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Nicotine-induced molecular alterations are modulated by $GABA_B$ receptor activity

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ABSTRACT

It has been demonstrated that $GABA_B$ receptors modulate nicotine (NIC) reward effect; nevertheless, the mechanism implicated is not well known. In this regard, we evaluated the involvement of $GABA_B$ receptors on the behavioral, neurochemical, biochemical and molecular alterations associated with the rewarding effects induced by NIC in mice, from a pharmacological and genetic approach. NIC-induced rewarding properties (0.5 mg/kg, subcutaneously, sc) were evaluated by conditioned place preference (CPP) paradigm. CPP has three phases: preconditioning, conditioning and postconditioning. $GABA_B$ receptor antagonist 2-hydroxysaclofen (0.25, 0.5 and 1 mg/kg; intraperitoneally, ip) or the $GABA_B$ receptor agonist baclofen (3 mg/kg; ip) was injected before NIC during the conditioning phase. $GABA_{B1}$ knockout ($GABA_{B1}$KO) mice received NIC during the conditioning phase. Vehicle and wild-type controls were employed. Neurochemical (dopamine, serotonin and their metabolites), biochemical (nicotinic receptor α4β2, α4β2nAChRs) and molecular (c-Fos) alterations induced by NIC were analyzed after the postconditioning phase by high-performance liquid chromatography (HPLC), receptor-ligand binding assays and immunohistochemistry, respectively, in nucleus accumbens (Acb), prefrontal cortex (PFC) and ventral tegmental area (VTA). NIC induced rewarding effects in the CPP paradigm and increased dopamine levels in Acb and PFC, α4β2nAChRs density in VTA and c-Fos expression in Acb shell (AcbSh), VTA and PFC. We showed that behavioral, neurochemical, biochemical and molecular alterations induced by NIC were prevented by baclofen. However, in 2-hydroxysaclofen pretreated and $GABA_{B1}$KO mice, these alterations were potentiated, suggesting that $GABA_B$ receptor activity is necessary to control alterations induced by NIC-induced rewarding effects. Therefore, the present findings provided important contributions to the mechanisms implicated in NIC-induced rewarding effects.

Keywords c-Fos, dopamine, $GABA_B$ receptors, nicotine, nicotinic receptors, reward.

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INTRODUCTION

Studies in laboratory animals strongly suggest that the neurotransmitter γ-aminobutyric acid (GABA) is critically involved in brain reward processes. Drugs of abuse powerfully stimulate the brain's reward pathways and provide the means to study how GABA transmission modulates circuits involved in reward processes (Vlachou & Markou 2010). Dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (Acb) and prefrontal cortex (PFC) have been implicated in reward processes (Koob & Volkow 2010). These dopaminergic neurons receive descending GABAergic inputs from the ventral pallidum and Acb which have an inhibitory effect on dopaminergic tone at the level of both the VTA and Acb (Pierce & Kumaresan 2006). There are GABA inhibitory afferents to dopaminergic VTA neurons, inhibitory GABA interneurons within the VTA and medium spiny GABA neurons in the Acb that also inhibit mesolimbic dopamine release (Pierce & Kumaresan 2006). GABA acts on two classes of receptors: ionotropic $GABA_A$ and $GABA_C$, and metabotropic $GABA_B$ receptors. The $GABA_A$ and $GABA_C$ receptors are located mostly postsynaptically (Barnard *et al.* 1998), while $GABA_B$

receptors are located both presynaptically and postsynaptically (Bowery *et al.* 2002). The $GABA_B$ receptors are coupled to G proteins and form a heteromer of $GABA_{B1}$ and $GABA_{B2}$ subunits, both of which are necessary for $GABA_B$ receptors to be functional (Marshall *et al.* 1999). Importantly, compounds that target $GABA_B$ receptors are unique as anti-abuse therapies because of their impact against multiple addictive drugs (Vacher *et al.* 2006; Kumar *et al.* 2013; Brown *et al.* 2015; Froger-Colléaux & Castagné 2016). It has been demonstrated that $GABA_B$ receptor activity can modulate the reward effect (Mombereau *et al.* 2007; Vlachou & Markou 2010; Vlachou *et al.* 2011; Falco, McDonald & Smith 2014; Filip *et al.* 2015) and other addictive properties induced by nicotine (NIC) (Tyacke *et al.* 2010; Phillips & Reed 2014). In that respect, we have observed that biochemical ($\alpha_4\beta_2$ nicotinic receptors, $\alpha_4\beta_2$nAChR), neurochemical [dopamine (DA) and serotonin concentrations], molecular (expression of c-Fos and brain-derived neurotrophic factor) changes induced by either acute or chronic behavioral responses of NIC 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 (Varani *et al.* 2015; 2014a,b,c,d; 2013; 2012; 2011; Varani & Balerio 2012).

Despite the strong evidences showing the involvement of $GABA_B$ receptors in rewarding properties induced by NIC, 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-induced rewarding properties. In particular, we analyzed alterations at behavioral (conditioned place preference), neurochemical (monoamines concentration), biochemical ($\alpha_4\beta_2$nAChRs density) and molecular (c-Fos expression) levels derived from the rewarding properties induced by NIC in mice pretreated with BAC or SAC and $GABA_{B1}$KO mice.

MATERIALS AND METHODS

Animals

For the pharmacological approach, we used adult male Swiss Webster mice. For the genetic approach, we used adult $GABA_{B1}$KO mice and their wild-type (WT) littermates. $GABA_{B1}$KO was generated in the laboratory of Dr Bernard Bettler, Department of Physiology, University of Basel, Switzerland (Schuler *et al.* 2001). We have developed our own $GABA_{B1}$KO mice colony in the Instituto de Investigaciones Farmacológicas (UBA-CONICET). The $GABA_{B1}$KO mice and their WT littermates are obtained by intercrossing heterozygous animals. Tail biopsies (performed for identification purposes) were used to isolate DNA for animal genotyping by polymerase chain reaction (PCR) as described (Schuler *et al.* 2001).

Care and handling conditions

The animals of both approaches were acclimatized to the laboratory conditions according to local regulation (SENASA, 2002) (12-hour light: 12-hour dark cycle, 21 ± 0.5°C room temperature, 65 ± 10 percent humidity). Mice weighing 22–26 g were housed five per cage, handled and habituated to the injections for 3 days prior to the experiment, in order to reduce the stress. Food and water were available *ad libitum*. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC and 2001–486/EEC) and approved by the local ethical committee: CICUAL (Institutional Committee for Care and Use of Laboratory Animals, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina). All experiments were performed with the investigators being blind to treatment conditions.

Drugs

(−)-Nicotine hydrogen tartrate salt ([−]-1- methyl-2-[3-pyridil]pyrrolidine) (NIC) (Sigma Chemical Co., Buenos Aires, Argentina), (±) BAC (Novartis, Basel, Switzerland) and SAC (Sigma Chemical Co., Buenos Aires, Argentina) were used in this study. NIC and BAC were dissolved in isotonic saline solution (NaCl 0.9 percent) (SAL), and SAC was dissolved in isotonic (five percent) glucose solution immediately before use. BAC and SAC were administered by (ip) route. NIC dose [0.5 mg/kg, (sc)] 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 (sc). The dose of NIC was chosen based on previous studies from our laboratory (Varani *et al.* 2014b). All drugs were administered in a volume of 10 ml/kg.

Place preference paradigm

The rewarding effects of NIC (0.5 mg/kg, sc) were evaluated by using the conditioned place preference paradigm, as previously described (Maldonado *et al.* 1997). The apparatus consisted of two main square conditioning compartments (15 × 15 × 15 cm) separated by a triangular central division. The compartments have different walls (striped and dotted) and also distinct floor textures (smooth floor in the striped compartment and rugged floor in the dotted one). During the preconditioning

phase, each mouse was placed in the middle of the central division and had free access to both compartments of the conditioning apparatus for 18 minutes, with the time spent in each compartment recorded by computerized monitored software (Vision Robot®, Buenos Aires, Argentina). Treatments were counterbalanced between compartments in order to use an unbiased procedure. No initial place preference or aversion for the different compartments was observed in any of the experiments. In the conditioning phase, SAL and NIC conditioning sessions were conducted daily over the next 4 days. Each day, animals were injected with SAL and placed into corresponding chamber for 20 minutes. Four hours later, animals were injected with NIC (0.5 mg/kg, sc) and placed in the opposite chamber for 20 minutes. Control animals received SAL every session in both chambers. The postconditioning phase was conducted exactly as the preconditioning phase, i.e., free access to both compartments during 18 minutes, and the time spent in each compartment was recorded. The time in the central area was proportionally shared and added to the time value of each compartment as previously described (Valverde *et al.* 1996). A score value was calculated for each mouse as the difference between the time spent in the drug-paired compartment during the postconditioning and preconditioning phases.

For the pharmacological approach, BAC (3 mg/kg, ip) or vehicle (VEH) (n = 12–17 per experimental group) were administered 45 minutes before NIC or SAL injection during the conditioning phase, whereas SAC (0.25, 0.5 and 1 mg/kg, ip) or VEH (n = 15 per experimental group) were administered 10 minutes before NIC or SAL injection. In a previous study from our laboratory by using a dose curve response for BAC (1, 2 and 3 mg/kg, ip), we observed that the high dose of BAC prevented the rewarding effects induced by NIC at behavioral level in mice (Varani *et al.* 2014b). Therefore, in the present study, we selected the effective dose of BAC (3 mg/kg, ip) in order to evaluate the neurochemical, biochemical and molecular alterations induced by NIC-induced rewarding effects. SAC doses were selected taking into account a previous behavioral results obtained from our laboratory (Varani & Balerio 2012).

