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$GABA_B$ receptors blockage modulates somatic and aversive manifestations induced by nicotine withdrawal

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ABSTRACT

There is substantial evidence that $GABA_B$ agonist, baclofen, prevents somatic and motivational responses induced by nicotine withdrawal and may target drug cue vulnerabilities in humans. In this context, we explored different aspects associated with the possible mechanisms whereby the $GABA_B$ receptors might influence nicotine withdrawal. Male mice received nicotine (2.5 mg/kg, s.c.) 4 times daily, for 7 consecutive days. Nicotine-treated mice received the nicotinic acetylcholine receptor antagonist, mecamylamine (MEC, 2 or 3.5 mg/kg, s.c.), to precipitate the withdrawal state. A second group of dependent mice received 2-hydroxysaclofen ($GABA_B$ receptor antagonist, 1 mg/kg, s.c.) before MEC-precipitated abstinence. Somatic signs of nicotine withdrawal were measured for 30 min. Anxiogenic-like response associated to nicotine withdrawal was assessed by the elevated plus maze test. The dysphoric/aversive effect induced by nicotine withdrawal was evaluated using conditioned place aversion paradigm. Dopamine, serotonin and its metabolites concentrations were determined by HPLC in the striatum, cortex and hippocampus. Finally, α4β2 nicotinic acetylcholine receptor density was determined in several brain regions using autoradiography assays. The results showed that MEC-precipitated nicotine withdrawal induced somatic manifestations, anxiogenic-like response and dysphoric/aversive effect, and 2-hydroxysaclofen potentiated these behavioral responses. Additionally, 2-hydroxysaclofen was able to change striatal dopamine levels and α4β2 nicotinic acetylcholine receptor density, both altered by MEC-precipitated nicotine withdrawal. These findings provide important contributions to elucidate neurobiological mechanisms implicated in nicotine withdrawal. We suggest that $GABA_B$ receptor activity is necessary to control alterations induced by nicotine withdrawal, which supports the idea of targeting $GABA_B$ receptors to treat tobacco addiction in humans.

1. Introduction

Preclinical and clinical studies have revealed that nicotine (NIC) withdrawal syndrome has both a somatic and emotional component [1,2]. The somatic component of NIC withdrawal in humans is characterized by symptoms such as bradycardia, gastrointestinal disturbances, hyperalgesia and increased appetite. On the other hand, symptoms such as depression, dysphoria, irritability, anxiety, frustration, difficulty concentrating and reactivity to environmental stimuli have been described and they are related to the emotional component of NIC withdrawal [1,3]. These symptoms appear in the first 6–12 h after cessation of consumption, reaching the maximum peak at 1–3 days, and returning to normal values within 7–30 days after cessation of tobacco use [4]. In experimental animals, the main somatic signs observed are:

Abbreviations: NIC, nicotine; SAL, isotonic saline solution; VEH, glucose solution; MEC, mecamylamine hydrochloride; GABA, gamma-aminobutyric acid; SAC, 2-hydroxysaclofen; BAC, baclofen; DA, dopamine; DOPAC, 3,4- dihydroxyphenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid; nAChR, nicotinic acetylcholine receptor; HPLC, high-performance liquid chromatography; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell, 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 área; Ip, interpeduncular nucleus; SN, substantia nigra; PAG, periaqueductal gray; CNS, central nervous system.

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Table 1
2-hydroxysaclofen potentiates the somatic expression of mecamylamine-precipitated nicotine withdrawal.

Signs	Two-way ANOVA						One-way ANOVA, Tukey post hoc					
	Pretreatment		Treatment		Interaction		VEH-SAL *vs* VEH-NIC		SAC-SAL *vs* SAC-NIC		VEH-NIC *vs* SAC-NIC	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Wet-dog shakes	$F_{(1,32)}$=4.831	<0.05	$F_{(1,32)}$=44.830	<0.001	$F_{(1,32)}$=1.401	NS	$F_{(3,35)}$=17.021	<0.01	$F_{(3,35)}$=17.021	<0.001	$F_{(3,35)}$=17.021	<0.05
Paw tremor	$F_{(1,32)}$=23.286	<0.001	$F_{(1,32)}$=268.319	<0.001	$F_{(1,32)}$=15.418	<0.001	$F_{(3,35)}$=102.341	<0.001	$F_{(3,35)}$=102.341	<0.001	$F_{(3,35)}$=102.341	<0.001
Body tremor	$F_{(1,32)}$ = 0.083	NS	$F_{(1,32)}$=0.750	NS	$F_{(1,32)}$=0.083	NS	$F_{(3,35)}$=0.306	NS	$F_{(3,35)}$=0.306	NS	$F_{(3,35)}$=0.306	NS
Locomotor activity	$F_{(1,32)}$=0.488	NS	$F_{(1,32)}$=0.956	NS	$F_{(1,32)}$=0.488	NS	$F_{(3,35)}$=0.644	NS	$F_{(3,35)}$=0.644	NS	$F_{(3,35)}$=0.644	NS
Scratches	$F_{(1,32)}$=0.000	NS	$F_{(1,32)}$=5.628	<0.05	$F_{(1,32)}$=0.419	NS	$F_{(3,35)}$=2.016	NS	$F_{(3,35)}$=2.016	NS	$F_{(3,35)}$=2.016	NS
Ptosis	$F_{(1,32)}$=2.560	NS	$F_{(1,32)}$=0.640	NS	$F_{(1,32)}$=0.640	NS	$F_{(3,35)}$=1.280	NS	$F_{(3,35)}$=1.280	NS	$F_{(3,35)}$=1.280	NS
Piloerection	$F_{(1,32)}$=1.029	NS	$F_{(1,32)}$=0.114	NS	$F_{(1,32)}$=1.029	NS	$F_{(3,35)}$=0.724	NS	$F_{(3,35)}$=0.724	NS	$F_{(3,35)}$=0.724	NS
Teeth chattering	$F_{(1,32)}$=0.000	NS	$F_{(1,32)}$=0.500	NS	$F_{(1,32)}$=0.000	NS	$F_{(3,35)}$=0.167	NS	$F_{(3,35)}$=0.167	NS	$F_{(3,35)}$=0.167	NS
Global score	$F_{(1,32)}$=18.865	<0.001	$F_{(1,32)}$=198.799	<0.001	$F_{(1,32)}$=9.164	<0.01	$F_{(3,35)}$=75.609	<0.001	$F_{(3,35)}$=75.609	<0.001	$F_{(3,35)}$=75.609	<0.001

Two-way ANOVA with treatment (SAL or NIC) and pretreatment (VEH or SAC) as between-subjects factors. When significant interaction between these factors was observed, the difference between two means was tested by one-way ANOVA and Tukey post hoc test. See Section 2 for details.
NIC, nicotine; SAL, saline; VEH, vehicle; SAC, 2-OH-saclofen.

body tremor, wet dog shakes, ptosis, piloerection, licking of genitals, attempted escape, paw tremor and teeth chattering [5].

In reference to the negative emotional component, studies in animals clearly demonstrated the dysphoric / aversive effects of NIC withdrawal [3]. It has been shown that the α4, β4 and α7 subunits of nicotinic acetylcholine receptor (nAChR) are involved in mediating NIC withdrawal [3,6,7]. In addition, it is known that nAChRs located both peripherally and centrally mediate the somatic and motivational signs of NIC withdrawal. Particularly, those expressed at the central nervous system (CNS) level, seem to participate in the negative emotional manifestations of NIC withdrawal [3]. Decreased dopaminergic activity has been reported in the accumbens nucleus (Acb), prefrontal cortex (PFC) and the amygdala, which would be responsible for the dysphoria and anxiety-like behavior characteristic of NIC withdrawal [3]. All these modifications seem to be related to the dysphoric state, impulsivity, irritability, and anxiety; and interestingly all of them are manifested during NIC withdrawal in humans [8].

GABA acts via ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors. $GABA_B$ receptors are potential therapeutic targets for the treatment of pain, anxiety, and depression. However, its role as a potential target in substance use disorders has received the most attention. Preclinical research has shown that the $GABA_B$ receptor plays a crucial role in mediating the behavioral and molecular effects of drugs of abuse, with activation of the $GABA_B$ receptor being identified as a potential anti-addictive therapeutic strategy [9]. It has been demonstrated that $GABA_B$ receptor activity can modulate the rewarding effect [10–13] and other addictive properties induced by NIC [14,15]. In that respect, we have observed that biochemical (α4β2 nicotinic receptors, α4β2nAChR), neurochemical [dopamine (DA) and serotonin (5-HT) concentrations], molecular (expression of c-Fos and brain-derived neurotrophic factor), changes in behavioral responses induced by either acute or chronic NIC administration can be modulated by the activation ($GABA_B$ receptor agonist baclofen, BAC), the blockade ($GABA_B$ receptor antagonist 2-hydroxysaclofen, SAC) or the lack ($GABA_{B1}$ knockout mice, $GABA_{B1}$ KO) of $GABA_B$ receptors [16–21].

Even though a bunch of studies revealed the modulatory effect elicited by $GABA_B$ receptors on NIC withdrawal, the mechanisms implicated behind this interaction remains poorly understood. In order to improve the understanding of this interaction, we explored different aspects associated with the possible mechanisms whereby the $GABA_B$ receptors might influence the NIC withdrawal. In particular, we analyzed alterations at behavioral (somatic expression and dysphoric/aversive responses), neurochemical (monoamines concentration) and biochemical (α4β2nAChRs density) levels derived from the NIC withdrawal in mice pretreated with SAC.

