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

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Background: A number of studies have shown that acetaldehyde synthesized in the brain is necessary to induce ethanol (EtOH) reinforcement in naïve animals (*acquisition phase*). However, after chronic intake is achieved (*maintenance phase*), EtOH intake becomes independent of acetaldehyde generation or its levels. Glutamate has been reported to be associated with the maintenance of chronic EtOH intake. The levels of brain extracellular glutamate are modulated by 2 glial processes: glutamate reabsorption via an Na^+-glutamate transporter (GLT1) and a cystine–glutamate exchanger. Chronic EtOH intake lowers GLT1 levels and increases extracellular glutamate. The administration of *N*-acetyl cysteine (NAC), a precursor of cystine, has been shown to reduce the relapse of several drugs of abuse, while NAC has not been tested on chronic EtOH intake or on EtOH's influence on the motivation for another drug. These were investigated in the present study.

Methods: (i) Rats bred for their high EtOH intake were allowed access to 10% EtOH and water up to 87 days. NAC was administered (30 and 60 mg/kg daily, intraperitoneally) for 14 consecutive days, either during the acquisition phase or the maintenance phase of EtOH drinking. (ii) In additional experiments, rats were allowed EtOH (10%) and water access for 61 days, after which EtOH was replaced by saccharin (0.3%) to determine both if chronic EtOH consumption influences saccharin intake and whether NAC modifies the post chronic EtOH saccharin intake.

Results: NAC did not influence the *acquisition* ("first hit") of chronic EtOH intake, but greatly inhibited (60 to 70%; $p < 0.0001$) EtOH intake when NAC was administered to animals that were consuming EtOH *chronically*. NAC did not influence saccharin intake in naïve animals. In animals that had consumed EtOH chronically and were thereafter offered a saccharin solution (0.3%), saccharin intake increased over 100% versus that of EtOH-untreated animals, an effect that was fully suppressed by NAC.

Conclusions: *N*-acetyl cysteine, a drug approved for use in humans, markedly reduces chronic EtOH intake and abolishes the increased intake of saccharin stimulated by chronic EtOH drinking.

Key Words: Voluntary Ethanol Drinking, *N*-Acetyl Cysteine Saccharin, Acquisition, Maintenance.

A NUMBER OF studies have shown that, in naïve animals, acetaldehyde synthesized in the brain (ventral tegmental area) is necessary to elicit ethanol (EtOH) reinforcement. The early development of reinforcement ("first hit") during the *acquisition* of voluntary EtOH intake or the operant EtOH self-administration can be blocked by: (i) inhibition of brain acetaldehyde generation (Aragon and Amit, 1992; Karahanian et al., 2011; Ledesma et al., 2014; Peana et al., 2015; Rotzinger et al., 1994; Tampier et al., 1995); (ii) increasing the degradation of brain acetaldehyde (Karahanian et al., 2015); or (iii) administration of acetaldehyde-trapping agents (Font et al., 2006; Orrico et al., 2013; Peana et al., 2015). However, on the *maintenance* phase, after a stable chronic EtOH intake or operant EtOH self-administration has already been reached, neither the inhibition of acetaldehyde generation (Peana et al., 2015; Quintanilla et al., 2012; Tampier et al., 2013), nor the increase in acetaldehyde degradation (Karahanian et al., 2015) or the administration of acetaldehyde-trapping agents (Peana et al., 2015) is able to reduce chronic EtOH intake. Thus, the chronic EtOH intake (*maintenance phase*) becomes *independent* of the early reinforcing mechanisms responsible for the "first hit" (Israel et al., 2015; Peana et al., 2015).

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Understanding the mechanisms that perpetuate chronic EtOH intake becomes clinically relevant.

The glutamate memory tone has been reported to be associated with the maintenance of chronic EtOH intake, as well as influencing the relapse of many drugs of abuse (McClure et al., 2014; Weiland et al., 2015). Animals consuming EtOH chronically present marked increases in extracellular glutamate levels in nucleus accumbens and the ventral tegmental area (Ding et al., 2012). These were associated with reduced levels of the Na^+-glutamate transporter (GLT1), which may constitute the mechanism responsible for an increased glutamatergic tone, the generation of a neurochemical memory trace of the rewarding circuits of the mesolimbic system (see Lüscher, 2013), and the maintenance of chronic EtOH intake (Chen et al., 2010; Sari et al., 2013b).

Sari and colleagues (2013b) determined EtOH intake in alcohol-preferring animals (P rats) that had consumed EtOH chronically for 5 weeks and had attained intakes of 7 g EtOH/kg/d. In these animals, the subsequent administration of ceftriaxone, a drug that up-regulates GLT1, resulted in a 60 to 70% reduction in voluntary EtOH intake. Das and colleagues (2015) confirmed a 2-fold elevation of extracellular glutamate in nucleus accumbens of rats exposed to EtOH chronically, and showed that ceftriaxone markedly inhibited chronic EtOH intake.

