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Evaluation of N-acetylcysteine on ethanol self-administration in ethanol-dependent rats

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Running title: NAC reduces ethanol seeking and relapse in dependent rats

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Introduction

Alcohol consumption is one of the major avoidable risk factors for chronic disease, injury and premature death, with an estimated 3.8% of all global deaths and 4.6% of global disability-adjusted life-years attributable to alcohol worldwide (Rehm et al., 2009). Among alcohol-induced pathologies, Alcohol Use Disorder (AUD) is a chronic disorder characterized by the compulsive use of alcohol, loss of control over intake and development of a negative emotional state when alcohol is unavailable (Koob, 2013, 2015). Given the high relapse rate when patients attempt abstinence and that not all patients respond to a specific treatment, research of new-treatments against relapse appears highly necessary especially in the context of future personalized therapies.

N-acetylcysteine (NAC) is a cystine prodrug that activates the cystine-glutamate exchanger, the system antiport $\text{xc}^-$, that maintains extrasynaptic glutamate concentrations (Millea 2009; Lewerenz et al. 2013). Numerous studies have shown that NAC decreases the craving for cocaine in both rodents and humans, via its stimulating action of the $\text{xc}^-$ antiport system (LaRowe et al., 2007, Kau et al., 2008, Amen et al., 2011, Kupchik et al., 2012, Brown et al., 2013, LaRowe et al., 2013, Ducret et al., 2016).

This effect was not only observed for cocaine but also for other substances. In rat, NAC decreases the relapse to nicotine self-administration (Ramirez-Nino et al., 2013, Moro et al., 2016). In humans, NAC is also promising since it has been shown to reduce craving for cannabis (Gray et al., 2012) and nicotine (Froeliger et al., 2015, Prado et al., 2015). NAC was accordingly proposed as an interesting therapeutic option in the treatment of addiction (Kalivas and Volkow, 2011, Asevedo et al., 2014).
Regarding ethanol, chronic NAC treatment inhibited ethanol intake in rats bred for their high ethanol intake when NAC was administered to animals displaying chronic ethanol consumption (Quintanilla et al., 2016). In a recent study (Lebourgeois et al., 2017), we demonstrated that a high dose (100 mg/kg) of NAC reduced ethanol consumption, motivation, seeking and relapse in rats self-administering 1.2 g/kg of 20% ethanol for 15 minutes per day and during several weeks, a model referred to as binge-drinking in rats (Jeanblanc et al., 2018). In human, NAC reduced the amount and frequency of alcohol use among participants with cannabis use disorders (Squeglia et al., 2018), and decreased subjective craving score in veterans with Posttraumatic Stress Disorders with co-occurring Substance Use Disorders (Back et al., 2016).

Here we investigated the efficacy of NAC in ethanol-dependent rats exposed for several weeks to repeated ethanol intoxication-withdrawal cycles using chronic intermittent ethanol vapor inhalation (CIE) (Aufrere et al., 1997, Gilpin et al., 2008, Houchi et al., 2013, Simon-O'Brien et al., 2014, Alaux-Cantin et al., 2015, Meinhardt and Sommer, 2015). This paradigm is highly relevant as a model of post-dependent state because of the induction of both behavioral (loss of control over intake, high motivation for intake, high level of extinction responding in the absence of alcohol, anxiety-like behavior, depression, compulsive use) and physical signs (tolerance, tremor, convulsive crisis, hyperactivity) (Vendruscolo and Roberts, 2014, Meinhardt and Sommer, 2015). In particular, acute withdrawal from CIE produces an increase in negative affect symptoms, which could facilitate excessive drinking (Somkuwar et al., 2017, Tunstall et al., 2017).

The glutamate release is enhanced in nucleus accumbens during cocaine (McFarland et al., 2003) nicotine (Gipson et al., 2013), opiates (LaLumiere and Kalivas 2008) and ethanol seeking (Gass et al., 2011). NAC is thought to reduce craving through the normalization of glutamatergic homeostasis, by the mean of an increase of negative feedback produced by
extrasynaptic glutamate on perisynaptic mGluR2/3 autoreceptors (Scofield et al., 2016). So we also tested the effect of NAC on the expression of xCT, the catalytic subunit of the $x_c^-$ system, and in the expression of GLT-1, the transporter that re-uptake glutamate from the extracellular fluids, in dependent and in nondependent (ethanol-naïve) rats.