2. Material and methods

2.1. Animals

Male Swiss Webster albino mice obtained from the 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 [22] (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 (NIC, [−]−1-methyl-2-[3-pyridil]pyrrolidine) (Sigma-Aldrich, USA), mecamylamine hydrochloride

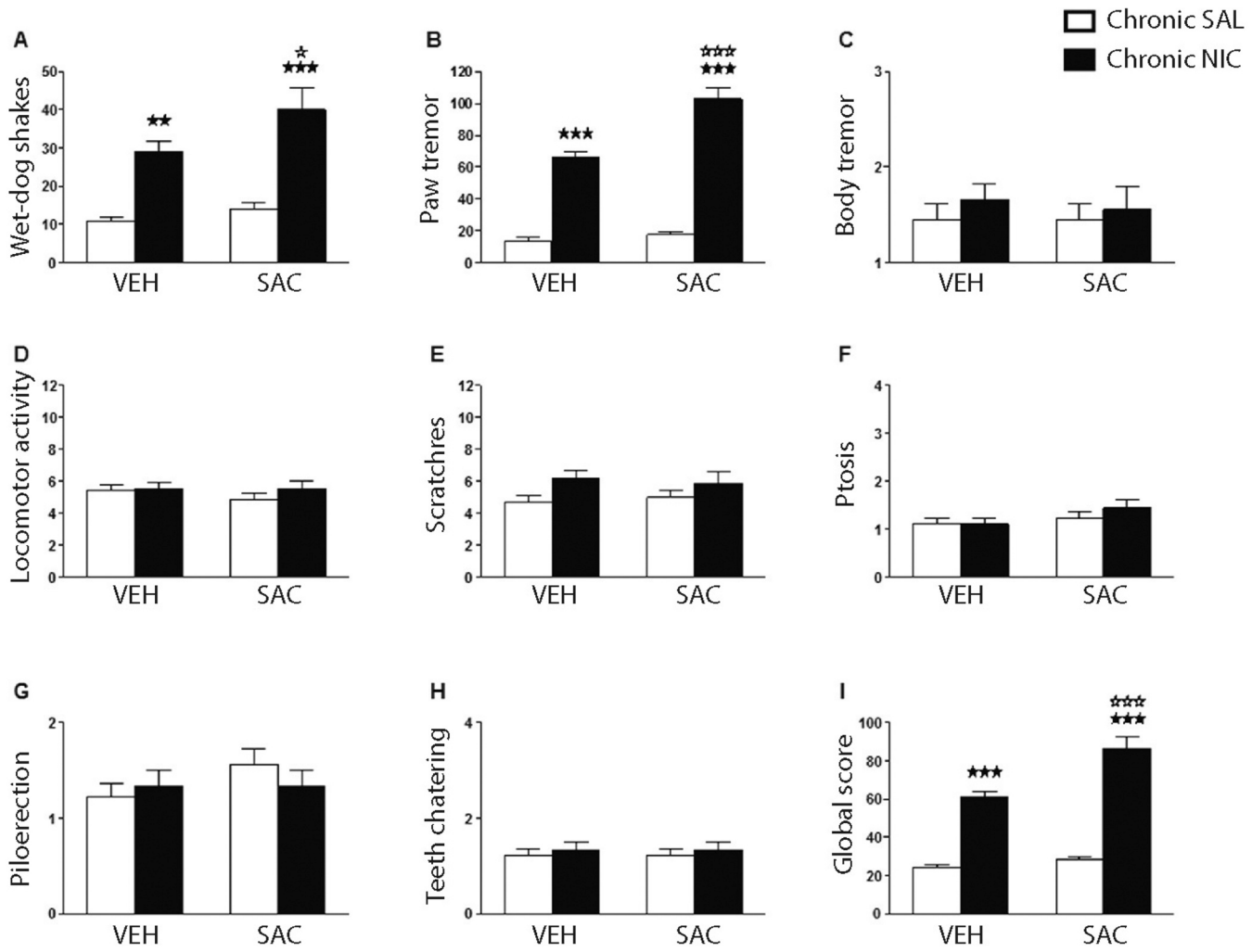

Fig. 1. Mecamylamine-precipitated nicotine withdrawal: behavioural signs. Each column represents the mean±SEM (n = 9 mice for each group) during 30 min. Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with nicotine (NIC, 2.5 mg/kg; s.c.) four times daily, for 7 days. On the day of the experiment (day 8), mice received the acute treatment: 50 min after the last injection of the chronic treatment, either 2-hydroxysaclofen (SAC, 1 mg/kg; s.c.) or vehicle (VEH) were administered. Sixty min after the last injection of the chronic treatment, mecamylamine (2 mg/kg; s.c.) was administered to all animals. ★★ $p < 0.01$, ★★★ $p < 0.001$ when compared to VEH group. ☆ $p < 0.05$, ☆☆☆ $p < 0.001$ comparison between similar groups receiving chronic NIC with or without SAC (two-way ANOVA followed by multiple comparison post hoc test).

(MEC) (Sigma-Aldrich, USA) and 2-hydroxysaclofen (SAC) (Sigma Chemical Co., Buenos Aires, Argentina) were used in this study. NIC and MEC were dissolved in isotonic saline solution (NaCl 0.9%) (SAL), and SAC was dissolved in isotonic (five percent) glucose solution (VEH) immediately before use. NIC, MEC and SAC were administered by subcutaneous (s.c.) route. NIC dose (2.5 mg/kg) used was calculated as NIC hydrogen tartrate salt (1 mg/kg of NIC hydrogen tartrate salt equals to 0.35087 mg/kg NIC free base); it was administered subcutaneously (s.c.). All drugs were administered in a volume of 10 ml/kg.

2.3. Chronic treatment

Mice were rendered dependent by 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 [23]. Control groups received SAL s.c., four times daily, for seven consecutive days.

2.4. Behavioral signs of mecamylamine-precipitated nicotine withdrawal

Seven days after the beginning of chronic treatment with NIC, on the day of the experiment (day 8), mice received SAC (1 mg/kg, s.c.) or VEH and 10 min after MEC (2 mg/kg, s.c.) or SAL were administered. After the last injection (MEC or SAL), mice were placed inside a circular clear plastic observation area, where the following abstinence signs were evaluated during 30 min, according to Castañé et al. [24]: locomotor activity, body tremor, ptosis, wet-dog shakes, teeth chattering, front paw tremor, scratching and piloerection. The number of wet-dog shakes, front paw tremor and scratches was counted. Ptosis, body tremor, piloerection and teeth chattering were scored 1 for appearance or 0 for nonappearance within each 5-min interval. The locomotor activity over 5-min periods was rated 0, 1 or 2 (0 for inactivity, 1 for low activity and 2 for normal activity). A quantitative value was obtained for these checked signs by adding up the individual values obtained for each 5-min period of the whole observation time. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight: 0.5 for each wet dog shake, front paw tremor and scratching; and 1 for presence of ptosis, body tremor, piloerection and teeth chattering during each 5-min observation interval. The relative weight of locomotor activity for each 5-min period was 0 normal activity, 0.5 low activity and 1 inactivity. Finally, a time course of the global withdrawal score was determined for each 5-min period of the whole observation time (30 min) for each animal [23].

Table 2
Somatic and motivational signs of control groups.

Signs	SAL-VEH-SAL	SAL-VEH-MEC	SAL-SAC-MEC	NIC-VEH-SAL
Somatic signs				
Wet-dog shakes	11.82 ± 0.47	10.98 ± 0.6	15.22 ± 1.43	12.13 ± 0.12
Paw tremor	15.36 ± 1.04	13.78 ± 2.30	17.56 ± 1.98	19.11 ± 1.33
Body tremor	1.35 ± 0.34	1.44 ± 0.17	1.44 ± 0.17	1.33 ± 0.12
Locomotor activity	6.13 ± 0.09	5.44 ± 0.33	4.88 ± 0.38	5.18 ± 0.37
Scratches	5.09 ± 0.30	4.66 ± 0.44	5.00 ± 0.47	4.95 ± 0.21
Ptosis	1.37 ± 0.11	1.11 ± 0.11	1.22 ± 0.14	1.61 ± 0.34
Piloerection	1.09 ± 1.09	1.22 ± 0.14	1.56 ± 0.17	1.18 ± 0.24
Teeth chattering	1.08 ± 0.17	1.22 ± 0.14	1.22 ± 0.14	1.29 ± 0.21
Global score	21.10 ± 1.44	24.06 ± 1.48	28.50 ± 1.21	25.23 ± 1.23
Motivational signs				
% of entries	13.3 ± 1.07	14.2 ± 0.87	11.1 ± 0.92	12.0 ± 0.10
% of time	4.13 ± 0.21	4.28 ± 0.29	3.99 ± 0.32	3.28 ± 0.23
CPA score	-15.3 ± 13.9	14.34 ± 17.3	13.24 ± 21.5	-9.20 ± 3.14

No significant differences were observed between control groups. Data represents the mean ± S.E.M. (n = 9 mice per group). NIC, nicotine; SAL, saline; VEH, vehicle; SAC, 2-hydroxysaclofen; MEC, mecamylamine.

2.5. Anxiety-like effects associated to mecamylamine-precipitated nicotine withdrawal

In order to evaluate the effect of SAC (1 mg/kg) on the anxiety-like effects associated to NIC withdrawal, NIC dependence was induced as mentioned in Section 2.3. and MEC (2 mg/kg, s.c.) was used to precipitate the NIC withdrawal. Immediately after MEC or SAL injection, mice (n = 7–9 per experimental group) were placed in the elevated plus-maze. SAC (1 mg/kg, s.c.) or VEH were administered 10 min before MEC injection. The elevated plus-maze [25,26] consisted of a black plastic apparatus with fours arms (16 × 5 cm) set in a cross from a neutral central square (5 × 5 cm). Two opposite arms were delimited by vertical walls (closed arms), while the other two opposite arms had unprotected edges (open arms). The maze was elevated 30 cm above the ground and illuminated from the top (100 lx). At the beginning of the 15-min observation session, each mouse was placed in the central neutral area, facing one of the open arms. The total number of visits to the closed and open arms, and the cumulative time spent in the open and closed arms were then observed on a monitor through a video camera system (Vision Robot, Buenos Aires, Argentina). An arm visit was recorded when the mouse moved both forepaws and the head into the arm, as we previously described [27].

