Commonality of Ethanol and Nicotine Reinforcement and Relapse in Wistar-Derived UChB Rats: Inhibition by *N*-Acetylcysteine

Maria Elena Quintanilla, Paola Morales, Fernando Ezquer, Marcelo Ezquer, Mario Herrera-Marschitz, and Yedy Israel

Background: Life expectancy is greatly reduced in individuals presenting alcohol use disorders and chronic smoking. Literature studies suggest that common mechanisms may apply to the chronic use and relapse of both alcohol and nicotine. It is hypothesized that an increased brain oxidative stress and neuroinflammation are involved in perpetuating these conditions and that a common treatment may be considered for both.

Methods: Rats bred as high ethanol (EtOH) drinkers (UChB) were allowed chronic access to EtOH solutions and water and were thereafter deprived of EtOH for a prolonged period, subsequently allowing reaccess to EtOH, which leads to marked relapse binge-like drinking. Separately, EtOH-naïve animals were chronically administered nicotine intraperitoneally and tested under either a conditioned place preference (CPP) reinstatement condition or allowed a free-choice drinking of nicotine solutions and water. Oral *N*-acetylcysteine (NAC) (100 mg/kg) was administered daily to the animals to determine its effect on both chronic voluntary EtOH and nicotine intake, on EtOH relapse and nicotine-CPP reinstatement. Oxidative stress was evaluated in hippocampus as the oxidized/reduced glutathione ratio (GSSG/GSH), and neuroinflammation by glial fibrillary acidic protein (GFAP) immunohistochemistry.

Results: Marked increases in hippocampal oxidative stress (GSSG/GSH) and neuroinflammation (astrocyte reactivity, GFAP) were observed after both chronic EtOH and chronic nicotine treatment. Oral NAC administration (i) fully abolished the increased oxidative stress and the neuroinflammation induced by both drugs, (ii) greatly inhibited EtOH intake (70%) and EtOH relapse binge-like drinking (76%), and (iii) markedly inhibited (90%) voluntary nicotine intake and fully suppressed nicotine-CPP reinstatement.

Conclusions: Data indicate that (i) oxidative stress and neuroinflammation are tightly associated with chronic EtOH and nicotine intake and drug relapse and (ii) NAC inhibits the relapse for both drugs, suggesting that the oral chronic administration of NAC may be of value in the concomitant treatment of alcohol and nicotine use disorders.

Key Words: Alcoholism, Nicotine, *N*-Acetylcysteine, Oxidative Stress, Neuroinflammation.

THE INABILITY TO cease smoking is often seen in subjects presenting alcohol use disorders (Cooney et al., 2009). A number of studies have shown that although altogether different mechanisms initiate and potentiate each other in initiating reinforcement for alcohol and nicotine (Doyon et al., 2013; Truitt et al., 2015), smoking remains a risk factor for alcoholism while continuous alcohol use is a risk factor to remain smoking (Lajtha and Sershen, 2010). DiFranza and Guerrera (1990) reported that 83% of alcoholics are smokers compared to 34% of the nonalcoholic subjects. Smoking also was highly associated with alcoholism in an outpatient Gastroenterology Unit (Pranke and Coral, 2017). A biological predisposition is further seen in rats bred as high-alcohol drinkers, which intravenously self-administer nicotine at higher rates than nondrinker rats (Lê et al., 2006).

The literature has provided a wealth of evidence indicating that although the mechanisms *initiate* the reinforcing action of different drugs of abuse proceed via distinct receptors, transporters, or signal transduction routes for each drug type (i.e., cocaine, heroin, ethanol [EtOH], nicotine), all drugs of abuse increase the release of dopamine in nucleus accumbens (see Volkow and Morales, 2015). Similarly, common mechanisms appear to *perpetuate* the chronic intake and relapse of

From the Molecular and Clinical Pharmacology Program (MEQ, PM, MH-M, YI), Institute of Biomedical Sciences, University of Chile, Santiago, Chile; Neuroscience Department (PM), Faculty of Medicine, University of Chile, Santiago, Chile; Facultad de Medicina Clínica (FE, ME), Centro de Medicina Regenerativa, Alemana-Universidad del Desarrollo, Santiago, Chile; and Facultad de Medicina (FE, ME), Centro de Medicina Regenerativa, Clinica Alemana-Universidad del Desarrollo, Santiago, Chile.

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Reprint requests: Yedy Israel, Molecular and Clinical Pharmacology Program, Faculty of Medicine, Institute of Biomedical Sciences, University of Chile, Independencia 1027, Santiago, Chile; Tel.: +56 22 978-6216; Fax: +56 22 737-2783; E-mail: yisrael@uchile.cl

MEQ and PM contributed equally to this work.

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these drugs (Kupchik et al., 2012; Mousavi et al., 2015; Quintanilla et al., 2016; Reissner et al., 2015; Volkow and Morales, 2015; Zhou and Kalivas, 2008). These common mechanisms (see Neuhofer and Kalivas, 2018) require investigation.

A number of studies have shown that EtOH administration and chronic EtOH intake markedly induce brain oxidative stress (Das and Vasudevan, 2007; Franke et al., 1977; Montoliu et al., 1994) and neuroinflammation (Crews and Vetreno, 2016; Montesinos et al., 2016; Schneider et al., 2017). Repeated nicotine administration to rats also increases brain oxidative stress (Bhagwat et al., 1998), while neuroinflammation has been reported in neonatal rats that received nicotine (Younes-Rapozo et al., 2015). Addition of nicotine to mesencephalic cells in culture also induces oxidative stress and activates the transcription of nuclear factor-*kappa B* (NF-κB), which is involved in pro-inflammatory cytokine production (Barr et al., 2007).

The interrelation between oxidative stress and neuroinflammation appears clear. In many tissues, even low concentrations of hydrogen peroxide (H_2O_2) and a number of stimuli, including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and lipopolysaccharide, activate the transcription NF-κB while various antioxidants prevent the NF-κB activation by H_2O_2, as well as that by several other inducers (Schreck et al., 1992). In nonstimulated cells, NF-κB resides in the cytoplasm in an inactive complex bound to the inhibitory IκB proteins (Baeuerle and Baltimore, 1988). It is well accepted that oxidative stress can inactivate IκB (Canty et al., 1999), allowing the NF-κB–mediated synthesis of TNF-α which, in turn, is a mitochondrial uncoupler that leads to the generation of the superoxide radical (Kastl et al., 2014; Schulze-Osthoff et al., 1992), a source of H_2O_2 and hydroxyl radicals. Thus, literature studies suggest the existence of a vicious cycle, generated by the mutual potentiation of pro-inflammatory factors and reactive oxygen species.

N-acetylcysteine (NAC), a well-known antioxidant, is used to treat acetaminophen overdoses (Smilkstein et al., 1988). The likely mechanism of action is the generation of cysteine, the rate-limiting amino acid for reduced glutathione (GSH) synthesis (Vincent et al., 1999), and its disulfide reducing effects (Samuni et al., 2013). It has been shown that daily intraperitoneal (i.p.) administration of NAC reduced chronic EtOH intake (Quintanilla et al., 2016).

