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Abstract:	Nicotine associated cues can trigger reinstatement in humans as well as in animal models of drug addiction. To date, no behavioral intervention or pharmacological treatment has been effective in preventing relapse in the long term. A large body of experimental and clinical evidence indicates that N-acetylcysteine (N-AC) blunts the activation of glutamatergic (GLUergic) neurons associated with reinstatement in the nucleus accumbens (Nacc). We decided to evaluated the effect of an experimental cue exposure therapy (eCET) alone or in combination with N-AC to verify whether restoring GLU homeostasis enhances extinction of nicotine related cues. Rats were trained to associate discriminative stimuli with intravenous nicotine or saline self-administration. Reinforced response was followed by cue signals. After rats met the self-administration criteria, the lasting anti-relapse activity of N-AC (60 and 100 mg/kg, i.p.) or vehicle was assessed in three different experimental conditions over 14 days: treatment + eCET; treatment + lever-presses extinction; and treatment + abstinence. Only N-AC 100 mg/kg + eCET induced anti-relapse activity that persisted 50 days after treatment. To identify potential mechanisms for behavioral results, separate groups of rats that received either N-AC or vehicle + eCET were killed at different time points for Western-blot analysis of the Nacc. Seven days after treatment, chronic N-AC restored the expression of proteins crucial for GLU homeostasis, while at 50 days it increased the expression of the type II metabotropic GLU receptors. These results suggest that N-AC treatment in combination with eCET may offer a novel, strategy to prevent relapse in nicotine addiction.

Lasting reduction of nicotine seeking-behavior by chronic N-acetylcysteine during experimental cue-exposure therapy

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Abstract

Nicotine associated cues can trigger reinstatement in humans as well as in animal models of drug addiction. To date, no behavioral intervention or pharmacological treatment has been effective in preventing relapse in the long term. A large body of experimental and clinical evidence indicates that N-acetylcysteine (N-AC) blunts the activation of glutamatergic (GLUergic) neurons associated with reinstatement in the nucleus accumbens (Nacc). We decided to evaluated the effect of an experimental cue exposure therapy (eCET) alone or in combination with N-AC to verify whether restoring GLU homeostasis enhances extinction of nicotine related cues.

Rats were trained to associate discriminative stimuli with intravenous nicotine or saline self-administration. Reinforced response was followed by cue signals. After rats met the self-administration criteria, the lasting anti-relapse activity of N-AC (60 and 100 mg/kg, i.p.) or vehicle was assessed in three different experimental conditions over 14 days:treatment + eCET; treatment + lever-presses extinction; and treatment + abstinence. Only N-AC 100 mg/kg + eCET induced anti-relapse activity that persisted 50 days after treatment.

To identify potential mechanisms for behavioral results, separate groups of rats that received either N-AC or vehicle + eCET were killed at different time points for Western-blot analysis of the Nacc. Seven days after treatment, chronic N-AC restored the expression of proteins crucial for GLU homeostasis, while at 50 days it increased the expression of the type II metabotropic GLU receptors. These results suggest that N-AC treatment in combination with eCET may offer a novel, strategy to prevent relapse in nicotine addiction.

Introduction

An important feature of susceptibility to relapse in drug abuse is the increased salience acquired by environmental stimuli strongly associated with the reinforcing properties of the drug. This has been observed also for nicotine whose consumption engages different Pavlovian and instrumental learning systems in the brain, causing neutral environmental cues and contexts to become strongly associated with its reinforcing properties (Henningfield and Goldberg, 1983).

A non-pharmacological strategy aiming to reduce the impact of nicotine-related cues may help to prevent relapse. Such strategy, known as cue exposure therapy(CET), relies on extinction training in which drug-associated cues are repeatedly presented in the absence of the abused drug to promote new learning that should counteract the motivating impact of the cues (Havermans and Jansen, 2003). Indeed, CET has been proposed to reduce cue-induced nicotine relapse (O'Brien et al., 1990). However, its efficacy might be limited by the context-dependent nature of extinction therapy (Conklin and Tiffany, 2002) and drug-induced dysfunction of the memory systems that are critical for extinction learning and consolidation (Quirk and Mueller, 2008; Peters et al., 2009). As a result, CET have shown limited effectiveness in preventing relapse (Conklin and Tiffany, 2002) and their clinical benefits for nicotine addiction are still debated (Hajek et al., 2013). To improve its efficacy, it has been proposed that a more ethological CET targeting drug-associated cues in the same context where the associative over-learning and consolidation took place (i.e. becoming "over-learned") could be a better strategy to improve extinction outcome. By using experimental animal models, it is possible to overcome the complexity of the human situation and evaluate whether exposing the animals to the full set of stimuli previously linked to the drug could produce extinction of drug-related memory.

Recent studies have highlighted that changes in glutamate (GLU) homeostasis in the circuitry from the prefrontal cortex (PFC) to the nucleus accumbens (Nacc) contribute to the reinstatement of drug-seeking behavior (Kalivas, 2009). In particular, cocaine and nicotine self-administration reduce the expression of the cystine/GLU exchanger (system Xc-), the glial GLU transporter GLT-1, and reduces the functionality of group II metabotropic GLU receptors (mGluR2/3) (Liechti et al., 2007), - proteins vital for regulating GLU transmission in the Nacc (Knackstedt et al., 2009).

Even though acute N-AC administration reduces nicotine-seeking behavior by activating mGluR2/3 and restoring GLU homeostasis (Moro et al., 2016), the effect of acute N-AC treatment was short-lasting suggesting that a drug regimen inducing long-lasting repair of nicotine-induced GLU-mediated neuroplasticity might have greater therapeutic value. It has been shown that repeated N-AC consistently restored non-synaptic GLU tone, normalizing the alterations in the cortico-accumbens synaptic transmission and glial cell activity produced by

chronic cocaine self-administration (Moussawi et al., 2011). Moreover, repeated N-AC markedly reduced relapse especially when given during the extinction of the instrumental response (Reichel et al., 2011).

For this reason, we hypothesized that restoring nicotine-induced dysfunction in the GLUergic system by repeated N-AC administration could help to increase extinction of the over-learned relationship between nicotine, conditioned-cues and instrumental response. In preclinical settings, extinction of drug-related memories has been mostly studied using the extinction/reinstatement model where extinction specifically refers to the extinguishing of the instrumental response used to self-administer the drug (Carter and Tiffany, 1999). To date, few preclinical studies have evaluated whether increasing the specificity of drug conditioned cues have an impact on cue extinction (Torregrossa and Taylor, 2013).