For the genetic approach, $GABA_{B1}$KO mice and their WT littermates (n = 9–15 per experimental group) received NIC (0.5 mg/kg) or SAL during the conditioning phase.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY EXPERIMENTS

High-performance liquid chromatography-coupled electrochemical detection (Heikkila, Hess & Duvoisin 1984) of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), 5-hydroxyindolacetic acid (5-HIAA) and norepinephrine was achieved using a Varian 5000 liquid chromatograph coupled to an electrochemical detector (BAS LC-4C). After postconditioning phase, mice (n = 3–9 per experimental group) were sacrificed by cervical dislocation; brains were quickly removed and placed in dry ice. When partially frozen, the VTA, Acb and PFC 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 NaH_2PO_4–H_2O 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.

c-Fos immunohistochemistry

After postconditioning phase, mice (n = 3–6 per experimental group) were deeply anesthetized using a mixture of ketamine (70 mg/kg, Holliday-Scott S.A., Argentina) and xylazine (10 mg/kg, König, Argentina). They were then transcardially perfused with heparinized PBS (0.1 M SAL phosphate buffer, pH 7.4), followed by a cold solution of four percent paraformaldehyde delivered with a peristaltic pump. Brains were removed and postfixed for 2 hours in the same fixative, and cryoprotected overnight in a 30 percent sucrose solution. Coronal frozen sections were made at 30 µm on a freezing microtome. They were collected in three serial groups of free-floating sections and stored at 4°C. The procedure for c-Fos immunohistochemistry was adapted from previously described protocols (Bester, De Felipe & Hunt 2001). All reactions were performed on floating sections agitated on a shaker. Sections from different experimental groups were processed in parallel to minimize the variations in immunohistochemical labeling. Free-floating sections were rinsed in 0.1 M phosphate buffered SAL with 0.15 percent Triton X-100 (PBS-T; pH 7.4) and then incubated with three percent hydrogen peroxide in PBS-T for a period of 30 minutes to remove endogenous peroxidase activity. After rinsing again in PBS-T, sections were incubated for 30 minutes in two percent normal goat serum in PBS-T. Then, sections were incubated overnight in a rabbit polyclonal antibody anti-c-Fos (Santa Cruz Biotechnology, USA) (1:1000 in PBS 0.1 M, thimerosal 0.02 percent, normal goat serum one percent) at 4°C. Sections were then

rinsed and incubated for 2 hours in a goat anti-rabbit biotinylated antibody (Vector Laboratories, USA) (1:250 in PBS-T). After being rinsed, sections were incubated for 2 hours in avidin-biotinylated horseradish peroxidase complex (1:125, ABC kit, Vector Laboratories). After successive washes in PBS-T and Tris buffer (0.25 M; pH 7.4), the antibody–antigen complex was developed with 0.05 percent m/v of 3,3′-diaminobenzidine (Sigma, USA) and 0.015 percent *v*/v of H_2O_2 in 20 ml Tris buffer 0.1 M. Sections were mounted on gelatin-coated slides, dehydrated and cover slipped. Controls for the specificity of primary antisera were carried out by substitution of primary antibody with PBS (Delfino *et al.* 2004).

For quantitative analysis, cells positive for c-Fos immunoreactivity were identified by the presence of dense immunohistochemical staining within the nuclei, under a light microscope. Digital images of the selected sections were taken at 200× on a Nikon Microscope (Eclipse 55i) equipped with a digital camera (Nikon DS, Control Unit DS-L1). For every area, the number of Fos-positive cells was counted within a grid under ImageJ 1.36b, provided by National Institutes of Health, USA (public domain software). The counting was performed bilaterally in each brain area, these counts were averaged into a single score for each region of each animal and finally the group mean ± SEM was calculated. c-Fos-positive nuclei were quantified in the following brain regions, identified according to the anatomic atlas of Paxinos & Franklin (2004): VTA, AcbSh, Acbc and PFC.

[3H]Epibatidine binding to membranes

Receptor-ligand binding assays were performed based on Chistyakov *et al.* (2010). After postconditioning phase, mice (n = 3–5 per experimental group) were sacrificed by cervical dislocation; brains were quickly removed and placed in dry ice. When partially frozen, the VTA, Acb and PFC were dissected under a dissecting microscope. The tissue samples were weighed and homogenized using glass Teflon homogenizer in ice-cold sucrose (0.32 M sucrose, pH 7.4) (VTA: 400 µl, Acb: 400 µl and PFC: 500 µl) and centrifuged at 27 000 *g*, for 20 minutes at 4°C. The pellet was washed twice by resuspension in phosphate buffer (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid, pH 7.4) and centrifuged (27 000 *g*, 20 minutes, 4°C). The final pellet was resuspended in phosphate buffer and kept frozen at −80°C until the binding experiments were performed. Before the receptor binding assay, the samples were thawed on ice, and their protein concentrations were determined using the technique based on the method of Lowry *et al.* (1951). Membrane samples (VTA: 100 µl, Acb: 100 µl and PFC: 50 µl) were incubated with 200pM [^{3}H]epibatidine (Specific Activity = 49 Ci/mmol; Amersham) for 2 hours in a total volume of 250 µl at room temperature. NIC (10 mM) was used to determine non-specific binding. Binding reactions were stopped by addition of ice-cold buffer phosphate, and the samples were filtered by using filters of glass fiber GF/B, which were washed three times with ice-cold buffer phosphate. Filters were transferred to vials containing scintillation cocktail (OptiPhase HiSafe 3, Wallac) and finally quantified on a liquid scintillation counter. Counts per minute were converted to disintegrations per minute (dpm) using the method of external standard. Specific binding was calculated as the difference between binding determined in absence and presence of NIC. Data are presented as amount of [^{3}H]epibatidine in femtomole bound specifically to milligram of protein. In order to calculate the density in femtomole per milligram, the following equation was applied: $[(X - NS)/T/Y/P/10] \times 1000$; X, dpm from filters samples; NS, dpm from non-specific filters; T, total radioactivity of [^{3}H]epibatidine 200 pM; Y, final volume (250 µl); P, protein concentration.

Statistical analysis

For the pharmacological approach, the results were analyzed using two-way ANOVA, with treatment (SAL or NIC) and the GABAergic ligand (VEH or BAC and VEH or SAC) administration as between-subjects factors of variation. When a significant interaction between these two factors was observed, the difference between two means was analyzed by Tukey's *post hoc* test. For the genetic approach, the results were analyzed by using two-way ANOVA (genotype and treatment) between subjects followed by Tukey's *post hoc* test after statistically significant changes were found. The level of significance was $P < 0.05$ in all experiments for both approaches.

RESULTS

Effect of the activation, blockage and lack of $GABA_B$ receptors on the rewarding effects induced by NIC

We evaluated the effect of $GABA_B$ receptor activation by using BAC (3 mg/kg, ip) during the rewarding effects induced by NIC. The results showed that NIC-induced rewarding effects in conditioned place preference paradigm and BAC (3 mg/kg) blocked this effect ($P < 0.001$, respectively) (Fig. 1a) (Varani *et al.* 2014d). SAC (0.25, 0.5 and 1 mg/kg, ip) was used in order to block the activity of $GABA_B$ receptors during NIC-induced rewarding effects. Importantly, we observed that SAC (1 mg/kg) pre-treatment increased the NIC-induced rewarding effects ($P < 0.05$) (Fig. 1b): similarly, NIC-induced rewarding effects in WT and $GABA_{B1}$KO ($P < 0.001$) (Fig. 1c) mice. However, NIC-induced rewarding effects is even greater in $GABA_{B1}$KO compared

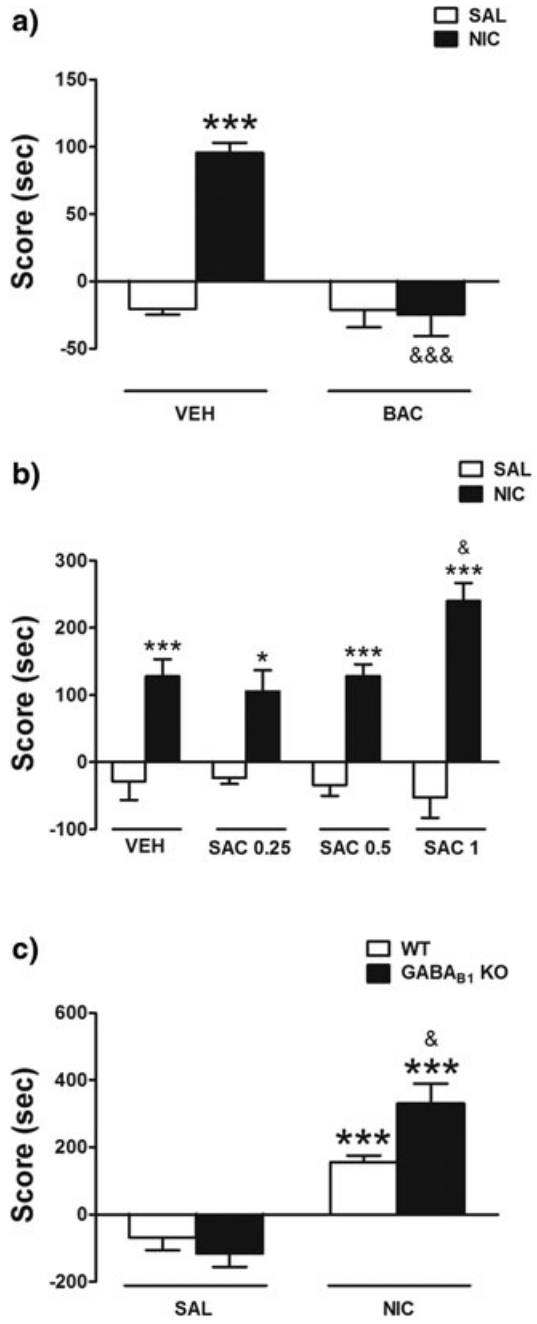

Figure 1 Effect of the activation, blockage and lack of $GABA_B$ receptors in the rewarding effects induced by nicotine in the conditioned place preference paradigm. Percent nicotine (NIC; 0.5 mg/kg, sc) rewarding effects was evaluated in baclofen (BAC) (3 mg/kg, ip), 2-hydroxysaclofen (SAC) (0.25, 0.5 and 1 mg/kg, ip) pretreated mice and $GABA_{B1}$ knockout (KO) mice in the conditioned place preference paradigm. Results are represented (mean ± SEM) as the difference between postconditioning and preconditioning time spent in the drug-paired compartment. The time in the central area was proportionally shared and added to the time value of each compartment. Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype [BAC or vehicle (VEH) and SAC or VEH/ wild type (WT) or KO] as factors of variation followed by *post hoc* comparisons using the Tukey test. $^{*}P < 0.05$, $^{***}P < 0.001$, when compared to respective SAL group; $^{\&}P < 0.05$, $^{\&\&\&}P < 0.001$ when compared to VEH/WT+NIC group

with WT mice ($P < 0.05$) (Fig. 1c). Statistical analysis is shown in Table 1.

