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N-acetylcysteine attenuates nicotine-induced kindling in female periadolescent rats

Adriana Mary Nunes Costa Okamura, Patrícia Xavier L. Gomes, Gersilene V. de Oliveira, Fernanda Yvelize R. de Araújo, Viviane S. Tomaz, Adriano José Maia Chaves Filho, Francisca Cléa F. de Sousa, Silvânia Maria Mendes Vasconcelos, David Freitas de Lucena, Danielle Macêdo *

Drug Research and Development Center, Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Fortaleza, CE, Brazil

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ABSTRACT

Kindling is a form of behavioral sensitization that is related to the progression of several neuropsychiatric disorders such as bipolar disorder. We recently demonstrated that female periadolescent rats are more vulnerable to nicotine (NIC)-induced kindling than their male counterparts. Furthermore, we evidenced that decreases in brain antioxidative defenses may contribute to this gender difference. Here we aimed to determine the preventive effects of the antioxidant N-acetyl cysteine (NAC) against NIC-kindling in female periadolescent rats. To do this female Wistar rats at postnatal day 30 received repeated injections of NIC 2 mg/kg, i.p. every weekday for up to 19 days. NAC90, 180 or 270 mg/kg, i.p. was administered 30 min before NIC. The levels of glutathione (GSH), superoxide dismutase (SOD) activity, lipid peroxidation (LP) and nitrite were determined in the prefrontal cortex (PFC), hippocampus (HC) and striatum (ST). The development of kindling occurred at a median time of 16.5 days with 87.5% of NIC animals presenting stage 5 seizures in the last day of drug administration. NAC270 prevented the occurrence of kindling. NIC-kindled animals presented decreased levels of GSH and increased LP in the PFC, HC and ST, while SOD activity was decreased in the ST. NAC180 or 270 prevented the alterations in GSH induced by NIC, but only NAC270 prevented the alterations in LP. Nitrite levels increased in the ST of NAC270 pretreated NIC-kindled animals. Taken together we demonstrated that NAC presents anti-kindling effects in female animals partially through the restoration of oxidative alterations.

1. Introduction

Smoke is highly addicting and exerts deleterious effects on the health of the fetus, newborn, child, and adolescent (Prokhorov et al. 2006; Rogers 2008). Adolescence is a period of increased vulnerability to the effects of drugs due to the numerous and dynamic alterations in the central nervous system, such as, synapse plasticity and elimination, maturation of numerous neurochemical systems and continuing myelination (Rakic et al. 1986; Lenroot and Giedd 2006).

Nicotine (NIC), the major substance from tobacco, is responsible for the addictive effects (Potts and Daniels). The effects of NIC are influenced by age and gender. This is reinforced by preclinical studies demonstrating that periadolescent rats are more vulnerable to the behavioral and neurochemical effects of NIC (Adriani et al. 2003). In line with this evidence, periadolescent mice when exposed to repeated NIC administration presented exacerbation of ethanol withdrawal seizures during adulthood (Riley et al. 2010). Differences in the subtypes and number of NIC acetylcholine receptors (nAChRs) helps to explain this age-related vulnerability to NIC's effects (Dwyer et al. 2009).

Regarding gender differences, NIC dependence rates are higher among females than males (31.6% compared with 27.4%) (Kandel and Chen 2000). The mechanisms underlying gender differences to NIC's effects remain elusive. Differences in the sensitivity to nicotine (Kandel and Chen 2000), nicotinic cholinergic receptor genes, psychological characteristics, among others could underpin these differences (Greenbaum et al. 2006).

The first evidence of kindling behavior induced by NIC came from the study of Bastlund et al. 2005. These authors showed that NIC kindling presented a different C-Fos immunoreactivity when compared to pentylenetetrazole (PTZ) kindling and that the anticonvulsants levetiracetam, tiagabine and phenytoin inhibited fully kindled seizures induced by NIC (Bastlund et al. 2005).

Kindling represents a form of behavioral sensitization, in which the repeated exposure to subthreshold electrical or chemical stimuli results in an increased responsiveness to these events along time culminating therefore to the development of aberrant behaviors as seizures (Goddard et al. 1969; Post et al. 1988). Kindling may also be induced by major life stress (Post et al. 1988). In other words, the repeated

* Corresponding author at: Drug Research and Development Center, Department of Physiology and Pharmacology, Federal University of Ceará, Rua Cel. Nunes de Melo 1000, 60430-270 Fortaleza, CE, Brazil.
E-mail addresses: daniellesilmacedo@gmail.com, danielle.macedo@ufc.br (D. Macêdo).

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exposure to originally innocuous or subthreshold stimulus, such as pharmacological (drugs of abuse) or environmental factors (emotional stress) may be related to the initiation of behavioral alterations, while after a sufficient number of exposures, a progression to spontaneity occurs leading to pathological processes (Kraus 2000). Thus, this model is a valuable tool to conceptualize the general mechanisms involved in illness progression for a wide range of neuropsychiatric disorders that presents recurrent and cycling symptoms (Post and Weiss 1998; Post 2007), such as mood, anxiety, obsessive–compulsive disorders and drug addiction. This is reinforced by the use of anticonvulsant drugs in the treatment of neuropsychiatric disorders that rely on the influence of kindling mechanisms in these disorders (Post 2002).

