Neuropharmacology 81 (2014) 31–41

Contents lists available at ScienceDirect

Neuropharmacology

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

Involvement of $GABA_B$ receptors in biochemical alterations induced by anxiety-related responses to nicotine in mice: Genetic and pharmacological approaches

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ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form
1 January 2014
Accepted 20 January 2014

Keywords:
Nicotine
$GABA_B$ receptor
2-OH-saclofen
Anxiety
Catecholamine
c-Fos

ABSTRACT

Previous studies from our laboratory showed that anxiety-related responses induced by nicotine (NIC), measured by the elevated plus maze, were abolished by 2-OH-saclofen ($GABA_B$ receptor antagonist) (1 mg/kg; ip) or the lack of $GABA_B$ receptors ($GABA_{B1}$ knockout mice). Based on these behavioral data, the aims of the present study were: 1) to evaluate the possible neurochemical changes (dopamine, DA, serotonin, 5-HT, 3,4-dihydroxyphenylacetic acid, DOPAC, 5-hydroxyindoleacetic acid, 5-HIAA and noradrenaline, NA) and the c-Fos expression induced by the anxiolytic (0.05 mg/kg) or anxiogenic (0.8 mg/kg) doses of NIC in the dorsal raphe (DRN) and lateral septal (LSN) nucleus; 2) to study the possible involvement of $GABA_B$ receptors on the neurochemical alterations and c-Fos expression induced by NIC (0.05 and 0.8 mg/kg), using both pharmacological (2-OH-saclofen) and genetic (mice $GABA_{B1}$ knockout) approaches. The results revealed that in wild-type mice, NIC (0.05 mg/kg) increased the concentration of 5-HT and 5-HIAA ($p < 0.05$) in the DRN, and NIC (0.8 mg/kg) increased the levels of 5-HT ($p < 0.01$) and NA ($p < 0.05$) in the LSN. Additionally, 2-OH-saclofen pretreatment (1 mg/kg, ip) or the lack of $GABA_B$ receptors abolished these neurochemical changes induced by NIC ($p < 0.01$, $p < 0.05$, respectively). On the other hand, NIC 0.05 and 0.8 mg/kg increased ($p < 0.01$) the c-Fos expression in the DRN and LSN respectively, in wild-type mice. In addition, 2-OH-saclofen pretreatment (1 mg/kg, ip) or the lack of $GABA_B$ receptors prevented the c-Fos alterations induced by NIC ($p < 0.01$). In summary, both approaches show that $GABA_B$ receptors would participate in the modulation of anxiolytic- and anxiogenic-like responses induced by NIC, suggesting the potential therapeutic target of these receptors for the tobacco addiction treatment.

1. Introduction

Human and animal studies have revealed that nicotine modifies anxiety-like behavior, which could participate in its addictive properties. Smokers report that tobacco consumption decreases anxiety (Ikard et al., 1969; Pomerleau, 1986; Kassel and Unrod, 2000), although other studies have reported that nicotine can also increase levels of anxiety (Newhouse et al., 1990; Foulds et al., 1997; Parrott and Garnham, 1998; Netter et al., 1998). In addition, several reports have shown that nicotine induces anxiogenic- or anxiolytic-like responses in rodents depending on the dose, the route of administration, the genetic strain and the baseline anxiety level (Cheeta et al., 2000a; File et al., 2000; Picciotto et al., 2002; Balerio et al., 2005). In this sense, it is well known that low doses of nicotine were anxiolytic, intermediate doses had no effect, and high doses were anxiogenic, in the elevated plus maze test (Biala et al., 2009; Biala and Kruk, 2009; Plaza-Zabala et al., 2010; Piri et al., 2012; Braun et al., 2011; Zarrindast et al., 2012, 2013). Accordingly, we previously found that nicotine 0.05 mg/kg elicited an anxiolytic-like response, whereas nicotine 0.8 mg/kg induced an anxiogenic-like effect in mice by the elevated plus maze test (Balerio et al., 2005, 2006; Varani and Balerio, 2012; Varani et al., 2012).

Abbreviations: NIC, nicotine; SAL, saline; GABA, gamma-aminobutyric acid; 2-OH-saclofen, 2-hydroxy-saclofen; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid; NA, norepinephrine; DRN, dorsal raphe nucleus; LSN, lateral septal nucleus; nAChR, nicotinic acetylcholine receptor; HPLC, high-performance liquid chromatography.

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http://dx.doi.org/10.1016/j.neuropharm.2014.01.030

The literature about the effects of nicotine on anxiety-related responses in humans and rodents is complex and difficult to interpret. It is likely that the large numbers of nicotinic acetylcholine receptor (nAChR) subtypes and neurotransmitter systems that can be regulated by nAChRs, are responsible for this complex situation. The neurobiological mechanisms underlying the effects of nicotine on anxiety remain to be clarified (Picciotto et al., 2002). Nicotine can induce the release of stimulatory (glutamate), inhibitory (gamma-aminobutyric acid, GABA) and modulatory (dopamine, DA; norepinephrine, NA and serotonin, 5-HT) neurotransmitters in different brain regions (Benowitz, 2008), thus the anxiety-related behaviors induced by nicotine administration could be the result of the interaction of these neurotransmitter systems. Local injection studies have been used to identify the sites of action of nicotine in behavioral models of anxiety. The dorsal raphe nucleus (DRN) has been identified as an important neuroanatomical substrate mediating nicotine's anxiolytic-like effect. Low doses of nicotine (2.5–10 ng) administered directly into the DRN induced an anxiolytic-like effect in mice (Cheeta et al., 2001a,b). In contrast to the pattern observed in the DRN with low doses of nicotine (anxiolytic-like effect), when injected into the lateral septum nucleus (LSN), nicotine at high doses (1 and 4 μg) induced anxiogenic-like effect in mice (Ouagazzal et al., 1999; File et al., 2000). Available evidence clearly showed that the noradrenergic and serotonergic systems could be implicated in mediating the anxiolytic- and anxiogenic-like effects of nicotine (Cheeta et al., 2000a,b; 2001a; Seth et al., 2002). However, other neurobiological substrates could modulate the nicotine anxiety-related responses (File et al., 2000). In this regard, previous results from our laboratory showed the involvement of the opioid and endocannabinoid systems in anxiety-related responses induced by nicotine (Balerio et al., 2005, 2006). In the present study we focused on the interaction between anxiety-related responses to nicotine and the GABAergic system. $GABA_B$ receptors have been reported to be involved in the modulation of anxiety (Dalvi and Rodgers, 1996; Mombereau et al., 2004; Gassmann and Bettler, 2007; Partyka et al., 2007; Jacobson and Cryan, 2008). Therefore, $GABA_B$ receptor compounds might provide a useful strategy in the treatment of nicotine anxiety-related responses. In this context, we have observed that 2-OH-saclofen, a $GABA_B$ receptor antagonist, abolished the anxiety-related responses induced by nicotine in the elevated plus maze test in mice (Varani and Balerio, 2012). In addition, we recently showed that the anxiolytic- but not the anxiogenic-like effect induced by nicotine was abolished in $GABA_{B1}$ knockout mice in the elevated plus maze test (Varani et al., 2012). Based on these behavioral data, the first aim of the present study was to evaluate the possible neurochemical changes (DA, NA and 5-HT) and the c-Fos expression pattern induced by the anxiolytic- and anxiogenic-like effect of nicotine in the DRN and LSN of mice. Finally, we explored the involvement of $GABA_B$ receptors in possible alterations (neurochemical changes and c-Fos expression) induced by anxiety-related responses of nicotine, using both pharmacological (2-OH-saclofen) and genetic ($GABA_{B1}$knockout mice) approaches.