The similarity in inducing oxidative stress and neuroinflammation by the continued EtOH and nicotine use suggests that NAC could inhibit the self-administration and relapse for both drugs. The relapse-like EtOH intake that follows a prolonged alcohol deprivation effect (ADE) followed by a short EtOH reaccess (Rodd et al., 2008; Spanagel and Hölter, 1999) is a good predictor of the therapeutic potency of antialcohol drugs in humans (Vengeliene et al., 2005). Another relapse testing technique is the post extinction–conditioned place preference (CPP) reinstatement paradigm, also referred to as motivation, used for different classes of drugs of abuse including EtOH and nicotine (Biala et al., 2009; Lee et al., 2016). In this paradigm, place preference for a recognizable environment is generated by administering the drug and placing the animals on 1 of the distinct sides of a 2-sided box. Place preference that has developed is subsequently extinguished by allowing animals to explore both sides of the box in the absence of the drug. After the extinction of CPP, a small priming dose of the drug is administered to determine whether CPP is reinstated.

This study addresses the effect of NAC administration on both reinstatement paradigms: (i) the alcohol relapse-like intake after an ADE and (ii) the reinstatement of extinguished nicotine-induced CPP.

Tobacco chewing (Gajalakshmi and Kanimozhi, 2015) and nicotine gums (Kostygina et al., 2016) have become public health concerns in Asia and the United States. While rats will not readily self-administer nicotine orally, we hypothesized that rats could be trained to voluntarily drink nicotine solutions if they became dependent on nicotine after its repeated i.p. administration.

Based on the existence of common mechanisms for the perpetuation of addictive drug intake indicated above, we propose that orally administered NAC will (i) prevent relapse-like EtOH binge drinking behavior, (ii) inhibit the reinstatement of nicotine-CPP, (iii) inhibit EtOH-induced and nicotine-induced hippocampal oxidized and reduced glutathione (GSSG/GSH) increase, a marker of oxidative stress, (iv) attenuate both EtOH-induced and nicotine-induced hippocampal astrocytosis, a marker of neuroinflammation, and (v) inhibit nicotine intake in animals trained to self-administer it chronically.

MATERIALS AND METHODS

Animals

Adult UChB female rats (Quintanilla et al., 2006) were used in the experiments. Animals were maintained on a 12-hour light/dark cycle (lights off at 7:00 PM). Experimental procedures were approved by the Ethics Committee for Experiments with Laboratory Animals at the Medical Faculty (Protocol CBA#0507, FMUCH) and by the Chilean Council for Science and Technology Research (CONICYT).

Drugs and Solutions

EtOH (Merck, Darmstadt, Germany) solutions (10 and 20% v/v) were prepared in distilled water. (−)-Nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, MO) was dissolved in saline and injected i.p. at a dose of 0.5 mg/kg (free base) (Biala et al., 2009). Drinking nicotine solutions (4 mg % w/v) were prepared each day. NAC (Sigma-Aldrich) dissolved in water was adjusted to pH 7.2 with NaOH and administered by oral gavage (100 mg/kg/d in 5.0 ml/kg volume). Glutathione reductase (G3664), NADPH (N1630), and DTNB (5, 5′-dithiobis-2-nitrobenzoic acid) (D-8130) were from Sigma-Aldrich.

Blood EtOH Levels and Tissue Glutathione Determination

Immediately after EtOH deprivation and 60 minutes of EtOH reaccess, blood (100 μl) was collected and EtOH levels were

determined by gas chromatography (Quintanilla et al., 2007). The redox ratio of GSSG/GSH was determined in hippocampal tissue samples as previously described (Ezquer et al., 2017).

Determination of Total Thiols in Plasma

Total thiols in plasma were determined by DTNB color generation. Absorbance was measured at 412 nm (Bell et al., 2016; Santos Bernardes et al., 2016). GSH was used as a standard.

Determination of Astrocyte Immunoreactivity

Immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP) was evaluated in hippocampus as previously reported (Ezquer et al., 2017). Total length and thickness of astrocyte primary processes were assessed for 6 GFAP-positive cells per Z-stack by confocal microscopy as previously reported (Ezquer et al., 2017).

Experiment 1: Effect of Oral NAC Administration on Chronic Alcohol Intake. Twelve female UChB rats weighing 190 to 270 g were housed in individual cages. For induction of chronic alcohol intake, animals were allowed continuous 24-hour free-choice access to 10% v/v EtOH and water for 16 weeks, followed by 10 days of concurrent access to 10 and 20% (v/v) of EtOH solutions and water. Daily consumption of EtOH solutions and water was recorded. After induction of chronic EtOH intake, the animals were divided into 2 groups and orally administered either NAC (100 mg/kg in a volume of 5 ml/kg) or water (n = 6 rats/group) once daily for 14 consecutive days. Twenty-four hours after the last NAC administration, the animals were anesthetized to obtain plasma for thiol analyses and thereafter the animals were euthanized to obtain hippocampal samples for GSSG/GSH ratio determination and astrocyte GFAP immunohistochemistry.

Experiment 2: Effect of NAC Treatment During EtOH Deprivation on Relapse-Like EtOH Drinking Behavior. Ten female UChB rats weighing 190 to 270 g were housed in individual cages. After induction of chronic EtOH intake as described above, the animals were deprived of EtOH for 17 days and after this allowed reaccess to EtOH solutions (10 and 20%) for only 60 minutes. Rats received 14 daily administrations of NAC (100 mg/kg) or vehicle (water) orally (n = 5 rats/group) *during only the first 14 days of EtOH deprivation*. Seventy-two hours after that last dose of NAC (EtOH deprivation on day 17), animals were offered a 60-minute reaccess to 10 and 20% of EtOH solutions. EtOH intake was expressed as gram of EtOH consumed/kg body weight/60 min. Immediately after the 60-minute EtOH reaccess, animals were anesthetized and subsequently euthanized to determine blood EtOH level and hippocampal GSSG/GSH levels.

Experiment 3: Effect of NAC on the Reinstatement of Nicotine CPP by Nicotine Priming. The studies determined whether NAC prevented the reinstatement of a previously extinguished nicotine place preference.

Preconditioning Phase—A "biased" place conditioning, in which the drug is administered in the less-preferred side of the conditioning box (Le Foll and Goldberg, 2005), was applied. Twenty naïve female UChB rats had an initial access to the 2 sides of the box, one side presenting white walls and other side presenting black walls (Quintanilla and Tampier, 2011). During 3 preconditioning days, rats were videotaped for 20 minutes to determine the animal nonpreferred side of the box.

Conditioning Phase—The 20 rats were assigned to 4 groups (n = 5 per group). Two nicotine groups (G3 and G4, 5 rats/group) were conditioned for nicotine (0.5 mg/kg, i.p.) administered daily and placed on the nonpreferred (white) side (morning sessions) and saline on the preferred (black) side (afternoon sessions). Two control groups (G1 and G2, 5 rats/group) received saline in the preferred side (morning sessions) and saline in the nonpreferred side (afternoon sessions). Sessions were conducted each day with an interval of 3 hours between sessions, for 14 consecutive days. This dose of nicotine has been reported to produce significant CPP after i.p. injection in the rat (Biala et al., 2009).

Postconditioning Phase (CPP Determination)—The postconditioning phase (no drug administered) was started 1 day after the last conditioning session. Each animal was tested for place preference by placing it in the box and allowing it to investigate both sides move to either side for 20 minutes. A video recording was used to determine the time spent in each compartment. Preference scores were calculated for each rat by subtracting the time (seconds) spent in the nonpreferred compartment, recorded during the preconditioning phase, from the time spent in the initially nonpreferred compartment during the postconditioning phase.