In this study, we demonstrated that a low dose of NAC significantly reduced ethanol consumption, motivation to ethanol, and extinction responding (i.e. seeking) during acute withdrawal (8 hours) from CIE, while dependent rats showed a reduced expression of xCT (the specific subunit of $x_c^-$ system) in the nucleus accumbens. NAC reduced the expression of GLT-1 with the same efficiency in dependent and nondependent rats. Since NAC was effective in dependent rats (25-50 mg/kg) at a lower dose than in binge-drinking rats (100 mg/kg), our results suggest that neuroadaptations underlying ethanol dependence may make rats respondent to lower doses. We also demonstrated that NAC reduces reacquisition of ethanol self-administration following protracted abstinence (1 month) in dependent rats. Finally, NAC (100 mg/kg) reduced 1% sucrose self-administration demonstrating the NAC efficacy on non-drug reward.

Methods and Materials

Animals:

Male Wistar rats, weighing 260-280g at the beginning of the experiment, were purchased from Janvier labs (Le Genest Saint Isle, France). Rats were individually housed in a controlled environment under a 12-h light/dark cycle (lights on at 8 a.m.) with food and water available ad libitum. All experiments were performed in conformity with the European Community guiding principles for the care and use of animals (2010/63/UE, CE Off. J. 20 October 2010), the French decree n° 2013-118 (French Republic Off. J., 2013) and approved
by the local ethics committee [Comité Régional d'Ethique en Matière d'Expérimentation Animale de Picardie (CREMEAP), University of Picardie Jules Verne].

Drugs:

For self-administration experiments, ethanol (VWR, Strasbourg, France) was diluted in tap water at the final concentration of 10 or 20% (v/v) and sucrose (VWR, Strasbourg, France) was diluted in tap water at the final concentration of 1% (w/v). For intraperitoneal (i.p.) injections, N-acetylcysteine (Sigma Aldrich, Saint Quentin Fallavier, France) was dissolved in sterile saline (0.9 % NaCl) and administered at the doses of 25, 50 and 100 mg/kg chosen accordingly to the literature, after pH adjustment to pH 7.4 (Kau et al., 2008, Reichel et al., 2011, Lebourgeois et al., 2017), 60 min before the start of self-administration sessions.

Behavioral acquisition of the self-administration task

The self-administration behavior was induced by a three-step paradigm described in details in Supplementary Materials and Methods. First, rats were exposed to intermittent access to 20% ethanol for 2 weeks to facilitate the acquisition of a high level of ethanol intake (Wise, 1973). Thereafter, rats were trained to self-administer ethanol during 30-min access operant sessions. Once rats reached a stable level of intake, they were placed in inhalation chambers to induce dependence. The effect of NAC was recorded during acute withdrawal (8-hour period after inhalation chambers were turned off). This timing was chosen according to a previous report showing that repeated withdrawal produced an increase in extracellular glutamate in hippocampus after 6-7 hours withdrawal, i.e. 6-7 hours after inhalation chambers were turned off (Dahchour and De Witte, 2003).
Behavioral testing: Effect of NAC on ethanol self-administration:

The timeline of the experimental procedures is given in figure 1 (Fig. 1).

Effect of NAC on ethanol self-administration during acute withdrawal:

Once rats reached a stable ethanol self-administration level, the effect of an acute injection of NAC (0, 25, 50 or 100 mg/kg; i.p., 60 min before the test) was tested on the level of ethanol 20% self-administration during a 30-min session, 8 hours after retrieval from the inhalation chambers. The doses and timing of injection doses were chosen depending on previous work (Baker et al., 2003, Moussawi et al., 2009, Amen et al., 2011, Reichel et al., 2011). Injections were performed according to a Latin square counterbalanced design with 1 day of wash-out between each injection.

Effect of NAC on the reinforcing properties of ethanol during acute withdrawal:

After a 2 week wash-out period were rats were submitted to five-day–a-week sessions of ethanol self-administration at 8-hours withdrawal, a progressive ratio (PR) schedule test was performed to evaluate the effect of NAC (0 or 25mg/kg) on the motivation to consume ethanol (i.e. its reinforcing properties). As the 25 mg/kg dose was the effective dose in the previous test, we choose this dose in the PR schedule test. In this test, the effort necessary to obtain one reward (i.e. the number of presses on the active lever) was continuously increased after each reward delivery (3, 4, 5, 7, 9, 12, 15, 18, 20, 23, 25, 28, 30, 33, 35). During the 30-min session, the maximum effort (breakpoint) produced to receive a single reward of ethanol was measured and reflected an index of motivation.

