Neuropharmacology 90 (2015) 90–101

Contents lists available at ScienceDirect

Neuropharmacology

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

Lack of $GABA_B$ receptors modifies behavioural and biochemical alterations induced by precipitated nicotine withdrawal

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ARTICLE INFO

Article history:
Received 18 July 2014
Received in revised form
19 November 2014
Accepted 22 November 2014
Available online 3 December 2014

Keywords:
Nicotine
$GABA_B$ receptor
Withdrawal
Anxiety
BDNF
Corticosterone
Epibatidine
Monoamines
Mice

ABSTRACT

The nicotine (NIC) withdrawal syndrome is considered to be a major cause of the high relapse rate among individuals undergoing smoking cessation. The aim of the present study was to evaluate a possible role of $GABA_B$ receptors in NIC withdrawal, by comparing $GABA_{B1}$ knockout mice and their wild-type littermates. We analysed the time course of the global withdrawal score, the anxiety-like effects, monoamine concentrations, the brain-derived neurotrophic factor (BDNF) expression, the corticosterone plasmatic levels and [^{3}H]epibatidine binding sites during NIC withdrawal precipitated by mecamylamine, a nicotinic receptor antagonist (MEC). In NIC withdrawn wild-type mice, we observed a global withdrawal score, an anxiety-like effect in the elevated plus maze, a decrease of the striatal dopamine and 3,4-dihydroxyphenylacetic acid concentrations, an increase of corticosterone plasma levels, a reduction of BDNF expression in several brain areas and an increase of [^{3}H]epibatidine binding sites in specific brain regions. Interestingly, the effects found in NIC withdrawn wild-type mice were absent in $GABA_{B1}$ knockout mice, suggesting that $GABA_{B1}$ subunit of the $GABA_B$ receptor is involved in the regulation of the behavioural and biochemical alterations induced by NIC withdrawal in mice. These results reveal an interaction between the $GABA_B$ receptors and the neurochemical systems through which NIC exerts its long-term effects.

1. Introduction

Smoking is a common addiction and is associated with health problems that result in significant morbidity and mortality throughout the world. Nicotine (NIC) is the main component of tobacco responsible for its addictive properties (Zaparoli and Galduróz, 2012). NIC withdrawal precipitates a characteristic abstinence syndrome, which includes increased NIC craving, anxiety-like behaviour, pain sensitivity, restlessness, appetite, and decreased cognitive abilities (Le Foll and Goldberg, 2009; Portugal and Gould, 2009). In addition, it is well known that NIC withdrawal induces alterations in neurotransmitters levels, nicotinic receptors density, neurotrophic factors expression and corticosterone plasma concentration (Markou, 2008; Kivinummi et al., 2011; Stoker and Markou, 2013; Ueno et al., 2014). Thus, the NIC withdrawal syndrome is considered to be one of the major causes of the high relapse rate among individuals undergoing smoking cessation (Le Foll and Goldberg, 2009). Therefore, it would be useful to identify pharmacological approaches that might ease the withdrawal syndrome associated with NIC dependence.

Our main interest has been the study of the GABAergic system, the major inhibitory neurotransmitter system in the mammalian central nervous system. Gamma-aminobutyric acid (GABA) acts on two classes of receptors: ionotropic $GABA_A$ and $GABA_C$, and

Abbreviations: NIC, nicotine; SAL, saline; MEC, mecamylamine; GABA, gamma-aminobutyric acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindolacetic acid; nAChR, nicotinic acetylcholine receptor; HPLC, high-performance liquid chromatography; BDNF, brain derived neurotrophic factor.

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http://dx.doi.org/10.1016/j.neuropharm.2014.11.013
0028-3908/

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 pre 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). It has been demonstrated that $GABA_B$ receptor activity can modulate biochemical and behavioural alterations produced by acute effects of NIC, as well as addictive properties of NIC (Mombereau et al., 2007; Lobina et al., 2011; Vlachou et al., 2011a,b; McClure-Begley et al., 2014). We have observed that the $GABA_B$ receptor agonist baclofen abolishes NIC-induced antinociceptive (Varani et al., 2014a) and rewarding (Varani et al., 2014b) effects in mice. Moreover, baclofen prevented biochemical (expression of c-Fos, brain-derived neurotrophic factor and $\alpha4\beta2$ nicotinic receptors) neurochemical (dopamine and serotonin concentrations) and behavioural (somatic and motivational manifestations) changes during NIC withdrawal in mice (Varani et al., 2011, 2013, 2014b, 2014c). Moreover, the $GABA_B$ receptor antagonist 2-hydroxysaclofen increased NIC-induced antinociceptive (Varani et al., 2014a) and rewarding effects (un-published results), and abolished NIC-induced hypolocomotion (Varani et al., 2014a) in mice. 2-hydroxysaclofen blocked the behavioural (anxiety-related responses), neurochemical (serotonin and noradrenalin concentrations) and biochemical (c-Fos expression) changes induced by an anxiolytic or anxiogenic dose of NIC in mice (Varani and Balerio, 2012; Varani et al., 2014d). Behavioural (antinociception, hypolocomotion and anxiety-related effect), neurochemical (serotonin and noradrenalin concentrations) and biochemical (c-Fos expression) changes induced by acute NIC administrations were modified in $GABA_{B1}$ knockout ($GABA_{B1}$ KO) mice, which lack functional $GABA_B$ receptors (Varani et al., 2012, 2014d). Finally, the biochemical (c-Fos expression) and behavioural (somatic manifestations) alterations induced by NIC withdrawal syndrome were also modified in $GABA_{B1}$ KO mice (Varani et al., 2012).

The aim of the present study was to demonstrate that $GABA_B$ receptors play a role in mediating the behavioural and biochemical alterations induced by precipitated NIC withdrawal, using $GABA_{B1}$ KO mice. In particular, we analysed the time course of the global score and the anxiety-like effects associated with NIC withdrawal syndrome precipitated by the antagonist of nicotinic receptors mecamylamine (MEC). In addition, we explored monoamine concentrations, brain-derived neurotrophic factor (BDNF) expression, corticosterone plasma levels and [^{3}H]epibatidine binding sites during MEC-precipitated NIC withdrawal.

2. Materials and methods

2.1. Animals

Male BALB/C mice lacking the $GABA_{B1}$ subunit of the $GABA_B$ receptor ($GABA_{B1}$ KO) (Schuler et al., 2001) and their wild-type littermates (WT) were obtained by crossing heterozygous animals. Fingertip biopsies were used to isolate DNA for PCR genotyping (Schuler et al., 2001). Animals weighing 20–30 g were housed five per cage and acclimatized to the laboratory conditions according to local regulations (SENASA, 2002) (12-h light: 12-h dark cycle, 21 ± 0.5 °C room temperature, 65 ± 10% humidity). The mice were manipulated 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*. Behavioural tests and animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, publication no. 85–23, revised 1985). All experiments were performed with investigators blinded to the genotype and treatment conditions. In order to validate the experimental protocols, we used wild-type BALB/C mice and the optimal range of NIC dose was based on previous studies (Castañe et al., 2002; Balerio et al., 2004, 2005; Berrendero et al., 2005; Varani et al., 2012).

2.2. Drugs

(−)-Nicotine hydrogen tartrate salt ([−]-1-methyl-2-[3-pyridil]pyrrolidine) (Sigma Chemical Co., USA) and mecamylamine hydrochloride (MEC) (Sigma–Aldrich, USA) were dissolved in isotonic saline solution (SAL, NaCl 0.9%) and administered subcutaneously (s.c.) in a volume of 10 ml/kg. NIC doses are reported as nicotine hydrogen tartrate salt (1 mg/kg of nicotine hydrogen tartrate salt equals to 0.35087 mg/kg nicotine free base).

2.3. Experimental protocol

NIC dependence was induced by using Alzet osmotic minipumps (Model, 2001; Alzet, Cupertino, CA) which delivered a constant subcutaneous flow at a rate of 1 μl/h. The minipumps containing SAL or NIC solutions were implanted subcutaneously in WT and $GABA_{B1}$ KO mice under brief anaesthesia. NIC concentration was adjusted to compensate for differences in subjects body weight. Thus, each average-weight mouse received a dose of approximately 25 mg/kg/day of NIC hydrogen tartrate salt. NIC withdrawal syndrome was precipitated 6 days after minipump implantation by injection of the nicotinic receptor antagonist, MEC (1 mg/kg, s.c.), as described in Castañé et al. (2002) and Balerio et al. (2004). NIC withdrawal syndrome was confirmed by the expression of somatic signs (wet dog shakes, front paw tremors, writhes, scratches, body tremor, ptosis, teeth chattering, genital licks, piloerection and locomotor activity) after MEC injection (Varani et al., 2012).

