

## Overexpression of the *CHRNA5/A3/B4* genomic cluster in mice increases the sensitivity to nicotine and modifies its reinforcing effects

Xavier Gallego · Susanna Molas · Alejandro Amador-Arjona · Michael J. Marks · Noemí Robles · Patricia Murtra · Lluís Armengol · Rubén D. Fernández-Montes · Mònica Gratacòs · Martí Pumarola · Roberto Cabrera · Rafael Maldonado · Josefa Sabrià · Xavier Estivill · Mara Dierssen

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**Abstract** Nicotinic acetylcholine receptors (nAChRs) are ligand-gated pentameric ion channels that account for the effects of nicotine. Recent genetic studies have highlighted the importance of variants of the *CHRNA5/A3/B4* genomic cluster in human nicotine dependence. Among these genetic variants those found in non-coding segments of the cluster may contribute to the pathophysiology of tobacco use through alterations in the expression of these genes. To discern the in vivo effects of the cluster, we generated a transgenic mouse overexpressing the human *CHRNA5/A3/B4* cluster using a bacterial artificial chromosome. Transgenic mice showed increased functional  $\alpha3\beta4$ -nAChRs in brain regions where these subunits are highly expressed under normal physiological conditions. Moreover, they exhibited increased sensitivity to the pharmacological effects of nicotine along with higher activation of the

medial habenula and reduced activation of dopaminergic neurons in the ventral tegmental area after acute nicotine administration. Importantly, transgenic mice showed increased acquisition of nicotine self-administration (0.015 mg/kg per infusion) and a differential response in the progressive ratio test. Our study provides the first in vivo evidence of the involvement of the *CHRNA5/A3/B4* genomic cluster in nicotine addiction through modifying the activity of brain regions responsible for the balance between the rewarding and the aversive properties of this drug.

**Keywords**  $\alpha5$  ·  $\alpha3$  ·  $\beta4$  Nicotinic receptor subunits · *CHRNA5/A3/B4* genomic cluster · VTA · MHb · Nicotine addiction

X. Gallego · S. Molas · A. Amador-Arjona · L. Armengol · R. D. Fernández-Montes · M. Gratacòs · X. Estivill · M. Dierssen (✉)  
Genes and Disease Program, Center for Genomic Regulation (CRG), Barcelona Biomedical Research Park (PRBB) Building, Room 522.04, C/Dr. Aiguader 88, 08003 Barcelona, Catalonia, Spain  
e-mail: mara.dierssen@crg.es

X. Gallego · S. Molas · A. Amador-Arjona · R. D. Fernández-Montes · M. Dierssen  
CIBER de Enfermedades Raras (CIBERER), CRG and UPF, Barcelona, Spain

X. Gallego · M. J. Marks  
Institute for Behavioral Genetics, University of Colorado at Boulder, Boulder, CO, USA

N. Robles · J. Sabrià  
Department of Biochemistry, Autonomous University of Barcelona (UAB), Cerdanyola del Vallès, Barcelona, Spain

P. Murtra · R. Cabrera · R. Maldonado  
Unit of Neuropharmacology, Pompeu Fabra University (UPF), Barcelona, Spain

P. Murtra  
Institute of Neuroscience, Miguel Hernández University, Consejo Superior de Investigaciones Científicas (CSIC), Alicante, Spain

L. Armengol · M. Gratacòs · X. Estivill  
Unit of Genetics, Pompeu Fabra University (UPF), Barcelona, Spain

M. Pumarola  
Department of Animal Medicine and Surgery, Autonomous University of Barcelona (UAB), Cerdanyola del Vallès, Barcelona, Spain