2. Materials and methods

2.1. Animals

2.1.1. Pharmacological approach: in Swiss Webster mice

We used male Swiss Webster mice obtained from Bioterio Central (Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina) weighing 22–26 g and housed five per cage. All experiments were performed with the investigators being blind to treatment conditions.

2.1.2. Genetic approach: in $GABA_{B1}$ knockout mice and their wild-type littermates

Mice lacking the $GABA_{B1}$ subunit of the $GABA_B$ receptor were generated in the laboratory of Dr Bernard Bettler, Department of Physiology, University of Basel, Switzerland (Schuler et al., 2001). We have developed our own $GABA_{B1}$ knockout mice colony in the Institute of Pharmacological Research (CONICET) located at the Faculty of Pharmacy and Biochemistry. The $GABA_{B1}$ knockout mice and their wild-type littermates are obtained by intercrossing heterozygous animals. Fingertip biopsies (performed for identification purposes) were used to isolate DNA for animal genotyping by PCR as described (Schuler et al., 2001). Mice weighing 22–26 g were housed five per cage. All experiments were performed with the investigators being blind to genotype and treatment conditions.

2.1.3. Care and handling conditions

The animals of both approaches were acclimatized to the laboratory conditions according to local regulation (SENASA, 2002) (12-h light:12-h dark cycle, 21 ± 0.5 °C room temperature, 65 ± 10% humidity). Mice were handled and habituated to the injections for three 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).

2.2. Drugs

(−)-Nicotine hydrogen tartrate salt ([−]-1- methyl-2-[3-pyridil]pyrrolidine) (Sigma Chemical Co., Buenos Aires, Argentina) and 2-hydroxisaclofen (2-OH-saclofen) (Sigma Chemical Co., Buenos Aires, Argentina) were used in this study. Nicotine was dissolved in isotonic (NaCl 0.9%) saline solution, and 2-OH-saclofen was dissolved in isotonic (5%) glucose solution immediately before use. Nicotine doses used were calculated as nicotine hydrogen tartrate salt; they were administered subcutaneously (s.c.). The doses of nicotine (0.05 and 0.8 mg/kg, s.c.) were chosen based on previous studies from our laboratory (Balerio et al., 2005, 2006). All drugs were administered in a volume of 10 ml/kg. Different groups of drug-naïve animals were used for each experiment.

For the pharmacological approach, 2-OH-saclofen (1 mg/kg) or glucose 5% were administered intraperitoneally (i.p.) 10 min before nicotine (0.05 and 0.8 mg/kg, s.c.) or saline injection in Swiss Webster mice. The 2-OH-saclofen dose (1 mg/kg, i.p.) was selected taking into account previous behavioral results obtained from our laboratory (Varani and Balerio, 2012).

For the genetic approach, $GABA_{B1}$ knockout mice and their wild-type littermates were injected subcutaneously with nicotine (0.05 and 0.8 mg/kg) or saline.

2.3. HPLC experiments

HPLC-coupled electrochemical detection (Heikkila et al., 1984) of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), 5-hydroxyindolacetic acid (5-HIAA) and norepinephrine (NA) was achieved using a Varian 5000 liquid chromatograph coupled to an electrochemical detector (BAS LC-4C). Fifteen minutes after nicotine or saline injection, brains (n = 4–5 per experimental group) were quickly removed and place in dry ice. When partially frozen, the DRN (extending between bregma −4.84–−4.24 mm, lateral ± 0.5 mm, and ventrally to the aqueduct) and the LSN (extending between bregma 0.14–1.10 mm, lateral ± 1 mm, and ventrally to the corpus callosum) 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, PICB85.24 ml/l, EDTA0.99 mM and 6% methanol, while the mobile phase for NA contained NaH_2PO_4–H_2O 0.076 M, PICB8 3.5 ml/l, EDTA 3.35 mM. 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.

2.4. c-Fos experiments

2.4.1. Tissue preparation

Fifty min after nicotine or saline injection, mice (n = 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 saline phosphate buffer, pH 7.4), followed by a cold solution of 4% paraformaldehyde delivered with a peristaltic pump. Brains were removed and postfixed for 2 h in the same fixative, and cryoprotected overnight in a 30% sucrose solution. Coronal frozen sections were made at 30 μm on a freezing microtome. They were collected in three serial groups of free-floating sections and stored at 4 °C.

2.4.2. c-Fos immunohistochemistry

The procedure was adapted from previously described protocols (Bester et al., 2001). All reactions were performed on floating sections agitated on a shaker. Sections from different experimental groups were processed in parallel to minimize the variations in immunohistochemical labeling. Free-floating sections were rinsed in 0.1 M phosphate buffered saline with 0.15% Triton X-100 (PBS-T; pH 7.4) and then

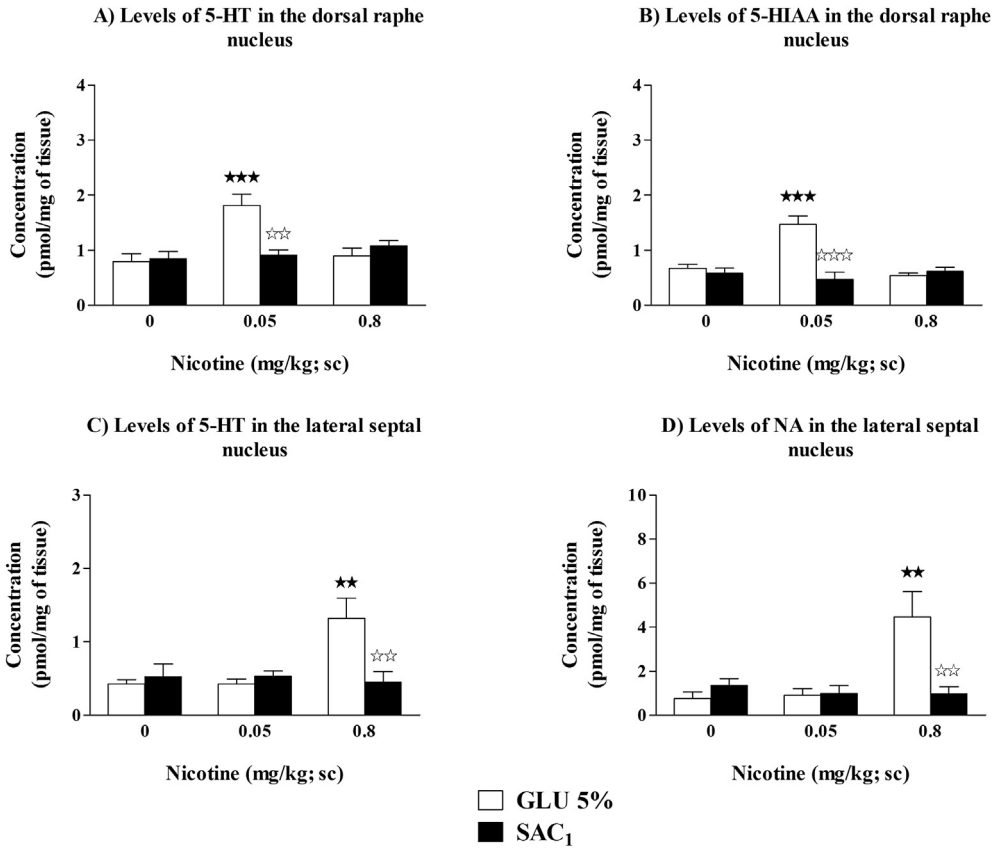


Fig. 1. 2-OH-saclofen (1 mg/kg, i.p.) abolished the increased levels of 5-HT (A), 5-HIAA (B) and 5-HT (C) and NA (D) induced by the anxiolytic (0.05 mg/kg, s.c.) and anxiogenic (0.8 mg/kg, s.c.) doses of nicotine in the dorsal raphe and lateral septal nucleus, respectively, in Swiss Webster mice. Data are expressed as mean ± SEM ($n = 4$–5 mice per group) of neurotransmitters levels, in glucose 5% (GLU 5%) (white bars) and 2-OH-saclofen (SAC_1) (black bars) pretreated mice. ★★ $P < 0.01$; ★★★ $P < 0.001$ when compared to respective vehicle group; ☆☆ $P < 0.01$; ☆☆☆ $P < 0.001$ when compared to GLU 5% + nicotine group (Tukey's *post hoc* test). 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid; NA, noradrenaline.

