Progress in Neuro-Psychopharmacology & Biological Psychiatry 44 (2013) 217–225

Contents lists available at SciVerse ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Mecamylamine-precipitated nicotine withdrawal syndrome and its prevention with baclofen: An autoradiographic study of $\alpha_4\beta_2$ nicotinic acetylcholine receptors in mice

Andrés P. Varani [a], Marta C. Antonelli [b], Graciela N. Balerio [a,c,*]

[a] Instituto de Investigaciones Farmacológicas (UBA-CONICET), Junín 956, 5° Piso, C1113AAD, Buenos Aires, Argentina
[b] Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina
[c] Cátedra de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 5° Piso, C1113AAD, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 11 October 2012
Received in revised form 13 February 2013
Accepted 27 February 2013
Available online 14 March 2013

Keywords:
[^{3}H]epibatidine
Baclofen
Nicotine
Withdrawal
$\alpha_4\beta_2$ nicotinic receptor

ABSTRACT

A previous study from our laboratory showed that baclofen (BAC, $GABA_B$ receptor agonist) was able to prevent the behavioral expression of nicotine (NIC) withdrawal syndrome. To further investigate the mechanisms underlying this effect, we conducted this study, with the aims of analyzing $\alpha_4\beta_2$ nicotinic receptor density during NIC withdrawal and, in case we found any changes, of determining whether they could be prevented by pretreatment with BAC. Swiss Webster albino mice received NIC (2.5 mg/kg, s.c.) 4 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 brain autoradiography with [^{3}H]epibatidine was carried out at five different anatomical levels. Autoradiographic mapping showed a significant increase of $\alpha_4\beta_2$ nicotinic receptor labeling during NIC withdrawal in the nucleus accumbens shell (AcbSh), medial habenular nucleus (HbM), thalamic nuclei, dorsal lateral geniculate (DLG) nucleus, fasciculus retroflexus (fr), ventral tegmental area, interpeduncular nucleus and superior colliculus. BAC pretreatment prevented the increased $\alpha_4\beta_2$ nicotinic receptor binding sites in the AcbSh, MHb, thalamic nuclei, DLG nucleus and fr. The present results suggest a relationship between BAC's preventive effect of the expression of NIC withdrawal signs, and its ability to restore the changes in $\alpha_4\beta_2$ nicotinic receptor labeling, evidenced in specific brain areas in NIC withdrawn animals.

1. Introduction

Nicotine (NIC), as delivered in tobacco smoke, is one of the most widely abused drugs worldwide (Wong and Licinio, 2001) and it is responsible for profound behavioral effects that can contribute to ongoing smoking (Stolerman and Jarvis, 1995). In rodents, NIC produces several behavioral responses, including changes in locomotion, nociception, anxiety, learning, memory, rewarding effects and physical dependence (Decker et al., 1995). The pharmacological effects of NIC are mediated by the activation of nicotinic acetylcholine receptors (nAChRs), which are widely distributed through the central nervous system (CNS). Neuronal nAChRs are pentameric ligand-gated ion channels, composed of either homomeric or heteromeric combinations of different subunits, (α_2–α_{10}) and (β_2–β_4), which generates a wide diversity of receptors with various electrical and binding properties (Millar and Gotti, 2009). The most abundant nAChRs subtypes in the CNS are homomeric α_7 and heteromeric $\alpha_4\beta_2$ (Millar and Gotti, 2009), and they have been proposed to play an important role in NIC addictive properties such as dependence (Benowitz, 2010) and withdrawal syndrome (De Biasi and Salas, 2008). Previous reports have shown the presence of $\alpha_4\beta_2$ nAChRs in the medial habenula (MHb), thalamus, dorsal lateral geniculate nucleus, fasciculus retroflexus, hippocampus, ventral tegmental area (VTA), interpeduncular (IP) nucleus, caudate putamen, superior colliculus, cortex (Cx) and striatum (Baddick and Marks, 2011; Champtiaux et al., 2003; Gotti and Clementi, 2004; Gotti et al., 2005a,b). Interestingly, several studies showed increased levels of nAChRs after NIC withdrawal in the same brain areas described above (Gould et al., 2012; Pauly et al., 1996; Slotkin et al., 2007). Although a number of preclinical studies have demonstrated that brain areas such as striatum, Cx (Varani et al., 2011), MHb, IP nucleus (Salas et al., 2009), hippocampus (Davis and Gould, 2009), and VTA (Nomikos et al., 1999) are implicated in the expression of NIC withdrawal syndrome, the specific brain regions and nAChRs subtypes that mediate the different somatic signs have not been firmly identified yet.

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,

Abbreviations: NIC, nicotine; BAC, baclofen; MEC, mecamylamine; SAL, saline; nAChRs, nicotinic acetylcholine receptors; Acb, nucleus accumbens; Cx, motor cortex; CPu, caudate putamen; BST, bed nucleus stria terminalis; MHb, medial habenula; DLG, dorsal lateral geniculate nucleus; fr, fasciculus retroflexus; VTA, ventral tegmental area; IP, interpeduncular nucleus; SN, substantia nigra; PAG, periaqueductal gray.

* Corresponding author at: Instituto de Investigaciones Farmacológicas (CONICET), Junín 956 5° Piso, Buenos Aires C1113AAD, Argentina. Tel.: +54 11 4961 5949; fax: +54 11 4963 8593.
E-mail address: gbalerio@ffyb.uba.ar (G.N. Balerio).

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http://dx.doi.org/10.1016/j.pnpbp.2013.02.016

given that the selective $GABA_B$ agonist, baclofen (BAC) was able to increase the hypolocomotive and decrease antinociceptive effects induced by NIC (data not published). In addition, we have recently observed that BAC prevented the expression of NIC withdrawal syndrome in mice (Varani et al., 2011). Moreover, we have found that BAC was able to reestablish the decreased cortical and striatal dopamine and serotonin levels during NIC withdrawal (Varani et al., 2011). On the other hand, we have also reported that the $GABA_B$ antagonist, 2-OH-saclofen, is able to block the anxiolytic and anxiogenic effects induced by NIC (Varani and Balerio, 2012). Finally, a recent study from our laboratory showed that acute behavioral responses to NIC (antinociception, hypolocomotion and anxiety-related effect) and mecamylamine(MEC)-precipitated NIC withdrawal syndrome are modified in $GABA_{B1}$ knockout mice (Varani et al., 2012).