## Introduction

Approximately 1.1 billion people use tobacco worldwide and it is the leading preventable cause of death. Each year, the global tobacco epidemic kills nearly 6 million people (<http://www.who.int/tobacco>). Nicotine is the major addictive component of tobacco and exerts its effects by binding to nicotinic acetylcholine receptors (nAChRs) (Benowitz 2010). Neuronal nAChRs are pentameric ligand-gated ion channels that mediate the addictive properties of nicotine (Greenbaum and Lerer 2009) and contribute to various neuropsychiatric disorders (Gotti and Clementi 2004; Greenbaum and Lerer 2009). To date, eight alpha ( $\alpha 2$ – $\alpha 7$  and  $\alpha 9$ – $\alpha 10$ ) and three beta ( $\beta 2$ – $\beta 4$ ) subunits have been localized in the mammalian nervous system. Neuronal nAChRs can be homopentamers or heteropentamers and their subunit composition and stoichiometry confer their pharmacological and functional properties (Albuquerque et al. 2009; Gotti et al. 2009). The high-affinity  $\alpha 4\beta 2$ -nAChR is the most abundant receptor in the brain and the principal mediator of nicotine dependence (Maskos et al. 2005; Mineur and Picciotto 2008; Tapper et al. 2004). Until recently, most research on the role of nAChRs in nicotine dependence has focused on these subunits. This has recently changed and new studies (mainly human genetics) have shown the importance of the *CHRNA5/A3/B4* cluster in nicotine dependence phenotypes (Baker et al. 2009; Berrettini et al. 2008; Bierut et al. 2008; Doyle et al. 2011; Li et al. 2010a, b; Saccone et al. 2009a, b; Schlaepfer et al. 2008; Wang et al. 2009). Evidence for association of genetic variation in the cluster with nicotine dependence-related phenotypes is more robust and convincing than for any other nAChR gene. Although the most plausible evidence for a risk variant is for the non-synonymous SNP (rs16969968) in the *CHRNA5* gene, multiple SNPs in the *CHRNA5/A3/B4* gene cluster are reported to influence the transition from smoking to nicotine dependence (Greenbaum and Lerer 2009; Saccone et al. 2009b). Some of these SNPs are located in non-coding sequences positioned in the  $\beta 4$  3'-untranslated region (McDonough and Deneris 1997; Schlaepfer et al. 2008) or in *CHRNA5* and *CHRNA3* promoter regions (Doyle et al. 2011), suggesting that rather than influence the functionality of a specific subunit these SNPs may alter the regulation of the entire cluster. Indeed, some genetic variants described in humans give rise to overexpression of the genomically clustered genes, found in postmortem brain and lung cancer, suggesting their coordinate regulation (Falvella et al. 2009; Wang et al. 2009). Previous studies in mouse have been carried out by knocking out or overexpressing individual nAChR subunits. These studies have demonstrated the role of  $\alpha 5$  and  $\beta 4$  in fear-related memories (Zhu et al. 2005), anxiety (Kedmi and Orr-Urtreger 2007; Salas et al. 2003b),

nicotine withdrawal (Salas et al. 2004), and nicotine intake (Fowler et al. 2011; Frahm et al. 2011), but currently there are no data regarding the role of the entire cluster in the reinforcing properties of nicotine.

To this end we have generated a BAC transgenic mouse model (Tg*CHRNA5/A3/B4*) that overexpresses the human *CHRNA5/A3/B4* cluster. These transgenic mice have increased epibatidine-binding sites and increased sensitivity to the pharmacological effects of nicotine. Moreover, overexpression of the *CHRNA5/A3/B4* cluster creates a behavioral profile prone to nicotine consumption.

## Methods

### Ethics statement

All animal work has been conducted according to relevant national and international guidelines. Experimental procedures were approved by the Ethical Committee of Animal Experimentation of the PRBB (Comitè Ètic d'Experimentació Animal del Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB, Catalan law 5/1995 and EU directives 86/609/2001-486) and met the Standards for Use of Laboratory Animals no A5388-01 (NIH). The local approval numbers are RML-09-1175-MDS MDS-09-1165.