With the aim of targeting nicotine-associated cues, we evaluated whether a model of experimental CET (eCET), carried out in a more ethological fashion, alone or in combination with chronic N-AC, could be an effective strategy to extinguish nicotine related memories and whether its effect could be long lasting. Finally, we also studied whether key components of GLU transmission in the Nacc were altered by the combination of N-AC + eCET.

Materials and Methods

Animals

Naïve male Wistar rats (Harlan Laboratories, San Pietro al Natisone, Udine, Italy) weighing 250-275 g were used for all experiments. They were housed individually at constant room temperature (21±1°C) and relative humidity (60%) under an inverted light/dark schedule (light on 7:30 PM-7:30 AM) with food and water *ad libitum*. All experimental work was done during the dark phase. After arriving at the facility, rats were allowed to adapt to the vivarium conditions for at least two weeks prior to the start of experiments and were handled daily during this period. After this, they received a maintenance diet of 20-25 g/rat of chow/day (Global Diet 2018S, Harlan Laboratories) for the duration of the experiments.

Animal care

Procedures involving animals were conducted at the IRCCS - Istituto di Ricerche Farmacologiche "Mario Negri" which adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate - UNI EN ISO 9001:2008 - Reg. No. 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01).

Drugs

Nicotine was dissolved in saline as previously reported (Moro et al., 2016). N-AC was prepared and administered daily for 14 days as previously described (Moro et al., 2016). See the Supporting Information for further details (S.I.).

Apparatus and nicotine self-administration training

Rats were trained in 16 identical operant chambers (ENV-007, MED Associates Inc., St Albans, VT, USA) as previously described (Moro et al., 2016). Rats were surgically prepared with jugular catheters and given a week of recovery before self-administration training (see Supporting Information).

One week after surgery, independent groups of rats were food-deprived overnight and trained to associate a white noise (20 dB above background) that lasted throughout the session as a discriminative stimulus (S^{D+}) for the availability of nicotine (0.03 mg/kg/65 μL/2-seconds/infusion). Rats were not trained for food at the beginning of the experiment, but they were required to immediately press the active lever under continuous reinforcement [fixed ratio 1 (FR1)] for nicotine self-administration. Sessions started with extension of active and inactive levers and reinforced response was followed by a light cue (6-seconds) on the active lever to signal a 20-seconds time out (CS^{+}). After ten days of self-administration training (see S.I. for further details) during which the fixed ratio 2 (FR2) was reached), rats were placed on a "discrimination learning" regimen comprising a seconds daily session without a reward. These sessions started with extension of the active and inactive levers together with illumination of the house light, which remained on throughout the session and served as

discriminative stimulus (S^{D-}) for no reward (65 µL/2-seconds/infusion of sterile saline). Reinforced response was followed by a 20-seconds intermittent tone (7 KHz, 70 dB) to signal a 20-seconds time out (CS^-).

The "discrimination learning" phase comprised two daily 1-h sessions, separated by 1-h rest in the home cage. Rats were exposed to nicotine and saline sessions in a random sequence. Responses on the inactive lever were recorded but had no programmed consequences. This training was conducted daily for five days/week until individual reinforced responding was stable (±15% over three consecutive sessions).

First reinstatement test

Twenty-four hours after the self-administration criterion was met, individual animals were tested a first time with either nicotine-associated cues (S^{D+}/CS^+) (half of the rats in each experiment) or saline-associated cues (S^{D-}/CS^-) (half the rats in each experiment). The next day the test order was switched (i.e. 1st day S^{D+}/CS^+, 2nd day S^{D-}/CS^- and vice versa). Test sessions lasted one hour during which rats were exposed to non-contingent S^{D+} or S^{D-}under conditions identical to the discrimination learning phase, except the reward (nicotine or saline) was unavailable. Two responses on the previously active lever were followed by activation of the pump motor followed by a 20-seconds CS^+ or CS^-presentation.

Effects of the treatments on reintroduction of nicotine-associated cues

Starting 24 h after the first reinstatement test, chronic treatments with vehicle (veh), 60 or 100 mg/kg N-AC were administered under different experimental conditions:

- eCET (*Instrumental and cue extinction)*: rats were exposed to the self-administration cage and S^{D+}/CS^+ under conditions identical to the first reinstatement test. Veh (n=8), N-AC 60 mg/kg (n=8) and N-AC 100 mg/kg (n=8) were given daily for two weeks 2.5 h before each reinstatement test.

- Lever press extinction ([LP-EXT], *instrumental extinction)*: rats were placed in the self-administration cage where no SDs were presented and the instrumental lever response produced neither the reinforcer nor the CSs (Cervo et al., 2013). Rats were exposed once a day for 14 consecutive days to an extinction session of the duration of 1 h and responding on either lever had no scheduled consequences. Veh (n=8), N-AC 60 mg/kg (n=8) and N-AC 100 mg/kg (n=8) were given 2.5 h before each LP-EXT test.

- Abstinence: rats were left in their home cage, handled, and injected daily for 14 days with veh (n=8 rats), N-AC 60 mg/kg (n=7) and N-AC 100 mg/kg (n=9) but were not placed in the self-administration cage.

On day 1, 6, 14 and 50 after the end of treatment, rats were tested with S^{D+}/CS^{+} to assess any effect on nicotine cue-induced reinstatement. To demonstrate the selectivity of nicotine-associated cues, on test days 14 and 50 half of the rats were tested with S^{D+}/CS^{+} and the other half with S^{D-}/CS^{-}. The day after the test, the order was switched (i.e. day 14 S^{D+}/CS^{+}, day 15 S^{D-}/CS^{-} and vice versa): thus, rats were also tested on days 15 and 51. For the sake of simplicity, we pooled the data from the same cues and indicated them as day 14 and 50 after the end of the treatment (i.e. not 14/15 days and 50/51 days).

To assess protein expression in the Nacc, independent groups of rats treated with N-AC during eCET were killed at 7 and 51 days after treatment. Fourteen rats (veh n=7; N-AC n=7) were killed 24 h after the reinstatement tests at 6 days. The same day, naïve rats that received N-AC 100 mg/kg (n=6) or veh (n=6) were also killed. Eighteen rats (veh n=9; N-AC n=9) were killed 24 h after the reinstatement tests at 50 days from the end of the treatment. The same day naïve rats that received N-AC 100 mg/kg (n=6) or veh (n=6) were also killed.