In mice, chronic EtOH vapor intoxication *plus* intermittent voluntary EtOH access lead to increases in EtOH intake compared with those of animals exposed to only intermittent EtOH access. The former group showed a 2-fold increase in glutamate levels in the nucleus accumbens versus those in animals exposed to only EtOH intermittent access (Griffin et al., 2014). The administration of a nonselective glutamate re-uptake antagonist (threo-β-benzyloxyaspartate) led to increases in EtOH consumption in both groups (Griffin et al., 2014).

Weiland and colleagues (2015) and Gass and colleagues (2011) trained rats to self-administer EtOH in an operant paradigm followed by extinction training and further exposure of the animals to the EtOH-associated cues (lights, sound, and odor). The cues induced an EtOH-seeking behavior (increased response of the previously active lever) (Gass et al., 2011; Weiland et al., 2015). This cue-induced reinstatement of EtOH-seeking was inhibited by ceftriaxone (Weiland et al., 2015). In this model, the animals *do not consume EtOH*, but are exposed to the memory of EtOH *effects* (and probably the neurochemical changes associated) during chronic operant drug self-administration. Thus, ceftriaxone disrupts the memory association by altering glutamate levels (Lüscher, 2013).

It is noted that the levels of extracellular glutamate in nucleus accumbens are regulated not only by the glial Na^+ gradient-dependent GLT1 but also by the exchange of cystine for glutamate via the cystine/glutamate exchanger (Baker et al., 2003; Herrera-Marschitz et al., 1996). As the cystine/glutamate exchanger is not energy dependent, the actual direction of the transport of cystine and glutamate will depend on the intra- and extracellular concentrations of both molecules (Baker et al., 2003; Warr et al., 1999). In cocaine dependence, the extracellular levels of glutamate in accumbens are greatly *reduced*, while *N*-acetyl cysteine (NAC) leads to a replenishment of extracellular glutamate (Baker et al., 2003; Moran et al., 2005). In animals that have consumed EtOH chronically, which present high extracellular levels of glutamate, the carrier may operate in a reverse action, which could be activated if intracellular cystine is elevated by NAC (i.e., to lower extracellular glutamate levels). However, neither the effect of NAC on early EtOH reinforcement (acquisition phase) nor during chronic EtOH intake (maintenance phase) has been studied. As NAC is an approved drug for clinical use, an examination of its effects on actual EtOH intake is of special interest.

Individuals presenting substance-use disorders often abuse drugs of a markedly different class than those that generated the initial reinforcement, for example, EtOH, nicotine, and caffeine (Hughes et al., 2000), and benzodiazepine and opiates (Browne et al., 1998). Alcoholics are known to display high preference for sweet substances (Kampov-Polevoy et al., 2004). Hauser and colleagues (2014) showed that a single cocaine administration increases both cued EtOH-seeking and relapse intake in animals that had learned to administer EtOH in operant conditions and were subjected to extinction sessions. While the study showed that cued EtOH-seeking and relapse drinking can be experimentally dissociated, it is clear that the neurochemical effects generated by one drug of abuse can contribute to the self-administration of another drug/reinforcer.

The aim of the present study is: (i) to determine if NAC administration can differentiate between the mechanisms that lead to early alcohol reinforcement ("first hit" or acquisition of voluntary EtOH intake) and those involved in the maintenance or perpetuation of chronic EtOH intake; (ii) to determine if the motivational/reinforcing properties of saccharin, another known rewarding substance, are increased after prolonged high levels of EtOH intake in line with a cross-reactivity for reinforcers and the possibility of other addiction liability in alcohol use disorders; and (iii) to determine if NAC differentiates between saccharin intake in naïve animals and in animals that have ingested alcohol chronically.

MATERIALS AND METHODS

Animals

Adult female Wistar-derived rats selectively bred as alcohol consumers (University of Chile Bibulous; UChB) (Mardones and Segovia-Riquelme, 1983; Quintanilla et al., 2006), weighing 180 to 280 g at the time of experiments, were used in the study. Two-month-old rats were housed in individual cages in a temperature- and humidity-controlled room under 12-hour light/dark cycle (lights off at 7 PM) for 1 week to acclimate them to the testing conditions. During this time, food and water were freely available ad libitum. All experimental procedures were conducted during the light phase. Animal experimental procedures were approved by the Ethics Committee

for Studies with Laboratory Animals at the Faculty of Medicine (Protocol CBA#0507, FMUCH) and by the Council for Science and Technology Research of Chile (CONICYT).