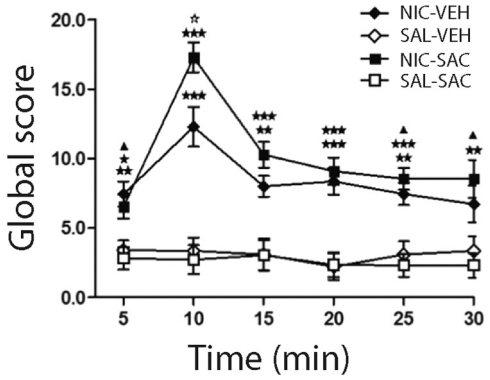

Fig. 2. Mecamylamine-precipitated nicotine withdrawal: time course of the global score. Each point represents the mean±SEM (n = 9 mice for each group) each 5-min period during the whole observation time (30 min). Empty symbol: chronic treatment with saline (SAL); filled symbol: chronic treatment with nicotine (NIC, 2.5 mg/kg; s.c.) four times daily, for 7 days. On the day of the experiment (day 8), mice received the acute treatment: 50 min after the last injection of the chronic treatment either 2-hydroxysaclofen (SAC, 1 mg/kg; s.c.) or vehicle (VEH) were administered. Sixty min after the last injection of the chronic treatment, mecamylamine (2 mg/kg; s.c.) was administered to all animals. ▲ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ when compared to VEH group. ☆ $p < 0.05$ comparison between similar groups receiving chronic NIC with or without SAC; ▲ $p < 0.05$ compared to the global score at 10 min (two-way ANOVA with one repeated measures variable followed by Tukey post hoc test).

2.6. Conditioned place aversion induced by mecamylamine-precipitated nicotine withdrawal

In a different set of animals, the place conditioning paradigm was used to evaluate the effect of SAC (1 mg/kg) on the dysphoric manifestations associated with nicotine withdrawal. The apparatus consisted of two main square conditioning compartments separated by a triangular neutral area [28]. The time spent by the mouse in each compartment was recorded by computerized monitoring software (Videotrack®, View Point, France). NIC dependence was induced as mentioned in Section 2.3. and MEC (3.5 mg/kg, s.c.) was used to precipitate the aversive manifestations of nicotine withdrawal. The place aversion conditioning paradigm consists of three phases: pre-conditioning, conditioning and post-conditioning. Pre-conditioning (day 8): after 7 days of treatment with NIC or SAL, each mouse was placed in the triangular area and had free access to both compartments for 20 min. Conditioning (day 9 and 10): Given the fact that none of the two compartments generates a place preference or aversion, MEC was administered indistinctly in one or another compartment. However, those animals that preferred one of the compartments received MEC in the initially preferred compartment. The procedure was based on previous studies with rats [55] and mice [29]. At 10:00 a.m., mice received SAL (s.c.) and were placed in the non-preferred compartment for 30 min. Four hours later, mice received an injection of MEC (3.5 mg / kg, s.c.) and were confined to the preferred compartment for 30 min. MEC dose was chosen taking into account previous studies [29,30]. SAC (1 mg/kg, s.c.) or VEH were administered 10 min before MEC injection. Mice were conditioned in this way for 2 consecutive days. Post-conditioning (day 11): This phase was carried out as in the pre-conditioning phase (the animals were placed in the neutral zone and had free access to both compartments for 20 min). The time spent in the central area was proportionally distributed and added to the time spent in each compartment, as previously reported [31]. A score was calculated for each mouse as the difference between the post-conditioning and pre-conditioning time spent in the drug-paired compartment.

2.7. Mecamylamine-precipitated nicotine withdrawal: concentration of monoamines and metabolites

High-performance liquid chromatography-coupled electrochemical detection [32] of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT, 5-hydroxyindolacetic acid (5-HIAA) was achieved using a Varian 5000 liquid chromatograph coupled to an electrochemical detector (BAS LC-4 C). Ten minutes after last injection on day 8 (the day of the experiment), mice (n = 3–6 per experimental group) were sacrificed by cervical dislocation; brains were quickly removed and placed in dry ice. When partially frozen, the striatum, hippocampus and cortex were dissected under a dissecting microscope. Brain tissues were weighed, homogenized, and deproteinezed in 0.2 N perchloric acid (1/20). Homogenates were centrifuged, and the supernatants were injected (50 µl) onto a 12.5 cm × 4 mm Nova-Pak C18 reverse phase column (Waters). Mobile phase for DA, DOPAC, 5-HT and 5-HIAA determinations contained NaH2-PO4–H2O 0.076 M, PICB8 5.24 ml/l, ethylenediaminetetraacetic acid (EDTA) 0.99 mM and six percent methanol. The electrode potential was set at 0.7 V. Peak heights were measured by Peak Simple Chromatography Data System (Model 302 Six Channel USB) and quantified based on standard curves using the same software. Concentrations of the monoamines and their metabolites were determined based on tissue wet weight.

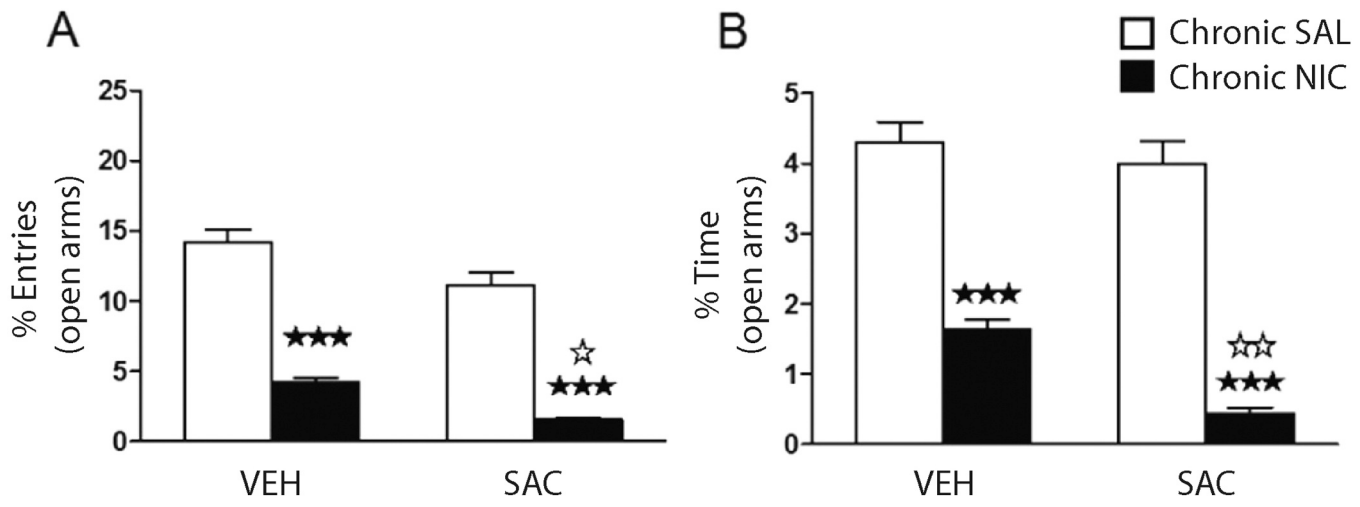

Fig. 3. Mecamylamine-precipitated nicotine withdrawal: dysphoric/aversive effect. Each column represents the mean±SEM (n = 9 mice for each group). Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with nicotine (NIC, 2.5 mg/kg; s.c.) four times daily, for 7 days. Mecamylamine (MEC, 3.5 mg/kg; s.c.) induced NIC withdrawal place aversion score and was calculated as the difference between the time (seconds) spent in the withdrawal associated compartment during the post-conditioning and the preconditioning phase. 2-hydroxysaclofen (SAC, 1 mg/kg, s.c.) or vehicle (VEH) were administered 10 min before MEC injection during the conditioning phase (see "Materials and methods" for details). ★★ $p < 0.01$, ★★★ $p < 0.001$ when compared to VEH group. ☆☆ $p < 0.01$ comparison between similar groups receiving chronic NIC with or without SAC (two-way ANOVA followed by multiple comparison post hoc test).

2.8. Mecamylamine-precipitated nicotine withdrawal: autoradiography assays

2.8.1. 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 [33].

Quantitative autoradiography of [^{3}H]epibatidine binding sections were processed for nicotinic autoradiography based on the technique previously described by Marks et al. [104]. Briefly, slides were thawed at room temperature. Slide-mounted tissue sections were first pre-incubated in binding buffer (NaCl, 144 mM; KCl, 1.5 mM; CaCl2, 2 mM; MgSO4, 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 α4β2-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.

2.8.2. 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 were 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 [34]. 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 Ip nucleus 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 unlabeled NIC. The specific binding was 60% since the nonspecific binding was around 40%.

2.9. Statistical analysis

For the statistical analysis we have excluded the outliers. We considered as outlier all values exceeding the mean ± [2 × SD] because is quite likely that these values could be consequence of other intervening variables rather than those analyzed in the present study. Results obtained for the time course of the global withdrawal score were analyzed by using two-way ANOVA (treatment × time) with one

Table 3
2-hydroxysaclofen potentiates the anxiogenic-like and dysphoric/aversive response of mecamylamine-precipitated nicotine withdrawal.

Signs	Two-way ANOVA						One-way ANOVA, Tukey post hoc					
	Pretreatment		Treatment		Interaction		VEH-SAL *vs* VEH-NIC		SAC-SAL *vs* SAC-NIC		VEH-NIC *vs* SAC-NIC	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
% of entries	$F_{(1,36)}$=215.191	<0.001	$F_{(1,36)}$=17.892	<0.001	$F_{(1,36)}$=0.125	NS	$F_{(3,35)}$=77.736	<0.001	$F_{(3,35)}$=77.736	<0.001	$F_{(3,35)}$=77.736	<0.05
% of time	$F_{(1,36)}$=172.882	<0.001	$F_{(1,36)}$=9.895	<0.01	$F_{(1,36)}$=3.612	NS	$F_{(3,35)}$=62.129	<0.001	$F_{(3,35)}$=62.129	<0.001	$F_{(3,35)}$=62.129	<0.01
CPA score	$F_{(1,36)}$=59.424	<0.001	$F_{(1,36)}$=6.562	<0.05	$F_{(1,32)}$=0.083	<0.05	$F_{(3,35)}$=24.128	<0.01	$F_{(3,35)}$=24.128	<0.001	$F_{(3,35)}$=24.128	<0.01

Two-way ANOVA with treatment (SAL or NIC) and pretreatment (VEH or SAC) as between-subjects factors. When significant interaction between these factors was observed, the difference between two means was tested by one-way ANOVA and Tukey post hoc test. See Section 2 for details.
NIC, nicotine; SAL, saline; VEH, vehicle; SAC, 2-OH-saclofen.