2.4. Time course of the global withdrawal score

The somatic signs of withdrawal were visually recorded by one observer during a period of 30 min after MEC or SAL injection ($n = 10–11$ per experimental group), as previously reported (Varani et al., 2012). A global withdrawal score was calculated for each animal by giving each individual sign a relative weight, as previously reported (Castañé et al., 2002; Balerio et al., 2004). Finally, a time course of the global withdrawal score was determined for each 5-min period of the whole observation time (30 min) for each animal (Varani et al., 2011).

2.5. Anxiety-like effects associated to withdrawal

Immediately after MEC or SAL injection, mice ($n = 7–9$ per experimental group) were placed in the elevated plus-maze. The elevated plus-maze (Pellow et al., 1985; File et al., 1992) consisted of a black plastic apparatus with fours arms (16 × 5 cm) set in a cross from a neutral central square (5 × 5 cm). Two opposite arms were delimited by vertical walls (closed arms), while the other two opposite arms had unprotected edges (open arms). The maze was elevated 30 cm above the ground and illuminated from the top (100 lx). At the beginning of the 15-min observation session, each mouse was placed in the central neutral area, facing one of the open arms. The total number of visits to the closed and open arms, and the cumulative time spent in the open and closed arms were then observed on a monitor through a video camera system (Vision Robot, Buenos Aires, Argentina). An arm visit was recorded when the mouse moved both forepaws and the head into the arm, as we previously described (Balerio et al., 2005).

2.6. Determinations of monoamines

HPLC-coupled electrochemical detection (Heikkila et al., 1984) of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) was achieved using a Varian 5000 liquid chromatograph coupled to an electrochemical detector (BAS LC-4C). Ten minutes after MEC or SAL injection, brains ($n = 5–6$ per experimental group) were quickly removed and place in dry ice. When partially frozen, the striatum, hippocampus and cortex were dissected under a dissecting microscope. Brain tissues were weighed, homogenized, and deproteinezed in 0.2 N perchloric acid (1/20). Homogenates were centrifuged, and the supernatants were injected (50 μl) onto a 12.5 cm × 4 mm Nova-Pak C18 reverse phase column (Waters). Mobile phase for DA, DOPAC, 5-HT and 5-HIAA determinations contained $NaH_2PO_4–H_2O$ 0.076 M, PICB8 5.24 ml/l, EDTA 0.99 mM and 6% 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.

2.7. Corticosterone determination

Ten minutes after MEC or SAL injection, blood samples ($n = 11$ per experimental group) were collected by cardiac puncture in a 1.5 ml tube with heparin. To avoid the influence of circadian rhythm on corticosterone levels, blood was collected between 8 and 12 a.m. (Jozsa et al., 2005). Plasma was separated by centrifugation (3000 × g, 15 min, 4 °C) and frozen at −70 °C. Plasmatic corticosterone concentration was determined by high performance liquid chromatography (HPLC). Corticosterone was extracted from 200 μl of plasma by adding 4 ml of diethyl ether-dicloromethane (60:40). Samples were vortexed and left at room temperature for 3 min and 100 μl of an internal standard (Fenitoin 1 mg/ml in methanol) was added to each tube. The organic phase was evaporated at 37 °C under nitrogen. Samples were resuspended with 150 μl of movil phase (acetonitrile-water 40:60), vortexed (15 s) and injected into the HPLC system. The column (Hypersil GOLD C18, particle size 5 μm, 250 × 4.6 mm; Thermo Fisher Scientific Inc.) was equilibrated using HPLC-grade acetonitrile-water (40:60 v/v) at a flow rate of 1 ml/min. A series of

standards (human normal plasma with corticosterone, SIGMA C2505) covering the range of 0.1–1 μg/ml were used in daily work in order to calibrate the HPLC system. A regression line between the peak heights and the concentration of corticosterone was calculated and used for determining the corticosterone concentration in the samples.

2.8. Immunohistochemistry experiments

2.8.1. Tissue preparation

Thirty min after MEC or SAL injection, mice ($n = 5$ 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). Mice 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, collected in three serial groups of free-floating sections and stored at 4 °C.

2.8.2. BDNF immunohistochemistry

The procedure was adapted from previously described protocols (Bester et al., 2001). All reactions were performed on floating sections agitated on a shaker. Sections from different experimental groups were processed in parallel to minimize the variations in immunohistochemical labelling. Free-floating sections were rinsed in 0.1 M phosphate buffered saline with 0.15% Triton X-100 (PBS-T; pH 7.4) and then incubated with 3% hydrogen peroxide in PBS-T for a period of 30 min to remove endogenous peroxidase activity. After rinsing again in PBS-T, sections were incubated for 30 min in 2% normal goat serum in PBS-T. Then, sections were incubated overnight in a rabbit polyclonal antibody anti-BDNF (Santa Cruz Biotechnology, USA, sc-20981) (1:50 in PBS 0.1 M, thimerosal 0.02%, normal goat serum 1%) (Santa Cruz Biotechnology, USA) 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 0.1 M (Delfino et al., 2004).

2.8.3. Data quantification

For quantitative analysis, cells positive for BDNF-like 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 BDNF-positive cells was counted within a grid under ImageJ 1.36 b, provided by National Institutes of Health, USA (public domain software). The counting was performed bilaterally in each brain area by an observer blind to genotype as well as treatment. These counts were averaged into a single score for each region of each animal and finally the group mean ± SEM was calculated. BDNF-positive cells were quantified in the following brain regions, identified according to the anatomic atlas of Paxinos and Franklin (2004): accumbens shell (AcbSh) and core (AcbC) nucleus, cingulate cortex area 1 and 2 (Cg), caudate putamen (CPu), the bed nucleus of the stria terminalis (BST), the basolateral amygdaloid nucleus (BLA), dentate gyrus (DG), CA1 and CA3 areas of the hippocampus and habenular nucleus (Hb).

2.9. Autoradiography assays

2.9.1. Tissue preparation

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

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

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

2.9.3. Film exposure and image analysis

Autoradiograms were obtained after exposing sections to Kodak BIOMAX MR-1 (Sigma) films at −4 °C for 1–4 months in light-tight cassettes. Radioactivity standards (American Radiolabeled Chemical Inc.) consisting of 14 sections of methacrylate plastic impregnated with tritium (0.14–489 μCi/g) were jointly exposed with the sections. Films were developed in Kodak Dektol developer (Sigma) and fixative. Autoradiography images were scanned in a conventional scanner, and analyses made using ImageJ software (developed at the U.S. National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/). Receptor binding levels were measured for the following regions: AcbSh, AcbC, motor cortex (deep layer; Cx), CPu, BST, Hb, thalamic nuclei, dorsal lateral geniculate nucleus (DLG), fasciculus retroflexus (fr), ventral tegmental area (VTA), interpeduncular nucleus (IP), superior colliculus, substantia nigra (SN) and periaqueductal grey (PAG). Structures were identified according to the corresponding outlines from the Mouse Atlas of Paxinos and Franklin (2004). The optimal plate was selected according to the images obtained from the film exposure. The limits of each brain area were defined taking into account some structures which can be easily identified such as corpus callosum, commissures, lateral ventricles, third ventricle, etc. The sections were obtained at five anatomical levels: bregma 1.10 mm, −1.22 mm, −2.70 mm, −2.92 mm, −3.52 mm. All experimental groups were processed together to ensure a paired protocol for binding, film apposition, and image analysis. The operator measuring optical densities was unaware of the experimental condition of each section. Optic density was converted to nCi/mg of tissue using the calibrated methacrylate tritium standards, and after subtracting nonspecific (background) from total binding, specific binding was expressed as fmol/mg tissue. For each anatomical level, left and right side of four contiguous sections (eight measurements per subject-brain) represented total binding; the eight determinations were averaged for each subject. The nonspecific binding was determined separately for each anatomical level using 4 sections. [^{3}H]epibatidine binding was at background levels in the presence of 10 mM unlabelled NIC. The specific binding was 60% since the nonspecific binding was around 40%.

2.10. Statistical analysis

For the statistical analysis we have excluded the outliers. We considered as outlier all values exceeding the mean ± [2 × SD] because is quite likely that these values could be consequence of other intervening variables rather than those analysed in the present study. Results obtained for the time course of the global withdrawal score were analysed by using two-way ANOVA (treatment × time) with one repeated-measures variable (time; within measurements). When a significant interaction between these factors was observed, the difference between two means was analysed by Tukey *post hoc* test. The remaining results were analysed 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. Statistical analysis was performed using SPSS 11.5 software.