Extinction Training—After the nicotine groups had acquired CPP, the rats were given extinction training sessions daily for 8 days. During this training, the nicotine group G3 received oral water, while the nicotine group G4 received NAC (100 mg/kg, by oral gavage) 2.5 hours before allowing the animals to explore both sides of the box for 20 minutes. The extinction training was also applied to the control groups: The control group G1 received oral water, and the control group G2 received NAC oral 2.5 hours before allowing the animals to explore both sides of the box for 20 minutes.

Reinstatement Phase—Following the extinction phase, to determine place preference on reinstatement the nicotine group G3 received oral water while the nicotine group G4 received oral NAC 2.5 hours before receiving a priming injection of nicotine (0.5 mg/kg, i.p.), allowing the animals to explore both sides of the box for 20 minutes. The control group G1 received oral water, and the control group G2 received oral NAC (100 mg/kg, i.p), 2.5 hours before administering saline (i.p.) also allowing the animals to explore both sides of the box for 20 minutes. Thereafter, the 4 groups of animals were anesthetized to obtain blood for assessment of plasma thiols, and thereafter, animals were euthanized to obtain hippocampal samples for GSSG/GSH ratio determination and astrocyte GFAP immunohistochemistry assay.

Experiment 4: Induction of Voluntary (Free Choice) Oral Nicotine Consumption: Effect of NAC Administration. A separate group (n = 16 rats) of adult naïve female UChB rats, housed in individual cages with water and food ad libitum, were administered a priming dose of 0.5 mg/kg i.p. of nicotine once a day for 14 consecutive days. After discontinuation of nicotine injections on day 15, each cage was fitted with a second drinking tube containing a solution of nicotine with increasing concentration on successive days. Initially, nicotine was 5 mg/l during the initial 2 days of nicotine exposure, 10 mg/l from day 3 to 7, and 20 mg/l from day 8 to 13. From day 14 to 20, nicotine concentration was 30 mg/l, and finally from day 21, nicotine concentration was raised to 40 mg/l and kept constant for the rest of the experiment. Nicotine and water intake were recorded daily.

Effect of Oral NAC on Voluntary Nicotine Intake—Following 9 weeks of oral nicotine self-administration, animals were divided into 2 groups (n = 8 rats/group) that received either NAC orally

(100 mg/kg/d) or water for 9 days, while voluntary oral nicotine intake was determined 10 days after NAC administrations were discontinued. Nicotine intake and water intake were recorded daily. Nicotine intake was expressed as mg nicotine consumed/kg body weight/d.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (San Diego, CA). Data are expressed as means ± SEM. Two-way (treatment × day) analysis of variance (ANOVA) followed by post hoc Tukey's *t*-test or repeated-measures ANOVA when required was conducted to compare EtOH, nicotine, or water intake between vehicle and nicotine control groups. One-way ANOVA followed by post hoc Tukey's *t*-test was used to analyze total length and thickness of primary processes of GFAP-positive astrocytes and for GSSG/GSH ratio data and differences between groups in the CPP test. When appropriate, Student's *t*-test was conducted. A level of $p < 0.05$ was considered for statistical significance.

RESULTS

Ethanol

NAC Administration to Rats Reduced Chronic Alcohol Consumption and Normalized Elevated Brain GSSG/GSH Redox Ratio and Astrocyte Activation. Two-way ANOVA

