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Acute behavioural responses to nicotine and nicotine withdrawal syndrome are modified in $GABA_{B1}$ knockout mice

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ABSTRACT

Nicotine is the main active component of tobacco, and has both acute and chronic pharmacological effects that can contribute to its abuse potential in humans. The aim of the present study was to evaluate a possible role of $GABA_B$ receptors in acute and chronic responses to nicotine administration, by comparing $GABA_{B1}$ knockout mice and their wild-type littermates. In wild-type mice, acute nicotine administration (0.5, 1, 3 and 6 mg/kg, sc) dose-dependently decreased locomotor activity, and induced antinociceptive responses in the tail-immersion and hot-plate tests. In $GABA_{B1}$ knockout mice, the hypolocomotive effect was observed only with the highest dose of nicotine, and the antinociceptive responses in both tests were significantly reduced in $GABA_{B1}$ knockout mice compared to their wild-type littermate. Additionally, nicotine elicited anxiolytic- (0.05 mg/kg) and anxiogenic-like (0.8 mg/kg) responses in the elevated plus-maze test in wild-type mice, while selectively the anxiolytic-like effect was abolished in $GABA_{B1}$ knockout mice. We further investigated nicotine withdrawal in mice chronically treated with nicotine (25 mg/kg/day, sc). Mecamylamine (1 mg/kg, sc) precipitated several somatic signs of nicotine withdrawal in wild-type mice. However, signs of nicotine withdrawal were missing in $GABA_{B1}$ knockout mice. Finally, there was a decreased immunoreactivity of Fos-positive nuclei in the bed nucleus of the stria terminalis, basolateral amygdaloid nucleus and hippocampal dentate gyrus in abstinent wild-type but not in $GABA_{B1}$ knockout mice. These results reveal an interaction between the $GABA_B$ system and the neurochemical systems through which nicotine exerts its acute and long-term effects.

1. Introduction

Tobacco dependence in the form of cigarette smoking is a major public health problem that results in significant morbidity and mortality throughout the world (Murray and Lopez, 1997). Among the components of tobacco, nicotine (NIC) is the main component responsible for its addictive properties. The effects of NIC have been widely studied; in rodents, for example, it has been shown to modify locomotion, anxiety, learning and memory, nociception, and to produce physical dependence after repeated administration (Clarke and Kumar, 1983; Hildebrand et al., 1999; Marubio et al., 1999; Picciotto et al., 1995). NIC exerts its pharmacological effects through the activation of nicotinic acetylcholine receptors (McGehee et al., 1995; Pontieri et al., 1996). This activation promotes the release of diverse neurotransmitters in the central nervous system (CNS), such as glutamate, γ-aminobutyric acid (GABA), acetylcholine, dopamine, norepinephrine and serotonin (Picciotto and Corrigall, 2002). Among these, our main interest has been the study of GABA, the major inhibitory neurotransmitter in the mammalian CNS. This amino acid acts on two classes of receptors: ionotropic $GABA_A$ and $GABA_C$, and metabotropic $GABA_B$ receptors. The $GABA_A$ and $GABA_C$ receptors are located mostly postsynaptically (Barnard et al., 1998), while $GABA_B$ receptors are located both pre and postsynaptically (Bowery et al., 2002). The $GABA_B$ receptors are coupled to G proteins and form a heterodimer of $GABA_{B1}$ and $GABA_{B2}$ subunits, both necessary for $GABA_B$ receptors to be functionally active (Marshall et al., 1999). It has been demonstrated that $GABA_B$ receptors can modulate NIC acute effects, as well as different addictive properties of NIC. In this sense, we have recently shown that the $GABA_B$ antagonist, 2-OH-saclofen, is able to block the anxiolytic and anxiogenic effects induced by NIC (Varani and Balerio, 2012). In

Abbreviations: NIC, nicotine; SAL, saline; GABA, gamma-aminobutyric acid; KO, $GABA_{B1}$ knockout; WT, wild-type; MEC, mecamylamine; 5-HT, 5-hydroxytryptamine; CNS, central nervous system.

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addition, the administration of baclofen, a $GABA_B$ receptor agonist, attenuates the antinociceptive, hypolocomotive (Aso et al., 2007) and rewarding effects induced by NIC (Le Foll et al., 2008). Also, we have previously found that baclofen prevents the neurochemical changes and behavioural manifestations of the mecamylamine-induced NIC withdrawal syndrome in mice (Varani et al., 2011). Furthermore, we have also shown that c-Fos expression was decreased in the caudate putamen and the dentate gyrus of hippocampus (Balerio et al., 2004) after mecamylamine-precipitated NIC withdrawal syndrome. Moreover, preliminary results from our laboratory showed that baclofen was able to normalize the altered c-Fos expression observed during NIC abstinence in the dentate gyrus of the hippocampus, medial habenular nucleus and bed nucleus of stria terminalis (unpublished results).

The neurobiological mechanisms underlying the acute and chronic effects of NIC have been extensively explored using pharmacological approaches in animal models (Markou, 2008). Recently, the availability of genetic engineering provides the generation of knockout mice lacking different components of the GABAergic system, supplying the opportunity to further explore new insights into the participation of this system on the effects of different drugs of abuse, such as NIC. We conducted this study with the aim of evaluating the role of $GABA_B$ receptors in the acute and chronic responses to NIC using knockout mice lacking the $GABA_{B1}$ subunit. We investigated the acute effects of NIC on locomotion, antinociception and anxiety. Moreover, we evaluated the behavioural expression of somatic withdrawal signs and the c-Fos immunoreactivity in NIC-dependent mice after precipitating the NIC withdrawal syndrome with the nicotinic antagonist mecamylamine.

2. Materials and methods

2.1. Animals

Mice lacking the $GABA_{B1}$ subunit of the $GABA_B$ receptor generated in the BALB/C inbred mouse strain and their wild-type littermates (Schuler et al., 2001) were 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). All animals weighing 20–30 g were housed five per cage 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) 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*. Behavioral tests and animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, publication no. 85–23, revised 1985). All experiments were performed with the investigators being blind to genotype and treatment conditions. In order to validate the different experimental protocols, wild-type BALB/C mice were used and the optimal range of NIC doses was based on previous studies (Castañé et al., 2002; Balerio et al., 2004, 2005; Berrendero et al., 2005).

2.2. Drugs

(−)-Nicotine hydrogen tartrate salt ([-]-1-methyl-2-[3-pyridil]pyrrolidine) (NIC) (Sigma Chemical Co., USA) and mecamylamine hydrochloride (Sigma–Aldrich, USA) were dissolved in isotonic (NaCl 0.9%) saline solution and administered subcutaneously (sc) in a volume of 10 ml/kg. All NIC doses are expressed as NIC hydrogen tartrate salt.

