N-acetylcysteine treatment blocks the development of ethanol-induced behavioural sensitization and related ΔFosB alterations

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ABSTRACT

Ethanol addiction is a serious public health problem that still needs more effective pharmacological treatment. A key factor in the development and maintenance of this disease is the advent of neuroadaptations in the mesocorticolimbic brain pathway upon chronic ethanol abuse. In general, these neuroadaptations are maladaptive and affect numerous neurotransmitter systems and intracellular molecules. One of these molecules is ΔFosB, a transcription factor that is altered after chronic drug use. Behavioural sensitization is a useful model for the study of the neuroadaptations related to addiction. Recent works have shown a role for the imbalance of glutamatergic neurotransmission in the symptoms found in addicted people. In this sense, the treatment with N-acetylcysteine, a L-cysteine prodrug that acts by restoring extrasynaptic concentrations of glutamate through the activation of cystine-glutamate antiporter, has shown promising results in the treatment of addiction. Thus, an animal model of behavioural sensitization was used to evaluate the effects of N-acetylcysteine treatment in the behavioural and molecular alterations induced by chronic ethanol administration. Swiss mice were subject to 13 days of daily ethanol administration to induce behavioural sensitization. Two hours before each ethanol administration and locomotor activity evaluation, the animals received intraperitoneally N-acetylcysteine injections. Immediately after the last test session, their brains were removed for ΔFosB and cystine-glutamate antiporter quantification. It was found that N-acetylcysteine treatment blocked ethanol-induced behavioural sensitization, the increase of ΔFosB content in the prefrontal cortex, and its reduction in the nucleus accumbens. The results suggest a possible use of N-acetylcysteine in ethanol-related disorders.

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1. Introduction

Ethanol addiction is a progressive disease characterised by the loss of control over ethanol use and its continuous intake despite adverse consequences (Koob, 2009). Upon chronic ethanol use, key brain areas undergo maladaptive neuroadaptations, leading to an excessive and compulsive intake pattern and craving after the discontinuation of the use (Most et al., 2014).

The mesocorticolimbic pathway is critical for the identification of relevant stimuli. It is comprised by the dopaminergic neurons of the ventral tegmental area (VTA) that projects to both the nucleus accumbens (Acb) and the medial prefrontal cortex (mPFC). It is activated, for example, when an individual experiences natural reinforcing stimuli, such as sex or food (Adinoff, 2004). Drugs of abuse also activate the mesocorticolimbic pathway that, upon chronic use, might cause maladaptive neuroadaptations related to addiction. Dysregulations in the homeostasis of mPFC and Acb are crucial in the transition from recreational to uncontrolled use of psychoactive substances (Koob and Volkow, 2010). Another important brain pathway related to addiction through the control of habit formation is the nigrostriatal pathway. It is comprised by the dopaminergic neurons of substantia nigra (SN) and their connections to the caudate-putamen (CPu) nucleus (Everitt and Robbins, 2013).

ΔFosB is a stable, truncated splice variant of the FosB gene, a transcription factor that is synthesised when a neuron is activated (Kovács, 1998). Acute challenge with ethanol causes an induction of FosB protein, whereas chronic ethanol exposure leads to the

accumulation of ΔFosB (Perrotti et al., 2008; Ryabinin and Wang, 1998). The formation of this molecule is one of the mechanisms by which addictive drugs produce stable changes in the brain. Its accumulation increases the motivational properties and psycho-motor effects of abuse drugs (Nestler et al., 2001).

Behavioural sensitization is a phenomenon used to study drug-induced neuroadaptations. It consists of a progressive increase of the psychostimulant effects of psychoactive substances upon repeated administrations and reflects the neuroadaptations related to addiction that occur in the mesocorticolimbic brain pathway (Robinson and Berridge, 2008; Vanderschuren and Kalivas, 2000).

Recent data have suggested that alterations of the glutamatergic system could be responsible for the signals and symptoms found in addicted people. In this sense, studies have focused on the glutamatergic hypothesis of addiction, which suggests that an imbalance between synaptic and extrasynaptic glutamate, altering the control of synaptic glutamate release and neuronal excitability by metabotropic glutamate receptors, are, at least in part, responsible for addiction (Kalivas, 2009). In line with this, chronic ethanol exposure elevates extracellular levels of glutamate in the Acb and the excitatory amino acid neurotransmission during withdrawal, in addition to changes in glutamate receptors and transporters (Rao et al., 2015a). The projections of glutamatergic neurons from mPFC to Acb are related to relapse induced by stress, drug priming, and drug-paired cues (Kalivas and Volkow, 2005). Restoration of glutamatergic homeostasis through the re-establishment of the extrasynaptic glutamate tonus acts by regulating synaptic glutamate release and the excitability of Acb neurons. Drugs that alter extrasynaptic glutamate concentrations (e.g. Ceftriaxone [CEF], N-acetylcysteine [N-AC]) are interesting candidates for addiction treatment (Rao et al., 2015a; Reissner and Kalivas, 2010). Promising results are being obtained from both preclinical and clinical work regarding the use of N-AC in the treatment of psychiatric disorders, including addiction (Berk et al., 2013).