Considering our previous results and in order to investigate if the behavioral and neurochemical effects of BAC pretreatment during MEC-precipitated NIC withdrawal (Varani et al., 2011) were related to adaptive changes of $\alpha_4\beta_2$ nAChRs, the aim of the present study was to analyze the $\alpha_4\beta_2$ nAChRs binding sites in several brain areas of mice during NIC dependence, MEC-precipitated NIC withdrawal, and BAC pretreatment.

2. Materials and methods

2.1. Animals

Male Swiss Webster mice obtained from Bioterio Central (Facultad de Farmacia y Bioquimica, 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 ± 10% humidity) and manipulated for three 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.

2.2. Drugs

(−)-Nicotine hydrogen tartrate salt ([−]-1-methyl-2-[3-pyridil] pyrrolidine) (Sigma-Aldrich, USA), mecamylamine hydrochloride (Sigma-Aldrich, USA) and (±)-baclofen (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.

2.3. Chronic treatment

Mice were rendered dependent by subcutaneous (s.c.) injection of NIC (2.5 mg/kg), four times daily, for seven consecutive days (injections were given at 04:00 AM, 10:00 AM, 16:00 PM, and 22:00 PM). 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 seven consecutive days.

2.4. Acute treatment

On day 8, dependent mice received the last dose of NIC at 10:00 AM and were then randomly divided into three groups ($n = 15$): 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, i.p.), 15 and 60 min after the last dose of NIC, respectively, in order 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 ($n = 15$): 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 and Balerio, 2012; Varani et al., 2011). 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). On the other hand, 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 made 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 two hours (Damaj et al., 2007).

2.5. Autoradiography assays

2.5.1. Preparation of brain sections

Thirty minutes after the last injection, mice (n = 5 per experimental group) were sacrificed and intact whole brains were removed immediately following cervical dislocation. Brains were rapidly frozen by immersion in freon (−40 °C) and stored at −80 °C. Frozen coronal sections (14 μm) were cut at five different anatomical levels in a cryostat at −20 °C, thawed, mounted onto gelatin-coated microscopic slides, and stored at −80 °C until use (Antonelli et al., 1989).

2.5.2. Quantitative autoradiography of [^{3}H]epibatidine binding

Sections were processed for nicotinic autoradiography based on the technique previously described by Marks et al. (1998). Briefly, slides were thawed at room temperature. Slide-mounted tissue sections were first preincubated in binding buffer (NaCl, 144 mM; KCl, 1.5 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; HEPES, 20 mM; pH = 7.5) for 10 min twice at room temperature. Sections were incubated for 120 min at 22 °C in binding buffer containing 400 pM (+)-[^{3}H] epibatidine (specific activity = 49 Ci/mmol; Amersham, UK) to label the $\alpha_4\beta_2$-nicotinic acetylcholine receptors. Nonspecific binding was determined with 10 mM NIC. After incubation, slides were washed as follows (all washes at 0 °C): 1× binding buffer for 10 s twice, 0.1× binding buffer for 10 s twice and 5 mM HEPES for 10 s twice. Sections were dried with a stream of air generated by 15-cm fans.

2.5.3. Film exposure and image analysis

Autoradiograms were obtained after exposing sections to Kodak BIOMAX MR-1 (Sigma) films at −4 °C for 1–4 months in light-tight cassettes. Radioactivity standards (American Radiolabeled Chemical Inc.) consisting of 14 sections of methacrylate plastic impregnated with tritium (0.14–489 μCi/g) were jointly exposed with the sections. Films were developed in Kodak Dektol developer (Sigma) and fixative. Autoradiography images were scanned in a conventional scanner, and analyses made using Image J software (developed at the U.S. National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/). Receptor binding levels were measured for the following regions: nucleus accumbens core (AcbC) and shell (AcbSh), motor cortex (deep layer; Cx), caudate putamen (CPu), bed nucleus stria terminalis (BST), medial

habenula (MHb), thalamic nuclei, dorsal lateral geniculate nucleus (DLG), fasciculus retroflexus (fr), ventral tegmental area (VTA), interpeduncular nucleus (IP), superior colliculus, substantia nigra (SN) and periaqueductal gray (PAG). Structures were identified according to the corresponding outlines from the Mouse Atlas of Paxinos and Franklin (2004). Firstly, the optimal plate was selected according to the images obtained from the film exposure. Finally, the limits of each brain area were defined taking into account some structures which can be easily identified such as corpus callosum, commissures, lateral ventricles, third ventricle, etc. The sections were obtained at five anatomical levels: bregma 1.10 mm, −1.22 mm, −2.70 mm, −2.92 mm, −3.52 mm. For the IPN the number of subjects was 4 in the SAL-SAL-MEC, SAL-SAL-SAL and NIC-SAL-SAL groups and 5 in the rest of the experimental groups. For the SN and PAG the number of subjects was 4 in the SAL-SAL-MEC, SAL-BAC-MEC and NIC-SAL-MEC groups and 5 in the rest of the experimental groups. For the thalamic nuclei the number of subjects was 4 in the SAL-SAL-SAL group and 5 in the rest of the experimental groups. In all remaining brain areas the number of subjects was 5 for each experimental group. The six different experimental groups were processed together to ensure a paired protocol for binding, film apposition, and image analysis. The operator measuring optical densities was unaware of the experimental condition of each section. Optic density was converted to nCi/mg of tissue using the calibrated methacrylate tritium standards, and after subtracting nonspecific (background) from total binding, specific binding was expressed as fmol/mg tissue. For each anatomical level, left and right side of four contiguous sections (eight measurements per subject-brain) represented total binding; the eight determinations were averaged for each subject. The nonspecific binding was determined separately for each anatomical level using 4 sections. [^{3}H]epibatidine binding was at background levels in the presence of 10 mM unlabelled NIC. The specific binding was 60% since the nonspecific binding was around 40%.