Neurochemical alterations induced by the rewarding effects of NIC in BAC and SAC pretreated mice and $GABA_{B1}$KO mice

On the basis of behavioral studies, neurochemical determinations were performed in those experimental groups that have shown statistical differences. NIC (0.5 mg/kg, sc) increased DA levels in Acb and PFC, and BAC (3 mg/kg) was able to prevent the increase in both brain areas ($P < 0.001$, respectively) (Fig. 2a). DOPAC levels increased with NIC (0.5 mg/kg, sc) treatment in Acb ($P < 0.001$) (Fig. 2b), and once again, BAC (3 mg/kg) prevented this neurochemical alteration ($P < 0.01$) (Fig. 2b). In those experiments where the antagonism effect was evaluated, NIC (0.5 mg/kg, sc) increased DA levels in Acb and PFC, and SAC (1 mg/kg) was able to potentiate such increase only in the Acb ($P < 0.05$, respectively) (Fig. 2c). DOPAC levels were increased by NIC (0.5 mg/kg, sc) treatment in Acb, and SAC (1 mg/kg) potentiated this neurochemical alteration ($P < 0.01$, respectively) (Fig. 2d). Lastly, the genetic approach showed that NIC (0.5 mg/kg, sc) increased DA levels in Acb ($P < 0.01$) and PFC ($p < 0.05$) in WT mice (Fig. 2e), while this effect was potentiated only in the Acb of $GABA_{B1}$KO mice ($P < 0.01$)(Fig. 2e). NIC (0.5 mg/kg, sc) increased DOPAC levels in the Acb of both genotypes (WT, $P < 0.01$; $GABA_{B1}$KO, $P < 0.001$) (Fig. 2f). Statistical analysis is shown in Table 2.

c-Fos expression changes induced by the rewarding effects of NIC in BAC and SAC pretreated mice and $GABA_{B1}$KO mice

The pharmacologic approach showed that NIC (0.5 mg/kg, sc) increased the number of c-Fos-positive nuclei in the AcbSh ($P < 0.01$), VTA ($P < 0.05$) and PFC ($P < 0.001$), and BAC (3 mg/kg) was able to prevent the increase only in AcbSh ($P < 0.01$) and VTA ($P < 0.01$) (Fig. 3a). In the experiments where SAC pretreatment was tested, NIC (0.5 mg/kg, sc) increased c-Fos expression in the AcbSh, VTA and PFC ($P < 0.05$, respectively), and SAC (1 mg/kg) potentiated such increase only in the AcbSh ($P < 0.01$) and VTA ($P < 0.05$) (Fig. 3b). The genetic approach revealed that NIC (0.5 mg/kg, sc) increased c-Fos expression in the AcbSh ($P < 0.05$), VTA ($P < 0.05$) and PFC ($P < 0.01$) of WT mice, and this effect was potentiated only in the AcbSh ($P < 0.05$) and VTA ($P < 0.05$) of $GABA_{B1}$KO mice (Fig. 3c). Statistical analysis is shown in Table 3.

Table 1 Rewarding effect induced by nicotine in baclofen (3 mg/kg), 2-hydroxysaclofen (0.25, 0.5 y 1 mg/kg) pretreated mice and $GABA_{B1}$ knockout mice.

	Two-way ANOVA						*Post hoc test (Tukey)*					
	Pre-treatment/genotype		*Treatment*		*Interaction*		*VEH/WT-SAL versus VEH/WT-NIC*		*BAC/SAC/KO-SAL versus BAC/SAC/KO-NIC*		*VEH/WT-NIC versus BAC/SAC/KO-NIC*	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*
Baclofen												
BAC 3	$F_{(1,\ 59)} = 37.298$	<0.001	$F_{(1,\ 59)} = 32.162$	<0.001	$F_{(1,\ 59)} = 36.628$	<0.001	$F_{(3,\ 58)} = 41.290$	<0.001	$F_{(3,\ 58)} = 41.290$	NS	$F_{(3,\ 58)} = 41.290$	<0.001
2-OH-SAC												
SAC 0.25	$F_{(3,\ 113)} = 2.019$	NS	$F_{(1,\ 113)} = 113.282$	<0.001	$F_{(3,\ 113)} = 4.624$	<0.01	$F_{(7,\ 112)} = 19.509$	<0.001	$F_{(7,\ 112)} = 19.509$	<0.05	$F_{(7,\ 112)} = 19.509$	NS
SAC 0.5	$F_{(3,\ 113)} = 2.019$	NS	$F_{(1,\ 113)} = 113.282$	<0.001	$F_{(3,\ 113)} = 4.624$	<0.01	$F_{(7,\ 112)} = 19.509$	<0.001	$F_{(7,\ 112)} = 19.509$	<0.001	$F_{(7,\ 112)} = 19.509$	NS
SAC 1	$F_{(3,\ 113)} = 2.019$	NS	$F_{(1,\ 113)} = 113.282$	<0.001	$F_{(3,\ 113)} = 4.624$	<0.01	$F_{(7,\ 112)} = 19.509$	<0.001	$F_{(7,\ 112)} = 19.509$	<0.001	$F_{(7,\ 112)} = 19.509$	<0.05
$GABA_{B1}$ KO	$F_{(1,\ 52)} = 2.665$	NS	$F_{(1,\ 52)} = 73.907$	<0.001	$F_{(1,\ 52)} = 8.138$	<0.01	$F_{(3,\ 51)} = 25.540$	<0.001	$F_{(3,\ 51)} = 25.540$	<0.001	$F_{(3,\ 51)} = 25.540$	<0.05

Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype (BAC or VEH and SAC or VEH/WT or KO) as factors of variation followed by *post hoc* comparisons using the Tukey test. In order to simplify the table, the comparison between VEH/WT-SAL and BAC/SAC/KO-SAL is not shown, because no significant differences were observed. BAC = baclofen; KO = $GABA_{B1}$ knockout; NIC = nicotine; SAC = 2-hydroxysaclofen; SAL = saline solution; VEH = vehicle; WT = wild type; NS = non significant.

$\alpha_4\beta_2$nAChR density alterations induced by the rewarding effects of NIC in BAC and SAC pretreated mice and $GABA_{B1}$KO mice

Nicotine (0.5 mg/kg, sc) increased $\alpha_4\beta_2$nAChR density in VTA ($P < 0.05$) but not in Acb and PFC (Fig. 4a–c). BAC (3 mg/kg) pre-treatment prevented such increase induced by NIC in VTA ($P < 0.01$) (Fig. 4a). Moreover, SAC (1 mg/kg) pre-treatment potentiated the increase induced by NIC in the VTA ($P < 0.05$) (Fig. 4b). Interestingly, the genetic approach revealed that the increase of $\alpha_4\beta_2$nAChR density induced by NIC in the VTA was even greater in $GABA_{B1}$KO mice ($P < 0.01$) (Fig. 4c). Statistical analysis is shown in Table 4.

DISCUSSION

The rewarding effects of drugs of abuse are responsible for the initiation of the addictive process (Koob & Le Moal 2001). We used the conditioned place preference in order to evaluate the rewarding properties of NIC in mice. NIC (0.5 mg/kg, sc) induced place preference in mice, as reported previously (Castañé *et al.* 2002; Berrendero *et al.* 2005; Castañé *et al.* 2006). Although it was demonstrated that stress prior to conditioned place preference potentiated NIC rewarding effects (Brielmaier, McDonald & Smith 2012), in our experimental design, control groups were included. The assessment of these control groups is essential in order to rule out that a possible stressful condition produced by the pre-treatment injections may interfere with the magnitude of the NIC-induced rewarding effects. Indeed, no significant differences were observed between VEH + SAL, BAC + SAL SAC + SAL, WT + SAL and $GABA_{B1}$KO + SAL control groups (Fig 1). These results allowed us to confirm that the NIC-induced regarding effect was not influenced by the prior pre-treatment injections, at least in our experimental conditions.