Drugs and Solutions

A 10% EtOH solution was obtained by dilution of EtOH (95%, Merck Darmstadt, Germany) with distilled water (10% v/v EtOH solution in 100 ml). Saccharin was prepared by dissolving 95% saccharin (Michelson Droguería, Santiago, Chile) in distilled water to a 0.3% (w/v) solution freshly prepared at least every 2 days. NAC (Sigma-Aldrich, St. Louis, MO) was prepared in saline, and injected at a volume of 5.0 ml/kg intraperitoneally (i.p.). The NAC doses used (30 and 60 mg/kg) were selected based on previous published studies (Baker et al., 2003; Ramirez-Niño et al., 2013).

Experimental Design

In a first series of experiments, the effect of repeated NAC administration on the acquisition and maintenance of voluntary EtOH drinking was studied.

Effect of NAC on the Acquisition of Voluntary EtOH Intake Using a 24-Hour Continuous Access Paradigm. In this experiment, 15 EtOH-naïve UChB rats were single-housed with food and water available ad libitum. After 1 week of acclimation, animals were exposed continuously (24 h/d) for 25 days to a choice between a 10% EtOH solution and water. On day 5 of free choice, rats were divided into 3 groups to evaluate the effect of 14 days of NAC administration on the early acquisition of voluntary EtOH intake. The control group received the saline vehicle (i.p., $n = 5$ rats); a second group was treated with 30 mg/kg NAC (i.p., $n = 5$ rats); and the third group received 60 mg/kg NAC (i.p., $n = 5$ rats) once daily for 14 days (days 5 to 18). Rats were allowed to self-administer 10% EtOH and water for 6 additional days after the 14 days of NAC/saline treatments.

Effect of NAC During the Acquisition of Voluntary EtOH Intake Using the 1-Hour Limited Access Paradigm. The plasma elimination half-life of NAC (approximately 34 minutes) (Zhou et al., 2015) might imply a short-lasting effect. Therefore, we tested whether NAC administered for 14 consecutive days was able to reduce EtOH intake under limited free-choice EtOH access conditions (1 hour a day). In this experiment, 10 naïve rats were used ($n = 5$ rats per group). Animals were offered limited access to 10% EtOH for 1 hour every day (from 2 to 3 PM), with food and water freely available for 23 consecutive days. Starting on day 5, the rats received saline vehicle (i.p., $n = 5$ rats, control group) or 60 mg/kg NAC (i.p., $n = 5$ rats), 2.5 hours before the onset of the 1-hour EtOH access period (Baker et al., 2003) once a day for 14 consecutive days (days 5 to 18). Rats were allowed to self-administer 10% EtOH for 1 hour every day for 4 additional days. EtOH consumption of each rat was expressed as g/kg weight/60 min.

Effect of NAC During the Maintenance of Voluntary EtOH Intake. Fifteen EtOH-naïve rats were given continuous (24 h/d) free-choice access to 10% EtOH and water for 87 days to evaluate the effect of NAC on the maintenance of voluntary EtOH intake. Starting on day 65, the rats received saline vehicle (i.p., $n = 5$ rats, control group), 30 mg/kg NAC (i.p., $n = 5$ rats), or 60 mg/kg NAC (i.p., $n = 5$ rats) once a day (2 PM) for 14 consecutive days (days 65 to 78). Rats were allowed to self-administer 10% EtOH and water for 9 additional days. Water and EtOH intake were read daily, immediately before the lights were turned off. EtOH consumption of each rat was expressed as grams per kilogram body weight per 24 hours.

Effects of NAC on Saccharin Intake

In a second series of experiments, the effect of NAC on the intake of saccharin, another known rewarding substance, was studied in naïve animals and in animals that had ingested alcohol chronically. Prior to studying the effect of NAC on saccharin intake, we determined if the motivational/reinforcing properties of saccharin were increased in animals that had previously ingested EtOH chronically. Thereafter, the amount of saccharin intake of both EtOH-naïve rats (control) versus that of rats with a previous history of chronic voluntary EtOH consumption was compared. The effect of NAC administration on saccharin consumption was further evaluated in new groups of animals.

Effect of Chronic EtOH Intake on Saccharin Consumption. Ten naïve rats were housed in individual cages under a 12-hour light/12-hour dark cycle and were given food and water available ad libitum. After 1 week of acclimation, rats were divided into 2 groups ($n = 5$ rats per group), a control group that had access to only water and an EtOH group that was given continuous free-choice access to both 10% EtOH and water for 2 months. On day 61, the EtOH bottle was removed and the 2 groups of rats were given continuous access to 0.3% saccharin solution and water for 30 days.