N-AC is a prodrug of L-cysteine clinically used as a mucolytic agent and for acetaminophen overdose (Dean et al., 2011). Once absorbed, N-AC is rapidly deacetylated to L-cysteine, which forms a cystine molecule by the formation of a disulfide bond between two L-cysteine molecules (Samuni et al., 2013). Besides being an anti-oxidant itself, it acts in the astrocytic cystine-glutamate antiporter (xCT), which transports one glutamate molecule to the extrasynaptic space at the same time as it transports one cystine to the intracellular space, increasing the extracellular glutamate concentrations (Karila et al., 2008). Preclinical and clinical data have shown some efficacy of N-AC against cocaine and nicotine addiction. N-AC treatment blocked the reinstatement of cocaine seeking in rats (Baker et al., 2003), as well as prevented behaviours caused by repeated cocaine administration, including escalation of cocaine intake and behavioural sensitization (Madayag et al., 2007). Nicotine withdrawal syndrome in rats is also attenuated by N-AC (Knackstedt et al., 2009). In humans, N-AC treatment in cocaine addicts decreased the desire for cocaine use, the interest in cues related to cocaine use (LaRowe et al., 2007), and the number of smoked cigarettes in nicotine addicts (Knackstedt et al., 2009). There are a few studies in the literature regarding N-AC and alcoholism, but none about ethanol-induced behavioural sensitization. The treatment with this prodrug blocked ethanol withdrawal and neuroendocrine alterations induced by chronic ethanol consumption in rats (Schneider et al., 2015; Seiva et al., 2009).

Considering the importance of new strategies for the treatment of ethanol addiction and the promising data regarding the use of N-AC in the treatment of neuropsychiatric disorders through its action on glutamate homeostasis, the present study evaluated the effect of N-AC on the development of ethanol-induced behavioural sensitization and the changes in ΔFosB and xCT content related to ethanol addiction.

2. Experimental procedure

2.1. Subjects

Sixty male Swiss mice (36–45 g at the beginning of the experiments) were obtained from the animal breeding facility of the Univ Estadual Paulista (UNESP) (Botucatu, SP, Brazil). They were transferred to our animal facility at least 7 days before the start of the experiments and housed within groups of 4 or 5 per cage (33 × 15 × 13 cm). The animals were maintained in a room at a temperature of 23 ± 2 °C on a 12:12 h light/dark cycle (lights on at 7 a.m.) with water and food access *ad libitum*. All experiments were performed during the light phase of the cycle, and animals were randomly tested across this time period (12 p.m.–5 p.m.). The experimental protocol was approved by the university ethics committee for animal care and use (CEUA/FCFAr 33/2014), and the experiments were conducted according to the principles of the National Council for Animal Experiments Control (CONCEA), based on NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. Drugs

N-Acetylcysteine (N-AC) (Sigma Aldrich, St. Louis, MO, USA) was diluted in physiological saline (6 or 12 mg/mL) and administered intraperitoneally (i.p.) (0.1 mL/10 g) at the doses of 60 and 120 mg/kg. Ethanol 99.8% (Labsynth, Diadema, SP, BR) was diluted in saline (20% vol/vol) and administered i.p. (0.125 mL/10 g) at the dose of 2.0 g/kg.

2.3. Behavioural sensitization

This protocol was recently standardised in our lab based on that described by Bahi and Dreyer (2012) and evaluated the development of the sensitization in mice to the ethanol-induced psychostimulation measured in the open field (OF) apparatus. The OF is a black-floor, circular arena, 26 cm in diameter, surrounded by opaque walls that are 30 cm high.

The procedure lasted for 15 days (Fig. 1A) as follows: during the first 2 days (H1-2), all animals received physiological saline i.p. and were placed in the OF for locomotor evaluation for 20 min. This was called the habituation phase and was necessary to eliminate the effect of novelty. Then, the animals underwent the sensitization phase, which lasted 13 days (S1-13). They received ethanol (2 g/kg, i.p.) or vehicle (NaCl 0.9%, control groups, i.p.) daily, after which they were immediately placed in the OF for behavioural evaluation for 20 min. Two hours before each saline or ethanol injection, the animals received N-AC (60 or 120 mg/kg, i.p.) or vehicle (NaCl 0.9%, control groups, i.p.). During habituation (H1-2) and days S1, S5, S9, and S13, distance travelled (in metres) by the animals in the OF was evaluated by a camera connected to a computer running the behavioural analysis software ANYmaze (Stoelting Co., Wood Dale, IL, USA).

2.4. Brain area dissection

Immediately after the last test session (S13), the animals were decapitated, and the brains were rapidly removed from the skull (less than 2 min) and frozen in methyl-butane on dry ice and then kept at −80 °C until dissection. In a cryostat at −20 °C, brains were coronally sectioned to find target areas according to stereotaxic coordinates from the Atlas of Paxinos and Franklin (2001) as follows: mPFC, rostral face, +2.34 mm from Bregma; Acb, rostral face, +1.54 mm from Bregma; CPu, rostral face, +1.10 mm from Bregma. Samples of 1 mm thickness were then removed with a flat-tipped needle of 1.4 mm internal diameter.