### Generation and genotyping of transgenic mice

We used a human BAC-clone (NCBI) containing the nicotinic receptor cluster. The presence of all promoter regions was assessed by PCR on maxiprep-extracted DNA from the RP11-335K5 (AC067863) clone, and rearrangements within the BAC were checked [EagI (BshTI) restriction pattern]. DNA plugs were digested using EagI and electrophoresed by Pulsed-Field Gel Electrophoresis. Transgenic mice were generated by standard pronucleus microinjection of the 111-kb fragment containing the three nicotinic receptor subunits ( $\alpha 5$ ,  $\alpha 3$ , and  $\beta 4$ ) on a hybrid B6/SJL-F1 J genetic background (Nucleis, France). Genotyping was performed routinely by PCR analysis using two primer pairs: CHRN4-F 5'-GAGCCAAGATCCCACCACTC-3' and CHRN4-R 5'-CCAGGCATCCGGATTTGTAT-3' and CHRNA5-F 5'-GAAAGACTTGAGTGGGCAGC-3' and CHRNA5-R 5'-CAACCCTGTCTGTCTCTAGC-3'. The presence of the transgene was tested on tail DNA by slot-blot analysis using a 342-bp 32P-labeled fragment against intronic sequence CHRNA3-CHRN4 inserted in the RP11-335K05 BAC (using the primer pairs F 5'-GGGGTGGGTGGAAGTTAGTT-3' and R 5'-TACA CCGCTTCCTTTCAACC-3') as a probe [Slot Blot Manifold, Hoefer (PR 648)]. DNA was quantified by Hoechst 33258 dye (Sigma-Aldrich Chemie, Germany) using a calf

thymus DNA standard curve (Sigma-Aldrich, USA) in a multi-label microplate luminescence reader (TECAN GmbH Infinite TM M200, Austria). Hybrid founders were crossed using B6/SJL-F1J females (F1–F5) to obtain transgenic mice. Non-transgenic (WT) littermates served as controls. Mice were housed 3–5 per cage in standard macrolon cages (40 × 25 × 20 cm) under a 12-h light/dark schedule.

#### mRNA expression analysis

For analysis of the human *CHRNA5*, *CHRNA3*, and *CHRN4* gene expression, total RNA from cortex, hippocampus and cerebellum of WT and Tg*CHRNA5/A3/B4* mice was extracted using TRIzol (Invitrogen, UK) and analyzed by RT-PCR. cDNA was synthesized from 1 µg of total RNA using Omniscript<sup>®</sup> RT Kit (Qiagen, Germany). The cDNA solution was subjected to 35 cycles of PCR amplification using specific primers against the transgene: CHRNA3-F 5'-TGAGCACCGTCTATTTGAGC-3' and CHRNA3-R 5'-ATGAACTCTGCCACCA-3'; CHRN4-F 5'-GCTCTACCCGGTGGCTAT-3' and CHRN4-R 5'-GTAGGGCCCTCAGAAGC-3'; CHRNA5-F 5'-AGAAAGAGAACTGAGAGTGGT-3' and CHRNA5-R 5'-AAAGCCCAAGAGATCCAACA-3'. Quantitative-PCR was performed in duplicates with cDNA (Tg*CHRNA5/A3/B4*  $n = 12$ ; WT  $n = 12$ ) using the LightCycler<sup>®</sup> 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Germany) in a LightCycler<sup>®</sup> 480 Real-Time PCR (Roche Diagnostics, Germany). 45 cycles of PCR amplification were run using specific primers for endogenous nAChR subunits: *Chrna3*-F 5'-AGCACCGCCTGTTCCAGT-3' and *Chrna3*-R 5'-CATGAACTCCACCCCTTGG-3'; *Chrn4*-F 5'-GCAGTCAGTCCACACG-3' and *Chrn4*-R 5'-TAGCCTAGAGGCCCTTGGAG-3'; *Chrna5*-F 5'-AGAAGAAGCCGAGAAAGACG-3' and *Chrna5*-R 5'-AGCCCTAGCGTCCCAATG-3'. *Pgk1* was used as reference gene. Duplicates with  $SD \geq 0.38$  were excluded and re-amplified.  $\Delta\Delta Ct$  method [Relative Expression Software Tool (REST)] was used to quantify the relative amount of mRNA in comparison with control samples and  $2^{-\Delta\Delta Ct}$  was transformed into logarithmic scale ( $\log_2$  ratio).