Oxidative imbalance is an alteration related to the pathophysiology of seizures (Aguiar et al. 2012), kindling (Frantseva et al. 2000) and neuropsychiatric disorders (Ng et al. 2008). This imbalance is triggered by factors such as increased activation of N-methyl-D-aspartate receptors (NMDAR) and/or mitochondrial respiratory chain (MRC) dysfunction among others (Waxman and Lynch 2005). While the activation of NMDA receptors leads to the synthesis of nitric oxide (NO), MRC dysfunction leads to an increased formation of superoxide. The combination of superoxide and NO makes peroxynitrite, a potent mediator of lipid peroxidation. Superoxide dismutase (SOD) breaks superoxide down to H_2O_2, while reduced glutathione (GSH), the main endogenous antioxidant, converts H_2O_2 to water. Of note, reduction of the antioxidant defenses SOD and/or GSH as well as increased lipid peroxidation were already demonstrated in seizure (Aguiar et al. 2012) and kindling models (Sashindranath et al. 2010; Zhu et al. 2015).

In line with the importance of oxidative alterations in seizures and kindling we recently observed that female periadolescent rats presented lower striatal levels of GSH and SOD and increased lipid peroxidation in the prefrontal cortex (PFC), hippocampus (HC) and striatum (ST) when compared to their male counterparts. These alterations may partially explain the earlier development of nicotine-induced full-blown kindled seizures by females periadolescent rats (median time of 19 days) when compared to age-matched males (median time of 24 days) (Gomes et al. 2013). Furthermore, we also observed the preventive effect of vitamin E against NIC-kindling development (Gomes et al. 2013).

N-acetyl cysteine (NAC) is an antioxidant whose mechanism involves the facilitation of GSH synthesis by the supply of the amino acid cysteine (Whillier et al. 2009). In the last years NAC has been extensively studied for the treatment of neuropsychiatric disorders (Dean et al. 2011; Deepmala et al. 2015). Additionally, this antioxidant seems to present anticonvulsant effects (Zaeri and Emamghoreishi 2015). Despite the great number of studies with NAC, this drug has not been evaluated yet as an anti-kindling drug. Since alterations in glutamatergic, cholinergic and dopaminergic neurotransmission as well as oxidative imbalance are involved in kindling progression (Frantseva et al. 2000) and NAC is able to restore alterations in all these mechanisms (Dodd et al. 2008), this drug seems to be a good candidate as an anti-kindling drug.

In the present study we evaluated the ability of NAC in attenuating NIC-induced kindling in female periadolescent rats. We also investigated if alterations in the brain antioxidants SOD and GSH, lipid peroxidation and nitrite (as an indirect measure of NO) were underlying the effects of NAC. We hypothesized that since oxidative mechanisms (i.e. decreased brain levels of GSH and increased lipid peroxidation) are related to the increased vulnerability of female periadolescent rats to the development of NIC-induced kindling (Gomes et al. 2013), NAC could prevent kindling development by the maintenance of brain oxidative balance.

2. Methods and materials

2.1. Animals

Female periadolescent Wistar rats (postnatal day 30, weight 50–60 g at the beginning of each experiment) were used. The age at the start and duration of NIC exposure in periadolescent rats (postnatal day 30–49) was selected because it roughly approximates adolescence (i.e., age 12–20 years) in humans (Andersen and Navalta; Spear 2000). The animals were housed four per cage in standard polycarbonate rat cages (42 × 20.5 × 20 cm) and standard environmental conditions (22 ± 1 °C; humidity 60 ± 5%; reversed 12-h light/dark cycle with lights on at 19:00) with access to food (FRI-LAB Rat II, FRI-Ribe) and water ad libitum. All experiments were conducted between 8:00 and 14:00 h. The experimental protocol was approved by the Institutional Animal Ethics Committee and conducted according to the guidelines of the Brazilian College of Animal Experimentation (COBEA) and the NIH Guide for Care and Use of Laboratory Animals (NIH 1996). The experiments were designed in order to minimize the number of rats and their suffering.

2.2. Kindling induction protocol

For kindling induction the rats were submitted to a daily (Monday to Friday) intraperitoneal administration of NIC 2 mg/kg (nicotine hemisulfate salt — Sigma-Aldrich, St. Louis, MO, USA). This dose was based on our previous study (Gomes et al. 2013), while the protocol for kindling induction was adapted from Bastlund et al. (2005).

Nicotine was administered every weekday for a period of 19 days. This time period was the best for the induction of kindling in female periadolescent animals (Gomes et al. 2013). Because at the beginning of the experiments when the rats were periadolescents we conducted a weekly evaluation to determine the occurrence of vaginal opening, which indicates the commencement of the estrous cycle. We observed no differences in the time of vaginal opening among animals allocated in the distinct treatment groups (described in Section 2.3) neither in the levels of estradiol as a function of NIC treatment (shown in the Results section).

After each NIC injection, rats were observed for 15 min by a trained observer blinded to the specific treatment with the drugs. The behavioral activity of the animals was classified according to Racine's scale (Racine 1972) modified by (Itzhak 1996). Briefly, the convulsant activity was assessed as follows: stage 1 — normal behavior; stage 2 — hyperactivity; stage 3 — repeated vertical movements that represent stereotypical-like behavior; stage 4 — forelimb clonus and rearing; and stage 5 — full motor seizures with loss of postural control. Complete kindling was considered whenever animals presented at least three consecutive stage 5 seizures on the same day (Itzhak 1996).

2.3. Treatment groups

In order to investigate the possible preventive effect of NAC against NIC-induced kindling, three different doses of NAC, 90, 180 or 270 mg/kg were administered intraperitoneally to distinct groups of animals 30 min before NIC or saline (SAL). The animals were randomly divided into the following groups (8 animals/group): SAL, NAC90 + SAL, NAC180 + SAL, NAC270 + SAL, NIC, NAC90 + NIC, NAC180 + NIC or NAC270 + NIC. The doses of NAC were adapted from previous studies (Busch et al. 1999; Chen et al. 2014).