Effect of NAC on ethanol seeking: test of extinction responding

In order to evaluate the effect of NAC on ethanol-seeking behavior, we performed a test in a single session of extinction. During this test, NAC was injected i.p. 60 min before a
30-min session test during which the light cue remained off and no alcohol was available. The persistence of drug-seeking behavior in the absence of the drug (i.e., craving) was assessed by recording the number of active lever presses. After a 2 weeks wash-out period where the rats were submitted to five-day-a-week sessions of ethanol self-administration, we performed a first test with the 25 mg/kg dose of NAC, followed by a second 2 weeks-wash out period and a second test at the 50 mg/kg dose.

**Effect of NAC on reacquisition after protracted abstinence:**

After a 2 weeks-wash out period, rats were exposed to one month of abstinence, during which rats were housed in the home cage and had no access to ethanol vapor chambers and self-administration boxes. After this abstinence period, we used the reacquisition model of relapse (Alaux-Cantin et al., 2013, Simon-O'Brien et al., 2014), which consists of the re-introduction of ethanol in the operant self-administration chambers. Briefly, a priming delivery of ethanol (0.1 ml of a 20% ethanol solution) was given, non-contingently, at the beginning of the re-acquisition session to provide an olfactory and gustatory cue. Ethanol was then available on an FR3 schedule for the duration of the 30-min session, and NAC or saline was injected i.p. 60 min before the beginning of the behavioral test. The experiment was preformed twice, first with the NAC 25 mg/kg and then with the 50 mg/kg dose.

**Behavioral testing: Effect of NAC on 1% sucrose self-administration in nondependent rats**

In a separate cohort of 14 Long Evans rats, the effect of NAC (100 mg/kg) was tested on 1% sucrose self-administration, after eight weeks of 20% ethanol self-administration during 15-min session (as described in Lebourgeois et al., 2017). It is noteworthy that rats were not dependent, since they were not exposed to the CIE.
Western-blotting assays

The effect of NAC on xCT and GLT1 proteins expression was evaluated in dependent rats exposed to 10 weeks of CIE following an 8-hour withdrawal period and in rats kept in the same conditions, but naïve to ethanol. NAC was administered i.p. one hour before euthanasia (n=6 rats per groups, 4 groups). After anaesthesia and decapitation, the nucleus accumbens was dissected in 4°C PBS. The details for western blot procedures are provided in Supplementary Materials and Methods.

Statistical analysis:

All results are expressed as mean ± standard error of the mean. For behavioral data, statistical differences were assessed using one- or two-way ANOVAs with or without repeated measures (RM). Significant main effects detected by ANOVAs were followed by a Tukey post-hoc test. For simple comparisons, data were analyzed with a Student’s t-test. For Western blotting data, the integrated optical density for xCT and GLT-1 was divided by the density of actin immunoreactivity within the same sample. This yielded a “ratio” for each rat and these values were compared between groups using two-way ANOVAs. Significant main effects detected by ANOVAs were followed by a Tukey post-hoc test. All statistical analyses were performed using the SigmaPlot software (version 11; Systat software, Inc., San Jose, CA USA) and a $p < 0.05$ was considered significant.

Results:

N-acetylcysteine reduced ethanol self-administration:
The effect of an acute injection of NAC (0, 25, 50 or 100 mg/kg; i.p.; 1h before the test) on mean numbers of active lever presses in the ethanol self-administration procedure was analyzed using a one way RM-ANOVA, which revealed a significant main effect of treatment \( (F[(3,48)=16.334, p<0.001) \). Subsequent post-hoc Tukey analyses showed that total active lever presses were significantly lower for the NAC 25 mg/kg group compared with the saline group (-58%). Similarly, total active lever presses were significantly lower for the 50 and 100 mg/kg doses of NAC compared with saline (respectively -48 et -95%) (Fig. 2A). Inactive lever presses were not statistically different between groups: \( F(3, 48)=1.153, p=0.337 \) (Fig. 2C). Analogous effects were observed regarding ethanol intake (Fig. 2B). Rats treated with 25, 50 or 100 mg/kg dose consumed significantly less ethanol than saline-treated rats (respectively: 64; 49 and 95% of inhibition) (treatment main effect, \( F[(3,48)=14.116, p<0.001] \)). Together, these data indicate that an acute injection of NAC at the doses of 25, 50 or 100 mg/kg, reduced ethanol self-administration.

**N-acetylcysteine was more effective in ethanol-dependent rats:**

Assuming that dependent animals have robust modifications of their glutamate homeostasis, and that NAC acts by restoring glutamatergic homeostasis, we next intended to test the hypothesis that NAC could be more effective in ethanol-dependent animals compared with those that are not dependent. To test this hypothesis, we compared our results presented here with data found in nondependent animals self-administering chronically large amount of ethanol during several weeks (Lebourgeois et al., 2017). Fig. 2D represents the percentage of baseline EtOH consumption in dependent and nondependent groups for 3 doses of N-acetylcysteine (25, 50 or 100 mg/kg; i.p., 1h before the self-administration test). Data were analyzed using a two-way ANOVA, which revealed a significant main effect of dependence \( (F[(1,90)=23.035, p<0.001]) \) and a significant main effect of treatment \( (F[(2,90)=8.049, p<0.001]) \).
Subsequent pairwise analyses (Tukey test) showed that NAC produced a greater relative reduction in ethanol consumption in dependent animals at all doses tested.