2.3. Acute responses to nicotine

In the first set of experiments, $GABA_{B1}$ knockout (KO) and wild-type (WT) littermates mice ($n = 87$) were injected with NIC (0.5, 1, 3, and 6 mg/kg, sc) or saline (SAL). Each mouse received only one injection with one of the doses of NIC, or with SAL. Locomotor activity and antinociceptiception (tail-immersion and hot-plate) were evaluated along a test battery scheme (Fig. 1). In preliminary experiments it has been demonstrated that when the tail-immersion test and the hot-plate test were applied consecutively, the former did not influence the results of the latter (Castañé et al., 2002).

2.3.1. Locomotor activity

Locomotor responses to NIC (0.5, 1, 3 and 6 mg/kg, sc) were evaluated by using a locomotor activity box (22 × 44 × 44 cm) (Infra Red ACTIMETER, Panlab, Spain). The box contained a line of photocells 2 cm above the floor to measure horizontal movements, and another line located 6 cm above the floor to measure vertical activity (rearing). Mice were individually placed in the box 5 min after NIC or SAL injection without previous exposure to the box. The horizontal and vertical activity was recorded for a period of 10 min in a low-luminosity environment (5 lx).

2.3.2. Antinociceptive responses

Two different nociceptive models, the tail-immersion and the hot-plate test were used to evaluate the antinociceptive responses elicited by NIC.

2.3.2.1. Tail-immersion. The tail-immersion test was conducted 15 min after NIC (0.5, 1, 3 and 6 mg/kg, sc) or SAL administration, as previously described (Simonin et al., 1998). The water temperature was maintained at 50 ± 0.5 °C using a thermo regulated water circuit-plating pump (Clifton, North Somerset UK). The trial was terminated once the animal flicked its tail. In the absence of tail-flick, a 10 s cut-off was used to prevent tissue damage.

2.3.2.2. Hot-plate. The hot-plate test was performed 16 min after NIC (0.5, 1, 3 and 6 mg/kg, sc) or SAL injection, as previously described (Simonin et al., 1998). The heated surface of the plate was kept at a temperature of 52 ± 0.1 °C (Ugo Basile, Italy, Model-DS 37). The nociceptive behaviour evaluated was the licking of forepaws or a jumping response. In absence of paw-licking or jump, a 15 s cut-off was used to prevent tissue damage.

2.3.3. Anxiety-related behaviour

Several doses of NIC have been previously tested to select those which produce anxiolytic- and anxiogenic-like responses (0.05 and 0.8 mg/kg, respectively) (Balerio et al., 2005). KO mice and their WT littermates ($n = 71$) were injected subcutaneously with NIC (0.05 and 0.8 mg/kg) or SAL. Elevated plus maze and locomotor activity were evaluated according to the timeline described in Fig. 2.

2.3.3.1. 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), whereas 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 5-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). Entries into the open and closed arms were recorded when the mouse moved both forepaws and the head into the arm, as we previously described (Balerio et al., 2005, 2006; Varani and Balerio, 2012). All observation sessions started 5 min after the acute injection of NIC or SAL.

Once the elevated plus-maze test was finished, the horizontal and vertical activity was evaluated 10 min after the acute injection of NIC or SAL for a period of 5 min, in order to confirm that the NIC doses used (0.05 and 0.8 mg/kg) did not have locomotor effects that could affect the results of the plus-maze test. The locomotor activity box used is the same which was previously described above (see Section 2.3.1).

2.4. Nicotine dependence and withdrawal

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 KO mice ($n = 24$) under brief anaesthesia. NIC concentration was adjusted to compensate for differences in subjects body weight. Thus, each average-weight

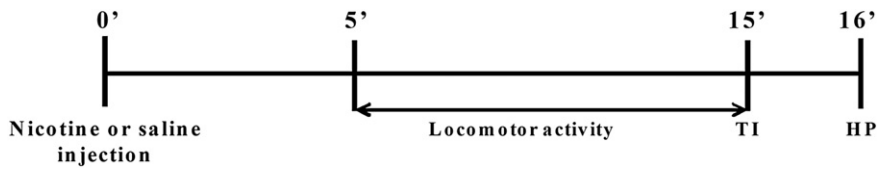

Fig. 1. Schematic representation of the procedure used for locomotor activity and nociceptive tests. TI: tail-immersion test, HP: hot-plate test. Numbers express time in minutes after nicotine or saline injection.

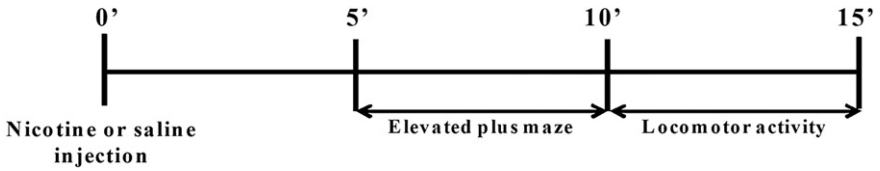

Fig. 2. Schematic representation of the procedure used for the elevated plus-maze test and locomotor activity. Numbers express time in minutes after nicotine or saline injection.

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, mecamylamine (1 mg/kg, sc) (MEC), as described in Castañé et al. (2002) and Balerio et al. (2004) Withdrawal somatic signs were evaluated immediately after MEC injection during a 30-min period, in turn sub divided into 5-min intervals, as previously reported (Castañé et al., 2002). The total number of wet-dog shakes and fore paw tremors was counted. Body tremor, ptosis, teeth chattering, genital licks, and piloerection were scored 1 for appearance or 0 for non-appearance, within each 5-min interval. The locomotor activity in each 5-min interval was rated 0 for inactivity, 1 for low activity, and 2 for normal activity. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight, as previously reported (Castañé et al., 2002).

2.5. c-Fos experiments

2.5.1. Tissue preparation

Six days after SAL or NIC minipump implantation, animals received an injection of either saline or MEC ($n = 12$). Thirty min after, mice 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.

2.5.2. 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 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).

2.5.3. Data quantification

For quantitative analysis, cells positive for Fos-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 Fos-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. Fos-positive cells were quantified in the following brain regions, identified according to the anatomic atlas of Paxinos and Franklin (2004): nucleus accumbens shell (AcbSh) and core (AcbC), cingulate cortex area 1 and 2 (Cg1 and Cg2), 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 medial habenular nucleus (MHb).

