two days followed by acetylsalicylic acid (15 mg/kg/day) given by oral

gavage, for the following 11 days and (4) N-acetylcysteine + acetylsalicylic (NAC+ASA) acid group: rats were given N-acetylcysteine (70 mg/kg/day), the first two days, followed by a daily combined N-acetylcysteine (40 mg/kg/day) plus acetylsalicylic acid (15 mg/kg/day) dose given by oral gavage, the following 11 days. Ethanol intake was recorded daily and expressed as g of ethanol consumed/kg body weight/day. Twenty-four hours after the last NAC, ASA or NAC+ASA administration and saline controls, the animals were anaesthetized with chloral hydrate (280 mg/Kg, i.p.) to obtain hippocampal samples for GSSG/GSH ratio determination, astrocyte (GFAP) and microglia (Iba-1) immunohistofluorescence and prefrontal cortex to determine GLT-1 levels (*vide infra*)

Experiment 2: Relapse-like ethanol drinking: Effect of oral administration of N-acetylcysteine, Acetylsalicylic acid or the combination of N-acetylcysteine + Acetylsalicylic acid during the alcohol deprivation period.

Twenty-four adult female UChB rats, weighing 190-250 g were housed in individual cages. Rats received continuous, concurrent free-choice access in the home cage to 10% (v/v) ethanol and water for 74 days. On day 75, rats were allowed to concurrent three-bottle choice access to 10% and 20% (v/v) ethanol and water, for 11 additional days. After induction of chronic ethanol intake, the animals were deprived of ethanol for 14 days and thereafter allowed re-access to ethanol solutions (10% and 20%), for five days. Rats received daily, a single dose of N-acetylcysteine (40 mg/kg/day); Acetylsalicylic acid (15 mg/kg/day) or N-acetylcysteine (40 mg/kg/day) + Acetylsalicylic acid (15 mg/kg/day) or vehicle (water) administered by oral gavage (n= 6 rats/group) during the last 9 days of ethanol deprivation. The last dose was administered 24 hours prior to offering the animals (on day 10) an initial 60-minute and further 24 hours re-access to 10% and 20% ethanol solutions, for five days. Ethanol intake was expressed as g of ethanol

consumed/kg body weight/day. Immediately after the first 60 minutes of ethanol re-access, blood samples were collected to determine blood ethanol levels. After the five-day ethanol re-access, animals were euthanized to determine hippocampal GSSG/GSH levels, astrocyte (GFAP) and microglia (Iba-1) immunohistofluorescence and GLT-1 protein levels in prefrontal cortex.

Statistical analyses. Statistical analyses were performed using GraphPad Prism (San Diego, CA). Data are expressed as means ± SEM. A two-way (treatment × day) analysis of variance (ANOVA), followed by Tukey post hoc test or ANOVA for repeated measures was conducted when required to compare the ethanol intake between vehicle and N-acetylcysteine, acetylsalicylic acid, or N-acetylcysteine/acetylsalicylic groups. One-way ANOVA followed by Tukey post hoc test was used to analyze total length and thickness of primary processes of GFAP positive astrocytes, microglial cell density, GSSG/GSH ratios, and GLT-1 protein levels data. A level of $P < 0.05$ was considered for statistical significance.

To facilitate text reading, full statistical ANOVA analyses are presented in the Legends to Figures.

RESULTS

Figure 1 shows that a marked inhibition of chronic ethanol intake was induced by the intraperitoneal administration of an initial dose of N-acetyl cysteine (70 mg/kg/day), for two days. This initial dose is equivalent to one-half of the initial dose administered systemically in the treatment of acetaminophen (paracetamol) hepatotoxicity in humans[54]. In order to avoid a possible "ceiling" of N-acetyl cysteine effect, when it is combined with aspirin (ASA), on day 3, the maintenance dose of N-acetyl cysteine (NAC) was reduced to 40 mg/kg/day and administered orally, for 11 days. Aspirin (ASA) administration at oral doses of 15 mg/kg/day also started on day 3 and was administered for 11 days. After such time ethanol intake was determined in the four groups: (Ethanol + Vehicle); (Ethanol + NAC); (Ethanol + ASA); (Ethanol + NAC + ASA). Ethanol intake was inhibited by 50-55% ($p<0.001$) in rats that received *either* N-acetyl cysteine or aspirin. The co-administration of N-acetylcysteine + aspirin showed a marked synergistic effect in inhibiting ethanol intake, such that chronic ethanol intake of the co-administered animals was inhibited by 70-75% ($p<0.0001$), which was significantly higher ($p<0.0001$) than the inhibition of ethanol intake of animals administered only N-acetyl cysteine or aspirin.

Figure 2 shows that chronic ethanol consumption led to significant ($p<0.001$) increases in both the length and thickness of hippocampal primary astrocyte processes versus water controls, determined by GFAP immunoreactivity. The density (cells/mm^3) of microglial cells determined by Iba-1 immunoreactivity was also significantly ($p<0.001$) increased in chronically ethanol consuming animals. Treatment of alcohol consuming animals with either (i) N-acetyl cysteine; (ii) aspirin or (iii) co-administration of both drugs fully normalized both astrocyte morphology and microglial tissue density ($p<0.001$).

Figure 3 shows that chronic ethanol consumption led to a 300% ($p<0.001$) increase in hippocampal oxidative stress, determined as the ratio of oxidized/reduced glutathione (GSSG/GSH). The oxidative stress was normalized by treatment with either (i) N-acetyl cysteine; (ii) aspirin or (iii) co-administration of both drugs ($p<0.001$), although treatment with only aspirin appeared somewhat less effective than that induced by only N-acetyl cysteine, but this difference did not attain statistical significance ($p>0.05$).

As proposed earlier, the literature implies that neuroinflammation and oxidative stress self-perpetuate each other for prolonged periods, which suggest such a protracted effect may be responsible for *relapse drug intake despite a prolonged drug abstinence*. Thus the present study determined whether the inhibitory effect on ethanol intake of N-acetyl cysteine and of aspirin is observed after *chronic ethanol consumption had ceased*. Figure 4A shows that rats that were alcohol deprived for 14 days after consuming ethanol chronically, consume ethanol to intoxicating levels when allowed ethanol re-access (vehicle group). Such a condition, first reported by Sinclair and Senter in 1968 [60], referred as the "alcohol deprivation effect" (ADE) is held as a clinical "proxi" for cued-intoxication and relapse binge-drinking, thus with relevant translational value (reviewed by Spanagel [61]). The increased ethanol intake upon ethanol re-access ($p<0.0001$) was observed for several days; although the greatest effect (in line with literature) was seen mostly on the first day of ethanol re-access. N-acetyl cysteine, aspirin or both drugs combined were administered daily for 9 days during the deprivation phase and discontinued 24-hours prior allowing ethanol re-access on day 10. Administration of N-acetyl cysteine (NAC: 40 mg/kg/day) or Aspirin (ASA: 15 mg/kg/day) during the deprivation phase significantly inhibited by 55% to 60% ($p<0.001$) ethanol intake following a 5-day ethanol re-access. The inhibition on ethanol intake was greater (75% $p<0.0001$) for the co-administration (NAC+ASA) group and significantly higher ($p<0.01$) than

intakes for NAC alone or ASA alone, respectively, thus indicating a synergy between the anti-inflammatory and antioxidant effects (Fig 4A right). Noteworthy, since neither N-acetyl cysteine nor aspirin or the combination of both drugs were administered *during the 5-day ethanol re-access time*, these drug effects were generated during the deprivation period. Figure 4B shows that after the deprivation period and upon re-access, the vehicle treated group consumed 1.87 ± 0.19 (mean ± SEM) g ethanol/kg in the first 60 minutes of alcohol re-access; an intake that was significantly reduced by about 55-60% ($p<0.001$) by pretreatment with either (i) N-acetyl cysteine (NAC) or (ii) aspirin (ASA) during alcohol deprivation. The 60-minute alcohol intake following (iii) the co-administration of (NAC+ASA) was reduced by 75% ($p<0.001$). Figure 4C shows that in vehicle-treated animals blood alcohol level after the 60-minute ethanol re-access was 97.0 ± 13 mg/dl (considered binge-drinking in humans; >80 mg/dl). Blood ethanol levels of animals treated with (i) N-acetyl cysteine (NAC) or aspirin (ASA) was 60% lower than those of the vehicle group ($p<0.001$). For animals pretreated with (NAC+ASA) blood ethanol level was 12.8± 4.3 mg/dl; thus, indicating that relapse-drinking was inhibited by 85% ($p<0.001$); resulting in blood alcohol levels akin to those seen in social drinking in humans.