incubated with 3% hydrogen peroxide in PBS-T for a period of 30 min to remove endogenous peroxidase activity. After rinsing again in PBS-T, sections were incubated for 30 min in 2% normal goat serum in PBS-T. Then, sections were incubated overnight in a rabbit polyclonal antibody anti-c-Fos (Santa Cruz Biotechnology, USA) (1:1000 in PBS 0.1 M, thimerosal 0.02%, normal goat serum 1%) at 4 °C. Sections were then rinsed and incubated for 2 h in a goat anti-rabbit biotinylated antibody (Vector Laboratories, USA) (1:250 in PBS-T). After being rinsed, sections were incubated for 2 h in avidin-biotinylated horseradish peroxidase complex (1:125, ABC kit, Vector Laboratories). After successive washes in PBS-T and Tris buffer (0.25 M; pH 7.4), the antibody–antigen complex was developed with 0.05% m/v of 3,3′-diaminobenzidine (Sigma, USA) and 0.015% v/v of H_2O_2 in 20 ml Tris buffer 0.1 M. Sections were mounted on gelatin-coated slides, dehydrated and coverslipped. Controls for the specificity of primary antisera used were carried out by substitution of primary antibody with PBS (Delfino et al., 2004).

2.4.3. Data quantification

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 200x 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 by an observer blind to treatment conditions. These counts were averaged into a single score for each region of each animal and finally the group mean ± SEM was calculated. Fos-positive nuclei were quantified in the following brain regions, identified according to the anatomic atlas of Paxinos and Franklin (2004): DRN and LSN.

2.5. Statistical analysis

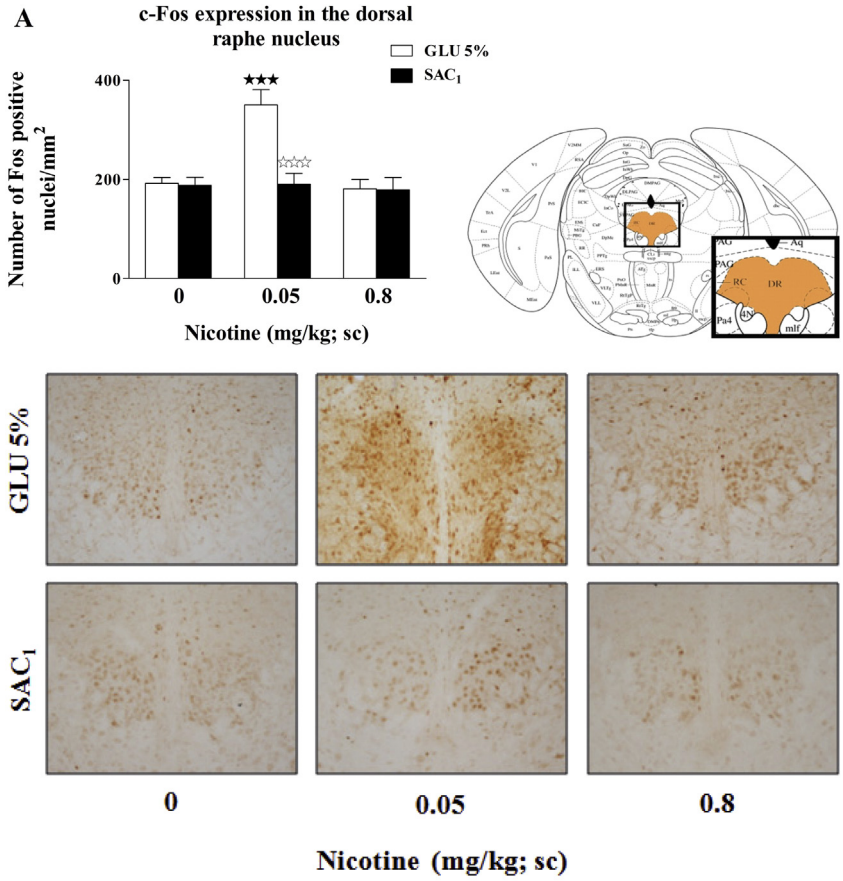
For the pharmacological approach, the results were analyzed using two-way ANOVA, with treatment (saline or nicotine) and the GABAergic ligand (vehicle or 2-OH-saclofen) administration as between-subjects factors of variation. When a significant interaction between these two factors was observed, the 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.