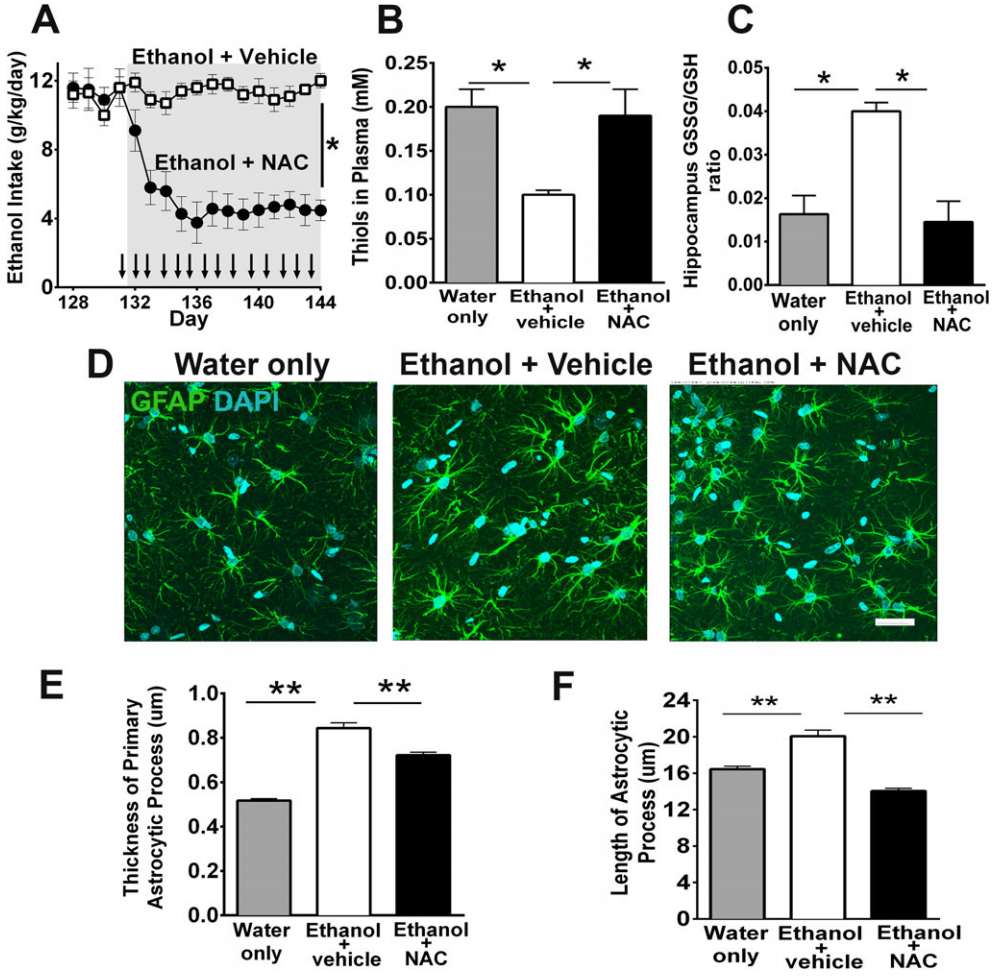

Fig. 1. Repeated oral administration of *N*-acetylcysteine (NAC; 100 mg/kg/d) inhibited chronic ethanol (EtOH) intake, normalized plasma thiols, and hippocampal oxidized/reduced glutathione (GSSG/GSH) ratio and reversed astrocytosis. Voluntary EtOH consumption of animals that following 18 weeks of chronic EtOH intake received a daily oral dose of 100 mg/kg of NAC for 14 days. Twenty-four hours after the last dose of NAC, blood samples were obtained for plasma thiol determination, and immediately thereafter, animals were euthanized for hippocampal immunohistochemistry (glial fibrillary acidic protein [GFAP]) and GSSG/GSH studies. (**A**) Data on alcohol consumption are expressed as g of EtOH consumed/kg body weight/d. NAC administration inhibited EtOH intake by 70%. (**B**) Total thiol levels (mM) were determined in plasma of chronic EtOH-drinking animals receiving the 14-day treatment with either oral NAC or vehicle. Animals consuming only water were used as controls. The 14-day administration of NAC to chronically EtOH ingesting animals markedly increased (80%) plasma thiol levels. (**C**) GSSG/GSH ratio in hippocampus of chronically EtOH-consuming animals that received the 14-day treatment with either oral NAC or vehicle. The hippocampal GSSG/GSH redox ratio was 150% higher in animals that ingested alcohol chronically than the GSSG/GSH ratio of animals that received NAC for 14 days. (**D**) Confocal microscopy microphotographs of GFAP immunoreactivity (**green**) counterstained with DAPI (nuclei marker, **blue**) from hippocampus of animals receiving a 14-day treatment with either oral NAC (**right** side) or vehicle (**center**). Animals consuming only water were used as controls. Scale bar 25 μm. (**E**) Thickness of primary process and (**F**) length of primary astrocytic process evaluated by confocal microscopy and FIJI image analysis software (University of Wisconsin, Madison WI). NAC administration significantly reduced the astrocyte processes of animals consuming alcohol chronically. Data are shown as mean ± SEM. $N = 6$ rats per experimental condition. All determinations: $^{*}p < 0.01$, $^{**}p < 0.001$.

(drug treatment × days) of data in Fig. 1*A* revealed that oral NAC (100 mg/kg/d) administration inhibited chronic EtOH intake by 70% compared to that of control animals, $F_{\text{treatment}}(1, 170) = 307.2$, $p < 0.0001$, $n = 6$ rats/group. The maximum inhibitory effect was observed 3 to 4 days after initiating the daily NAC administration. Twenty-four hours after the last dose of oral NAC, blood was taken for the assay of total thiols in plasma. Figure 1*B* shows that chronically EtOH-drinking animals (white bar) exhibited lower plasma thiols (R-SH mainly in plasma proteins) than the water control group (gray bar), $F(2, 15) = 6.86$, $p < 0.01$. Repeated administration of oral NAC markedly increased the plasma thiol levels (post hoc Tukey's *t*-test, $p < 0.05$). Following blood sampling, animals were euthanized to determine hippocampal GSSG/GSH ratio and astrocyte immunoreactivity. One-way ANOVA of data in Fig. 1*C* revealed that the hippocampal GSSG/GSH ratio was significantly (150%) higher in the chronically EtOH-drinking animals (white bar) compared to that in the water control group (gray bar), 1-way ANOVA, $F(2, 15) = 13.34$, $p < 0.0005$, indicating that chronic EtOH intake leads to brain oxidative stress and increase in the GSSG/GSH ratio that was fully reversed by the oral NAC administration (black bar) (post hoc Tukey's *t*-test, $p < 0.001$).

The histologic hallmark of alcohol-induced neuroinflammation is an enlargement and increased thickness of astrocytic processes, assessed by immunoreactivity against GFAP (Franke et al., 1977). Figure 1*D* shows that chronic EtOH-drinking animals (central image of Fig. 1*D*) exhibited a significant increase in both the thickness of the primary processes (Fig. 1*E*), $F(2, 638) = 105.0$, $p < 0.0001$, and total length (Fig. 1*F*), $F(2, 3135) = 40.35$, $p < 0.0001$, of the astrocytic processes compared with the control group that had ingested only water (left image of Fig. 1*D*). Repeated oral administration of NAC (100 mg/kg/d) for 14 days (right image of Fig. 1*D*) reversed the increases in GFAP-positive cells induced by chronic EtOH intake (black bars in Fig. 1*E, F*) (post hoc Tukey's *t*-test, $p < 0.0001$).

NAC Administration to Alcohol-Deprived Rats Following Chronic EtOH Intake Prevented Relapse-Like EtOH Drinking Upon EtOH Reaccess. As indicated above, a protracted vicious cycle perpetuates the oxidative stress–neuroinflammation condition. The aim of experiments in Fig. 2 was to test the hypothesis that a normalized oxidative stress following the administration NAC would reduce EtOH relapse–intake in chronically alcohol-treated rats, even when NAC was administered *during the EtOH deprivation* period, prior to allowing alcohol reaccess. Thus, the study addresses the action of NAC on protracted biological changes induced by EtOH and leading to relapse *after EtOH intake has been discontinued,* changes associative with earlier alcohol-induced biological effects. To test this hypothesis rats that had ingested alcohol chronically for 140 days were deprived of EtOH for 17 days. On the initial 14 days of EtOH deprivation, animals received a daily oral dose of NAC (100 mg/kg)

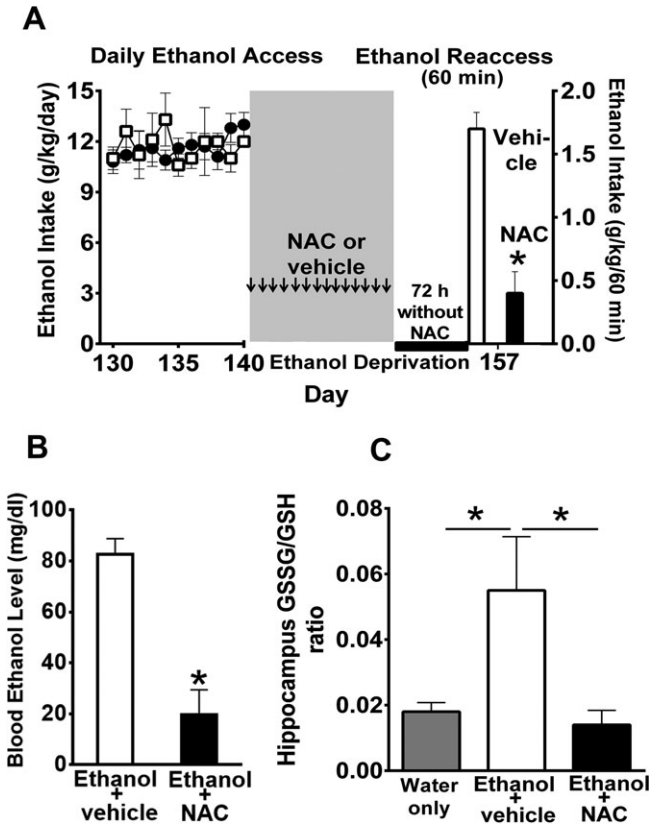

Fig. 2. Protracted effect of *N*-acetylcysteine (NAC) exerted during ethanol (EtOH) deprivation. (**A left side**) Rats that had consumed EtOH for 12 weeks followed by 17-day EtOH deprivation period, received a daily dose of 100 mg/kg of NAC or vehicle via oral during the first 14 days of EtOH deprivation. Relapse drinking upon EtOH reaccess after the deprivation period was determined on day 17, thus 72 hours after discontinuing NAC administration. (**A right**) EtOH intake during a 60-minute EtOH reaccess, expressed as g EtOH consumed/kg body weight/60 min, was significantly inhibited by 76%. Blood EtOH levels (**B**) determined after the 60-minute relapse-like EtOH consumption were reduced by 75% following NAC treatment. The hippocampal oxidized/reduced glutathione (GSSG/GSH) ratio (**C**) of NAC-treated animals determined immediately after the 60-minute relapse-like EtOH consumption was inhibited by 70%. Animals consuming only water were used as controls. Data are shown as mean ± SEM. $N = 5$ rats per experimental condition. All determinations: $^*p < 0.02$.