Figure 5A shows the effects of ethanol deprivation period to rats previously consuming alcohol chronically (101 days) and allowed a 5-day ethanol re-access on the morphology of astrocytes (GFAP-immunoreactivity, red) and microglia (IBA-1-immunoreactivity, green) counterstained with nuclear marker (DAPI, blue) in hippocampus. Chronic ethanol consumption was followed by a 14-day alcohol deprivation and a subsequent 5-day alcohol re-access (Ethanol + vehicle), resulting in significant increases both in the length (Figure 5B) ($p<0.001$) and thickness ($p<0.001$) (Figure 5C) of astrocytic processes versus Water controls. The density (cells/mm^3) of microglial cells (Figure 5D) was also significantly increased ($p<0.01$) in Ethanol +vehicle animals versus Water controls. The

antioxidant or/and anti-inflammatory drug administration during 9-days of the alcohol deprivation-period and ethanol re-access on day 10 : (i) N-acetyl cysteine (Ethanol+NAC) ; (ii) aspirin (Ethanol +ASA) and (iii) co-administration of both drugs (Ethanol +NAC +ASA) ,led to the full normalization of the ethanol-induced increase in astrocyte Length (Fig 5B) (NAC $p < 0.001$; ASA $p < 0.0001$; NAC+ASA $p < 0.001$) thickness of astrocyte processes (NAC $p < 0.001$; ASA $p < 0.001$; NAC+ASA $p < 0.0001$) (Fig 5C) and ethanol-induced increase in microglial tissue density (NAC $p < 0.01$; ASA $p < 0.05$; NAC+ASA $p < 0.01$] (Fig. 5D).

Figure 6 shows the oxidative stress (GSSG/GSH) in the hippocampus of rats following chronic ethanol intake-deprivation and alcohol re-access. A 300% increase in the GSSG/GSH ratio ($p<0.001$) was again observed for ethanol re-access animals (ethanol-vehicle versus water naive), an effect that was reduced 60% to 70% ($p<0.001$) by either (i) N-acetyl cysteine (NAC); (ii) aspirin (ASA) or (iii) N-acetyl cysteine +aspirin (NAC+ASA), administered during the deprivation period prior to the 5-day ethanol re-access. Following treatment with NAC, ASA or NAC+ASA, the smaller ethanol intakes (spread over 24 hours) did not significantly increase the GSSG/GSH ratio, in line with literature studies (*vide infra*) that indicate that neuroinflammation and oxidative stress are generated either following either binge-like ethanol intake or prolonged chronic voluntary alcohol intake.

Figure 7A shows that chronic alcohol treatment significantly reduced by 50% ($p<0.01$) the levels of the glutamate transporter GLT-1 in prefrontal cortex (area, where changes in GLT-1 levels are relevant to ethanol intake [39]). In line with studies by Lebourgeois et al [34], treatment with N-acetyl cysteine did not modify the reduction of GLT-1 levels induced by chronic ethanol intake (N.S.). However, aspirin administration, whether by itself or co-administered with N-acetyl cysteine, fully

normalized the GLT-1 levels ($p<0.05$). Figure 7B shows the GLT-1 levels in animals that had received NAC, ASA or NAC+ASA only during the alcohol deprivation phase, while these drugs were discontinued for 24 hours prior to the 5-days ethanol re-access. In line with the protracted effects of ethanol, once again chronically ethanol treated animals showed a marked reduction in GLT-1 levels (50% $p<0.01$) versus untreated animals, while N-acetyl cysteine administration did not affect the ethanol-induced GLT-1 reduction. Nevertheless, treatment with aspirin during the deprivation period markedly increased ($p< 0.01$) GLT-1 levels, an effect that was consistent with the reduction in ethanol relapse intake induced by aspirin shown above. The GLT-1 level following the combination aspirin+ N-acetyl cysteine was not significantly different from that of the aspirin-only group.

In line with studies by Wallace et al [53], the administration of 15 mg/kg of aspirin (ASA) did not generate gastric injury, as determined by mucosal myeloperoxidase levels- a marker of neutrophil inflammation (data not shown). None of the groups whether treated with ethanol alone; N-acetyl cysteine, aspirin or both showed abnormal serum liver enzyme activities or serum albumin levels (Figure S1; Supporting Information).

DISCUSSION

Studies presented show the existence of both oxidative stress and neuro-inflammation in the brain of rats that had consumed ethanol for a prolonged period (3 to 4 months). Changes in hippocampal astrocyte morphology (GFAP) and microglial (Iba-1) density, were accompanied by increases in oxidative stress determined as the ratio of oxidized/reduced glutathione (GSSG/GSH). Neuroinflammation has been reported following long term (5-months) ethanol intake by C57/BL alcohol-preferring mice [2,62]. For animals that do not readily ingest ethanol, other paradigms are used in which animals are slowly introduced to increasing concentrations of ethanol for prolonged periods or, to obtain faster effects ethanol injection or gavage to achieve intoxicating alcohol levels have also demonstrated the generation of neuroinflammation[4,63-65]. Similar doses and time of ethanol intake (administration have been reported for the generation of brain oxidative stress [5,6, 11-13]. Both neuroinflammation [66] and oxidative stress [67] are found in post-mortem brain of alcoholics.

Studies presented are in line with the existence of a neuroinflammation-oxidative stress self-perpetuating cycle, since the inhibition of one of these conditions inhibits the other condition. The study shows that the administration of an antioxidant (N-acetyl cysteine) reversed the GSSG/GSH ratio and fully reversed neuroinflammation, as shown by the normalization of astrocyte and microglial changes. Similarly, aspirin with anti-inflammatory actions significantly inhibited oxidative stress. A mechanism perpetuating brain neuroinflammation is in line with studies by Crews and associates [69] who reported that a single administration of systemic LPS to mice led to neuroinflammation lasting several months. A self-perpetuating mechanism of neuroinflammation is seen beyond the chronic ethanol intake period in which neuroinflammation -along with an

increased oxidative stress- were seen in the brain of rats that had chronically consumed ethanol and were further deprived of ethanol for 10 days.

As indicated above, the presentation of visual or olfactory cues associated with drugs of abuse is the main factor inducing drug relapse[36]. Drug-related cues, including ethanol's, activate the release of glutamate in several brain areas including medial prefrontal cortex, nucleus accumbens, amygdala, and hippocampus [35,68]. In the "alcohol deprivation effect" (ADE) paradigm observed following the re-installation of the ethanol solutions to chronic alcohol consuming rats that had been deprived of alcohol for a long period, alcohol odor is likely a main cue eliciting alcohol relapse, as it is also seen in humans who are heavy alcohol users [69]. In a protracted oxidative stress condition in animals that have consumed ethanol for prolonged periods, the removal of cued-released glutamate from the tripartite synapse(s) is inhibited due to an inefficient GLT-1 glutamate transporter following the oxidation and cross-linking of relevant sulfhydryl moieties [38], which magnifies a cued-induced glutamatergic tone. In the present study, the levels of GLT-1 were reduced by 50% following chronic ethanol intake.

Administration of N-acetyl cysteine markedly reduced chronic alcohol intake as well as the relapse-drinking that followed alcohol deprivation and its re-admission. In line with studies by Lebourgeois et al [34], N-acetyl cysteine administration did not modify the alcohol-induced reduction of GLT-1 levels; likely because a stronger reducing treatment is needed to reverse the disulfide bridges of protein bonding in oxidized GLT-1[38]. It has been proposed [37] that the inhibitory effect of N-acetyl cysteine in reducing the cue-induced relapse for a drug such a cocaine is due to an increased activity of the cystine-glutamate xCT transporter, by increases in the exchange of cystine

(the oxidized form of cysteine generated by N-acetyl cysteine) for glutamate; paradoxically increasing extracellular glutamate while activating the presynaptic (inhibitory) mGlu2/3 glutamate receptor, thus reducing glutamate release at the tripartite synapse. An increased exchange of cystine for glutamate and a presynaptic inhibitory effect of glutamate likely occurs in areas distant from the nucleus accumbens, as recent studies place the xCT transporter mainly away from the tripartite GFAP+ astrocytes [70] and close to the juncture with the blood brain barrier [71].