2.6. Statistical analysis

Results obtained for [^{3}H]epibatidine binding sites were analyzed by using two-way analysis of variance (ANOVA) with chronic treatment (saline or NIC) and acute treatment (BAC or saline) 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.

3. Results

There were no differences between NIC-SAL-SAL, SAL-SAL-SAL control, SAL-BAC-MEC control and SAL-SAL-MEC control groups for [^{3}H]epibatidine binding sites in all brain areas studied after the last injection (Tables 1 and 2).

Two-way ANOVA (Table 2) showed a significant interaction between chronic treatment (saline or NIC) and acute treatment (BAC or saline) in the AcbSh ($F_{(2,24)} = 9.82$; $P < 0.001$), MHb ($F_{(2,24)} = 11.31$; $P < 0.001$), thalamic nuclei ($F_{(2,23)} = 4.10$; $P < 0.05$), DLG ($F_{(2,24)} = 8.18$; $P < 0.01$), fr ($F_{(2,24)} = 11.08$; $P < 0.001$), IP ($F_{(2,21)} = 4.58$; $P < 0.05$), VTA ($F_{(2,24)} = 6.54$; $P < 0.01$) and superior colliculus ($F_{(2,24)} = 8.21$; $P < 0.01$), while there were no significant interactions for the rest of the brain areas. In addition, two-way ANOVA revealed significant main effects for chronic treatment only in the AcbSh ($F_{(1,24)} = 10.88$; $P < 0.01$), MHb ($F_{(1,24)} = 16.75$; $P < 0.001$), DLG ($F_{(1,24)} = 7.94$; $P < 0.01$), fr ($F_{(1,24)} = 6.95$; $P < 0.05$), IP ($F_{(1,21)} = 33.51$; $P < 0.001$) and VTA ($F_{(1,21)} = 42.12$; $P < 0.001$). Two-way ANOVA also showed significant main effects for acute treatment only in the AcbSh ($F_{(2,24)} = 16.59$; $P < 0.001$), MHb ($F_{(2,24)} = 9.24$; $P < 0.001$), thalamic nuclei ($F_{(2,23)} = 5.31$; $P < 0.05$), DLG ($F_{(2,24)} = 11.01$; $P < 0.001$), fr ($F_{(2,24)} = 15.93$; $P < 0.001$), VTA ($F_{(2,24)} = 8.26$; $P < 0.01$) and superior colliculus ($F_{(2,24)} = 5.37$; $P < 0.01$).

The multiple comparison post hoc test revealed that binding levels in NIC-withdrawn (NIC-SAL-MEC) mice significantly increased in the AcbSh ($F_{(5,24)} = 12.74$; $P < 0.001$), MHb ($F_{(5,24)} = 11.57$; $P < 0.001$), thalamic nuclei ($F_{(5,23)} = 4.26$; $P < 0.05$), DLG nucleus ($F_{(5,24)} = 9.26$; $P < 0.001$), fr ($F_{(5,24)} = 12.19$; $P < 0.001$), IP nucleus ($F_{(5,21)} = 10.66$; $P < 0.01$), VTA ($F_{(5,24)} = 14.34$; $P < 0.001$) and superior colliculus ($F_{(5,24)} = 6.17$; $P < 0.01$) compared with SAL-SAL-MEC control group. Additionally, BAC pretreatment (NIC-BAC-MEC group) induced a significant decrease in binding levels of AcbSh ($F_{(5,24)} = 12.74$; $P < 0.001$), MHb ($F_{(5,24)} = 11.57$; $P < 0.01$), thalamic nuclei ($F_{(5,23)} = 4.26$; $P < 0.05$), DLG nucleus ($F_{(5,24)} = 9.26$; $P < 0.001$) and fr ($F_{(5,24)} = 12.19$; $P < 0.001$), compared with NIC-SAL-MEC group (Figs. 1, 2 and Tables 1, 2).

No significant changes were observed between the experimental groups for binding levels in any of the other brain areas studied (AcbC, motor cortex, CPu, BST, SN and PAG) (Tables 1 and 2).

4. Discussion

The present results show that MEC-precipitated NIC withdrawal dramatically affects $\alpha_4\beta_2$ binding sites in specific brain regions. We also report that the $GABA_B$ agonist BAC is able to prevent the increase

Table 1
[^{3}H]epibatidine autoradiography of α4β2 nAChR binding sites (fmol/mg of tissue) 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	58.2 ± 10.0	80.4 ± 4.3	56.4 ± 11.5	70.9 ± 13.2	57.0 ± 10.7 ★★★	63.3 ± 10.1
Nucleus accumbens shell	74.4 ± 12.4	85.8 ± 8.7	70.9 ± 11.5	80.7 ± 11.9	182.3 ± 19.5	66.2 ± 5.8 ☆☆☆
Motor cortex (deep layers)	52.3 ± 6.6	71.6 ± 7.2	66.8 ± 9.8	50.0 ± 6.7	57.3 ± 9.0	74.7 ± 5.4
Caudate putamen	81.6 ± 9.4	78.1 ± 9.9	92.8 ± 2.7	95.3 ± 15.3	76.3 ± 12.9	100.1 ± 7.3
Bed nucleus stria terminalis	97.8 ± 11.8	75.8 ± 16.3	87.5 ± 7.1	86.1 ± 4.1	86.6 ± 21.4	77.6 ± 16.3
Medial habenula	54.1 ± 3.8	50.5 ± 5.7	55.9 ± 6.4	47.2 ± 5.5	130.2 ± 17.8 ★★★	76.3 ± 9.0 ☆☆
Thalamic nuclei	196.9 ± 16.4	213.3 ± 13.2	212.5 ± 29.1	178.9 ± 43.8	326.9 ± 10.8 ★	208.2 ± 23.3 ☆
Dorsal lateral geniculate nucleus	349.2 ± 7.0	364.6 ± 15.6	360.7 ± 29.3	342.7 ± 27.1	500.0 ± 14.2 ★★★	366.2 ± 13.1 ☆☆☆
Fasciculus retroflexus	369.2 ± 20.4	357.6 ± 20.3	317.2 ± 27.6	338.1 ± 19.2	502.6 ± 12.8 ★★★	329.3 ± 12.3 ☆☆☆
Ventral tegmental area	450.4 ± 136.3	512.0 ± 83.6	522.3 ± 72.0	597.8 ± 59.8	1271.6 ± 64.2 ★★★	1081.8 ± 103.2 ★★
Interpeduncular nucleus	97.4 ± 2.7	92.9 ± 7.0	78.0 ± 13.3	124.3 ± 25.4	239.3 ± 24.8 ★★	234.3 ± 36.1 ★★★
Superior colliculus	303.0 ± 47.9	275.0 ± 37.0	338.3 ± 13.4	232.6 ± 29.0	496.0 ± 21.2 ★★	359.8 ± 54.3
Substantia nigra	101.5 ± 6.4	86.6 ± 5.7	107.5 ± 12.6	87.9 ± 6.3	112.2 ± 8.5	94.2 ± 5.1
Periaqueductal gray	57.9 ± 3.5	47.2 ± 5.6	58.6 ± 3.5	64.7 ± 2.4	60.9 ± 1.7	56.6 ± 2.1

