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

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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 In NIC withdrawn wild-type mice, we observed a global withdrawal score, an anxiety-like effect in the elevated plus maze, a decrease of the striatal dopamine and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 Interestingly, the effects found in NIC withdrawn wild-type mice were absent in $GABA_{B1}$ knockout mice, suggesting that $GABA_{B1}$ subunit of the $GABA_B$ receptor is involved in the regulation of the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.

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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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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 α4β2 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 $[^3H]$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

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