3. Results

3.1. Mecamylamine-precipitated nicotine withdrawal: time course of the global withdrawal score

The time course of the global withdrawal score was evaluated in $GABA_{B1}$ KO mice and WT littermates. NIC withdrawal induced an increase of the global score in WT but not in $GABA_{B1}$ KO mice. The NIC withdrawal score was highest 10 min after MEC injection in WT mice (Fig. 1). No significant differences were observed in SAL- or NIC-dependent WT and KO mice (data not shown).

The two-way ANOVA with repeated measures test showed a significant effect of treatment [$F_{3,40} = 67.394$; $P < 0.001$], time [$F_{5,200} = 2.905$, $P < 0.05$], and interaction between treatment × time [$F_{5,200} = 3.864$, $P < 0.001$]. Post hoc comparisons revealed a significant increase of the global score in NIC-treated WT mice compared to the SAL group, at 5, 10, 15, 20, 25 ($P < 0.001$) but not at 30 min after MEC injection (Fig. 1). Moreover, this test also showed that the increase of the global score was higher at 10 min compared to the global score at 5 min ($P < 0.05$), 25 ($P < 0.05$) or 30 min ($P < 0.05$) after MEC-precipitated NIC withdrawal (Fig. 1). When post hoc comparisons were made in $GABA_{B1}$ KO mice, there were no significant differences between NIC and SAL treatment groups for the global score during the whole observation time (Fig. 1). Post

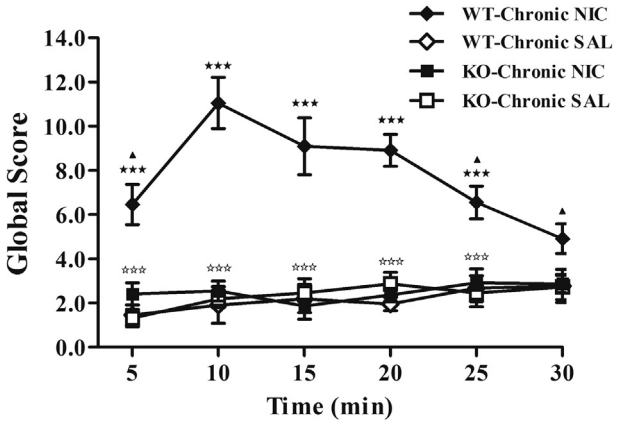

Fig. 1. Time course of the global score of nicotine (NIC) withdrawal in wild-type (WT) and $GABA_{B1}$ knockout (KO) mice. Each point represents the mean ± SEM (n = 8–11 mice per experimental group) of the global score to each 5-min period during the whole observation time (30 min). Empty symbol: chronic treatment with saline (SAL); filled symbol: chronic treatment with nicotine (NIC). Rhombus represents wild-type (WT) and square $GABA_{B1}$ knockout (KO) mice. ★ ★ ★ $P < 0.001$ when compared to vehicle group of the same genotype. ☆ ☆ ☆ $P < 0.001$ for between-genotype comparisons (two-way ANOVA with treatment and genotype as factors of variation, followed by corresponding one-way ANOVA and post hoc comparisons using the Tukey test). ▲ $P < 0.05$ compared to the global score at 10 min (two-way ANOVA with one repeated measures variable followed by Tukey post hoc test).

hoc comparisons also revealed significant differences between genotypes in the global score at 5, 10, 15, 20, 25 ($P < 0.001$) but not at 30 min after MEC injection (Fig. 1). No significant differences were observed between genotypes in SAL-treated mice during the whole observation time (Fig. 1).

3.2. Mecamylamine-precipitated nicotine withdrawal: anxiety-like effects

The anxiety-like effects associated to NIC withdrawal were evaluated in $GABA_{B1}$ KO mice and WT littermates. NIC withdrawal induced an anxiety-like effect in WT but not in $GABA_{B1}$ KO mice measured by the elevated plus maze test (Fig. 2). No significant differences were observed in SAL- or NIC-dependent WT and KO mice (data not shown).

Two-way ANOVA revealed a significant effect of treatment (NIC-SAL) in the case of the percentage of entries [$F_{(1,32)} = 3.924$, $P < 0.05$] but not in the time spent [$F_{(1,32)} = 0.093$, N.S.] in the open arms, and a significant effect of genotype (WT and $GABA_{B1}$ KO mice) in the case of the percentage of entries [$F_{(1,32)} = 11.166$, $P < 0.01$] but not in the time spent [$F_{(1,32)} = 9.296$, N.S.] in the open arms. Significant interaction between treatment and genotype was observed in the percentage of entries [$F_{(1,32)} = 5.253$, $P < 0.05$] and in the time spent [$F_{(1,32)} = 4.999$, $P < 0.05$] in the open arms. Subsequent one-way ANOVA showed significant effects of NIC treatment in WT and $GABA_{B1}$ KO mice in the case of the percentage of entries [$F_{(3,31)} = 7.310$, $P < 0.001$] but not in the time spent [$F_{(3,31)} = 2.625$, N.S.] in the open arms. Post hoc comparisons revealed a significant decrease of the percentage of entries ($P < 0.05$) (Fig. 2A) but not in the percentage of time spent ($P = 0.269$) (Fig. 2B) in the open arms, in NIC-treated WT mice compared to the SAL group. When the same analysis was made for $GABA_{B1}$ KO mice, there were no significant differences between the NIC and SAL groups, for the percentage of entries and time spent in the open arms found to be altered in WT mice (Fig. 2). Post hoc comparisons also revealed significant differences between genotypes in the case of the percentage of entries ($P < 0.01$) (Fig. 2A) but not in the time spent ($P = 0.051$) (Fig. 2B) in the open arms. No significant differences were observed between genotypes in SAL-treated mice (Fig. 2).

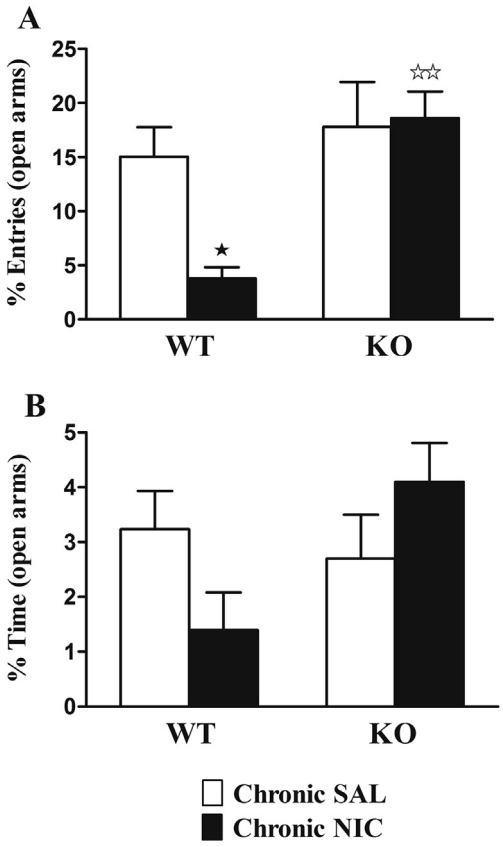

Fig. 2. Anxiety-like effects in the elevated plus maze test were observed during nicotine (NIC) withdrawal in wild-type (WT) but not in $GABA_{B1}$ knockout (KO) mice. Results are expressed as mean ± SEM (n = 7–9 mice per experimental group) of the percentage of entries into (A) and time spent (B) in the open arms of the elevated plus maze test. Bars represent chronic saline-treated (white bars) and chronic nicotine-treated (black bars) wild-type and $GABA_{B1}$ knockout mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post hoc comparisons using the Tukey test. ★ $P < 0.05$ when compared to vehicle group of the same genotype. ☆ ☆ $P < 0.01$ for between-genotype comparisons.

3.3. Mecamylamine-precipitated nicotine withdrawal: monoamines concentrations

Monoamines concentrations were analysed during NIC withdrawal in $GABA_{B1}$ KO mice and WT littermates. NIC withdrawal induced a decrease of the striatal DA and DOPAC concentrations in both WT and $GABA_{B1}$ KO mice (Fig. 3). Monoamines concentrations were not altered either in SAL- or NIC-dependent WT and KO mice in any of the brain areas studied (data not shown).