Brain micro-dissection

Rats were killed by decapitation 7 or 51 days after the end of chronic N-AC treatment. Whole brains were frozen on dry ice and stored at -80°C for later micro-dissection. Microdissection was done as previously described (Giannotti et al., 2016). Briefly, coronal sections (220 µm thickness) were obtained in a cryostat at -15°C, mounted on glass slides and rapidly cooled with dry ice. Nacc core and shell were micro-dissected from bregma +2.76 to bregma +0.84 mm according to Paxinos and Watson rat brain atlas (2007) a sharp cutting tip of 1 mm diameter (Harris Uni-Core, Ted Pella inc.), snap frozen and stored at -80°C.

Protein extraction and Western-blot analysis

Protein extraction and Western-blot analyses were performed as previously described (Caffino et al., 2016). See S.I. for more details and uncropped immunoblot data (Figure S1, S2, S3, S4).

Statistical analysis

Sample size for each experiment was determined by a combination of power analysis and our previous work in similar models. Animals were randomized to experimental procedures and treatments. Experimenters were blinded to treatment allocation. All data are expressed as mean ± standard error of the mean (SEM) of active and inactive lever presses during the training period, extinction and reinstatement phases. Only data from rats that completed all experimental phases were included in the statistical analysis. Six rats were excluded, two because of lack of catheter patency, two because the self-administration training criterion was not reached, and two rats were excluded from all the analyses because they were identified as outliers using Grubb's test.

In the training period, the number of lever presses during the last three sessions of nicotine self-administration/no-reward were analyzed separately by one-way analysis of variance (ANOVA) with repeated measures. Since there were no differences between sessions, the last three days for each condition were pooled for further statistical analysis.

A mixed-factorial ANOVA with treatment as between-subject factor and sessions as within-subject factor was done to analyze the experiments. When appropriate, post-hoc comparisons were made with the Newman-Keuls test. Protein levels were analyzed by two-way ANOVA with treatment and self-administration history as main factors. When appropriate, post-hoccomparisons were done with Tukey's multiple comparisons test. Assumptions of normality were checked using the D'Agostino-Pearson normality test. Standard software packages were utilised throughout (GraphPad Prism 7, La Jolla, CA, USA).

Results

Training and first reinstatement tests before the beginning of the N-AC treatment

All rats in the different experiments developed stable nicotine self-administration, and lever presses during the last three saline sessions were less than nicotine sessions ($P<0.05$, Newman-Keuls test) and similar across the different groups ($P>0.05$, Newman-Keuls test) (Table 1). During the last three self-administration training sessions, all experimental groups earned similar amounts of nicotine, as demonstrated by the similar number of infusions. During the 1st reinstatement test S^{D+}/CS^{+}, but not S^{D-}/CS^{-}, renewed active lever presses ($P<0.05$, Newman-Keuls test) to similar extents in the different groups ($P>0.05$, Newman-Keuls test). The revived active lever presses were similar to those during the nicotine self-administration sessions and significantly higher than

during the saline self-administration ($P<0.05$, Newman-Keuls test). Inactive lever responses remained negligible throughout all experimental phases.

Effect of eCET alone or with chronic N-AC

Figure 1 shows the responses on the active lever during self-administration (mean of the last three sessions), the 1st reinstatement test, the 14 eCET sessions after veh or N-AC and reinstatement tests at the end of the treatment. Mixed-factorial ANOVA showed significant effects of session [$F(23,414)=19.42$, $P<0.01$], treatment [$F(2,18)=1.47$, $P<0.001$] and interaction treatment x sessions [$F(46,414)=2.74$, $P<0.01$]. Responses during the last three nicotine self-administration sessions were similar between groups ($P>0.05$, Newman-Keuls test) and significantly higher than the last three saline self-administration sessions ($P<0.05$ vs. nicotine self-administration, Newman-Keuls test). During eCET, S^{D+}/CS^{+} always revived the number of active lever presses with veh and 60, but not 100, mg/kg N-AC ($P<0.05$ vs. S^{D-}/CS^{-} at the 1st reinstatement, Newman-Keuls test). In fact, except on test days 7-10 and 12, N-AC 100 mg/kg significantly reduced the number of active lever presses, with no tolerance ($P<0.05$ vs. S^{D+}/CS^{+} and $P>0.05$ vs. S^{D-}/CS^{-} at 1st reinstatement and $P<0.05$ vs. respective veh, Newman-Keuls test). After the eCET-sessions, the reintroduction of S^{D+}/CS^{+}, but not S^{D-}/CS^{-}, always revived active presses in veh and N-AC 60 mg/kg treated rats ($P<0.05$ vs. S^{D-}/CS^{-}before and after eCET+treatment, Newman-Keuls test). N-AC 100 mg/kg completely blocked the renewed active lever presses induced by nicotine-associated cues ($P<0.05$ vs. respective veh, $P>0.05$ vs. S^{D-}/CS^{-}before and after eCET+treatment, Newman-Keuls test).

Chronic N-AC treatment during LP-EXT

Figure 2 shows the responses on the active lever during the self-administration, the 1st reinstatement test, the 14 LP-EXT sessions of rats pre-treated daily with veh or N-AC, and reinstatement tests at the end of treatment. Mixed-factorial ANOVA found a significant effect of sessions [$F(23,460)=88.20$, $P<0.01$] and interaction treatment x sessions [$F(46,460)=2.04$, $P<0.05$] with no effect of treatment [$F(2,20)=2.34$, $P>0.05$]. Responses during the last three nicotine self-administration sessions were similar between groups (P>0.05, Newman-Keuls) and significantly higher than the means of last three saline self-administration sessions ($P<0.05$, Newman-Keuls test). During LP-EXT sessions, the numbers of active lever presses between days were similar in all groups of

rats and always similar to those during S^{D-}/CS^{-} and lower than in presence of S^{D+}/CS^{+} ($P<0.05$, Newman-Keuls test) at the 1st reinstatement test. No effect of N-AC was detectable during the treatment. After the LP-EXT sessions S^{D+}/CS^{+} but not S^{D-}/CS^{-}, revived active presses in all groups ($P>0.05$ vs. S^{D+}/CS^{+} and $P<0.05$ vs. S^{D-}/CS^{-} at 1st reinstatement test, Newman-Keuls test). The number of active lever presses after N-AC 100 mg/kg was partially reduced at 24 h, 6 and 50 days, but not at 14 days, after the end of the treatment ($P<0.05$ vs. respective veh, Newman-Keuls test). In veh and N-AC 60 mg/kg treated rats, the active lever presses were significantly higher 6, 14 and 50 days after the end of treatment than during the 1st reinstatement test ($P<0.05$, Newman-Keuls test), indicating a potential "drug-seeking incubation".