2.4. Sample collection and preparation

On the 19th day of drug administration, after the behavioral determination, blood from SAL and NIC-kindled animals was collected into pre-chilled tubes containing sodium-EDTA (Sigma-Aldrich) and centrifuged at 1000 ×g for 15 min to obtain plasma. Immediately after blood collection the animals were euthanized by decapitation. For neurochemical studies, the prefrontal cortex (PFC), hippocampus (HC) and striatum (ST) of all experimental groups were immediately dissected. All samples were stored at −70 °C until assayed. For the analysis the brain areas were homogenized ten times (*w*/*v*) with ice-cold 0.1 M phosphate buffer, pH 7.4. The homogenates were centrifuged for

10 min at 3600 rpm at 4 °C. The supernatants were then collected and used for neurochemical analysis. Protein content was determined by the method described elsewhere (Bradford 1976).

2.5. Neurochemical determinations

2.5.1. Determination of GSH levels

GSH levels were determined to estimate the endogenous antioxidant defenses (Sedlák and L'Hanus 1982). The method was based on the Ellman's reagent (DTNB) reaction with free thiol groups. Briefly, the samples were mixed with 0.4 M Tris–HCl buffer, pH 8.9 and 0.01 M DTNB. Reduced glutathione levels were determined using a microplate reader set at 412 nm. A standard glutathione curve was used for data calculation. The results are expressed as ng of GSH/g wet tissue.

2.5.2. Measurement of SOD activity

The activity of this enzyme was evaluated by measuring its capacity to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) (Sun et al. 1988). In this assay, the photochemical reduction of riboflavin generates O^{2-} that reduces the NBT to produce formazan salt, which absorbs at a wavelength of 560 nm. In the presence of SOD, the reduction of the NBT is inhibited because the enzyme converts the superoxide radical to peroxide. The results are expressed as the quantity of SOD necessary to inhibit the rate of reduction of the NBT by 50% in units of the enzyme per gram of protein. Briefly, in a dark chamber, 1 mL of the reactant (50 mM phosphate buffer, 100 nM EDTA and 13 mM L-methionine, pH 7.8) was mixed with 30 μL of the sample, 150 μL of 75 μM NBT and 300 μL of 2 μM riboflavin. The tubes containing the resulting solution were exposed to fluorescent light bulbs (15 W) for 15 min and then read using a spectrophotometer at 560 nm. Results are expressed as U/mg protein.

2.5.3. Measurement of membrane lipid peroxidation

The rate of lipoperoxidation was estimated by the determination of malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) test (Ohkawa et al. 1979). The samples were mixed with 100 μl of 35% perchloric acid and were centrifuged at 5000 rpm for 10 min. 150 μl of the supernatants were removed, mixed with 50 μl of 1.2% thiobarbituric acid, and then heated in a boiling water bath for 30 min. After cooling, the lipid peroxidation was determined using a microplate reader set at 535 nm and expressed as μmol MDA/g wet tissue.

2.5.4. Measurement of nitrite levels

Nitrite levels, an indirect measure of NO, were determined based on Griess reaction and expressed as μM/g wet tissue (Green and Goldman 1981).

2.5.5. Determination of plasma 17-β-estradiol levels

For this measure we used a chemiluminescence method with the ADVIA Centaur analyzer (Bayer Diagnostics, Tarrytown, NY, USA), according to the manufacturer's instructions. Plasma 17-β-estradiol levels are reported as pg/ml.

2.6. Statistical analysis

Differences in the percentage of stage 5 seizures over time among the groups were analyzed through survival analysis using the log-rank (Mantel–Cox) test.

The results of neurochemical studies are expressed as the means ± SEM (standard errors of the mean) and were analyzed with a regular two-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test. The factors were "NIC kindling" (SAL and NIC) and "NAC administration" (SAL, NAC 90, 180 and 270 mg/kg).

Student's *t*-test was used for the analysis of 17-β-estradiol levels.

For all analyses, the significance level was set at α 0.05. Statistical analyses were performed with GraphPad Prism 6.0 for Mac, GraphPad Software (San Diego, CA, USA).

3. Results

Kindling behavior is characterized by the occurrence of stage 5 seizures (Itzhak 1996). In a previous study we demonstrated that female periadolescent animals administered NIC 2 mg/kg presented a progressive increase in the stage of seizure along time, with the median time for stage 5 seizure occurrence of 19 days (Gomes et al. 2013). For this reason, in the present study, we decided to evaluate the percent of stage 5 seizure along time in animals administered NIC combined or not with NAC, as presented in Fig. 1. The analysis of the results by survival curve showed that the median time for stage 5 seizures in SAL + NIC treated animals was 16.5 days with 87.5% of a total of 8 animals presenting stage 5 seizures in the last day of drug administration. The median time for stage 5 seizures in the animals administered NAC90 + NIC or NAC180 + NIC was 9 or 18.5 days, respectively. On the contrary, the animals administered NAC270 + NIC or SAL-treated ones presented an undefined median time for the occurrence of stage 5 seizures. In the group administered NAC270 + NIC only 12.5% of a total of 8 animals presented stage 5 seizures in the last day of drug administration. The comparison of the survival curves by log-rank (Mantel–Cox) test revealed that the co-administration of NAC270 + NIC significantly prevented the occurrence of stage 5 seizures induced by SAL + NIC ($P < 0.01$).