2.6. Statistical analysis

Results of all experiments were analyzed by using a two-way ANOVA (genotype and treatment) between subjects followed by a corresponding one-way ANOVA, and post hoc test (Tukey test) where 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. Nicotine hypolocomotion was attenuated in $GABA_{B1}$ knockout mice

The locomotor effects of NIC (0.5, 1, 3 and 6 mg/kg) were evaluated in KO mice and their WT littermates. NIC at the doses of 3 and 6 mg/kg, dose-dependently decreased locomotor activity in WT mice, while in KO mice it only exhibited an attenuated hypolocomotor effect at the highest dose tested (6 mg/kg) (Fig. 3). For horizontal locomotor activity, two-way ANOVA revealed significant effects of treatment ($F_{(4,77)} = 20.716$, $p < 0.001$) and genotype ($F_{(1,77)} = 17.143$, $p < 0.001$), and significant interaction between these two factors ($F_{(4,77)} = 2.856$, $p < 0.05$). Subsequent one-way ANOVA showed a significant effect of NIC treatment in both genotypes ($F_{(9,77)} = 14.752$, $p < 0.001$). Post hoc analysis showed a significant reduction of horizontal locomotor activity at the doses of 3 and 6 mg/kg ($p < 0.001$) in WT mice, but only at the dose of 6 mg/kg in KO mice ($p < 0.05$). Significant differences between genotypes were observed at the doses of 3 ($p < 0.01$) and 6 ($p < 0.05$) mg/kg of NIC. No significant differences were observed between genotypes in mice treated with saline (SAL) or with the two low doses of NIC (0.5 and 1 mg/kg) (Fig. 3A).

For vertical locomotor activity, two-way ANOVA, showed a significant effect of treatment ($F_{(4,77)} = 15.066$, $p < 0.001$), but not of genotype ($F_{(1,77)} = 0.001$, $p = 0.979$), without interaction between treatment and genotype ($F_{(4,77)} = 1.924$, $p = 0.115$). Subsequent one-way ANOVA revealed a significant effect of NIC treatment in both genotypes ($F_{(9,77)} = 9.817$, $p < 0.001$). Post hoc analysis showed a significant decrease of vertical locomotor activity at the doses of 3 and 6 mg/kg ($p < 0.001$) in WT mice, but only at the dose of 6 mg/kg in KO mice ($p < 0.01$). No significant differences between genotypes were observed in SAL- or NIC-treated mice (Fig. 3B).

3.2. Nicotine antinociceptive effect was abolished in $GABA_{B1}$ knockout mice

The antinociceptive effect of NIC (0.5, 1, 3 and 6 mg/kg) was evaluated in KO mice and their WT littermates (Fig. 4). In KO mice, there was no significant antinociception at any of the NIC doses used, neither in the tail-immersion nor in the hot-plate test. In the tail-immersion test (tail-flick response), two-way ANOVA revealed a significant effect of treatment ($F_{(4,77)} = 16.197$, $p < 0.001$), genotype ($F_{(1,77)} = 26.863$, $p < 0.001$), and interaction between these two factors ($F_{(4,77)} = 2.563$, $p < 0.05$). Subsequent one-way ANOVA showed significant effects of NIC treatment ($F_{(9,77)} = 12.504$, $p < 0.001$). Post hoc comparisons revealed a significant effect of NIC treatment at doses of 3 and 6 mg/kg ($p < 0.001$) when compared to the SAL group in WT, but not in KO mice. Significant differences between genotypes were observed at doses 3 and 6 mg/kg ($p < 0.01$). No significant differences were observed between

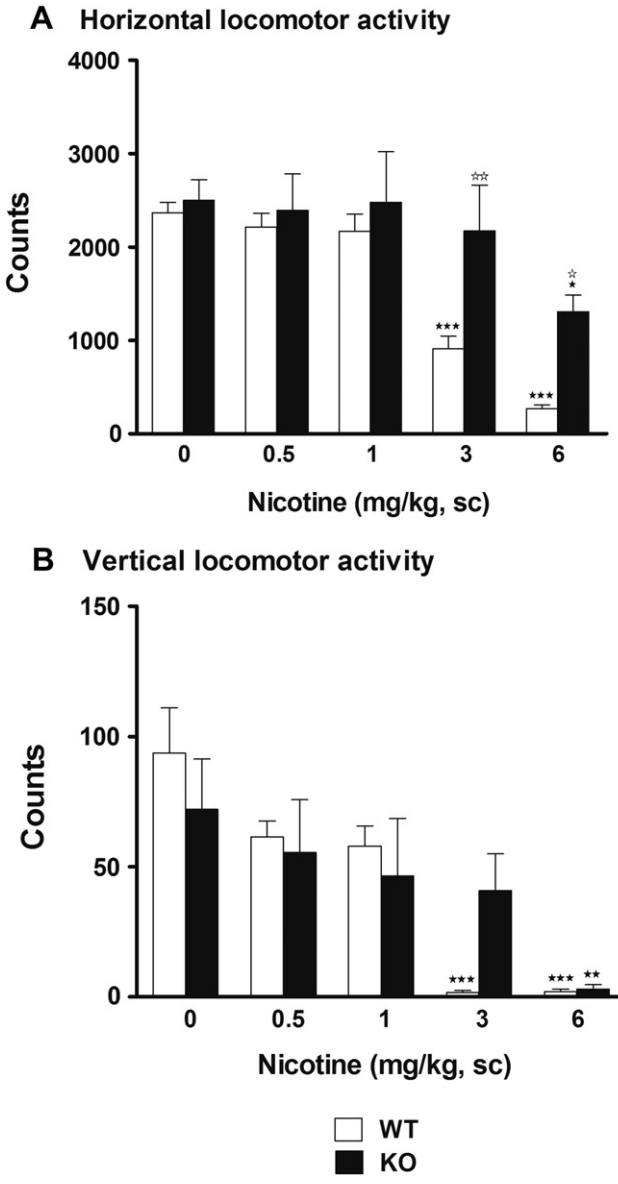

Fig. 3. Nicotine (NIC) hypolocomotion was attenuated in $GABA_{B1}$ knockout mice. Horizontal (A) and vertical (B) activity were measured 5 min after acute nicotine administration (0.5, 1, 3 and 6 mg/kg, sc). Results are expressed as mean ± SEM of photocell counts during a 10-min period in wild-type (WT) (white bars) (saline, $n = 11$; NIC, 0.5 mg/kg, $n = 13$; NIC, 1 mg/kg, $n = 12$; NIC, 3 mg/kg, $n = 14$; NIC, 6 mg/kg, $n = 9$) and $GABA_{B1}$ knockout (KO) (black bars) (saline, $n = 6$; NIC, 0.5 mg/kg, $n = 6$; NIC, 1 mg/kg, $n = 4$; NIC, 3 mg/kg, $n = 5$; NIC, 6 mg/kg, $n = 7$) 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. ★$p < 0.05$; ★★$p < 0.01$; ★★★$p < 0.001$ when compared to vehicle group of the same genotype. ☆$p < 0.05$; ☆☆$p < 0.01$ for between-genotype comparisons.