3.3.1. Striatum

Two-way ANOVA revealed a significant effect of treatment (NIC or SAL) in the striatal DA [$F_{(1,22)} = 18.492$, $P < 0.001$], DOPAC [$F_{(1,22)} = 21.520$, $P < 0.001$], but not in 5-HT [$F_{(1,22)} = 0.021$, N.S.] and 5-HIAA [$F_{(1,22)} = 0.046$, N.S.], and a significant effect of genotype (WT and $GABA_{B1}$ KO mice) in the DA [$F_{(1,22)} = 23.123$, $P < 0.001$], DOPAC [$F_{(1,22)} = 19.847$, $P < 0.001$], but not in 5-HT [$F_{(1,22)} = 0.249$, N.S.] and 5-HIAA [$F_{(1,22)} = 0.133$, N.S.]. No significant interaction between treatment and genotype was observed in the DA [$F_{(1,22)} = 0.080$, N.S.], DOPAC [$F_{(1,22)} = 0.012$, N.S.], 5-HT [$F_{(1,22)} = 0.191$, N.S.] and 5-HIAA [$F_{(1,22)} = 1.127$, N.S.]. Subsequent one-way ANOVA for treatment showed significant effect in both genotypes in DA ($F_{(3,21)} = 13.880$, $P < 0.001$) and DOPAC ($F_{(3,21)} = 13.818$, $P < 0.001$) but not in 5-HT ($F_{(3,21)} = 0.168$, $P = 0.916$) and 5-HIAA ($F_{(3,21)} = 0.383$, $P = 0.767$). Post hoc comparisons revealed a significant decrease of the striatal

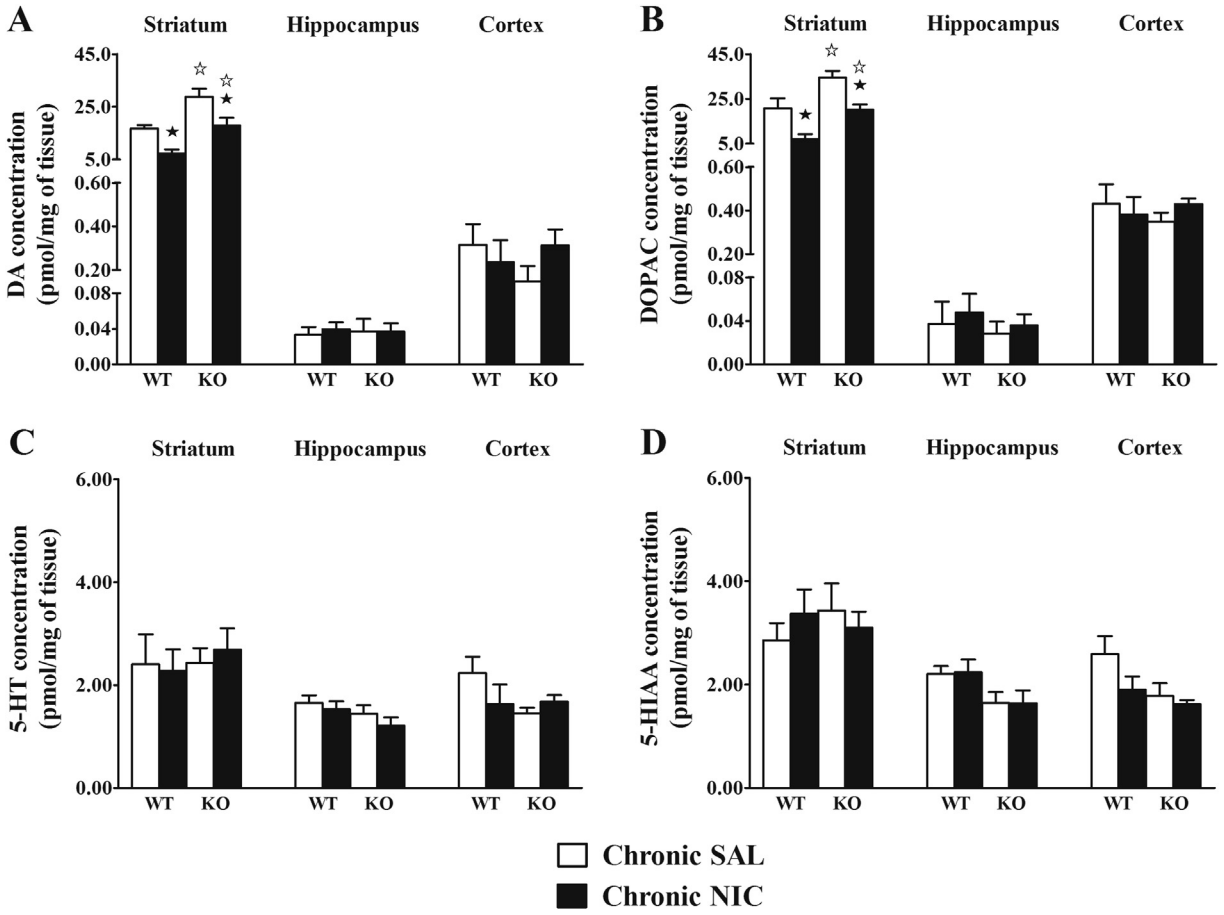

Fig. 3. Striatal dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations were decreased during nicotine (NIC) withdrawal in wild-type (WT) and $GABA_{B1}$ knockout (KO) mice. Results are expressed as mean ± SEM (n = 5–6 mice per experimental group) of DA (A), DOPAC (B), serotonin (5-HT) (C) and 5-hydroxyindolacetic acid (5-HIAA) (D) concentrations (pmol/mg of tissue) in the striatum, hippocampus and cortex. Bars represent chronic saline-treated (white bars) and chronic nicotine-treated (black bars) wild-type and $GABA_{B1}$ knockout mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post hoc comparisons using the Tukey test. ★ $P < 0.05$ when compared to vehicle group of the same genotype. ☆ $P < 0.05$ for between-genotype comparisons.

DA ($P < 0.05$) (Fig. 3A) and DOPAC ($P < 0.05$) (Fig. 3B) in NIC-treated WT mice compared to the SAL group, but not in 5-HT ($P = 0.997$) (Fig. 3C) and 5-HIAA ($P = 0.821$) (Fig. 3D). When the same analysis was made in $GABA_{B1}$ KO mice, post hoc comparisons revealed a significant decrease of the striatal DA ($P < 0.05$) (Fig. 3A) and DOPAC ($P < 0.05$) (Fig. 3B) in NIC-treated $GABA_{B1}$ KO mice compared to the SAL group, but not in 5-HT ($P = 0.976$) (Fig. 3C) and 5-HIAA ($P = 0.941$) (Fig. 3D). Post hoc comparisons also revealed significant differences between genotypes in DA ($P < 0.05$) (Fig. 3A) and DOPAC ($P < 0.05$) (Fig. 3A), but not 5-HT ($P = 0.898$) (Fig. 3A) and 5-HIAA ($P = 0.962$) (Fig. 3A) in NIC-treated mice. Significant differences were observed between genotypes in DA ($P < 0.05$) (Fig. 3A) and DOPAC ($P < 0.05$) (Fig. 3A), but not 5-HT ($P = 1.000$) (Fig. 3A) and 5-HIAA ($P = 0.788$) (Fig. 3A) in SAL-treated mice.

3.3.2. Hippocampus

Two-way ANOVA revealed no significant effect of treatment (NIC or SAL) in the hippocampal DA [$F_{(1,22)} = 0.086$, N.S.], DOPAC [$F_{(1,22)} = 0.358$, N.S.], 5-HT [$F_{(1,22)} = 1.258$, N.S.] and 5-HIAA [$F_{(1,22)} = 0.004$, N.S.], and a significant effect of genotype (WT and $GABA_{B1}$ KO mice) in the 5-HIAA [$F_{(1,22)} = 6.657$, $P < 0.05$] but not in the DA [$F_{(1,22)} = 0.002$, N.S.], DOPAC [$F_{(1,22)} = 0.441$, N.S.] and 5-HT [$F_{(1,22)} = 0.249$, N.S.]. No significant interaction between treatment and genotype was observed in the DA [$F_{(1,22)} = 0.096$, N.S.], DOPAC [$F_{(1,22)} = 0.010$, N.S.], 5-HT [$F_{(1,22)} = 0.114$, N.S.] and 5-HIAA [$F_{(1,22)} = 0.009$, N.S.]. Subsequent one-way ANOVA for treatment showed no significant effect in both genotypes in DA ($F_{(3,21)} = 0.061$, $P = 0.980$), DOPAC ($F_{(3,21)} = 0.275$, $P = 0.843$), 5-HT ($F_{(3,21)} = 1.461$, $P = 0.258$) and 5-HIAA ($F_{(3,21)} = 2.256$, $P = 0.117$).