Chronic N-AC treatment during abstinence

Figure 3 shows the responses on the active lever during the self-administration and reinstatement tests before and after the end of veh or N-AC treatment. Mixed-factorial ANOVA found a significant effect of session [$F(9,171)=40.94$, $P<0.001$] with no effect of treatment [$F(2,19)=0.08$, $P>0.05$] or interaction treatment x sessions [$F(18,171)=0.53$, $P>0.05$]. The number of responses after the reintroduction of S^{D-}/CS^{-} and S^{D+}/CS^{+} differed, as revealed by the main effect of the sessions. N-AC never altered the number of responses during reinstatement tests.

Behavioral results and protein analyses of rats killed seven days after N-AC + eCET

Figure 4 shows the responses on the active lever during self-administration, the 1st reinstatement test, the 14 eCET sessions of rats pre-treated with veh or N-AC 100 mg/kg i.p. and reinstatement tests. Behavioral results are discussed more in details in S.I, briefly we replicated the results of figure 1 by showing that during treatment and and later reinstatement sessions N-AC 100 mg/kg (but not veh) significantly reduced the number of active lever presses,($P<0.05$ vs. S^{D+}/CS^{+}, $P>0.05$ vs. S^{D-}/CS^{-}vs. 1st reinstatement; and $P<0.05$ vs. respective veh, Newman-Keuls test).

Figure 4c indicates that the effects of N-AC on xCT expression in the Nacc core of naïve and nicotine self-administered rats revealed a significant main effect of N-AC [$F(1,22)=7.93$, $P<0.05$] and the main effect of the nicotine self-condition [$F(1,22)=9.18$,$P<0.01$] but no nicotine self-condition x treatment interaction [$F(1,22)=0.29$, $P>0.05$]. NAC had an effect on GLT-1 expression in the Nacc shell [$F_{(1,22)}$self-

condition=2.95, P>0.05; $F_{(1,22)}$treatment=17.13, P<0.05; $F_{(1,22)}$interaction=5.01, P<0.05]. Compared to naïve/veh, the expression of GLT-1 in nicotine self-administered rats treated with veh + eCET was lower (P<0.05 vs. naïve/veh, Tukey's test) and N-AC (nic/N-AC group) restored protein expression to that of naïve rats (P>0.05 vs. naïve/veh, Tukey's test). Moreover, the expression of GluN2B was affected by N-AC in the Nacc shell [$F_{(1,22)}$self-condition=2.76, P>0.05; $F_{(1,22)}$treatment=4.11, P>0.05; $F_{(1,22)}$interaction=5.19, P<0.05]. Compared to naïve rats, the expression of GluN2B in nicotine self-administered rats treated with veh + eCET increased (P<0.05 vs. naïve/veh group, Tukey's test) and N-AC (nic/N-AC group) restored protein expression back to the level of naïve rats (P>0.05 vs. naïve/veh, Tukey's test) (Figure 4c). N-AC did not significantly alter protein expression in naïve animals (Figure 4c).

Behavioral results and proteinanalyses in rats killed 51 days after N-AC + eCET

Figure 5 shows the responses on the active lever during self-administration, 1st reinstatement test, 14 eCET sessions of rats pre-treated with veh or N-AC 100 mg/kg i.p. and reinstatement tests. We replicated the behavioral results of figure 1 and 4 by showing that N-AC 100 mg/kg (but not veh) reduced active lever presses during treatment and up to 50 days after the end of the treatment (see S.I. for more more details).

Figure 5c illustrate the protein expression of some determinants of GLU transmission in rats killed 51 days after N-AC + eCET. N-AC affected on the expression of GLT-1 [$F_{(1,26)}$self-condition=2.53, P>0.05; $F_{(1,26)}$treatment=1.07, P>0.05; $F_{(1,26)}$interaction=6.07, P<0.05] and mGluR2 [$F_{(1,26)}$self-condition=2.90, P>0.05; $F_{(1,26)}$treatment=3.39, P>0.05; $F_{(1,26)}$interaction=4.3, P<0.05] in the Nacc shell. Compared to the nic/veh, the expression of GLT-1 and mGluR2 in nicotine self-administered rats treated with N-AC + eCET was higher (P<0.05, Tukey's test). Moreover, the expression of mGluR2 was higher in the Nacc core [$F_{(1,26)}$self-condition=0.37, P<0.05; $F_{(1,26)}$treatment=7.25, P<0.05; $F_{(1,26)}$interaction=4.28, P<0.05] of Nic/N-AC groups (P<0.05 vs. nic/veh, Tukey's test). N-AC had no effects on proteins expression in naïve animals (P>0.05, Tukey's test) (Figure 5c).

Discussion

This study examined the effects of 14 days of behavioral treatments either alone or in combination with chronic N-AC on cue-induced nicotine-seeking behavior and molecular determinants of GLUergic homeostasis in the Nacc. The main findings were: i) 14 days of eCET, LP-EXT or abstinence in the home cage did not alter the seeking behavior induced by reintroduction of nicotine-associated cues; ii) 14 days of N-AC (100 mg/kg) treatment during eCET completely blocked nicotine-seeking behavior by associated cues, an effect that lasted at least 50 days after the treatment; iii) 14 days of N-AC (100 mg/kg) treatment during LP-EXT attenuated cue-induced nicotine-seeking behavior up to 50 days after N-AC treatment; iv) 14 days of N-AC treatment during abstinence in the home cage did not alter cue-induced nicotine-seeking behavior; and v) 14 days of N-AC (100 mg/kg) treatment during eCET changed the expression of proteins vital for regulating GLU homeostasis in the Nacc.