There is evidence to support that the GABAergic system participates in the rewarding effects of drugs of abuse such as morphine, alcohol, cocaine and among others (Tyacke *et al.* 2010). Few of these studies show the involvement of $GABA_B$ receptors in NIC-induced rewarding properties. Our pharmacological approach showed that BAC (3 mg/kg, ip) blocked the rewarding effects induced by NIC. The dose of BAC (3 mg/kg, ip) used did not produce any response by itself in the paradigm of conditioned place preference, in agreement with previous studies (Heinrichs *et al.* 2010; Varani *et al.* 2014d). BAC alone did not have specific locomotor effects, as previously reported (Holstein & Phillips 2006; Frankowska, Filip & Przegaliński 2007; Varani & Balerio 2012). We also previously observed that BAC alone did not induce motor incoordination or sedation in the rota-rod test

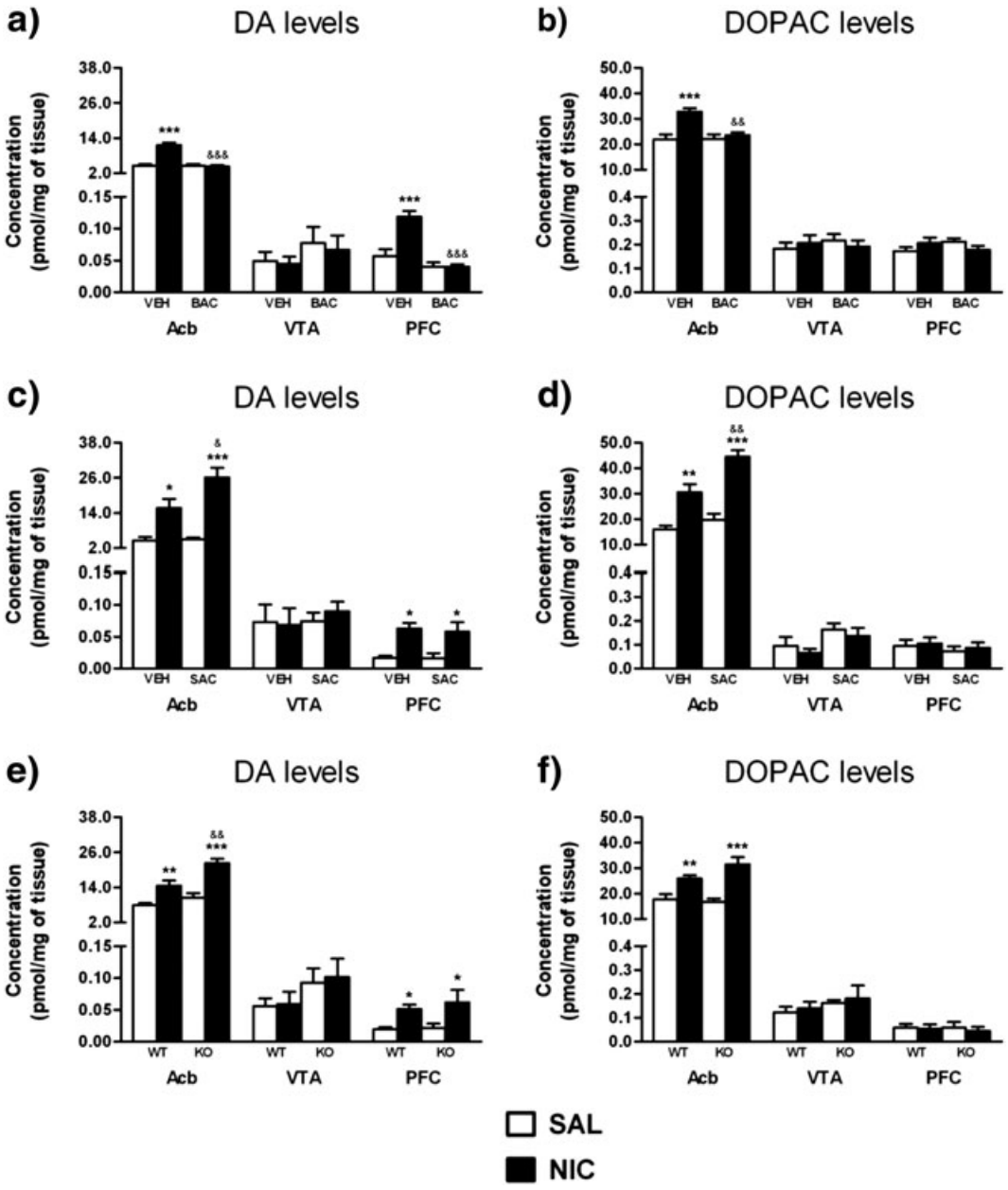

Figure 2 Neurochemical alterations induced by the rewarding effects of nicotine in baclofen (BAC) and 2-hydroxysaclofen (SAC) pretreated mice and GABAB1 knockout (KO) mice. Dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) alterations induced by nicotine (NIC) were evaluated in the nucleus accumbens (Acb), ventral tegmental area (VTA) and prefrontal cortex (PFC) of BAC (3 mg/kg, ip), SAC (0.25, 0.5 and 1 mg/kg, ip) pretreated mice and GABAB1 KO mice. Results are expressed as mean ± SEM of DA and DOPAC concentrations (pmol/mg of tissue) in the Acb, VTA and PFC. Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype (BAC or VEH and SAC or VEH/WT or KO) as factors of variation followed by *post hoc* comparisons using the Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to respective SAL group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ when compared to VEH/WT+NIC group

(Balerio & Rubio 2002). Our findings confirm that $GABA_B$ receptors activation prevent the rewarding effects of NIC, a phenomenon previously demonstrated. Indeed, it was reported that BAC prevents the rewarding effects of NIC in the paradigm of conditioned place preference in rats (Le Foll, Wertheim & Goldberg 2008). Mombereau *et al.* (2007) showed that a positive allosteric modulator of $GABA_B$ receptors also blocks NIC-induced place preference in rats. Moreover, it has been suggested that BAC is able to reduce the rewarding effects induced by NIC in a self-administration paradigm in rats (Fattore *et al.* 2009). BAC also prevents the reestablishment of NIC-induced rewarding effects previously extinguished in mice (Fattore *et al.* 2009). Based on the evidences mentioned earlier and our current results, we concluded that the activation of $GABA_B$ receptors modulates NIC

Table 2 Effect of nicotine (0.5 mg/kg) administration on neurotransmitters levels in the nucleus accumbens, ventral tegmental area and prefrontal cortex of baclofen (3 mg/kg), 2-hydroxysaclofen (0.25, 0.5 y 1 mg/kg) pretreated mice and $GABA_{B1}$ knockout mice.

