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Baclofen Prevented the Changes in c-Fos and Brain-Derived Neutrophic Factor Expressions During Mecamylamine-Precipitated Nicotine Withdrawal in Mice

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ABSTRACT Previous studies from our laboratory showed that baclofen (BAC, $GABA_B$ receptor agonist) prevented the behavioral and neurochemical alterations of nicotine (NIC) withdrawal syndrome. To further investigate the mechanisms underlying these effects, we analyzed the c-Fos and brain-derived neutrophic factor (BDNF) expression during NIC withdrawal and its prevention with BAC. Swiss-Webster mice received NIC (2.5 mg/kg, sc) four times daily, for 7 days. On the 8th day, NIC-treated mice received the nicotinic antagonist mecamylamine (MEC; 2 mg/kg, i.p.) 1 h after the last dose of NIC. A second group of NIC-treated mice received BAC (2 mg/kg, i.p.) prior to MEC administration. Thirty minutes after MEC, mice were sacrificed and the immunohistochemistry assays (c-Fos and BDNF) were performed at different anatomical levels. c-Fos expression decreased in the dentate gyrus of the hippocampus (DG) and the bed nucleus of the stria terminalis (BST), and increased in the habenular (Hb), accumbens shell (AcbSh) nuclei during NIC withdrawal. BAC re-established the modified c-Fos expression only in the DG, BST and AcbSh during NIC withdrawal. Conversely, BDNF expression decreased in the CA1 and CA3 area of the hippocampus, the Hb, and caudate putamen (CPu) during NIC withdrawal. Finally, BAC restored the decreased BDNF expression during NIC withdrawal in the CA1, CA3, Hb, and CPu. The results suggest a relationship between BAC's preventive effect of the expression of NIC withdrawal signs, and its ability to restore the changes in c-Fos and BDNF expression, observed in specific brain areas of NIC-withdrawn mice. **Synapse 68:508–517, 2014.**

INTRODUCTION

Nicotine (NIC) withdrawal is a collection of somatic and affective symptoms that is observed within a few hours after discontinuation of NIC intake (De Biasi and Salas, 2008). Withdrawal reflects the abrupt disruption of the equilibrium maintained in the presence of NIC, and withdrawal triggers new neuroadaptations that counteract the negative state (De Biasi and Dani, 2011). In this sense, it is well known that both spontaneous and mecamylamine-precipitated NIC withdrawals are associated with a neurochemical alteration within the brain (Carboni et al., 2000; Gäddnäs et al., 2002; Rada et al., 2001; Rahman et al., 2004). However, little is known about the possible molecular alterations that could be induced by the NIC withdrawal. Thus, a better understanding of the mechanisms involved in NIC dependence could lead to develop new treatments.

c-Fos is a transcription factor considered to be a marker of neuronal activity (Chao and Nestler, 2004; Dragunow and Faull, 1989; Morgan and Curran, 1991) that has been used to identify the brain areas involved in the actions induced by psychostimulants

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(Graybiel et al., 1990). In fact, acute NIC treatment induces an increased expression of c-Fos in several brain regions, including areas such as: caudate putamen (CPu), accumbens core nucleus (AcbC), cingulate cortex (Cg), and amygdala (Marttila et al., 2006). Studies report an increase in c-Fos expression in rats trained to self-administer NIC (Pagliusi et al., 1996). In reference to chronic NIC treatment and withdrawal, Marttila et al. (2006) found that the expression of ΔFosB and FosB (Fos family members) increased in AcbC during a chronic NIC treatment for 2 weeks. This same study reported that expression of ΔFosB, FosB, and c-Fos increased in the AcbC of rats treated with NIC in drinking water for 5 days. This increased expression was not maintained after cessation of NIC treatment.

The brain-derived neurotrophic factor (BDNF) is present throughout the adult central nervous system (Conner et al., 1997; Ernfors et al., 1990), promoting cell survival (Ghosh et al., 1994), and regulating dendrite (Xu et al., 2000), and synaptic plasticity (McAllister et al., 1999; Pattwell et al., 2012). BDNF may facilitate or inhibit drug-seeking behaviors depending on the drug type, the brain site, and the addiction phase (initiation, maintenance, or abstinence/relapse) (Ghitza et al., 2010). Regarding NIC, few studies show the role of BDNF in any stage related to the addictive process. NIC acute administration reduced the BDNF levels in the hippocampus 2 h and 24 h after administration. In addition, Kivinummi et al. (2011) reported that chronically treated mice with NIC in their drinking water increased the BDNF expression in the Acb after 24 h and 29 days of NIC abstinence.

Our laboratory has extensively explored the behavioral and neurochemical effects of NIC addiction. Indeed, we have previously observed an interaction between the GABAergic and nicotinic cholinergic systems, since the selective $GABA_B$ receptor agonist baclofen (BAC) prevented the somatic expression of NIC withdrawal syndrome in mice (Varani et al., 2011, 2014). Moreover, BAC re-established the decreased cortical and striatal dopamine and serotonin levels during NIC withdrawal (Varani et al., 2011). Furthermore, we observed that BAC restored the increased $\alpha_4\beta_2$ nicotinic receptor labeling, evidenced in specific brain areas in NIC-withdrawn mice (Varani et al., 2013). Conversely, we have also reported that the $GABA_B$ antagonist, 2-OH-saclofen, blocked the anxiolytic- and anxiogenic-like effects induced by NIC (Varani and Balerio, 2012). Finally, we showed that acute behavioral responses to NIC (antinociception, hypolocomotion, and anxiety-related effect) and mecamylamine-precipitated NIC withdrawal syndrome are modified in $GABA_{B1}$ knockout mice (Varani et al., 2012). Taking into account these previous results from our laboratory the aim of this study was investigate the variations in c-Fos and BDNF expression during NIC withdrawal and its prevention with BAC.