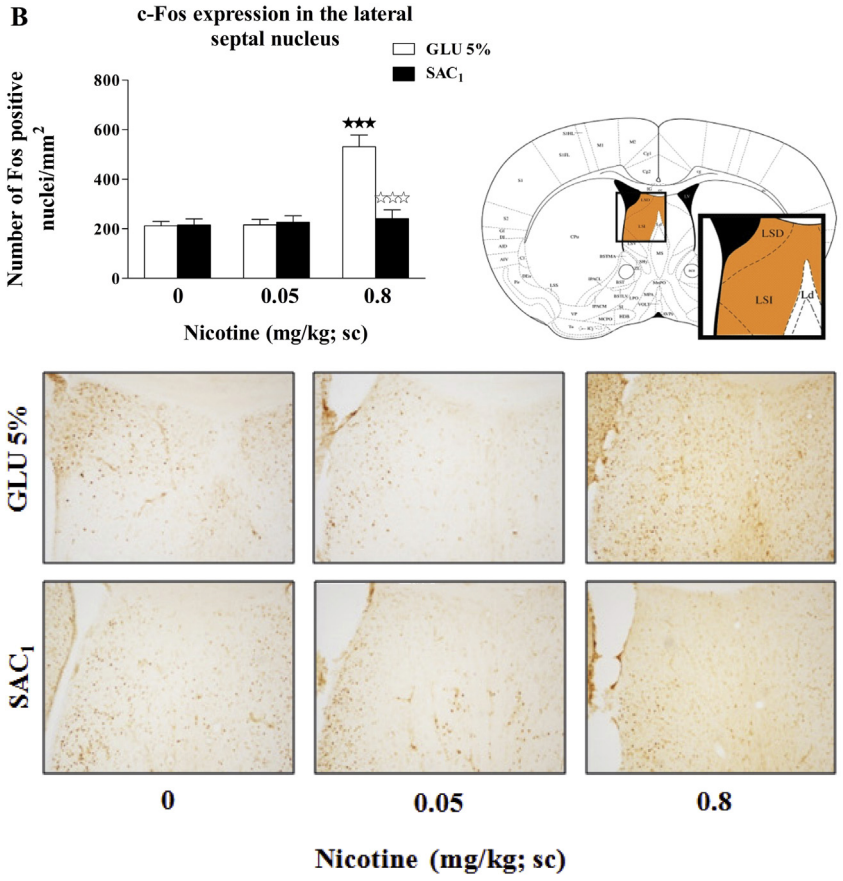
3. Results

3.1. Pharmacological approach: in Swiss Webster mice

3.1.1. Effects of 2-OH-saclofen on anxiety-related responses induced by nicotine: neurochemical changes

3.1.1.1. Dorsal raphe nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) in the 5-HT ($F_{(2,18)} = 8.020$; $p < 0.01$) and 5-HIAA ($F_{(2,18)} = 8.903$; $p < 0.01$) concentrations of the DRN, and a significant effect of 2-OH-saclofen treatment (glucose 5% or 2-OH-saclofen) only in the 5-HIAA ($F_{(1,18)} = 16.455$; $p < 0.01$) concentrations of the DRN. Significant interaction between the two factors was observed in the 5-HT ($F_{(2,18)} = 9.214$; $p < 0.01$) and 5-HIAA ($F_{(2,18)} = 16.215$; $p < 0.001$) concentrations, while there were not significant interactions for the rest of neurotransmitters and metabolites. Tukey's *post hoc* test revealed: Nicotine at the lowest dose tested (0.05 mg/kg) significantly increased the levels of 5-HT ($p < 0.001$) (Fig. 1A) and 5-HIAA ($p < 0.001$) (Fig. 1B) compared to control group, in the DRN. 2-OH-saclofen (1 mg/kg) abolished the increased levels of 5-HT ($p < 0.01$) and 5-HIAA ($p < 0.001$) induced by nicotine (0.05 mg/kg) in DRN (Fig. 1A, B, respectively). Nicotine at the highest dose tested


A
c-Fos expression in the dorsal raphe nucleus
GLU 5%
SAC1
Number of Fos positive nuclei/mm2
400
200
0
★★★
☆☆☆
0
0.05
0.8
Nicotine (mg/kg; sc)
AQ
Aq
PAG
RC
DR
Pa4
dtn
mlf
GLU 5%
SAC1
0
0.05
0.8
Nicotine (mg/kg; sc)


B
c-Fos expression in the lateral septal nucleus
GLU 5%
SAC1
Number of Fos positive nuclei/mm2
800
600
400
200
0
★★★
☆☆☆
0
0.05
0.8
Nicotine (mg/kg; sc)
LSD
LSI
Ld
GLU 5%
SAC1
0
0.05
0.8
Nicotine (mg/kg; sc)

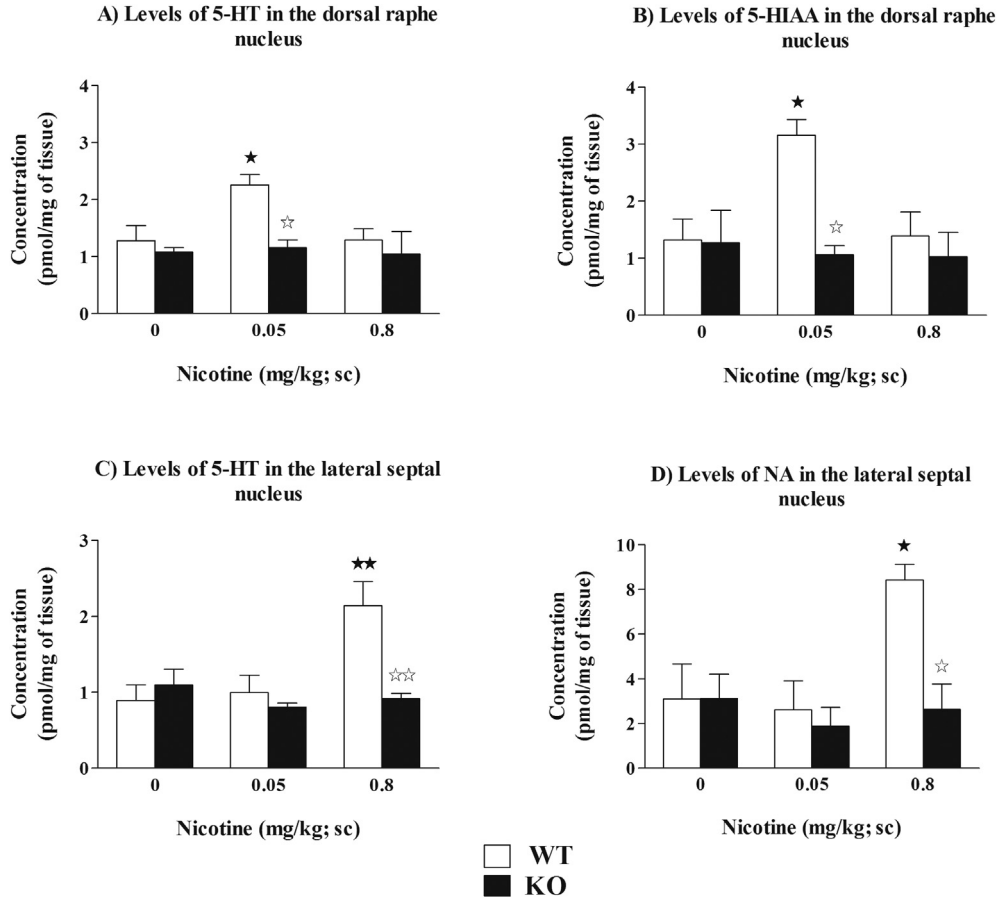


Fig. 3. The anxiolytic (0.05 mg/kg, s.c.) and anxiogenic (0.8 mg/kg, s.c.) doses of nicotine increased the levels of 5-HT (A), 5-HIAA (B) and 5-HT (C), NA (D) in the dorsal raphe and lateral septal nucleus, respectively; in wild-type, but not in $GABA_{B1}$ knockout mice. Data are expressed as mean ± SEM ($n = 4$–5) of neurotransmitters levels, in wild-type (WT) (white bars) and $GABA_{B1}$ knockout (KO) (black bars) mice. ★ $P < 0.05$; ★★ $P < 0.01$ when compared to vehicle group of the same genotype. ☆ $P < 0.05$; ☆☆ $P < 0.01$ for between-genotype comparisons (Tukey's *post hoc* test). 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid; NA, noradrenaline.

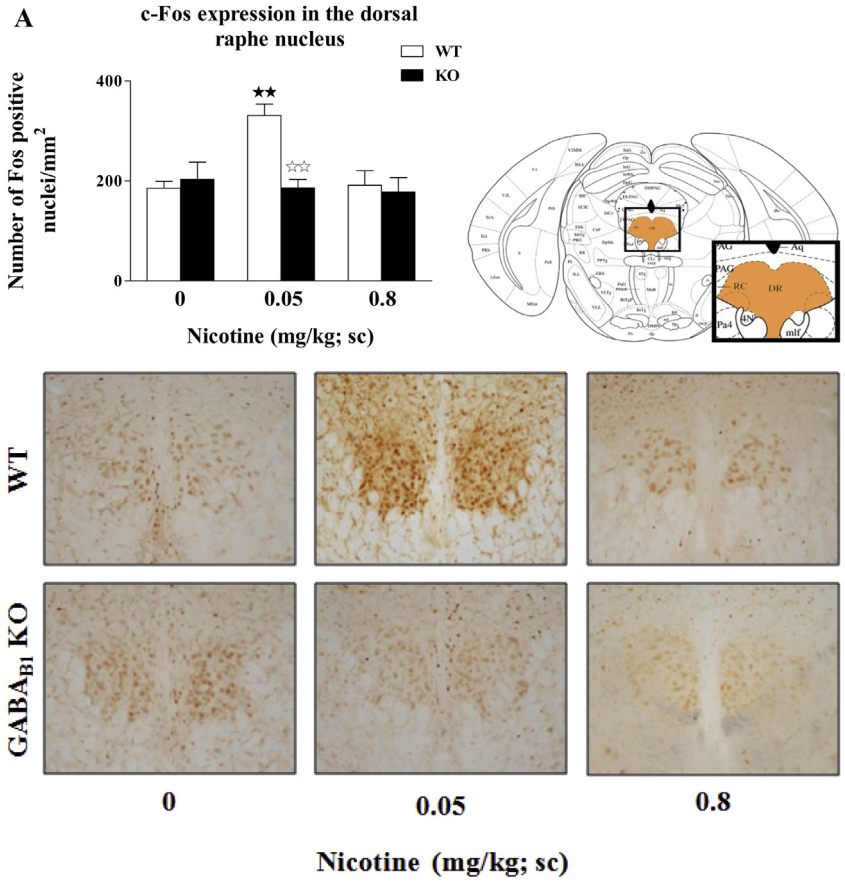
(0.8 mg/kg) was unable to modify the levels of 5-HT and its metabolite (5-HIAA) in the DRN (Fig. 1A, B, respectively). 2-OH-saclofen did not modify *per se* the levels of the neurotransmitters and their metabolites measured in the DRN. The levels of DA, DOPAC and NA were not modified in any of the experimental groups in the DRN.