3.3.3. Cortex

Two-way ANOVA revealed no significant effect of treatment (NIC or SAL) in the cortical DA [$F_{(1,22)} = 0.086$, N.S.], DOPAC [$F_{(1,22)} = 0.057$, N.S.], 5-HT [$F_{(1,22)} = 0.500$, N.S.] and 5-HIAA [$F_{(1,22)} = 2.992$, N.S.], and a significant effect of genotype (WT and $GABA_{B1}$ KO mice) in the 5-HIAA [$F_{(1,22)} = 4.961$, $P < 0.05$] but not in the DA [$F_{(1,22)} = 0.101$, N.S.], DOPAC [$F_{(1,22)} = 0.063$, N.S.] and 5-HT [$F_{(1,22)} = 1.909$, N.S.]. No significant interaction between treatment and genotype was observed in the DA [$F_{(1,22)} = 1.561$, N.S.], DOPAC [$F_{(1,22)} = 1.026$, N.S.], 5-HT [$F_{(1,22)} = 2.404$, N.S.] and 5-HIAA [$F_{(1,22)} = 1.141$, N.S.]. Subsequent one-way ANOVA for treatment showed no significant effect in both genotypes in DA ($F_{(3,21)} = 0.563$, $P = 0.646$), DOPAC ($F_{(3,21)} = 0.369$, $P = 0.776$), 5-HT ($F_{(3,21)} = 1.485$, $P = 0.252$) and 5-HIAA ($F_{(3,21)} = 2.903$, $P = 0.063$).

3.4. Mecamylamine-precipitated nicotine withdrawal: corticosterone plasma levels

Corticosterone plasma levels were analysed during NIC withdrawal in $GABA_{B1}$ KO mice and WT littermates. NIC withdrawal

induced an increase of corticosterone plasma levels in WT but not $GABA_{B1}$ KO mice (Fig. 4). Corticosterone plasma levels were not altered either in SAL- or NIC-dependent WT and KO mice (data not shown).

Two-way ANOVA revealed a significant effect of treatment (NIC-SAL) [$F_{(1,44)} = 8.225$, $P < 0.01$], genotype (WT and $GABA_{B1}$ KO mice) [$F_{(1,44)} = 8.254$, $P < 0.01$] and interaction between these two factors [$F_{(1,44)} = 9.309$, $P < 0.01$] in the corticosterone plasma levels. Subsequent one-way ANOVA showed significant effects of treatment in WT and $GABA_{B1}$ KO mice [$F_{(3,43)} = 8.596$, $P < 0.001$] in the corticosterone plasma levels. Post hoc comparisons revealed a significant increase of the corticosterone plasma levels ($P < 0.001$) (Fig. 4) in NIC-treated WT mice compared to the SAL group. When the same analysis was made in $GABA_{B1}$ KO mice, there were no significant differences between NIC and SAL treatment groups for the corticosterone plasma levels found to be altered in WT mice (Fig. 4). Post hoc comparisons also revealed significant differences between genotypes in the corticosterone plasma levels ($P < 0.001$) (Fig. 4). No significant differences were observed between genotypes in SAL-treated mice (Fig. 4).

3.5. Mecamylamine-precipitated nicotine withdrawal: BDNF expression

BDNF expressions were analysed during NIC withdrawal in $GABA_{B1}$ KO mice and WT littermates. NIC withdrawal induced a decrease of BDNF expression in WT but not $GABA_{B1}$ KO mice (Fig. 5). BDNF expression was not altered either in SAL- or NIC-dependent WT and KO mice in any of the brain areas studied (data not shown).

Two-way ANOVA revealed a significant effect of treatment (NIC or SAL) in the BDNF expression in CPu [$F_{(1,20)} = 7.950$, $P < 0.05$], BST [$F_{(1,20)} = 13.688$, $P < 0.01$], Hb [$F_{(1,20)} = 7.157$, $P < 0.05$], CA1 [$F_{(1,20)} = 17.315$, $P < 0.001$] and CA3 [$F_{(1,20)} = 6.602$, $P < 0.05$], and a significant effect of genotype (WT and $GABA_{B1}$ KO mice) in CPu [$F_{(1,20)} = 7.576$, $P < 0.05$], BST [$F_{(1,20)} = 4.963$, $P < 0.05$], Hb [$F_{(1,20)} = 10.949$, $P < 0.01$], CA1 [$F_{(1,20)} = 10.908$, $P < 0.01$] and CA3 [$F_{(1,20)} = 7.946$, $P < 0.05$]. Significant interaction between treatment and genotype was observed in CPu [$F_{(1,20)} = 13.634$, $P < 0.01$], BST [$F_{(1,20)} = 7.535$, $P < 0.05$], Hb [$F_{(1,20)} = 7.931$, $P < 0.05$], CA1 [$F_{(1,20)} = 4.931$, $P < 0.05$] and CA3 [$F_{(1,20)} = 4.929$, $P < 0.05$]. Subsequent one-way ANOVA for treatment showed significant effect in both genotypes in CPu [$F_{(3,19)} = 8.729$, $P < 0.001$], BST [$F_{(3,19)} = 9.720$, $P < 0.001$], Hb [$F_{(3,19)} = 8.679$, $P < 0.001$], CA1 [$F_{(3,19)} = 11.051$, $P < 0.001$] and CA3 [$F_{(3,19)} = 6.492$, $P < 0.01$]. Post hoc comparisons revealed a significant decrease of BDNF expression in CPu ($P < 0.01$) (Fig. 5A), BST ($P < 0.01$) (Fig. 5B), Hb ($P < 0.01$) (Fig. 5C), CA1 ($P < 0.01$) (Fig. 5D) and CA3 ($P < 0.05$) (Fig. 5E) in NIC-treated WT mice compared to the SAL group, while the same analysis for $GABA_{B1}$ KO mice showed no significant differences between NIC and SAL treated groups in any of the brain areas analysed. Post hoc comparisons also revealed significant differences between genotypes in CPu ($P < 0.01$) (Fig. 5A), BST ($P < 0.05$) (Fig. 5B), Hb ($P < 0.01$) (Fig. 5C), CA1 ($P < 0.01$) (Fig. 5D) and CA3 ($P < 0.05$) (Fig. 5E). No significant differences were observed between genotypes in SAL-treated mice, in any of the brain areas analysed (Fig. 5A–E).

No significant changes in BDNF expression were observed in the other brain areas studied.

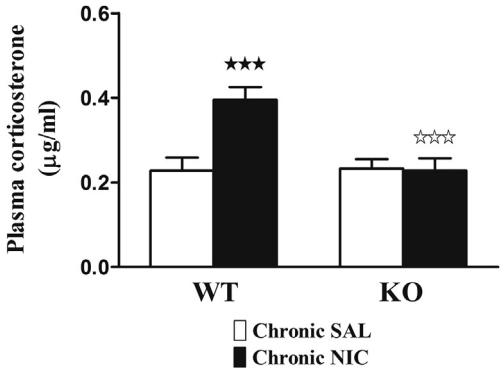

Fig. 4. Corticosterone plasma concentration was increased during nicotine (NIC) withdrawal in wild-type (WT), but not in $GABA_{B1}$ knockout (KO) mice. Results are expressed as mean ± SEM (n = 11 mice per experimental group) of corticosterone plasma concentration per μg/ml. Bars represent chronic saline-treated (white bars) and chronic nicotine-treated (black bars) wild-type and $GABA_{B1}$ knockout mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post hoc comparisons using the Tukey test. ★ ★ ★ $P < 0.001$ when compared to vehicle group of the same genotype. ☆ ☆ ☆ $P < 0.001$ for between-genotype comparisons.

3.6. Mecamylamine-precipitated nicotine withdrawal: [^{3}H] epibatidine binding levels

[^{3}H]epibatidine binding levels were analysed during NIC withdrawal in $GABA_{B1}$ KO mice and WT littermates. NIC withdrawal induced an increase of [^{3}H]epibatidine binding levels in WT but not $GABA_{B1}$ KO mice (Figs. 6 and 7). [^{3}H]epibatidine binding levels were not altered either in SAL- or NIC-dependent WT and KO mice in any of the brain areas studied (data not shown).