2.5. Western blotting

After dissection, samples were sonicated in 1% sodium dode-cylsulfate (SDS), and the protein concentration was determined by Bio-Rad DC protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). Thirty micrograms of protein of each sample were boiled for 5 min in a loading buffer containing 5% of β-Mercaptoethanol and 0.002% of Bromophenol Blue and subjected to electrophoresis in 9% SDS-polyacrylamide minigels for 50—60 min at 200 V using the Bio-Rad Mini-PROTEAN tetra cell with Bio-Rad Powerpac basic supply (Bio-Rad Laboratories Inc.). Proteins were electrophoretically trans-ferred to low-fluorescence polyvinylidene fluoride membranes (LF-PVDF) using the Trans-Blot Turbo system (Bio-Rad Laboratories Inc.).

The membranes were then incubated in blocking buffer con-taining 5% of non-fat milk in T-TBS (0.05% of tween-20 in tris-buffered saline) at room temperature. After that, membranes were washed three times and incubated overnight at 4 °C in T-TBS containing the primary antibodies against FosB/ΔFosB (1:500, cat. No. 102: sc-48, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA, 35/37 kDa) or xCT (1:1000, cat. No. ab37185, Abcam Inc., Cam-bridge, MA, USA, 35 kDa). Membranes were washed again and then incubated for 1 h at room temperature in T-TBS containing the anti-rabbit secondary antibody Cy3 (1:2000, cat. No. 28901107, GE Heathcare, Little Chalfont, BUX-ENG). Bands fluorescence was assessed directly in dry membrane using a TyphoonTrio® scanner and quantified using Image Quant TL software (GE Heathcare). The amount of ΔFosB and xCT was expressed as % relative to the control group (saline/saline). Loading control was performed by staining PVDF membranes with Coomassie blue followed by quantification of total protein in the entire lane.

Two points are important to be highlighted. First, xCT detection was done at a molecular weight different from that predicted by the manufacturer (55 kDa). The use of adult brain tissue and the addition of β-Mercaptoethanol shift the band to a molecular weight of 35 kDa (Shih et al., 2006), which was used here for xCT quanti-fication. Finally, the antibody used to detect ΔFosB also detects FosB protein. However, the time point chosen for brain removal (20 min after ethanol administration) is too short to induce any significant protein translation, including FosB. Another point is that the splicing that occurs in FosB mRNA originates a lighter protein, with ΔFosB detected at 35 and 37 kDa and FosB at 46—50 kDa (Kovács, 1998; Nestler et al., 2001). In our study, only the protein detected at 35/37 kDa was quantified. It appears as a single band probably because samples were run in minigels and 35 and 37 kDa ΔFosB bands are very close to each other. Thus, our quantification was specific for the chronic Fos-related antigen ΔFosB.

2.6. Statistics

Statistical analysis was performed using Statistica software 13 (Dell Inc., Round Rock, TX, USA), and graphs were made using GraphPad Prism 5 software (GraphPad software Inc., La Jolla, CA, USA). All values were expressed as mean +SE. Data of the habituation and sensitization phases of the behavioural sensitization protocol were analysed by three-way analysis of variance (ANOVA) considering the factors ethanol (ethanol × saline), treatment (N-AC × vehicle) and days (H1 × H2 or S1 × S5 × S9 × S13). Data of western blotting were analysed by two-way ANOVA considering the factors ethanol and treatment. In cases where ANOVA showed significant differences ($p \leq 0.05$), the Duncan post hoc test was performed.

3. Results

Habituation to the novelty of the OF was seen in the second day of the experiment, as shown by decreased locomotor activity on day H2 relative to H1 (Fig. 1B). The three-way ANOVA showed the main effect for the factor days ($F_{1,54} = 68.68$, $p < 0.001$). Thus, animals were habituated to the OF before the beginning of the sensitization phase.

N-AC treatment and ethanol administration altered mice loco-motor activity (Fig. 1B). The three-way ANOVA showed effect for ethanol factor ($F_{1,54} = 25.91$, $p < 0.001$), treatment factor ($F_{2,54} = 5.41$, $p < 0.01$), days factor ($F_{3,162} = 8.10$, $p < 0.001$) and interaction for ethanol and treatment factors ($F_{2,54} = 3.39$, $p < 0.05$), ethanol and days ($F_{3,162} = 2.61$, $p < 0.05$), and for all the factors ($F_{6,162} = 2.39$, $p < 0.05$). Duncan post hoc test revealed that vehicle/ethanol mice showed an increase in distance travelled in OF after acute ethanol administration when compared to vehicle/sa-line mice (S1 day, $p < 0.05$).