#### Protein expression analysis

Cortex and hippocampus extracts from adult (3 month) Tg*CHRNA5/A3/B4* ( $n = 6$ ) and WT ( $n = 6$ ) mice were prepared in ice-cold RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS 0.1 M, pH 7.4). Samples were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad, USA), blocked and incubated with anti-nAChR $\alpha 3$  (1:200, Santa Cruz, USA) anti-nAChR $\alpha 5$  (1:200, Santa Cruz,

USA) and anti-nAChR $\beta 4$  (1:200, Abcam, UK) antibodies at 4°C overnight. Specific secondary IgG/HRP (1:2000, Dako Denmark) was followed by ECL assay (Pierce, USA). Tubulin was used as internal standard (1:2000, Upstate, USA).

#### Immunohistochemistry

Mice were habituated to the injection protocol 3 days prior to the experiment. For each experiment two groups per genotype ( $n = 8$ ) were injected subcutaneously (0.1 mL/10 g weight) with saline or nicotine tartrate (Sigma-Aldrich, USA). 120 min after injection mice were euthanized with isoflurane and perfused with 50 mL of PBS 0.1 M and pH 7.4, followed by 150 mL of chilled 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA). To check the activation of VTA dopaminergic neurons, we used a non-aversive dose of 0.5 mg/kg of nicotine, previously described in the literature to induce conditioned place preference in mice and increase the firing rate and burst firing of dopaminergic neurons. Serial coronal frozen sections (30 µm; VTA Bregma  $-2.92$  and  $-4.04$  mm) were incubated with Tyrosine Hydroxylase (TH, 1:4000; Sigma, USA) and c-Fos (1:500, Santa Cruz, USA) antibodies in 1% FBS/PBS overnight at 4°C, followed by incubation with secondary fluorescent-labeled antibodies (1:300, goat anti-rabbit Alexa<sup>®</sup> Fluor 488 and goat anti-mouse Alexa<sup>®</sup> Fluor 594; Molecular Probes, USA) at room temperature 1 h in dark. The same dose of 0.5 mg/kg of nicotine was used to check the activation of the MHb in serial coronal frozen sections (30 µm; MHb Bregma  $-0.82$  and  $-2.30$  mm) incubated with c-Fos primary antibody. The color reaction was developed with a biotinylated secondary antibody, addition of avidin/biotinylated complex followed by incubation with 3,3-diamio-benzide-tetra-hydrochloride (DAB) solution, and counterstained with hematoxylin to delimitate the area of the MHb. Since the dose of 0.5 mg/kg of nicotine was not able to significantly activate c-Fos expression in the MHb of any genotype, we further examined if the MHb/IPN circuit was altered in our transgenic mice using a dose of nicotine of 6 mg/kg. Serial coronal frozen sections (30 µm; MHb Bregma  $-0.82$  and  $-2.30$  mm) were incubated with c-Fos primary antibody following the above-mentioned protocol and using a secondary fluorescent-labeled antibody (1:300, goat anti-rabbit Alexa<sup>®</sup> Fluor 488; Molecular Probes, USA). Hoechst staining was used to delimitate the area of the MHb. The number of positive neurons was counted under fluorescence/bright microscope at 40× and the area of the MHb was delimited using the ImageJ Software.

## Receptor-binding autoradiography

Mice ( $n = 3$  per genotype) were killed by cervical dislocation; the brain was rapidly ( $<1$  min) removed from the skull and quickly frozen by immersion in isopentane ( $-35^{\circ}\text{C}$ ) for 10 s, and stored at  $-70^{\circ}\text{C}$  until sectioning. Coronal sections ( $14\ \mu\text{m}$  thick) were obtained using a Leica CM 1850 cryostat/microtome (Leica, Germany) and thaw-mounted on Fisher Suprafrost/Plus microscope slides. A series of ten sets of sections was prepared from each brain to allow comparison of results for several different experiments on adjacent or near-adjacent sections. Slides containing the brain sections were desiccated and stored, at  $-70^{\circ}\text{C}$  until use.