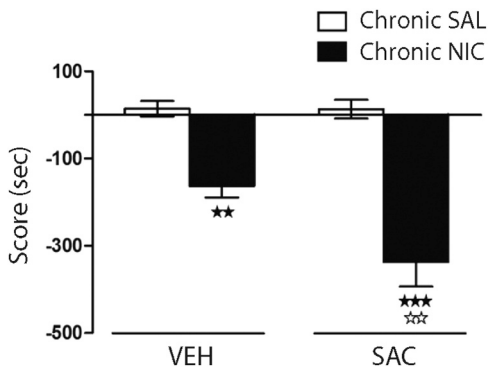

Fig. 4. Mecamylamine-precipitated nicotine withdrawal: anxiogenic-like effect. Each column represents the mean±SEM (n = 9 mice for each group). Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with nicotine (NIC, 2.5 mg/kg; s.c.) four times daily, for 7 days. Percentage of entries into (a) and time spent in the open arms were observed during 15 min immediately after mecamylamine (MEC, 2 mg/kg; s.c.) administration. 2-hydroxysaclofen (SAC, 1 mg/kg, s.c.) or vehicle (VEH) were administered 10 min before MEC injection. ★★★ $p < 0.001$ when compared to VEH group. ☆ $p < 0.05$, ☆☆ $p < 0.01$ comparison between similar groups receiving chronic NIC with or without SAC (two-way ANOVA followed by multiple comparison post hoc test).

repeated-measures variable (time; within measurements). When a significant interaction between these factors was observed, the difference between two means was analyzed by Tukey post hoc test. The remaining results were analyzed by using two-way analysis of variance (ANOVA) with chronic treatment (SAL or NIC) and acute treatment (SAC or VEH) 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. The level of significance was $p < 0.05$ in all experiments. Statistical analysis was performed using SPSS 26 software.

3. Results

3.1. Effect of pretreatment with SAC on the somatic expression of NIC withdrawal

The following somatic signs were significantly revealed in the different experimental groups: paw tremor, body tremor, teeth chattering, wet dog shakes, scratches, ptosis, piloerection, and locomotor activity (see Table 1 for two-way ANOVA and Tukey's post hoc test). The administration of MEC in mice chronically treated with NIC produced an increase in the expression of certain behavioral signs such as wet dog shakes ($p < 0.01$; Fig. 1A) and paw tremor ($p < 0.001$; Fig. 1B). SAC pretreatment potentiated these behavioral signs induced by the NIC withdrawal (wet dog shakes $p < 0.05$, paw tremor $p < 0.001$; Fig. 1A, B, respectively). The analysis of global abstinence revealed a significant increase in the somatic expression of NIC withdrawal ($p < 0.001$) and the SAC was able to potentiate this increase ($p < 0.001$; Fig. 1I). There were no significant differences between control groups (see Table 2). We also analyzed the time curve of global abstinence for 30 min at 5 min intervals. As shown in Fig. 2, Tukey post hoc test showed that the global score was increased in the abstinence group (NIC-VEH) compared to the SAL-VEH control group ($p < 0.05$) during the whole observation time. Moreover, this test also showed that the increase of the global score was higher at 10 min compared to the global score at 5 min ($p < 0.05$), 25 min ($p < 0.05$) or 30 min ($p < 0.05$) after MEC precipitated NIC withdrawal (Fig. 2). On the other hand, Tukey post hoc test showed that the global score was also increased in the abstinence group pretreated with SAC (NIC-SAC) compared to the SAL-SAC control group ($p < 0.01$) during the whole observation time (Fig. 2). However, the pretreatment with SAC showed that the increase of the global score was much higher

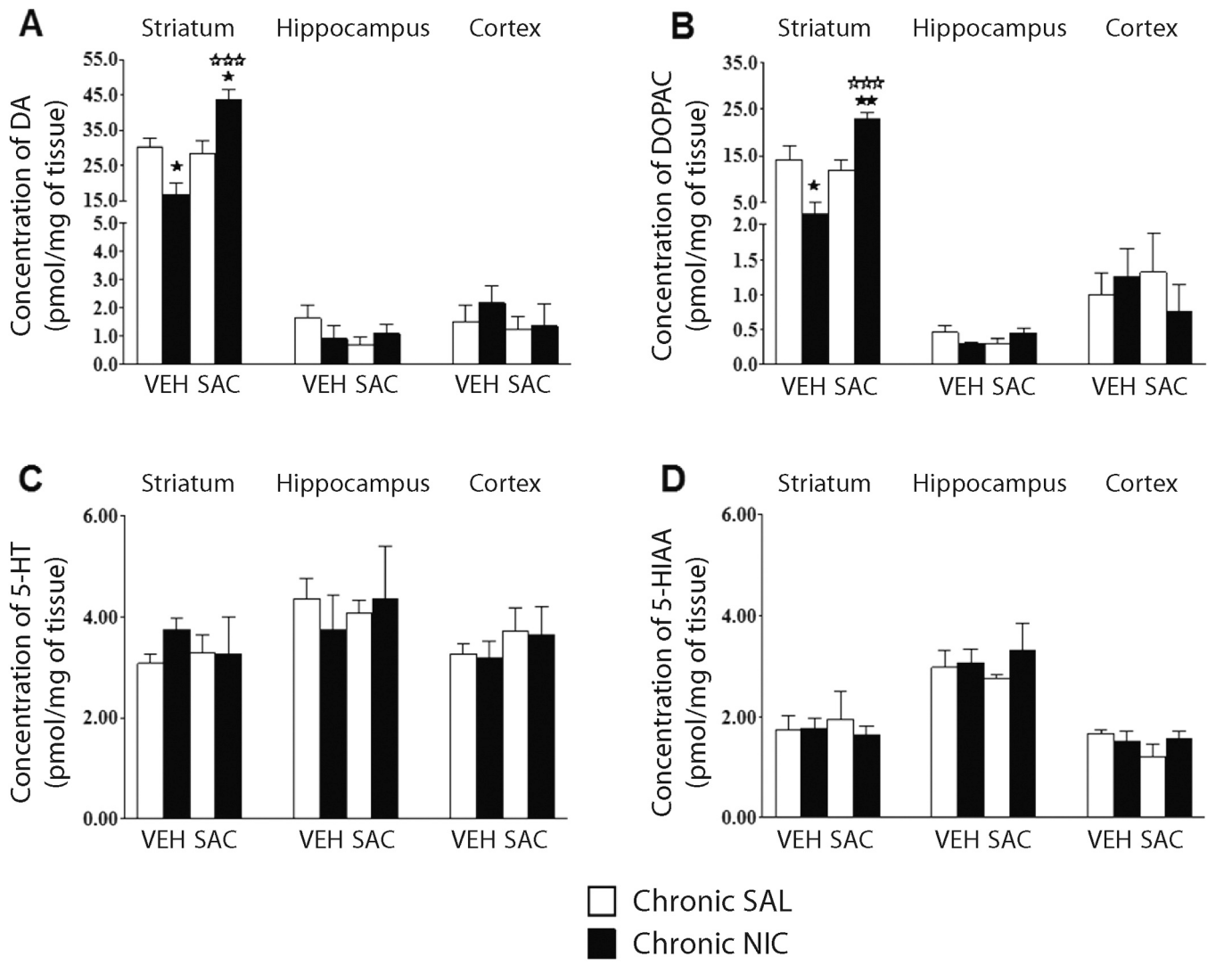

Fig. 5. Mecamylamine-precipitated nicotine withdrawal: concentrations of striatal, cortical and hippocampal monoamines and metabolites. Each column represents the mean±SEM (n = 4–6 mice for each group) of dopamine (DA; A), 3,4-dihydroxyphenylacetic acid (DOPAC; B), serotonin (5-HT; C), 5-hydroxyindolacetic acid (5-HIAA; D) concentrations. Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with nicotine (NIC, 2.5 mg/kg; s.c.) four times daily, for 7 days. On the day of the experiment (day 8), mice received the last injection of the chronic treatment and 50 min after, either 2-hydroxysaclofen (SAC, 1 mg/kg, s.c.) or vehicle (VEH) were administered. Sixty min after the last injection of the chronic treatment, mecamylamine (MEC, 2 mg/kg; s.c.) was administered to all animals. Ten minutes after the MEC injection brains were collected and monoamines and metabolites were measured. ★ $p < 0.05$, ★★ $p < 0.01$ when compared to VEH group. ☆☆☆ $p < 0.001$ comparison between similar groups receiving chronic NIC with or without SAC (two-way ANOVA followed by multiple comparison post hoc test).

at 10 min ($p < 0.01$) after MEC administration compared to the abstinence group (NIC-VEH) (Fig. 2). There were no significant differences between control groups for each 5-min interval of the global score time course.

3.2. Effect of SAC pretreatment on the anxiogenic-like response associated with NIC withdrawal

The results showed that the injection of MEC in NIC-dependent mice (NIC-VEH) induced an anxiogenic-like response, characterized by a decrease in the percentage of entries ($p < 0.001$: Fig. 3A) and time spent ($p < 0.001$; Fig. 3B) in the open arms of the elevated plus maze test. Similarly, a decrease in the percentage of entries ($p < 0.001$; Fig. 3A) and time spent ($p < 0.001$; Fig. 3B) in the open arms was observed in the abstinence group pretreated with SAC (NIC-SAC) compared to SAL-SAC control group. However, the pretreatment with SAC (NIC-SAC) potentiated the effect induced by NIC withdrawal for both the percentage of entries ($p < 0.05$; Fig. 3A) as well as time spent ($p < 0.01$; Fig. 3B) in the open arms compared to NIC-VEH control group. See Table 3 for two-way ANOVA and Tukey's post hoc test. There were no significant differences between control groups (see Table 2).

3.3. Effect of SAC pretreatment on the dysphoric/aversive response of NIC withdrawal

The results revealed that the MEC administration produced conditioned place aversion associated with withdrawal syndrome in mice treated chronically with NIC (NIC-VEH) ($p < 0.01$; Fig. 4). Similarly, MEC administration also induced conditioned place aversion associated with withdrawal syndrome in mice pretreated with SAC and chronically treated with NIC (NIC-SAC) ($p < 0.001$; Fig. 4) compared to SAL-SAC control group. In addition, we observed that SAC pretreatment (NIC-SAC) potentiated the dysphoric/aversive effect associated with NIC withdrawal ($p < 0.001$; Fig. 4) compared to NIC-VEH control group. See Table 3 for two-way ANOVA and Tukey's post hoc test. There were no significant differences between control groups (see Table 2).