One of the anti-inflammatory mediators induced by aspirin may be responsible for normalizing the GLT-1 levels in brain cortex, thus becoming the likely mechanism by which aspirin reduces chronic ethanol intake and relapse-drinking. A number of studies have shown that aspirin activates the synthesis of the peroxisome proliferator-activated receptor-gamma (PPAR-γ)[45,46], known to have both strong anti-inflammatory effects [72-74] and to activate brain GLT-1 transcription, increasing GLT-1 protein levels[75]. Six PPAR response elements (PPREs) exist in the GLT1 (EAAT2) promoter [75]. In the present study, aspirin administration led to marked increases in GLT-1 levels. In another study[76], ibudilast, an anti-inflammatory phosphodiesterase inhibitor, was shown to inhibit ethanol intake and to reduce relapse in alcohol-preferring P rats, to the same extent as that observed for aspirin.

It is noteworthy that administration of the beta-lactam antibiotic ceftriaxone to alcohol preferring rats (P-rats) was shown[39] to increase the levels of GLT-1 both in prefrontal cortex and nucleus accumbens. However the increases in GLT-1 in prefrontal cortex were associated with an 80% reduction of chronic ethanol intake, while this effect was observed prior to GLT-1 increases in nucleus accumbens , which followed a longer administration of ceftriaxone. In the present study,

changes in GLT-1 levels in the prefrontal cortex correlated well with both chronic alcohol intake and relapse-drinking. The prefrontal cortex receives hippocampal outputs which confirm the "context" of interrelated memories [77]. Ethanol odor-cues might require such a hippocampal-prefrontal cortex context confirmation to elicit the drinking behavior.

Overall studies presented show that, in an animal model of spontaneous chronic alcohol intake and relapse, both an antioxidant (N-acetyl cysteine) and an anti-inflammatory (aspirin) drug, administered in well accepted clinical doses, are effective individually and when combined these act synergistically in reducing chronic ethanol intake and relapse binge-like drinking. The study further supports the existence of a neuroinflammation-oxidative stress self-perpetuating cycle, which may explain the fact that relapse can occur at times that are distant from those of alcohol abstinence.

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REFERENCES

1. Crews FT, Vetreno RP. Mechanisms of neuroimmune gene induction in alcoholism. *Psychopharmacology* 2016; 233:1543–1557.
2. Alfonso-Loeches S, Pascual-Lucas M, Blanco AM, Sanchez-Vera I, Guerri C. Pivotal Role of TLR4 Receptors in Alcohol-Induced Neuroinflammation and Brain Damage. *J. Neuroscience* 2010; 30: 8285– 8295.
3. Montesinos J, Alfonso-Loeches S, Guerri C. Impact of Innate Immune Response in the Actions of Ethanol on the Central Nervous System. *Alcohol Clin Exp Res* 2016; 40: 2260–2270.
4. Schneider Jr R · Bandiera S, Souza DG ·Bellaver B, Caletti G, Quincozes-Santos A, Elisabetsky E, Gomez R. N-acetylcysteine Prevents Alcohol Related Neuroinflammation in Rats. *Neurochem Res* 2017; 42:2135–2141.
5. Quintanilla ME, Morales P, Ezquer F, Ezquer M, Herrera-Marschitz M, Israel Y. Commonality of Ethanol and Nicotine Reinforcement and Relapse in Wistar-Derived UChB Rats: Inhibition by N-Acetylcysteine. *Alcohol Clin Exp Res*. 2018; 42:1988-1999.
6. Ezquer F, Morales P, Quintanilla ME, Santapau D, Lespay-Rebolledo C, Ezquer M, Herrera-Marschitz M, Israel Y. Intravenous administration of anti-inflammatory mesenchymal stem cell spheroids reduces chronic alcohol intake and abolishes binge drinking. *Scientific Reports* 2018; 8:4325. DOI:10.1038/s41598-018-22750-7.
7. Erickson EK, Grantham EK, Warden AS, Harris RA. Neuroimmune signaling in alcohol use disorder. *Pharmacol Biochem Behav*. 2019; 177:34-60.
8. Ferrier L, Bérard F, Debrauwer L, Chabo C, Langella P, Bue L, Fioramonti J. Impairment of the Intestinal Barrier by Ethanol Involves Enteric Microflora and Mast Cell Activation in Rodents. *Am J Pathol* 2006; 168: 1148–1154.

9. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong J-S, Knapp DJ, Crews FT. Systemic LPS Causes Chronic Neuroinflammation and Progressive Neurodegeneration *Glia. 2007; 55: 453–462.*

10. Leclercq S, de Timary P, Delzenne NM, Stärkel P The link between inflammation, bugs, the intestine and the brain in alcohol dependence *Transl Psychiatry* 2017; 7, e1048; doi:10.1038/tp.2017.15

11. Montoliu C, Valles S, Renau-Piqueras J, Guerri, C. Ethanol-induced oxygen radical formation and lipid peroxidation in rat brain: effect of chronic alcohol consumption. *J Neurochem* 1994; 63: 1855-1862.

12. Das SK, Vasudevan DM. Alcohol-induced oxidative stress. *Life Sci*. 2007; 81:177-187.

13. Amin FU, Shah SA, Kim MO. Glycine inhibits ethanol-induced oxidative stress, neuroinflammation and apoptotic neurodegeneration in postnatal rat brain. N*eurochem Int*. 2016; 96:1-12

14. Cunha-Oliveira T, Rego AC, Oliveira CR. Oxidative stress and drugs of abuse: an update. *Org Chem* 2013; 10: 1–14.

15. Dietrich JB, Mangeol A, Revel MO, Burgun C, Aunis D, Zwiller J. Acute or repeated cocaine administration generates reactive oxygen species and induces antioxidant enzyme activity in dopaminergic rat brain structures. *Neuropharmacol.* 2005; 48:965-974.

16. Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci U S A*. 1988; 85: 5274-5278.

17. Quintanilla ME, Bustamante D, Tampier L, Israel Y, Herrera-Marschitz M Dopamine release in the nucleus accumbens (shell) of two lines of rats selectively bred to prefer or avoid ethanol. *Eur J Pharmacol*. 200; 573:84-92.

18. Karahanian E, Quintanilla ME, Tampier L, Rivera-Meza M, Bustamante D, Gonzalez-Lira V, Morales P, Herrera-Marschitz M, Israel Y. Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol Clin Exp Res*. 2011; 35:606-612.
19. Graham DG, Tiffany SM, Bell WR Jr, Gutknecht WF. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. *Mol Pharmacol.* 1978; 14:644-653
20. Howard LA1, Miksys S, Hoffmann E, Mash D, Tyndale RF Brain CYP2E1 is induced by nicotine and ethanol in rat and is higher in smokers and alcoholics. *Br J Pharmacol*. 2003; 138:1376-1386.
21. Persson JO1, Terelius Y, Ingelman-Sundberg M. Cytochrome P-450-dependent formation of reactive oxygen radicals: isozyme-specific inhibition of P-450-mediated reduction of oxygen and carbon tetrachloride. *Xenobiotica.* 1990; 20:887-900.
22. Gloire G, Legrand-Poels S, Piette J. NF-kB activation by reactive oxygen species: Fifteen years later *Biochem Pharmacol* 2006; 72:1463-1510.
23. Kim Y-S, Morgan MJ, Choksi S, Liu Z-g. TNF-Induced Activation of the Nox NADPH Oxidase and Its Role in the Induction of Necrotic Cell Death. *Molecular Cell* 2007; 26: 675–687.
24. Basuroy S, Bhattacharya S, Leffler CW, Parfenova H. Nox4 NADPH oxidase mediates oxidative stress and apoptosis caused by TNFα in cerebral vascular endothelial cells. *Am J Physiol Cell Physiol* 2009 296: C422–C432.
25. Kastl L, Sauer SW, Ruppert T, Beissbarth T, BeckerMS, Süss D, Krammer PH, Gülow K. TNF–α mediates mitochondrial uncoupling and enhances ROS-dependent cell migration via NF-kB activation in liver cells. *FEBS Letters* 2014; 588: 175–183.
26. Cui C, Shurtleff D, Harris RA. Neuroimmune mechanisms of alcohol and drug addiction. *Int Rev Neurobio*l. 2014; 118: 1-12.

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

28. Robinson G, Most D, Ferguson LB, Mayfield J, Harris RA, Blednov YA. Neuroimmune pathways in alcohol consumption: evidence from behavioral and genetic studies in rodents and humans. *Int Rev Neurobiol*. 2014; 118:13-39.

29. Montesinos J, Gil A, Guerri C. Nalmefene Prevents Alcohol-Induced Neuroinflammation and Alcohol Drinking Preference in Adolescent Female Mice: Role of TLR4. *Alcohol Clin Exp Res.* 2017; 41:1257-1270.

30. Warden AS, Azzam M, DaCosta A, Mason S, Blednov YA, Messing RO, Mayfield RD, Harris RA. Toll-like receptor 3 activation increases voluntary alcohol intake in C57BL/6J male mice. *Brain Behav Immun.* 2019; 77:55-65.