MATERIALS AND METHODS

Animals

Male Swiss Webster mice obtained from Bioterio Central (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina) weighing 22–24 g were housed five per cage, acclimatized to laboratory conditions according to local regulation (SENASA, 2002) (12-h light:12-h dark cycle, 21 ± 0.5°C room temperature, $65 \pm 10\%$ humidity) and manipulated for 3 days prior to the experiment. Food and water were available ad libitum. Behavioral tests and animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, publication no. 85-23, revised 1985). All experiments were performed with the investigators being blind to treatment conditions.

Drugs

(−)-Nicotine hydrogen tartrate salt ([−]-1-methyl-2-[3-pyridil]pyrrolidine) (Sigma–Aldrich, N5260), mecamylamine hydrochloride (Sigma–Aldrich, M9020), and (±)-baclofen (donated by Novartis, Basel, Switzerland) were dissolved in isotonic (NaCl 0.9%) saline (SAL) solution. The dose of NIC refers to the salt form. All drugs were administered in a volume of 10 ml/kg.

Chronic treatment

Mice were rendered dependent by subcutaneous (s.c.) injection of NIC (2.5 mg/kg), four times daily, for 7 consecutive days (injections were given at 04:00 a.m., 10:00 a.m., 16:00 p.m., and 22:00 p.m.). The dose of NIC (2.5 mg/kg, s.c.) was chosen based on previous studies from our group (Varani et al., 2011). Control groups received SAL s.c., four times daily, for 7 consecutive days.

Acute treatment

On day 8, dependent mice received the last dose of NIC at 10:00 a.m. and were then randomly divided into three groups ($n = 7$ per experimental group): in the "NIC-SAL-SAL" group the animals received SAL 15 and 60 min after the last dose of NIC; in the "NIC-SAL-MEC" group mice received SAL and MEC (2 mg/kg; intraperitoneal, i.p.), 15 and 60 min after the last dose of NIC, respectively, to precipitate NIC withdrawal; in the "NIC-BAC-MEC" group mice received BAC (2 mg/kg, i.p.) and MEC (2 mg/kg, i.p.), 15 and 60 min after the last dose of NIC, respectively.

On day 8, control animals received the last injection of SAL at 10:00 h and were randomly divided into three control groups ($n = 7$ per experimental group): in the "SAL-SAL-SAL" group the animals

received SAL 15 and 60 min after the last dose of SAL; in the "SAL-SAL-MEC" group mice received SAL and MEC (2 mg/kg, i.p.), 15 and 60 min after the last dose of SAL, respectively; in the "SAL-BAC-MEC" group mice received BAC (2 mg/kg, i.p.) and MEC (2 mg/kg, i.p.), 15 and 60 min after the last dose of SAL, respectively. The dose of BAC (2 mg/kg, i.p.) was selected based on our previous reports and did not have intrinsic effect in nondependent animals (Diaz et al., 2001, 2003, 2004, 2006; Varani et al., 2011, 2013, 2014; Varani and Balerio, 2012). BAC was administered 15 min after the last NIC injection to ensure that its maximal effect occurs 15 min after MEC. This time was chosen taking into account a previous study from our laboratory where it was observed that the maximal antinociceptive response of BAC occurred 1 h after its administration (Balerio and Rubio, 2002). Conversely, we previously observed that NIC withdrawal syndrome shows an increase of behavioral signs at 10–15 min after MEC administration (Varani et al., 2011). Therefore, with the present experimental design we make sure that the maximal effect of BAC takes place when NIC withdrawal syndrome seems to be more intense (10–15 min).

MEC was injected 60 min after the last NIC injection to ensure optimal plasmatic NIC concentrations since the absorption half-life is nearby to 15 min while its elimination half-life is around 2 h (Damaj et al., 2007).

c-Fos and BDNF experiments

The brains used in this study were obtained from mice tested in a previous behavioral study from our laboratory under the experimental procedure mentioned above (Varani et al., 2011).

Tissue preparation

Thirty minutes after the injection of MEC or SAL (day 8), mice were deeply anesthetized using a mixture of ketamine (70 mg/kg, Holliday-Scott S.A., Argentina) and xylazine (10 mg/kg, König, Argentina). They were then transcardially perfused with heparinized PBS (0.1 M saline phosphate buffer, pH 7.4), followed by a cold solution of 4% paraformaldehyde delivered with a peristaltic pump. Brains were removed and postfixed for 2 h in the same fixative, and cryoprotected overnight in a 30% sucrose solution. Coronal frozen sections were made at 30 μm on a freezing microtome. They were collected in three serial groups of free-floating sections and stored at 4°C.