### $[^{125}\text{I}]$ Epibatidine autoradiography

Slides were warmed to room temperature and subsequently transferred to Bel-Art slide racks and rehydrated by incubation at  $22^{\circ}\text{C}$  for 15 min in isotonic buffer (NaCl, 144 mM; KCl, 2.2 mM,  $\text{CaCl}_2$ , 2.0 mM,  $\text{MgSO}_4$ , 1.0 mM; HEPES, 25 mM;  $\text{pH} = 7.5$ ). The racks containing the rehydrated slides were subsequently transferred to the isotonic buffer containing 200 pM  $[^{125}\text{I}]$ -epibatidine.  $[^{125}\text{I}]$ -Epibatidine (Perkin-Elmer NEN, Boston, MA, original specific activity 2200 Ci/mmol) was mixed with unlabeled I-epibatidine to yield a final specific activity of 110 Ci/mmol (20-fold dilution). Samples were incubated for 2 h at  $22^{\circ}$ . A second set of incubations with  $[^{125}\text{I}]$ -epibatidine also included 10 nM 3-((2S-azetidylmethoxy)-5-iodo)-pyridine (5I-A-85380, Tocris Bioscience, Ellisville, MO) to selectively inhibit binding to  $\beta 2^*$ -nAChRs (Mukhin et al. 2000) such that the residual signal represented binding of  $\beta 4^*$ -nAChRs. Following the incubation the slides were redistributed to slide racks containing 25 slides and washed as follows (all solutions at  $4^{\circ}\text{C}$ ): Twice for 30 s in isotonic buffer, twice for 5 s in hypotonic buffer ( $0.1\times$ ) and twice for 5 s in 10 mM HEPES,  $\text{pH} = 7.5$ . The samples were then dried and stored desiccated at room temperature in vacuum overnight before exposure to Packard Super Resolution Cyclone Storage Phosphor Screens to yield images for quantitation and subsequently to Kodak MR autoradiography film to yield higher resolution images for photography. Each Phosphor Screen was also simultaneously exposed to a series of tissue paste standards containing measured amounts of  $^{125}\text{I}$  to allow quantitation of the image intensity. Images were captured using a Cyclone Storage Phosphor Screen (Perkin-Elmer).

### Quantitation

Tissue paste samples prepared from whole brain homogenates were used to construct standard curves. The

Phosphor screens yield a linear relationship between signal intensity and tissue radioactivity content over several orders of magnitude. The regression line calculated for the standard curve was used to convert the measure value of pixels/ $\text{mm}^2$  to the cpm/mg wet weight from which signal intensity in fmol/mg wet weight was estimated from the specific activity of each ligand. Brain regions were identified using the mouse brain atlas (Franklin and Paxinos 1997). Several measurements were made in each brain region of each mouse and the average of these measurements defined the signal intensity for each region.

### Behavioral characterization

Male Tg $CHRNA5/A3/B4$  and WT littermates (2–6 months of age; see number of animals per each experiment below) from 6–8 different litters were used. 30 mice (15 per genotype) were tested at 2 months of age on behavioral measurements of gait, balance, reflexes, and startle response using SHIRPA protocol (Nolan et al. 2000). Behaviors were scored to provide a semi-quantitative assessment. In the open-field apparatus ( $70 \times 70 \times 60$  cm) (Altafaj et al. 2001; Escorihuela et al. 1995), 14–18 mice per genotype were individually placed in the center of the field (500 Lux; 5 min) and total distance travelled, speed, time in each zone and number of rearing, grooming and fecal boli (Maccarrone et al. 2002) were recorded (SMART, Panlab, Spain). Locomotor activity was evaluated in activity cages (LE 881 Panlab, Spain;  $45 \times 45$  cm) for 10 min (Actitrack software, Panlab, Spain). Randomly assigned groups of Tg $CHRNA5/A3/B4$  and WT ( $n = 16$  per treatment and genotype) were injected with saline or nicotine (0.5 or 1 mg/kg s.c.). Five minutes after injection mice were placed in the activity cages (20–25 Lux). For nicotine-induced seizures mice received either nicotine tartrate (Sigma-Aldrich, USA dissolved in 0.9% NaCl;  $n = 24$ ) or saline (s.c. 0.1 mL/10 g of body weight,  $n = 22$ ). We used different nicotine doses per genotype (WT: 12, 25 and 50 mg/kg vs. Tg $CHRNA3/B4/A5$ : 4, 6 and 12 mg/kg). After injection, behavior was recorded for 5 min. The effects of nicotine were scored from 0 to 7: 0, no effects; 1, locomotor alterations; 2, tachypnea and tremors; 3, back arching; 4 myoclonic seizures; 5, clonic seizures; 6, tonic seizures; and 7, death. Concentration–response curves were fitted with a logistic curve to determine the  $\text{ED}_{50}$ .