Data represents the mean ± S.E.M. (n = 4–5 mice per group). ★ $P < 0.05$; ★★ $P < 0.01$; ★★★ $P < 0.001$ compared with chronic saline group. ☆ $P < 0.05$; ☆☆ $P < 0.01$; ☆☆☆ $P < 0.001$ comparison between similar groups receiving chronic nicotine with or without BAC (two-way ANOVA; multiple comparison post hoc test).

Table 2
Statistical analysis of [^{3}H]epibatidine autoradiographic study of α4β2 nAChR binding in different brain regions.

	Two-way ANOVA						Multiple comparison	
	Chronic treatment		Acute treatment		Interaction		BAC pretreatment	
Brain region	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Nucleus accumbens core	$F_{(1,24)} = 0.006$	NS	$F_{(2,24)} = 0.37$	NS	$F_{(2,24)} = 1.87$	NS	$F_{(5,24)} = 0.89$	NS
Nucleus accumbens shell	$F_{(1,24)} = 10.88$	<0.01	$F_{(2,24)} = 16.59$	<0.001	$F_{(2,24)} = 9.82$	<0.001	$F_{(5,24)} = 12.74$	<0.001
Motor cortex (deep layers)	$F_{(1,24)} = 0.26$	NS	$F_{(2,24)} = 3.26$	NS	$F_{(2,24)} = 1.05$	NS	$F_{(5,24)} = 1.77$	NS
Caudate putamen	$F_{(1,24)} = 0.26$	NS	$F_{(2,24)} = 1.84$	NS	$F_{(2,24)} = 0.13$	NS	$F_{(5,24)} = 0.84$	NS
Bed nucleus stria terminalis	$F_{(1,24)} = 0.33$	NS	$F_{(2,24)} = 0.73$	NS	$F_{(2,24)} = 0.65$	NS	$F_{(5,24)} = 0.62$	NS
Medial habenula	$F_{(1,24)} = 16.75$	<0.001	$F_{(2,24)} = 9.24$	<0.001	$F_{(2,24)} = 11.31$	<0.001	$F_{(5,24)} = 11.57$	<0.01
Thalamic nuclei	$F_{(1,23)} = 1.97$	NS	$F_{(2,23)} = 5.31$	<0.05	$F_{(2,23)} = 4.10$	<0.05	$F_{(5,23)} = 4.26$	<0.05
Dorsal lateral geniculate nucleus	$F_{(1,24)} = 7.94$	<0.01	$F_{(2,24)} = 11.01$	<0.001	$F_{(2,24)} = 8.18$	<0.01	$F_{(5,24)} = 9.26$	<0.001
Fasciculus retroflexus	$F_{(1,24)} = 6.95$	<0.05	$F_{(2,24)} = 15.93$	<0.001	$F_{(2,24)} = 11.08$	<0.001	$F_{(5,24)} = 12.19$	<0.001
Interpeduncular nucleus	$F_{(1,21)} = 33.51$	<0.001	$F_{(2,21)} = 3.02$	NS	$F_{(2,21)} = 4.58$	<0.05	$F_{(5,21)} = 10.66$	NS
Ventral tegmental area	$F_{(1,21)} = 42.12$	<0.001	$F_{(2,24)} = 8.26$	<0.01	$F_{(2,24)} = 6.54$	<0.01	$F_{(5,24)} = 14.34$	NS
Superior colliculus	$F_{(1,24)} = 3.65$	NS	$F_{(2,24)} = 5.37$	<0.01	$F_{(2,24)} = 8.21$	<0.01	$F_{(5,24)} = 6.17$	NS
Substantia nigra	$F_{(1,21)} = 1.38$	NS	$F_{(1,21)} = 0.26$	NS	$F_{(1,21)} = 0.62$	NS	$F_{(5,21)} = 0.60$	NS
Periaqueductal gray	$F_{(1,21)} = 1.11$	NS	$F_{(2,21)} = 0.68$	NS	$F_{(1,21)} = 0.97$	NS	$F_{(5,21)} = 0.92$	NS

Two-way ANOVA with chronic treatment and acute treatment as between-subjects factors. When significant interaction between these factors was observed, the difference between two means was tested by multiple comparison post hoc test. See Materials and methods for details.

of $\alpha_4\beta_2$ nAChRs levels induced by MEC-precipitated NIC withdrawal in several brain areas, suggesting they could play a role in preventing the expression of NIC withdrawal signs, an effect previously reported for BAC (Varani et al., 2011).