c-Fos and BDNF immunohistochemistry

The procedure was adapted from previously described protocols (Bester et al., 2001). All reactions were performed on floating sections agitated on a shaker. Sections from different experimental groups were processed in parallel to minimize the variations in immunohistochemical labeling. Free-floating sections were rinsed in 0.1 M phosphate buffered saline with 0.15% Triton X-100 (PBS-T; pH 7.4) and then incubated with 3% hydrogen peroxide in PBS-T for a period of 30 min to remove endogenous peroxidase activity. After rinsing again in PBS-T, sections were incubated for 30 min in 2% normal goat serum in PBS-T. Then, sections were incubated overnight in a rabbit polyclonal antibody anti-c-Fos (Santa Cruz Biotechnology, sc-253) (1:1000 in PBS 0.1 M, thimerosal 0.02%, normal goat serum 1%) or anti-BDNF (Santa Cruz Biotechnology, sc-20981) (1:50 in PBS 0.1 M, thimerosal 0.02%, normal goat serum 1%) at 4°C. Sections were then rinsed and incubated for 2 h in a goat anti-rabbit biotinylated antibody (Vector Laboratories) (1:250 in PBS-T). After being rinsed, sections were incubated for 2 h in avidin-biotinylated horseradish peroxidase complex (1:125, ABC kit, Vector Laboratories). After successive washes in PBS-T and Tris buffer (0.25 M; pH 7.4), the antibody–antigen complex was developed with 0.05% (m/v) of 3,3′-diaminobenzidine (Sigma) and 0.015% (v/v) of H_2O_2 in 20 ml Tris buffer 0.1 M. Sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. Controls for the specificity of primary antisera used were performed by substitution of primary antibody with PBS (Delfino et al., 2004).

Data quantification

For quantitative analysis, cells positive for c-Fos and BDNF immunoreactivity were identified by the presence of dense immunohistochemical staining within the nuclei, under a light microscope. Digital images of the selected sections were taken at 200× on a Nikon Microscope (Eclipse 55i) equipped with a digital camera (Nikon DS, Control Unit DS-L1).

For every area, the number of c-Fos and BDNF positive cells was counted within a grid under ImageJ 1.36 b, provided by National Institutes of Health (public domain software). The counting was performed bilaterally in each brain area by an observer blind to the treatment. These counts were averaged into a single score for each region of each animal and finally the group mean ± SEM was calculated. c-Fos and BDNF positive cells were quantified in the following brain regions, identified according to the anatomic atlas of Paxinos and Franklin (2004): nucleus accumbens shell (AcbSh) and core (AcbC), cingulate cortex (Cg), caudate putamen (CPu), the bed nucleus of the stria terminalis (BST), the basolateral amygdaloid nucleus (BLA), dentate gyrus (DG), CA1 and CA3 areas of the hippocampus, and habenular nucleus (Hb).

Statistical analysis

Results obtained for c-Fos and BDNF expression were analyzed using two-way analysis of variance (ANOVA) with chronic treatment (SAL or NIC) and

TABLE I. c-Fos expression (number of c-Fos positive nuclei/mm^2) in different brain regions of mice

	SAL-SAL-SAL	SAL-SAL-MEC	SAL-BAC-MEC	NIC-SAL-SAL	NIC-SAL-MEC	NIC-BAC-MEC
Nucleus accumbens core	705.8 ± 75.16	789 ± 122	640 ± 39.89	956 ± 114.6	1136 ±166.7	725 ± 3.72
Nucleus accumbens shell	1600 ± 303.5	921.2 ± 123.4	1043 ± 218.5	1263 ± 182.2	2003 ± 358.1 ★	877.2 ± 158.8 ☆
Cingulate cortex	1000 ± 203.5	1308 ± 121.3	931.2 ± 149.8	1407 ± 92.32	1356 ± 127.8	953.7 ± 162
Caudate putamen	864.3 ± 197.5	789.7 ± 94.46	633.7 ± 101	804 ± 143.8	945.4 ± 99	661.5 ± 134.9
Bed nucleus of the stria terminalis	716.3 ± 15.36	684.8 ± 16.82	522.4 ± 77.72	574.3 ± 31.41	291.2 ± 23.81 ★	687.3 ± 102.7 ☆☆
Basolateral amygdaloid nucleus	518.2 ± 79.61	517.7 ± 32	439.4 ± 52.16	625.6 ± 75.37	571.3 ± 59.43	683.7 ± 69.13
Dentate gyrus	1161 ± 31.55	1178 ± 157.3	989.3 ± 35.20	811.3 ± 89.19	718.4 ± 48.09 ★	1346 ± 108.7 ☆☆
CA1	1469 ± 116.1	1474 ± 82.65	1481 ± 171.2	1535 ± 119.6	1647 ± 314.2	1615 ± 131.5
CA3	1467 ± 284.4	969.4 ± 176.4	1223 ± 235	1079 ± 116.2	1216 ± 140.5	1317 ± 62.67
Habenular nucleus	164.2 ± 23.33	175.1 ± 31	181.6 ± 18.37	206.5 ± 2.25	345.5 ± 23.46 ★★	231.6 ± 44.30

Data represents the mean ± S.E.M. (n = 3–7 mice per group). ★ $P < 0.05$; ★★ $P < 0.01$ compared to chronic saline group. ☆ $P < 0.05$; ☆☆ $P < 0.01$ comparison between similar groups receiving chronic nicotine with or without baclofen (two-way ANOVA; multiple comparison post hoc test). SAL, saline; MEC, mecamylamine; BAC, baclofen; NIC, nicotine.

acute treatment (SAL or BAC) as between-subjects factors of variation. When a significant interaction between these factors was observed, the difference between two means was analyzed by multiple comparison post hoc test for each experimental group. In all cases, $P < 0.05$ was considered statistically significant. The statistical analysis was performed with three to seven mice per group because outliers showing a large variation in immunostaining (mean ± [2 × SD]) were excluded.

RESULTS

The data were not corrected for multiple comparisons, given that so many analyses were performed on each individual.

c-Fos expression

There were no differences between NIC-SAL-SAL, SAL-SAL-SAL control, SAL-BAC-MEC control, and SAL-SAL-MEC control groups in the number of c-Fos positive nuclei in all brain areas studied after the last injection (Table I).