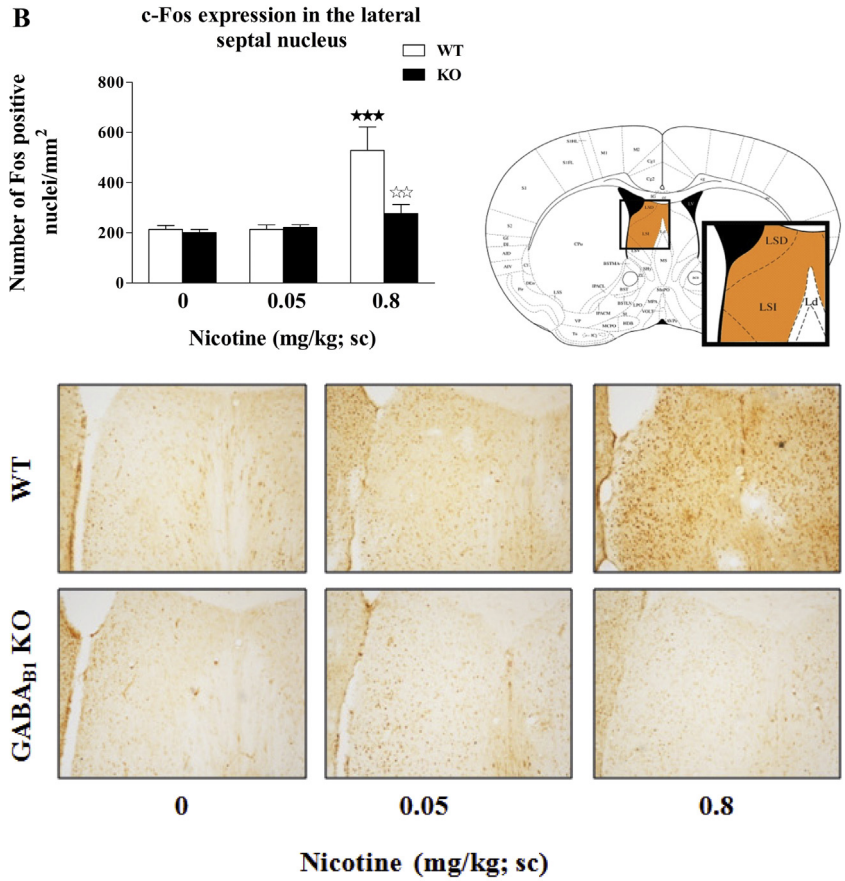
3.1.1.2. Lateral septal nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) only in the 5-HT ($F_{(2,19)} = 5.127$; $p < 0.05$) and NA ($F_{(2,19)} = 6.695$; $p < 0.01$) concentrations of the LSN, and a significant effect of 2-OH-saclofen treatment (glucose 5% or 2-OH-saclofen) only in the NA ($F_{(1,19)} = 4.375$; $p < 0.05$) concentrations of the LSN. Significant interaction between the two factors was observed in the 5-HT ($F_{(2,19)} = 7.254$; $p < 0.01$) and NA ($F_{(2,19)} = 8.257$; $p < 0.01$) concentrations, while there were not significant interactions for the rest of neurotransmitters and metabolites. Tukey's *post hoc* test revealed: Nicotine (0.8 mg/kg) significantly increased the levels of 5-HT ($p < 0.01$) (Fig. 1C) and NA ($p < 0.01$) (Fig. 1D) compared to control group, in the LSN. 2-OH-saclofen (1 mg/kg) abolished the increased levels of 5-HT ($p < 0.01$) and NA ($p < 0.01$) induced by nicotine (0.8 mg/kg) in the LSN (Fig. 1C, D, respectively). Nicotine (0.05 mg/kg) was unable to modify the levels of 5-HT and NA in the LSN (Fig. 1C, D, respectively). 2-OH-saclofen did not modify *per se* the levels of the neurotransmitters measured in the LSN. The levels of DA, DOPAC and 5-HIAA were not modified in any of the experimental groups in the LSN.

3.1.2. Effects of 2-OH-saclofen on anxiety-related responses induced by nicotine: c-Fos expression

3.1.2.1. Dorsal raphe nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) ($F_{(2,30)} = 10.340$; $p < 0.001$), 2-OH-saclofen treatment (glucose 5% or 2-OH-saclofen) ($F_{(1,30)} = 9.650$; $p < 0.01$) and an interaction between the two factors ($F_{(2,30)} = 8.632$; $p < 0.001$) in the number of Fos-positive nuclei of the DRN. Tukey's *post hoc* test revealed: Nicotine (0.05 mg/kg) significantly increased c-Fos expression ($p < 0.001$) compared to control group, in the DRN (Fig. 2A). This effect was abolished by 2-OH-saclofen (1 mg/kg) ($p < 0.001$) (Fig. 2A). Nicotine (0.8 mg/kg) was unable to modify c-Fos

Fig. 2. 2-OH-saclofen (1 mg/kg, i.p.) abolished the increased c-Fos expression induced by the anxiolytic (0.05 mg/kg, s.c.) and anxiogenic (0.8 mg/kg, s.c.) doses of nicotine in the dorsal raphe (A) (bregma 0.74 mm) and lateral septal nucleus (B) (bregma −4.36 mm), respectively, in Swiss Webster mice. Data are expressed as mean ± SEM ($n = 6$ mice per group) of Fos positive nuclei/mm^2, in glucose 5% (GLU 5%) (white bars) and 2-OH-saclofen (SAC_1) (black bars) pretreated mice. ★★★ $P < 0.001$ when compared to respective vehicle group; ☆☆☆ $P < 0.001$ when compared to GLU 5% + nicotine group (Tukey's *post hoc* test).


A
c-Fos expression in the dorsal raphe nucleus
WT
KO
Number of Fos positive nuclei/mm2
400
200
0
★★
☆☆
0
0.05
0.8
Nicotine (mg/kg; sc)
AG
Aq
PAG
RC
DR
Pa4
mlf
WT
GABAB1 KO
0
0.05
0.8
Nicotine (mg/kg; sc)


B
c-Fos expression in the lateral septal nucleus
WT
KO
Number of Fos positive nuclei/mm2
800
600
400
200
0
★★★
☆☆
0
0.05
0.8
Nicotine (mg/kg; sc)
LSD
LSI
Ld
WT
GABAB1 KO
0
0.05
0.8
Nicotine (mg/kg; sc)

expression in the DRN (Fig. 2A). 2-OH-saclofen did not modify *per se* c-Fos expression in the DRN.