Table 4
Effect of 2-hydroxysaclofen on monoamines and metabolites concentration in the striatum, cortex and hippocampus during mecamylamine-precipitated nicotine withdrawal.

	Two-way ANOVA						One-way ANOVA, Tukey post hoc			
	Pretreatment		Treatment		Interaction		VEH-SAL *vs* VEH-NIC		SAC-SAL *vs* SAC-NIC		VEH-NIC *vs* SAC-NIC	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Striatum												
DA	$F_{(1,20)}$=16.691	<0.001	$F_{(1,20)}$=0.085	NS	$F_{(1,20)}$=22.005	<0.001	$F_{(3,20)}$=12.927	<0.05	$F_{(3,20)}$=12.927	<0.05	$F_{(3,20)}$=12.927	<0.001
DOPAC	$F_{(1,20)}$=15.402	<0.001	$F_{(1,20)}$=0.145	NS	$F_{(1,20)}$=26.019	<0.001	$F_{(3,20)}$=13.855	<0.05	$F_{(3,20)}$=13.855	<0.01	$F_{(3,20)}$=13.855	<0.001
5-HT	$F_{(1,20)}$=0.095	NS	$F_{(1,20)}$=0.524	NS	$F_{(1,20)}$=0.680	NS	$F_{(3,20)}$=0.433	NS	$F_{(3,20)}$=0.433	NS	$F_{(3,20)}$=0.433	NS
5-HIAA	$F_{(1,20)}$=0.017	NS	$F_{(1,20)}$=0.158	NS	$F_{(1,20)}$=0.271	NS	$F_{(3,20)}$=0.149	NS	$F_{(3,20)}$=0.149	NS	$F_{(3,20)}$=0.149	NS
Cortex												
DA	$F_{(1,16)}$=0.728	NS	$F_{(1,16)}$=0.380	NS	$F_{(1,16)}$=0.192	NS	$F_{(3,16)}$=0.479	NS	$F_{(3,16)}$=0.479	NS	$F_{(3,16)}$=0.479	NS
DOPAC	$F_{(1,16)}$=0.035	NS	$F_{(1,16)}$=0.127	NS	$F_{(1,16)}$=0.988	NS	$F_{(3,16)}$=0.380	NS	$F_{(3,16)}$=0.380	NS	$F_{(3,16)}$=0.380	NS
5-HT	$F_{(1,16)}$=1.234	NS	$F_{(1,16)}$=0.032	NS	$F_{(1,16)}$=0.000	NS	$F_{(3,16)}$=0.426	NS	$F_{(3,16)}$=0.426	NS	$F_{(3,16)}$=0.426	NS
5-HIAA	$F_{(1,16)}$=1.395	NS	$F_{(1,16)}$=0.318	NS	$F_{(1,16)}$=1.883	NS	$F_{(3,16)}$=1.051	NS	$F_{(3,16)}$=1.051	NS	$F_{(3,16)}$=1.051	NS
Hippocampus												
DA	$F_{(1,19)}$=0.907	NS	$F_{(1,19)}$=0.190	NS	$F_{(1,19)}$=2.055	NS	$F_{(3,19)}$=1.091	NS	$F_{(3,19)}$=1.091	NS	$F_{(3,19)}$=1.091	NS
DOPAC	$F_{(1,19)}$=0.001	NS	$F_{(1,19)}$=0.024	NS	$F_{(1,19)}$=4.330	<0.05	$F_{(3,19)}$=1.451	NS	$F_{(3,19)}$=1.451	NS	$F_{(3,19)}$=1.451	NS
5-HT	$F_{(1,19)}$=0.061	NS	$F_{(1,19)}$=0.072	NS	$F_{(1,19)}$=0.425	NS	$F_{(3,19)}$=0.175	NS	$F_{(3,19)}$=0.175	NS	$F_{(3,19)}$=0.175	NS
5-HIAA	$F_{(1,19)}$=0.002	NS	$F_{(1,19)}$=0.875	NS	$F_{(1,19)}$=0.508	NS	$F_{(3,19)}$=0.484	NS	$F_{(3,19)}$=0.484	NS	$F_{(3,19)}$=0.484	NS

Two-way ANOVA with treatment (SAL or NIC) and pretreatment (VEH or SAC) as between-subjects factors. When significant interaction between these factors was observed, the difference between two means was tested by one-way ANOVA and Tukey post hoc test. See Section 2 for details. NIC, nicotine; SAL, saline; VEH, vehicle; SAC, 2-hydroxysaclofen; DA, dopamine; DOPAC, 3,4-Dihydroxyphenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid

Table 5
Neurotransmitters concentration (pmol/mg of tissue) and α4β2 nAChRs binding sites (fmol/mg of tissue) of control groups.

	SAL-VEH-SAL	SAL-VEH-MEC	SAL-SAC-MEC	NIC-VEH-SAL
Neurotransmitters				
Cortex				
DA	1.23 ± 0.51	1.53 ± 0.58	1.27 ± 0.45	1.41 ± 0.55
DOPAC	1.24 ± 0.50	0.99 ± 0.31	1.33 ± 0.56	1.22 ± 0.40
5-HT	3.57 ± 0.25	3.27 ± 0.18	3.71 ± 0.46	3.51 ± 0.45
5-HIAA	1.52 ± 0.21	1.67 ± 0.08	1.20 ± 0.25	1.41 ± 0.21
Striatum				
DA	29.41 ± 1.80	30.20 ± 2.42	28.33± 3.69	26.76 ± 3.29
DOPAC	10.50 ± 1.98	14.14 ± 2.91	11.78 ± 2.26	13.32 ± 2.11
5-HT	3.51 ± 0.30	3.07 ± 0.18	3.29 ± 0.36	2.86 ± 0.19
5-HIAA	2.44 ± 0.48	1.73 ± 0.30	1.95 ± 0.54	1.41 ± 0.19
Hippocampus				
DA	0.88 ± 0.35	1.65 ± 0.47	0.72 ± 0.27	1.06 ± 0.40
DOPAC	0.30 ± 0.07	0.46 ± 0.09	0.30 ± 0.08	0.43 ± 0.11
5-HT	4.34 ± 0.31	4.65± 0.41	4.08 ± 0.26	4.38 ± 0.43
5-HIAA	2.99 ± 0.25	2.98 ± 0.32	2.75 ± 0.09	3.03 ± 0.31
α4β2 nAChRs				
AcbC	122.08 ± 16.9	121.7 ± 28.6	77.44 ± 12.54	104.68 ± 10.3
AcbSh	77.0 ± 53.32	79.44 ± 8.33	76.77 ± 11.45	72.9 ± 34.15
Cx	78.20 ± 14.81	137.9 ± 27.5	83.54 ± 8.96	71.98 ± 8.56
CPu	44.09 ± 17.71	70.13 ± 14.1	58.83 ± 15.54	54.78 ± 15.21
BST	70.15 ± 18.55	85.78 ± 12.9	70.19 ± 12.99	77.22 ± 20.30
MHb	56.4 ± 31.22	48.52 ± 5.38	55.92 ± 7.09	79.03 ± 19.34
Thalamic nuclei	249.8 ± 20.58	202.2 ± 33.2	266.6 ± 15.85	192.0 ± 32.29
DLG	305.9 ± 30.82	244.7 ± 8.83	256.1 ± 51.74	252.8 ± 45.63
fr	444.2 ± 106.2	222.8 ± 27.1	207.2 ± 25.09	316.0 ± 70.11
VTA	472 ± 237.5	440.7 ± 81.2	512.1 ± 63.07	396 ± 321.0
Ip	92.1 ± 42.15	79.75 ± 8.26	77.44 ± 12.54	81.6 ± 60.51
Superior Colliculus	309.5 ± 70.38	255.0 ± 35.8	298.3 ± 54.60	296.3 ± 49.1
SN	158.6 ± 17.91	134.2 ± 25.9	147.5 ± 18.28	143.1 ± 16.2
PAG	39.97 ± 7.75	44.73 ± 8.84	46.19 ± 4.09	40.25 ± 7.88

No significant differences were observed between control groups. Data represents the mean ± S.E.M. (n = 4-6 mice per group). NIC, nicotine; SAL, saline; VEH, vehicle; SAC, 2-hydroxysaclofen; MEC, mecamylamine; DA, dopamine; DOPAC, 3,4-Dihydroxyphenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Cx, deep layer of 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.

3.4. Effect of SAC pretreatment on the possible neurochemical alterations induced by NIC withdrawal

The results showed a decrease in the striatal DA ($p < 0.05$) and DOPAC ($p < 0.05$) levels during NIC withdrawal (NIC-VEH) (Fig. 5A and B, respectively) compared to SAL-VEH control group. On the other hand, MEC administration increased striatal DA ($p < 0.05$) and DOPAC ($p < 0.01$) levels in mice pretreated with SAC and chronically treated with NIC (NIC-SAC) (Fig. 5A and B, respectively) compared to SAL-SAC control. Comparison between NIC-SAC and NIC-VEH also showed significant differences in the striatal DA ($p < 0.001$) and DOPAC ($p < 0.001$) concentrations (Fig. 5A and B, respectively).

There were no significant differences in 5-HT and 5-HIAA endogenous striatal concentrations between the experimental groups. In addition, there were no significant differences in cortical and hippocampal DA, DOPAC, 5-HT and 5-HIAA endogenous concentrations. See Table 4 for two-way ANOVA and Tukey's post hoc test. No significant differences between control groups were observed (see Table 5).