Effect of NAC on Saccharin Intake in EtOH-Naïve Rats. In this study, naïve rats were provided free choice of continuous access to a 0.3% saccharin solution and water for 24 days (Tampier and Quintanilla, 2005). On day 5 of free-choice saccharin intake, rats were divided into 2 groups to evaluate for 14 days the effect of NAC administration on saccharin intake. The control group received the saline vehicle (i.p., $n = 5$ rats) and the second group was treated with 60 mg/kg NAC (i.p., $n = 5$ rats), once a day for 14 days (days 5 to 18).

Effect of NAC on Saccharin Intake in Rats that Have Consumed Alcohol Chronically. For these experiments, rats ($n = 10$) that had received 60 consecutive days of continuous (24 h/d) free choice of 10% (v/v) EtOH versus water consumption were studied. On day 61, the 10% EtOH bottle was removed and the animals were exposed to the choice between 0.3% saccharin solution and water for 35 days. Following 15 days of saccharin intake, the effect of NAC administration was evaluated for 14 days in 2 groups of rats. One group received the saline vehicle (i.p., $n = 5$ rats) and a second group was treated with 60 mg/kg NAC (i.p., $n = 5$ rats), once daily.

Statistical Methods

Statistical analysis was performed using GraphPad Prism (San Diego, CA). Data are expressed as means ± SEM. Statistical differences are analyzed by a 2-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A level of $p < 0.05$ is considered for statistical significance.

RESULTS

NAC reduces voluntary EtOH consumption during the maintenance, but not during the acquisition, of voluntary EtOH drinking.

Effect of NAC During the Acquisition of Voluntary EtOH Intake Using the 24-Hour Continuous Access Paradigm

To determine whether a dose of 30 or 60 mg/kg of NAC administered for 14 consecutive days could affect the voluntary EtOH intake during the acquisition phase, treatment started when rats had been given only 5 days of continuous

(24 h/d) access to 10% EtOH and water, and consequently had not reached a steady-state level of EtOH intake.

Two-way ANOVA (treatment × day) of results for groups shown in Fig. 1*A* (saline, 30 or 60 mg/kg NAC, i.p.) revealed that there is no effect of NAC treatment on EtOH intake, $F_{\text{treatment}}$ (2, 252) = 2.708, $p = 0.06$, N.S., during the acquisition phase when a continuous (24 h/d) free-choice access paradigm was used. A significant effect of days on EtOH intake, F_{day} (20, 252) = 3.604, $p < 0.0001$, indicates that EtOH intake is increasing throughout the days of this acquisition phase.

Effect of NAC During the Acquisition of Voluntary EtOH Intake Using the 1-Hour Limited Access Paradigm

One possible explanation for the fact that NAC treatment did not alter the acquisition of voluntary EtOH intake on the 24-hour free-choice paradigm is the short plasma elimination half-life of NAC, reported to be of 34 minutes (Zhou et al., 2015). Therefore, we tested whether NAC (60 mg/kg/d, administered i.p. shortly before EtOH is presented) administered for 14 consecutive days was able to reduce the amount of EtOH consumed during 1 h/d under such limited EtOH access condition.

The effect of NAC administered for 14 consecutive days compared with saline on the EtOH intake under a 1-hour limited access is shown in Fig. S1. A 2-way ANOVA (treatment × day) of data indicated that NAC treatment (60 mg/kg/d, i.p., administered from days 5 to 18) did not significantly alter the overall acquisition of voluntary EtOH intake, $F_{\text{treatment}}$ (1, 176) = 2.746, $p = 0.099$, but intake increased with time, F_{day} (21, 176) = 2.884, $p < 0.0001$, indicating that the EtOH consumption of all groups was augmented across the acquisition days.