	Two-way ANOVA						*Post hoc test (Tukey)*					
	Pre-treatment/genotype		*Treatment*		*Interaction*		*VEH/WT-SAL versus VEH/WT-NIC*		*BAC/SAC/KO-SAL versus BAC/SAC/KO-NIC*		*VEH/WT-NIC versus BAC/SAC/KO-NIC*	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*
Baclofen												
Acb												
DA	$F_{(1, 26)} = 32.903$	<0.001	$F_{(1, 26)} = 26.940$	<0.001	$F_{(1, 26)} = 31.760$	<0.001	$F_{(3, 25)} = 29.142$	<0.001	$F_{(3, 25)} = 29.142$	NS	$F_{(3, 25)} = 29.142$	<0.001
DOPAC	$F_{(1, 26)} = 8.033$	<0.01	$F_{(1, 26)} = 15.303$	<0.001	$F_{(1, 26)} = 8.739$	<0.01	$F_{(3, 25)} = 10.143$	<0.001	$F_{(3, 25)} = 10.143$	NS	$F_{(3, 25)} = 10.143$	<0.01
5-HT	$F_{(1, 26)} = 0.135$	NS	$F_{(1, 26)} = 0.382$	NS	$F_{(1, 26)} = 0.422$	NS	$F_{(3, 25)} = 0.335$	NS	$F_{(3, 25)} = 0.335$	NS	$F_{(3, 25)} = 0.335$	NS
5-HIAA	$F_{(1, 26)} = 0.910$	NS	$F_{(1, 26)} = 2.218$	NS	$F_{(1, 26)} = 2.648$	NS	$F_{(3, 25)=}1.810$	NS	$F_{(3, 25)=}1.810$	NS	$F_{(3, 25)=}1.810$	NS
VTA												
DA	$F_{(1, 26)} = 1.533$	NS	$F_{(1, 26)} = 0.146$	NS	$F_{(1, 26)} = 0.021$	NS	$F_{(3, 25)} = 0.569$	NS	$F_{(3, 25)} = 0.569$	NS	$F_{(3, 25)} = 0.569$	NS
DOPAC	$F_{(1, 26)} = 0.104$	NS	$F_{(1, 26)} = 0.000$	NS	$F_{(1, 26)} = 0.783$	NS	$F_{(3, 25)} = 0.298$	NS	$F_{(3, 25)} = 0.298$	NS	$F_{(3, 25)} = 0.298$	NS
5-HT	$F_{(1, 26)} = 2.576$	NS	$F_{(1, 26)} = 0.012$	NS	$F_{(1, 26)} = 2.561$	NS	$F_{(3, 25)} = 1.731$	NS	$F_{(3, 25)} = 1.731$	NS	$F_{(3, 25)} = 1.731$	NS
5-HIAA	$F_{(1, 26)} = 1.496$	NS	$F_{(1, 26)} = 2.490$	NS	$F_{(1, 26)} = 1.625$	NS	$F_{(3, 25)} = 1.775$	NS	$F_{(3, 25)} = 1.775$	NS	$F_{(3, 25)} = 1.775$	NS
PFC												
DA	$F_{(1, 26)} = 36.261$	<0.001	$F_{(1, 26)} = 15.304$	<0.001	$F_{(1, 26)} = 15.311$	<0.001	$F_{(3, 25)} = 21.563$	<0.001	$F_{(3, 25)} = 21.563$	NS	$F_{(3,25)} = 21.563$	<0.001
DOPAC	$F_{(1, 26)} = 0.072$	NS	$F_{(1, 26)} = 0.005$	NS	$F_{(1, 26)} = 3.446$	NS	$F_{(3, 25)} = 1.187$	NS	$F_{(3, 25)} = 1.187$	NS	$F_{(3,25)} = 1.187$	NS
5-HT	$F_{(1, 26)} = 0.485$	NS	$F_{(1, 26)} = 0.503$	NS	$F_{(1, 26)} = 0.022$	NS	$F_{(3, 25)} = 0.332$	NS	$F_{(3, 25)} = 0.332$	NS	$F_{(3,25)} = 0.332$	NS
5-HIAA	$F_{(1, 26)} = 0.130$	NS	$F_{(1, 26)} = 0.953$	NS	$F_{(1, 26)} = 1.625$	NS	$F_{(3, 25)=}$ 0.844	NS	$F_{(3, 25)=}$ 0.844	NS	$F_{(3,25)=}$ 0.844	NS
2-hydroxysaclofen												
Acb												
DA	$F_{(1, 28)} = 5.130$	<0.05	$F_{(1, 28)} = 43.963$	<0.001	$F_{(1, 28)} = 4.301$	<0.05	$F_{(3, 27)} = 12.729$	<0.05	$F_{(3, 27)} = 12.729$	<0.001	$F_{(3, 27)} = 12.729$	<0.05
DOPAC	$F_{(1, 28)} = 12.354$	<0.01	$F_{(1, 28)} = 61.973$	<0.001	$F_{(1, 28)} = 4.226$	NS	$F_{(3, 27)} = 19.783$	<0.01	$F_{(3, 27)} = 19.783$	<0.001	$F_{(3, 27)} = 19.783$	<0.01
5-HT	$F_{(1, 28)} = 3.381$	NS	$F_{(1, 28)} = 1.263$	NS	$F_{(1, 28)} = 2.101$	NS	$F_{(3, 27)} = 2.344$	NS	$F_{(3, 27)} = 2.344$	NS	$F_{(3, 27)} = 2.344$	NS
5-HIAA	$F_{(1, 28)} = 0.082$	NS	$F_{(1, 28)} = 1.241$	NS	$F_{(1, 28)} = 0.982$	NS	$F_{(3, 27)} = 0.542$	NS	$F_{(3, 27)} = 0.542$	NS	$F_{(3, 27)} = 0.542$	NS
VTA												
DA	$F_{(1, 28)} = 0.285$	NS	$F_{(1, 28)} = 0.057$	NS	$F_{(1, 28)} = 0.220$	NS	$F_{(3, 27)} = 0.240$	NS	$F_{(3, 27)} = 0.240$	NS	$F_{(3, 27)} = 0.240$	NS
DOPAC	$F_{(1, 28)} = 5.487$	<0.05	$F_{(1, 28)} = 0.916$	NS	$F_{(1, 28)} = 0.003$	NS	$F_{(3, 27)} = 1.855$	NS	$F_{(3, 27)} = 1.855$	NS	$F_{(3, 27)} = 1.855$	NS
5-HT	$F_{(1, 28)} = 1.810$	NS	$F_{(1, 28)} = 0.243$	NS	$F_{(1, 28)} = 1.067$	NS	$F_{(3, 27)} = 0.587$	NS	$F_{(3, 27)} = 0.587$	NS	$F_{(3, 27)} = 0.587$	NS
5-HIAA	$F_{(1, 28)} = 1.531$	NS	$F_{(1, 28)} = 0.084$	NS	$F_{(1, 28)} = 4.219$	NS	$F_{(3, 27)} = 1.954$	NS	$F_{(3, 27)} = 1.954$	NS	$F_{(3, 27)} = 1.954$	NS
PFC												
DA	$F_{(1, 28)} = 0.084$	NS	$F_{(1, 28)} = 19.806$	<0.001	$F_{(1, 28)} = 0.053$	NS	$F_{(3, 27)} = 7.729$	<0.01	$F_{(3, 27)} = 7.729$	NS	$F_{(3, 27)} = 7.729$	NS

Table 2. (Continued)

	Two-way ANOVA						*Post hoc test (Tukey)*					
	Pre-treatment/genotype		*Treatment*		*Interaction*		*VEH/WT-SAL versus VEH/WT-NIC*		*BAC/SAC/KO-SAL versus BAC/SAC/KO-NIC*		*VEH/WT-NIC versus BAC/SAC/KO-NIC*	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*
DOPAC	$F_{(1, 28)} = 0.817$	NS	$F_{(1, 28)} = 0.289$	NS	$F_{(1, 28)} = 0.004$	NS	$F_{(3, 27)} = 0.404$	NS	$F_{(3, 27)} = 0.404$	NS	$F_{(3, 27)} = 0.404$	NS
5-HT	$F_{(1, 28)} = 8.359$	<0.01	$F_{(1, 28)} = 0.519$	NS	$F_{(1, 28)} = 0.071$	NS	$F_{(3, 27)} = 4.186$	NS	$F_{(3, 27)} = 4.186$	NS	$F_{(3, 27)} = 4.186$	NS
5-HIAA	$F_{(1, 28)} = 8.811$	<0.01	$F_{(1, 28)} = 0.476$	NS	$F_{(1, 28)} = 0.014$	NS	$F_{(3, 27)} = 2.486$	NS	$F_{(3, 27)} = 2.486$	NS	$F_{(3, 27)} = 2.486$	NS
GABA$_{B1}$ KO												
Acb												
DA	$F_{(1, 28)} = 11.209$	<0.01	$F_{(1, 28)} = 36.649$	<0.001	$F_{(1, 28)} = 2.840$	NS	$F_{(3, 27)} = 15.953$	<0.01	$F_{(3, 27)} = 15.953$	<0.001	$F_{(3, 27)} = 15.953$	<0.01
DOPAC	$F_{(1, 28)} = 1.318$	NS	$F_{(1, 28)} = 32.796$	<0.001	$F_{(1, 28)} = 2.712$	NS	$F_{(3, 27)} = 11.371$	<0.01	$F_{(3, 27)} = 11.371$	<0.001	$F_{(3, 27)} = 11.371$	NS
5-HT	$F_{(1, 28)} = 0.013$	NS	$F_{(1, 28)} = 1.668$	NS	$F_{(1, 28)} = 3.163$	NS	$F_{(3, 27)} = 2.234$	NS	$F_{(3, 27)} = 2.234$	NS	$F_{(3, 27)} = 2.234$	NS
5-HIAA	$F_{(1, 28)} = 0.426$	NS	$F_{(1, 28)} = 2.944$	NS	$F_{(1, 28)} = 0.347$	NS	$F_{(3, 27)} = 1.546$	NS	$F_{(3, 27)} = 1.546$	NS	$F_{(3, 27)} = 1.546$	NS
VTA												
DA	$F_{(1, 28)} = 3.828$	NS	$F_{(1, 28)} = 0.094$	NS	$F_{(1, 28)} = 0.019$	NS	$F_{(3, 27)} = 1.308$	NS	$F_{(3, 27)} = 1.308$	NS	$F_{(3, 27)} = 1.308$	NS
DOPAC	$F_{(1, 28)} = 1.607$	NS	$F_{(1, 28)} = 0.321$	NS	$F_{(1, 28)} = 0.000$	NS	$F_{(3, 27)} = 0.651$	NS	$F_{(3, 27)} = 0.651$	NS	$F_{(3, 27)} = 0.651$	NS
5-HT	$F_{(1, 28)} = 1.663$	NS	$F_{(1, 28)} = 0.044$	NS	$F_{(1, 28)} = 0.032$	NS	$F_{(3, 27)} = 0.574$	NS	$F_{(3, 27)} = 0.574$	NS	$F_{(3, 27)} = 0.574$	NS
5-HIAA	$F_{(1, 28)} = 0.022$	NS	$F_{(1, 28)} = 0.004$	NS	$F_{(1, 28)} = 0.034$	NS	$F_{(3, 27)} = 0.019$	NS	$F_{(3, 27)} = 0.019$	NS	$F_{(3, 27)} = 0.019$	NS
PFC												
DA	$F_{(1, 28)} = 0.496$	NS	$F_{(1, 28)} = 16.262$	<0.001	$F_{(1, 28)} = 0.191$	NS	$F_{(3, 27)} = 5.772$	<0.05	$F_{(3, 27)} = 5.772$	<0.05	$F_{(3, 27)} = 5.772$	NS
DOPAC	$F_{(1, 28)} = 0.052$	NS	$F_{(1, 28)} = 0.210$	NS	$F_{(1, 28)} = 0.037$	NS	$F_{(3, 27)} = 0.089$	NS	$F_{(3, 27)} = 0.089$	NS	$F_{(3, 27)} = 0.089$	NS
5-HT	$F_{(1, 28)} = 0.595$	NS	$F_{(1, 28)} = 0.024$	NS	$F_{(1, 28)} = 0.453$	NS	$F_{(3, 27)} = 0.350$	NS	$F_{(3, 27)} = 0.350$	NS	$F_{(3, 27)} = 0.350$	NS
5-HIAA	$F_{(1, 28)} = 2.539$	NS	$F_{(1, 28)} = 0.122$	NS	$F_{(1, 28)} = 0.786$	NS	$F_{(3, 27)} = 1.112$	NS	$F_{(3, 27)} = 1.112$	NS	$F_{(3, 27)} = 1.112$	NS

Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype (BAC or VEH and SAC or VEH/WT or KO) as factors of variation followed by *post hoc* comparisons using the Tukey test. In order to simplify the table, the comparison between VEH/WT-SAL and BAC/SAC/KO-SAL is not shown, because no significant differences were observed. Acb = nucleus accumbens; BAC = baclofen; DA = dopamine; DOPAC = 3,4-dihydroxyphenylacetic acid; KO = GABA$_{B1}$ knockout; NIC = nicotine; PFC = prefrontal cortex; SAC = 2-hydroxysaclofen; SAL = saline solution; VEH = vehicle; VT = ventral tegmental area; WT = wild type; 5-HIAA = 5-hydroxyindolacetic acid; 5-HT = serotonin; NS = non significant.