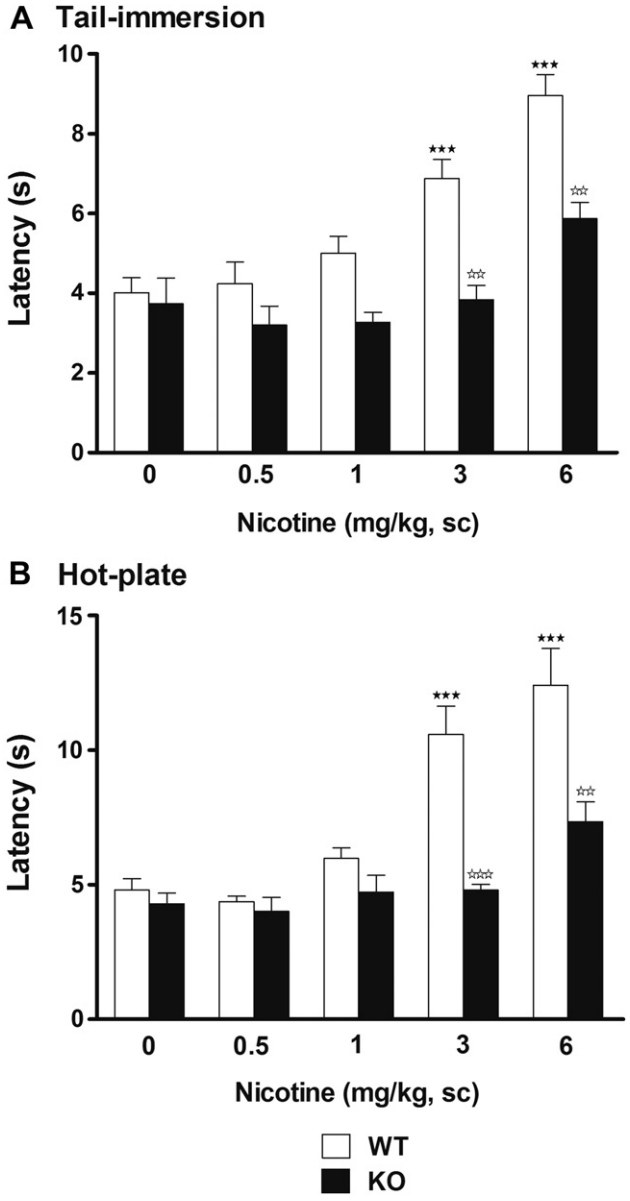

Fig. 4. Nicotine (NIC) antinociception was abolished in $GABA_{B1}$ knockout mice. Antinociceptive responses in the tail-immersion (A) and hot-plate (B) test were measured 15 and 16 min respectively after nicotine administration (0.5, 1, 3 and 6 mg/kg, sc). Results are expressed as mean ± SEM of latency time (in seconds) in wild-type (WT) (white bars) (saline, $n = 11$; NIC, 0.5 mg/kg, $n = 13$; NIC, 1 mg/kg, $n = 12$; NIC, 3 mg/kg, $n = 14$; NIC, 6 mg/kg, $n = 9$) and $GABA_{B1}$ knockout (KO) (black bars) (saline, $n = 6$; NIC, 0.5 mg/kg, $n = 6$; NIC, 1 mg/kg, $n = 4$; NIC, 3 mg/kg, $n = 5$; NIC, 6 mg/kg, $n = 7$) 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.

genotypes in mice treated with SAL or with the two low doses of NIC (0.5 and 1 mg/kg) (Fig. 4A).

In the hot-plate test (jumping and licking response), two-way ANOVA revealed a significant effect of treatment ($F_{(4,77)} = 16.365$, $p < 0.001$), genotype ($F_{(1,77)} = 22.474$, $p < 0.001$), and interaction between these two factors ($F_{(4,77)} = 4.727$, $p < 0.01$). Subsequent one-way ANOVA showed significant effects of NIC treatment ($F_{(9,77)} = 14.810$, $p < 0.001$). Post hoc comparisons revealed a significant effect of NIC treatment at doses 3 and 6 mg/kg ($p < 0.001$) when compared to the SAL group in WT, but not in KO mice. Significant differences between genotypes were observed using doses 3 ($p < 0.001$) and 6 ($p < 0.01$) mg/kg. No significant differences were observed between genotypes in mice treated with SAL or with the two low doses of NIC (0.5 and 1 mg/kg) (Fig. 4B).

3.3. Nicotine anxiolytic- but not anxiogenic-like effect was abolished in $GABA_{B1}$ knockout mice

Results are expressed as percentage of time spent and number of entries into the open arms, two parameters which provide a measure of anxiety. NIC at a dose of 0.05 mg/kg induced

anxiolytic-like responses in WT but not in KO mice, while at 0.8 mg/kg, NIC-induced anxiogenic-like effects in both genotypes (Fig. 5A,B). When a two-way ANOVA was applied to the percentage of entries in open arms results, it revealed a significant effect of treatment ($F_{(2,65)} = 56.532$, $p < 0.001$), genotype ($F_{(1,65)} = 10.962$, $p < 0.01$), and interaction between these two factors ($F_{(2,65)} = 9.962$, $p < 0.001$). Subsequent one-way ANOVA showed significant effects of NIC treatment in WT and KO ($F_{(5,65)} = 3.589$, $p < 0.001$) mice. For the percentage of time spent in open arms results, two-way ANOVA revealed a significant effect of treatment ($F_{(2,65)} = 29.244$, $p < 0.001$), genotype ($F_{(1,65)} = 6.148$, $p < 0.05$), and interaction between these two factors ($F_{(2,65)} = 3.900$, $p < 0.05$). Subsequent one-way ANOVA showed significant effects of NIC treatment in WT and KO ($F_{(5,65)} = 17.792$, $p < 0.001$) mice. Post hoc comparisons revealed that NIC at the dose of 0.05 mg/kg significantly increased the percentage of entries ($p < 0.001$) and time spent ($p < 0.001$) in the open arms in WT but not in KO mice (Fig. 5A and B). On the other hand, NIC (0.8 mg/kg) significantly decreased the percentage of entries ($p < 0.001$) (Fig. 5A) and time spent ($p < 0.01$) (Fig. 5B) in the open arms in WT mice. The same dose also decreased the percentage of entries ($p < 0.05$) (Fig. 5A) and time spent ($p < 0.05$) (Fig. 5B) in the open arms in KO mice. Significant differences between genotypes were only observed at the dose of 0.05 mg/kg, for both the percentage of entries ($p < 0.001$) and time spent ($p < 0.01$) in the open arms. No significant differences were observed between genotypes in SAL-treated mice (Fig. 5A,B).