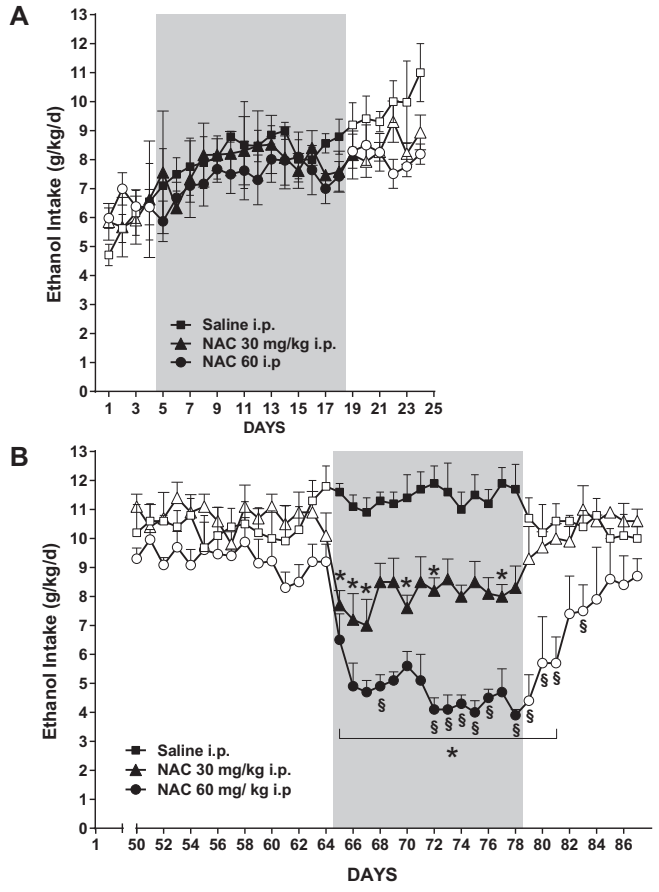

Fig. 1. (**A**) Chronic *N*-acetyl cysteine (NAC) administration (30 or 60 mg/kg, intraperitoneally [i.p.]) during the acquisition phase did not reduce ethanol (EtOH) intake, but given during the maintenance phase (**B**) markedly reduced EtOH intake. Three groups of rats under continuous access to 10% EtOH and water were treated with either saline (black square), 30 mg/kg NAC (black triangles), or 60 mg/kg NAC (black circles) from days 5 to 18 (acquisition phase) of EtOH intake (**A**). Different groups of rats under continuous access to 10% EtOH and water were treated with either saline (black square), 30 mg/kg NAC (black triangles), or 60 mg/kg NAC (black circles) from days 65 to 78 (maintenance phase) of EtOH intake (**B**). Data are means ± SEM of daily EtOH intake. Asterisk symbol (*) $p < 0.05$, indicates that the EtOH intake is significantly lower than that of the saline control group of the same day. (§) symbol $p < 0.001$, indicates that the EtOH intake of NAC 60 mg/kg group is lower than that of NAC 30 mg/kg group.

Effect of NAC During the Maintenance of Voluntary EtOH Intake

To determine in a new group of rats whether a dose of 30 or 60 mg/kg of NAC administered for 14 consecutive days (from days 65 to 78) could affect the EtOH intake during the maintenance phase, NAC treatment started when rats had reached a steady-state level of EtOH intake, of the order of 11 g EtOH/kg/d.

Two-way ANOVA (treatment × day) of saline, 30 and 60 mg/kg NAC group data in Fig. 1*B* show a marked effect of NAC treatment on EtOH intake compared with saline during the maintenance phase, $F_{\text{treatment}}$ (2, 456) = 272.3, $p < 0.0001$; in addition, significant treatment × days interaction was found, $F(74, 456) = 3.993$ $p < 0.0001$. When both NAC doses were compared, it was found that the effects of both doses were significantly different, $F_{\text{treatment}}$ (1, 456) = 243.9, $p < 0.0001$. Bonferroni's post hoc test revealed that 30 mg/kg NAC (Fig. 1*B*, black triangles) significantly decreased EtOH intake during 6 (65 to 67, 70, 72, and 77) of the 14 treatment days, while the 60 mg/kg dose (Fig. 1*B*, black circles) decreased EtOH intake on each of the 14 treatment days compared with the saline group ($p < 0.01$). The magnitude of reduction compared with the saline-treated rats and calculated over the entire treatment period averaged approximately 25 to 30% in the 30 mg/kg and 60 to 70% in the 60 mg/kg NAC rats. The effect of treatment with 60 mg/kg NAC was long lasting, as the EtOH intake remained significantly reduced ($p < 0.01$) for 4 days after the treatment ended (Fig. 1*B*, empty circles). It is noteworthy that the half-life of NAC in the circulation is less than 1 hour, thus indicating that chronic NAC administration generates a neurochemical effect that extends beyond the molecule's half-life. NAC treatment during 14 consecutive days did not reduce body weight (means ± SEM, $n = 5$; 254 ± 14 g, the first treatment day, vs. 267 ± 11 g, the last day of treatment with 60 mg/kg NAC), suggesting its safety and tolerability. The NAC-induced reduction in the volume of 10% EtOH

consumed was fully compensated by an increase in water intake, thus maintaining the animal's total fluid intake (water + 10% EtOH) homeostasis (data not shown).

Subsequent studies were conducted to determine whether chronic EtOH intake increases saccharin intake and if NAC is able to differentiate between saccharin intake in naïve animals and in animals that have ingested alcohol chronically.