The treatment with N-AC was able to block ethanol-induced psychostimulation after an acute dose of ethanol. The N-AC 120 mg/kg/ethanol group did not show increased locomotor ac-tivity after acute ethanol administration when compared to the vehicle/saline group (S1 day, $p = 0.39$), whereas the N-AC 60 mg/kg/ethanol group showed a trend to increase its locomotor activity when compared to the vehicle/saline group (S1 day, $p = 0.09$). The treatment with N-AC at both doses did not alter the locomotor activity of the animals per se (followed by saline injection) on any of the days (S1, S5, S9, and S13 days, $p > 0.1$ compared to vehicle/saline group).

The vehicle/ethanol and N-AC 60 mg/kg/ethanol groups devel-oped ethanol behavioural sensitization. The vehicle/ethanol group showed increased locomotor activity on days S5, S9, and S13 rela-tive to its locomotion on day S1 (S1 × S5, $p \leq 0.05$; S1 × S9, $p < 0.05$; S1 × S13, $p \leq 0.001$). The N-AC 60 mg/kg/ethanol group showed increased locomotor activity on days S5, S9, and S13 rela-tive to its locomotion on day S1 (S1 × S5, $p \leq 0.001$; S1 × S9, $p < 0.001$; S1 × S13, $p < 0.05$). The N-AC 120 mg/kg/ethanol group did not develop ethanol behavioural sensitization, as shown by its similar distance travelled on days S1, S5, S9, and S13 (S1 × S5, $p = 0.70$; S1 × S9, $p = 0.48$; S1 × S13, $p = 0.61$), showing that the treatment with N-AC 120 mg/kg blocked the development of ethanol-induced behavioural sensitization.

Considering that only N-AC treatment at the highest dose blocked the development of ethanol-induced behavioural sensiti-zation, the groups that received N-AC at the dose of 60 mg/kg were not used for western blotting. Western blotting analysis showed that in the mPFC, N-AC treatment blocked ethanol-induced in-crease on ΔFosB content (Fig. 2). The two-way ANOVA showed a significant effect of ethanol factor ($F_{1,25} = 5.92$, $p < 0.05$) and treatment factor ($F_{1,25} = 5.28$, $p < 0.05$). The Duncan post hoc test revealed that the vehicle/ethanol group showed increased ΔFosB content relative to the vehicle/saline ($p < 0.05$), N-AC/saline ($p < 0.01$), and N-AC/ethanol ($p < 0.05$) groups. No changes were found in the N-AC/saline ($p = 0.46$) and N-AC/ethanol groups ($p = 0.92$) relative to the vehicle/saline group. xCT content in the mPFC was not altered in any of the experimental groups (Fig. 2).

In the Acb, N-AC treatment blocked ethanol-induced decrease on ΔFosB content (Fig. 3). The two-way ANOVA showed a signifi-cant effect of the interaction between ethanol and treatment fac-tors ($F_{1,29} = 5.36$, $p < 0.05$). The vehicle/ethanol group showed a decrease in ΔFosB relative to the vehicle/saline group ($p < 0.05$), with no changes when compared to the N-AC/saline group ($p = 0.77$) and the N-AC/ethanol group ($p = 0.23$). No changes were found in the N-AC/saline group ($p = 0.07$) and the N-AC/ethanol group ($p = 0.36$) relative to the vehicle/saline group. xCT in the Acb content was not altered in any of the experimental groups (Fig. 3).