while controls received water. Further, EtOH reaccess was allowed 72 hours *after* NAC administration had been discontinued (see Discussion for the *biological* half-life of NAC and Quintanilla et al., 2016). As shown in Fig. 2*A* (right side) during the 60-minute EtOH reaccess, animals of the EtOH group (treated with vehicle) showed a relapse (binge-like) EtOH intake of 1.69 ± 0.13 g EtOH/kg/60 min. Conversely, animals that had consumed EtOH chronically and received oral NAC (100 mg/kg/d) for 14 days, and 3 days without NAC, showed a markedly reduced EtOH intake of 0.40 ± 0.17 g EtOH/kg/60 min, 76% lower than the alcohol intake of the alcohol control (vehicle) group (*t*-test: 6.08, $p < 0.001$, $n = 5$/group). Immediately after the 60-minute EtOH reaccess session, blood samples were obtained to determine EtOH levels. Figure 2*B* shows that the alcohol group treated with vehicle exhibited blood EtOH levels of

82.6 ± 6.13 mg/dl, while the alcohol group treated with NAC showed blood EtOH levels of 19.7 ± 9.8 mg/dl, 76% lower than the control group (*t*-test: 5.46, $p < 0.001$, $n = 5$ rats/group). Immediately after blood sampling, animals were euthanized to determine hippocampal GSSG/GSH ratio. Figure 2*C* shows, although the animals ingesting alcohol chronically had a 17 days of EtOH deprivation before the 60 minutes of EtOH reaccess, the GSSG/GSH ratio was 200% higher than that of control animals that had ingested only water (gray bar), $F(2, 12) = 5.17$, $p < 0.02$. This increase was normalized by the 14-day oral NAC administration, which was discontinued 72 hours prior to hippocampal collection (black bar Fig. 2*C*) (post hoc Tukey's *t*-test, $p < 0.0001$).

Nicotine

Pretreatment with Oral NAC Prevented the Reinstatement of Nicotine-Induced CPP. Figure 3 shows the timeline of the CPP study. As it has been shown that long-term nicotine continuous treatment (1 to 3 weeks) is necessary to induce oxidative stress in rat brain (Bhagwat et al., 1998), rats were conditioned with nicotine by 14 daily sessions.

Figure 4*A* shows that on the CPP test day (day 18), rats of the G3 (striped bar) and G4 (black bar) groups that had been conditioned with nicotine i.p. (0.5 mg/kg/d) for 14 consecutive days ($n = 5$/group) exhibited a marked CPP, compared to the control groups G1 (white bar) and G2 (gray bar), $F(3, 16) = 31.63$, $p < 0.0001$. During 8 extinction days (Fig. 4*A*), the 2 nicotine groups G3 and G4 that had acquired marked CPP were pretreated orally with either water or NAC (100 mg/kg), respectively. The 2 control groups G1 and G2 also received water or NAC (100 mg/kg), respectively. In all cases, the water or nicotine was administered 2.5 hours before allowing the animals to explore both sides of the box for 20 minutes. The finding that during the extinction period the 2 groups, G3 and G4 gradually, while not differently, $F_{\text{Groups}}(1, 56) = 0.34$, $p = 0.55$, N.S., reduced the time spent in the nicotine-paired side of the box indicates that pretreatment with oral NAC (100 mg/kg/d) did not accelerate the extinction of CPP (Fig. 4*A*, see extinction days 18, 23, 26). On the last extinction day (day 26), the 2 groups, G3 (striped bar) and G4 (black bar), that had previously acquired nicotine CPP did not show preference for the side previously conditioned with nicotine, and was similar to the control groups G1 (white bar) and G2 (gray bar), $F(3, 16) = 1.0$ $p < 0.2$, N.S., indicates that nicotine CPP had been extinguished. On reinstatement day 27, Fig. 4*A* shows that the initially priming nicotine dose (0.5 mg/kg, i.p.) administered to rats of group G3 (striped bar) reinstated the previously extinguished CPP (*t*-test: 4.94, $p < 0.001$, $n = 10$). By contrast, pretreatment with nicotine (0.5 mg/kg) of rats of group G4 (black bar) that had chronically received oral NAC (100 mg/kg) showed no nicotine-induced CPP reinstatement (*t*-tests: 1.67, $p = 0.11$, vs. control group G2, $n = 5$ animals per group).

Immediately after the above CPP reinstatement experiment, blood samples were taken for the assay of total plasma thiol group. Figure 4*B* shows that rats displaying nicotine reinstatement pretreated with water, group G3 (striped bar), exhibited a decrease in reduced total thiols (SH) in plasma compared with control rats (G1 white bar) that received a saline injection, $F(2, 12) = 8.24$, $p < 0.005$, suggesting that repeated nicotine administration is (as shown above for EtOH) also associated with peripheral oxidative stress. The oral administration of NAC during extinction and reinstatement days normalized the nicotine-induced decrease in total thiols (R-SH) in plasma (black bar, Fig. 4*B*) (post hoc Tukey's *t*-test, $p < 0.001$). Immediately after blood sampling for thiol assays, animals were euthanized to determine the GSSG/GSH ratio (Fig. 4*C*) and astrocyte immunoreactivity in hippocampus. Figure 4*C* shows that rats displaying nicotine reinstatement pretreated with water, group G3 (striped bar), exhibited a higher GSSG/GSH ratio compared with control rats (G1 white bar) that received a saline priming injection (white bar), $F(2, 12) = 24.02$, $p < 0.0001$. This increase was fully normalized by the administration of oral NAC (black bar) (post hoc Tukey's *t*-test, $p < 0.0001$).

Figure 5*A* shows GFAP immunoreactivity of hippocampus immediately following the above reinstatement studies after chronic nicotine administration (center, G3 rats) or

Session (days):	1 - 3	4 - 17	18	19 - 26	27
Phase:	Pre-Conditioning	Conditioning	Test	Extinction	Reinstatement
Injection:	None	Alternating Nic/Sal or Sal/Sal	None	Sal or NAC	Nic-Sal or Nic-NAC

Fig. 3. Schematic representation of the conditioned place preference study design. In sessions 1 to 3 of *preconditioning,* no drug or vehicle injection was administered and animals have open access to the entire box. In *conditioning*, sessions 4 to 17, alternating nicotine and saline or saline was administered, pairing nicotine with the nonpreferred side and saline with the preferred side. Session 18, *testing*, consists of no drug or vehicle injections while allowing animals open access to the 2 sides of the box. In *extinction* sessions 19 to 26, rats that have been pretreated with *N*-acetylcysteine (NAC) or water 2.5 hours before box placing had access to the both sides of the box. In *reinstatement* session on day 27, rats were pretreated with oral NAC or vehicle 2.5 hours before injecting them with their initial training dose (nicotine 0.5 mg/kg i.p. or saline) and allowed to roam throughout the box.