In order to determine the possible mechanisms related to the protective effects of NAC and based on our previous determination of an involvement of oxidative mechanisms in female susceptibility to NIC-induced kindling (Gomes et al. 2013) we decided to determine the levels of GSH, SOD, MDA and nitrite in the PFC, HC and ST of female rats.

The evaluation of GSH levels in the PFC (Fig. 2A) and HC (Fig. 2B) by two-way ANOVA revealed a significant interaction between "NIC kindling" × "NAC administration" with a significant main effect of "NAC administration" in the PFC and of both factors in the HC. In the ST (Fig. 2C) we detected only a significant main effect of "NAC administration" (PFC — "NIC kindling" × "NAC administration" [$F(3, 39) = 11.26$, $P < 0.0001$]; "NAC administration" [$F(3, 39) = 22.48$, $P < 0.0001$]; HC — "NIC kindling" × "NAC administration" interaction [$F(3, 46) = 3.852$, $P = 0.0153$]; "NIC kindling" [$F(1, 46) = 5.830$, $P = 0.0198$]; "NAC administration" [$F(3, 46) = 17.98$, $P < 0.0001$]; ST — "NAC administration" [$F(3, 46) = 26.27$, $P < 0.0001$]).

Tukey post hoc test showed that NIC-kindled animals (group SAL + NIC) presented significant decreased levels of GSH in the PFC (Fig. 2A), HC (Fig. 2B) and ST (Fig. 2C) when compared to SAL-treated

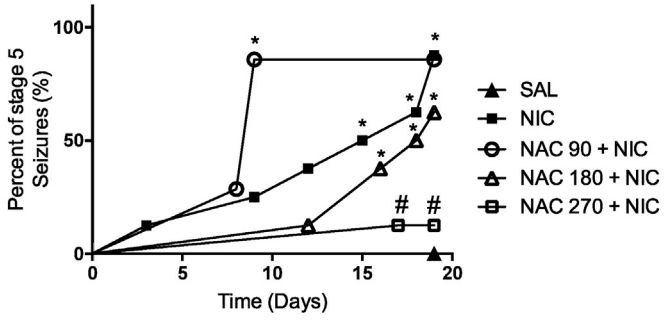

Fig. 1. Development of nicotine (NIC)-induced stage 5 seizures in female periadolescent rats following NIC administration alone or pretreated with N-acetyl cysteine (NAC 90, 180 or 270 mg/kg). Each point of the line represents a percentage of animals (n = 8 animals/group) that presented stage 5 seizures following NIC administration. All groups of animals were administered daily injections of NIC 2 mg/kg, i.p. for up to 19 days during the weekdays. *P < 0.05 vs. saline (SAL), #P < 0.05 vs. SAL + NIC compared to survival curves using the log-rank (Mantel–Cox) test.

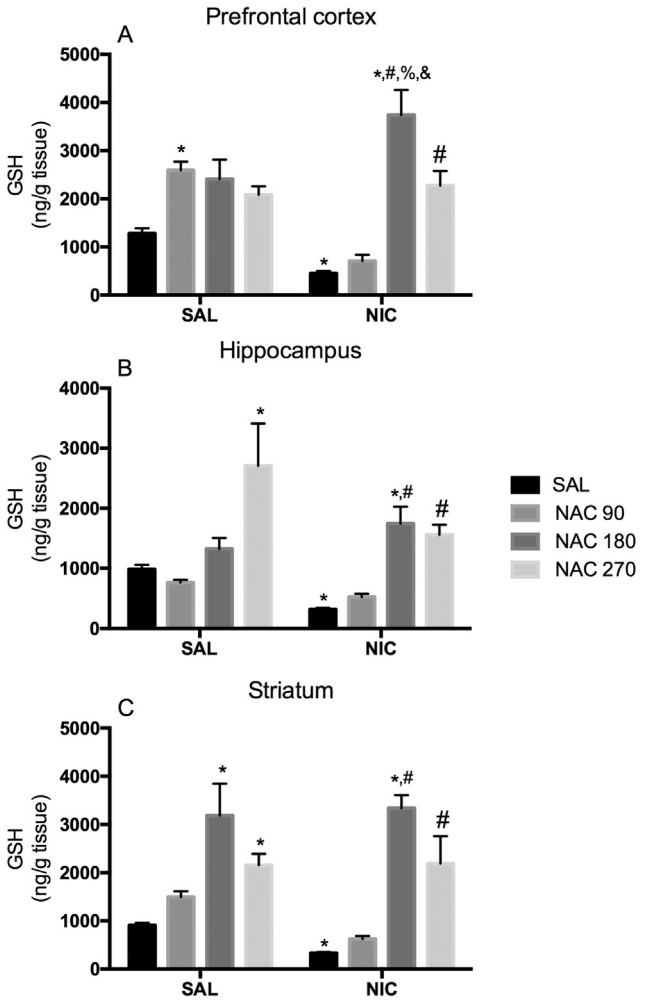

Fig. 2. Reduced glutathione (GSH) levels in the prefrontal cortex (A), hippocampus (B) and striatum (C) of female periadolescent nicotine (NIC)-kindled animals and N-acetyl cysteine (NAC 90, 180 or 270 mg/kg) pretreated rats. Each bar represents the mean ± SEM of 6–8 animals/group. *P < 0.05 vs. saline (SAL), #P < 0.05 vs. SAL + NIC compared using two-way ANOVA followed by post-hoc Tukey's test.