31. Quintanilla ME, Rivera-Meza M, Berríos-Cárcamo P, Salinas-Luypaert C, Herrera-Marschitz M, Israel Y. Beyond the "First Hit": Marked Inhibition by N-Acetyl Cysteine of Chronic Ethanol Intake But Not of Early Ethanol Intake. Parallel Effects on Ethanol-Induced Saccharin Motivation. *Alcohol Clin Exp Res.* 2016;40:1044-1051.

32. Quintanilla ME, Morales P, Ezquer F, Ezquer M, Herrera-Marschitz M, Israel Y. Commonality of Ethanol and Nicotine Reinforcement and Relapse in Wistar-Derived UChB Rats: Inhibition by N-Acetylcysteine. *Alcohol Clin Exp Res*. 2018; 42:1988-1999.

33. Lebourgeois S, González-Marín MC, Jeanblanc J, Naassila M, Vilpoux C. Effect of N-acetylcysteine on motivation, seeking and relapse to ethanol self-administration. *Addict Biol.* 2018; 23:643-652.

34. Lebourgeois S, González-Marín MC, Antol J, Naassila M, Vilpoux C. Evaluation of N-acetylcysteine on ethanol self-administration in ethanol-dependent rats. *Neuropharmacol.* 2019;150:112-120.

35. Gass JT, Sinclair CM, Cleva RM, John J. Widholm JJ, Foster, Olive MF. Alcohol-seeking behavior is associated with increased glutamate transmission in basolateral amygdala and nucleus accumbens as measured by glutamate-oxidase-coated biosensors *Addiction Biol* 2011; 16:215-228.

36. Kalivas PW The glutamate homeostasis hypothesis of addiction. *Nat Rev Neurosci*. 2009; 10:561-572

37. Scofield MD, Heinsbroek JA, Gipson CD, Kupchik YM, Spencer SW, Smith AC, Roberts-Wolfe, Kalivas PW. The Nucleus Accumbens: Mechanisms of Addiction across Drug Classes Reflect the Importance of Glutamate Homeostasis. *Pharmacol Rev* 2016; 68:816-871.

38. Trotti D, Danbolt NC, Volterra A.Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? *TiPS* 1998; 19. 329-334.

39. Rao PSS, Saternos H, Goodwani S, Sari Y. Effects of ceftriaxone on GLT1 isoforms, xCT and associated signaling pathways in P rats exposed to ethanol. *Psychopharmacology* (Berl). 2015; 232: 2333–2342.

40. Bechard AR, Hamor PU, Schwendt M2, Knackstedt LA. The effects of ceftriaxone on cue-primed reinstatement of cocaine-seeking in male and female rats: estrous cycle effects on behavior and protein expression in the nucleus accumbens. *Psychopharmacol* (Berl). 2018; 235:837-848.

41. Sari Y, Toalston JE, Rao PSS, Bell RL. Effects of ceftriaxone on ethanol, nicotine or sucrose intake by alcohol-preferring (P) rats and its association with GLT-1 expression. *Neuroscience*. 2016; 326:117-125.

42. Ezquer F, Morales P, Quintanilla ME, Santapau D, Lespay-Rebolledo C, Ezquer M, Herrera-Marschitz M, Israel Y. Intravenous administration of anti-inflammatory mesenchymal stem cell spheroids reduces chronic alcohol intake and abolishes binge-drinking. *Sci Rep*. 2018a; 8:432. doi: 10.1038/s41598-018-22750-7

43. Ezquer F, Quintanilla ME, Morales P, Santapau D, Ezquer M, Kogan MJ, Salas-Huenuleo E, Herrera-Marschitz M, Israel Y. Intranasal delivery of mesenchymal stem cell-derived exosomes reduces oxidative stress and markedly inhibits ethanol consumption and post-deprivation relapse drinking. *Addict Biol.* 2018b doi: 10.1111/adb.12675.

44. Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol Therapy* 2012; 20:14–20.

45. Tang J, Xiong J, Wu T, Tang Z, Ding G, Zhang C, Wang S, Liu Y. Aspirin Treatment Improved Mesenchymal Stem Cell Immunomodulatory Properties via the 15d-PGJ2/PPARg/TGF-b1 Pathway. *Stem cells and development 2014;* 23: 2093-2103.

46. Yiqin Y, Meilin X, Jie X, Keping Z. Aspirin Inhibits MMP-2 and MMP-9 Expression and Activity Through PPARα/γ and TIMP-1-Mediated Mechanisms in Cultured Mouse Celiac Macrophages. I*nflammation*, 2009; 32: 233-241.

47. Pascual G, Sullivan AL, Ogawa S, Gamliel A, Perissi V, Rosenfeld MG, Glass CK. Anti-inflammatory and antidiabetic roles of PPAR-gamma. *Novartis Found Symp.* 2007; 286:183-96 and 196-203.

48. József L, Zouki C, Petasis NA, Serhan CN, Filep JG Lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 inhibit peroxynitrite formation, NF-kappa B and AP-1 activation, and IL-8 gene expression in human leukocytes. *Proc Natl Acad Sci* U S A. 2002 ;99:13266-13271.

49. Romano M, Cianci E, Simiele F, Recchiuti A. Lipoxins and aspirin-triggered lipoxins in resolution of inflammation. *Eur J Pharmacol*. 2015; 760:49-63.

50. Kopp E, Ghosh S. Inhibition of NF-kB by Sodium Salicylate and Aspirin *Science* 1994; 265: 556-958.

51. Eisen DP, Moore EM, Leder K, Lockery J, McBryde ES, McNeil JJ, Pilcher D, Wolfe R, Woods R Aspirin to Inhibit sepsis (ANTISEPSIS) randomised controlled trial protocol. *BMJ Open.* 2017 Jan 20;7(1):e013636. doi: 10.1136/bmjopen-2016-013636.

52. Bauer IE, Green C, Colpo GD, Texeira A, SElvaraj S, Durkin K, Zuta-Soares K, Soares JC. A Double blind randomized placebo controlled study of Aspirin and Na-cetyl cysteine as adjunctive treatments for bipolar depression. *J Clin Psychiatry 2019;* 80:1 January February and online: Psychiatrist.com e1-e9 2019.

53. Wallace JL, Zamune ZR, McKnight W, Dicay M, Mencarelli A, del Soldato P, Fiorucci S. Aspirin, but not NO-releasing aspirin (NCX-4016), interacts with selective COX-2 inhibitors to aggravate gastric damage and inflammation *Am J Physiol Gastrointest Liver Physiol* 2004; 286: G76–G81.

54. Waring WS. Novel acetylcysteine regimens for treatment of paracetamol overdose. *Ther Adv Drug Safety* (2012) 3(6) 305–315.

55. La Rowe SD, Kalivas, PW, Nicholas JS, Randall PK, Mardikian PN, Malcolm RJ, Ralph H. A Double-Blind Placebo-Controlled Trial of N-Acetylcysteine in the Treatment of Cocaine Dependence. *American J Addiction 2013;,* 22: 443–452.

56. Quintanilla ME, Israel Y, Sapag A, Tampier L (2006): The UChA and UChB rat lines: metabolic and genetic differences influencing alcohol intake. *Addict Biol*. 11: 310-323.

57. Israel Y, Karahanian E, Ezquer F, Morales P, Ezquer M, Rivera-Meza M, Herrera-Marschitz M, Quintanilla ME (2017) Acquisition, Maintenance and Relapse-Like Alcohol Drinking: Lessons from the UChB Rat Line. *Front Behav Neurosci.* 2017 Apr 4;11:57. doi: 10.3389/fnbeh.2017.00057. eCollection 2017. Review.

58. Quintanilla ME, Tampier L, Sapag A, Gerdtzen Z, Israel Y. Sex differences, alcohol dehydrogenase, acetaldehyde burst, and aversion to ethanol in the rat: a systems perspective. *Am J Physiol Endocrinol Metab* 2007; 293:E531-7.

59. Ezquer F, Quintanilla ME, Morales P, Ezquer M, Lespay-Rebolledo C, Herrera-Marschitz M, Israel Y. Activated mesenchymal stem cell administration inhibits chronic alcohol drinking and suppresses relapse-like drinking in high-alcohol drinker rats. *Addict Biol* 2019; 24:17-27.

60. Sinclair JD, Senter RJ. Development of an alcohol-deprivation effect in rats. Q J Stud Alcohol. 1968; 29:863-867

61. Spanagel R. Animal models of addiction *Dialogues in Clinical Neuroscience* 2017; 19: 247-257.

62. Pascual M, Baliño P, Aragón CM, Guerri C. Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: role of TLR4 and TLR2 *Neuropharmacol,* 2015; 89:352-359.