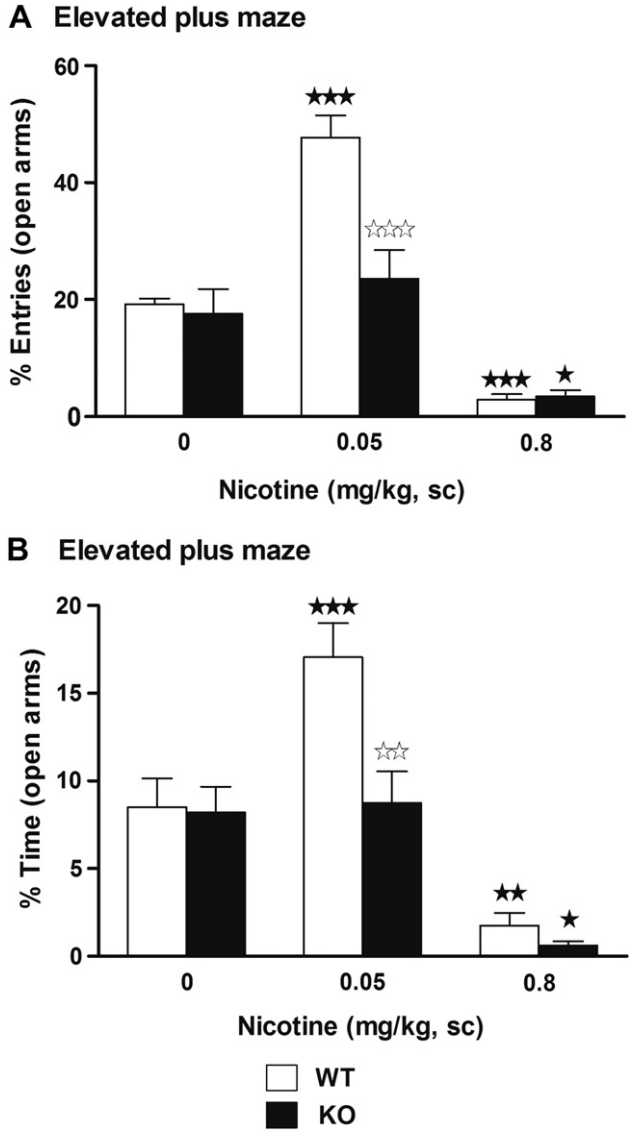

Fig. 5. Nicotine (NIC) anxiolytic- but not the anxiogenic-like effect was abolished in $GABA_{B1}$ knockout mice in the elevated plus-maze test. A) Percentage of entries into in the open arms; B) Percentage of time spent in the open arms. Nicotine (0.05 and 0.8 mg/kg, sc) was administered 5 min before the test. Data are expressed as mean ± SEM of percentage of entries and time in the open arms, in wild-type (WT) (white bars) (saline, $n = 15$; NIC, 0.05 mg/kg, $n = 17$; NIC, 0.8 mg/kg, $n = 14$) and $GABA_{B1}$ knockout (KO) (black bars) (saline, $n = 8$; NIC, 0.05 mg/kg, $n = 8$; NIC, 0.8 mg/kg, $n = 9$) 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. ★$p < 0.05$; ★★$p < 0.01$; ★★★$p < 0.001$ when compared to vehicle group of the same genotype. ☆☆$p < 0.01$; ☆☆☆$p < 0.001$ for between-genotype comparisons.

3.4. *Mecamylamine-precipitated nicotine withdrawal was abolished in $GABA_{B1}$ knockout mice*

No behavioural signs related to NIC withdrawal were observed before mecamylamine (MEC) injection either in NIC-dependent WT or in KO mice (data not shown). After MEC injection, NIC-dependent WT, but not KO mice displayed the constellation of somatic signs previously described by Castañé et al. (2002) and Balerio et al., 2004 to characterize the withdrawal syndrome (Fig. 6A–I).

Two-way ANOVA revealed a significant effect of treatment (NIC–SAL) in the case of body tremor ($F_{(1,20)} = 5.664$, $p < 0.05$), ptosis ($F_{(1,20)} = 10.753$, $p < 0.01$), wet-dog shakes ($F_{(1,20)} = 9.366$, $p < 0.01$), paw tremor ($F_{(1,20)} = 13.167$, $p < 0.01$) and the global score ($F_{(1,20)} = 28.558$, $p < 0.001$), and a significant effect of genotype (WT and KO mice) in the case of body tremor ($F_{(1,20)} = 7.168$, $p < 0.05$), ptosis ($F_{(1,20)} = 6.882$, $p < 0.05$), wet-dog shakes ($F_{(1,20)} = 11.147$, $p < 0.01$), paw tremor ($F_{(1,20)} = 8.930$, $p < 0.01$) and the global score ($F_{(1,20)} = 22.403$, $p < 0.001$). Significant interaction between treatment and genotype was observed in ptosis ($F_{(1,20)} = 8.710$, $p < 0.01$), wet-dog shakes ($F_{(1,20)} = 14.635$, $p < 0.001$) paw tremor ($F_{(1,20)} = 12.859$, $p < 0.01$) and the global score ($F_{(1,20)} = 29.215$, $p < 0.001$). Subsequent one-way ANOVA showed significant effects of NIC treatment in WT and KO mice in the case of body tremor ($F_{(3,20)} = 5.339$, $p < 0.01$), ptosis ($F_{(3,20)} = 8.781$, $p < 0.001$), wet-dog shakes ($F_{(3,20)} = 11.716$, $p < 0.001$), paw tremor ($F_{(3,20)} = 11.652$, $p < 0.001$) and the global score ($F_{(3,20)} = 26.725$, $p < 0.001$). Post hoc comparisons revealed a significant increase of body tremor ($p < 0.05$) (Fig. 6A), ptosis ($p < 0.001$) (Fig. 6B), wet-dog shakes ($p < 0.001$) (Fig. 6C), paw tremor ($p < 0.001$) (Fig. 6D) and the global score ($p < 0.001$) (Fig. 6I) in NIC-treated WT mice compared to the SAL group. When the same analysis was made in KO mice, there were no significant differences between NIC and SAL treatment groups, for any of the withdrawal signs found to be altered in WT mice. Post hoc comparisons also revealed significant differences between genotypes in the case of body tremor ($p < 0.05$) (Fig. 6A), ptosis ($p < 0.01$) (Fig. 6B), wet-dog shakes ($p < 0.001$) (Fig. 6C), paw tremor ($p < 0.001$) (Fig. 6D) and the global score ($p < 0.001$) (Fig. 6I). No significant differences were observed between genotypes in SAL-treated mice (Fig. 6A–I).

3.5. *c-Fos expression was re-established in abstinent $GABA_{B1}$ knockout mice*

c-Fos expression was not altered before mecamylamine (MEC) injection, either in SAL- or NIC-dependent WT and KO mice (data not shown). After MEC injection, NIC-dependent WT, but not KO mice showed a significant reduction in c-Fos expression in bed nucleus of the stria terminalis (BST), basolateral amygdaloid nucleus (BLA) and hippocampal dentate gyrus (DG) (Fig. 7A–C).