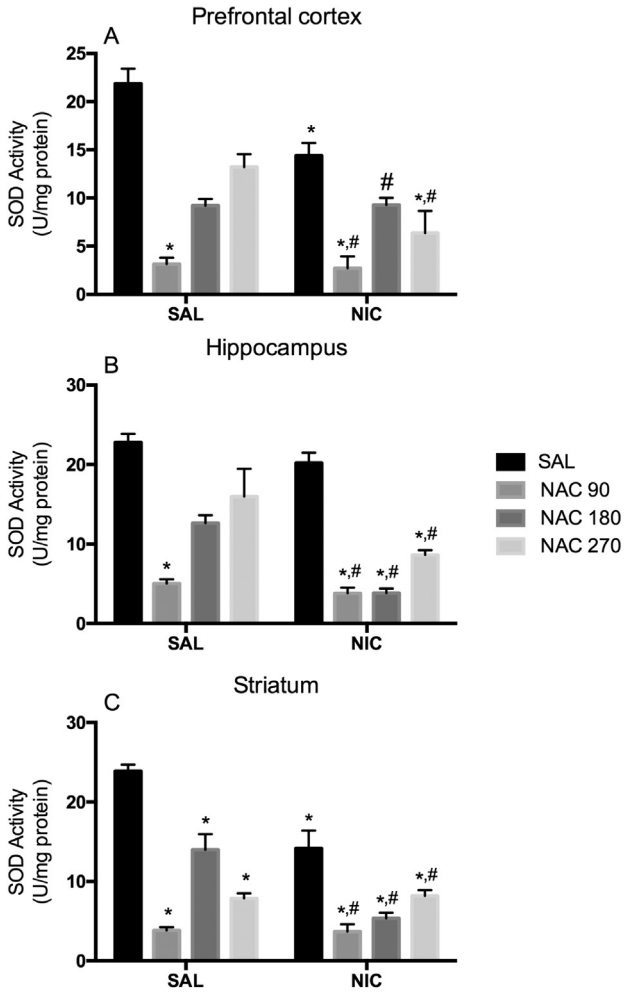

Fig. 3. Superoxide dismutase (SOD) activity in the prefrontal cortex (A), hippocampus (B) and striatum (C) of female periadolescent nicotine (NIC)-kindled animals and N-acetyl cysteine (NAC 90, 180 or 270 mg/kg) pretreated rats. Each bar represents the mean ± SEM of 6–8 animals/group. *P < 0.05 vs. saline (SAL), #P < 0.05 vs. SAL + NIC compared using two-way ANOVA followed by post-hoc Tukey's test.

animals ($P < 0.05$). In all brain areas studied the groups co-administered NAC180 + NIC or NAC270 + NIC presented significant increased levels of GSH when compared to NIC-kindled animals. Interestingly, the animals administered NAC180 + NIC presented increased levels of GSH also when compared to SAL-treated animals in the PFC ($P < 0.0001$), HC ($P < 0.001$) and ST ($P < 0.0001$). The animals administered NAC90 + SAL in the PFC, NAC270 + SAL in the HC and NAC180 + SAL in the ST presented increased levels of GSH when compared to SAL-treated animals ($P < 0.05$).

In relation to SOD activity in the PFC (Fig. 3A), HC (Fig. 3B) and ST (Fig. 3C) there was a significant interaction between "NIC kindling" × "NAC administration", with a significant main effect of "NAC administration" in the PFC, while in the HC and ST significant main effects of both factors were observed (PFC — "NIC kindling" × "NAC administration" [$F(3, 44) = 5.209$, $P = 0.0036$]; "NAC administration" [$F(3, 44) = 31.34$, $P < 0.0001$]; HC — "NIC kindling" × "NAC administration" [$F(3, 48) = 17.25$, $P < 0.0001$]; "NIC kindling" — [$F(1, 48) = 4.661$, $P = 0.0359$]; "NAC administration" — [$F(3, 48) = 64.84$, $P < 0.0001$]; ST — "NIC kindling" × "NAC administration" [$F(3, 45) = 9.874$, $P < 0.0001$]).

Post hoc test revealed that in the PFC and HC of NIC-kindled animals there was no alteration in SOD activity when compared to SAL-treated ones. On the other hand, in the ST ($P < 0.0001$) there was a significant decrease of SOD activity in NIC-kindled rats when compared to SAL-treated ones. The co-administration of NAC in all three doses studied to NIC-treated animals significantly decreased SOD activity when compared to NIC-kindled and SAL-treated animals. This decrease in relation to SAL-treated animals was not significant only in the PFC of animals administered NAC180 + NIC. The animals administered NAC90 + SAL presented significant decreased activity of SOD in all brain areas studied. The same decrease in SOD activity was observed in the ST of the animals administered NAC180 + SAL and NAC270 + SAL ($P < 0.0001$).

Lipid peroxidation levels (Fig. 4), evaluated here as alterations in MDA equivalents, presented a significant interaction between "NIC kindling" × "NAC administration" in all brain areas studied (PFC — [$F(3, 40) = 7.343$, $P = 0.0006$], HC — [$F(3, 40) = 9.345$, $P = 0.0001$], ST — [$F(3, 41) = 5.185$, $P = 0.0040$].

Post hoc test showed that NIC-kindled animals presented increased levels of lipid peroxidation when compared to SAL-treated ones ($P < 0.001$). In the PFC (Fig. 4A), HC (Fig. 4B) and ST (Fig. 4C), the animals administered NAC270 + NIC presented significant decreased levels of lipid peroxidation when compared to NIC-kindled rats ($P < 0.05$). A significant decrease in lipid peroxidation was also observed in the PFC of NAC90 + NIC animals when compared to NIC-kindled animals ($P < 0.01$).