Two-way ANOVA showed a significant interaction between chronic treatment (SAL or NIC) and acute treatment (SAL or BAC) in the AcbSh ($F_{(2,25)}$=5.402; $P < 0.05$), BST ($F_{(2,21)} = 9.408$; $P < 0.01$), DG ($F_{(2,22)}$ = 11.399; $P < 0.001$), and Hb ($F_{(2,19)}$ = 3.510; $P < 0.05$), while there were not significant interactions for the rest of the brain areas. In addition, two-way ANOVA revealed significant main effects for chronic treatment only in the AcbC ($F_{(1,21)} = 5.965$; $P < 0.05$); BST ($F_{(1,21)} = 5.422$; $P < 0.05$), BLA ($F_{(1,20)} = 6.455$; $P < 0.05$), DG ($F_{(1,22)} = 4.220$; $P < 0.05$), and Hb ($F_{(1,19)} = 15.909$; $P < 0.01$). Two-way ANOVA also showed significant main effects for acute treatment only in the Hb ($F_{(2,19)}$ = 4.141; $P < 0.05$).

The multiple comparison post hoc test revealed that c-Fos positive nuclei in NIC-withdrawn (NIC-SAL-MEC) mice significantly increased in the AcbSh ($P < 0.05$) and Hb ($P < 0.01$) and decreased in the BST ($P < 0.05$) and DG ($P < 0.05$); compared with SAL-SAL-MEC control group (Fig. 1). Additionally, BAC pre-treatment (NIC-BAC-MEC group) re-established the modified c-Fos expression in the AcbSh ($P < 0.05$), BST ($P < 0.01$), and DG ($P < 0.01$), compared with NIC-SAL-MEC group (Fig. 1).

No significant changes were observed between the experimental groups in the number of c-Fos positive nuclei in any of the other brain areas studied (AcbC, Cg, CPu, BLA, CA1, and CA3) (Table I).

BDNF expression

There were no differences between NIC-SAL-SAL, SAL-SAL-SAL control, SAL-BAC-MEC control, and SAL-SAL-MEC control groups in the number of BDNF-positive nuclei in all brain areas studied after the last injection (Table II).

Two-way ANOVA showed a significant interaction between chronic treatment (SAL or NIC) and acute treatment (SAL or BAC) in the CPu ($F_{(2,28)} = 12.701$; $P < 0.001$), CA1 ($F_{(2,30)}$ = 6.549; $P < 0.01$), CA3 ($F_{(2,24)}$ = 38.265; $P < 0.001$), and Hb ($F_{(2,25)}$ = 5.600; $P < 0.05$), while there were not significant interactions for the rest of the brain areas. In addition, two-way ANOVA revealed significant main effects for chronic treatment only in the CA1 ($F_{(1,30)}$ = 4.335; $P < 0.05$), CA3 ($F_{(1,24)}$ = 20.361; $P < 0.001$), and Hb ($F_{(1,25)} = 10.391$; $P < 0.01$). Two-way ANOVA also showed significant main effects for acute treatment only in the Cg ($F_{(2,29)}$ = 4.720; $P < 0.05$), CPu ($F_{(2,28)}$ = 19.573; $P < 0.001$), CA3 ($F_{(2,24)} = 31.250$; $P < 0.001$), and Hb ($F_{(2,25)} = 3.386$; $P < 0.05$).

The multiple comparison post hoc test revealed that BDNF-positive nuclei in NIC-withdrawn (NIC-SAL-MEC) mice significantly decreased in the Cpu ($P < 0.001$), Hb ($P < 0.01$), CA1 ($P < 0.01$), and CA3 ($P < 0.001$) compared with SAL-SAL-MEC control group (Fig. 2). Additionally, BAC pre-treatment (NIC-BAC-MEC group) re-established the modified BDNF expression in the CPu ($P < 0.01$), Hb ($P < 0.05$), CA1 ($P < 0.05$), and CA3 ($P < 0.001$) compared with NIC-SAL-MEC group (Fig. 2).

No significant changes were observed between the experimental groups in the number of BDNF-positive nuclei in any of the other brain areas studied (AcbSh, AcbC, Cg, BST, BLA, and DG) (Table II).