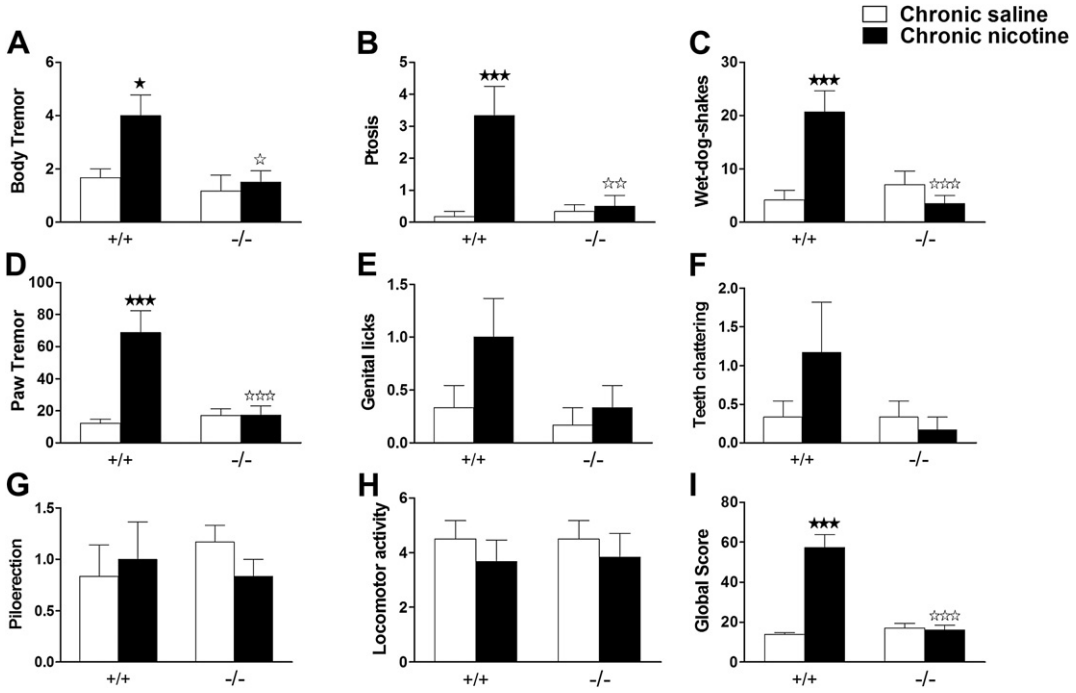

Fig. 6. Nicotine (NIC) withdrawal was abolished in $GABA_{B1}$ knockout mice. Abstinence was precipitated acute administration of the nicotinic antagonist mecamylamine (1 mg/kg, sc) (MEC) after a 6-day period of NIC (25 mg/kg/day) or saline (SAL) infusion by means of subcutaneous minipumps. Counted (wet-dog shakes and paw tremor) and checked (body tremor, ptosis, genital licks, teeth chattering, and piloerection) somatic signs of withdrawal were observed during 30 min immediately after MEC administration. A global withdrawal score was calculated for each animal, as described in the Materials and methods section. Results are expressed as mean ± SEM in chronic saline-treated (white bars) and chronic nicotine-treated (black bars) wild-type (+/+) (SAL, $n = 6$; NIC, $n = 6$) and $GABA_{B1}$ knockout (−/−) (SAL, $n = 6$; NIC, $n = 6$) 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. ★$p < 0.05$; ★★★$p < 0.001$ when compared to vehicle group of the same genotype. ☆$p < 0.05$; ☆☆$p < 0.01$; ☆☆☆$p < 0.001$ for between-genotype comparisons.

Two-way ANOVA revealed a significant effect of treatment (NIC or SAL) in the number of Fos-positive nuclei in BST ($F_{(1,8)} = 5.850$, $p < 0.05$), BLA ($F_{(1,8)} = 15.858$, $p < 0.01$) and DG ($F_{(1,7)} = 5.329$, $p < 0.05$), and a significant effect of genotype (WT and KO mice) in BST ($F_{(1,8)} = 6.042$, $p < 0.05$), BLA ($F_{(1,8)} = 10.794$, $p < 0.01$) and DG ($F_{(1,7)} = 6.220$, $p < 0.05$). Significant interaction between treatment and genotype was observed in BLA ($F_{(1,8)} = 7.095$, $p < 0.05$) and DG ($F_{(1,7)} = 6.001$, $p < 0.05$). Subsequent one-way ANOVA for NIC treatment showed significant effect in both genotypes in BST ($F_{(3,8)} = 5.291$, $p < 0.05$), BLA ($F_{(3,8)} = 11.249$, $p < 0.01$) and DG ($F_{(3,7)} = 6.393$, $p < 0.05$). Post hoc comparisons revealed a significant reduction of Fos-positive nuclei in BST ($p < 0.05$) (Fig. 7A), BLA ($p < 0.01$) (Fig. 7B) and DG ($p < 0.05$) (Fig. 7C) in NIC-treated WT mice compared to the SAL group, while the same analysis for KO mice showed no significant differences between NIC and SAL-treated groups in any of the brain areas analyzed. Post hoc comparisons also revealed significant differences between genotypes in BST ($p < 0.05$) (Fig. 7A), BLA ($p < 0.05$) (Fig. 7B) and DG ($p < 0.05$) (Fig. 7C). No significant differences were observed between genotypes in SAL-treated mice, in any of the brain areas analyzed (Fig. 7A–C).

No significant changes in c-Fos expression were observed in the other brain areas studied (Table 1).

4. Discussion

This study provides further evidence for an involvement of $GABA_B$ receptors in the acute and long-term pharmacological effects of NIC. We examined the behavioural effects of NIC on locomotor activity, nociception, anxiety and dependence and these effects were modified in mice lacking $GABA_B$ receptors. Firstly, acute NIC administration decreased locomotor activity in WT mice. Similarly, several studies showed a dose dependent hypolocomotive effect of NIC in WT mice using the same range of doses we used in the present study (Castañé et al., 2002; Berrendero et al., 2005). However, in KO mice, NIC-induced hypolocomotion was attenuated, since the effect was only seen at the highest dose tested. This result suggests that $GABA_B$ receptors play a role, at least partially, in mediating the hypolocomotor effect induced by NIC. In addition, a previous study from our laboratory using a pharmacological approach showed that the $GABA_B$ receptor agonist, baclofen, was able to prevent the NIC-induced hypolocomotive effect (Aso et al., 2007). Therefore, our present findings together with the study previously mentioned (Aso et al., 2007) would support the idea of a potential relationship between the GABAergic and cholinergic nicotinic systems. On the other hand, our results showed that the spontaneous locomotor activity of KO mice was similar to that observed in WT littermates. Conversely, previous studies revealed that KO mice exhibit a pronounced hyperlocomotor activity when mice were exposed to a new testing environment for a duration of 1–2 h (Schuler et al., 2001; Gassmann et al., 2004). However, Mombereau et al. (2004) observed that the locomotor activity of KO mice can be divided into three phases: a short 'low activity' period (0–5 min), a 'rebound' phase associated with a large increase in locomotor activity (10–45 min), and finally a period of hypoactivity (45–120 min). The fact that in our study the locomotor activity was similar in both genotypes could be due that it was measured for 10 min which corresponds to the first phase described by Mombereau et al. (2004) where the locomotor activity between WT and KO mice seems to be no different.