N-acetylcysteine decreased the reinforcing efficacy of ethanol

To further assess the effect of NAC (25 mg/kg) on the motivation to consume ethanol, we performed a PR test. The number of active lever presses and the breakpoint values during the 30-min session are depicted in Fig. 3A and 3B. Statistical analyses using Student t-tests revealed that NAC-treated rats pressed significantly fewer times during the PR session (-55%; p<0.05) and displayed a significantly lower breakpoint value compared with saline-treated rats (-43%; p<0.01). Inactive lever presses were statistically not different between groups (p=0.591) (Fig. 3C).

N-acetylcysteine reduced extinction responding (ethanol seeking)

Next, we assessed the effect of an acute injection of NAC (25 and 50 mg/kg; i.p., 1h before the test) on ethanol seeking during extinction. In this test, rats were placed in ethanol self-administration chambers and each press on the active lever was counted, but had no consequences (neither ethanol nor light cue). We performed two separate experiments at 2 weeks interval with the 25 (Fig. 4A and 4C) and 50 mg/kg dose (Fig 4B and 4D). The total active lever presses were analyzed using a Student t-test. NAC 25 mg/kg did not produce any alteration of extinction responding (p=0.59) while the 50 mg/kg dose of NAC significantly reduced by 92% the persistence of drug-seeking behavior in the absence of alcohol (p=0.0004). However, the total number of inactive lever presses was not significantly different between groups.
N-acetylcysteine limited reacquisition after abstinence:

Addiction is a highly relapsing disorder, so we next examined the effect of an acute injection of NAC (25 and 50 mg/kg; i.p., 1h before the test) on relapse after 1 month of protracted abstinence. We performed two separate experiments at 1.5 months interval with the 25 (Fig. 5A, C, E) and 50 mg/kg dose (Fig. 5B, D and F). NAC 25 mg/kg did not produce any alteration in the total active lever presses (p=0.35) (Fig. 5A), while the NAC 50 mg/kg decreased significantly the total active lever presses compared with saline group (p=0.037) (i.e., a reduction of 55%) (Fig. 5B). As expected, analogous effects were observed regarding ethanol intake. Rats treated with 50 mg/kg NAC consumed significantly less ethanol than saline-treated rats (p=0.027) (55% of reduction) (Fig 5F). Inactive lever presses were statistically not different between groups (Fig 5 C and D).

Behavioral testing: Effect of NAC on 1% sucrose self-administration in nondependent rats:

We tested the effect of NAC on 1% sucrose self-administration in rats that were submitted before sucrose experiment to 2 months of 5 day-a-week 20% ethanol self-administration during 15 minutes sessions (Lebourgeois et al., 2017). In those rats (Fig. 6), NAC 100 mg/kg significantly decreased the 1% sucrose self-administration from -83% (101+/− 12.1 active lever presses for NaCl rats versus 17+/− 13.5 for NAC treated rats, paired Student t-test p=0.0003).

xCT protein levels were decreased in nucleus accumbens of dependent rats:

Fig. 7A shows the expression of xCT measured using western blot in ethanol-dependent and ethanol-naïve rats following treatment with NAC (100 mg/kg; i.p.,
administered 1h before euthanasia). The expression of Xc-system was investigated through the expression of its xCT subunit. As indicated by the Abcam 175186 manufacturer, the expected molecular weight for xCT protein was 55 kDa. Data were analyzed using a two-way ANOVA, which revealed a significant main effect of dependence ($F(1,21)=7.697, p=0.012$), no significant main effect of NAC treatment ($F(1,21)=0.0997, p=0.756$) and no significant interaction ($F(1,19)=0.149, p=0.704$). Dependent rats showed a -18% decrease in xCT expression compared with ethanol-naïve rats, irrespective of NAC treatment ($p<0.05$).