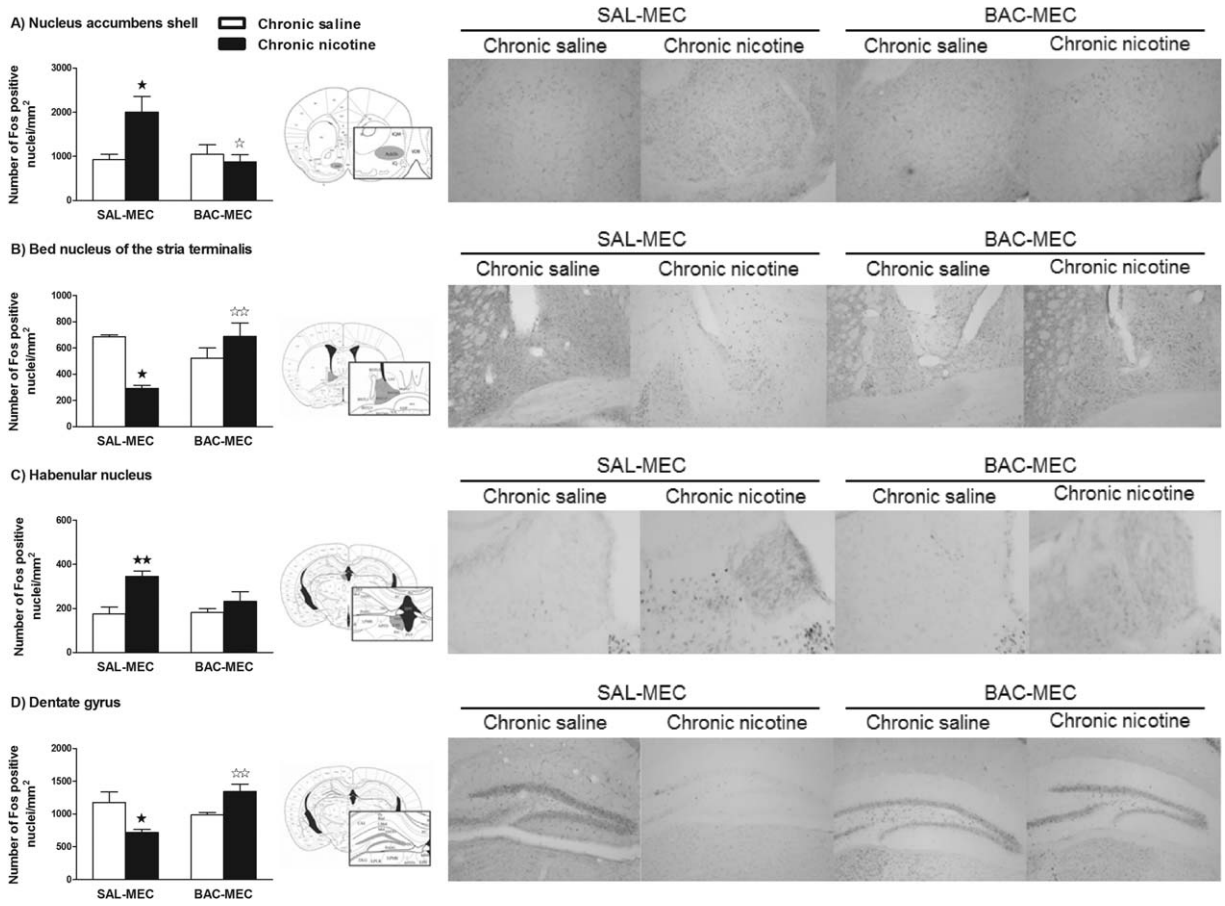

Fig. 1. c-Fos expression (number of c-Fos positive nuclei/mm^2) in mice following nicotine (NIC) withdrawal and its prevention with baclofen (BAC) in nucleus accumbens shell (**A**, $n = 3$–5), bed nucleus of the stria terminalis (**B**, $n = 3$–4), habenular nucleus (**C**, $n = 3$–4), and dentate gyrus (**D**, $n = 3$–5). Each column represents the mean + SEM. Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with NIC (2.5 mg/kg; sc) four times daily, during 7 days. On the day of the experiment (day 8) mice received the acute treatment: 15 min after the last injection of chronic treatment either BAC (2 mg/kg; i.p.) or saline was administered. Sixty minutes after the last injection of chronic treatment, mecamylamine (MEC; 2 mg/kg; i.p.) was administered to all animals. ★ $P < 0.05$; ★★ $P < 0.01$ compared to chronic saline group. ☆ $P < 0.05$; ☆☆ $P < 0.01$ comparison between similar groups receiving chronic NIC with or without BAC (two-way ANOVA followed by multiple comparison test).

TABLE II. BDNF expression (number of BDNF positive nuclei/mm^2) in different brain regions of mice

	SAL-SAL-SAL	SAL-SAL-MEC	SAL-BAC-MEC	NIC-SAL-SAL	NIC-SAL-MEC	NIC-BAC-MEC
Nucleus accumbens core	36.86 ± 5.99	35 ± 1.91	36.86 ± 3.63	36 ± 1.13	32 ±5.82	39.67 ± 2.75
Nucleus accumbens shell	46.43 ± 3.04	42.71 ± 3.46	39.71 ± 3.24	42.43 ± 2.93	43.71 ± 3.31	44.50 ± 0.92
Cingulate cortex	45.40 ± 2.69	43 ± 1.05	43.40 ± 1.66	47 ± 3.34	38.20 ± 1.35	38.20 ± 1.74
Caudate putamen	29 ± 2.05	26.67 ± 0.66	26 ± 0.70	31.60 ± 2.15	15.50 ± 1.71 ★★★	27.33 ± 0.33 ☆☆
Bed nucleus of the stria terminalis	43.67 ± 5.82	45.14 ± 3.24	44.71 ± 4.91	45.86 ± 1.74	33.20 ± 4.04	41.43 ± 5.52
Basolateral amygdaloid nucleus	805.6 ± 94.95	836 ± 75.37	753.6 ± 62.06	816.4 ± 55.59	795.6 ± 151.9	883 ± 119.1
Dentate gyrus	83.25 ± 2.09	88 ± 10.30	76.75 ± 6.76	86.67 ± 5.48	63 ± 6.29	70 ± 9.05
CA1	123 ± 2.64	144 ± 14.51	127.4 ± 9.04	114.6 ± 9.98	86.50 ± 0.56 ★★	139.2 ± 11.83 ☆
CA3	68.75 ± 2.72	66.25 ± 2.86	59 ± 1.87	67.25 ± 3.19	32.60 ± 1.72 ★★★	66.33 ± 2.40 ☆☆☆
Habenular nucleus	58 ± 1.52	57 ± 4.73	53 ± 1.35	53.75 ± 1.18	44.20 ± 0.86 ★★	54 ± 2.64 ☆

Data represents the mean ± S.E.M. ($n = 3$–7 mice per group). ★★ $P < 0.01$; ★★★ $P < 0.001$ compared to chronic saline group. ☆ $P < 0.05$; ☆☆ $P < 0.01$; ☆☆☆ $P < 0.001$ comparison between similar groups receiving chronic nicotine with or without baclofen (two-way ANOVA; multiple comparison post hoc test). SAL, saline; MEC, mecamylamine; BAC, baclofen; NIC, nicotine.