63. Tiwari V, Chopra K Resveratrol prevents alcohol-induced cognitive deficits and brain damage by blocking inflammatory signaling and cell death cascade in neonatal rat brain. J *Neurochem* 2011; 117: 678-690.

64. Qin L, Crews FT. NADPH oxidase and reactive oxygen species contribute to alcohol-induced microglial activation and neurodegeneration. *J Neuroinflammation*. 2012; 9: 2-19.

65. Walter TJ, Crews FT. Microglial depletion alters the brain neuroimmune response to acute binge ethanol. *J Neuroinflammation*. **2017**; 14:86. doi: 10.1186/s12974-017-0856-z.

66. Yang JY, Xue X, Tian H, Wang XX, Dong YX, Wang F, Zhao YN, Yao XC, Cui W, Wu CF.Role of microglia in ethanol-induced neurodegenerative disease: Pathological and behavioral dysfunction at different developmental stages. *Pharmacol Ther*. 2014; 144:321-337.

67. Flatscher-Bader T1, van der Brug MP, Landis N, Hwang JW, Harrison E, Wilce PA. ROS Comparative gene expression in brain regions of human alcoholics. Genes Brain Behav 2006; 6 Suppl 1: 78-84

68. Bell RL Hauser SR, McClintick J, Rahman S, Edenberg HJ, Szumlinski KK, McBride WJ Ethanol-Associated Changes in Glutamate Reward Neurocircuitry: A Minireview of Clinical and Preclinical Genetic Findings. *Molecular Basis of Drug Addiction* 2016; 137 ISSN 1877-11-73 doi 10.1016/bs.pmbts.2015.10.018

69. Kareken DA, Claus ED, Sabri M, Dzemidzic M, Kosobud AE, Radnovich AJ, Hector D, Ramchandani VA, O'Connor SJ, Lowe M, Li TK. Alcohol-related olfactory cues activate the nucleus accumbens and ventral tegmental area in high-risk drinkers: preliminary findings. *Alcohol Clin Exp Res*. 2004; 28:550-557.

70. Soria FN, Zabala A, Pampliega O, Palomino A, Miguelez C, Ugedo L, Sato H, Matute C, Domercq M. Cystine/Glutamate Antiporter Blockage Induces Myelin Degeneration. *Glia* 2016; 64:1381–1395

71. Sigrid Ottestad-Hansen, Qiu Xiang Hu, Virgine Veronique Follin-Arbelet, Eduard Bentea, Hideyo Sato, Ann Massie, Yun Zhou, Niels Christian Danbolt. The cystine-glutamate

exchanger (xCT, Slc7a11) is expressed in significant concentrations in a subpopulation of astrocytes in the mouse brain. *Glia,* 2 January 2018.

72. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 391:82-88 (1988)

73. Ricote M, Huang JT, Welch JS, Glass CK. The peroxisome proliferator activated receptor (PPAR gamma) as a regulator of monocyte macrophage function. *J Leukoc Biol*. 1999; 66:733-739. Review

74. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK. A SUMOylation- dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature*. 2005; 437:759-63.

75. Romera C, Hurtado O, Mallolas JPereira MO, Morales JR, Romera A,, Joaquı´n Serena J, Vivancos J, Nombela F, Lorenzo P, Lizasoain I, Moro MA. Ischemic preconditioning reveals that GLT1/EAAT2 glutamate transporter is a novel PPARg target gen involved in neuroprotection. *Journal of Cerebral Blood Flow & Metabolism* 2007; 27: 1327–1338.

76. Bell RL, Lopez MF, Cui C, Egli M, Johnson KW, Franklin KM and Becker HC. Ibudilast reduces alcohol drinking in multiple animal models of alcohol dependence *Addiction Biol,* 2013, 20, 38–42

77. Preston AR, Eichenbaum H. Interplay of hippocampus and prefrontal cortex in memory *Curr Biol.* 2013; 23 :R 764-773. Review.

LEGENDS TO FIGURES

Figure 1. Significant inhibition of chronic alcohol intake following the oral administration of N-acetyl cysteine (NAC 40 mg/kg/day) or aspirin (ASA 15 mg/kg/day) versus vehicle group (one-way ANOVA: $F_{3,48}$ = 168, ****P < 0.0001; Tukey post hoc: vehicle versus NAC and versus ASA treated groups, ****P < 0.0001, n= 6 rats/group). At the doses administered orally, NAC and ASA administered separately inhibited chronic ethanol intake (****p<0.0001). Inhibition was synergistically increased by the co-administration of N-acetyl cysteine plus aspirin (NAC+ASA), when compared with either NAC or ASA groups (**** p< 0.0001, NAC +ASA versus NAC; ****p< 0.0001, NAC + ASA versus ASA). The shaded area corresponds to two days on which animals received an i.p dose/day of N-acetyl cysteine (NAC 70 mg/kg).

Figure 2. Chronic ethanol intake (101 days) by rats treated with vehicle (Ethanol+vehicle) led to an increase in length (one way ANOVA $F_{(4,1835)}$=42.57;*** p<0.001) and thickness ($F_{(4,868)}$= 36.82; p<0.0001) of astrocyte processes (GFAP immunoreactivity, red; DAPI blue., Fig 2A top, Fig 2B,C) and an increase of microglial density ($F_{(4,46)}$=7.206;*** p< 0.001). (IBA-1 immunoreactivity, green, depicted by white arrows, Fig 2A, center, Fig 2D) compared to (naïve) rats drinking Water. Figs 2B and 2C: The administration of either N-acetyl cysteine (Ethanol +NAC), aspirin (Ethanol +ASA) or co-administration of N-acetyl cysteine plus aspirin (ethanol +NAC+ASA) fully normalized ethanol-induced increase in length (Tukey post-hoc: NAC ***p < 0.001; ASA ***p < 0.001; NAC+ASA ***p < 0.001) ; ethanol-induced increase in thickness (Tukey post-hoc: NAC***p < 0.001; ASA ***p < 0.001; NAC+ASA,*** p < 0.001) and the ethanol-induced increase in microglial density (Fig 2D) (Tukey post-hoc: NAC ***p < 0.001; ASA *p < 0.05; NAC+ASA *p < 0.05).

Figure 3. Marked increase in oxidative stress in the hippocampus of animals that had consumed ethanol for 3 to 4 months was reversed by the administration of N-acetyl cysteine (NAC 40 mg/kg), aspirin (ASA 15 mg/kg) or the co-administration of equal doses both drugs (NAC+ASA). Oxidative stress determined as the ratio of oxidized/reduced glutathione (GSSG/GSH) is markedly increased (300%) by chronic ethanol intake (101 days, ethanol vehicle versus water group) (one-way ANOVA: $F_{3,15}$ = 22, P < 0.0001; Tukey post hoc: water versus ethanol vehicle ***P < 0.001, n= 6 rats/group), whereas N-acetyl cysteine (Ethanol NAC), ASA and NAC + ASA animals fully normalized GSSG/GSH ratio versus the ethanol vehicle group, ***P < 0.0001). Aspirin appeared to be less effective in lowering the GSSG/GSH ratio than N-acetyl cysteine; however, the difference between NAC vs ASA did not attain statistical significance.