Effect of Chronic EtOH Intake on Saccharin Consumption

A 2-way ANOVA of saccharin intake (Fig. 2*A*) in naïve rats versus that of rats preexposed to continuous voluntary EtOH consumption for 2 months and thereafter offered a saccharin solution (0.3%) indicated a significant elevation of saccharin intake due to previous EtOH consumption, $F_{\text{treatment}}$ (1, 240) = 431.3, $p < 0.0001$. Post hoc analysis revealed that saccharin intake of rats preexposed to chronic voluntary EtOH was significantly higher than that of EtOH-naïve rats from days 4 to 30 ($p < 0.01$). The enhanced saccharin intake suggests a potentiation/sensitization between 2 reinforcers.

Effect of NAC on Saccharin Intake in EtOH-Naïve Rats

A 2-way ANOVA (treatment × day) of data in Fig. 2*B* (saline, or 60 mg/kg NAC, i.p.) revealed that there was no effect of NAC treatment on saccharin intake in naïve animals, $F_{\text{treatment}}$ (1, 192) = 0.79, $p = 0.37$, N.S.

Effect of NAC on Saccharin Intake in Rats that Have Ingested Alcohol Chronically

A 2-way ANOVA (treatment × day) of data shown in Fig. 2*C* shows a significant inhibition of saccharin intake in rats that had ingested EtOH chronically and were treated with NAC (60 mg/kg, i.p.), $F_{\text{treatment}}$ (1, 280) = 198.7, $p < 0.0001$; in addition, significant treatment × day interaction was found, $F(34, 280) = 3.989$, $p < 0.0001$; Bonferroni's post hoc test revealed that 60 mg/kg NAC significantly decreased saccharin intake during all days of treatment. The magnitude of the reduction compared to the saline-treated rats and calculated over the entire treatment period was 55 to 60% in NAC rats. It is noteworthy that the intake of saccharin after NAC administration was virtually identical to that of EtOH-naïve rats (dotted line in Fig. 2*C*).