DISCUSSION

These results showed that c-Fos and BDNF expression were modified in several brain areas during MEC-precipitated NIC withdrawal syndrome in mice. In addition, BAC pre-treatment re-established the c-Fos and BDNF expression modified in specific brain regions during MEC-precipitated NIC withdrawal. These results suggest that BAC effect could be related to its ability to prevent the somatic signs expression (wet-dog-shakes and paw tremor) of NIC withdrawal, previously reported by our laboratory (Varani et al., 2011).

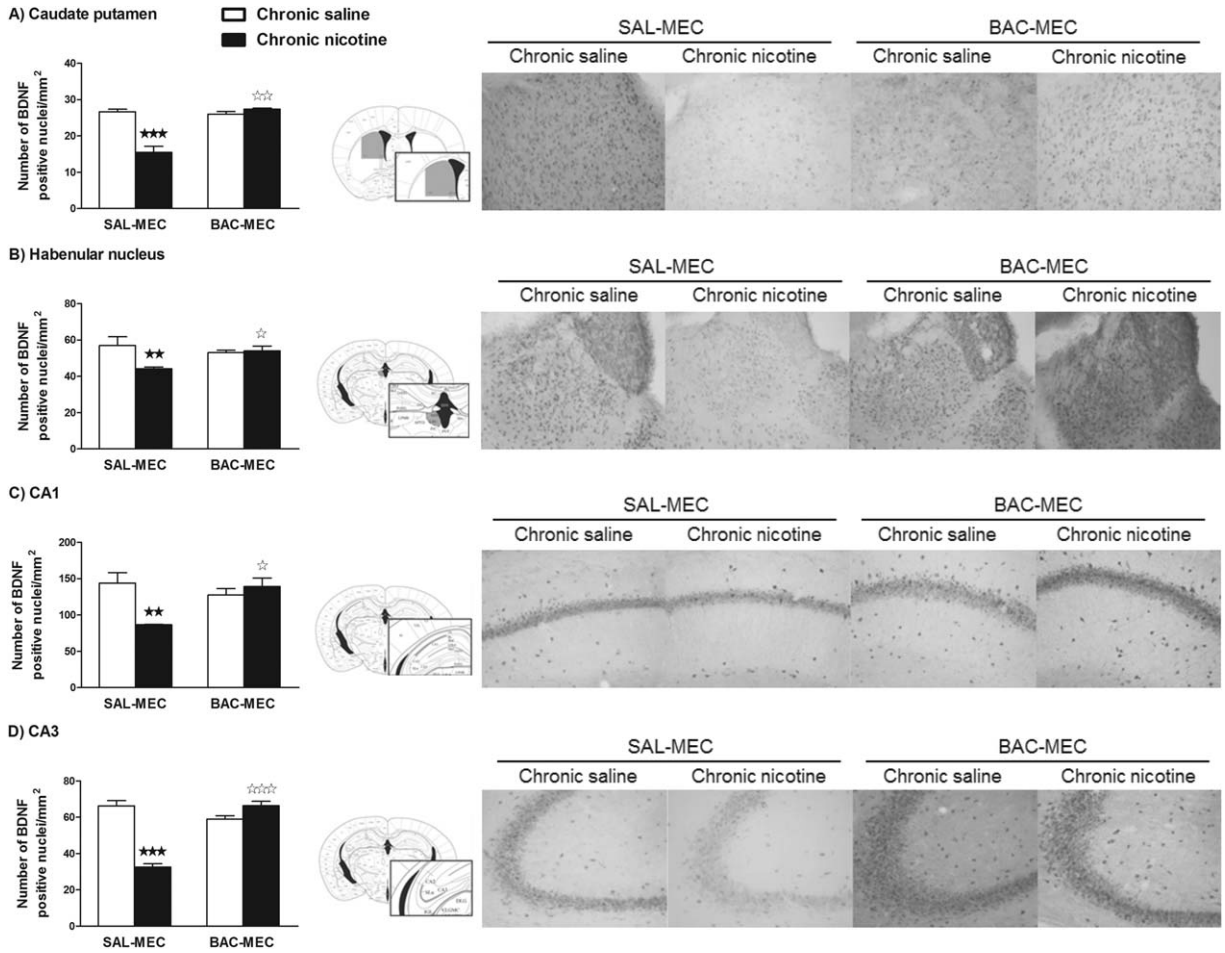

Fig. 2. BDNF expression (number of BDNF positive nuclei/mm^2) in mice following nicotine (NIC) withdrawal and its prevention with baclofen (BAC) in caudate putamen (**A**, $n = 5$–7), habenular nucleus (**B**, $n = 3$–5), CA1 (**C**, $n = 3$–7) and CA3 (**D**, $n = 3$–5). Each column represents the mean + SEM. Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with NIC (2.5 mg/kg; sc) four times daily, during 7 days. On the day of the experiment (day 8) mice received the acute treatment: 15 min after the last injection of chronic treatment either BAC (2 mg/kg; i.p.) or saline was administered. Sixty minutes after the last injection of chronic treatment, mecamylamine (MEC; 2 mg/kg; i.p.) was administered to all animals. ★★ $P < 0.01$; ★★★ $P < 0.001$ compared to chronic saline group. ☆ $P < 0.05$; ☆☆ $P < 0.01$; ☆☆☆ $P < 0.001$ comparison between similar groups receiving chronic NIC with or without BAC (two-way ANOVA followed by multiple comparison test).