In this study we induced NIC chronic treatment in mice by implementing a previously reported experimental protocol, which consisted in a chronic treatment of NIC (2.5 mg/kg; s.c.), four times a day, for 7 days (Varani et al., 2011). The dependence state did not

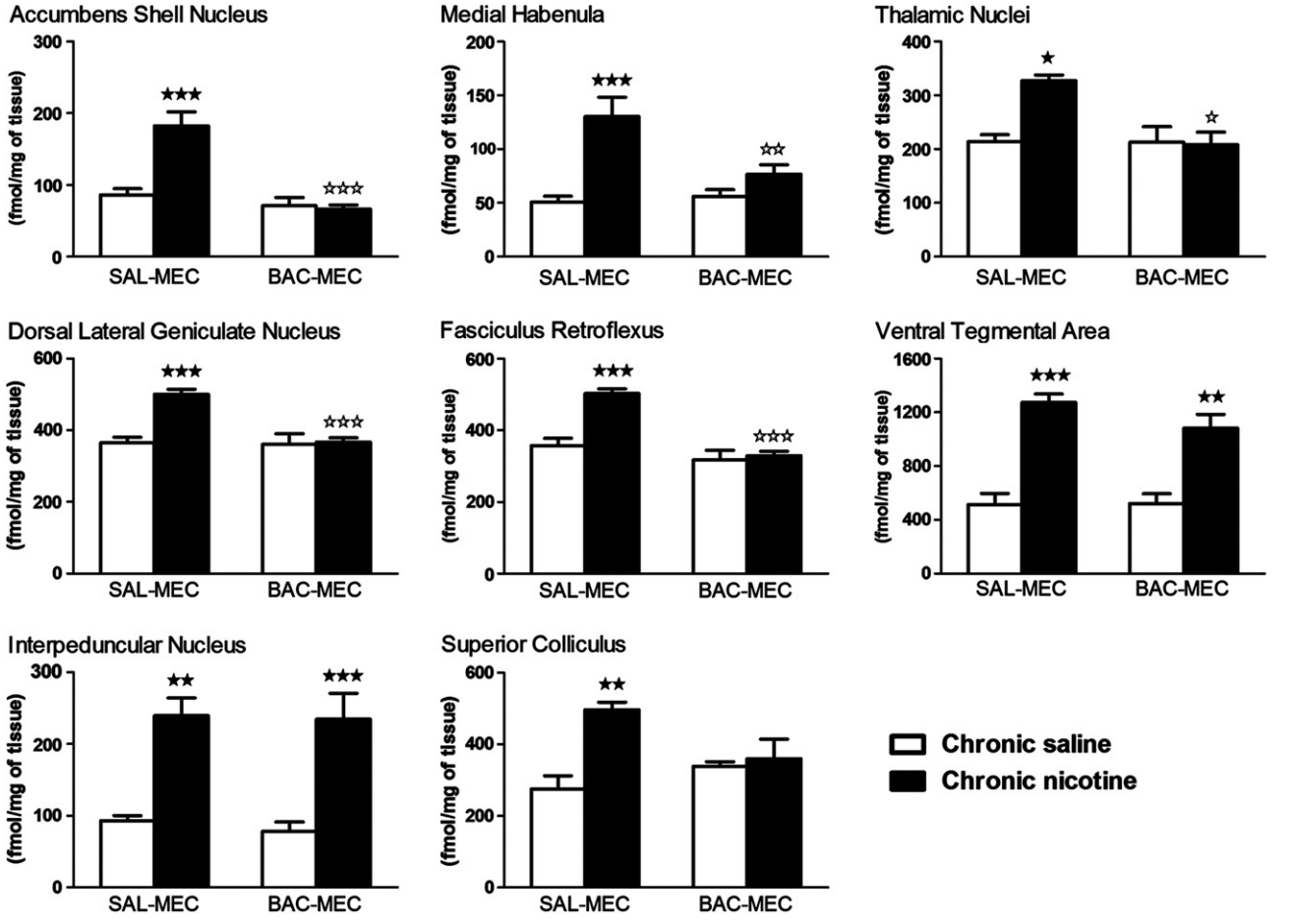

Fig. 1. [^{3}H]epibatidine autoradiographic study of α4β2 nAChR binding sites (fmol/mg of tissue) in mice following nicotine (NIC) withdrawal and its prevention with baclofen (BAC) in accumbens shell nucleus, medial habenula, thalamic nuclei, dorsal lateral geniculate nucleus, fasciculus retroflexus, ventral tegmental area, interpeduncular nucleus and superior colliculus. Each column represents the mean + SEM (n = 4–5 mice for each group). Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with NIC (2.5 mg/kg; s.c.) 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 were administered. Sixty minutes after the last injection of chronic treatment, mecamylamine (MEC; 2 mg/kg; i.p.) was administered to all animals. ★ $P < 0.05$; ★★ $P < 0.01$; ★★★ $P < 0.001$ compared with 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).