We found that the reintroduction of stimuli predictive of, and associated with, nicotine infusion during self-administration sessions induced strong and lasting drug-seeking behavior in abstinent rats, as demonstrated in our previous studies (Cervo et al., 2013; Moro et al., 2016). This effect cannot be attributed to non-specific arousal or spontaneous recovery, because responding on the inactive lever and with S^{D-}/CS^{-}remained negligible.

To evaluate the efficacy of our eCET, we refined the procedure we previously used for evaluating anti-relapse treatments (Moro et al., 2016) by removing the extinction phases before and between the reinstatement sessions. During eCET, rats were exposed to the full set of stimuli (instrumental response and S^{D+}/CS^{+}) and associated to nicotine self-administration in a condition identical to that of the reinstatement sessions with the only difference that the treatment was delivered 2.5 hours before the beginning of the sessions. The double self-administration training allowed us to verify at different time points (24 h after nicotine self-administration, 14 days and 50 days after treatment) whether nicotine-related cues (S^{D+}/CS^{+}) induced seeking behavior by comparing the reinstatement level produced by the re-introduction of saline-related cues (S^{D-}/CS^{-}). Indeed, in the 1st and in all the other reinstatement sessions, the number of active lever presses after reintroduction of nicotine-associated cues were always higher than that of saline-associated cues. The evidence that the limited period of nicotine self-administration (20–22 1-hour sessions) induced such strong and lasting associations with cues predictive of, and associated with, nicotine availability might beunexpected. However, as with other drugs of abuse (Ciccocioppo et al., 2001; Gracy et al., 2000; Weiss et al., 2001), drug-associated cues can induce strong,

persistent drug-seeking behavior. This is probably because during self-administration sessions, rats were not only exposed non-contingently to the nicotine S^{D+}, signaling drug availability, but infusions were also paired with a response cue marking the 20-seconds time-out period acting as CS^{+}. Thus, re-introduction of $S^{D+}$by signaling drug availability may favour the search of the substance, and the contingent CS^{+} may subsequently have maintained drug-seeking behavior (Cervo et al., 2013; Weiss et al., 2001). Moreover, nicotine act not only as a primary reinforcer but also as a reinforcement enhancer as demonstrated in both preclinical (Caggiula et al., 2001; Chaudhri et al., 2006; Donny et al., 2003)and clinical studies (Perkins et al., 2017), this may explain the increased salience for nicotine-related stimuli.

A further interesting result of our study was that, even though eCET was carried out in "a more ethological way" (i.e. with the same stimuli presented in the same context), it was not sufficient to reduce the salience of nicotine-associated cues during the 14 days of treatment nor during the later reinstatement sessions. This was in line with clinical studies reporting that CET for tobacco cessation was less successful in promoting cessation when compared to other drug dependencies (Conklin and Tiffany, 2002; Bounton, 2009). By contrast, when eCET was performed in combination with repeated N-AC 100 mg/kg, nicotine-seeking behavior was reduced throughout the 14 treatment days and this anti-relapse action lasted for at least 50 days after treatment. One possible explanation may lie in the nicotine-induced alteration of GLUergic homeostasis in brain regions known to affect cue-induced reinstatement, thus impairing the ability to extinguish nicotine-related cues. Thus, it is possible that repeated N-AC treatment restores the "top-down" GLUergic control over seeking behaviors, promoting the extinction of nicotine-associated stimuli only when N-AC treatment is paired with the cues. This hypothesis is further supported by the lack of effect of chronic N-AC treatment when administered during abstinence and by the fact that chronic N-AC during LP-EXT only attenuated drug-seeking behavior. Supporting this interpretation, repeated N-AC treatment during extinction sessions after cocaine self-administration has previously demonstrated lasting anti-relapse activity (Knackstedt et al., 2010; Moussawi et al., 2011; Reichel et al., 2011). A second interpretation could be that nicotine has enhanced responding for the unconditioned reinforcing stimuli (S^{D+} and/or CS^{+}) and that N-AC-induced decrease of S^{D+}/CS^{+}responding during and after eCET, would not necessarily result from a facilitated extinction but rather by acutely reducing the reinforcing value of S^{D+}/CS^{+}. However, this seems to be unlikely since N-AC was found to still be effective 14 and 50 days after the end of the treatment and previous work has shown that chronic N-AC also reduced heroin (Zhou and Kalivas, 2008)and cocaine (Riechel et al., 2011) cue-induced seeking behavior.

To the best of our knowledge, this study describes the first attempt to dissect the long-term anti-relapse effect of repeated N-AC when given during eCET, rather than LP-EXT, on nicotine-seeking behavior. Our results seem

in agreement with those obtained after cocaine (Moussawi et al., 2011) and heroin (Zhou et al., 2008) self-administration.

To investigate the molecular correlates mediating the long-lasting anti-relapse effect of N-AC in combination with eCET treatment, we examined the expression of proteins associated with GLU homeostasis in the Nacc, since evidence exists of overlapping effects on GLU transmission in this brain region after extinction training (Myers et al., 2011) and after repeated N-AC (Knackstedt et al., 2010; Moussawi et al., 2011). First, even in this cohort of rats we replicated behavioral findings, showing that N-AC + eCET decreased cue-induced reinstatement during the treatment while maintaining anti-craving activity. Next, we sought to quantify changes in proteins crucial for GLU homeostasis as these are consistently altered across different drugs of abuse and might account for the lasting behavioral responses produced by drug-related cues (Scofield et al., 2016). Previous studies have reported a decrease in the expression of xCT (Knackstedt et al., 2009) and GLT-1 (Knackstedt et al., 2009; Gipson et al., 2013), and high expression of the GluN2B subunit of NMDARs (Gipson et al., 2013) in the Nacc after withdrawal from nicotine self-administration. xCT and GLT-1 were also lowered during withdrawal from cocaine (Madayag et al., 2007) and ethanol (Alhaddad et al., 2014) self-administration.