Regarding nitrite levels (Fig. 5) we observed in the PFC (Fig. 5A) and HC (Fig. 5B) a significant interaction between "NIC kindling" × "NAC

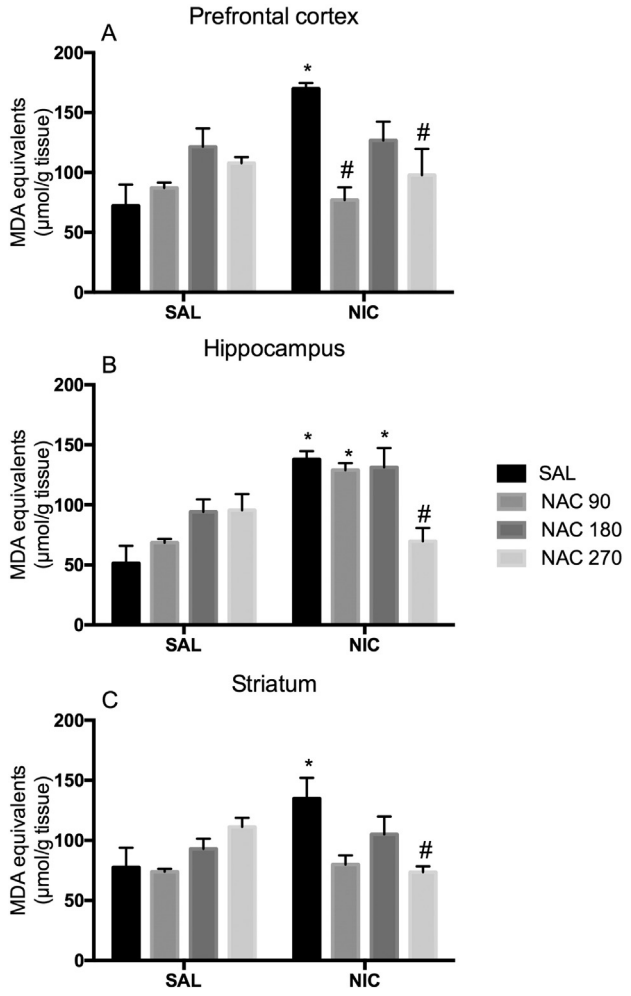

Fig. 4. Lipid peroxidation, determined by the levels of the marker malondialdehyde (MDA), in the prefrontal cortex (A), hippocampus (B) and striatum (C) of female periadolescent nicotine (NIC)-kindled animals and N-acetyl cysteine (NAC 90, 180 or 270 mg/kg) pretreated rats. Each bar represents the mean ± SEM of 6–8 animals/group. *P < 0.05 vs. saline (SAL), #P < 0.05 vs. SAL + NIC compared using two-way ANOVA followed by post-hoc Tukey's test.

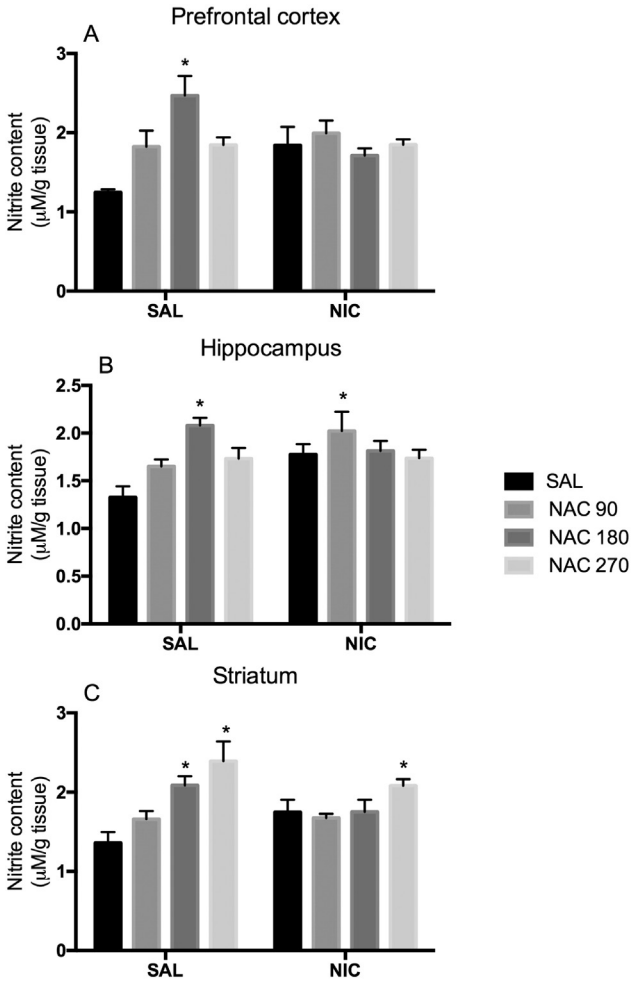

Fig. 5. Nitrite levels in the prefrontal cortex (A), hippocampus (B) and striatum (C) of female periadolescent nicotine (NIC)-kindled animals and N-acetyl cysteine (NAC 90, 180 or 270 mg/kg) pretreated rats. Each bar represents the mean ± SEM of 6–8 animals/group. *P < 0.05 vs. saline (SAL), #P < 0.05 vs. SAL + NIC compared using two-way ANOVA followed by post-hoc Tukey's test.

administration", with a significant main effect of "NAC administration". In the ST (Fig. 5C) there was only a significant main effect of "NAC administration" (PFC —"NIC kindling" × "NAC administration" [F (3, 48) = 4.667, P = 0.0061]; "NAC administration" (PFC — [F (3, 48) = 3.176, P = 0.0324]; HC — "NIC kindling" × "NAC administration" [F (3, 55) = 4.078, P = 0.0110]; "NAC administration" [F (3, 55) = 4.420, P = 0.0074]; ST — "NAC administration" [F (3, 53) = 8.132, P = 0.0002]).