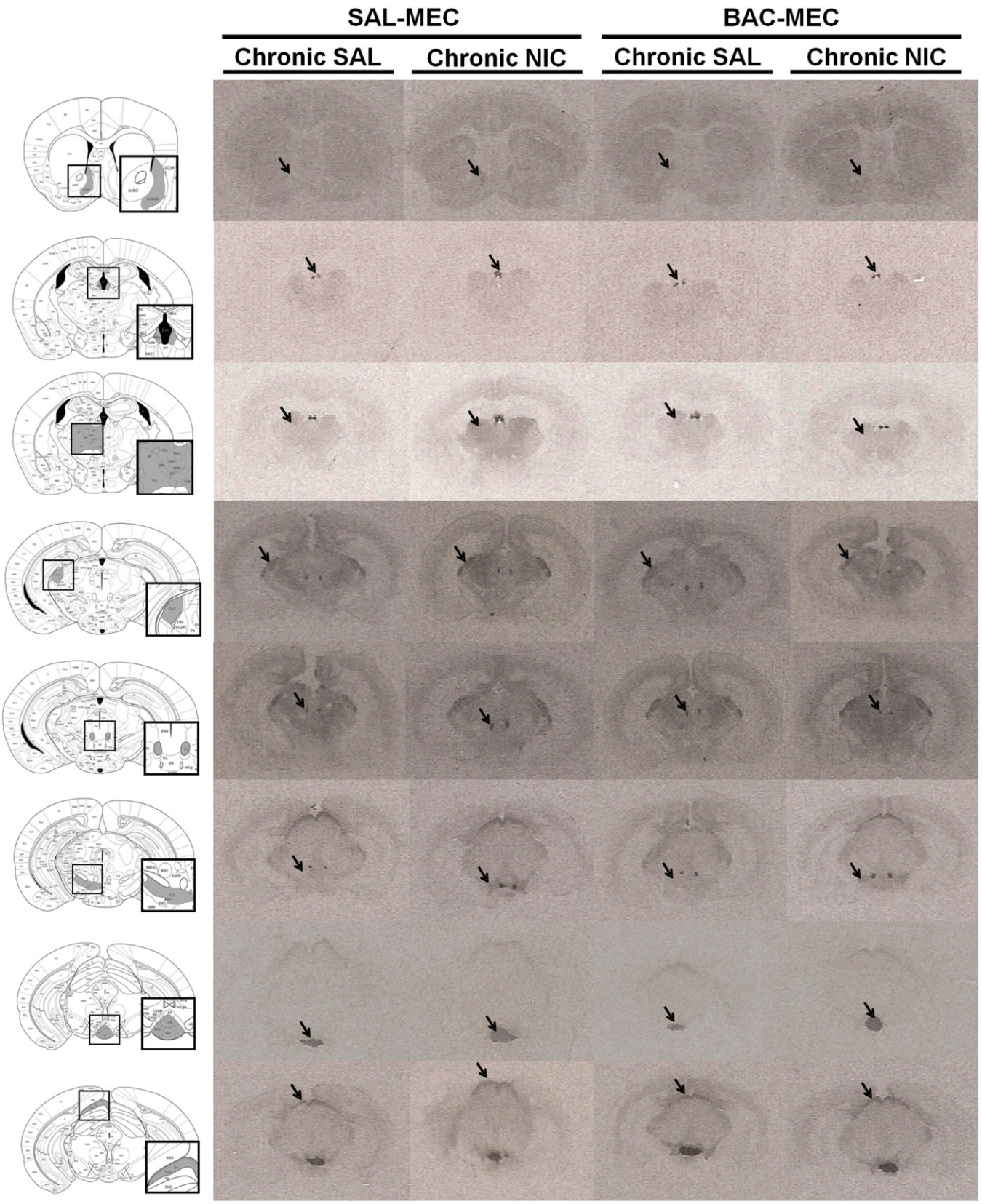

Fig. 2. [^{3}H]epibatidine autoradiograms of α4β2 nAChR binding in mice of SAL-SAL-MEC control, SAL-BAC-MEC control, NIC-SAL-MEC and NIC-BAC-MEC groups. The first and second columns show the SAL-SAL-MEC control and NIC-SAL-MEC groups, respectively while the third and fourth columns show the SAL-BAC-MEC control and NIC-BAC-MEC groups, respectively. The first line shows sections cut at the nucleus accumbens shell level (bregma 1.10 mm). The second and third lines show sections cut at the medial habenula and thalamic nuclei levels, respectively (bregma −1.22). The fourth and fifth lines show sections cut at the dorsal lateral geniculate nucleus and fasciculus retroflexus levels, respectively (bregma −2.70). The sixth line shows sections cut at the ventral tegmental area level (bregma −2.92). The seventh and eighth lines show sections cut at the interpeduncular nucleus and superior colliculus levels, respectively (bregma −3.52). The arrows indicate the brain areas measured.

modify $\alpha_4\beta_2$ binding sites in any of the brain areas studied, as evaluated by autoradiography with [^{3}H]epibatidine, a specific ligand of the $\alpha_4\beta_2$ heteropentamer (Marks et al., 2006; Metaxas et al., 2010; Traynor, 1998; Whiteaker et al., 2000). These results are in agreement with a previous study which reported that mice treated with NIC (2.4 mg/kg/day) for 24 h, 5 and 15 days showed no changes in [^{125}I]epibatidine binding sites in CPu, Acb, motor cortex, thalamic nuclei, MHb and VTA (Even et al., 2008). In addition, early studies using L-[^{3}H]-nicotine ligand showed that nAChRs levels were not altered in brain areas such as thalamic nuclei, MHb, DLG and IP nucleus of NIC-treated mice during 7 (Pauly et al., 1996) or 10 (Marks et al., 1992) days. Moreover, it has been demonstrated in rats that tobacco smoke exposure did not affect [^{125}I]epibatidine binding sites in cortex, MHb, SN, fr and superior colliculus (Small et al., 2010). Conversely, it is known that chronic NIC exposure elicits increases in high-affinity nAChRs binding sites (upregulation) in brains of mice (Marks et al., 1983, 2004, 2011). The mechanisms behind this upregulation are not totally clear and remain controversial; however some theories state that strong desensitization is a necessary requirement for upregulation (Buisson and Bertrand, 2002; Paterson and Nordberg, 2000). In spite of this proposed desensitization, electrophysiological studies showed small residual responses of nAChRs following long-term exposure to low doses of NIC in oocytes (Hsu et al., 1996), HEK293 (Buisson and Bertrand, 2001) and SH-SY5Y (Sokolova et al., 2005) cells. It could then be speculated that such a strong desensitization could not be achieved, as these few remaining functional receptors would be less prone to desensitization (Fenster et al., 1997; Olale et al., 1997; Paterson and Nordberg, 2000), thus no consequent upregulation could occur. This would explain the fact that, in our present study, $\alpha_4\beta_2$ binding sites were not increased in the NIC-SAL-SAL group. In this sense, it is well known that NIC dose, route (self-administered, experimenter delivered, minipumps, etc.), rate, frequency and duration of administration are possible factors that would alter the balance of nAChRs desensitization and resensitization, suggesting that changes on nAChRs density could depend on the protocol used (Matta et al., 2007). Finally, another alternative explanation is that nAChR expression patterns may differ depending upon whether an animal experiences spontaneous NIC withdrawal versus MEC-precipitated NIC withdrawal.