Interestingly, 7 days after eCET, we found lower expression of xCT and GLT-1 as well as higher expression of GluN2B in the Nacc shell of the nicotine self-administered rats when compared to naïve rats. Notably, this time point represents more than 21 days after the last nicotine self-administration session, thus suggesting a long-lasting effect of eCET. Repeated treatment with N-AC, with eCET, restored the GLUergic deficits mediated by nicotine self-administration. It is important to note that protein expression showed no significant changes in the Nacc core of the same animals. The fact that the expression of these proteins was different in the two subregions of the Nacc is not surprising since Nacc is a very complex area that mediates the reinforcing effect of drugs of abuse and integrates cognitive and affective information processed by cortical regions (Floresco, 2015). The Nacc shell receives GLUergic afferents from the infralimbic cortex and activation of this pathway promotes extinction (Peters et al., 2008) alongside attenuating cue-induced reinstatement of cocaine-seeking behavior (Augur et al., 2016). The Nacc core receives GLUergic afferents from the prelimbic cortex and this pathway is activated during cue-induced drug-seeking behavior (McFarland and Kalivas, 2001). Although the Nacc core is the region that has been implicated mostly in drug-seeking behavior (Kalivas, 2009), recently the Nacc shell has emerged as the subregion involved in reward devaluation and extinction of drug-related cues (West and Carelli, 2016). Thus, restored GLU homeostasis in the Nacc shell may account for the extinguishing/attenuation of the tendency of nicotine-associated cues to induce seeking behavior in rats treated with N-AC + eCET. In the present experiment, rats were not only treated with N-AC, but were also exposed

daily to eCET: accordingly, the results should be viewed as a combination of a pharmacological (N-AC) and behavioral (eCET) treatment.

Interestingly, 51 days after the end of the N-AC + eCET treatment (i.e. more than two months after the last nicotine self-administration session), the protein profile was drastically different in the Nacc with no nicotine-related changes present. In contrast, N-AC treated rats displayed a steep increase in mGluR2 and GLT-1 expression, perhaps as an attempt to tone down the increased GLU release.

The differences in protein expression at different time points suggest that behavioral and molecular results are not directly correlated. To the best of our knowledge, protein levels in the Nacc at chronic time points after nicotine self-administration and N-AC + eCET have not yet been investigated. It is possible that 7 days after the end of the treatment, nicotine-induced changes in protein expression were still present while the treatment was reversing them. Conversely, 51 days later, the over-expression of mGluR2 might counteract the increased GLU release in the Nacc caused by the activation of PFC afferents during cue-induced reinstatement. Moreover, the increased expression of GLT-1 in the Nacc shell might further prevent activation of post-synaptic receptors, thus blunting GLU transmission.

In conclusion, the present results support the validity of N-AC treatment innicotine addiction and identify eCET as a major contributor to the mechanisms that improve the outcome of N-AC in preventing relapse. In addition, taking into account that evidence for N-AC treatment for nicotine addiction in humans is still limited (Deepmala et al., 2015) and that, so far, human studies have evaluated the efficacy of repeated N-AC treatment during abstinence (Schmaal et al., 2011) or while the subjects were still taking nicotine (Grant et al., 2014; Knackstedt et al., 2009; McClure et al., 2014; Prado et al., 2015), our data pave the way for a novel approach of N-AC, in combination with CET, for clinical trials.

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Table 1.

		Self-administration sessions (mean of last 3 days)					1st Reinstatement test			
			Act. Lever Presses		Inact. Lever Presses		Act. Lever Presses		Inact. Lever Presses	
Experiments	Sessions to criteria	Number of Nic. Infusions	Nic	Sal	Nic	Sal	S^{D+}/CS^{+}	S^{D-}/CS^{-}	S^{D+}/CS^{+}	S^{D-}/CS^{-}
N-AC + experimental cue exposure therapy (eCET)										
Vehicle	20.3±0.2	13.9±2.5	34.7±5.5*	5.0±1.1	3.3±1.9	5.5±1.7	37.6±5.0*	7.8±0.6	6.4±1.5	8.1±3.0
N-AC 60 mg/kg	20.5±0.2	13.2±2.5	34.7±6.3*	8.1±2.1	1.6±0.4	3.8±1.3	29.3±5.7*	5.5±1.2	3.8±0.9	5.2±3.0
N-AC 100 mg/kg	20.2±0.2	13.5±2.2	33.3±6.2*	5.9±1.3	1.2±0.3	2.5±0.6	31.4±5.5*	5.4±1.4	3.4±1.3	2.4±1.2
N-AC + lever press-extinction (LP-EXT)										
Vehicle	21.5±0.2	16.4±2.1	56.8±9.4*	18.0±4.2	4.4±0.8	3.9±1.1	37.8±4.8*	6.1±1.5	3.3±0.6	4.9±1.5
N-AC 60 mg/kg	21.3±0.2	15.5±1.8	54.4±11.9*	14.5±3.7	5.3±1.9	6.2±1.9	38.4±6.6*	6.1±1.2	6.0±1.7	4.1±1.0
N-AC 100 mg/kg	21.1±0.4	13.8±2.2	58.0±13.3*	16.9±4.2	5.7±1.5	5.8±2.0	36.8±3.3*	6.1±1.2	5.9±1.5	4.9±2.8
N-AC + abstinence										
Vehicle	19.4±0.3	13.8±1.8	35.6±4.5*	6.1±0.6	5.5±1.2	3.6±1.6	33.3±5.5*	5.9±1.3	5.5±1.2	3.4±1.7
N-AC 60 mg/kg	19.7±0.2	15.2±1.5	37.8±3.2*	5.8±1.0	11.1±2.0	5.5±2.9	35.2±4.5*	8.5±2.2	6.6±3.1	3.8±1.8
N-AC 100 mg/kg	19.4±0.3	13.7±2.2	35.6±4.1*	6.3±1.1	6.5±3.1	5.1±1.1	43.0±5.8*	7.7±1.0	11.1±2.0	4.8±1.1
Proteins expression										
N-AC + cues-devaluation (7 d)										
Vehicle	20.3±0.2	14.6±1.4	37.8±4.4*	5.1±0.8	3.3±1.5	4.7±0.8	42.4±10.2*	6.2±1.2	8.4±3.2	4.6±1.0
N-AC 100 mg/kg	20.6±0.2	13.0±2.2	32.4±5.2*	5.3±1.3	2.2±0.5	2.3±0.5	32.9±5.0*	7.0±1.7	3.0±0.8	3.0±0.9
N-AC + cues-devaluation (51 d)										
Vehicle	20.3±0.2	13.7±2.1	35.1±5.0*	6.0±0.9	1.1±0.5	1.9±0.6	35.6±5.0*	7.0±1.1	1.6±0.4	2.0±0.7
N-AC 100 mg/kg	20.9±0.3	12.9±1.7	31.2±3.6*	5.1±0.7	2.4±0.5	2.4±0.3	30.9±2.8*	5.0±1.1	4.4±2.0	2.9±1.0