Post hoc test showed no significant alterations in brain nitrite levels in NIC-kindled animals. The administration of NAC90 + NIC or NAC270 + NIC respectively in the HC and ST increased nitrite levels when compared to SAL-treated animals (P < 0.05). In the PFC, HC and ST of NAC180 + SAL animals as well as in the ST of NAC270 + SAL rats there was a significant increase in nitrite content when compared to SAL-treated animals.

As shown in Fig. 6, the analysis of plasma levels of 17-β-estradiol in NIC-kindled rats was not significantly different from SAL-treated animals (t = 0.02610 df = 8).

4. Discussion

In the present study, we demonstrated that the administration of NAC at the dose of 270 mg/kg 30 min before NIC attenuated the occurrence of kindling behavior in female periadolescent rats. This attenuation was accompanied by restoration of GSH and MDA levels in the PFC, HC and ST of NIC-kindled animals. Remarkably, as far as we know, this is the first evidence for an anti-kindling effect of NAC.

Here we replicated our previous findings on NIC-induced kindling (Gomes et al. 2013) with the exception that, in the experiments shown here, the median time for stage 5 seizure occurrence in NIC-treated female periadolescent animals was 16.5 days and not 19 days as observed in our previous study.

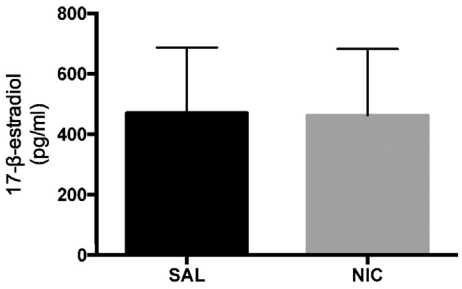

Fig. 6. Plasma levels of 17-β-estradiol on the day of kindling development in female periadolescent rats. Each bar represents the mean ± SEM of 6–8 animals/group. Student's *t*-test was used for comparisons.

The activation of nAChRs α4β2 and α7 underlies the convulsant effects of NIC (Damaj et al. 1999a,b). nAChRs are present at the presynaptic level and the chronic exposure to NIC upregulates these receptors (Dani and Bertrand 2007). nAChRs facilitate dopaminergic (Threlfell and Cragg 2011; Graupner et al. 2013) and glutamatergic neurotransmission (Girod et al. 2000). A recent electrophysiological study showed that NIC administration during 14 days increased NMDA currents in PFC neurons and the core part of the nucleus accumbens when compared to those of control rats (Ávila-Ruiz et al. 2014). Besides nAChRs, dopamine release in the nucleus accumbens is also under the control of NMDA receptors (Kelley and Throne 1992). Of note, stimulation of NMDA receptors is related to NIC's convulsant action (Damaj et al. 1999a) and kindling progression (Sutula et al. 1996), but has not yet been evaluated in NIC kindling.

As previously mentioned, nAChRs modulate dopaminergic neurotransmission (Threlfell and Cragg 2011) and NMDA receptor function (Ávila-Ruiz et al. 2014). The excessive stimulation of NMDA receptors leads to the production of reactive oxygen species (ROS) (Love 1999), while the exposure to ROS induces an upregulation of functional NMDA receptors (Betzen et al. 2009). Similarly, hyperdopaminergic states and dopamine metabolism are related to ROS production (Yamato et al. 2010).

In our experiments, female periadolescent NIC-kindled rats presented decreased levels of the major endogenous antioxidant GSH, decreased SOD activity and increased levels of lipid peroxidation in the PFC, HC and ST. Similarly, in our previous study, we observed an oxidative imbalance in brain areas of NIC kindled female periadolescent animals (Gomes et al. 2013).

Neuronal GSH deficiency is related to increased oxidant levels and increased susceptibility to oxidant injury (Aoyama et al. 2006). Furthermore, oxidative mechanisms are strongly implicated in seizures (Aguiar et al. 2012) and kindling development (Frantseva et al. 2000), being one of the mechanisms related to cell death. The involvement of oxidative imbalance in seizure generation is reinforced by the findings showing that increased oxygen tension (Hauser and Annegers 1991) and local infusion of redox-active iron salts (Willmore et al. 1978) or mitochondrial toxins (Zuchora et al. 2001) were able to enhance mitochondrial free radicals and induce seizure activity.

GSH deficiency also seems to precede later changes in the activities of mitochondrial complex I and aconitase in vulnerable hippocampal sub-regions following limbic status epilepticus (SE) (Sleven et al. 2006). This cited study concluded that strategies to boost GSH levels and/or otherwise reduce oxidative stress following seizures, deserve further study, both in terms of preventing the biochemical consequences of SE, the neuronal dysfunction and clinical consequences (Sleven et al. 2006).

Here we demonstrated that NAC 270 mg/kg attenuates NIC-induced kindling. This effect was accompanied by increases in GSH levels in all brain areas studied. Indeed, NAC is a precursor of the antioxidant GSH, whose mechanism involves the modulation of glutamatergic, neurotrophic, and inflammatory pathways (Dean et al. 2011). The decision to use NAC as a preventive approach came from our previous evidences for decreased GSH levels in brain areas of NIC-kindled female animals (Gomes et al. 2013). From the amino acids that constitute GSH structure, i.e. glutamate, glycine, and cysteine, this later has the lower intracellular concentration (Aruoma et al. 1989) that can limit GSH synthesis. Since cysteine is usually the limiting precursor of GSH synthesis, NAC being an acetylated cysteine residue is the major contributor to the maintenance of the cellular GSH status and consequently to the cell protection against oxidative stress (Aruoma et al. 1989).