All the groups showed decreased ΔFosB content in the CPu relative to the vehicle/saline group (Fig. 4). The two-way ANOVA revealed a significant effect of ethanol factor ($F_{1,28} = 6.97$, $p < 0.01$)
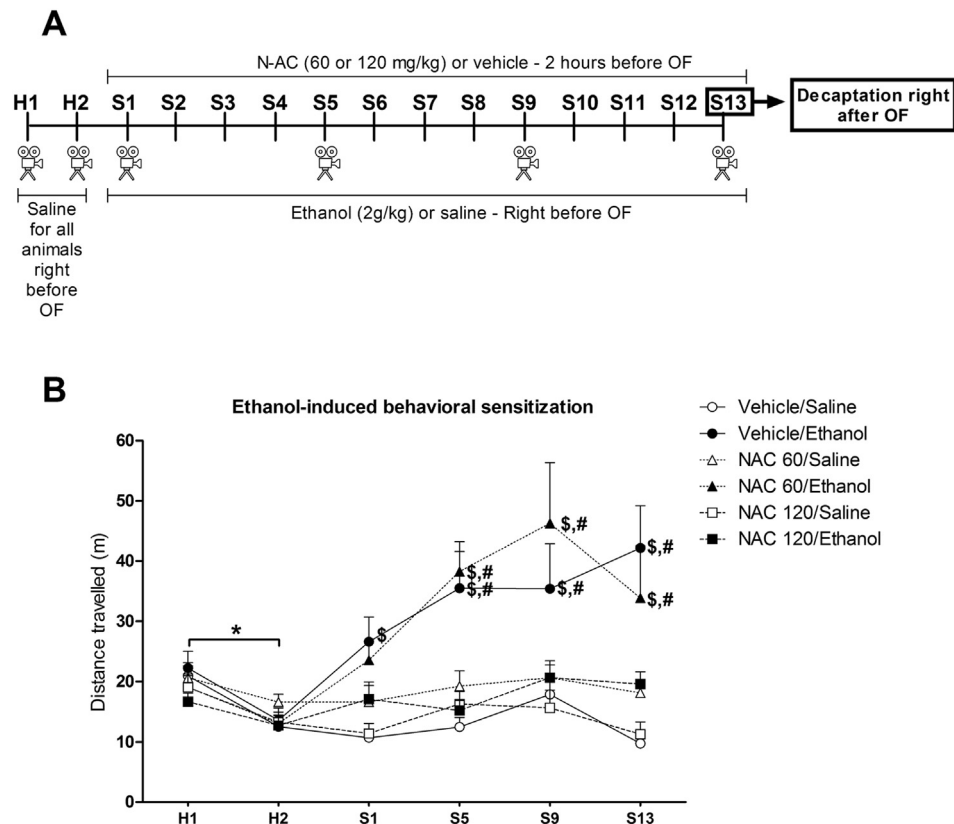
Fig. 1. Experimental procedure and ethanol-induced behavioural sensitization. A, experimental procedure timeline. Animals underwent the behavioural sensitization protocol for 15 days, and the locomotor activity was evaluated in the days marked with a camera drawing. Two hours before ethanol administration (2 g/kg) in the sensitization phase (days S1-13), animals received an i.p. injection of N-AC (60 or 120 mg/kg) or vehicle. H1-2, habituation days; S1-13, sensitization days; OF, open field; N-AC, n-acetylcysteine. B, effect of the N-AC treatment on ethanol-induced behavioural sensitization. Points represent means + SE of the distance travelled in the apparatus (N = 10 animals per group). *, p < 0.05 when comparing H1 × H2; $, p < 0.05 relative to the vehicle/saline group; #, p < 0.05 relative to the same group on day S1.

and a trend of interaction between ethanol and treatment factor ($F_{1,28} = 3.21$, p = 0.08). The *post hoc* test showed that the vehicle/ethanol (p < 0.01), N-AC/saline (p < 0.05), and N-AC/ethanol groups (p < 0.01) had decreased ΔFosB content relative to the vehicle/saline group. Regarding xCT in the CPu, two-way ANOVA showed a trend of significant effect of ethanol factor ($F_{1,29} = 3.78$, p = 0.06) inducing a decrease in xCT content in the ethanol- and N-AC-treated groups (Fig. 4).

4. Discussion

Our results suggest a possible use of N-AC in ethanol-related disorders. The treatment with N-AC blocked the development of ethanol-induced behavioural sensitization and related molecular changes in two important brain areas intrinsically related to addiction: mPCF and Acb.

As postulated by the incentive-sensitization theory (Robinson and Berridge, 1993), the behavioural sensitization paradigm offers a valuable method to study the neuroadaptations associated with craving and relapse, which are key factors in the maintenance of addiction. These neuroadaptations appear only in susceptible individuals upon chronic drug exposure and lead to the main characteristics of addiction: the loss of control over the drug use and the continuous use despite adverse consequences (Robinson and Berridge, 2008). In line with this theory, sensitized animals show enhanced ethanol consumption (Abrahao et al., 2013; Lessov et al., 2001) and an increased basal firing rate of dopaminergic neurons (Didone et al., 2015).

The chronic ethanol administration in our study induced ΔFosB accumulation in the mPFC and behavioural sensitization. Behavioural sensitization to opioids (Kaplan et al., 2011), methamphetamine (McDaid et al., 2006), and even sodium depletion (Hurley et al., 2014) also induces FosB/ΔFosB accumulation in various brain regions. However, FosB/ΔFosB alterations are not universally correlated to behavioural alterations. Different mice strains have distinct FosB/ΔFosB responses with the same behavioural response to amphetamine (Conversi et al., 2011).

The alterations in the ΔFosB signalling pathway found in mPFC and Acb could be responsible for the behavioural alterations induced by ethanol. Indeed, according to Li et al. (2010), increased voluntary ethanol consumption is correlated to the accumulation of FosB/ΔFosB in the core of the Acb. According to these same authors, naltrexone treatment blocked this protein accumulation and decreased voluntary ethanol consumption (Li et al., 2010). Similarly, electroacupuncture treatment decreases voluntary ethanol consumption and blocks the accumulation of FosB/ΔFosB in the Acb, mPFC, and VTA (Li et al., 2012). Cytisine, a neuronal nicotinic acetylcholine receptor partial agonist, modulates ethanol intake and FosB/ΔFosB expression in striatum (Sajja and Rahman, 2013). Increased ΔFosB expression in the Acb during adulthood increases the locomotor and rewarding effects of cocaine (Colby et al., 2003; Kelz et al., 1999). Thus, FosB/ΔFosB alterations may well have a role in behavioural changes induced by drugs of abuse, and their blockade prevents behavioural changes.