3.1.2.2. Lateral septal nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) ($F_{(2,30)} = 20.461$; $p < 0.001$), 2-OH-saclofen treatment (glucose 5% or 2-OH-saclofen) ($F_{(1,30)} = 13.743$; $p < 0.001$) and an interaction between the two factors ($F_{(2,30)} = 15.804$; $p < 0.001$) in the number of Fos-positive nuclei of the DRN. Tukey's *post hoc* test revealed: Nicotine (0.8 mg/kg) significantly increased c-Fos expression ($p < 0.001$) compared to control group, in the LSN (Fig. 2B). This effect was abolished by 2-OH-saclofen (1 mg/kg) ($p < 0.001$) (Fig. 2B). Nicotine (0.05 mg/kg) was unable to modify c-Fos expression in the LSN (Fig. 2B). 2-OH-saclofen did not modify *per se* c-Fos expression in the LSN.

3.2. Genetic approach: in $GABA_{B1}$ knockout mice and their wild-type littermates

3.2.1. Anxiolytic- and anxiogenic-like responses induced by nicotine: Neurochemical changes

3.2.1.1. Dorsal raphe nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) only in the 5-HT ($F_{(2,21)} = 3.487$; $p < 0.05$) concentrations of the DRN, and a significant effect of genotype (wild-type or $GABA_{B1}$ knockout mice) only in the 5-HT ($F_{(1,21)} = 7.324$; $p < 0.05$) and 5-HIAA ($F_{(1,21)} = 6.904$; $p < 0.05$) concentrations of the DRN. Significant interaction between the two factors was observed in the 5-HIAA ($F_{(2,21)} = 3.998$; $p < 0.05$) concentrations, while there were not significant interactions for the rest of neurotransmitters and metabolites. Tukey's *post hoc* test revealed: Nicotine (0.05 mg/kg) significantly increased the levels of 5-HT ($p < 0.05$) and 5-HIAA ($p < 0.05$) in the DRN of wild-type, but not of $GABA_{B1}$ knockout mice (Fig. 3A, B, respectively). Nicotine (0.8 mg/kg) was unable to modify the levels of the neurotransmitters and their metabolites in the DRN of both genotypes (Fig. 3A, B). Significant differences between genotypes were only observed at the dose of 0.05 mg/kg, for both the levels of 5-HT ($p < 0.05$) and 5-HIAA ($p < 0.05$) in the DRN (Fig. 3A, B, respectively). No significant differences were observed between genotypes in saline-treated mice (Fig. 3A,B). The levels of DA, DOPAC and NA were not modified in any of the experimental groups in the DRN.

3.2.1.2. Lateral septal nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) in the 5-HT ($F_{(2,21)} = 4.654$; $p < 0.05$) and NA ($F_{(2,19)} = 4.282$; $p < 0.05$) concentrations of the LSN, and a significant effect of genotype (wild-type or $GABA_{B1}$ knockout mice) only in the 5-HT ($F_{(1,21)} = 4.969$; $p < 0.05$) and NA ($F_{(1,21)} = 5.194$; $p < 0.05$) concentrations of the DRN. Significant interaction between the two factors was observed in the 5-HT ($F_{(2,21)} = 5.508$; $p < 0.01$) and NA ($F_{(2,19)} = 3.597$; $p < 0.05$) concentrations, while there were not significant interactions for the rest of neurotransmitters and metabolites. Tukey's *post hoc* test revealed: Nicotine (0.8 mg/kg) significantly increased the levels of 5-HT ($p < 0.01$) and NA ($p < 0.05$) in the LSN of wild-type, but not of $GABA_{B1}$ knockout mice (Fig. 3C, D, respectively). Nicotine (0.05 mg/kg) was unable to modify the levels of 5-HT and NA in the LSN of both genotypes (Fig. 3C, D, respectively). Significant differences between genotypes were only observed at the dose of 0.8 mg/kg, for the levels of 5-HT ($p < 0.01$) and NA ($p < 0.05$) in the LSN (Fig. 3C, D, respectively). No significant differences were observed between genotypes in saline-treated mice (Fig. 3C, D). The levels of DA, DOPAC and 5-HIAA were not modified in any of the experimental groups in the LSN.

3.2.2. Anxiolytic- and anxiogenic-like responses induced by nicotine: c-Fos expression

3.2.2.1. Dorsal raphe nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) ($F_{(2,30)} = 4.971$; $p < 0.05$), genotype (wild-type or $GABA_{B1}$ knockout mice) ($F_{(1,30)} = 4.988$; $p < 0.05$) and an interaction between the two factors ($F_{(2,30)} = 5.718$; $p < 0.01$) in the number of Fos-positive nuclei of the DRN. Tukey's *post hoc* test revealed: Nicotine (0.05 mg/kg) significantly increased c-Fos expression ($p < 0.01$) in the DRN of wild-type, but not in $GABA_{B1}$ knockout mice (Fig. 4A). Nicotine (0.8 mg/kg) was unable to modify c-Fos expression in the DRN of both genotypes (Fig. 4A). Significant differences between genotypes were only observed at the dose of 0.05 mg/kg, for c-Fos expression ($p < 0.01$) in the DRN (Fig. 4A). No significant differences were observed between genotypes in saline-treated mice (Fig. 4A).

3.2.2.2. Lateral septal nucleus. Two-way ANOVA revealed a significant effect of nicotine treatment (saline or nicotine) ($F_{(2,30)} = 13.313$; $p < 0.001$), genotype (wild-type or $GABA_{B1}$ knockout mice) ($F_{(1,30)} = 6.117$; $p < 0.05$) and an interaction between the two factors ($F_{(2,30)} = 5.758$; $p < 0.01$) in the number of Fos-positive nuclei of the DRN. Tukey's *post hoc* test revealed: Nicotine (0.8 mg/kg) significantly increased c-Fos expression ($p < 0.001$) in the LSN of wild-type, but not in $GABA_{B1}$ knockout mice (Fig. 4B). Nicotine (0.05 mg/kg) was unable to modify c-Fos expression in the LSN of both genotypes (Fig. 4B). Significant differences between genotypes were only observed at the dose of 0.8 mg/kg, for c-Fos expression ($p < 0.01$) in the LSN (Fig. 4B). No significant differences were observed between genotypes in saline-treated mice (Fig. 4B).

4. Discussion

In the present study the pharmacological approach revealed that 2-OH-saclofen, $GABA_B$ receptor antagonist, blocked the increased levels of 5-HT, 5-HIAA and the neuronal activity (c-Fos expression) induced by a low dose of nicotine (0.05 mg/kg) in the DRN. In addition, 2-OH-saclofen abolished the increased levels of NA, 5-HT and the neuronal activity induced by a high dose of nicotine (0.8 mg/kg) in the LSN. Likewise, the genetic approach showed that genetic deletion of the $GABA_{B1}$ subunit blocked the increased levels of 5-HT, 5-HIAA and the neuronal activity induced by nicotine (0.05 mg/kg) in the DRN. Moreover, the increased levels of NA, 5-HT and the neuronal activity induced by nicotine (0.8 mg/kg) were abolished in the LSN of $GABA_{B1}$ knockout mice.