Fig. 7B shows the expression of GLT-1 measured using western blot in ethanol-dependent and ethanol-naïve rats following treatment with NAC (100 mg/kg; i.p., administered 1h before euthanasia). As indicated by the Abcam ab 41621 manufacturer the expected molecular weight for GLT-1 protein was 63 kDa. Data were analyzed using a two-way ANOVA, which revealed no significant main effect of dependence ($F(1,21)=0.474; p=0.499$), a significant main effect of NAC treatment ($F(1,21)=4.456, p=0.048$) and no significant interaction ($F(1,19)=0.114; p=0.739$). NAC treated rats showed a -28% decrease in GLT-1 expression compared with saline group, in dependent rats as well as in ethanol naive rats.

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Discussion:

We have previously demonstrated that a high dose (100mg/kg) of NAC reduced excessive ethanol intake, motivation, seeking and relapse in rats displaying a binge drinking-like behavior for several weeks (Lebourgeois et al., 2017). In the present study we investigated the effect of NAC in rats displaying ethanol dependence following CIE to ethanol vapor. One of the primary findings of the present study is that a low dose (25mg/kg) of NAC reduced ethanol self-administration and motivation to consume ethanol and that a
higher dose (50mg/kg) reduced both extinction responding and reacquisition after abstinence. We also showed that NAC (100 mg/kg) reduced 1% sucrose self-administration in non-dependent rats, demonstrating the NAC efficacy on non-drug reward. When examining glutamate-related neuroadaptations in this addiction model, we found a 18% decrease in the xCT protein levels in the nucleus accumbens of ethanol-dependent rats compared with those of nondependent rats, and we found that NAC induced -28% decrease in GLT-1 expression in both groups, dependent or not.

**NAC efficacy on ethanol self-administration and motivation: relevance of long-term ethanol exposure**

NAC was tested in rats exposed to ethanol for several weeks via the CIE paradigm, i.e. in ethanol dependent animals. The dose of 25mg/kg decreased both ethanol intake and motivation (evaluated in the progressive ratio paradigm) to consume ethanol, assessed after 8-h of withdrawal. This finding complements our prior results in rats engaging in binge-like drinking consumption for several weeks. A main finding of the present study is that NAC is much more effective in ethanol-dependent animals compared with animals displaying binge-like drinking behavior. While the NAC dose of 25 mg/kg significantly reduced intake and motivation in ethanol-dependent rats, a higher dose of 100 mg/kg was necessary to reduce them in rats displaying binge-drinking behavior (Lebourgeois et al., 2017).

It appears that the extended exposure to ethanol is important for NAC efficacy. In rats bred for their high ethanol intake and given free access to ethanol beverage for three months, NAC decreased ethanol intake. However, in rats that drank for only 5 days, NAC did not reduce ethanol intake (Quintanilla et al., 2016). These results and ours suggest that NAC acts by counteracting some of the chronic ethanol-induced neuroadaptations that are responsible for the transition to ethanol addiction.
The NAC dose-response data highlight ethanol-dependence-induced neuroadaptations of glutamatergic transmission. We hypothesized that NAC would be more effective in dependent rats because the hyperglutamatergic state would be higher than in rats self-administrating large quantities of ethanol, but not exposed to the ethanol vapor regimen. In the ethanol withdrawal condition (i.e. the condition in which we tested NAC efficacy here), there are alcohol-induced alterations of the glutamate homeostasis: Chronic alcohol exposure leads to increased extracellular concentrations of glutamate in the nucleus accumbens during withdrawal (Dahchour and De Witte, 2003, Melendez et al., 2005, Griffin et al., 2015), revealing an hyperglutamatergic state in the mesocorticolimbic system following withdrawal (Ding et al., 2013, Rao et al., 2015a). The CIE of ethanol vapor has been described as a model inducing a post-dependent state (Heilig and Koob, 2007, Meinhardt and Sommer, 2015), resulting in long-term changes toward compulsive alcohol intake and hypersensitivity to stress. The use of this model allows the evaluation of NAC impact on the chronic negative affective state experienced by these rats during acute withdrawal (Meinhardt and Sommer, 2015). Herman et al. demonstrated that the hyperglutamatergic state observed during acute withdrawal in these rats parallels the same hyperglutamatergic state observed in alcoholic patients (Hermann et al., 2012). This observation lends support for the CIE model to test glutamate-targeting substances, as we did for NAC here.

Potential role of NAC to reduce craving

The 50mg/kg dose of NAC completely abolished the extinction responding in ethanol-dependent rats. This result indicates that NAC is effective not only when ethanol is available, but also during periods of drug seeking (a behavioral correlate of craving) when ethanol is not available. This finding also suggests that the NAC effect on negative-reinforcement-induced
extinction responding during acute withdrawal we observed in ethanol dependent rats could also be obtained in abstinent patients treated with NAC.