Two-way ANOVA revealed a significant effect of treatment (NIC or SAL) in the [^{3}H]epibatidine binding levels in AcbSh [$F_{(1,20)} = 23.462$, $P < 0.001$], Hb [$F_{(1,20)} = 13.105$, $P < 0.01$], VTA [$F_{(1,20)} = 64.252$, $P < 0.001$], IP [$F_{(1,20)} = 19.207$, $P < 0.001$] and superior culliculus [$F_{(1,20)} = 13.104$, $P < 0.01$], and a significant effect of genotype (WT and $GABA_{B1}$ KO mice) in AcbSh [$F_{(1,20)} = 25.384$, $P < 0.001$], Cx [$F_{(1,20)} = 5.174$, $P < 0.05$], Hb [$F_{(1,20)} = 6.932$, $P < 0.05$], VTA [$F_{(1,20)} = 81.692$, $P < 0.001$], IP [$F_{(1,20)} = 13.646$, $P < 0.01$] and superior culliculus [$F_{(1,20)} = 9.132$, $P < 0.01$]. Significant interaction between treatment and genotype was observed in AcbSh [$F_{(1,20)} = 14.988$, $P < 0.001$], BST [$F_{(1,20)} = 6.311$, $P < 0.05$], Hb [$F_{(1,20)} = 18.857$, $P < 0.01$], VTA [$F_{(1,20)} = 96.342$, $P < 0.001$], IP [$F_{(1,20)} = 6.344$, $P < 0.05$] and superior culliculus [$F_{(1,20)} = 24.152$, $P < 0.001$]. Subsequent one-way ANOVA for treatment showed significant effect in both genotypes in AcbSh [$F_{(3,19)} = 21.279$, $P < 0.001$], Hb [$F_{(3,19)} = 12.964$, $P < 0.001$], VTA [$F_{(3,19)} = 80.762$, $P < 0.001$], IP [$F_{(3,19)} = 13.066$, $P < 0.001$] and superior culliculus [$F_{(3,19)} = 15.463$, $P < 0.001$]. Post hoc comparisons revealed a significant increase of [^{3}H]epibatidine binding levels in AcbSh ($P < 0.001$) (Figs. 6A and 7), Hb ($P < 0.001$) (Figs. 6B and 7), VTA ($P < 0.001$) (Figs. 6C and 7), superior culliculus ($P < 0.001$) (Figs. 6D and 7) and IP ($P < 0.001$) (Figs. 6E and 7) in NIC-treated WT mice compared to the SAL group, while the same analysis for $GABA_{B1}$ KO mice showed no significant differences between NIC and SAL treated groups in any of the brain areas analysed. Post hoc comparisons also revealed significant differences between genotypes in AcbSh ($P < 0.001$) (Figs. 6A and 7), Hb ($P < 0.001$) (Figs. 6B and 7), VTA ($P < 0.001$) (Figs. 6C and 7), superior culliculus ($P < 0.001$) (Figs. 6D and 7) and IP ($P < 0.01$) (Figs. 6E and 7). No significant differences were observed between genotypes in SAL-treated mice, in any of the brain areas analysed (Figs. 6A–E, 7).

No significant changes in [^{3}H]epibatidine binding levels were observed in the other brain areas studied.

4. Discussion

The present study provides further evidence for an involvement of $GABA_B$ receptors in the behavioural, neurochemical and

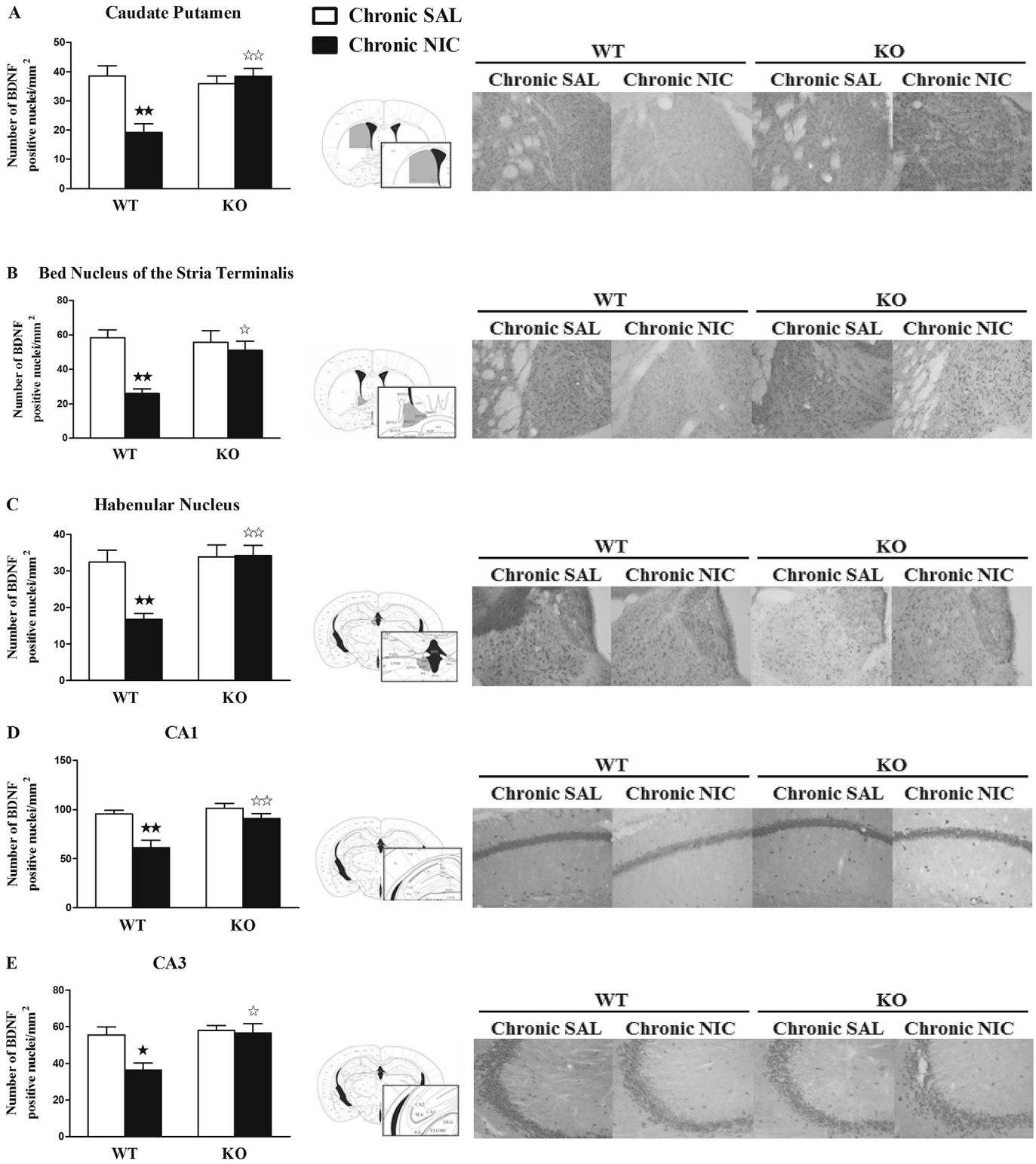

Fig. 5. BDNF expression was decreased during nicotine (NIC) withdrawal in wild-type (WT), but not in $GABA_{B1}$ knockout (KO) mice. Results are expressed as mean ± SEM ($n = 5$ mice per experimental group) of BDNF-positive nuclei per mm^2 in the caudate putamen (A), bed nucleus of the stria terminalis (B), habenular nucleus (C), CA1 (D) and CA3 (E). Bars represent chronic saline-treated (white bars) and chronic nicotine-treated (black bars) wild-type and $GABA_{B1}$ knockout mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post hoc comparisons using the Tukey test. ★ $P < 0.05$; ★ ★ $P < 0.01$ when compared to vehicle group of the same genotype. ☆ $P < 0.05$; ☆ ☆ $P < 0.01$ for between-genotype comparisons.

biochemical alterations induced by NIC withdrawal. In NIC withdrawn WT mice, we observed a global withdrawal score, an anxiety-like effect in the elevated plus maze, a decrease of the striatal DA and DOPAC concentrations, an increase of corticosterone plasma levels, a reduction of BDNF expression in several brain areas and an increase of [^{3}H]epibatidine binding sites in specific brain regions. Interestingly, the effects found in NIC withdrawn WT mice were absent in $GABA_{B1}$ KO mice.