Figure 4. N-acetyl cysteine (NAC), aspirin (ASA) or co administration of NAC +ASA during the alcohol deprivation period (shaded area) after chronic alcohol consumption, markedly inhibited relapse drinking induced by alcohol re-access. Figure 4A shows the basal chronic ethanol intake of animals consuming ethanol for 85 days prior to a 14-day alcohol deprivation. The alcohol re-access intake was determined on day 100 in 4-groups (vehicle oral water); (oral ASA 15 mg/kg/day); (oral NAC 40 mg/kg/day); (oral ASA 15mg/kg/day + oral NAC 40 mg/kg/day). Vehicle treated animals consumed significantly more ethanol following alcohol re-access than their baseline intake prior to deprivation (Two-way ANOVA (deprivation × day): $F_{1,140}$ = 35.85, ****P < 0.0001; Tukey post hoc: pre-deprivation versus post-deprivation, ****P < 0.0001). A 9-day administration of ASA, NAC or ASA+NAC during the ethanol deprivation period significantly inhibited alcohol relapse upon re-access on day 10 versus the vehicle treated group (one-way ANOVA: $F_{3,106}$ = 120, P

< 0.0001; Tukey post hoc: vehicle versus ASA, ****P < 0.0001, vehicle versus NAC, ****p < 0.0001, vehicle versus NAC + ASA, ****P < 0.0001, = 6 rats/group). Administration of NAC was more effective in inhibiting re-access intake than ASA (**P<0.01). Combined NAC + ASA was more effective in inhibiting re-access intake than NAC, **p < 0.01 and more effective than ASA ****P < 0.0001. To avoid an acute effect of ASA, NAC or (ASA+NAC) on relapse, ethanol intake was determined upon ethanol re-access on day 10, namely 24 hours after the last ASA, NAC or (ASA+NAC) administration. Compared with the Vehicle group, the (ASA+NAC) co-administration inhibited alcohol intake upon re-access by 70%-75% for the 5-days. Figure 4B shows ethanol intake on the first 60-minutes of ethanol re-access. Vehicle treated animals consumed 170 mg ethanol/kg/60 minutes, an intake that was reduced by 50% to 75% (***p<0.001) by ASA, NAC or ASA+NAC (one-way ANOVA: $F_{3,20}$ = 12.49, P < 0.0001; Tukey post hoc: vehicle versus NAC, ***p< 0.001, vehicle versus ASA, ***p < 0.001, vehicle versus NAC + ASA, ***p < 0.001, = 6 rats/group). Figure 4C shows the blood alcohol concentrations (BAC) at the end of the 60-minute alcohol re-access intake. The vehicle group showed a (binge-like) BAC of 97 mg/dl, which was reduced by 60% to 70% by ASA and NAC. The (ASA+NAC) treated group showed a BAC of 13 mg/dl (one-way ANOVA: $F_{3,20}$ = 13.94, P < 0.0001; Tukey post hoc: vehicle versus NAC, ***p< 0.001, vehicle versus ASA, ***p < 0.001, vehicle versus NAC + ASA, ***p < 0.001, = 6 rats/group).

Figure 5. The ethanol deprivation period imposed on rats previously consuming alcohol chronically and allowed a 5-day ethanol re-access induced in Ethanol +vehicle treated animals an increase in length (one way-ANOVA $F_{(4,2205)}$=95,23 p<0.0001) and thickness ($F_{(4,1018)}$=58,53 p<0.0001) of astrocyte processes (GFAP immunoreactivity, green, Fig 5A top, Fig 5B,C) and an increase of microglial density ($F_{(4,39)}$=15.83 p<0.001) (IBA-1

immunoreactivity, green, depicted by white arrows Fig 5A, center, Fig 5D), compared to those in rats drinking water. Compared with ethanol-vehicle group either N-acetyl cysteine (ethanol +NAC), aspirin (ethanol +ASA) or co-administration of N-acetyl cysteine plus aspirin (ethanol +NAC+ASA) fully inhibited the increase in length [Tukey post-hoc: NAC ***p < 0.001; ASA ***p < 0.001; NAC+ASA ***p < 0.001, Fig 5B] and thickness [Tukey post-hoc: NAC ***p < 0.001; ASA p < 0.001; NAC+ASA ***p < 0.001, Fig 5C] of astrocyte processes and the microglial density increase (Fig 5D) [Tukey post-hoc: NAC ***p < 0.001; ASA , *p < 0.05; NAC+ASA **p < 0.01] in comparison to rats treated with vehicle (Ethanol+ vehicle).

Figure 6. Marked hippocampal oxidative stress (GSSG/GSH) in chronically alcohol-ingesting animals that were alcohol deprived for 14 days and were subsequently allowed ethanol access for 5 days (+300% ***p<0.001) (Ethanol +vehicle) (one-way ANOVA: $F_{4,21}$ = 5.5, P < 0.0036, Tukey post hoc: water versus ethanol vehicle P < 0.0001, n= 6 rats/group). Animals were administered NAC, ASA or NAC + ASA for 9-days during alcohol deprivation phase and euthanized on day 10. Administration of NAC (Ethanol +NAC), aspirin (Ethanol + ASA) or the combination of both (Ethanol +NAC +ASA) significantly inhibited (60%-65% ***p<0.001) the hippocampal GGSG/GSH ratios (***p < 0.001).

Figure 7. Fig 7A shows that chronic alcohol treatment significantly reduced by 50% the level of the glutamate transporter GLT-1 in prefrontal cortex (Ethanol-Vehicle versus Water; ANOVA **p<0.01) (one-way ANOVA: $F_{4,20}$ = 6.22, P < 0.002) Tukey post hoc: Water versus Ethanol-Vehicle **P < 0.01, n= 6 rats/group).Treatment with N-acetyl cysteine (Ethanol + NAC) did not modify the reduction of GLT-1 levels induced by chronic ethanol

intake (N.S.). However, aspirin (Ethanol +ASA) administration, whether by itself or when co-administered with N-acetyl cysteine (NAC+ASA), fully normalized the GLT-1 levels (Tukey post hoc: Ethanol-Vehicle versus ASA, *$p < 0.05$, Ethanol-Vehicle versus NAC + ASA, *$p< 0.05$). Figure 7B shows the GLT-1 level in animals that received NAC, ASA or NAC+ASA during 9 days of the alcohol deprivation phase and discontinued 24 hours prior to the 5-day ethanol re-access. Once again, ethanol treated animals (Ethanol-Vehicle versus Water naive) showed a marked reduction in GLT-1 levels (one-way ANOVA: $F_{4,22} = 4.8$, $P < 0.006$, Tukey post hoc: Water versus Ethanol-Vehicle **p < 0.01, n= 6 rats/group), while N-acetyl cysteine administration did not alter the ethanol-induced GLT-1 reduction. Prior treatment with aspirin increased GLT-1 toward normal levels (*p< 0.05). The GLT-1 level following the combination aspirin+ N-acetyl cysteine (Ethanol +NAC +ASA) was not significantly different from the Ethanol-ASA group.

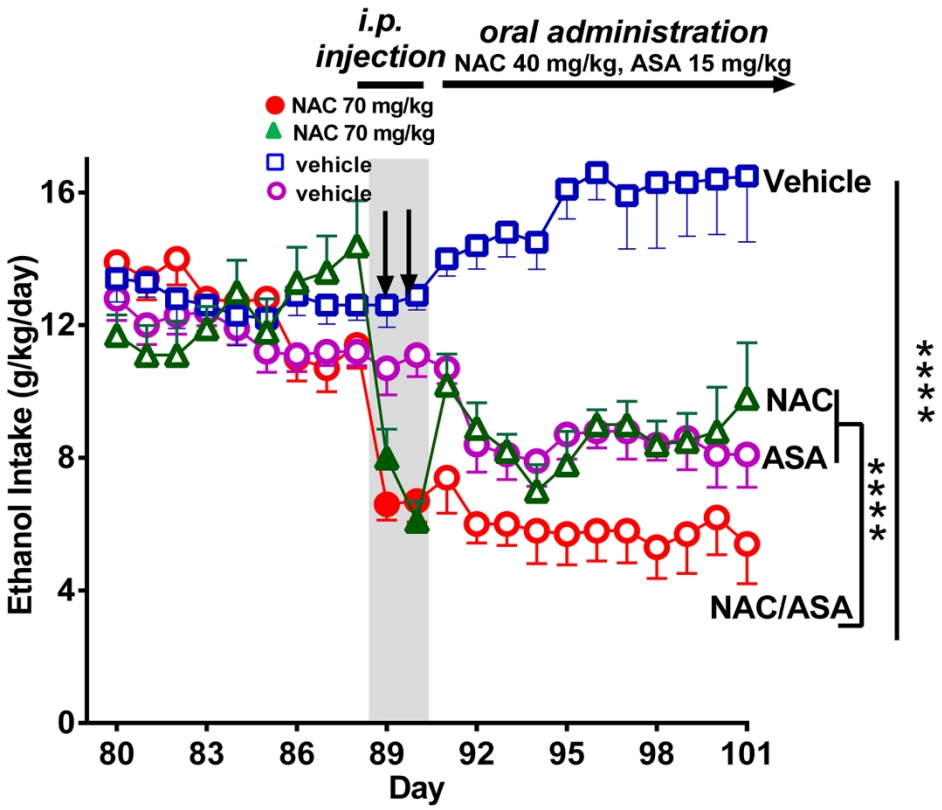
i.p.
injection
oral administration
NAC 40 mg/kg, ASA 15 mg/kg
NAC 70 mg/kg
NAC 70 mg/kg
vehicle
vehicle
Vehicle
NAC
ASA
NAC/ASA
****
****
Ethanol Intake (g/kg/day)
16
12
8
4
0
80
83
86
89
92
95
98
101
Day