In this study, NIC dependence was induced using a chronic treatment of NIC (2.5 mg/kg, sc), four times daily, for 7 days. On day 8th, dependence was demonstrated by the injection of MEC (2 mg/kg, i.p.) and subsequent appearance of behavioral somatic signs (wet-dog-shakes and paw tremor) (Varani et al., 2011). No significant differences were observed in the c-Fos and BDNF expression between the SAL-SAL-MEC and SAL-SAL-SAL control groups, indicating that MEC is not able to induce changes in c-Fos and BDNF immunoreactivity. These results are in agreement with previous studies (Balerio et al., 2004; Castrén et al., 1993; Panagis et al., 2000; Plaza-Zabala et al., 2012; Schilström et al., 2000; Varani et al., 2012, Varani et al., Unpublished work). Therefore, the modified c-Fos and BDNF expression observed in this study may be attributed solely to the combination of chronic NIC treatment and acute MEC administration. In addition, no changes were observed in the c-Fos and BDNF expression of the NIC-SAL-SAL group in any of the brain areas studied. Accordingly, we previously reported that NIC chronic treatment did not modify the c-Fos and BDNF expression in several brain areas (Balerio et al., 2004; Varani et al., 2012, Varani et al., Unpublished work). Moreover, other authors reported that NIC chronically treated rodents did not affect the c-Fos (Salminen et al., 1999, 2000; Schroeder et al., 2001) and BDNF (Alzoubi and Alkadhi, 2014; Kivinummi et al., 2011; Ortega et al., 2013) expression in several brain areas compared to SAL treated rodents.

These results showed a significant decrease in the number of c-Fos positive nuclei in the DG of the hippocampus. Accordingly, we recently observed a

decrease in the c-Fos immunoreactivity in the DG during MEC-precipitated NIC withdrawal (Varani et al., 2012). Similarly, the c-Fos expression decreased in DG after a naloxone (μ-opioid receptor antagonist) acute administration in mice chronically treated with NIC (Balerio et al., 2004). c-Fos is a transcription factor considered to be a marker of neuronal activity (Dragunow and Faull, 1989), thus these results would indicate that the neuronal activity could decrease in the hippocampus during NIC withdrawal in mice. In addition, we found herein a marked reduction in BDNF expression in the CA1 and CA3 area of the hippocampus during MEC-precipitated NIC withdrawal. These results are in agreement with a previous study from our laboratory (Varani et al., Unpublished work). Given the fact that BDNF is involved in the regulation of synaptic plasticity (Lipsky and Marini, 2007), the decrease in the level of BDNF immunoreactivity of hippocampal CA1 and CA3 during NIC withdrawal, could suggest alterations in plasticity in these brain areas during withdrawal. Taken together, we suggest that a decrease of the neuronal activity and synaptic plasticity in the hippocampus could play an important role in the modulation of the somatic and motivational components of NIC withdrawal.

Several studies have shown that the striatum could also be involved in NIC withdrawal syndrome (Fung et al., 1996; Gäddnäs et al., 2000; Hildebrand et al., 1998; Slotkin and Seidler, 2007). Moreover, we have shown that MEC-precipitated NIC withdrawal syndrome induced striatal neurochemical alterations, together with an increase of the behavioral signs in mice (Varani et al., 2011). The striatum is composed of three anatomical divisions, AcbSh, AcbC, and Cpu (Paxinos and Franklin, 2004). It is well known that AcbSh mediates the addictive properties of NIC, while the contribution of Cpu and AcbC in addiction is still not fully understood (Balfour, 2009). Our results showed a significant increase of c-Fos immunoreactivity in AcbSh but not in AcbC and Cpu during MEC-precipitated NIC withdrawal. In contrast, we have previously reported a decrease of c-Fos expression in Cpu during naloxone-precipitated NIC withdrawal (Balerio et al., 2004). Furthermore, Marttila et al. (2006) observed that in rodents treated with NIC in drinking water for 5 days there was an increase of c-Fos expression in the AcbC but not in AcbSh and Cpu. However, this increase of c-Fos expression was not maintained 24 h or 7 weeks after cessation of NIC treatment. This discrepancy could be related to the different experimental protocols, the dose of NIC and the different method to precipitate the withdrawal. Interestingly, an increase of striatal nAChRs density has been observed after cessation of chronic NIC treatment (Slotkin et al., 2007; Turner et al., 2011). Accordingly, an autoradiography study from our laboratory revealed an increase of $\alpha_4\beta_2$ nAChRs binding sites in the AcbSh nucleus during MEC-precipitated NIC withdrawal (Varani et al., 2013). The increase of c-Fos expression in the AcbSh nucleus observed herein could reflect an increase of the neuronal activity, as a consequence of a compensatory mechanism to alleviate the abrupt absence of reinforcing stimulus. Regarding the BDNF immunoreactivity, we observed a decrease in BDNF expression in the Cpu but not AcbC and AcbSh during MEC-precipitated NIC withdrawal in mice, suggesting a possible alteration in the synaptic plasticity in Cpu. Accordingly, we recently observed a decrease in BDNF expression in the Cpu of NIC-withdrawn mice (Varani et al., Unpublished work). Conversely, Kivinummi et al., (2011) reported an increase in the BDNF expression in the Acb of NIC-withdrawn mice. In addition, we previously reported a decrease in the striatal dopamine and serotonin levels in NIC-withdrawn mice (Varani et al., 2011), which could support that the plasticity would be affected during NIC withdrawal in the Cpu.