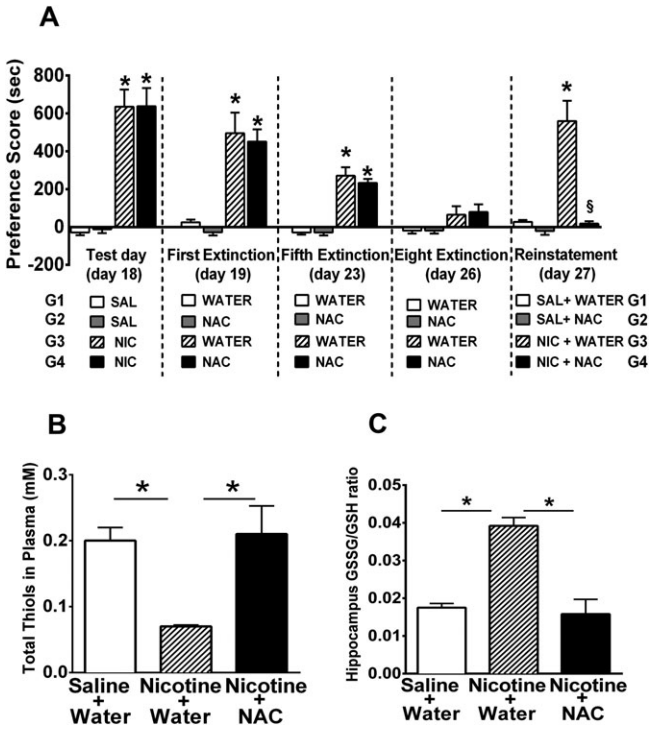

Fig. 4. Pretreatment with oral *N*-acetylcysteine (NAC) prevented the reinstatement of nicotine-induced conditioned place preference (CPP), normalized both the decreased plasma thiols and the increased hippocampus oxidized/reduced glutathione (GSSG/GSH) ratio. (**A**) Rats of groups shown as G3 (striped bars) and G4 (black bars), both conditioned with 0.5 mg/kg nicotine (i.p.), showed a significant CPP on test day (day 18). The nicotine CPP of rats of group G3 and G4 was extinguished along the 8 extinction days (until day 26). On day 27, CPP for nicotine in group G3 (striped bar) was clearly reinstated by a priming nicotine dose (0.5 mg/kg, i.p.). Oral NAC (100 mg/kg) pretreatment for 7 days fully prevented the reinstatement of nicotine CPP in group G4 (black bar). (**B**) Total thiol levels (mM) in plasma were evaluated in both the nicotine water group (group G3, striped bar) and in the nicotine NAC group (group G4, black bar), in which NAC prevented nicotine reinstatement. The saline control group, injected with saline and pretreated with water (group G1, white bar), was used as control. The hippocampal GSSG/GSH ratio (**C**) was evaluated in nicotine water group, which showed nicotine reinstatement of CPP (group G3, striped bar), in nicotine NAC group, in which NAC prevented nicotine reinstatement (group G4, black bar), and in saline control group, injected with saline and pretreated with water (group G1, white bar). Each bar represents the mean ± SEM of 5 rats per group (**A**, *$p < 0.001$ vs. the corresponding control group; §$p < 0.0002$ vs. the nicotine water group). Figure **B** and **C** all determinations: *$p < 0.001$. NIC, nicotine; SAL, saline.

nicotine administration plus NAC (right, G4 rats), versus saline controls (left, G1 rats). Figure 5*B* shows that G3 rats displaying nicotine reinstatement (striped bar) exhibited a significant increase in the number of astrocytes/mm^3, $F(2, 30) = 5.66$, $p < 0.01$, increased process lengths, Fig. 5*C*, striped bar, $F(2, 3135) = 40.35$, $p < 0.0001$, and increased thickness of primary astrocyte processes, Fig. 5*D*, striped bar, $F(2, 638) = 105$, $p < 0.0001$, compared with control rats (G1 white bars in Fig. 5*B–D*) that received a saline priming injection. Overall, the repeated oral administration of NAC (100 mg/kg/d) during extinction and 2.5 hours before the nicotine priming dose fully reversed the astrocyte morphological changes induced by nicotine (black bars vs. striped bars in Fig. 5*B–D*) (post hoc Tukey's *t*-test, $p < 0.01$).

Induction of Voluntary Free-Choice Nicotine Consumption: Inhibition by Oral NAC Administration. Figure 6*A* shows the change in voluntary nicotine solution intake observed following i.p injections of 0.5 mg of nicotine/kg/d for 14 successive days, and allowed the choice of consuming increasing concentrations of nicotine solution (5 to 40 mg/l) and water. Following 62 days of voluntary nicotine intake, animals reached a stable nicotine intake of 4.72 ± 0.17 mg/kg/d, a daily average which is substantially higher than the weekly average of 4.66 ± 0.35 mg/kg/wk of nicotine intake observed by Nesil and colleagues (2011), who did not administer systemic nicotine priming doses. On day 63, rats were divided into 2 groups that received either a daily oral dose of NAC (100 mg/kg) or water for 9 days. Results in Fig. 6*B* indicate that oral NAC induced a 90% reduction in nicotine intake compared with the control animals treated with water, $F_{\text{treatment}}(1, 200) = 1{,}344$, $p < 0.0001$. Noteworthy, upon discontinuation of the NAC administration, nicotine intake remained below controls for 6 days after treatment ended (Fig. 6*B*, 73 to 78 days post treatment), suggesting the existence of long-acting internal reduced sulfhydryl "buffering," likely in blood and tissue proteins. Figure 6*C* shows that NAC treatment did not modify total water intake (water + water contained in the nicotine solution) or animal body weights.

DISCUSSION

While the mechanisms that mediate the activation of mesolimbic dopaminergic transmission involved in the initiation of the reinforcing actions of EtOH and nicotine may be different (Israel et al., 2015; Truitt et al., 2015), the perpetuation of both EtOH intake and nicotine intake appears to be maintained by a common mechanism. Present studies showed that chronic intake of both drugs leads to brain oxidative stress and neuroinflammation, as shown by an elevated hippocampal GSSG/GSH ratio and astrocyte immunoreactivity, respectively. The hippocampus is the brain area most pathologically affected by chronic EtOH intake (Franke et al., 1977; Walker et al., 1980), which in humans has been proposed to be responsible for the loss of control over drinking and severe intoxication (see Crews, 2008; Hermens et al., 2015). For nicotine, hippocampal function is responsible for the associative-learned drug cues that perpetuate drug intake (Davis and Gould, 2008).

A number of crossover points exist between the effect of EtOH and nicotine. *Not only both drugs* activate the release of dopamine in nucleus accumbens during the initiation of their intake (see Di Chiara, 2002), but following long-term exposure, they share the several effects that could be associated with the perpetuation of intake and with the relapse behavior: (i) chronic EtOH and nicotine *potentiate each* other in increasing EtOH relapse self-administration (Truitt et al., 2015); (ii) both *chronic alcohol* (Montoliu et al., 1994) and *chronic nicotine* (Bhagwat et al., 1998) result in marked increases in brain oxidative stress; (iii) both have a common