Interestingly, we observed that NAC despite having a protective mechanism by increasing the levels of GSH at the doses of 180 and 270 mg/kg, decreased SOD activity in all doses and brain areas studied. It was previously demonstrated that the intravenous administration of SOD-1 increased the seizure threshold in amygdala kindling rat models of epilepsy (Gusakov et al. 1995). On the other hand, experiments with SOD-2 knockout mice demonstrated that these animals are more susceptible to kainate induced neurodegeneration and neuronal cell death (Liang and Patel 2004). Sod2(−/+) mice also showed an age-related decrease in the expression of glial glutamate transporters (GLT-1 and GLAST), suggesting that oxidant-induced inhibition of glutamate transport may play a mechanistic role in rendering some Sod2(−/+) mice susceptible to seizures (Liang and Patel 2004). SOD-1, or CuZn-SOD is found almost exclusively in intracellular cytoplasmic spaces, while SOD2, or Mn-SOD is found exclusively in the mitochondrial spaces (Zelko et al. 2002).

The oxidant-induced inhibition of glial glutamate transport (Liang and Patel 2004) may be one possible mechanism related to the exacerbation of kindling behavior in NAC90 + NIC group that was observed in the present study. In fact, the rats administered NAC90 + NIC presented decreased SOD activity associated with decreased levels of GSH that may be indicative of superoxide radical accumulation. On the contrary, the animals administered NAC180 + NAC and NAC270 + NIC presented decreased SOD activity, but increased levels of GSH. GSH reacts with superoxide being converted to its oxidized form, GSSG (Winterbourn 1993). Therefore we can suggest that the increase in GSH levels observed in NIC180 + NAC and NAC270 + NIC groups has the ability to scavenge the superoxide radical thus leading to a redox homeostasis. The fact that decreases in SOD activity were also observed by the administration of the anti-kindling drugs lithium and valproate (Jornada et al. 2011; Kiełczykowska et al. 2015) deserves mention. Overall, our results suggest that GSH is an important parameter related to the protection against NIC kindling.

We also observed decreased brain lipid peroxidation in NAC270 + NIC treated animals. Lipid peroxidation is a process of lipid damage caused by an increased production of ROS or the inefficiency of scavenging mechanisms (Ayala et al. 2014). In our previous study we showed the occurrence of lipid peroxidation in the PFC, HC and ST of female periadolescent NIC-kindled rats, but not in male ones, this alteration being prevented by vitamin E.

Besides NAC's antioxidant effects, this drug acts as a vasodilator by facilitating the production and action of NO (Lopez et al. 1998). Increases in the activity of brain neuronal NO synthase were observed in normotensive and hypertensive animals chronically administrated NAC (Pechanova et al. 2009).

We observed increased brain levels of nitrite in animals administered only NAC as well as in the ST of NAC270 + NIC treated animals. Numerous evidences indicate that increases in GSH and NO levels, as observed in the ST of NAC270 + NIC treated rats, leads to the production of S-nitrosogluthatione (GSNO). This compound presents antioxidant properties. Furthermore, it was previously demonstrated that GSNO can protect brain dopamine neurons from iron-induced oxidative stress and degeneration (Rauhala et al. 1998). As NIC is known to increase brain dopamine content and consequently cause oxidative alterations, the synthesis of GSNO in the ST of NAC270 + NIC treated animals would be a possible mechanism related to the protective effects of NAC against NIC induced deleterious effects in the brain. Therefore, future studies evaluating the role of GSNO in kindling behavior need to be conducted.

Regarding sexual hormone levels, 17-β-estradiol did not vary between NIC-kindled and saline-treated rats. Moreover, there are no evidences in the literature of alterations in female sexual hormone levels by the administration of NAC either in rats or in humans (Fulghesu 2002; Badawy et al. 2006; Abu Hashim et al. 2010; Salehpour et al. 2012; Amorim et al. 2014).

A limitation of the present study is that we did not evaluate the occurrence of other behavioral alterations such as hyperlocomotion and anxiety in NIC-kindled animals. This would help us to find a possible relation between NIC kindling and the development of behavioral alterations that resemble neuropsychiatric disorders. A second limitation is that we did not evaluate the influence of glutamatergic and dopaminergic neurotransmissions in NIC kindling. Indeed, alterations in these neurotransmissions could underpin NAC's anti-kindling effects.

In conclusion, NAC at the dose of 270 mg/kg was able to attenuate the behavioral and brain pro-oxidative alterations observed in female periadolescent NIC-kindled animals. NAC attenuated the alterations in brain GSH and lipid peroxidation observed in NIC-kindled animals. This is of importance since we have demonstrated that oxidative alterations were underlying the vulnerability of female periadolescent animals to NIC kindling. Future studies must be conducted to explore the involvement of other mechanisms underlying NAC attenuation of NIC kindling, such as alterations in glutamatergic and dopaminergic neurotransmissions, that may be upstream to the oxidative alterations observed in the present study. Furthermore, since kindling mechanisms are related to some neuropsychiatric disorders, this anti-kindling effect of NAC needs to be further explored for a better characterization of the important therapeutic effects of NAC in these disorders (Dean et al. 2011; Berk et al. 2013).

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