Previous results from our laboratory provide clear evidence for the involvement of $GABA_B$ receptors in the effects induced by nicotine on anxiety-related responses. Indeed, we have found that nicotine-induced anxiolytic- and anxiogenic-like effects are blocked in a dose-dependent manner by the $GABA_B$ receptor antagonist 2-OH-saclofen (Varani and Balerio, 2012). Similarly, we recently observed that the anxiolytic- but not the anxiogenic-like effect induced by nicotine was abolished in $GABA_{B1}$ knockout

Fig. 4. The anxiolytic (0.05 mg/kg, s.c.) and anxiogenic (0.8 mg/kg, s.c.) doses of nicotine increased the c-Fos expression in the dorsal raphe (A) (bregma −4.36 mm) and lateral septal nucleus (B) (bregma 0.74 mm), respectively; in wild-type, but not in $GABA_{B1}$ knockout mice. Data are expressed as mean ± SEM ($n = 6$) of Fos positive nuclei/mm^2, in wild-type (WT) (white bars) and $GABA_{B1}$ knockout (KO) (black bars) mice. ★★ $P < 0.01$; ★★★ $P < 0.001$ when compared to vehicle group of the same genotype. ☆☆ $P < 0.01$ for between-genotype comparisons (Tukey's *post hoc* test).

mice (Varani et al., 2012). Together with these previous findings, the data of this study imply a direct involvement of $GABA_B$ receptors in the neurochemical alterations induced by the anxiety-related responses to nicotine.

Little is known about the different brain regions that could mediate the anxiolytic- and anxiogenic-like effects induced by nicotine. It has been reported that local administration of nicotine into the DRN and the LSN induced anxiolytic- (File et al., 1999) and anxiogenic-like (Ouagazzal et al., 1999) responses, respectively. It is well established that nAChRs within the brain are predominantly situated pre-synaptically, and their activation modulates the release of acetylcholine and other neurotransmitters such as, for example, NA, 5-HT, DA, GABA, and glutamate (Hurst et al., 2013). The regulation of neurotransmitters release, acting on various post-synaptic receptors, is the usual mechanism by which nicotine modulates behavior (Benowitz, 2008).

Several reports have demonstrated that the serotonergic system play a role in many of the behavioral effects produced by nicotine (Damaj et al., 1994; Riekkinen et al., 1994; Suzuki et al., 1997; Seth et al., 2002). Nicotine (0.05 mg/kg) increased the levels of 5-HT and 5-HIAA but not NA, DA and DOPAC, in the DRN of wild-type mice. Serotonergic neurons located in the DRN provide the majority of serotonergic innervation to the forebrain and control the affective state (Steinbusch et al., 1980). The DRN has been identified as an important neuroanatomical substrate mediating nicotine's anxiolytic effect. Indeed, low doses of nicotine (2.5–10 ng) administered directly into the DRN induced anxiolytic-like effects (Cheeta et al., 2001a). Nicotine stimulates the release of 5-HT in the DRN (Garduño et al., 2012; Hernandez-Lopez et al., 2013) by acting on somatodendritic and presynaptic nAChRs (Chang et al., 2011). In this regard, the majority of serotonergic neurons of the DRN increase their action potential firing in response to nicotine, and this leads to an increase of 5-HT release within the DRN and projecting brain areas (Mihailescu et al., 2001, 2002; Guzmán-Marín et al., 2001; Garduño et al., 2012). It is known that there are many interconnections between the different raphe nuclei (Frazer and Hensler, 1990) as well as local collateral axons of serotonergic neurons which travel for some distance within the DRN (Li et al., 2001). Therefore, these evidences would explain the fact that in the present study nicotine increased the 5-HT and 5-HIAA levels within the DRN. Additionally, we might suppose that nicotine (0.05 mg/kg) administration would induce an increase of 5-HT release in the DRN since its metabolite (5-HIAA) was also increased, suggesting that increased 5-HT levels in the DRN could be responsible in mediating the anxiolytic-like effect induced by nicotine (Seth et al., 2002). In addition, Cheeta et al. (2001a) have shown that the anxiolytic-like effect of nicotine was completely antagonized by the administration of the 5-HT_{1A} receptor antagonist WAY100635 into the DRN. These results suggest that nicotine indirectly stimulates somatodendritic 5-HT_{1A} autoreceptors, leading to a reduction in 5-HT neuronal firing and a subsequent decrease in 5-HT release in terminal regions of the limbic system (Engberg et al., 2000). Although Cheeta et al. (2001a) have also suggested that the DRN could be involved in the anxiogenic-like effect, in our present study the high dose of nicotine (0.8 mg/kg) was not able to induce neurochemical changes in the DRN. Based on the behavioral results obtained in our previous studies (Varani and Balerio, 2012; Varani et al., 2012), we have explored herein the effect of 2-OH-saclofen (1 mg/kg) or the lack of $GABA_B$ receptor on the brain neurotransmitters' content of mice treated with nicotine. Interestingly, in the present study both approaches showed that the increased levels of 5-HT and 5-HIAA induced by nicotine (0.05 mg/kg) in the DRN were re-established by 2-OH-saclofen or by the lack of $GABA_B$ receptor. Although the DRN is well known to be major source of 5-HT in the brain, it has been reported that the DRN also contains afferent neurons with GABA, DA, NA, substance P, and acetylcholine (Okada et al., 2011). We suggest that the stimulation of nAChRs located pre-synaptically on GABAergic neurons (Mihailescu et al., 2002) which are directly connected to GABAergic interneurons in the DRN (Harsing, 2006; Hernandez-Lopez et al., 2013), would induce an increase of 5-HT levels with the resulting anxiolytic-like effect. However, in 2-OH-saclofen pretreated mice or $GABA_{B1}$ knockout mice the released GABA induced by nicotine should not be able to exert its inhibitory effect because of the blockade or the lack of $GABA_B$ receptors on the GABAergic interneurons, respectively. Thus, the GABA released from GABAergic interneurons would inhibit the serotonergic neurons, leading to the re-establishment of 5-HT levels, with the resulting abolishment of the nicotine's anxiolytic-like effect (Fig. 5A).

Regarding the anxiogenic dose of nicotine (0.8 mg/kg), our present neurochemical data revealed that NA, 5-HT but not 5-HIAA, DA and DOPAC levels were increased in the LSN of wild-type mice. Accordingly, it was observed that a subcutaneous injection of nicotine increased NA concentration in the LSN of rodents (Shearman et al., 2008). Several studies have also suggested that, after nicotine administration, an increase of NA and 5-HT levels would elicit an anxiogenic-like effect in the LSN (Cheeta et al., 2000a; Picciotto et al., 2002; Seth et al., 2002). Interestingly, in

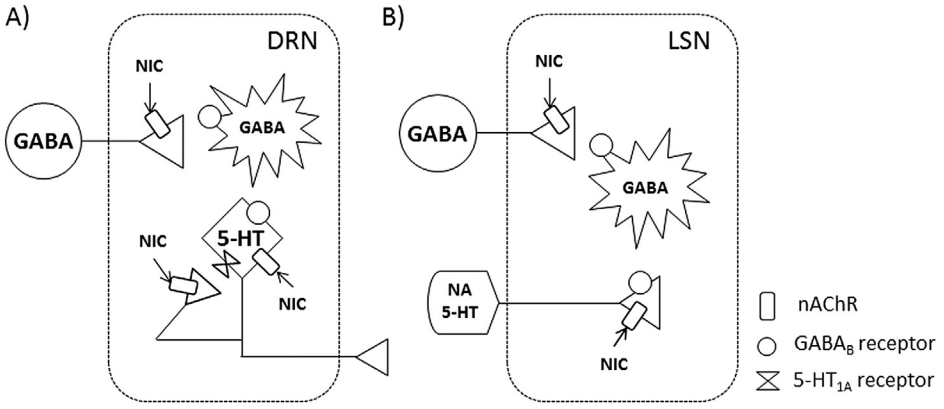


Fig. 5. Diagram showing the possible role of $GABA_B$ receptors in the neurochemical changes induced by a low or high dose of nicotine in the dorsal raphe (A) and lateral septal (B) nucleus, respectively. The arrows indicate the main possible sites of action of nicotine to explain its modulatory effects on the GABAergic, noradrenercic and serotonergic transmission. NIC, nicotine; GABA, γ-aminobutyric acid; 5-HT, serotonin; NA, noradrenaline; nAChR, nicotine acetylcholine receptor; DRN, dorsal raphe nucleus; LSN, lateral septal nucleus.