3.5. Effect of SAC pretreatment on the possible variations in the α4β2 nAChR binding induced by NIC withdrawal

The results showed an increase in α4β2 nAChR density during NIC withdrawal (NIC-VEH) in the AcSh nucleus ($p < 0.001$, Fig. 6A), habenular nucleus ($p < 0.05$, Fig. 6B), VTA ($p < 0.001$; Fig. 6C), fr

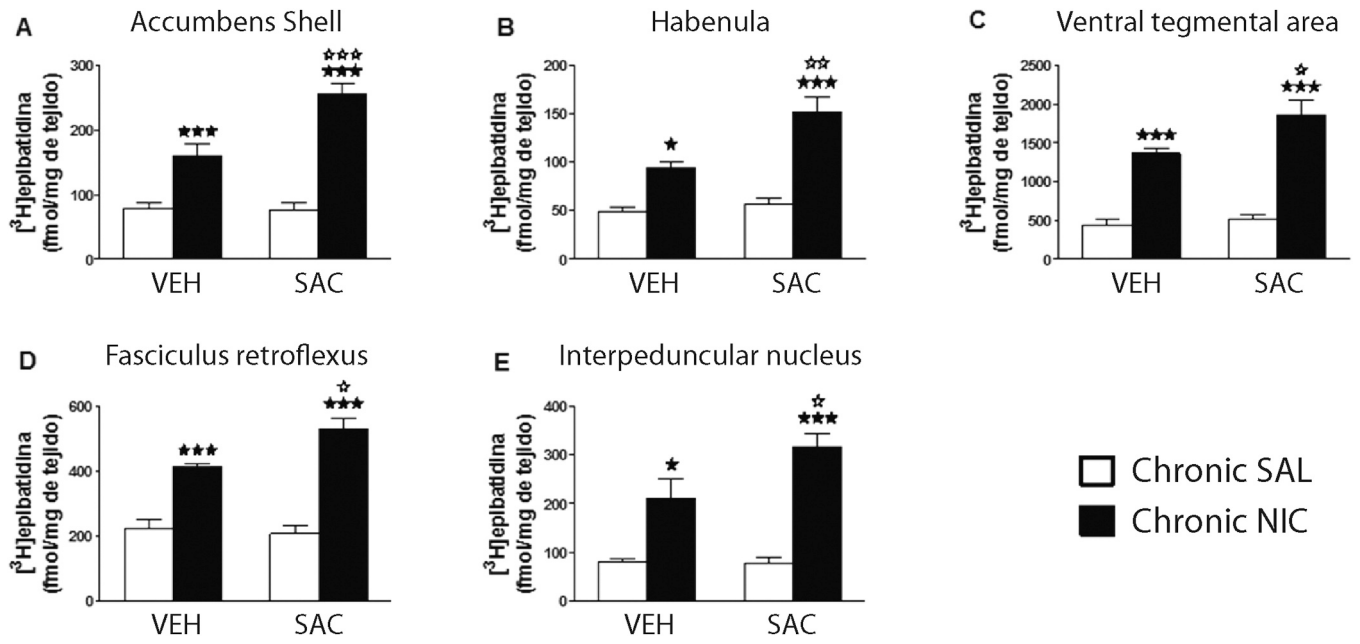

Fig. 6. Mecamylamine-precipitated nicotine withdrawal: α4β2 nAChR binding sites. Each column represents the mean±SEM (n = 4–6 mice for each group) of [3 H] epibatidine binding sites (fmol/mg of tissue) in accumbens shell, habenula, ventral tegmental area, fasciculus retroflexus, and interpeduncular nucleus. Empty column: chronic treatment with saline (SAL); filled column: chronic treatment with nicotine (NIC, 2.5 mg/kg; s.c.) four times daily, for 7 days. On the day of the experiment (day 8), mice received the last injection of the chronic treatment and 50 min after, either 2-hydroxysaclofen (SAC, 1 mg/kg, s.c.) or vehicle (VEH) were administered. Sixty min after the last injection of the chronic treatment, mecamylamine (MEC, 2 mg/kg; s.c.) was administered to all animals. Thirty minutes after the MEC injection brains were collected and α4β2 nAChR binding sites were measured. ★★★ $p < 0.001$ when compared to VEH group. ☆ $p < 0.05$, ☆☆ $p < 0.01$ ☆☆☆ $p < 0.001$ comparison between similar groups receiving chronic NIC with or without SAC (two-way ANOVA followed by multiple comparison post hoc test).

($p < 0.001$, Fig. 6D) and Ip nucleus ($p < 0.05$, Fig. 6E) compared to SAL-VEH control group. On the other hand, MEC administration also increased α4β2 nAChR density in the AcSh nucleus, habenular nucleus, VTA, fr and Ip nucleus ($p < 0.001$, Fig. 6A-E, respectively) in mice pretreated with SAC and chronically treated with NIC (NIC-SAC) compared to SAL-SAC control. Comparison between NIC-SAC and NIC-VEH also showed significant differences in the AcSh nucleus ($p < 0.001$, Fig. 6A), habenular nucleus ($p < 0.01$, Fig. 6B), VTA ($p < 0.05$, Fig. 6C), fr ($p < 0.05$; Fig. 6D) and Ip nucleus ($p < 0.05$; Fig. 6E). See Table 6 for two-way ANOVA and Tukey's post hoc test. There were no significant differences between control groups (see Table 5). Representative images of the different brain structures for each experimental group are shown in Fig. 7.

4. Discussion

4.1. Somatic manifestations of NIC withdrawal: participation of $GABA_B$ receptors

In the present study we evaluate the somatic manifestations of NIC withdrawal and its pretreatment with SAC. NIC withdrawal was pharmacologically precipitated with MEC administration [31,35,36]. The induction of NIC dependence was performed by means of a chronic treatment (7 days), four times a day, with a NIC dose of 2.5 mg / kg (s.c.) [37]. The dependence state was demonstrated by the injection of MEC and the subsequent appearance of somatic signs. The dose of MEC (2 mg / kg, s.c.) was selected after testing increasing doses of the antagonist in mice chronically treated with NIC (data not shown). The control group treated with MEC alone did not show significant differences with respect to the control treated with SAL (see Table 2). This indicates that the dose of MEC is not capable of precipitating a withdrawal syndrome by itself, in non-dependent animals [24].

Despite the years in the area of Biomedical research, the time curve of NIC withdrawal has not been widely studied [38]. Studies in humans indicate that the effects of NIC withdrawal syndrome are transient [1,4, 39], while other studies show that NIC withdrawal is characterized by a long and variable time course [40–42]. However, some reports in animals show a progression of NIC withdrawal over time [43]. In this sense, our study showed an increase in global withdrawal at 10 min after the administration of MEC in NIC-dependent mice. The intensity of withdrawal syndrome decreased gradually as a function of time, and finally stabilized at 25–30 min [23]. Together these findings suggest that the temporary course of NIC withdrawal in mice could be short and transient, at least in our experimental conditions.

Regarding the effect of SAC, our results showed that this drug can potentiate the somatic manifestations of NIC withdrawal, such as paw tremor and wet dog shakes. The observed effect of SAC is not due to changes in locomotion, since there were no significant differences between the control groups treated with MEC and those treated with SAC + MEC (see Table 2) [16]. Interestingly, in previous studies from our group we observed that the acute administration of the agonist BAC (2 mg / kg, i.p.) prevents the somatic expression of NIC withdrawal syndrome [21,23]. Therefore, the pharmacological stimulation of $GABA_B$ receptors with BAC prevents somatic manifestations of NIC withdrawal while the blockade with SAC enhances them, suggesting that somatic component of NIC withdrawal can be positively or negatively modulated by $GABA_B$ receptors. In addition, clinical studies revealed that BAC does not modify the number of cigarettes smoked, nor the desire to smoke. However, this same study showed that BAC alters the sensory properties of smokers, increasing the unpleasant effects and decreasing the pleasurable effects [44]. On the contrary, Franklin et al. [45] reported that BAC administered in high doses significantly reduces the number of cigarettes smoked per day in humans.

4.2. Anxiogenic-like effect of NIC withdrawal: participation of $GABA_B$ receptors

Studies in animals [46,47] and humans [48] revealed that NIC withdrawal causes an increase in anxiety levels, which has been proposed as one of the motivational components of abstinence. Our results

Table 6
Effect of 2-hydroxysaclofen on α4β2 nAChR binding sites in different brain regions during mecamylamine-precipitated nicotine withdrawal.

	Two-way ANOVA						One-way ANOVA, Tukey post hoc					
	Pretreatment		Treatment		Interaction		VEH-SAL *vs* VEH-NIC		SAC-SAL *vs* SAC-NIC		VEH-NIC *vs* SAC-NIC	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
AcbC	$F_{(1,16)}$=9.574	<0.01	$F_{(1,16)}$=4.227	<0.05	$F_{(1,16)}$=0.105	NS	$F_{(3,16)}$=4.636	NS	$F_{(3,16)}$=4.636	NS	$F_{(3,16)}$=4.636	NS
AcbSh	$F_{(1,16)}$=11.868	<0.01	$F_{(1,16)}$=92.685	<0.001	$F_{(1,16)}$=13.270	<0.01	$F_{(3,16)}$=39.275	<0.001	$F_{(3,16)}$=39.275	<0.001	$F_{(3,16)}$=39.275	<0.001
Cx	$F_{(1,16)}$=8.946	<0.01	$F_{(1,16)}$=0.556	NS	$F_{(1,16)}$=0.400	NS	$F_{(3,16)}$=3.301	NS	$F_{(3,16)}$=3.301	NS	$F_{(3,16)}$=3.301	NS
CPu	$F_{(1,16)}$=0.560	NS	$F_{(1,16)}$=1.195	NS	$F_{(1,16)}$=0.003	NS	$F_{(3,16)}$=0.586	NS	$F_{(3,16)}$=0.586	NS	$F_{(3,16)}$=0.586	NS
BST	$F_{(1,16)}$=0.061	NS	$F_{(1,16)}$=0.247	NS	$F_{(1,16)}$=0.000	NS	$F_{(3,16)}$=0.480	NS	$F_{(3,16)}$=0.480	NS	$F_{(3,16)}$=0.480	NS
MHb	$F_{(1,16)}$=12.950	<0.01	$F_{(1,16)}$=60.643	<0.001	$F_{(1,16)}$=7.763	<0.05	$F_{(3,16)}$=27.119	<0.05	$F_{(3,16)}$=27.119	<0.001	$F_{(3,16)}$=27.119	<0.01
Thalamic nuclei	$F_{(1,16)}$=0.344	NS	$F_{(1,16)}$=0.840	NS	$F_{(1,16)}$=2.441	NS	$F_{(3,16)}$=1.208	NS	$F_{(3,16)}$=1.208	NS	$F_{(3,16)}$=1.208	NS
DLG	$F_{(1,16)}$=0.530	NS	$F_{(1,16)}$=0.691	NS	$F_{(1,16)}$=0.184	NS	$F_{(3,16)}$=0.468	NS	$F_{(3,16)}$=0.468	NS	$F_{(3,16)}$=0.468	NS
fr	$F_{(1,16)}$=3.860	NS	$F_{(1,16)}$=100.142	<0.001	$F_{(1,16)}$=6.616	<0.05	$F_{(3,16)}$=36.873	<0.001	$F_{(3,16)}$=36.873	<0.001	$F_{(3,16)}$=36.873	<0.05
VTA	$F_{(1,16)}$=6.547	<0.05	$F_{(1,16)}$=104.302	<0.001	$F_{(1,16)}$=3.674	NS	$F_{(3,16)}$=38.174	<0.001	$F_{(3,16)}$=38.174	<0.001	$F_{(3,16)}$=38.174	<0.05
Ip	$F_{(1,16)}$=4.075	NS	$F_{(1,16)}$=51.767	<0.001	$F_{(1,16)}$=4.445	<0.05	$F_{(3,16)}$=20.096	<0.05	$F_{(3,16)}$=20.096	<0.001	$F_{(3,16)}$=20.096	<0.05
Superior Colliculus	$F_{(1,16)}$=0.107	NS	$F_{(1,16)}$=0.178	NS	$F_{(1,16)}$=1.411	NS	$F_{(3,16)}$=0.565	NS	$F_{(3,16)}$=0.565	NS	$F_{(3,16)}$=0.565	NS
SN	$F_{(1,13)}$=0.008	NS	$F_{(1,13)}$=0.080	NS	$F_{(1,13)}$=0.304	NS	$F_{(3,13)}$=0.134	NS	$F_{(3,13)}$=0.134	NS	$F_{(3,13)}$=0.134	NS
PAG	$F_{(1,13)}$=0.013	NS	$F_{(1,13)}$=0.019	NS	$F_{(1,13)}$=0.086	NS	$F_{(3,13)}$=0.043	NS	$F_{(3,13)}$=0.043	NS	$F_{(3,13)}$=0.043	NS