Secondly, the NIC antinociceptive effect was evaluated in the tail-immersion and hot-plate tests, two models with different neuronal pathways involved in the processing of nociceptive

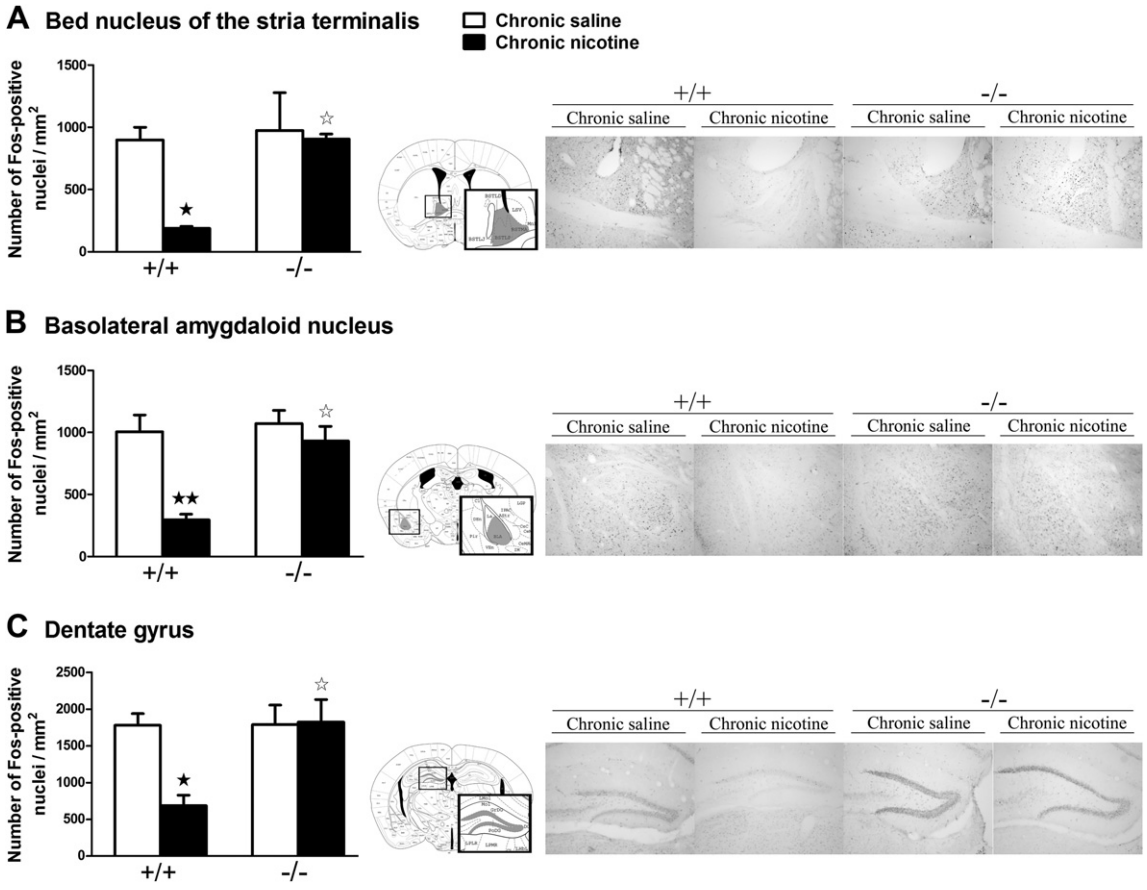

Fig. 7. c-Fos expression was modified during mecamylamine (MEC)-precipitated nicotine (NIC) withdrawal in wild-type (+/+), but not in $GABA_{B1}$ knockout (−/−) mice. Results are expressed as mean ± SEM of Fos-positive nuclei number per mm^2 in bed nucleus of the stria terminalis (BST) (saline, $n = 3$; NIC, $n = 3$; in both genotypes), basolateral amygdaloid nucleus (BLA) (saline, $n = 3$; NIC, $n = 3$; in both genotypes) and dentate gyrus (DG) of the hippocampus (+/+; saline, $n = 3$; NIC, $n = 3$) (−/−; saline, $n = 2$; NIC, $n = 3$). 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. ☆$p < 0.05$ for between-genotype comparisons.

signals. The tail-immersion test evokes a response mediated by a spinal reflex (Caggiula et al., 1995), whereas responses to the hot-plate test require supraspinal integration (Caggiula et al., 1995; Rubinstein et al., 1996) of the nociceptive stimuli. Our results showed that NIC induced an antinociceptive effect in WT mice in both tests. In line with this, previous studies demonstrated antinociceptive effects of acute NIC administration in WT mice tested in the same behavioural tests described above (Galeote et al., 2008; Trigo et al., 2009). However, the present findings revealed that in KO mice NIC-induced antinociception was abolished in both tests. In connection to this, we previously found that systemic administration of baclofen in WT mice was able to decrease the antinociceptive effect induced by NIC in the hot-plate test (Aso et al., 2007). Taken together, these findings also imply an interaction between GABAergic and cholinergic nicotinic systems in the antinociceptive effect induced by NIC. Serotonin (5-HT) releasing spinal neurons from the nucleus raphe magnus have been shown to modulate nociceptive inputs, and activation of these projections mediates NIC-elicited analgesia (Iwamoto, 1991; Bannon et al., 1998). Based on these results, we suggest that NIC increases the levels of endogenous GABA, presumably by stimulating nicotinic acetylcholine receptors (nAChRs) located on GABAergic terminals, which in turn could have a role in the antinociceptive effects induced by NIC. Moreover, Cordero-Erausquin and Changeux (2001) found the existence of a population of nAChRs that exerts a tonic negative modulation on [^{3}H]-5-HT release through the activation of GABAergic interneurons. Therefore, in our current study, the lack of $GABA_B$ receptors would prevent the released GABA from modulating 5-HT release in the spinal cord, with the subsequent abolishment of NIC antinociceptive effect. On the other hand, we observed that the spontaneous nociceptive threshold was similar in both genotypes, exhibiting response latencies in the hot-plate (Blaszczyk et al., 2010; Paudel et al., 2011) and tail-immersion (Castañé et al., 2008; Park et al., 2011) test similar to those described by previous studies. It is well known that KO mice exhibited hyperalgesia in several behavioural tests such as the hot-plate, tail-flick, paw-pressure (Schuler et al., 2001) and formalin

Table 1
c-Fos expression in several brain areas during mecamylamine-precipitated nicotine withdrawal syndrome.

	+/+		−/−	
	Chronic saline	Chronic nicotine	Chronic saline	Chronic nicotine
Nucleus accumbens shell	1105 ± 71	1132 ± 114	1084 ± 55	1129 ± 126
Nucleus accumbens core	654 ± 21	764 ± 99	692 ± 131	709 ± 85
Cingulate cortex	868 ± 179	912 ± 296	891 ± 153	788 ± 208
Caudate putamen	1026 ± 45	945 ± 187	1102 ± 90	1008 ± 263
CA1 of the hippocampus	411 ± 45	472 ± 139	575 ± 264	368 ± 70
CA3 of the hippocampus	1272 ± 173	1236 ± 331	994 ± 218	1105 ± 394
Medial habenular nucleus	363 ± 83	211 ± 71	318 ± 39	366 ± 177

No significant differences were observed between experimental groups. Data represents the mean ± SEM ($n = 2$–3 mice per group).

test (Gangadharan et al., 2009). However, in our conditions the spontaneous nociceptive threshold was not significant different between both genotypes. This discrepancy could be due to different laboratory environment factors (Chesler et al., 2002) such as experimenter, season, cage density, sex, humidity and order of testing. In addition, other influencing factors can be the hot-plate temperature (Imamachi et al., 2009), the animal's age (Tajima et al., 2009; Berry et al., 2007), the time day of testing (Jeong et al., 2000; Konecka and Sroczynska, 1998) and the fact that nociceptive threshold in the tail-flick, paw-pressure and formalin tests could be different.