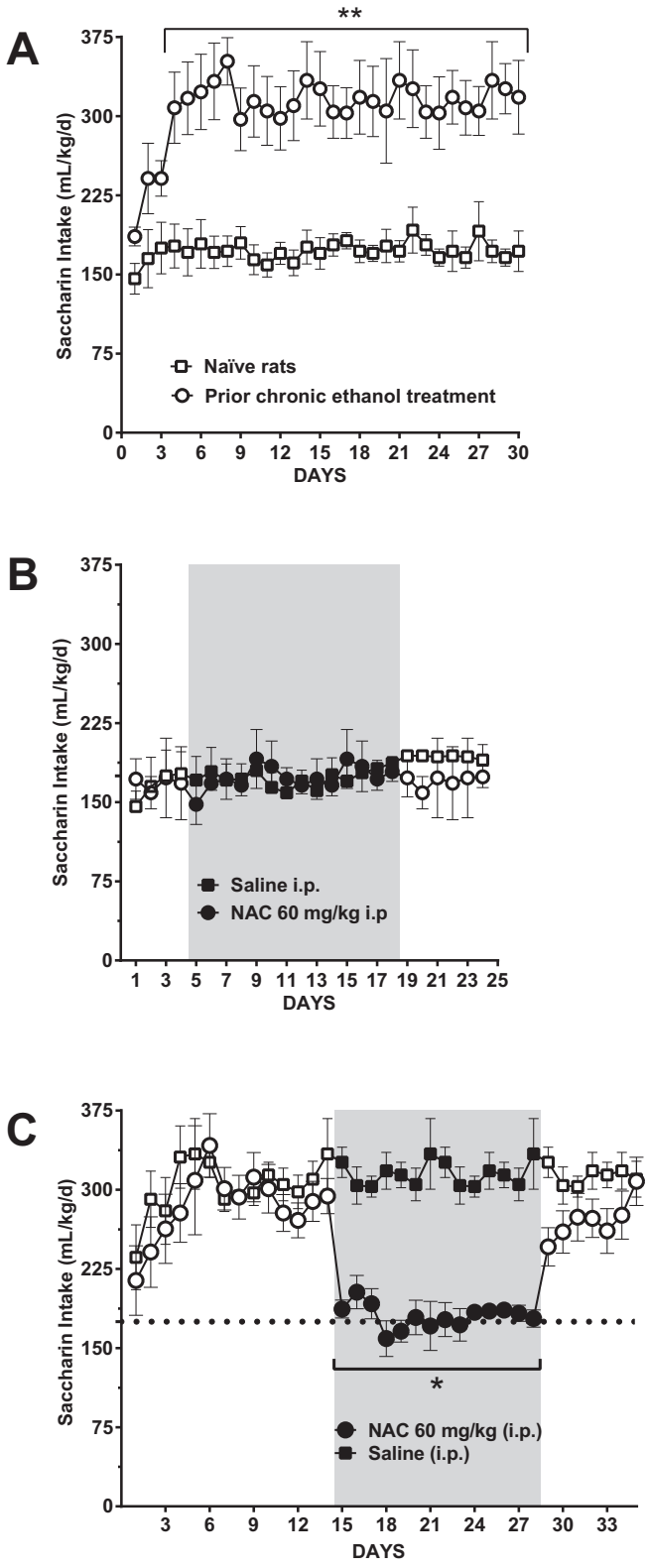

Fig. 2. (**A**) Prior chronic ethanol (EtOH) intake increases saccharin intake. (**B**) Daily *N*-acetyl cysteine (NAC) administration (60 mg/kg, intraperitoneally [i.p.]) did not reduce saccharin intake in EtOH-naïve rats, but (**C**) suppressed the increased intake of saccharin stimulated by chronic EtOH intake. (**A**) Before the presentation of saccharin solutions, rats were given access to either water (empty squares) or free choice between 10% EtOH and water (empty circles) for 60 days. $^{**}p < 0.001$. (**B**) Different groups of EtOH-naïve rats given continuous access to 0.3% (w/v) saccharin and water were treated with either saline (black square) or 60 mg/kg NAC (black circles) from days 5 to 18 of saccharin intake. (**C**) Two new groups of rats were allowed continuous access to either water or free choice between 10% EtOH and water, after which on day 61 (day zero in Fig. 2), the EtOH bottle was removed and exchanged for saccharin (0.3%) (black squares), or saccharin plus daily 60 mg/kg NAC (black circles) on days 15 to 28 of saccharin intake. Data are means ± SEM of daily saccharin intake. Asterisk symbol (*) $p < 0.05$, indicates that the saccharin intake is significantly lower than that of the saline control group of the same day. The dotted line in (**C**) indicates the saccharin intake of EtOH-naïve rats in (**B**).

DISCUSSION

Data presented indicate that NAC does not affect EtOH volition in rats during the acquisition period of EtOH intake, but markedly inhibits EtOH intake of rats that had attained high levels of EtOH consumption on a chronic maintenance schedule. This effect showed no tolerance development.

The different effects of NAC during the maintenance and acquisition of EtOH intake are consistent with studies reporting the effect of NAC on cocaine self-administration. Chronic NAC does not affect cocaine self-administration under a short-access condition (2 h/d) (Amen et al., 2011; Madayag et al., 2007), but does reduce and block escalation of intake in an extended 6 h/d access condition (Kau et al., 2008; Madayag et al., 2007). These findings are in agreement with those of Sari and colleagues (2013a), who found that ceftriaxone, which also restores glutamate homeostasis, exerts more profound effects in reducing voluntary EtOH intake during the maintenance than during the acquisition phase. Reduction in EtOH intake by ceftriaxone was paralleled by an up-regulation of GLT1 protein levels (Sari et al., 2013b). Supporting our findings that NAC reduces EtOH intake during the maintenance, but not during the acquisition, of EtOH intake, Baker and colleagues (2003) showed that NAC failed to increase the cystine/glutamate exchanger in drug-naïve control, indicating that NAC selectively alters extracellular glutamate in pathological states.

Peana and colleagues (2014) showed marked increases of the cystine/glutamate exchanger in nucleus accumbens of rats under forced chronic EtOH intake (reaching EtOH intakes of 6 to 14 g/EtOH/kg/d, in line with the voluntary intakes of rats in the present study). Such an increase in the cystine/glutamate exchanger may constitute a compensatory mechanism, although not efficient under normal or low cystine levels, to lower extracellular glutamate levels to normal. Indeed, animals that consume these high EtOH levels show marked increases in extracellular glutamate (Das et al., 2015; Ding et al., 2012). However, the exogenous administration of the cysteine/cystine precursor NAC could potentiate an increased cystine/glutamate exchanger level, which might return extracellular glutamate levels to normal. Further studies should be conducted to test this possibility.

Data also show that chronic EtOH consumption markedly increases saccharin volition, which is abolished by NAC administration, while NAC does not alter the intake of saccharin of EtOH-naïve rats. Taken together, these studies indicate that *different* neurochemical mechanisms account for the initiation of reinforcement (acquisition) versus the perpetuation (maintenance) of drug intake. Also, that some common mechanisms appear to transfer the sensitivity from one reinforcer to another reinforcer.

In most studies on drugs other than EtOH, NAC was administered to regulate the levels of extracellular glutamate and to reduce drug-seeking behavior (Baker et al., 2003; Corbit et al., 2014; see McClure et al., 2014). In clinical studies, LaRowe and colleagues (2013) showed that the daily administration of 2,400 mg of NAC to abstinent cocaine users reduced cocaine relapse by 80% for 50 days. It has been shown that administration of NAC reverses the lowering of GLT1 induced by cocaine in the nucleus accumbens of rats (Ducret et al., 2015; Knackstedt et al., 2010).