Table 1. ‼┼Data are expressed as means±S.E.M. The number of self-administration sessions required to meet the criteria (stable reinforced responding ±15% over 3 consecutive sessions), the mean of nicotine infusions, active and inactive lever presses in the last three days of self-administration sessions are reported. Twenty-four hours after the end of the self-administration training half the rats were exposed to stimuli predictive of (SD+) and associated with nicotine availability (CS+) and half to stimuli predictive of (SD-) and saline availability (CS-). The next day rats tested with SD+/CS+ were tested with SD-/CS-, and vice versa. Numbers of active and inactive lever presses during the 1st reinstatement test are reported for all the experimental groups‼┼* p<0.01 vs. respective saline or SD-/CS-, Newman-Keuls post hoc comparison‼┼

254x190mm (72 x 72 DPI)

Fig. 1

Moro et al

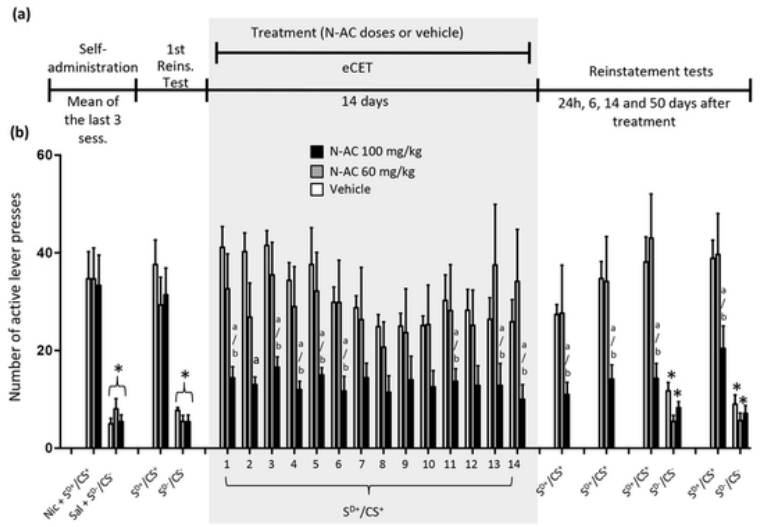


Figure 1. ‼┼Effects of chronic treatment with 60 mg/kg (n=6), 100 mg/kg (n=7) N-acetylcysteine (N-AC) or vehicle (n=8) during eCET on repeated reintroduction of stimuli predictive of (SD+) and associated with nicotine availability (CS+). (a) Time course of the experiment. After a first reinstatement test rats were treated daily during eCET. At the end of the treatment rats were tested with SD+/CS+ and SD-/CS- at different time points. (b) Mean±SEM number of presses on the active lever in a within/between-subject design. Also shown is the number of lever presses during self-administration training (mean±SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD-/CS-). ‼┼* P<0.05 vs. respective SD+/CS+, aP <0.05 vs. vehicle, bP <0.05 vs 60 mg/kg N-AC -treated group, Newman-Keuls post hoc comparison ‼┼

254x190mm (72 x 72 DPI)

Fig. 2

Moro et al

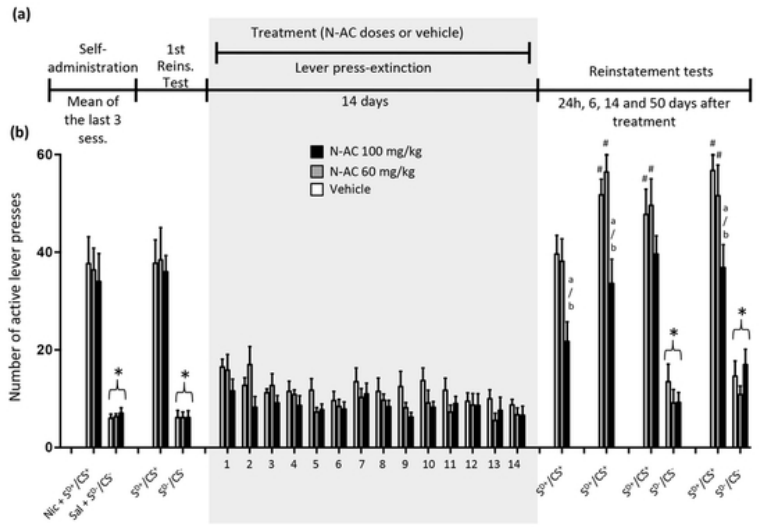


Figure 2. ‼┼Effects of chronic treatment with 60 mg/kg (n=7), 100 mg/kg (n=8) N-acetylcysteine (N-AC) or vehicle (n=8) during LP-EXT on repeated reintroduction of stimuli predictive of (SD+) and associated with nicotine availability (CS+). (a) Time course of the experiment. After a first reinstatement test rats were treated daily during LP-EXT. At the end of the treatment rats were tested with SD+/CS+ and SD+/CS+ at different time points. (b) Mean±SEM number of presses on the active lever in a within/between-subject design. Also shown is the number of lever presses during self-administration training (mean SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD-/CS-). ‼┼* P<0.05 vs. respective SD+/CS+, #P<0.05 vs. respective 1st SD+/CS+, aP <0.05 vs. vehicle, bP <0.05 vs 60 mg/kg N-AC-treated group, Newman-Keuls post hoc comparison‼┼