Figure 1

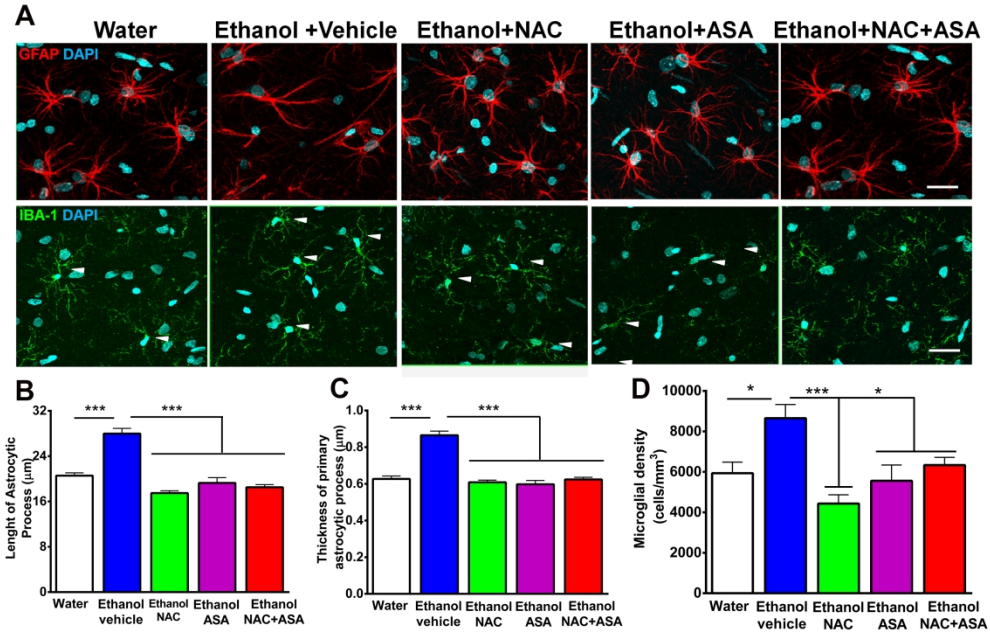
A
Water
Ethanol +Vehicle
Ethanol+NAC
Ethanol+ASA
Ethanol+NAC+ASA
GFAP DAPI
IBA-1 DAPI
B
Lenght of Astrocytic Process (µm)
C
Thickness of primary astrocytic process (µm)
D
Microglial density (cells/mm3)
Water
Ethanol vehicle
Ethanol NAC
Ethanol ASA
Ethanol NAC+ASA

Figure 2

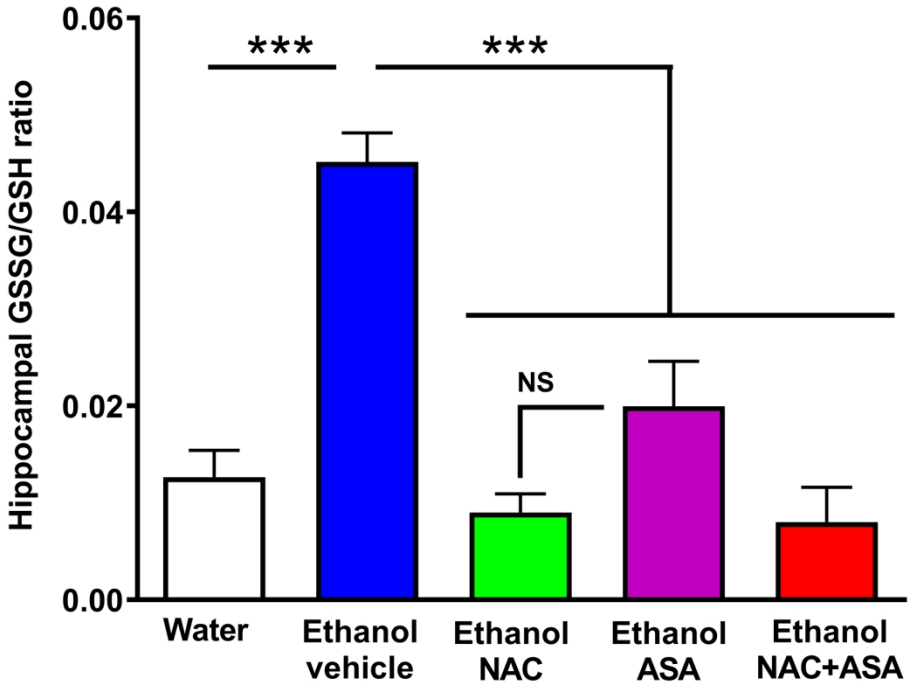
Hippocampal GSSG/GSH ratio
0.06
0.04
0.02
0.00
***
***
NS
Water
Ethanol vehicle
Ethanol NAC
Ethanol ASA
Ethanol NAC+ASA