**Potential efficacy of NAC to reduce relapse**

Furthermore, the 50mg/kg dose of NAC is effective in reducing reacquisition of ethanol self-administration (induced by both ethanol and visual cues) after long-term abstinence. Taken together, our results demonstrate the efficacy of NAC both during acute withdrawal and after long-term abstinence and these findings are consistent with the fact that enduring changes in glutamatergic transmissions underlie long-term vulnerability to relapse to drug use and drug-associated memories (Kalivas, 2009).

**NAC effect on the reinforcing properties of a non-drug reward**

In another experiment, we investigated whether the effect of NAC on operant self-administration was specific to ethanol by measuring its effects on the reinforcing properties of sucrose, a non-drug appetitive reward. The 1% sucrose concentration was chosen to achieve the same level of response as that observed in rats self-administering ethanol (about 100 deliveries). Our data show that NAC 100 mg/kg is very effective in reducing lever pressing for sucrose. These data suggest that NAC affects general rewarding and motivational mechanisms. Other molecules that are effective in reducing ethanol self-administration have also been shown to have an effect on self-administration of natural reinforcers such as the serotonergic and noradrenergic transporter inhibitor milnacipran (Simon O’Brien et al., 2011), the CB1R antagonist SR-141716A (Economidou et al., 2006), the A2R agonist CGS21680 (Houchi et al., 2013) and even substances used to treat AUD patients such as naltrexone (Stromberg et al., 2002) and the γ-aminobutyric (GABA) B receptors agonist baclofen (Anstrom et al., 2003).
CIE-induced decrease in the $x_c^-$ antiport system

The $x_c^-$ system is expressed on glial cells and its role is to export glutamate in the extrasynaptic space at the presynaptic level. As the glial $x_c^-$ system plays a crucial role in maintaining glutamate homeostasis, its expression has previously been monitored in the context of substance abuse: rats withdrawn from chronically self-administering nicotine showed decreased xCT expression in the nucleus accumbens, 12 hours after the last nicotine exposure (Knackstedt et al., 2009).

There is a debate over the correct western band that has to be chosen for xCT Western analysis (Van Liefferinge et al., 2016). Using the 55 kDa band in our study, as mentioned by the manufacturer for Acam 175186, we found a 18 % decrease in xCT expression in the nucleus accumbens of ethanol-dependent animals compared with ethanol-naïve animals, at the 8h-withdrawal period. Interestingly, the increase in XCT expression has been related to a five-fold decreased ethanol consumption in alcohol-preferring rats (Rao et al., 2015b). A dynamic regulation of the xCT transporter in the nucleus accumbens along ethanol chronic exposure or withdrawal has been already described. The xCT transporter is strongly over-expressed in ethanol-dependent rat along exposure, and strongly down-regulated during withdrawal (Peana et al., 2014). In the nucleus accumbens, around 60% of the basal extracellular glutamate is derived from the activity of the $x_c^-$ antiport system (Baker et al., 2003). So the withdrawal-induced decrease in $x_c^-$ could have an impact on glutamate homeostasis, leading to an ethanol withdrawal-induced decrease in extracellular glutamate level. However, this hypothesis is difficult to reconcile with the observation that chronic alcohol exposure leads to increased extracellular concentrations of glutamate in the nucleus accumbens that persists for 24h hours after withdrawal (Melendez et al., 2005) or 7 days after withdrawal (Griffin et al., 2014).
The NAC treatment administered 1 hour before western blot sampling was unable to modify the xCT expression in the nucleus accumbens. This is concordant with the proposal that the acute NAC effect would not be due to a direct, rapid increase in the xCT system expression, but rather, increased activity of the xCT system.

However, another actor plays a major role in drug-induced alteration of glutamate homeostasis: the glial glutamate transporter GLT1, that regulates glutamatergic signaling by removing excess glutamate from the extrasynaptic space (Roberts-Wolfe and Kalivas, 2015). For example chronic alcohol intake for five weeks in P (alcohol preferring) rats resulted in downregulation of GLT1 expression in the nucleus accumbens as compared to ethanol-naive animals (Sari and Sreemantula, 2012), thus demonstrating a link between ethanol intake and GLT-1 expression. Besides, it has been shown that the ceftriaxone-induced reduction in ethanol intake by P rats was associated with significantly enhanced expression of GLT1, in the nucleus accumbens (Rao et al., 2015b). Altogether these data obtained in P rats suggest an inverse correlation between GLT-1 expression and ethanol intake.

However here we did not observe any difference in GLT-1 expression during acute withdrawal dependent rats compared with non dependent animals. In summary, our results suggest that the higher efficacy of NAC observed in dependent rats would be related to the decreased expression of the xCT transporter and not to a modification of GLT-1. This assumption is based on the fact that we found only a decrease in xCT but not GLT-1 in the dependent rats and no difference between our two groups of rats regarding the effects of NAC.