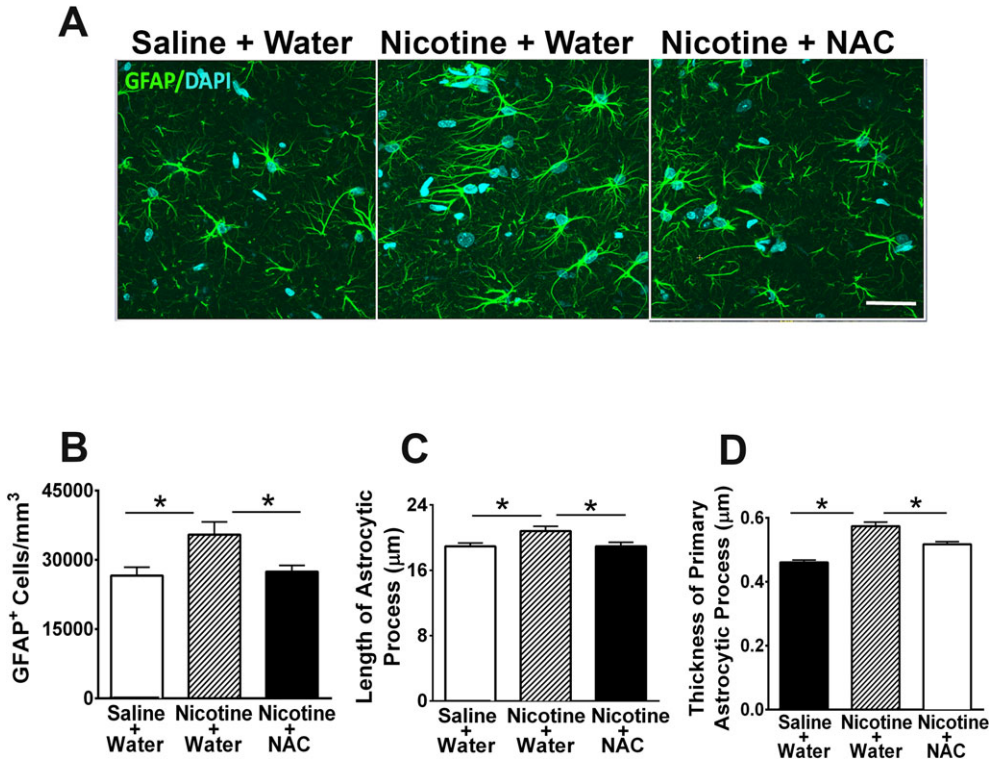

Fig. 5. Pretreatment with oral *N*-acetylcysteine (NAC) normalizes the nicotine-induced activation of hippocampal astrocytes. (**A**) Confocal microscopy microphotographs of glial fibrillary acidic protein (GFAP) immunoreactivity (**green**) counterstained with DAPI (**blue**) in hippocampal astrocytes of saline water group (**left** image, G1 of Fig. 4), nicotine water animals (**center**, G4 of Fig. 3), and nicotine NAC group (**right** image, G4 of Fig. 4). Scale bar 25 μm. (**B**) $GFAP^+$ cells/mm^3. (**C**) Total length and (**D**) thickness of primary astrocytic process (evaluated by confocal microscopy and FIJI image analysis software). Saline control group (white bar, G1), nicotine water animals that had shown nicotine reinstatement of conditioned place preference (striped bar, G3 of Fig. 4), and nicotine NAC group in which NAC had prevented nicotine reinstatement (black bar, G4) are shown. NAC administration significantly reduced the astrocyte processes of animals consuming nicotine chronically. Data are shown as mean ± SEM. $N = 5$ rats per experimental condition. All determinations: $^*p < 0.01$.

mechanism, namely the induction of cytochrome P450 2E1 (Joshi and Tyndale, 2006; Montoliu et al., 1994); (iv) EtOH and nicotine combined *potentiate* each other in elevating brain cytochrome P4502E1 (Ferguson et al., 2013); and (v) as shown in the present study, the intake of EtOH or nicotine alone results in neuroinflammation and oxidative stress, which in turn are associated with a high intake of both drugs and their relapse behavior.

As indicated earlier, oxidative stress and neuroinflammation potentiate and perpetuate each other (Kastl et al., 2014; Schulze-Osthoff et al., 1992). A long-acting vicious cycle is in line with the protracted neuroinflammation—exceeding 10 months—induced by a single administration of systemic lipopolysaccharide (Qin et al., 2007), which promotes both neuroinflammation and oxidative stress (see Crews and Vetreno, 2016).

NAC orally administered to rats ingesting both EtOH and nicotine chronically (i) inhibited oxidative stress, as shown by the normalization of an elevated hippocampal GSSG/GSH ratio, (ii) normalized the EtOH- and nicotine-induced astrocytosis, a marker of neuroinflammation, and (iii) inhibited both voluntary EtOH intake and nicotine intake. The inhibitory effect of NAC on neuroinflammation is in line with studies by Schneider and colleagues (2017) who showed that chronic EtOH administration to rats elevated pro-inflammatory cytokines, TNF-α, and IL-1α levels in brain, changes which were reversed by a short-term i.p. administration of (90 mg/kg) NAC.

For EtOH, 2 additional mechanisms to increases in cytochrome P450 2E1 appear to be involved in generating a neuroinflammation–oxidative stress cycle: (i) the alcohol/acetaldehyde-induced entry of gut lipopolysaccharide into systemic circulation (Ferrier et al., 2006), and (ii) H_2O_2-generated by monoamine oxidase catalyzed metabolism of dopamine released in nucleus accumbens (Cunha-Oliveira et al., 2013). The latter mechanism is common for all drugs of abuse and may constitute the common link involved in the relapse of several addictive drugs, including alcohol and nicotine.

Following chronic intake of an addictive drug, and long-term abstinence, relapse is triggered by the presentation of cues previously associated with drug use/effect, which activate glutamate release in several brain areas involved in the memory coding and transmission of addictive drug cues to the nucleus accumbens and the frontal cortex (Bell et al., 2016; Gass et al., 2011; Rao et al., 2015). The odor may constitute a cue in EtOH relapse and likely in chronic