Dependent subjects are exposed to withdrawal effects upon cessation of drug intake. NIC withdrawal is a collection of affective and somatic symptoms that reflect an imbalance in brain neurochemistry (Paolini and De Biasi, 2011), created by removing the NIC source, or precipitated by administration of a nAChR antagonist such as MEC (Balerio et al., 2004; De Biasi and Salas, 2008; Malin and Goyarzu, 2009; Salas et al., 2009). In this study, we used a dose of MEC of 2 mg/kg, i.p. in order to precipitate withdrawal in NIC-treated mice, as we previously reported (Varani et al., 2011). The SAL-SAL-MEC control group did not show significant differences with respect to the SAL-SAL-SAL control group, indicating that the dose of MEC used was not by itself able to induce changes in [^{3}H]epibatidine binding sites in SAL-treated mice. Accordingly, Salas et al. (2004) have shown that an acute injection of MEC in mice chronically treated with saline was not able to modify [^{3}H]epibatidine binding sites in several brain areas. Therefore, in our study the changes observed in NIC-withdrawn mice can be solely attributed to the combination of the NIC-dependent state plus MEC administration.

The $\alpha_4\beta_2$ nAChRs subtypes are widely distributed and highly expressed in several brain areas such as the MHb, thalamic nuclei, DLG nucleus, fr, hippocampus, VTA, IP nucleus, CPu, superior colliculus, Cx and striatum (Baddick and Marks, 2011; Champtiaux et al., 2003; Gotti and Clementi, 2004; Gotti et al., 2005a,b; Huang and Winzer-Serhan, 2006; Whiteaker et al., 2000). Changes in nAChRs expression has been suggested to mediate tolerance, locomotor sensitization and addiction to NIC (Changeux, 2010; Govind et al., 2009; Hilario et al., 2012; Nashmi et al., 2007; Wecker et al., 2010), even though these are associations only. Interestingly, several studies have also found increased levels of nAChRs after NIC withdrawal in some of the brain areas described above (Gould et al., 2012; Pauly et al., 1996; Slotkin et al., 2007). Accordingly, in our present study we observed a pronounced increase of [^{3}H]epibatidine binding sites in the AcbSh, MHb, thalamic nuclei, DLG nucleus, fr, VTA, IP nucleus and superior colliculus during the MEC-precipitated NIC withdrawal syndrome. Considering that affective and somatic symptoms characteristic of NIC abstinence have been mapped partly to the mesolimbic dopaminergic system, which originates from dopaminergic neurons in the VTA and projects to the Acb nucleus and other forebrain limbic structures (De Biasi and Dani, 2011), the increase in $\alpha_4\beta_2$ nAChRs labeling in VTA could be regarded as a compensatory mechanism tending to alleviate the abrupt absence of the reinforcing stimulus. In line with our results, it has been established that in NIC withdrawn rodents, the $\alpha_4\beta_2$ nAChRs binding sites were increased in the midbrain (Ribeiro-Carvalho et al., 2009; Slotkin et al., 2007), a brain region that includes several brain areas such as VTA, SN, PAG, superior colliculus, among others (Eapen et al., 2011; Peltopuro et al., 2010). Notably, despite the lack of reports showing a participation of superior colliculus in NIC withdrawal syndrome, we also found an increased $\alpha_4\beta_2$ nAChRs density in this area, suggesting it could also be important to elicit some of the withdrawal behavioral signs. However, further studies should be conducted to conclusively demonstrate this hypothesis.

Regarding the other areas under analysis, Hb and IP nucleus are two small nuclei known to be connected by an axon bundle, the fr. In rodents, the Hb–IP axis has been implicated in a variety of brain functions and behaviors (Klemm, 2004). In addition, Hb has also been shown to modulate dopaminergic activity in striatal areas (Matsumoto and Hikosaka, 2007), which are known to be important for the effects of drugs of addiction, including NIC (Hyman et al., 2006). Our present study shows that $\alpha_4\beta_2$ nAChRs binding sites increase in Hb, IP and fr during MEC-precipitated NIC withdrawal. In agreement with this, Salas et al. (2004) have shown that [^{3}H]epibatidine binding sites increased during NIC withdrawal in the Hb and IP of wild-type mice. Reports on several lines of mutant mice pointed to the Hb–IP axis as a critical mediator of NIC withdrawal signs and several other effects of NIC (Baldwin et al., 2011). Furthermore, Salas et al. (2009) observed that in mice chronically treated with NIC, MEC microinjected into the MHb or IP nucleus was able to precipitate NIC withdrawal, which implies a relevant role of this axis in mediating the expression of NIC abstinence signs.

Even though much attention has been given to the effects of NIC on the mesolimbic dopaminergic system with the aim of elucidating its role in drug reward and reinforcement, 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. 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). Regarding nAChRs striatal density in mice, an increase has been observed after cessation of chronic NIC treatment (Slotkin et al., 2007; Turner et al., 2011). Accordingly, in the present study the autoradiography mapping performed in the striatum revealed an increase of $\alpha_4\beta_2$ nAChRs binding sites in the AcbSh, but not in AcbC and CPu, during MEC-precipitated NIC withdrawal. The upregulation observed in the AcbSh could be the consequence of a compensatory mechanism in order to alleviate the abrupt absence of reinforcing stimulus. However, further studies should be necessary to reveal the role of this brain area during the somatic expression of MEC-precipitated NIC abstinence.

Despite the lack of literature showing the role of thalamic nuclei and DLG nucleus in mediating the behavioral signs of NIC withdrawal syndrome, it is known that NIC exposure affects neuronal circuits involving these brain areas. Indeed, the activation of nAChRs in these

areas may be responsible for the effects of smoking seen in tobacco dependent subjects, such as improvements in attentional performance, mood, anxiety, and irritability (Brody, 2006). In this regard, we observed herein that an injection of MEC in mice chronically treated with NIC induced an increase of $\alpha_4\beta_2$ nAChRs binding sites in the thalamic nuclei and DLG nucleus. Similarly, Pauly et al. (1996) showed that combined chronic administration of NIC and MEC in mice increased nAChRs levels in the same brain areas. Therefore, although additional experiments would be necessary, our present results suggest that the thalamic nuclei and DLG nucleus may play an important part in mediating the motivational component of MEC-precipitated NIC withdrawal syndrome.