In our study, only mPFC showed an accumulation of ΔFosB after repeated ethanol treatment. In the Acb and CPu, we found

Fig. 2. Effect of the *N*-AC treatment on the expression of ΔFosB and xCT in the medial prefrontal cortex (mPFC). Twenty min after the last ethanol administration in the behavioural sensitization protocol, the animals were decapitated, and the mPFC were removed for western blotting analyses. A, quantification of ΔFosB and xCT proteins. Bars represent means + SE of the % protein expression relative to the vehicle/saline group (n = 7—9 animals per group). Representative blots of each group are shown below each bar. *N*-AC, *n*-acetylcysteine; xCT, cystine/glutamate antiporter; *, $p < 0.05$ relative to the vehicle/saline group; #, $p < 0.05$ relative to the *N*-AC/ethanol group. B, coronal section of the extraction region of the mPFC for western blotting according to the atlas of stereotaxic coordinates of Paxinos and Franklin (2001). The black circle represents the extraction site. Cg1, cingulate cortex area 1; Prl, prelimbic cortex.

Fig. 3. Effect of the *N*-AC treatment on the expression of ΔFosB and xCT in the nucleus accumbens (Acb). Twenty min after the last ethanol administration in the behavioural sensitization protocol, the animals were decapitated, and the Acb were removed for western blotting analyses. A, quantification of ΔFosB and xCT proteins. Bars represent means + SE of the % protein expression relative to the vehicle/saline group (n = 7—9 animals per group). Representative blots of each group are shown below each bar. *N*-AC, *n*-acetylcysteine; xCT, cystine/glutamate antiporter; *, $p < 0.05$ relative to the vehicle/saline group. B, coronal section of the region of extraction of the Acb for western blotting according to the atlas of stereotaxic coordinates of Paxinos and Franklin (2001). The black circles represent the extraction sites. AcbSh, nucleus accumbens shell part; AcbC, nucleus accumbens core part; aca, anterior commissure.

decreased ΔFosB content in vehicle/ethanol-treated animals. A possible explanation arises from the neuronal ensembles theory of addiction. This relatively new theory states that the learned associations between drug and environment occur only in a minority of neurons in a brain area related to drug stimuli (Cruz et al., 2014). Thus, since behavioural sensitization is a process that is dependent on drug/environment associations (Koya et al., 2009; Marin et al., 2009; Mattson et al., 2007; Zancheta et al., 2012), only a small number of neurons is repeatedly activated by chronic drug administration. In our case, the use of the western blotting technique to evaluate ΔFosB accumulation did not allow us to differentiate activated neurons from non-activated neurons. Most studies that have found accumulation of this molecule used immunohistochemistry in brain slices instead of western blotting of tissue homogenate, which could explain our contradictory results. Indeed, the levels of an activated transcription factor, p-CREB, in the Acb are diminished in cocaine-sensitized animals when using western blotting and increased when using immunohistochemistry and counting the number of positive neurons for p-CREB (Marin et al., 2009). So, we hypothesised that the depressor effects of ethanol in the majority of Acb and CPu neurons masked the accumulation of ΔFosB in the minority of neurons that are related to behavioural sensitization. In the mPFC, the number of activated neurons probably was big enough to overcome the ethanol depressor effect in the majority of neurons (Pfarr et al., 2015; Whitaker et al., 2015).

Accumulation of ΔFosB has been found after chronic ethanol treatment (Lobo et al., 2013) and after protocols of ethanol-induced behavioural sensitization (De Pauli et al., 2014). Although at first sight these results appear to be contrary to ours, these findings help in the interpretation of our data. Lobo et al. (2013) found that only a specific group of striatal neurons (D1 medium spiny neurons) shows ΔFosB accumulation after chronic ethanol consumption. De Pauli et al. (2014) showed that 5 days of withdrawal are necessary for ΔFosB induction in the Acb, and this is restricted to animals that were resistant to ethanol-induced locomotor sensitization. Thus, in our study, once the animals showed locomotor sensitization and our protocol did not allow us to differentiate specific groups of neurons, no ΔFosB accumulation was predicted in Acb.