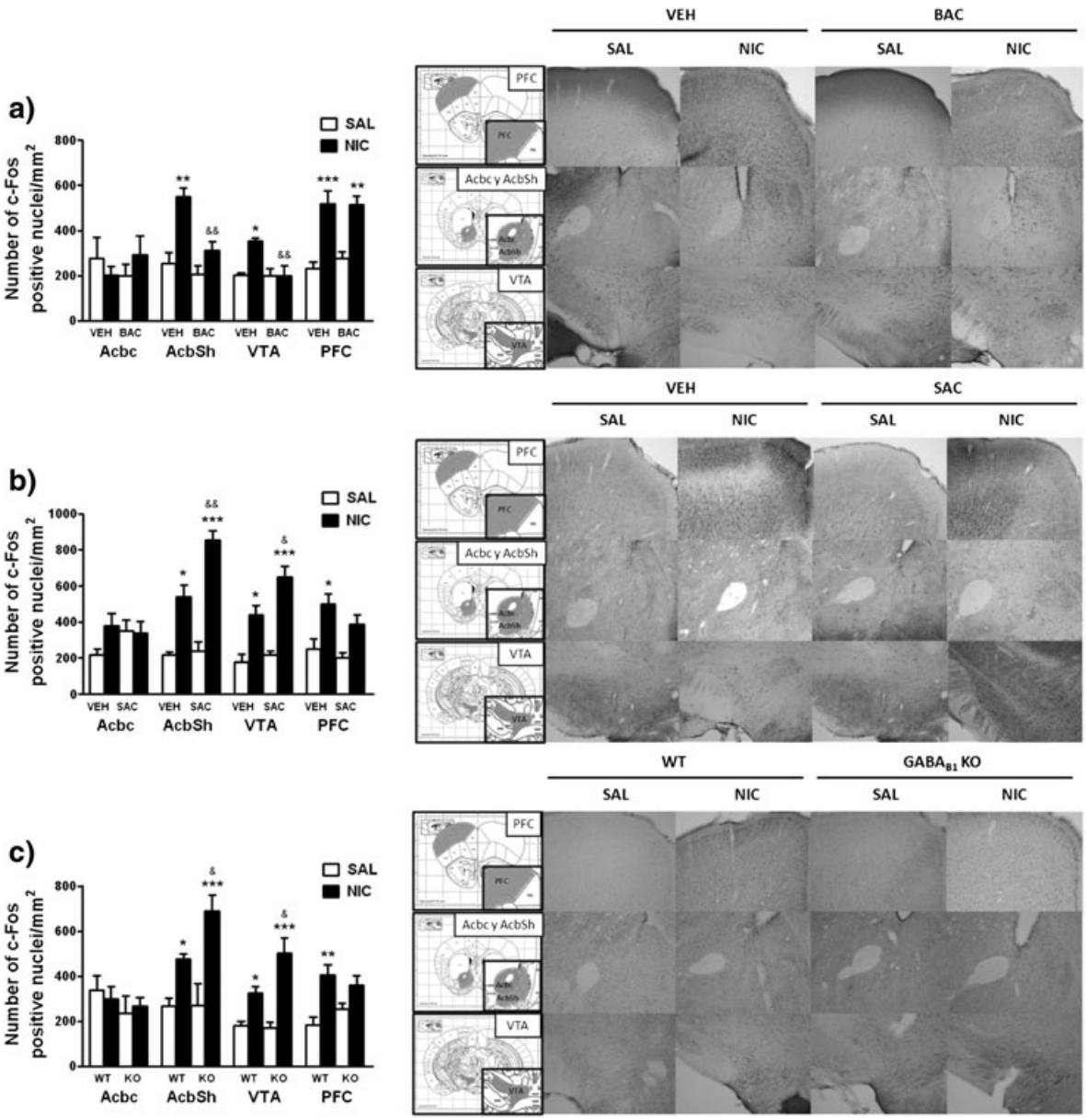

Figure 3 c-Fos expression changes induced by the rewarding effects of nicotine in baclofen (BAC) and 2-hydroxysaclofen (SAC) pretreated mice and $GABA_{B1}$ knockout (KO) mice. Changes in c-Fos expression induced by nicotine (NIC) were evaluated in the nucleus accumbens shell (AcbSh) and core (Acbc), ventral tegmental area (VTA) and prefrontal cortex (PFC) of BAC (3 mg/kg, ip), SAC (0.25, 0.5 and 1 mg/kg, ip) pretreated mice and $GABA_{B1}$ KO mice. Results are expressed as mean ± SEM of c-Fos-positive nuclei per square millimeter in the Acb, VTA and PFC. Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype (BAC or VEH and SAC or VEH/WT or KO) as factors of variation followed by *post hoc* comparisons using the Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to respective SAL group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ when compared to VEH/WT+NIC group

self-administration, preventing the rewarding effects of NIC and its development. Conversely, Falco *et al.* (2014) observed that BAC potentiated NIC-induced rewarding effects. However, there are some important procedural differences in comparison with our study: firstly, Falco *et al*. used adolescent rats, while we used adult mice. Secondly, Falco *et al.* used a single-trial conditioned place preference paradigm, while we used a four-trial design. Finally, Falco *et al*. counterbalanced the order of drug administration, while in our experiments, the animals were firstly injected with SAL and 4 hours later with NIC on each day. Our genetic approach revealed that the NIC-induced rewarding effects are significantly increased in $GABA_{B1}$KO mice. In line with this result, pre-treatment of WT mice with SAC (1 mg/kg, ip) potentiated the NIC-induced rewarding effects. The SAC doses used (0.25, 0.5 and 1 mg/kg; ip) did not produce any response by itself in the conditioned place preference paradigm. In addition, SAC given at different doses did not modify the locomotor activity, in agreement with previous studies

Table 3 Effect of nicotine (0.5 mg/kg) administration on c-Fos expression in the nucleus accumbens, ventral tegmental area and prefrontal cortex of baclofen (3 mg/kg), 2-hydroxysaclofen (0.25, 0.5 y 1 mg/kg) pretreated mice and GABA$_{B1}$ knockout mice.

	Two-way ANOVA						*Post hoc test (Tukey)*					
	Pre-treatment/genotype		*Treatment*		*Interaction*		*VEH/WT-SAL versus VEH/WT-NIC*		*BAC/SAC/KO-SAL versus BAC/SAC/KO-NIC*		*VEH/WT-NIC versus BAC/SAC/KO-NIC*	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*
Baclofen												
Acbc	$F_{(1, 10)} = 0.010$	NS	$F_{(1, 10)} = 0.015$	NS	$F_{(1, 10)} = 0.418$	NS	$F_{(3, 13)} = 0.505$	NS	$F_{(3, 13)} = 0.505$	NS	$F_{(3, 13)} = 0.505$	NS
AcbSh	$F_{(1, 10)} = 12.586$	<0.01	$F_{(1, 10)} = 24.752$	<0.01	$F_{(1, 10)} = 5.419$	<0.05	$F_{(3, 13)} = 13.336$	<0.01	$F_{(3, 13)} = 13.336$	NS	$F_{(3, 13)} = 13.336$	<0.01
VTA	$F_{(1, 14)} = 7.087$	<0.05	$F_{(1, 14)} = 6.592$	<0.05	$F_{(1, 14)} = 6.69$	<0.05	$F_{(3, 17)} = 8.591$	<0.05	$F_{(3, 17)} = 8.591$	NS	$F_{(3, 17)} = 8.591$	<0.01
PFC	$F_{(1, 20)} = 0.0277$	NS	$F_{(1, 20)} = 41.185$	<0.001	$F_{(1, 20)} = 0.319$	NS	$F_{(3, 23)=}13.927$	<0.001	$F_{(3, 23)=}13.927$	<0.01	$F_{(3, 23)=}13.927$	NS
2-hydroxysaclofen												
Acbc	$F_{(1, 14)} = 0.463$	NS	$F_{(1, 14)} = 1.258$	NS	$F_{(1, 14)} = 1.611$	NS	$F_{(3, 17)} = 0.936$	NS	$F_{(3, 17)} = 0.936$	NS	$F_{(3, 17)} = 0.936$	NS
AcbSh	$F_{(1, 14)} = 7.866$	<0.05	$F_{(1, 14)} = 60.340$	<0.001	$F_{(1, 14)} = 5.914$	<0.05	$F_{(3, 17)} = 25.005$	<0.05	$F_{(3, 17)} = 25.005$	<0.001	$F_{(3, 17)} = 25.005$	<0.01
VTA	$F_{(1, 15)} = 5.739$	<0.05	$F_{(1, 15)} = 44.113$	<0.001	$F_{(1, 15)} = 2.848$	NS	$F_{(3, 18)} = 17.299$	<0.05	$F_{(3, 18)} = 17.299$	<0.001	$F_{(3, 18)} = 17.299$	<0.05
PFC	$F_{(1, 17)} = 2.308$	NS	$F_{(1, 17)} = 16.750$	<0.001	$F_{(1, 17)} = 0.385$	NS	$F_{(3, 20)} = 6.775$	<0.05	$F_{(3, 20)} = 6.775$	NS	$F_{(3, 20)} = 6.775$	NS
GABA$_{B1}$ KO												
Acbc	$F_{(1, 15)} = 1.083$	NS	$F_{(1, 15)} = 0.001$	NS	$F_{(1, 15)} = 0.279$	NS	$F_{(3, 18)} = 0.446$	NS	$F_{(3, 18)} = 0.446$	NS	$F_{(3, 18)} = 0.446$	NS
AcbSh	$F_{(1, 15)} = 4.698$	<0.05	$F_{(1, 15)} = 39.262$	<0.001	$F_{(1, 15)} = 4.378$	NS	$F_{(3, 18)} = 15.722$	<0.05	$F_{(3, 18)} = 15.722$	<0.001	$F_{(3, 18)} = 15.722$	<0.05
VTA	$F_{(1, 16)} = 5.477$	<0.05	$F_{(1, 16)} = 44.049$	<0.001	$F_{(1, 16)} = 6.545$	<0.05	$F_{(3, 19)} = 17.035$	<0.05	$F_{(3, 19)} = 17.035$	<0.001	$F_{(3, 19)} = 17.035$	<0.05
PFC	$F_{(1, 16)} = 0.092$	NS	$F_{(1, 16)} = 16.573$	<0.001	$F_{(1, 16)} = 2.007$	NS	$F_{(3, 19)=}7.283$	<0.01	$F_{(3, 19)=}7.283$	NS	$F_{(3, 19)=}7.283$	NS

Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype (BAC or VEH and SAC or VEH/WT or KO) as factors of variation followed by *post hoc* comparisons using the Tukey test. In order to simplify the table the comparison between VEH/WT-SAL and BAC/SAC/KO-SAL is not shown, since no significant differences were observed. Acbc = nucleus accumbens core; AcbSh = nucleus accumbens shell; BAC = baclofen; KO = GABA$_{B1}$ knochout; NIC = nicotine; PFC = prefrontal cortex; SAC = 2-hydroxysaclofen; SAL = saline solution; VTA = ventral tegmental area; VEH = vehicle; WT = wild type; NS = non significant.

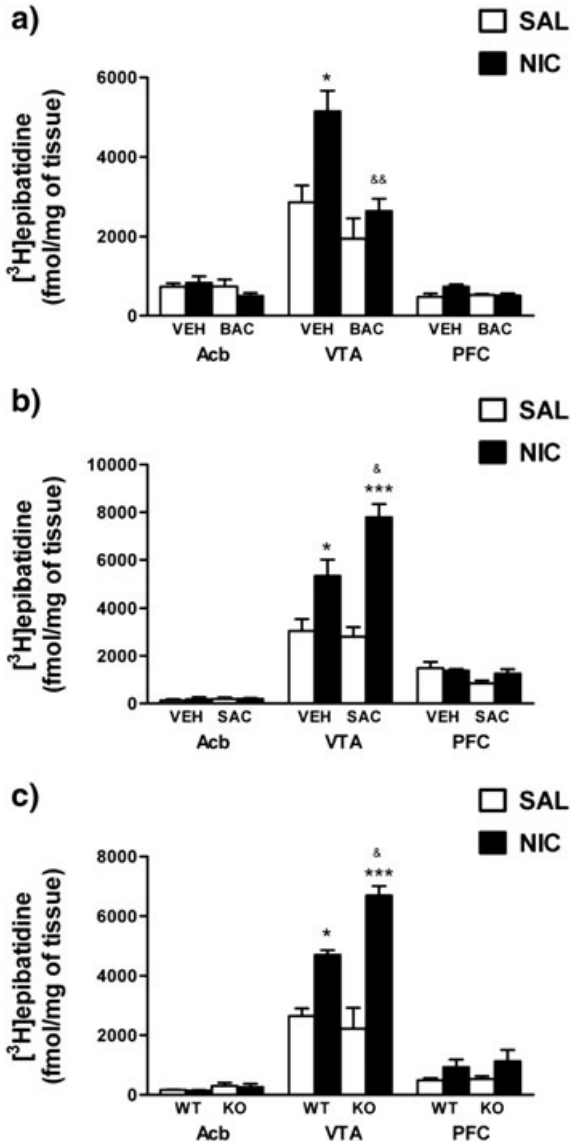

Figure 4 Nicotinic receptors α4β2 (nAChR α4β2) density alterations induced by the rewarding effects of nicotine in baclofen (BAC) and 2-hydroxysaclofen (SAC) pretreated mice and $GABA_{B1}$ knockout (KO) mice. Alterations in nicotinic receptors α4β2 (nAChR α4β2) density induced by NIC were evaluated in the nucleus accumbens (Acb), ventral tegmental area (VTA) and prefrontal cortex (PFC) of BAC (3 mg/kg, ip), SAC (0.25, 0.5 and 1 mg/kg, ip) pretreated mice, and $GABA_{B1}$ KO mice results are expressed as mean ± SEM of [3H] epibatidine binding levels (fmol/mg of tissue) in the Acb, VTA and PFC. Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype (BAC or VEH and SAC or VEH/WT or KO) as factors of variation followed by *post hoc* comparisons using the Tukey test. $^{*}P < 0.05$, $^{***}P < 0.001$ when compared to respective SAL group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ when compared toVEH/WT+NIC group

(Kalivas *et al.* 2001; Abraini *et al.* 2003; Varani & Balerio 2012). In our study, locomotor activity between WT and $GABA_{B1}$KO mice seems to be no different. Similarly, we have shown that the spontaneous locomotor activity of $GABA_{B1}$KO mice was similar to that observed in WT littermates (Varani *et al.* 2012). Conversely, previous studies revealed that $GABA_{B1}$KO mice exhibit a pronounced hyperlocomotor activity when mice were exposed to a new testing environment (Schuler *et al.* 2001; Gassmann *et al.* 2004). However, in our conditions, mice were exposed to the testing environment during 4 days before the postconditioning phase (testing day).

Nicotine increases extracellular DA levels in the Acb, which underlies its rewarding properties (Marubio *et al.* 2003). By using HPLC, we determined the role of $GABA_B$ receptors in the alteration of the DA concentration induced by NIC in the mesocorticolimbic pathway. Both the pharmacological and genetic approach revealed that NIC (0.5 mg/kg, sc) induces an increase of DA levels in the Acb and PFC. Berrendero *et al.* (2005) showed that the same dose of NIC induces an increase of extracellular DA concentrations in the Acb of mice, measured using microdialysis. Our neurochemical data are in line with the results obtained at the behavioral level. Indeed, the dose of NIC (0.5 mg/kg, sc) that increased DA concentrations in the Acb and the PFC also induced rewarding effects. In fact, the rewarding effects induced by NIC in mice occur only at an intermediate dose (0.5 mg/kg) but not at low (0.25 mg/kg) or high (1 mg/kg) doses (Castañé *et al.* 2002). The reason behind this U-shaped dose response curve is presumably due to a lack of NIC reward at low doses and the emergence of aversive effects produced by high NIC doses (Torres *et al.* 2008). The increase of DA concentration in the Acb and PFC induced by NIC is due to the modulation exerted on excitatory (glutamatergic neurons) and inhibitory (GABAergic neurons) inputs in the VTA. These inputs are connected with dopaminergic neurons that innervate the Acb and PFC from the VTA. NIC stimulates nAChRs located on dopaminergic, glutamatergic and GABAergic neurons of the VTA, causing the release of DA in the Acb that is responsible for the NIC-induced rewarding properties (Paterson 2009; De Biasi & Dani 2011; Polosa & Benowitz 2011). The activity of dopaminergic neurons in the VTA is partly controlled by local GABAergic interneurons, and particularly, the expression of $GABA_B$ receptors in this area is limited to somatodendritic localization in dopaminergic and GABAergic neurons (Cruz *et al.* 2004; Laviolette & Van Der Kooy 2004). In our study, BAC (3 mg/kg, ip) pre-treatment blocked the increase of DA concentration induced by NIC in the Acb and had no effects by itself on neurotransmitter concentration in any of the brain areas studied. BAC may activate $GABA_B$ receptors expressed in dopaminergic and GABAergic neurons of the VTA, causing a decrease in DA release in the Acb by inhibition of these neurons. This would explain the fact that BAC was able to block the rewarding effects induced by NIC in the conditioned place preference test.

Table 4 Effect of nicotine (0.5 mg/kg) administration on nicotinic receptors α4β2 density in the nucleus accumbens, ventral tegmental area and prefrontal cortex of baclofen (3 mg/kg), 2-hydroxysaclofen (0.25, 0.5 y 1 mg/kg) pretreated mice and GABA$_{B1}$ knockout mice.