Weiland and colleagues (2015) reported that NAC did not inhibit cued EtOH-seeking in rats that had learned to self-administer EtOH in an operant paradigm and were subjected to at least 10 daily extinction sessions, during which EtOH was removed. However, in these studies, NAC (30 to 60 mg/kg) was given primarily during the extinction-training sessions, while only 1 dose of NAC was administered 2 hours prior to a single 45-minute cued EtOH-seeking test. In the present study, NAC (60 mg/kg) was administered 5 hours before determining its first effect on EtOH intake, while its maximum effect was seen following daily NAC administration for 14 days (see Fig. 1*B*). An additional difference is that, in the present study, the animals were not EtOH deprived as in the study of Weiland and colleagues (2015).

It is of interest that, in the present study, the administration of NAC (30 or 60 mg/kg) *did not inhibit* the acquisition of chronic EtOH intake, while in the studies of Peana and colleagues (2010), the administration of cysteine (20 to 40 mg/kg) blocked the acquisition of oral EtOH self-administration on an operant paradigm. The acetaldehyde-binding ability of cysteine is probably responsible for the inhibition of acquisition of EtOH intake. Indeed Peana and colleagues (2015) have reported that penicillamine, a synthetic amino acid that strongly binds acetaldehyde, inhibits the acquisition of oral EtOH self-administration in the same model. It is possible that the administration of the prodrug NAC in the present study may result in lower levels of brain cysteine/cystine than those obtained after the direct administration of cysteine. However, cysteine (40 mg/kg), which fully blocked the *acquisition* of EtOH self-administration, had no effect on the *maintenance* of EtOH self-administration (Peana et al., 2010). Doses of 60 to 100 mg cysteine were required to marginally reduce EtOH intake (Peana et al., 2010). Overall, the studies support the idea that NAC and cysteine act by different mechanisms on the acquisition and maintenance of EtOH intake on the experimental paradigms used in these studies.

It is noted that the maintenance phase in the present study (chronic EtOH intake without deprivation) differs from EtOH intake following a deprivation condition (the "alcohol deprivation effect" termed ADE) where cysteine inhibits the postdeprivation reinstatement condition (Peana et al., 2013). It has been shown that brain acetaldehyde is again required to elicit the ADE condition (Karahanian et al., 2015; Tampier et al., 2013). Furthermore, the synthetic amino acid penicillamine, which strongly binds acetaldehyde, significantly inhibits ADE (Orrico et al., 2013).

A finding that deserves special mention is that a general mechanism appears to exist that leads a drug-experienced

animal to consume a drug that is of a different class from the drug that initiated the reinforcement, suggesting codependency due to common neurocircuits (see Hauser et al., 2014; Pastor et al., 2010). Pastor and colleagues (2010) showed in mice that sensitization to EtOH consumption led to an increased preference for sucrose. Present studies further indicate that following chronic intake, not only one reinforcing molecule can be replaced by a second reinforcing molecule, but that the motivational/reinforcing effect of the second drug is *sensitized* by the first drug, suggesting that drug-abuse disorders may have common mechanisms of action that might be tackled in concert.

It can be asked whether the withdrawal reaction from the first drug of abuse explains the marked sensitization for a second one. This is unlikely, as Tampier and Quintanilla (2009) described that chronic EtOH consumption increases saccharin intake while the animals *continue drinking* EtOH at high levels. In addition, UChB animals develop blood EtOH levels of only 20 to 50 mg/dl (mean of 32 mg/dl) in the dark period of the circadian cycle (M. Rivera-Meza and M.E. Quintanilla, personal communication) and do not show behavioral changes when EtOH administration is discontinued.

Overall, data presented indicate that (i) NAC does not affect EtOH volition in rats during the acquisition period of EtOH intake, but it markedly inhibits EtOH intake of rats that had attained high levels of EtOH consumption on a chronic maintenance schedule; (ii) support the view that different neurochemical mechanisms exist to account for the initiation of reinforcement versus the perpetuation of drug intake; (iii) the latter probably having a common mechanism for different reinforcers; and (iv) NAC may be considered as an adjunct in the treatment of chronic alcohol use disorders.

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AUTHORS' CONTRIBUTION

MEQ and YI designed the study. MR-M, PB-C, CS-L, and MH-M contributed to interpretation and presentation of the data. YI and MEQ wrote the manuscript. All authors critically reviewed the content and approved the final version for publication.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Chronic NAC administration (60 mg/kg, i.p.) given during the acquisition phase of voluntary EtOH intake using the 1-hour limited access paradigm did not reduce EtOH intake.