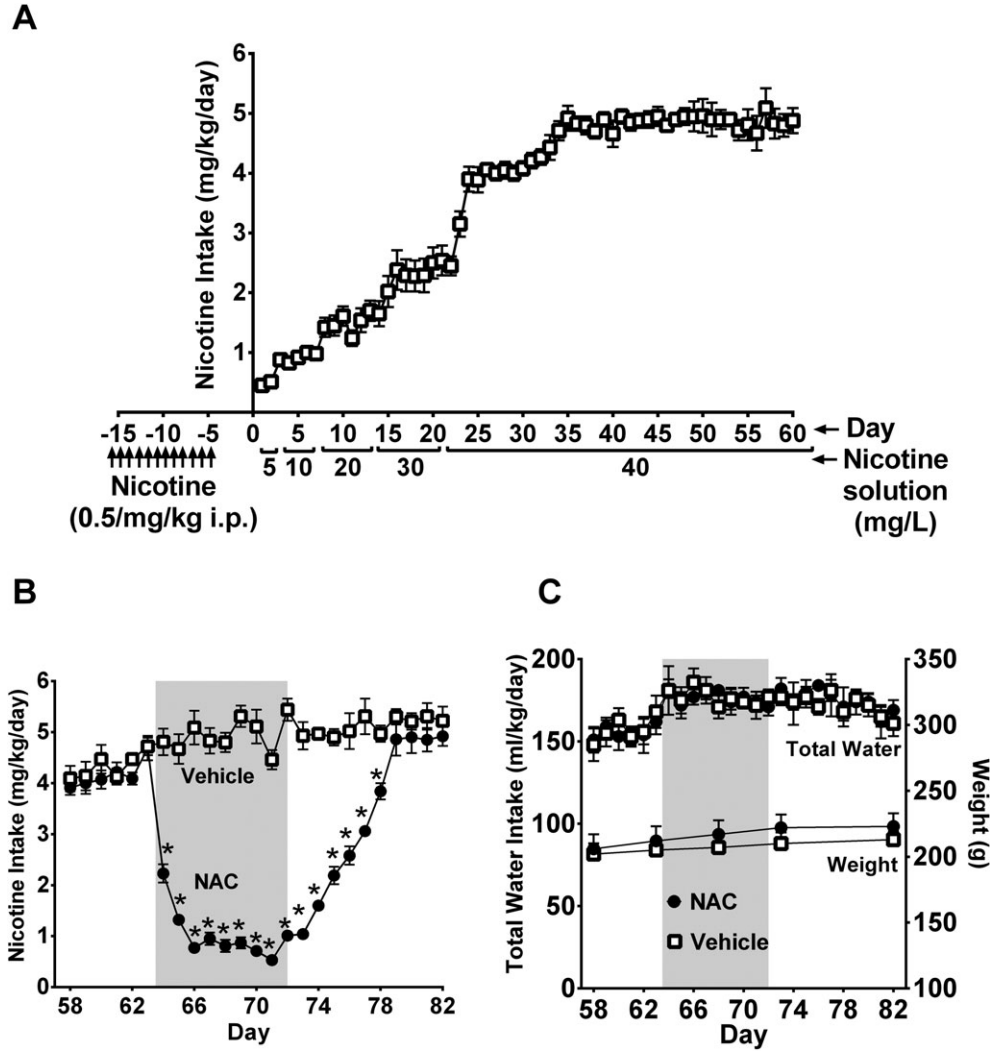

Fig. 6. Induction of voluntary oral nicotine consumption following the systemic administration of nicotine: inhibition of voluntary intake by oral *N*-acetylcysteine (NAC) administration. (**A**) Change in voluntary oral nicotine solution (5 to 40 mg/l) after the administration of daily doses of 0.5 mg of nicotine/kg/d administered i.p. for 14 days. Following 35 days under free choice of water or nicotine solution intake, animals reach a stable nicotine intake. (**B**) Following 62 days of voluntary nicotine intake, rats received either a daily oral dose of NAC (100 mg/kg, by oral gavage) or water during the next 9 days. Shaded area shows a 90% inhibition of nicotine intake (ANOVA, $p < 0.0001$) versus the corresponding control group achieved after the third daily administration of NAC. After the oral NAC (or water) administration was discontinued, the animals remained for the next 10 days under the nicotine and water choice condition (*$p < 0.01$). Total water intake (**C top**) and body weight (**C bottom**) of animals had consumed nicotine for 9 weeks and were orally administered a daily dose of 100 mg/kg NAC or vehicle for 9 days. Total water intake is expressed as ml of water + water contained in the nicotine solution consumed/kg body weight/d. Neither total water intake nor body weight of nicotine solution-consuming animals was affected by NAC administration (NS).

self-administration (Kareken et al., 2004). For nicotine, the cue upon place preference reinstatement after the injection of a small i.p dose of nicotine is akin to the presentation of an interoceptive cue, which leads to relapse-like motivational seeking ("wanting") for the place where the drug was originally received (Biala et al., 2009).

In vitro studies have shown that in oxidative stress conditions, the astrocyte removal of glutamate from the tripartite synapse is impaired due to sulfhydryl oxidation of cysteines, leading to the inactivation of the astrocyte (GLT-1) carrier protein (Sari, 2014; Trotti et al., 1998). This carrier inactivation is expected to also occur due to the increased oxidative stress following both chronic EtOH intake and chronic nicotine intake/administration.

In cocaine-cued relapse studies in animals, NAC administration has been shown to increase GLT-1 levels and to inhibit cocaine self-administration (Reissner et al., 2015). NAC administration was also reported to inhibit cued nicotine-seeking behavior (Ramirez-Niño et al., 2013), in line with the present studies. Increases in glial GLT-1 glutamate carrier by i.p administration of ceftriaxone (Rao and Sari, 2014; Sari et al., 2016) reduce nicotine and EtOH intake; additionally, GLT-1 increases by intravenous mesenchymal stem cell administration reduced alcohol intake and relapse (Ezquer et al., 2018).

The effectiveness of NAC in inhibiting nicotine intake for 6 days after discontinuing NAC administration is noteworthy, as the half-life of intravenously administered NAC is 5

to 6 hours (see Samuni et al., 2013). Samuni and colleagues (2013) postulated that cysteine generated from NAC can reduce disulfide (S-S) bonds in many proteins. This reaction might generate a long-acting reservoir (Protein-SH) that could become antioxidants by reacting with reactive oxygen species, thus recovering their original S-S structures. An extended inhibitory effect of NAC was also observed on chronic EtOH administration after NAC discontinuation (Quintanilla et al., 2016). Data in the present study show that NAC lowered the alcohol-elevated GSSG/GSH ratio observed 72 hours after discontinuing NAC administration. Further, plasma thiols remained elevated after such a discontinuation period, most likely in plasma proteins (but possibly throughout the body), suggesting the existence of a long-acting sulfhydryl reducing buffer system.

After the administration of a daily dose of oral NAC (100 mg/kg/d), the inhibition of both chronic voluntary EtOH intake and nicotine intake was maximal by the third to fourth daily administration. For the treatment of acetaminophen hepatotoxicity in humans, the Food and Drug Administration–approved dose of NAC is a loading dose of 140 mg/kg of body weight, with maintenance doses of 70 mg/kg that are repeated every 4 hours for a total of 17 doses (Smilkstein et al., 1988). A dose of 2,400 mg NAC/d was used for 50 days in a successful placebo-controlled clinical trial against cocaine relapse in drug abstinent patients (La Rowe et al., 2013).

In well-conducted studies, Lebourgeois and colleagues (2017) showed that an i.p. of NAC (100 mg/kg) administered 60 minutes before operant-cued relapse testing markedly inhibited EtOH relapse. In such a study, the NAC bolus may generate high levels of cysteine, which binds acetaldehyde, known to play a role in EtOH relapse-like drinking (Orrico et al., 2013; Tampier et al., 2013). In the present studies, the relapse-like EtOH intake in the ADE condition was studied 72 hours after the last oral administration of NAC, thus suggesting that different mechanisms of relapse inhibition are likely invoked in these 2 different designs.

It is noted that NAC has been used with some success in clinical conditions and experimental models in which there are increases in brain oxidative stress (Halliwell, 1989), namely Alzheimer's disease (Adair et al., 2001; Pocernich and Butterfield, 2012), stroke/ischemia (Uemura et al., 2018), and Parkinson's disease (Monti et al., 2016; Rahimmi et al., 2015). In line with preclinical work (Quintanilla et al., 2016), a recent clinical study showed that NAC treatment significantly reduced alcohol intake in patients who had also consumed cannabis (Squeglia et al., 2018).

Overall, data presented show strong similarities between the mechanisms that perpetuate chronic EtOH intake and nicotine self-administration: Both are inhibited by chronic administration of NAC, while the reduction in reactive oxygen species and neuroinflammation induced by NAC treatment are associated with both a reduction in EtOH intake–relapse and nicotine self-administration. Data suggest that the use of NAC may have translational value in the concomitant treatment of drug-related disorders generated by alcohol and tobacco.

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