We have previously reported that 2 mg/kg of the $GABA_B$ agonist BAC, administered before MEC, prevents the expression of NIC induced withdrawal signs (Varani et al., 2011). In this study, BAC was able to prevent the rise of $\alpha_4\beta_2$ nAChRs levels, produced by the MEC-precipitated withdrawal syndrome, in AcbSh, MHb, thalamic nuclei, DLG nucleus and fr. However, it was not able to normalize receptor levels in VTA, IP nucleus and superior colliculus. The dose of BAC (2 mg/kg) was selected based on our previous reports and did not have intrinsic effect in saline-treated mice (Diaz et al., 2001, 2003, 2004, 2006; Varani and Balerio, 2012; Varani et al., 2011). In the present study, the SAL-SAL-MEC group did not produce changes on $\alpha_4\beta_2$ nAChRs in non-dependent mice as well as the combination between BAC and MEC (SAL-BAC-MEC control group). On the other hand, there were no significant differences between these two groups (SAL-SAL-MEC and SAL-BAC-MEC) and the control group (SAL-SAL-SAL). Therefore, we could assume that BAC given alone would not affect the nAChR population, but this remains to be established.

Regarding the regional distribution, as we mentioned above, a high distribution of $\alpha_4\beta_2$ nAChRs has been reported in several brain structures. Interestingly, neuroanatomical studies in mammalian brains have shown high density of $GABA_B$ receptors in neurons of AcbSh, MHb, thalamic nuclei, DLG nucleus and fr (Bowery et al., 1987; Sander et al., 2009; Young and Chu, 1990). These reports clearly show an overlapping distribution of $GABA_B$ receptors and $\alpha_4\beta_2$ nAChRs, a fact that supports the hypothesis of a possible functional interaction between these two systems. On the one hand, this overlapping distribution could explain the fact that BAC was able to abolish the upregulation of $\alpha_4\beta_2$ nAChRs induced by MEC-precipitated NIC withdrawal in some of the brain areas studied. On the other hand, it is worth noting that nAChRs are localized prominently on GABA-containing neurons of thalamic nuclei, striatum (Grady et al., 2012; Grilli et al., 2009; Lu et al., 1998; McClure-Begley et al., 2009) and lateral geniculate nucleus (Cox and Sherman, 2000; Guo et al., 1998, 2005). In addition, it has been established that MHb and fr are brain areas enriched in GABAergic and cholinergic neurons (Lecourtier and Kelly, 2007), suggesting that the $\alpha_4\beta_2$ nAChRs could be located on GABAergic neurons. Therefore, taking into account the preceding evidence, it is possible to speculate that the effect of BAC observed in our present study is achieved by its interaction with GABA-containing neurons located in the different brain areas.

Preclinical and clinical studies support BAC as a promising drug to treat NIC addiction (Corrigall et al., 2000; Cousins et al., 2000; Fattore et al., 2009; Franklin et al., 2009). We recently showed that MEC-precipitated NIC withdrawal was abolished in $GABA_{B1}$ knockout mice (Varani et al., 2012), suggesting a relevant role of $GABA_B$ receptors in mediating the expression of somatic withdrawal signs. Hence, our present results suggest that BAC's ability to prevent the expression of NIC withdrawal signs could be partially explained by its ability to decrease the $\alpha_4\beta_2$ nAChRs levels available to bind MEC. To our knowledge, no mechanism has been proposed to date to explain how this $GABA_B$ agonist prevents the levels of upregulated $\alpha_4\beta_2$ nAChRs. Although the mechanisms causing this upregulation are not totally clear, there is general agreement that the effect is post-transcriptional, since NIC treatment does not alter subunit mRNA levels in rat and mouse brains (Bencherif et al., 1995; Marks et al., 1992; Peng et al., 1994). Six different post-transcriptional mechanisms have been proposed to account for NIC-induced upregulation: slow cell surface turnover, increased receptor trafficking to the surface, enhanced subunit maturation and assembly in the endoplasmic reticulum (ER), changes in subunit stoichiometry, blockade of subunit degradation in the ER, and nAChR conformational changes (Govind et al., 2009). The reported effect of BAC could be attributed to interferences in any of these six mechanisms. However, additional experiments should be conducted to elucidate this matter.

5. Conclusion

In conclusion, the present results showed that $\alpha_4\beta_2$ binding sites were affected in specific brain regions during MEC-precipitated NIC withdrawal syndrome. In addition, BAC was able to prevent an increase in $\alpha_4\beta_2$ nAChRs levels induced by MEC-precipitated NIC withdrawal in several of the brain areas studied. Taken together, our current information suggests that the preventive effect of BAC on the expression of MEC-precipitated NIC withdrawal could be associated with its ability to prevent the $\alpha_4\beta_2$ nicotinic receptor labeling in certain brain areas related to behavioral signs of NIC withdrawal. Finally, the present data support the idea that BAC may be a potential agent to treat NIC withdrawal.

Contributors

A. Varani executed the experimental protocols, statistical analyses and wrote the first draft of the manuscript. Dr Antonelli contributed to design and provided her expertise for the autoradiography assays. Dr Balerio designed the study, wrote the research questions and revised the manuscript draft. Dr Balerio is the Principal Investigator of the study.

Acknowledgment

This study was supported by grant UBACyT no. B016 and PIP 11420090100303 from the University of Buenos Aires and CONICET, respectively (P.I.: Dr Balerio) and ANPCYT (PICT 0040) (Dr. Antonelli). Andrés Varani is supported by a doctoral fellowship from the University of Buenos Aires (677/10). Founding sources had no further role in study design; in the collection, analysis and interpretation of the data; in the writing of the report; or in the decision to submit the paper for publication.

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