ΔFosB signalling pathways appear to have a role in numerous psychiatric disorders. Its accumulation after chronic stimuli is differently associated with behavioural outcomes, and it is also different depending on the studied brain area (Nestler et al., 1999). For example, mice exposed to chronic social defeat stress protocol can be classified as resilient or susceptible, and each phenotype presents different behavioural alterations (Golden et al., 2011). Accumulation of ΔFosB is seen in the Acb of resilient animals (Vialou et al., 2010b) and in the mPFC of susceptible mice (Vialou et al., 2014). Overexpression of ΔFosB in Acb promotes stress resilience and is correlated to antidepressant action (Donahue et al., 2014; Vialou et al., 2010a, 2010b). Also, ΔFosB induction in mPFC by
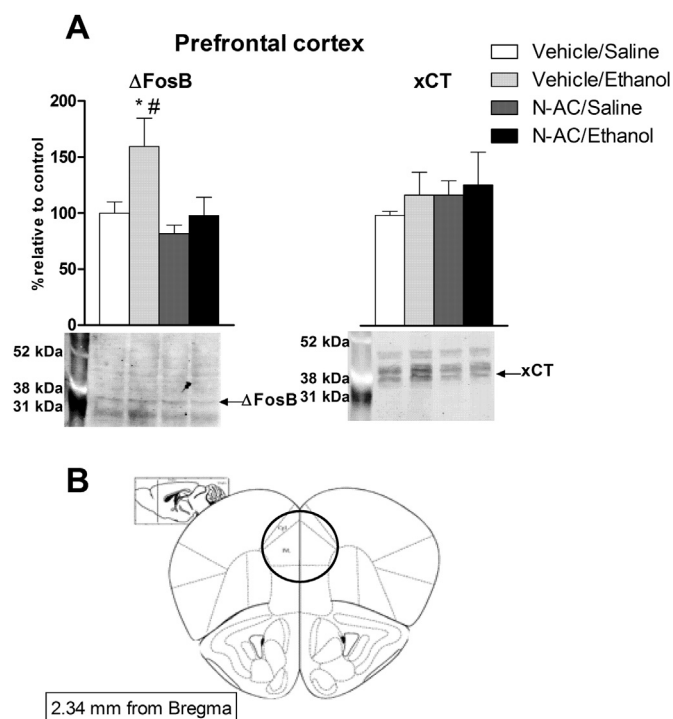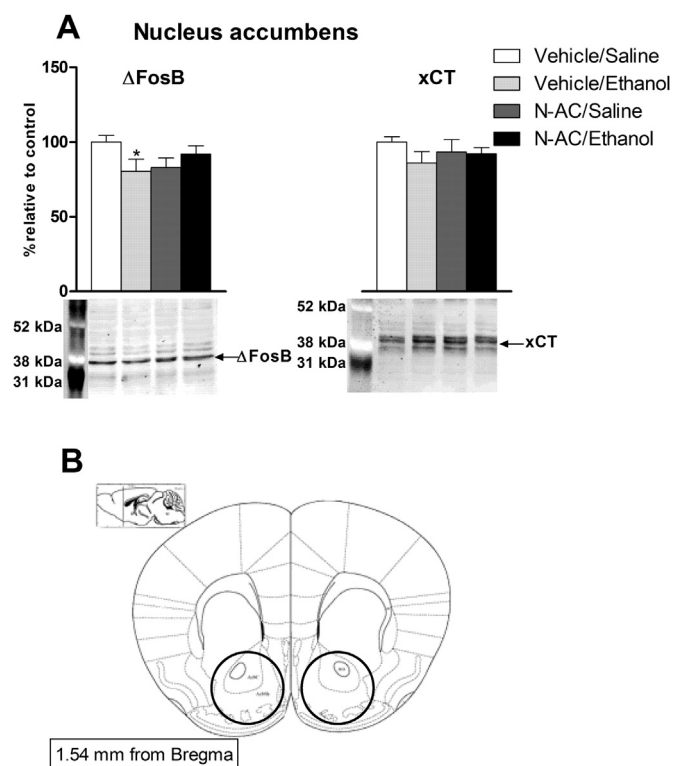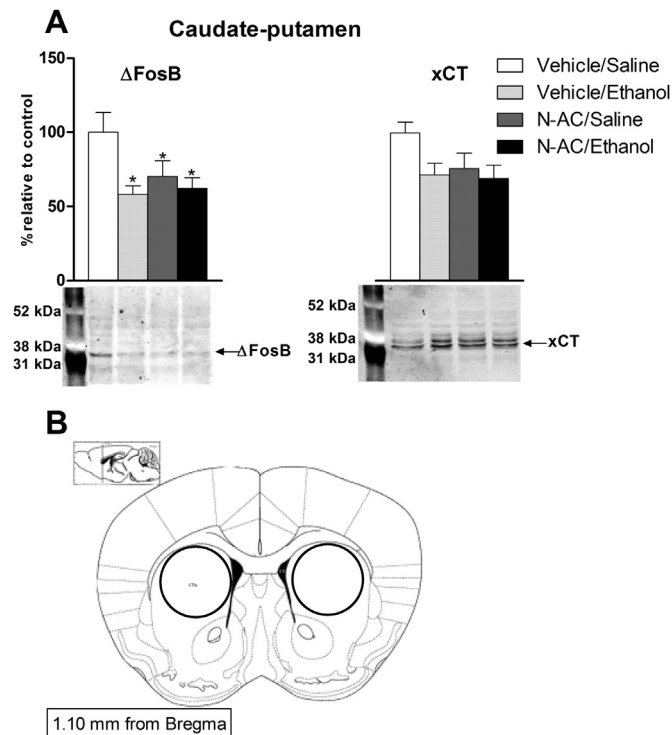
Fig. 4. Effect of the *N*-AC treatment on the expression of ΔFosB and xCT in the caudate-putamen (CPu). Twenty min after the last ethanol administration in the behavioural sensitization protocol, the animals were decapitated, and the CPu were removed for western blotting analyses. A, quantification of ΔFosB and xCT proteins. Bars represent means + SE of the % protein expression relative to the vehicle/saline group (n = 7–9 animals per group). Representative blots of each group are shown below each bar. *N*-AC, *n*-acetylcysteine; xCT, cystine/glutamate antiporter; *, p < 0.05 relative to the vehicle/saline group. B, coronal section of the region of extraction of the CPu for western blotting according to the atlas of stereotaxic coordinates of Paxinos and Franklin (2001). The black circles represent the extraction sites.