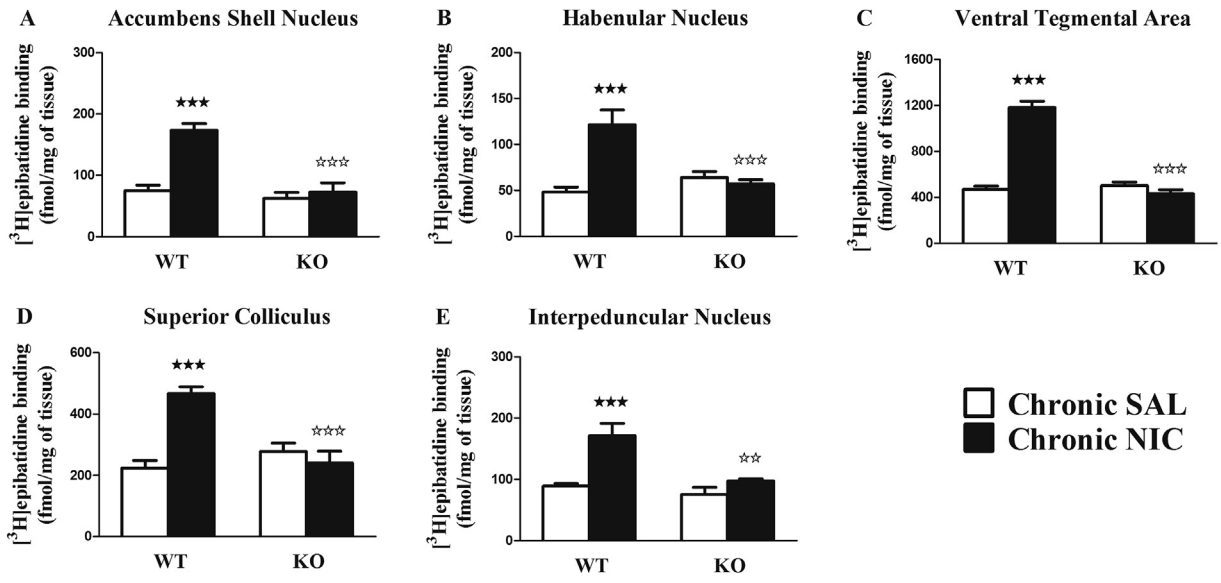

Fig. 6. [^{3}H]epibatidine binding levels were increased during nicotine (NIC) withdrawal in wild-type (WT), but not in $GABA_{B1}$ knockout (KO) mice. Results are expressed as mean ± SEM ($n = 5$ mice per experimental group) of [^{3}H]epibatidine binding levels (fmol/mg of tissue) in the accumbens shell nucleus (A), medial habenula (B), ventral tegmental area (C), superior culliculus (D) and interpeduncular nucleus (E). Bars represent chronic saline-treated (white bars) and chronic nicotine-treated (black bars) wild-type and $GABA_{B1}$ knockout mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post hoc comparisons using the Tukey test. ★ ★ ★ $P < 0.001$ when compared to vehicle group of the same genotype. ☆ ☆ $P < 0.01$; ☆ ☆ ☆ $P < 0.001$ for between-genotype comparisons.

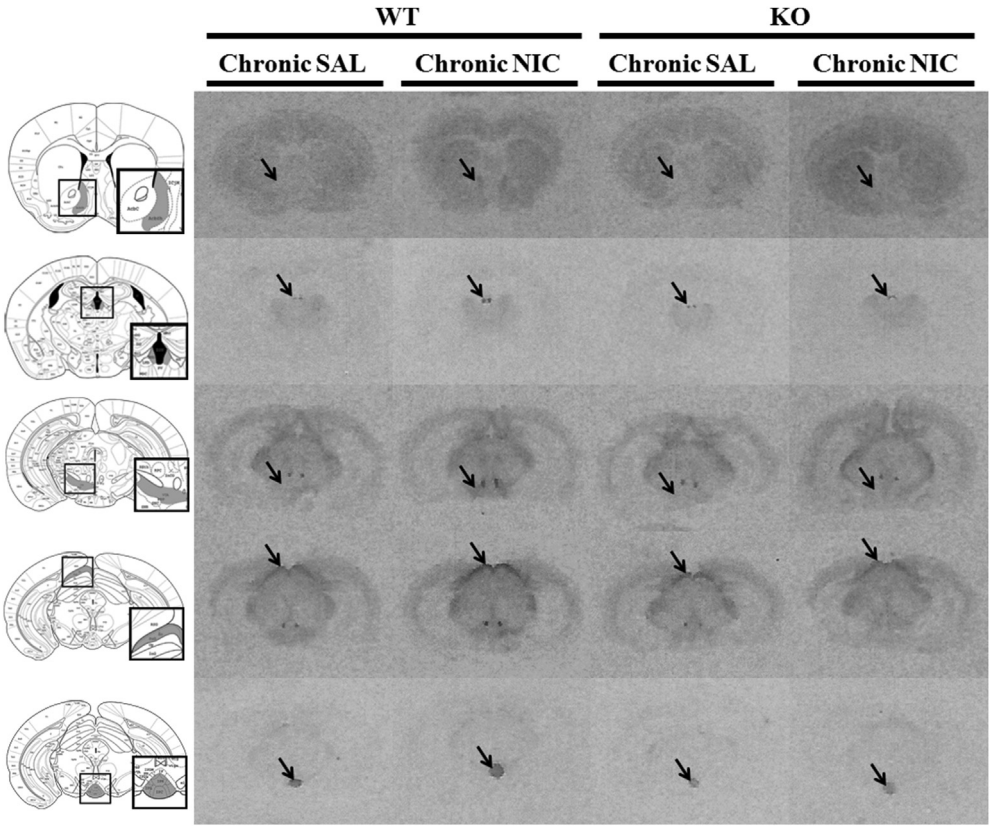

Fig. 7. [^{3}H]epibatidine autoradiograms of α4β2 nAChR binding of chronic saline (SAL)-treated and chronic nicotine (NIC)-treated wild-type (WT) and $GABA_{B1}$ knockout (KO) mice. The first and second columns show SAL-treated and chronic NIC-treated WT mice, respectively while the third and fourth columns show SAL-treated and chronic NIC-treated KO mice, respectively. The first line show sections cut at level of the accumbens shell nucleus (bregma 1.10 mm). The second line show sections cut at level of the medial habenula (bregma −1.22). The third line show sections cut at level of the ventral tegmental area (bregma −2.92). The fourth and fifth lines show sections cut at level of the superior colliculus and interpeduncular nucleus, respectively (bregma −3.52).

The MEC control groups (WT and $GABA_{B1}$ KO mice) did not show significant differences with respect to their corresponding saline control groups (data not shown), indicating that the dose of MEC used was unable to induce alterations in non-dependent animals (Varani et al., 2011, 2013, 2014c) in any of the experimental procedures.

Despite several decades of research, the time course of NIC withdrawal has not been fully established (Gilbert and McClernon, 2000; Jorenby et al., 1996). Previous studies in humans showed transient withdrawal effects (Hughes et al., 1990, 1991; Hughes and Hatsukami, 1992; Shiffman et al., 2006), while other works indicated a substantially longer and more variable time course of NIC withdrawal (Gilbert et al., 1998, 1999, 2002; Piasecki et al., 1998, 2000, 2003a,b). Most of the studies were conducted in humans, nevertheless some reports showed the progression of NIC withdrawal along the time in animals (Irvine et al., 1999; Malin et al., 2010). In this sense, we previously observed that the global score of NIC withdrawal increase within the first 10 min after MEC injection in mice, and gradually decline and stabilize within 25–30 min (Varani et al., 2011). In the present study, the analysis of the time course of global withdrawal score confirmed that WT withdrawn mice show an increase of somatic signs within 5–25 min but not 30 min after MEC injection. However, this increase was bigger 10 min after MEC injection, suggesting that in mice the time course of NIC withdrawal could be short and transient, at least in our experimental conditions. Our current results also revealed that the global score of NIC withdrawal was not observed in $GABA_{B1}$ KO mice, at any of the times evaluated. These results indicate that $GABA_B$ receptors may control NIC withdrawal in mice. Interestingly, a previous study from our laboratory demonstrated the ability of baclofen to prevent the incidence of somatic signs during 30 min of NIC withdrawal syndrome (Varani et al., 2011).

Animal (Cheeta et al., 2001; Irvine et al., 2001) and human (Parrott and Garnham, 1998) studies have revealed that NIC withdrawal results in an increased anxiety-like effect, which has been proposed as an affective aspect of NIC withdrawal. The present results also showed that NIC withdrawal induced an anxiety-like effect in WT mice, in the elevated plus maze. These results are in agreement with a previous study from our laboratory (Varani et al., 2014b). Similarly, it has been reported that MEC-precipitated (Jackson et al., 2008; Rehni et al., 2012; Singh et al., 2013) or spontaneous NIC withdrawal (Abreu-Villaça et al., 2008; Jackson et al., 2009; Manhães et al., 2008) induce a reduction in exploration of the open arms in the elevated plus maze in mice. Our study revealed that MEC alone did not change the elevated plus maze responses when compared to littermate control mice, in accordance to previous reports (Roni and Rahman, 2011; Singh et al., 2013). Importantly, we herein observed that the anxiety-like effects induced by MEC-precipitated NIC withdrawal were abolished in $GABA_{B1}$ KO mice, suggesting that these effects could be minimized by the lack of $GABA_B$ receptors. In this respect, we recently observed that the activation of $GABA_B$ receptors by baclofen prevents the anxiety-like responses associated with NIC withdrawal precipitated by naloxone (opioid receptor antagonist) in the elevated plus maze test (Varani et al., 2014b). Thus, our findings reveal that $GABA_B$ receptors could modulate affective aspects of NIC withdrawal in mice.