contrast to the pattern observed in the DRN with low doses of nicotine, when injected into the LSN, nicotine (1 and 4 μg) had anxiogenic-like effects in the elevated plus maze test (Ouagazzal et al., 1999; File et al., 2000; Cheeta et al., 2000a). It has been shown that the LSN receives dense noradrenergic and serotonergic innervations from different brain regions (Antonopoulos et al., 2004; Sheehan et al., 2004). Thereby, nicotine could stimulate nAChRs located pre-synaptically on noradrenergic and serotonergic terminals causing an increase of NA and 5-HT release in the LSN. In addition, several authors demonstrated that the LSN abundantly expresses both local interneurons and afferent neurons containing GABA (Castañeda et al., 2005; Garrido Sanabria et al., 2006; Zhao et al., 2013; Sheehan et al., 2004). In this context, we suggest that stimulation of nAChRs located on GABAergic neurons (probably directly connected to GABAergic interneurons) might increase NA and 5-HT content in the LSN, with the subsequent anxiogenic-like effects. Therefore, $GABA_B$ receptor's blockade by 2-OH-saclofen could abolish noradrenergic and serotonergic activity in the LSN (Fig. 5B). Thus, nicotine's anxiogenic-like effect would be abolished by 2-OH-saclofen, as we previously observed (Varani and Balerio, 2012). Interestingly, we previously reported using a genetic approach, that $GABA_B$ receptors would not be involved in the anxiogenic-like response induced by nicotine in the elevated plus maze test, since this effect was not abolished in $GABA_{B1}$ knockout mice (Varani et al., 2012). However, our present neurochemical results revealed that the increased levels of NA and 5-HT induced by the anxiogenic dose of nicotine (0.8 mg/kg) in the LSN of wild-type mice were not observed in $GABA_{B1}$ knockout mice. Therefore, these results suggest that $GABA_B$ receptors could be partially involved in the neurochemical alterations induced by nicotine (0.8 mg/kg) in the LSN. The fact that the neurochemical alterations, but not the behavioral responses induced by the anxiogenic dose of nicotine were abolished in $GABA_{B1}$ knockout mice might reflect a compensatory regulation of other neurobiological mechanisms involved in the anxiogenic responses to nicotine. In this sense, previous studies from our laboratory showed that the opioid and cannabinoid systems were involved in the anxiety-related behaviors in the elevated plus maze test in mice (Balerio et al., 2005, 2006). Hence, we suggest that at least these two systems might exert a compensatory mechanism in order to compensate the lack of $GABA_B$ receptors in the $GABA_{B1}$ knockout mice.

Finally in order to examine whether nicotine-induced anxiolytic- and anxiogenic-like responses could induce an increase of neuronal activity in the DRN and LSN, respectively, we have evaluated the c-Fos expression in both pharmacological and genetic approaches. The earlier expression gene c-Fos is a transcription factor considered to be a marker of neuronal activity (Dragunow and Faull, 1989). It is known that addictive related behaviors are associated to different molecular adaptations, such as gene regulation, which are observed in specific brain areas (Berke and Hyman, 2000; Nestler, 2001). In line with this, several authors have shown that acute nicotine (Salminen et al., 1996), chronic nicotine (Soderstrom et al., 2007), nicotine self-administration (Pagliusi et al., 1996) and nicotine rewarding effects (Mombereau et al., 2007) induced an increase in Fos-like immunoreactivity in diverse brain regions.

As it was expected, in the pharmacological approach of the present study, we found that the anxiolytic dose of nicotine (0.05 mg/kg) increased c-Fos expression in the DRN, but not in the LSN. Therefore, these results together with the neurochemical data confirm that the DRN might be involved in mediating the anxiolytic-like effects induced by nicotine. Interestingly, it has been demonstrated that acute nicotine activated c-Fos expression in a subset of serotonergic neurons in the DRN (Sperling and Commons, 2011; Bang and Commons, 2011), suggesting that nicotine increases the firing rate of DRN neurons (Mihailescu et al., 2001, 2002; Guzmán-Marín et al., 2001; Garduño et al., 2012). Thereby, our present immunohistochemical results support the idea that the anxiolytic-like effect of nicotine, assessed by the elevated plus maze test, might be mediated by an increase of 5-HT levels in the DRN. On the other hand, both approaches revealed that either 2-OH-saclofen or the lack of $GABA_B$ receptor re-established the increased c-Fos expression induced by the anxiolytic dose of nicotine (0.05 mg/kg) in the DRN, suggesting that $GABA_B$ receptors would be involved in the anxiolytic-like effect induced by nicotine.

The anxiogenic dose of nicotine (0.8 mg/kg) increased the c-Fos expression in the LSN, but not in the DRN. These findings show that the LSN could participate in mediating the anxiogenic effect of nicotine measured by the elevated plus maze test. In addition, we suggest that nicotine's anxiogenic-like effect would produce an increase of neuronal activity in the LSN. In accordance, the subcutaneous nicotine administration, at a high dose (0.35 mg/kg), induced an increase of c-Fos expression in the LSN of rats (Mathieu-Kia et al., 1998). Similarly, acute nicotine administration (0.4 and 0.8 mg/kg, sc) increased c-Fos mRNA expression in LSN of adolescent and adult rats (Shram et al., 2007). Even though there are no available evidences showing that nicotine activate c-Fos expression exclusively in noradrenergic and serotonergic neurons of the LSN, our present neurochemical data provide information suggesting that the augmented c-Fos expression could be associated to an increase of NA and 5-HT levels in the LSN. Thus, we could conclude that the anxiogenic-like effect induced by nicotine, assessed by the elevated plus maze test in mice, might be mediated by an increase of NA and 5-HT content in the LSN. Regarding the role of $GABA_B$ receptors in this effect, we showed that either 2-OH-saclofen or the lack of $GABA_B$ receptor were able to re-establish the increased c-Fos expression induced by the anxiogenic dose of nicotine (0.8 mg/kg) in the LSN, suggesting that $GABA_B$ receptors could be involved in the anxiogenic-like effect induced by nicotine.

Nicotine's anxiety-related effects are expressed as complex behavioral phenomena, and involve several neurotransmitters systems and neuroanatomical substrates. Information to completely understand these behavioral responses is still missing (Picciotto et al., 2002). The present results shed light on the hypothesis which postulates that the GABAergic system has an important role in mediating the anxiety-related effects induced by nicotine, as do other systems such as the opioid and the endocannabinoid systems (Balerio et al., 2005, 2006). In summary, we provide the first pharmacological and genetic evidence for a specific involvement of $GABA_B$ receptors in the regulation of the effects induced by nicotine on anxiety-related behavior in mice. The elucidation of this new interaction between nicotine and the GABAergic system provides a further step towards a better understanding of the complex behavioral responses to nicotine. Additionally, the present studies on the effects of $GABA_B$ ligand over nicotine's anxiety-related behavior may contribute to the development of treatments that target nicotine addiction.

Conflict of interest

All the authors declare that they have no conflicts of interest.

Acknowledgments

This work has been supported by grants from University of Buenos Aires (UBACyT B016), CONICET (PIP 11420090100303) and Swiss National Science Foundation (3100A0-117816). Andrés Varani and Valeria T. Pedrón are supported by a doctoral fellowship from the University of Buenos Aires (677/10) and CONICET,

respectively. The authors wish to thank Fernanda De Fino and Lidia Caballero for their excellent HPLC technical assistance.

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