Two-way ANOVA with treatment (SAL or NIC) and pretreatment (VEH or SAC) as between-subjects factors. When significant interaction between these factors was observed, the difference between two means was tested by one-way ANOVA and Tukey post hoc test. See Section 2 for details. NIC, nicotine; SAL, saline; VEH, vehicle; SAC, 2-hydroxysaclofen; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Cx, deep layer of 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

showed that NIC withdrawal induces an anxiogenic-like effect in the elevated plus maze test. In accordance with these results, it has been reported that spontaneous [29,49] or precipitated [29,50] NIC withdrawal causes a reduction in the exploration of the open arms of the elevated plus maze test. In addition, we observed that MEC by itself did not modify the responses in the elevated plus maze test compared to the control mice, as shown in Singh et al. [51]. SAC pretreatment potentiated the anxiogenic-like effect associated with NIC withdrawal. Conversely, pre-treatment with BAC (2 mg / kg; i.p.) prevented the anxiogenic-like effect associated with NIC withdrawal in mice [52]. On the other hand, no intrinsic effects of SAC on anxiety levels were observed (see Table 2) [53,54]. Therefore, our results revealed that $GABA_B$ receptors could produce a bidirectional modulation of affective aspects of NIC withdrawal in mice.

4.3. Dysphoric/aversive effect associated to NIC withdrawal: participation of $GABA_B$ receptors

In order to evaluate the aversive state associated with NIC withdrawal, the conditioned place aversion was used (Jackson et al.). Previous studies have shown that NIC withdrawal syndrome is associated with a negative affective state [29,31,55]. Our results showed that MEC administration induced a dysphoric/aversive effect in NIC-dependent mice, which is in accordance with previous studies carried out in both mice [29,30] and rats [56,57].

To elucidate the role of $GABA_B$ receptors on the dysphoric/aversive effect of NIC withdrawal we also used the SAC antagonist. The dose of SAC did not change the responses in the conditioned place aversion paradigm by itself (see Table 2). We also observed that SAC pretreatment potentiated the dysphoric/aversive manifestations of the NIC withdrawal. These results suggest that blocking one of the components of the GABAergic system ($GABA_B$ receptors) it could regulate the aversive state associated to NIC withdrawal in mice. It has been previously reported a decrease in DA concentration during NIC withdrawal in the Acb nucleus, CPu and prefrontal cortex [58–60]. Presynaptic $GABA_B$ receptors located in the dopaminergic VTA neurons, which project to the brain areas mentioned above, are involved in the control of DA release [61]. In this context, we propose that blocking $GABA_B$ receptors would produce a disinhibition of dopaminergic neurons, causing an increase in DA concentration during NIC withdrawal compared to baseline levels. This could explain the fact that we have detected an increase of the dysphoric/aversive effect associated to NIC withdrawal in animals pretreated with SAC. Given these results, we could assume that $GABA_B$ receptors would be involved in the control of the aversive states induced by NIC withdrawal. The effects of SAC pretreatment observed in these experiments are not due to changes in locomotion since the dose used did not alter the locomotor activity (see Table 2). Similarly, it has been shown that the cerebral administration of SAC does not induce changes in locomotor activity [62]. Interestingly, we previously observed that the aversive manifestations of NIC withdrawal precipitated by naloxone (opioid antagonist) was not modified by BAC pretreatment [63]. This could indicate that opioidergic activity would be necessary in order to allow BAC to generate a preventive effect during the withdrawal. In this sense, it has been reported that the opioid system is clearly involved in both the somatic and the motivational component of NIC withdrawal [64,65]. In addition, somatic manifestations of NIC withdrawal are attenuated in mice deficient in the μ-opioid receptor [2] and preproencephalin [66]. In summary, these results show the participation of $GABA_B$ receptors in the dysphoric/aversive effect associated to NIC withdrawal, thus suggesting the existence of a possible interaction between the GABAergic system and the nicotinic cholinergic system [3,67,68].

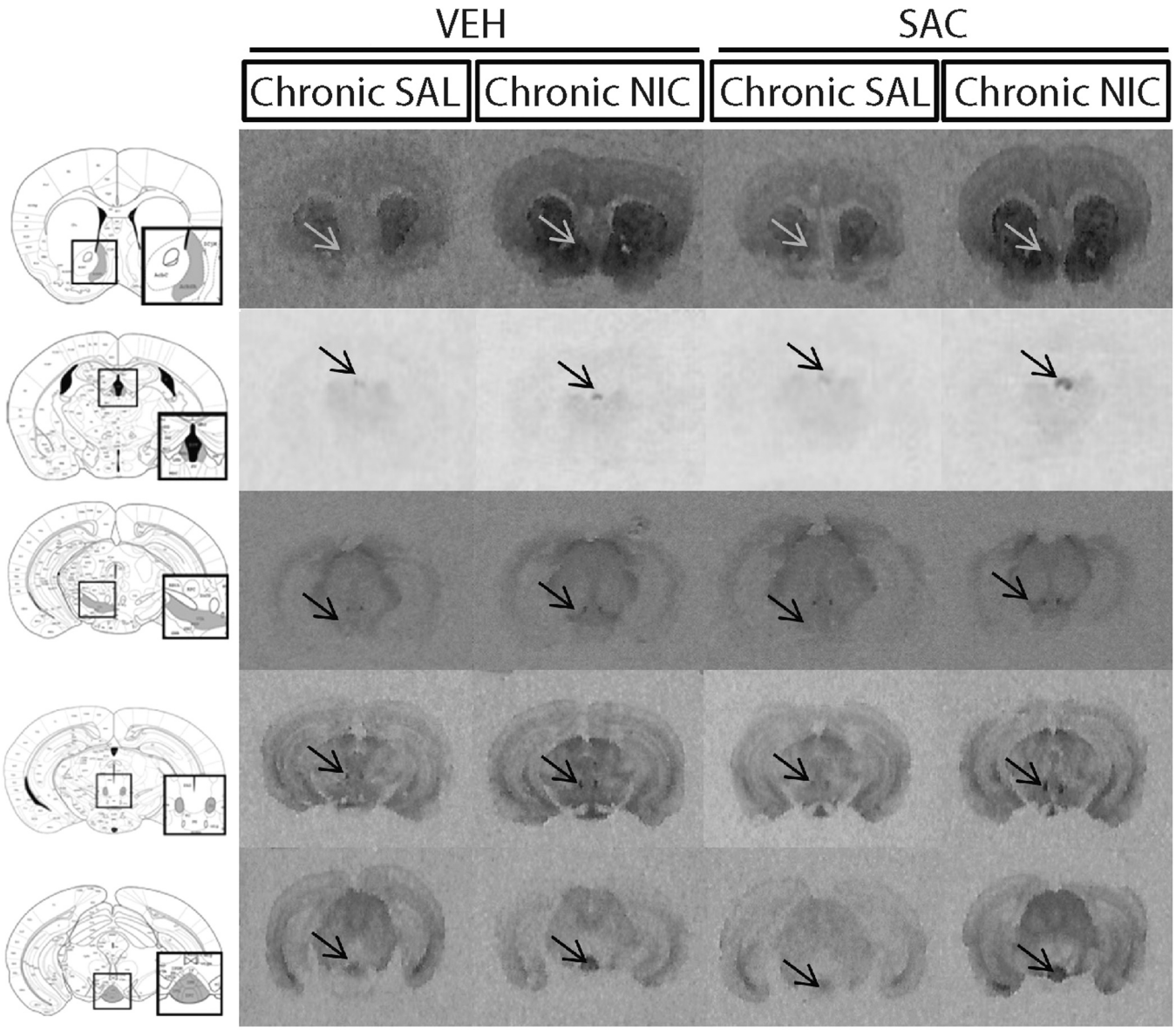

Fig. 7. [3 H]epibatidine autoradiograms of α4β2 nAChR binding in mice of SAL-VEH-MEC control, SAL-SAC-MEC control, NIC-VEH-MEC and NIC-SAC-MEC groups. The first and second columns show the SAL-VEH-MEC control and NIC-VEH-MEC groups, respectively while the third and fourth columns show the SAL-SAC-MEC control and NIC-SAC-MEC groups, respectively. The first line shows sections cut at the nucleus accumbens shell level (bregma 1.10 mm). The second line shows sections cut at the habenula (bregma −1.22). The third line shows sections cut at the ventral tegmental area level (bregma −2.92). The fourth line shows sections cut at the fasciculus retroflexus level (bregma−2.70). The fifth line shows sections cut at the interpeduncular nucleus (bregma −3.52). The arrows indicate the brain areas measured.