antipsychotic drugs is correlated to cognitive deficits (Dietz et al., 2014). Transposing these results to our findings, the accumulation of ΔFosB in the mPFC and the decreased expression in the Acb could be markers of addiction vulnerability in our sensitized animals. These alterations could also be responsible for the behavioural alterations found in our study.

Some studies suggest the hypoactivity of the Acb as a contributor to cocaine addiction. Preclinical and clinical findings have demonstrated decreased Acb activity after repeated cocaine exposure. Cocaine exposure results in differential neuroplasticity in neurons that receive strong excitatory inputs and in neurons that do not (Peoples et al., 2007). Although the contribution of this hypoactivity to addiction is not clearly elucidated, it could be an important factor for addiction and for the development of behavioural sensitization in our study. Independent from the direction of molecular alteration (increase or decrease), *N*-AC treatment blocked the behavioural alteration caused by chronic ethanol exposure and the molecular neuroadaptation on the ΔFosB pathway in two important regions for addiction: mPFC and Acb. This result shows that our treatment prevents ethanol-induced behavioural sensitization through the prevention of the maladaptive neuroadaptations that occur in these brain regions.

The effects of *N*-AC are, presumably, by the restoration of extrasynaptic glutamatergic tonus on metabotropic glutamate receptors, especially mGluR2/3, within the *N*-AC. First, chronic ethanol exposure increases extracellular glutamate levels in several brain areas involved in addiction (Ding et al., 2013; Hermann et al.,

2012; Melendez et al., 2005). The expression of the glutamate transporters GLT-1 and xCT, important proteins in the maintenance of glutamatergic homeostasis, is downregulated in ethanol-preferring rats (P rats) after chronic ethanol consumption. The treatment with CEF, which acts by restoring glutamate uptake, attenuates ethanol intake and upregulates GLT-1 and xCT (Alhaddad et al., 2014). In this same rat strain, the chronic treatment with CEF, ampicillin, cefazolin, cefoperazone, and the synthetic compound (R)-(−)-5-methyl-1-nicotinoyl-2-pyrazoline upregulates GLT-1 and xCT and prevents increases in ethanol intake (Aal-Aaboda et al., 2015; Alasmari et al., 2015; Rao and Sari, 2014; Rao et al., 2015b). *N*-AC prevents cocaine relapse in rats, an effect which is related to restoration of xCT activity in the Acb (Baker et al., 2003). *N*-AC also reverts cocaine-induced synaptic plasticity (Madayag et al., 2007) and cocaine-induced synaptic potentiation through restoration of mGluR2/3 tonus (Moussawi et al., 2011).

Some alterations of the glutamatergic neurotransmission caused by chronic drug abuse could be mediated by ΔFosB. For example, this molecule increases the expression of AMPA receptor subunit GluA2 (Kelz et al., 1999). Based on this, we suggest that ΔFosB accumulation in the mPFC would lead to alterations in AMPA receptors within this region, contributing to ethanol-induced behavioural sensitization, and the blockade of these alterations by *N*-AC treatment could prevent behavioural sensitization.

The use of L-cysteine (product of the *N*-AC metabolism) in preclinical models also supports our work. This drug blocked ethanol and acetaldehyde conditioned place preference (Peana et al., 2009), ethanol self-administration and relapse (Peana et al., 2010), ethanol-induced dopamine release in Acb (Sirca et al., 2011) and ethanol-seeking, relapse, and correlated neuroadaptations (Peana et al., 2013). In this series of studies, authors also discuss the ability of L-cysteine as an acetaldehyde-sequestering agent, another possible effect of *N*-AC.

Apparently, prolonged periods of ethanol exposure are necessary to elicit changes in xCT expression. Some authors have found decreased levels of xCT expression after at least three weeks of chronic ethanol exposure (Alhaddad et al., 2014; Peana et al., 2014). In our study, we did not find alterations in xCT content, probably due to our relatively short period (less than two weeks) of ethanol exposure.

Recent work has focused on glutamatergic homeostasis as a target for the treatment of ethanol addiction. Exciting preclinical results have emerged using mainly antibiotics that also act in glutamate reuptake as possible treatments against alcoholism. Although promising, the clinical use of antibiotics is dangerous due to the selection of resistant bacterial strains (Wang and Chou, 2013). On the other hand, *N*-AC is a safe and well-tolerated drug that has typically been used in clinics since 1978. Thus, the positive results presented here provide support for future studies evaluating the use of *N*-AC in the treatment of ethanol addiction.

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