254x190mm (72 x 72 DPI

Fig. 3

Moro et al

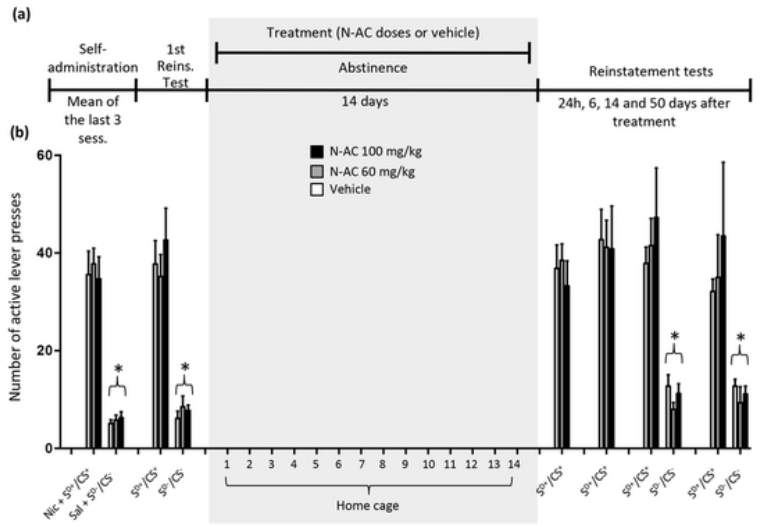
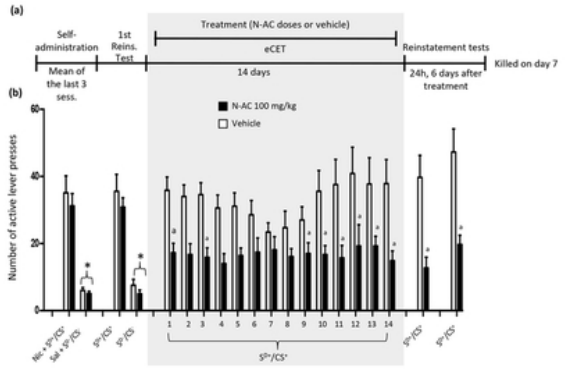


Figure 3.‼†Effects of chronic treatment with 60 mg/kg (n=6), 100 mg/kg (n=8) N-acetylcysteine (N-AC) or vehicle (n=8) during abstinence on repeated reintroduction of stimuli predictive of (SD+) and associated with nicotine availability (CS+). (a) Time course the experiment. After a first reinstatement test rats were treated daily during abstinence. At the end of the treatment rats were tested with SD+/CS+ and SD-/CS- at different time points. (b) Mean±SEM number of presses on the active lever in a within/between-subject design. Also shown is the number of lever presses during self-administration training (mean SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD-/CS-). ‼†*P<0.05 vs. respective SD+/CS+ Newman-Keuls post hoc comparison ‼†

254x190mm (72 x 72 DPI



(c)

Exp. Group	Brain Area	Protein expressions					
		GLT-1	xCT	mGluR2	GluN1	GluN2B	GluN2A
Naïve/Veh	Nacc-shell	100±5	100±7	100±39	100±10	100±4	100±10
	Nacc-core	100±8	100±8	100±25	100±10	100±2	100±8
Naïve/N-AC	Nacc-shell	111±9	106±17	135±45	109±16	105±8	109±11
	Nacc-core	103±13	118±12	106±28	109±15	90±12	84±14
nic/Veh	Nacc-shell	77±2#	99±16	86±19	136±17	136±11#	111±11
	Nacc-core	94±5	72±6	90±35	111±17	78±2	90±7
nic/N-AC	Nacc-shell	114±4***	91±12	69±5	121±10	103±8*	120±9
	Nacc-core	99±7	98±5	54±6	108±16	72±7	93±12

Figure 4. ‼┼Effects of chronic treatment with 100 mg/kg N-acetylcysteine (N-AC) (n=7) or vehicle (n=7) during eCET on repeated reintroduction of stimuli predictive of (SD+) and associated with nicotine availability (CS+). (a) Time course of the experiment. (b) Mean±SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean±SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD-/CS-). (c) Western blot analysis of rats killed 24h after the reinstatement test on day 6 after treatment [Naïve/Veh (n=6), Naïve/N-AC (n=6), Nic/Veh (n=7), Nic/N-AC (n=7). Behavioral data were analyzed by mixed-factorial ANOVA followed by Newman-Keuls test. Protein data were analyzed by two-way ANOVA followed by Tukey's test. ‼┼**P<0.05 vs. respective SD+/CS+, aP<0.05 vs. Veh. Panel c *P<0.05, and *** P<0.001 vs. Nic- Veh; # P<0.05 vs. Naïve-Veh‼┼

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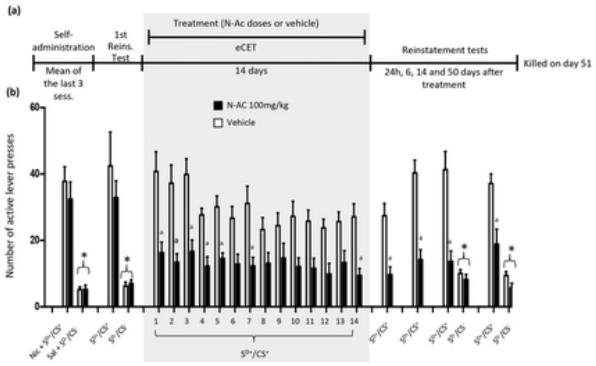

(c)

Exp. Group	Brain Area	Protein expressions					
		GLT-1	xCT	mGluR2	GluN1	GluN2B	GluN2A
Naïve/Veh	Nacc-shell	100±8	100±3	100±5	100±12	100±6	100±9
	Nacc-core	100±7	100±3	100±5	100±5	100±10	100±8
Naïve/N-AC	Nacc-shell	95±7	93±3	93±9	96±13	105±18	87±7
	Nacc-core	105±9	100±3	110±9	106±11	96±13	126±20
nic/Veh	Nacc-shell	93±3	100±6	96±11	90±8	97±9	86±7
	Nacc-core	105±7	85±5	76±6	108±8	101±9	93±6
nic/N-AC	Nacc-shell	115±5#	110±5	165±28*	84±11	112±5	111±7
	Nacc-core	105±6	92±10	154±25**	103±13	107±8	87±6

Figure 5.‼┼Effects of chronic treatment with 100 mg/kg N-acetylcysteine (N-AC) (n=9) or vehicle (n=9) during experimental eCET on repeated reintroduction of stimuli predictive of (SD+) and associated with nicotine availability (CS+). (a) Time course of the experiment. (b) Mean±SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean±SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD-/CS-). (c) Western blot analysis of rats killed 24h after the reinstatement test on day 50 after treatment [Naïve/Veh (n=6), Naïve/N-AC (n=6), Nic/Veh (n=9), Nic/N-AC (n=8)]. Behavioral data were analyzed by mixed-factorial ANOVA followed by Newman-Keuls test. Proteins data were analyzed by two-way ANOVA followed by Tukey's test.‼┼*P<0.05 vs. respective SD+/CS+, aP<0.05 vs. Veh. Panel c *P<0.05, and ** P<0.01 vs. Nic- Veh; # P<0.05 vs. Naïve-Veh‼┼

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