4.4. Neurochemical alterations induced by NIC withdrawal: participation of $GABA_B$ receptors

Various brain structures and neurotransmitters systems are implicated in NIC withdrawal syndrome [67]. Previous studies showed that a deficit in dopaminergic and serotonergic transmission in the striatum and cortex could play a role in the somatic expression of NIC withdrawal syndrome [58,69]. Based on the results obtained in the time curve of global abstinence, we decided to explore the concentration of monoamines within 10 min after precipitating the NIC withdrawal. We observed a decrease in the striatal DA concentration 10 min after MEC injection in NIC-dependent mice. This decrease should not be due to an increase in DA metabolism, since we have also observed a decrease in the concentration of DOPAC in the striatum. These findings are in agreement with previous studies, which show that the DA content in the striatum decreases significantly during NIC withdrawal in mice [23] and rats [58]. Regarding the striatal 5-HT concentration, no changes were found between the different experimental groups. Conversely, we previously observed that an intraperitoneal injection of MEC in chronically NIC treated mice decreased striatal 5-HT concentrations [23]. Similarly, it has been shown a decrease of the striatal 5-HT contents during withdrawal after 17 days of continuous NIC exposure [69]. The discrepancies with these studies could be due to a number of methodological differences such as the route of MEC administration (subcutaneous vs intraperitoneal) and the schedule of NIC treatment (7 vs 17 days). In the present study, no changes in DA, 5-HT and its metabolites levels were observed in the cortex during NIC withdrawal. On the contrary, we have previously shown that cortical DA and DOPAC concentrations decreased after an intraperitoneal injection of MEC in NIC-dependent mice [23]. Furthermore, Hildebrand et al. [59] did not observe changes in cortical DA levels in rats undergoing MEC-precipitated NIC withdrawal. Available evidence indicates that

neither chronic NIC administration nor its spontaneous withdrawal affected the 5-HT and 5-HIAA concentrations in the cortex of mice [60]. These discrepancies could be due to a number of methodological differences between these studies: species (mouse vs rats), differences in sampling (microdialysis vs tissue homogenate), cortical region (prefrontal cortex vs whole cortex) and more importantly route of MEC administration (subcutaneous vs intraperitoneal). Finally, we also showed that hippocampal DA, 5-HT and its metabolites concentrations were not modified during NIC withdrawal. Accordingly, there is no available evidence related to hippocampal neurochemical changes produced during NIC withdrawal syndrome.

The present results confirm that the dopaminergic system is involved in NIC withdrawal. The stimulation of nAChRs by NIC induced an increase in the release of neurotransmitters in different areas of the brain and their antagonism produced a decrease in the release of these neurotransmitters [67]. Therefore, our findings could be the result of direct blockage of nAChRs, which results in a decrease of DA release in the striatum during NIC withdrawal. Brain monoamines participate in the reinforcing effect of most drugs of abuse. Particularly, the activation of the dopaminergic mesocorticolimbic system is responsible for the reinforcing effect of NIC [70]. Kuhar et al. [71] showed that almost 80% of all DA in the brain is found in the striatum. On the other hand, Guyton [72] suggests that DA can act as an inhibitory neurotransmitter in this area. Therefore, the decrease of striatal DA levels could be related to the increase in the intensity of the somatic signs observed at 10 min after withdrawal. In a previous study from our group, we observed that BAC was able to restore the striatal DA concentrations modified during NIC withdrawal in mice [23]. In accordance with our results, Fadda et al. [73] indicated that BAC has the capacity to modulate the mesolimbic dopaminergic transmission. In fact, BAC pretreatment (1.25 and 2.5 mg / kg; i.p.) prevented the increase in DA release induced by acute NIC, morphine and cocaine in the striatum [73]. Therefore, we can speculate that the restoration of striatal DA concentrations induced by BAC could be related to the decrease in somatic signs during NIC withdrawal. Based on these results we suggest that the activation of $GABA_B$ receptors by BAC would modulate GABAergic neurons directly connected with dopaminergic neurons in the striatum. In this context, it should be noted that in the present study the pretreatment with SAC increased the neurochemical alterations induced by NIC withdrawal. Thus, blocking the $GABA_B$ receptor would prevent the action of the released GABA, which would lead to a disinhibition of the dopaminergic neurons. Statistical analysis showed that there were no significant differences between the control groups (see Table 5). This suggests that the potentiation of neurochemical alterations caused by SAC could be due to the interaction of SAC and the NIC withdrawal state.

4.5. *Variations in α4β2 nAChR density induced during NIC withdrawal: participation of $GABA_B$ receptors*

nAChRs (pentameric ion channels) are made up of various combinations of subunits (α2-α10 and β2-β4), which gives rise to a wide variety of subtypes [74]. In the CNS, the most abundant subtypes of nAChRs are α7 (homomeric) and α4β2 (heteromeric) [74]. These two subtypes of receptors play an important role in the addictive properties of NIC, such as dependence [75] and withdrawal [76]. In the present study, we observed that chronic treatment with NIC did not modify the binding sites to α4β2 nAChR in any of the brain areas studied compared with control levels (see Table 5), at least, in our experimental conditions. To evaluate the density of nAChRs we carried out an autoradiography study using [^{3}H]epibatidine. This drug is a specific ligand of the α4β2 nAChR [77,78]. Our results are in agreement with previous studies in which mice chronically treated with NIC did not show changes in the epibatidine binding sites in several brain structures [79,80]. On the other hand, it is known that chronic exposure to NIC causes an increase in nAChR binding sites (upregulation) in mouse brains [81,82]. The mechanisms that underlie this upregulation are not entirely clear and remain controversial [83].

NIC withdrawal syndrome is a set of somatic and motivational signs that reflects an imbalance in the neurochemical equilibrium of the brain [84]. This imbalance can be generated by the cessation of the administration of the NIC or the administration of an antagonist of the nAChRs [76]. In the present study MEC injection in mice chronically treated with SAL showed no significant differences between the control group VEH + MEC and VEH + SAL (see Table 5). This indicates that MEC itself is not capable of inducing changes in the [^{3}H]epibatidine binding of mice chronically treated with SAL [7]. The α4β2 nAChRs are widely distributed and expressed in brain structures such as the habenular nucleus, thalamic nuclei, DLG, fr, hippocampus, VTA, Ip nucleus, CPu, superior colliculus, cortex and striatum [85–88]. It has been suggested that changes in the expression of nAChR are responsible for mediating NIC effects, such as tolerance and locomotor sensitization [89–91].

Several studies have shown that NIC withdrawal induces an increase in nAChR levels in different brain structures [69,83,92]. In agreement, our current results revealed that NIC withdrawal dramatically affects the density of α4β2 nAChRs in specific regions of the brain. Particularly, we have observed that the administration of MEC in mice chronically treated with NIC produced a significant increase in [^{3}H]epibatidine binding sites in the striatum, especialy the AcbSh nucleus, habenular nucleus, fr, VTA and Ip nucleus. In agreement, it has been reported that after the interruption of chronic NIC treatment there is an increase in the density of nAChRs in the striatum of mice [69,93]. The striatum consists of three brain structures, the AcbSh, AcbC and CPu. It is well known that the AcbSh mediates some of the addictive properties of NIC, while the contribution of the CPu and the AcbC in addiction has not being clarified [94]. In addition, it has been established that NIC withdrawal increases the density of α4β2 nAChRs in the midbrain [95,96], a brain structure that includes the VTA [97]. Salas et al. [7] observed an increase of [^{3}H] epibatidine binding sites during NIC withdrawal in the habenular nucleus and Ip nucleus in mice. The habenular and Ip nucleus are two small nuclei connected by a group of axons, the fr. In rodents, the habenulo-interpeduncular axis has been implicated in a variety of brain alterations and behaviors induced by NIC [98,99].

Regarding to the participation of $GABA_B$ receptors, it is important to highlight that SAC potentiated the increase in nAChRs induced by NIC withdrawal in the AcbSh nucleus, habenular nucleus, VTA, fr and Ip nucleus. These results show that the activity of $GABA_B$ receptors is a fundamental requirement to maintain the balance of nAChRs during NIC withdrawal. The dose of SAC had no intrinsic effects in mice treated with SAL (see Table 5). Control groups treated with MEC, with SAC + MEC or SAL did not induce changes on the levels of α4β2 nAChR in non-dependent mice. In addition, no significant differences were observed between these groups (see Table 5), indicating that SAC itself does not modify the population of nAChRs. Importantly, we have previously found that BAC is capable of restoring increased levels of nAChR α4β2 during NIC withdrawal syndrome in AcbSh, habenular nucleus, thalamic nuclei, DLG and fr, but not in the VTA, Ip nucleus and superior colliculus [83].

As we mentioned earlier, α4β2 nAChRs are widely distributed in various brain structures. Neuroanatomic studies demonstrated a high density of $GABA_B$ receptors in AcbSh neurons, habenular nucleus, thalamic nuclei, DLG and fr of mammalian brains [100,101]. These reports support the idea about a possible functional interaction between the nicotinic cholinergic and the GABAergic systems, since there is clearly a co-localization of $GABA_B$ receptors and α4β2 nAChRs. Thus, we could speculate that the observed effect of SAC on the density of α4β2 nAChRs is due to its interaction with $GABA_B$ receptors located in GABAergic neurons, which also express nAChRs α4β2.

5. Conclusion

Preclinical studies [45,102] and clinical studies [44,103] support the idea that $GABA_B$ receptors are a promising drug to treat tobacco

addiction. In this sense, the behavioral, neurochemical and biochemical experiments carried out in this study showed the ability of SAC to modulate alterations induced by NIC withdrawal in mice. Together with the findings related to BAC, we confirmed that somatic and motivational manifestations of NIC withdrawal could be modulated bidirectionally by $GABA_B$ receptors. Therefore, we proposed that targeting $GABA_B$ receptors could ensure an encouraging impact to treat NIC addiction.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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