	Two-way ANOVA						*Post hoc test (Tukey)*					
	Pre-treatment/genotype		*Treatment*		*Interaction*		*VEH/WT-SAL versus VEH/WT-NIC*		*BAC/SAC/KO-SAL versus BAC/SAC/KO-NIC*		*VEH/WT-NIC versus BAC/SAC/KO-NIC*	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*
Baclofen												
Acb	$F_{(1, 20)} = 1.430$	NS	$F_{(1, 20)} = 0.260$	NS	$F_{(1, 20)} = 1.558$	NS	$F_{(3, 19)} = 1.083$	NS	$F_{(3, 19)} = 1.083$	NS	$F_{(3, 19)} = 1.083$	NS
VTA	$F_{(1, 20)} = 14.569$	<0.01	$F_{(1, 20)} = 11.000$	<0.01	$F_{(1, 20)} = 3.189$	NS	$F_{(3, 19)} = 9.586$	<0.05	$F_{(3, 19)} = 9.586$	NS	$F_{(3, 19)} = 9.586$	<0.01
PFC	$F_{(1, 20)} = 1.770$	NS	$F_{(1, 20)} = 3.665$	NS	$F_{(1, 20)} = 4.536$	<0.05	$F_{(3, 19)} = 3.324$	NS	$F_{(3, 19)} = 3.324$	NS	$F_{(3, 19)} = 3.324$	NS
2-hydroxysaclofen												
Acb	$F_{(1, 19)} = 0.453$	NS	$F_{(1, 19)} = 0.208$	NS	$F_{(1, 19)} = 0.121$	NS	$F_{(3, 18)} = 0.236$	NS	$F_{(3, 18)} = 0.236$	NS	$F_{(3, 18)} = 0.236$	NS
VTA	$F_{(1, 19)} = 3.769$	NS	$F_{(1, 19)} = 40.134$	<0.001	$F_{(1, 19)} = 5.328$	<0.05	$F_{(3, 18)} = 16.772$	<0.05	$F_{(3, 18)} = 16.772$	<0.001	$F_{(3, 18)} = 16.772$	<0.05
PFC	$F_{(1, 19)} = 8.514$	<0.05	$F_{(1, 19)} = 0.247$	NS	$F_{(1, 19)} = 4.608$	<0.05	$F_{(3, 18)} = 4.346$	NS	$F_{(3, 18)} = 4.346$	NS	$F_{(3, 18)} = 4.346$	NS
GABA$_{B1}$ KO												
Acb	$F_{(1, 20)} = 2.576$	NS	$F_{(1, 20)} = 0.158$	NS	$F_{(1, 20)} = 0.011$	NS	$F_{(3, 17)} = 1.527$	NS	$F_{(3, 17)} = 1.527$	NS	$F_{(3, 17)} = 1.527$	NS
VTA	$F_{(1, 20)} = 3.651$	NS	$F_{(1, 20)} = 62.692$	<0.001	$F_{(1, 20)} = 8.630$	<0.05	$F_{(3, 17)} = 18.465$	<0.05	$F_{(3, 17)} = 18.465$	<0.001	$F_{(3, 17)} = 18.465$	<0.05
PFC	$F_{(1, 20)} = 0.229$	NS	$F_{(1, 20)} = 4.763$	<0.05	$F_{(1, 20)} = 0.117$	NS	$F_{(3, 17)} = 2.145$	NS	$F_{(3, 17)} = 2.145$	NS	$F_{(3, 17)} = 2.145$	NS

Statistical analysis was performed using two-way ANOVA with treatment (NIC or SAL) and pre-treatment/genotype (BAC or VEH and SAC or VEH/WT or KO) as factors of variation followed by *post hoc* comparisons using the Tukey test. In order to simplify the table, the comparison between VEH/WT-SAL and BAC/SAC/KO-SAL is not shown, because no significant differences were observed. Acb = nucleus accumbens; BAC = baclofen; KO = GABA$_{B1}$ knockout; NIC = nicotine; PFC = prefrontal cortex; SAL = saline solution; VEH = vehicle; SAC = 2-hydroxysaclofen; WT = wild type; VTA = ventral tegmental area; NS = non significant.

Cruz *et al*. (2004) have shown in rodent VTA slices that a low concentration of the canonical agonist BAC caused increased activity, whereas higher doses eventually inhibited DA neurons. It has been shown that the stimulation of $GABA_B$ receptors in the VTA attenuates the rewarding properties induced by NIC (Cousins, Roberts & de Wit 2002). In addition, BAC prevents DA release induced by NIC in the Acb of rats (Fadda *et al.* 2003). The pharmacological approach of the present study showed that the increase in DA concentration in the Acb was significantly higher in mice pretreated with SAC (1 mg/kg; ip). The dose of SAC showed no effects by itself on the neurotransmitters concentration in the brain areas studied. We found that increased DA concentration induced by NIC in the Acb was even greater in $GABA_{B1}$KO mice. We therefore propose that the genetic lack or the pharmacological blockage of $GABA_B$ receptors in dopaminergic neurons of the VTA disinhibits these neurons and increases DA release in the Acb.

In order to determine a possible correlation with the neurochemical data, we determined c-Fos expression, a marker of neuronal activity (Dragunow & Faull 1989). Our results show that NIC (0.5 mg/kg; sc) induces an increase in c-Fos expression in AcbSh, VTA and PFC, which is in agreement with previous reports (Di *et al.* 2012; Dehkordi *et al.* 2015). These findings are in line with the results obtained at the behavioral and neurochemical level. Indeed, an NIC dose (0.5 mg/kg, sc) produces place preference, increases DA concentrations in Acb and PFC as well as Fos expression in the mesocorticolimbic circuit. We confirm that the NIC-induced rewarding effects lead to an increase in neuronal activity in the reward pathway. Regarding the involvement of $GABA_B$ receptors, BAC (3 mg/kg, ip) blocked the increased c-Fos expression induced by NIC in the AcbSh and VTA, but not in the PFC. On the other hand, BAC by itself did not modify the c-Fos expression in any of the brain areas studied. These results are in agreement with previous results from our laboratory (Pedrón *et al.* 2013). In line with our results, a previous study from our group showed that BAC is able to restore altered c-Fos expression during NIC withdrawal in mice (Varani *et al*. 2014b). Furthermore, Mombereau *et al.* (2007) demonstrated that a positive allosteric modulator of $GABA_B$ receptors prevents the increase of Fos immunoreactivity induced by NIC in the Acb of rats. On the other hand, SAC (1 mg/kg, ip) by itself did not alter the c-Fos expression in any of the brain areas studied. However, SAC was able to potentiate the increase of c-Fos expression induced by NIC in the Acb and the VTA, but not in the PFC. Similarly, the genetic approach revealed that the increase of c-Fos expression induced by NIC in the AcbSh and VTA of WT mice is even greater in $GABA_{B1}$KO mice. We previously showed that NIC withdrawal induces alterations in c-Fos expression in WT mice but not in $GABA_{B1}$KO mice (Varani *et al.* 2012). In both approaches, the increase of c-Fos expression in the PFC was not modified. This could be explained by a differential density of $GABA_B$ receptors in the PFC, compared with the VTA and AcbSh. Given the aforementioned background and our results, we assume that $GABA_B$ receptors modulate neuronal activity in the mesocorticolimbic circuit during the NIC-induced rewarding effects.

Finally, we analyzed the density of $\alpha_4\beta_2$nAChRs by binding ligand-receptor assays in tissue homogenates. These experiments were conducted in order to determine if the changes observed at behavioral and neurochemical level could be due to possible alterations in $\alpha_4\beta_2$nAChRs density. The results of both approaches showed that NIC (0.5 mg/kg, sc) induces an increase in the density of $\alpha_4\beta_2$nAChRs in the VTA but not in the Acb and the PFC, in agreement with previous studies (Dehkordi *et al.* 2015). In this context, it is noteworthy that NIC induces the rewarding effects through the stimulation of nAChRs in the VTA (Wu *et al.* 2013). The data obtained in the present study are consistent with the results at behavioral and neurochemical level. In addition as previously mentioned, NIC also increased the c-Fos expression in the VTA. Therefore, NIC could stimulate $\alpha_4\beta_2$nAChRs expressed in dopaminergic neurons of the VTA, increasing DA release in the Acb and PFC and consequently inducing the rewarding effects. Importantly, BAC (3 mg/kg, ip) pre-treatment blocked the increase of $\alpha_4\beta_2$nAChRs density induced by NIC in VTA, but not in the PFC and Acb. On the other hand, BAC by itself did not alter the expression of $\alpha_4\beta_2$nAChRs in the brain areas studied. Consistent with our results, it has been shown that BAC modifies the activity of nAChRs located on dopaminergic neurons (McClure-Begley *et al.* 2014). Moreover, SAC (1 mg/kg, ip) alone did not affect the $\alpha_4\beta_2$nAChRs density in any of the brain areas studied. However, SAC was able to potentiate the increase of the $\alpha_4\beta_2$nAChRs density induced by NIC in the VTA, but not in the PFC and Acb. SAC and BAC may have had no effects on $\alpha_4\beta_2$nAChRs density in the PFC or Acb because NIC had no effect there either. The genetic approach revealed that the increased $\alpha_4\beta_2$nAChRs density induced by NIC in the VTA of WT mice is even greater in $GABA_{B1}$KO mice. Based on our results, we can assume that $GABA_B$ receptors are involved in the control of $\alpha_4\beta_2$nAChRs synthesis in VTA during the rewarding effects induced by NIC. In fact, it has been well established that the expression, activity and function of $\alpha_4\beta_2$nAChRs in the VTA is regulated by $GABA_B$ receptors (McClure-Begley *et al.* 2014; Pitman, Puil & Borgland 2014; Ngolab *et al.* 2015). Therefore, we confirm that an increase of $\alpha_4\beta_2$nAChRs density in the VTA would be responsible, at least in part, of NIC-induced rewarding effects, and $GABA_B$ receptors would modulate these alterations.

The present results support the hypothesis that $GABA_B$ receptors play a role in mediating the behavioral, neurochemical, molecular and biochemical alterations induced by the rewarding effects of NIC. Our study emphasizes the relevance of $GABA_B$ receptor activity in the dopaminergic mesocortical pathway in mediating these alterations. Finally, this work further supports a role for the GABAergic system, in particular $GABA_B$ receptors, in behaviors that contribute to the development of NIC addiction.

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Authors Contribution

VAP was responsible for the study concept and design, executed the experimental protocols, analyzed the data, interpreted the results and wrote the first draft of the manuscript. PVT performed brain's perfusion, edited the manuscript and prepared the figures. AAJ performed the genotyping, the protein determination and the immunohistochemistry experiments. HC performed the HPLC determinations. AGB assisted with the receptor-ligand binding assays. BB provided critical revision of the manuscript for important intellectual content. BGN wrote the research questions and was responsible for the design of the research, edition and revision of the manuscript. All authors critically reviewed the content and approved the final version for publication.

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