NAC properties on modulation of oxidative stress and inflammatory system

In addition to its action on the glutamatergic system, NAC is also able to modulate the oxidative stress and the inflammatory system. NAC administration leads to an increase in intracellular antioxydant glutathione (GSH) and reduces oxidative stress. Indeed, NAC has
been found to restore a cocaine-induced decrease of glutathion levels in glial cells (Badisa et al., 2015), while ethanol abstinence and NAC intake interact synergistically, improving serum lipids and hepatic antioxidant defenses (Ferreira Seiva et al., 2009). The expression of the glutathione-S-transferases, the enzyme that catalyzes the conjugation of the reduced form of GSH to ROS for the purpose of detoxification, is altered in ethanol-naive ethanol-preferring rats compared with non-preferring ones (Liang et al., 2004, Bjork et al., 2006). Moreover, the activity of blood glutathione-S-transferase is reduced in alcohol-dependent male patients (Peter et al., 2013), revealing the importance of the redox balance in ethanol addiction (for a review see (Uys et al., 2014). Moreover, an anti-oxidative effect could be linked to an anti-addictive effect. For example, it has been demonstrated that oxidative stress plays a role in the reinforcing properties of substances of abuse as enhancement of ROS in the nucleus accumbens contributes to the reinforcing effect of cocaine (Jang et al., 2015) and methamphetamine (Jang et al., 2016).

Regarding ethanol–induced neuroinflammation, elevation of TNFα and IL-6 are correlated with high levels of craving in humans (Leclercq et al., 2012) and studies have shown that chronic administration of NAC during abstinence helps to prevent increasing proinflammatory cytokines (Schneider et al., 2017). Therefore, NAC could limit relapse and somatic disorders by acting simultaneously on these 3 systems: glutamate homeostasis, oxidative stress and inflammation.

Two other recent preclinical studies underscore the relevance of NAC in the context of ethanol addiction. First, NAC treatment blocks the development of ethanol-induced behavioral sensitization (Morais-Silva et al., 2016). Second, abstinence from ethanol induces an increase in leptin and corticosterone levels in rats consuming alcohol by gavage for 30 days (2g/kg; twice a day), while NAC treatment prevents these biochemical changes (Schneider et al., 2015). Thus, given that increased corticosterone concentration is a stress
indicator and that elevated levels of leptin are correlated with a high level of craving in patients (Kiefer et al., 2001), this suggests that NAC could act to reduce stress and craving observed during alcohol abstinence.

**Conclusion**

Overall, the current findings demonstrate that NAC reduces ethanol self-administration, motivation, craving and reacquisition after abstinence in relevant preclinical model of alcohol addiction. NAC was also effective in reducing sucrose self-administration thus indicating that it may also have a more general effect on the reward system. The present data confirm and extend previous studies showing that NAC is a promising pharmacological therapy for addiction in general, but especially for ethanol addiction, which is characterized by enduring changes in glutamatergic transmission. Our results strongly support clinical development of NAC for alcohol use disorders and this is further supported by recent findings indicating that NAC may be effective at reducing consumption of alcohol by ≥30% among treatment-seeking adults with cannabis use disorder (Squeglia et al., 2018).

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**Author's contribution:**

SL, CV, MCGM and MN were responsible for the study concept and design. SL, MCGM and CV contributed to the acquisition of animal data. SL, MCGM and CV assisted with data analysis and interpretation of findings. SL and CV drafted the manuscript. MN provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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References


Figure 1: Timeline of experimental procedures on the effect of NAC on ethanol self-administration: The procedure was performed in dependent rat following CIE procedure for 10 weeks. NAC or saline was always injected i.p. one hour before the 30-min session ethanol 20% self-administrations, 8 hours after retrieval from the inhalation chambers. All along the experiments, during the two weeks recovery and during NAC testing, rats were maintained under the CIE procedure and were submitted to sessions of ethanol self-administration five-days a week, at 8h withdrawal. The sole periods without the CIE procedure was during the two one-month abstinence period. PR: Progressive Ratio.