Several brain areas and neurotransmitter systems are involved in NIC withdrawal (Markou, 2008). Previous studies have shown that deficits in DA and 5-HT transmission in the striatum and cortex could play a role in mediating the somatic expression of NIC withdrawal (Fung et al., 1996; Slotkin and Seidler, 2007; Mannucci et al., 2007). Similarly, we recently found that striatal and cortical DA and 5-HT levels were decreased during NIC withdrawal (Varani et al., 2011). Accordingly, the neurochemical determination performed in the present study revealed that the striatal DA and DOPAC levels decreased in withdrawn WT mice. On the other hand, a previous report from our laboratory showed that baclofen prevents the neurochemical changes induced by NIC withdrawal. These results suggest that the activation of $GABA_B$ receptors by baclofen would modulate GABAergic inputs directly connected with 5-HTergic and DAergic neurons in the striatum and cortex during NIC withdrawal (Varani et al., 2011). Remarkably, we also found a decrease of striatal DA and DOPAC levels in withdrawn $GABA_{B1}$ KO mice, suggesting that the lack of $GABA_B$ would not affect the neurochemical alterations induced by NIC withdrawal in mice.

Although tobacco is used to alleviate the anxiety, long-term tobacco use is also motivated by avoiding negative affective states, such as stress, that emerge during withdrawal (Aronson et al., 2008; Hughes and Callas, 2010; Parrott and Murphy, 2012; Perkins et al., 2012). In fact, stress is a major factor that promotes tobacco use and relapse during withdrawal (Torres et al., 2013). Studies comparing biological indices of stress produced by nicotine withdrawal have demonstrated that plasma levels of corticosterone are increased in animals undergoing NIC withdrawal (Rhodes et al., 2004; Semba et al., 2004; Lutfy et al., 2006). The present results also show that corticosterone plasma levels are increased in WT withdrawn mice. Similarly, it has been shown that NIC withdrawal increased corticosterone plasma levels in rats (Torres et al., 2013) and mice (Ueno et al., 2014). On the other hand, we suggest that $GABA_B$ receptors could be implicated in controlling the increase of corticosterone plasma levels in mice undergoing NIC withdrawal, since this effect was abolished in $GABA_{B1}$ KO mice. Even though additional experiments would be required, our findings provide information about the possible involvement of $GABA_B$ receptors in negative affective states, such as stress, that emerge during NIC withdrawal in mice.

BDNF is present throughout the adult central nervous system (Conner et al., 1997; Erntors et al., 1990), promoting cell survival (Ghosh et al., 1994), regulating dendrite (Xu et al., 2000) and synaptic plasticity (McAllister, 1999; Pattwell et al., 2012). BDNF may facilitate or inhibit drug-seeking behaviours depending on the drug type, the brain site, the addiction phase (initiation, maintenance, or abstinence/relapse) (Ghitza et al., 2010). Regarding NIC, few studies show the role of BDNF in any stage related to the addictive process (Alzoubi and Alkadhi, 2013; Kivinummi et al., 2011; Ortega et al., 2013). In reference to NIC withdrawal, Kivinummi et al. (2011) reported an increase in the BDNF expression only in the Acb of NIC-withdrawn mice. However, our present immunohistochemical analysis revealed that NIC withdrawal decreased the number of BDNF-positive nuclei in the Cpu, BST, Hb, CA1 and CA3 in WT mice, whereas no significant changes in BDNF expression were observed in the other areas studied. Similarly, we previously observed that BDNF expression was decreased in the Cpu, Hb, CA1 and CA3 during NIC withdrawal syndrome in mice (Varani et al., 2014c). Given the fact that BDNF is involved in the regulation of synaptic plasticity (Lipsky and Marini, 2007), the decrease of BDNF immunoreactivity in the Cpu, BST, Hb, CA1 and CA3 during NIC withdrawal, could suggest plasticity alterations in these brain areas during NIC withdrawal. In addition, the decrease in BDNF expression might be attributed to the corticosterone plasma levels which increased in NIC withdrawn mice (Mao et al., 2014; Hill et al., 2014). Taken together, we suggest that a decrease of the synaptic plasticity in these brain areas could play an important role in the modulation of the somatic and motivational components of NIC withdrawal. On the other hand, we found that BDNF expression was no affected in NIC-treated $GABA_{B1}$ KO mice in any of the brain areas studied. These results indicate that the decreased BDNF expression during NIC withdrawal in WT mice could be modulated by $GABA_B$ receptors. In this sense, we have shown that baclofen restored the

decreased BDNF expression during NIC withdrawal in the CPu, Hb, CA1 and CA3 (Varani et al., 2014c). Thus, $GABA_B$ receptors could modulate possible alterations of the synaptic plasticity during NIC withdrawal in specific brain regions.

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels, composed of either homomeric or heteromeric combinations of different subunits, (α2–α10) and (β2–β4), which generates a wide diversity of receptors with various electrical and binding properties (Millar and Gotti, 2009). The most abundant nAChRs subtypes in the central nervous system are homomeric α7 and heteromeric α4β2 (Millar and Gotti, 2009), and they have been proposed to play an important role in NIC addictive properties such as dependence (Benowitz, 2010) and withdrawal syndrome (De Biasi and Salas, 2008). Several studies showed increased levels of nAChRs after NIC withdrawal in the Hb, thalamus, DLG, fr, hippocampus, VTA, IP nucleus, caudate putamen, superior colliculus, Cx and striatum (Gould et al., 2012; Pauly et al., 1996; Slotkin et al., 2007). In accordance, our present autoradiography experiments revealed that NIC withdrawal increased the [^{3}H]epibatidine binding sites in the AcbSh, Hb, VTA, superior colliculus and IP nucleus in WT mice, whereas no significant changes in BDNF expression were observed in the other areas studied. In addition, we previously observed a pronounced increase of [^{3}H]epibatidine binding sites in the AcbSh, Hb, thalamic nuclei, DLG nucleus, fr, VTA, IP nucleus and superior colliculus during the MEC-precipitated NIC withdrawal syndrome in mice (Varani et al., 2013). Additionally, this study provides further information about the specific brain regions and nAChRs subtypes that could mediate the NIC withdrawal syndrome in mice. On the other hand, we found that [^{3}H]epibatidine binding sites was not affected in NIC-treated $GABA_{B1}$ KO mice, in AcbSh, Hb, VTA, superior colliculus and IP nucleus. These findings suggest that the increase of nAChRs levels during NIC withdrawal in WT mice, could be modulated by $GABA_B$ receptors. In this sense, we also reported that baclofen is able to prevent the increase of α4β2 nAChRs levels induced by MEC-precipitated NIC withdrawal in mice (Varani et al., 2013). Taken together, $GABA_B$ receptors could modulate the alterations in the nAChRs levels induced during NIC withdrawal in specific brain regions.

In summary, the present results support the hypothesis that $GABA_B$ receptors play a role in mediating the behavioural and biochemical alterations induced by precipitated NIC withdrawal. By studying $GABA_{B1}$ KO mice we now provide genetic evidence for a specific involvement of $GABA_B$ receptors in the regulation of the behavioural and biochemical effects induced by NIC withdrawal in mice. In this context, we suggest that the lack of $GABA_{B1}$ subunit would prevent the action of the released GABA, leading to a disinhibition of neurons in several brain areas and subsequently to avoid the behavioural and biochemical alterations induced by NIC withdrawal. In addition, our neurochemical and biochemical findings identify possible brain regions that are involved in NIC withdrawal. On the other hand, the fact that similar effects were observed upon activation of $GABA_B$ receptors using baclofen (Varani et al., 2011, 2013; 2014b,c) and $GABA_{B1}$ KO mice, might reveal a compensatory mechanism in order to compensate the lack of $GABA_{B1}$ subunit. Finally, our work supports that $GABA_B$ receptors represent promising targets to treat NIC withdrawal.

Acknowledgements

This work has been supported by grants from University of Buenos Aires (UBACyT 2013-2016, N° 20020120100244BA), CONICET (PIP N° 11420090100303 and N° 2065) and Swiss National Science Foundation (31003A-152970). Andrés P. Varani and Valeria T. Pedrón are supported by a doctoral fellowship from the University of Buenos Aires (6936/2013) and CONICET, respectively. The authors wish to thank Fernanda De Fino and Lidia Caballero for their excellent HPLC technical assistance.

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