Figure 3

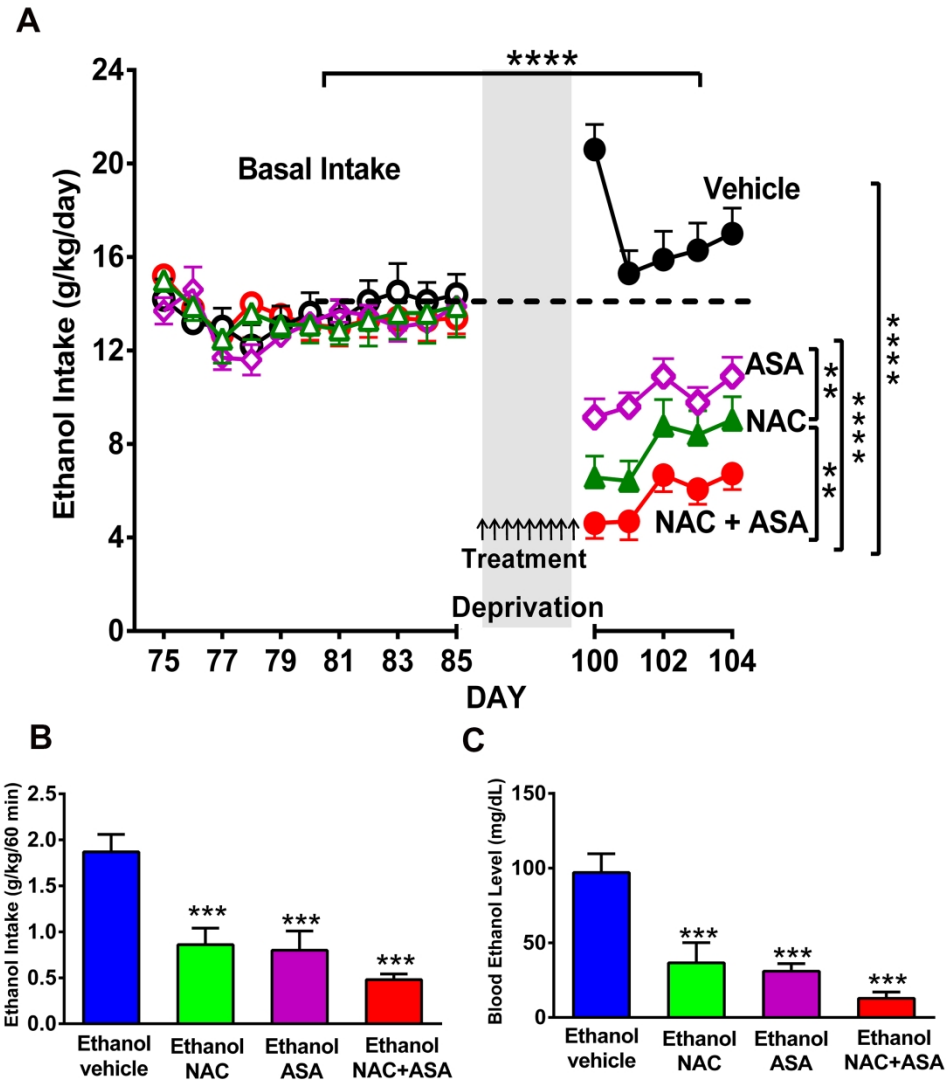

Figure 4

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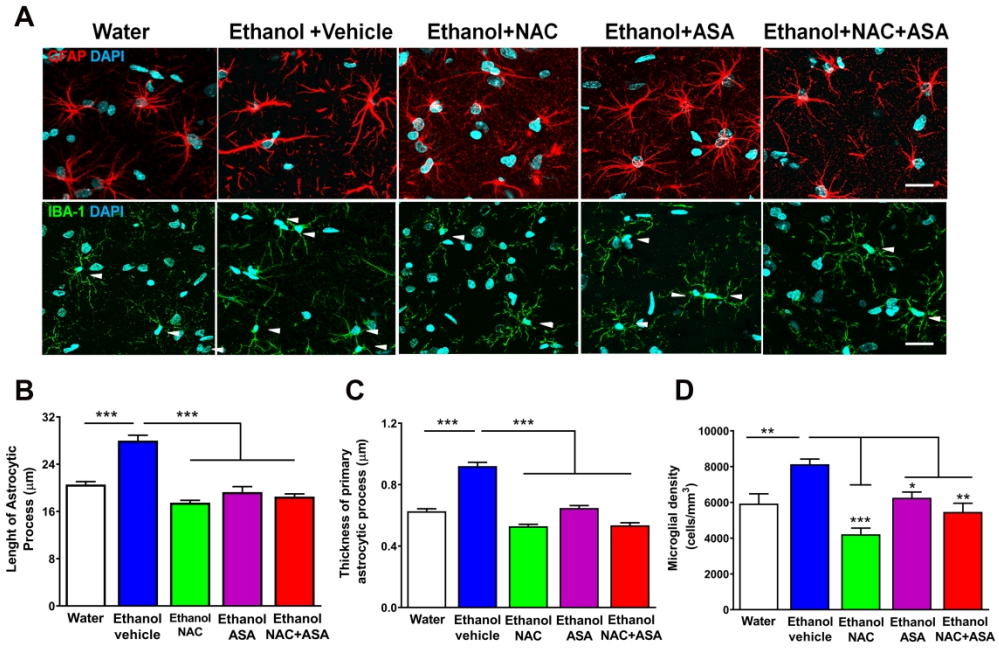
A
Water
Ethanol +Vehicle
Ethanol+NAC
Ethanol+ASA
Ethanol+NAC+ASA
GFAP DAPI
IBA-1 DAPI
B
Lenght of Astrocytic Process (µm)
C
Thickness of primary astrocytic process (µm)
D
Microglial density (cells/mm3)
Water
Ethanol vehicle
Ethanol NAC
Ethanol ASA
Ethanol NAC+ASA

Figure 5

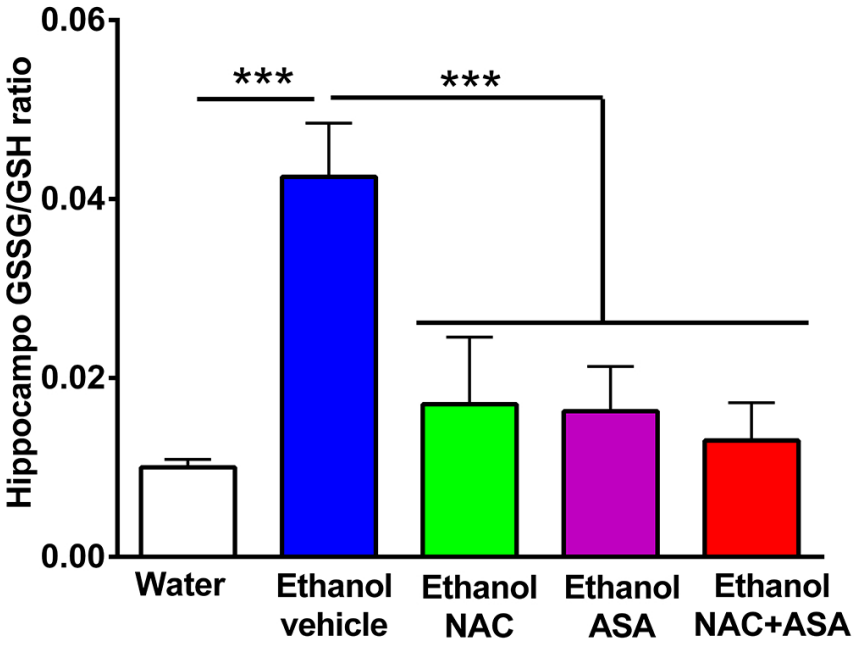

Figure 6

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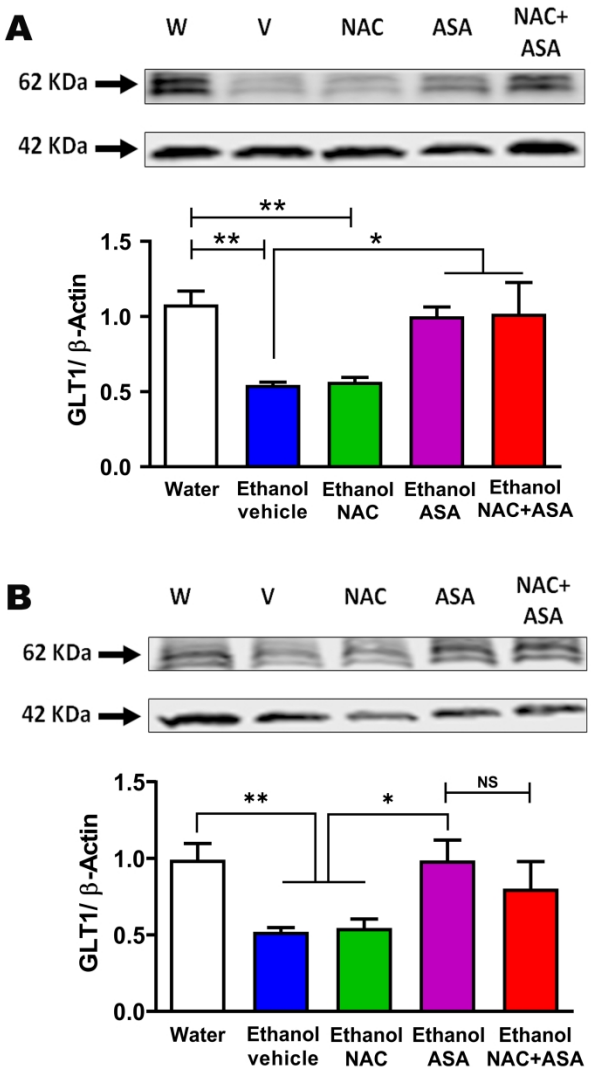
A
W
V
NAC
ASA
NAC+ ASA
62 KDa
42 KDa
**
**
*
GLT1/ β-Actin
1.5
1.0
0.5
0.0
Water
Ethanol vehicle
Ethanol NAC
Ethanol ASA
Ethanol NAC+ASA
B
W
V
NAC
ASA
NAC+ ASA
62 KDa
42 KDa
**
*
NS
GLT1/ β-Actin
1.5
1.0
0.5
0.0
Water
Ethanol vehicle
Ethanol NAC
Ethanol ASA
Ethanol NAC+ASA

Figure 7

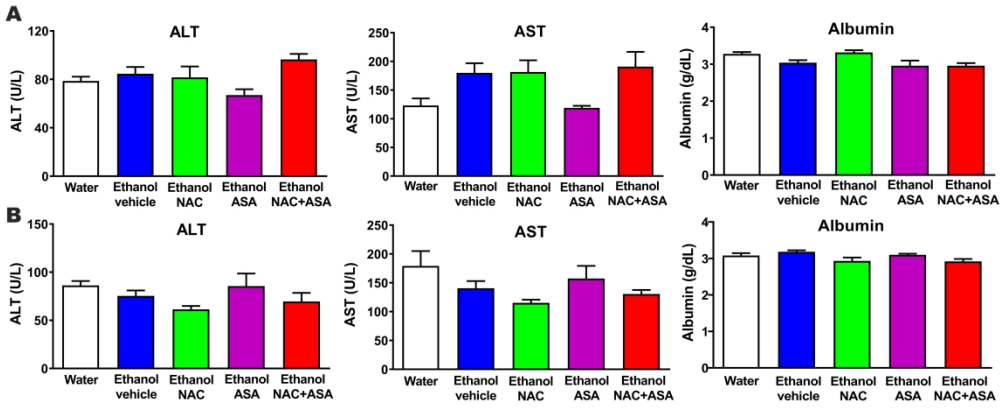

Figure S1. Chronic administration of N-acetylcysteine (NAC), acetyl salicylic acid (ASA) or the combination of NAC+ASA did not induce hepatic damage in chronically alcohol ingesting animals. No differences were observed between the experimental groups in plasma levels of markers associated with hepatic damage (aspartate aminotransferase AST; alanine aminotransferase ALT; albumin). Samples were analyzed after 101 days of chronic alcohol intake (Figure S1A) (ANOVA *$p<0.05$) and after deprivation and a 5-day alcohol re-access (Figure S1B). Data are shown as mean ± SEM. N=6 per experimental condition.