Figure 2: N-acetylcysteine reduced ethanol self-administration: Effect of an i.p. injection of NAC (0, 25, 50 or 100 mg/kg; 1h before the test; n=17/group) on 20% ethanol self-administration. A. Total active lever presses were significantly lower for the NAC 25, 50 and 100 mg/kg group compared with saline group, and there was no effect on inactive lever presses. B. Rats treated with NAC 25, 50 and 100 mg/kg consumed significantly less ethanol than saline-treated rats. ***: p<0.001, **: p<0.01. C. Total inactive lever presses were not different for the NAC 25, 50 and 100 mg/kg group compared with saline group. D. N-acetylcysteine was more effective in dependent rats than in binge-drinking rats: Efficacy of an acute injection of NAC (25, 50 or 100 mg/kg; i.p., 1h before the test) on ethanol self-administration in dependent (n=17) vs binge-drinking rats (n=15). For each dose tested, the percent of baseline EtOH consumption was significantly lower in dependent compared with binge-drinking rats. In binge-drinking rats, an acute injection of 100 mg/kg NAC was necessary to observe a reduction in EtOH consumption whereas in dependent rats, an injection of 25 mg/kg was sufficient. **: p<0.01, *: p<0.05 compared with binge-drinking group.

Figure 3: N-acetylcysteine decreased the reinforcing efficacy of ethanol: A. NAC 25 mg/kg-treated rats (n=8) pressed significantly fewer on the active lever during the PR session (-55%) compared to saline-treated rats (n=9), while there was no effect on inactive lever presses. B. NAC 25 mg/kg-treated rats displayed a significantly lower breaking point value compared to saline-treated rats (-43%) **: p<0.01, *: p<0.05. C. Total inactive lever presses were not different between groups.

Figure 4: N-acetylcysteine 50 mg/kg decreased extinction responding: A. NAC 25 mg/kg (n=9) treated rats showed no alteration in extinction responding compared with saline (n=9). B. NAC 50 mg/kg-treated rats (n=8) pressed significantly fewer on the active lever compared to saline-treated rats. There was no effect of NAC 25 mg/kg on inactive lever presses D. There was no effect of NAC 50 mg/kg on inactive lever presses. ***: p<0.001

Figure 5: N-acetylcysteine 50 mg/kg reduced reacquisition after abstinence: A. During the reacquisition procedure, NAC 25 mg/kg (n=9) treated rats showed no alteration of reacquisition of operant self-administration behavior compared with saline. B. NAC 50mg/kg (n=11) treated rats showed a reduced reacquisition of operant self-administration behavior.
compared with saline (-57%). C. There was no effect of NAC 25 mg/kg on inactive lever presses. D. There was no effect of NAC 50 mg/kg on inactive lever presses. E. NAC 25 mg/kg treated rats showed no alteration in ethanol intake during the reacquisition session compared with Saline Group. F. NAC 50 mg/kg treated rats showed a decrease in ethanol intake during the reacquisition session (-59%). *: p<0.05 compared with Saline Group.

**Figure 6:** N-acetylcysteine 100 mg/kg reduced 1% sucrose self-administration. In 14 rats that self-administered 20% ethanol for eight weeks during 15-min daily sessions (as described in Lebourgeois et al., 2017), NAC (100 mg/kg) was tested on 1% sucrose self-administration. NAC treatment was very effective in reducing the number of active lever presses for 1% sucrose, ***p<0.001.

**Figure 7:** Western blot of xCT and GLT-1 expression in nucleus accumbens of dependent versus naive rats, following N-acetylcysteine 100 mg/kg treatment. A. Dependent rats showed a -18% decrease of xCT expression compared with nondependent rats that have never been exposed to ethanol. There was no effect of NAC treatment. n = 6 rats per groups. *: p<0.05: Main “Dependence” effect, dependent versus non dependent rats. Immunoblot for xCT protein yielded a band of approximately 56 kDa. Below are representative xCT and actin blots. B. NAC-treated rats showed a -28% decrease of GLT-1 expression compared with non-treated rats, whatever they were dependent or not. n = 6 rats per groups. *: p<0.05: Main “NAC” effects, NAC-treated rats versus non-treated rats. Immunoblot for xCT protein yielded a band of approximately 63 kDa. Below are representative GLT-1 and actin blots.
NAC Doses mg/kg: 25, 50, 100

Dose effect 2 wks recovery Motivation PR 2 wks recovery Extinction responding 2 wks recovery Extinction responding 2 wks recovery 1 month withdrawal

Reacquisition 2 wks recovery 1 month withdrawal Reacquisition
Figure 1: Effects of NAC doses on active and inactive lever presses.

A. Active lever presses: 0 mg/kg versus 25 mg/kg.
B. Active lever presses: 0 mg/kg versus 50 mg/kg.
C. Inactive lever presses: 0 mg/kg versus 25 mg/kg.
D. Inactive lever presses: 0 mg/kg versus 50 mg/kg.
E. g/kg body weight: 0 mg/kg versus 25 mg/kg.
F. g/kg body weight: 0 mg/kg versus 50 mg/kg.

*Denotes significant difference.