The present results also provide clear evidence for the involvement of $GABA_B$ receptors in the effects induced by NIC on anxiety-related responses. We have found that the anxiolytic- but not anxiogenic-like effect of NIC was abolished in KO mice. The anxiolytic- and anxiogenic-like effect induced by NIC observed in the present study is in accordance with a previous study from our laboratory using the same doses of NIC (Balerio et al., 2005). The present data demonstrated that anxiety-related behaviour induced by NIC is not related to alterations in locomotor activity, since no changes were observed in locomotor response. As mentioned before, we showed that NIC anxiolytic-like effect was selectively abolished in KO mice. Accordingly, the administration of the $GABA_B$ receptor antagonist 2-OH-saclofen was able to block the anxiolytic-like effects of NIC in mice (Varani and Balerio, 2012). Therefore, the abolishment of NIC-induced anxiolytic-like response in KO mice appears to be due to the absence of GABA effects on $GABA_B$ receptors. Previous studies showed that local administration of NIC into the dorsal raphe nucleus (DRN) induced an anxiolytic-like response (File et al., 1999) apparently by inhibiting the firing of 5-HTergic neurons in this brain area (Engberg et al., 2000), which suggests that NIC anxiolytic-like effect could be related to the decreased 5-HTergic firing. Taken together, we propose that NIC exposure would induce GABA release in the DRN, but in KO mice GABA would not be able to exert its inhibitory effect because of the lack of $GABA_B$ receptors on 5-HTergic neurons. As GABA inhibition of the 5-HTergic neurons does not occur, there would be an increase of 5-HT release, with the resulting abolishment of the anxiolytic-like effect. On the other hand, the present findings showed that the anxiogenic-like effect induced by NIC was not modified in KO mice. This result might reflect a compensatory regulation of other neurobiological mechanisms involved in the anxiogenic responses to NIC. Furthermore, our current results revealed no significant differences in the spontaneous anxiety-related responses between both genotypes. However, Mombereau et al. (2004) showed that the genetic disruption of $GABA_{B1}$ subunit of $GABA_B$ receptors induced anxiogenic-like responses in rodents, in anxiety-related tests such as the light–dark box and staircase test. In contrast, using a pharmacological approach, the $GABA_B$ receptor antagonist SCH 50911 produced anxiolytic-like effects in the elevated zero maze (Frankowska et al., 2007). Thus, the observed discrepancies could be due to differences in the test used, suggesting that the type of anxiety evoked by each test is completely different (Picciotto et al., 2002).

Our findings also revealed that mecamylamine-precipitated NIC withdrawal syndrome was abolished in KO mice. The present results showed that somatic manifestations observed during NIC abstinence syndrome in WT mice, are in accordance with the ones previously described under similar experimental conditions (Castañé et al., 2002; Balerio et al., 2004; Trigo et al., 2009). However, the NIC withdrawal syndrome was abolished in KO mice, suggesting a relevant role of $GABA_B$ receptors in mediating the expression of the somatic signs of NIC withdrawal. Several brain areas and neurotransmitter systems are involved in NIC withdrawal syndrome (Markou, 2008). In this sense, previous studies have shown that deficits in dopamine (DA) and 5-HT transmission in the striatum and cortex could play a role in mediating the somatic expression of NIC withdrawal syndrome (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). On the other hand, baclofen prevented the somatic manifestations and neurochemical changes induced by NIC withdrawal, suggesting a modulation of GABAergic inputs directly connected with 5-HTergic and DAergic neurons in the striatum and cortex (Varani et al., 2011). Thereby, in the current study the lack of $GABA_B$ receptors would prevent the action of the released GABA, leading to a disinhibition of 5-HTergic and DAergic neurons. Thus, NIC withdrawal syndrome would be abolished in KO mice.

Finally, it is known that addictive related behaviours are associated to different molecular adaptations, such as gene regulation, which are observed in specific brain areas (Berke and Hyman, 2000; Nestler, 2000). In line with this, several authors have shown that acute NIC (Salminen et al., 1996), chronic NIC (Soderstrom et al., 2007), NIC self-administration (Pagliusi et al., 1996) and NIC rewarding effects (Mombereau et al., 2007) induced an increase in Fos-like immunoreactivity in diverse brain regions. c-Fos is a transcription factor considered to be a marker of neuronal activity (Dragunow and Faull, 1989). Our current data showed that c-Fos expression was not modified in the BST, BLA and DG of NIC-treated KO and WT mice (data not shown). In agreement, a previous study from our laboratory revealed that chronic NIC treatment is not able to alter the c-Fos expression in the hippocampus and amygdala in mice (Balerio et al., 2004). In addition, other authors observed that in NIC-treated rodents does not change the c-Fos expression in limbic areas compared with control animals (Salminen et al., 1999, 2000; Schroeder et al., 2001). On the other hand, few reports have previously studied the immediate early gene induction following NIC withdrawal. Panagis et al. (2000) found that NIC withdrawal selectively increases the number of Fos-positive nuclei in the central nucleus of the amygdale, but not in other brain areas. In contrast, Marttila et al. (2006) showed that after 48 h of NIC withdrawal, Fos-like immunoreactivity in the nucleus accumbens core and the caudate putamen did not significantly differ compared to the control group. However, our present histochemical analysis revealed that NIC withdrawal syndrome decreased the number of Fos-positive nuclei in the BST, BLA and DG in WT mice, whereas no significant changes in c-Fos expression were observed in the other areas. Similarly, we previously observed that c-Fos expression was decreased in the DG and caudate putamen during NIC withdrawal syndrome in mice (Balerio et al., 2004). On the other hand, in abstinent KO mice, we found that c-Fos expression was re-established, indicating that decreased c-Fos expression during NIC withdrawal in WT mice would be modulated by $GABA_B$ receptors. The fact that the behavioural manifestations of NIC withdrawal syndrome were abolished in KO mice could be related to the re-establishment of c-Fos expression.

In summary, the present results provide solid evidence that supports the involvement of the $GABA_B$ receptors in a variety of pharmacological responses induced by acute and chronic NIC administration, namely hypolocomotion, antinociception, anxiety and dependence. Our behavioural and neurochemical findings highlight some of the neurobiological substrates involved in NIC addiction.

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