The Hb and interpeduncular (IP) nuclei are two small regions known to be connected by an axon bundle, the fasciculus retroflexus. In rodents, the Hb–IP axis has been implicated in a variety of brain functions and behaviors (Klemm, 2004). There is evidence indicating that the Hb is a brain area involved in mediating the NIC withdrawal syndrome in mice (Baldwin et al., 2011; Salas et al., 2004). These results show a significant increase in c-Fos immunoreactivity in the Hb during MEC-precipitated NIC withdrawal. Salas et al. (2009) observed that NIC withdrawal syndrome induces an increase of nAChRs density in the Hb, suggesting that the withdrawal state lead to neuronal adaptations. Consistent with this, we recently found an increase of α4β2 nAChR density during NIC withdrawal in the Hb (Varani et al., 2013). We also found a reduction in BDNF expression in the Hb during MEC-precipitated NIC withdrawal, which could indicate that the synaptic plasticity is affected in the Hb of NIC-withdrawn mice. These results are in accord with a previous study from our laboratory (Varani et al., Unpublished work). Therefore, the increased c-Fos expression and α4β2 nAChR density as well as the decreased BDNF expression could reflect neuronal adaptations in the Hb during NIC withdrawal.

The BST, BLA, and central amygdala are structures that are part of the amygdaloid complex (Paxinos and Franklin, 2004). Several studies suggest that some of the amygdaloid complex structures would be associated with both somatic and motivational components of NIC withdrawal (Bruijnzeel et al., 2012; Marcinkiewcz et al., 2009; Markou, 2008). We observed herein a significant decrease in the c-Fos expression in BST during MEC-precipitated NIC

withdrawal. These results are in agreement with a previous study from our laboratory (Varani et al., 2012). Although, in this study the c-Fos expression was not modified in the BLA and the central amygdala, we previously observed a decrease of c-Fos immunoreactivity in the BLA in NIC-withdrawn mice (Varani et al., 2012). In addition, Panagis et al. (2000) found that the c-Fos expression increases in the central amygdala, but not in other brain areas. These discrepancies could be related to several experimental factors such as: the species and strain used, the dose and route of NIC administration as well as the method used to precipitate the withdrawal. However, more importantly it has been reported that NIC withdrawal can generate activation or inhibition of Fos gene expression in several brain areas (Frenois et al., 2002). Therefore, we suggest that MEC-precipitated NIC withdrawal could increase or decrease c-Fos expression in specific limbic areas, which could be related to alterations in the neuronal activity in the amygdaloid complex.

Even though there is evidence showing the involvement of $GABA_B$ receptors in NIC addiction, the specific role of $GABA_B$ receptors on the possible molecular alterations induced by NIC withdrawal remains yet to be clarified. In this context, these results revealed that $GABA_B$ receptors would be involved in the c-Fos and BDNF alterations produced during NIC withdrawal. Thus, in this study we observed that BAC ($GABA_B$ receptor agonist) was able to re-establish the c-Fos expression which was increased in the AcbSh and decreased in the DG and BST during MEC-precipitated NIC withdrawal. BAC did not modify the increased c-Fos expression during NIC withdrawal in the Hb. This result could be explained by a decrease of $GABA_B$ receptors expression in this brain area, but this remains to be established. Conversely, BAC restored the decreased BDNF expression in the CA1 and CA3 areas of the hippocampus, the Hb, and Cpu during MEC-precipitated NIC withdrawal. Together, these results showed that the activation of $GABA_B$ receptor by baclofen would be necessary to prevent the alterations observed in the neuronal activity (c-Fos expression) and synaptic plasticity (BDNF expression) during MEC-precipitated NIC withdrawal in mice. In this study, there were no significant differences in c-Fos and BDNF expressions in SAL-SAL-MEC, SAL-BAC-MEC, NIC-SAL-SAL, and NIC-BAC-MEC groups compared to SAL-SAL-SAL control group. Although additional experiments would be required due to the constraints of this study, we could assume that baclofen given alone would not affect the c-Fos and BDNF expressions. Similarly, the same dose of BAC (2 mg/kg, i.p.) did not have intrinsic effect on c-Fos and BDNF expressions in nondependent animals (Pedrón et al., 2013, unpublished work). Therefore, the preventive effect of BAC on the c-Fos and BDNF alterations observed during NIC withdrawal could be due to the interaction between BAC and NIC withdrawal state. In this sense, we recently showed that c-Fos and BDNF expressions altered in NIC-withdrawn mice were restored in $GABA_{B1}$ knockout mice (Varani et al., 2012, Unpublished work), suggesting the involvement of $GABA_B$ receptors in the effects induced by MEC-precipitated NIC withdrawal. Interestingly, our previous studies reported the ability of BAC to re-establish the striatal and cortical dopamine and serotonin levels as well as the $\alpha4\beta2$ nAChR density modified during MEC-precipitated NIC withdrawal (Varani et al., 2011, 2013). Furthermore, we have shown that BAC prevents the behavioral signs observed during MEC-precipitated NIC withdrawal in mice (Varani et al., 2011), suggesting a relevant role of $GABA_B$ receptors in mediating the expression of somatic withdrawal signs.

In conclusion, taken into account our previous and these result, the ability of BAC in preventing the expression of NIC withdrawal signs could be partially explained by its ability to re-establish the dopamine and serotonin levels, the $\alpha_4\beta_2$ nAChRs density, and the expression of c-Fos and BDNF. This study provides new evidences regarding the involvement of $GABA_B$ receptors in the biochemical alterations induced during the MEC-precipitated NIC withdrawal in mice.

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