### Nicotine self-administration

Adult mice, WT ( $n = 14$ ) and Tg $CHRNA5/A3/B4$  ( $n = 16$ ) were implanted with a cannula into the right jugular vein. Nicotine self-administration sessions (Soria

et al. 2005) started 3 days after surgery. Responding was maintained by nicotine (0.015 mg/kg per infusion) delivered in 23.5  $\mu$ l over 2 s. Nicotine was infused via a syringe mounted on a microinfusion pump (PHM-100A, Med-Associates, USA) connected to a single-channel liquid swivel (375/25, Instech Laboratories, USA). Two hours daily self-administration sessions were conducted 6 days per week. The house light was “on” at the beginning of the session for 3 s and then remained “off”. Each daily session started with a priming injection of the drug. Mice were trained under a fixed ratio 1 (FR1) schedule of reinforcement. A 10-s time-out period was established after reinforcement. Responses on inactive hole and all responses during the 10-s time-out period were recorded. Session terminated after 50 reinforcers were delivered or after 2 h. The criteria for acquisition was achieved when mice maintained a stable response with less than 20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability), with at least 65% responding on the active hole, and a minimum of four reinforcers per session. Once achieved acquisition, mice were moved from FR1 to progressive ratio (PR) with response requirement to earn an injection escalating according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The PR session lasted for 3 h or until mice did not complete the ratio for delivery of one reinforcer within 1 h.

#### Data analysis

Parametric data are reported as mean  $\pm$  standard error of mean (SEM). Whenever differences were observed per transgenic line, data were analyzed separately. For the open field we used Student's *t* test for comparison between genotypes. In the SHIRPA protocol, we used the Fisher non-parametric test since data did not fit parametric assumptions. Data analysis for nicotine effect was performed using non-linear regression analysis and two-way ANOVA to analyze genotype X treatment interaction. qRT-PCR results were analyzed using Pair Wise Reallocation Randomisation Test©. For the binding assays and western blot, values were compared by one-way ANOVA, using Tukey HSD post hoc test. Nicotine self-administration was analyzed using repeated measures analysis. Student's *t* test was used to analyze differences in number of nose-pokes between the active and inactive hole, and differences between genotypes for the progressive ratio and day of criteria acquisition. The statistical analysis was performed using the SPSS 12.0 software, except for the qRT-PCR analysis that was performed using Relative Expression Software Tool—384 (REST-384© v.2).

## Results

### Generation of *TgCHRNA5/A3/B4* mice

For the generation of *TgCHRNA5/A3/B4* mice we used a BAC inserted with the human cluster (Fig. 1a). Two lines were generated carrying between 16–18 (*Tg22*) and 4–5 copies (*Tg30*) of the transgene, respectively, as detected by Slot-blot (Fig. 1b). Similar mRNA expression of the  $\alpha$ 3,  $\beta$ 4, and  $\alpha$ 5 subunits was confirmed in both lines by RT-PCR (Fig. 1c). Western blot analysis (Fig. 1d, e) confirmed increased expression of the  $\beta$ 4 subunit in transgenic hippocampus ( $F_{(2,10)} = 4.90$ ,  $p < 0.05$ , one-way ANOVA), while expression of the  $\alpha$ 3 and  $\alpha$ 5 subunits was similar to WT. Conversely, in the cerebral cortex a significant increase of the  $\alpha$ 3 subunit was observed in both transgenic lines compared with WT ( $p < 0.05$ , one-way ANOVA).

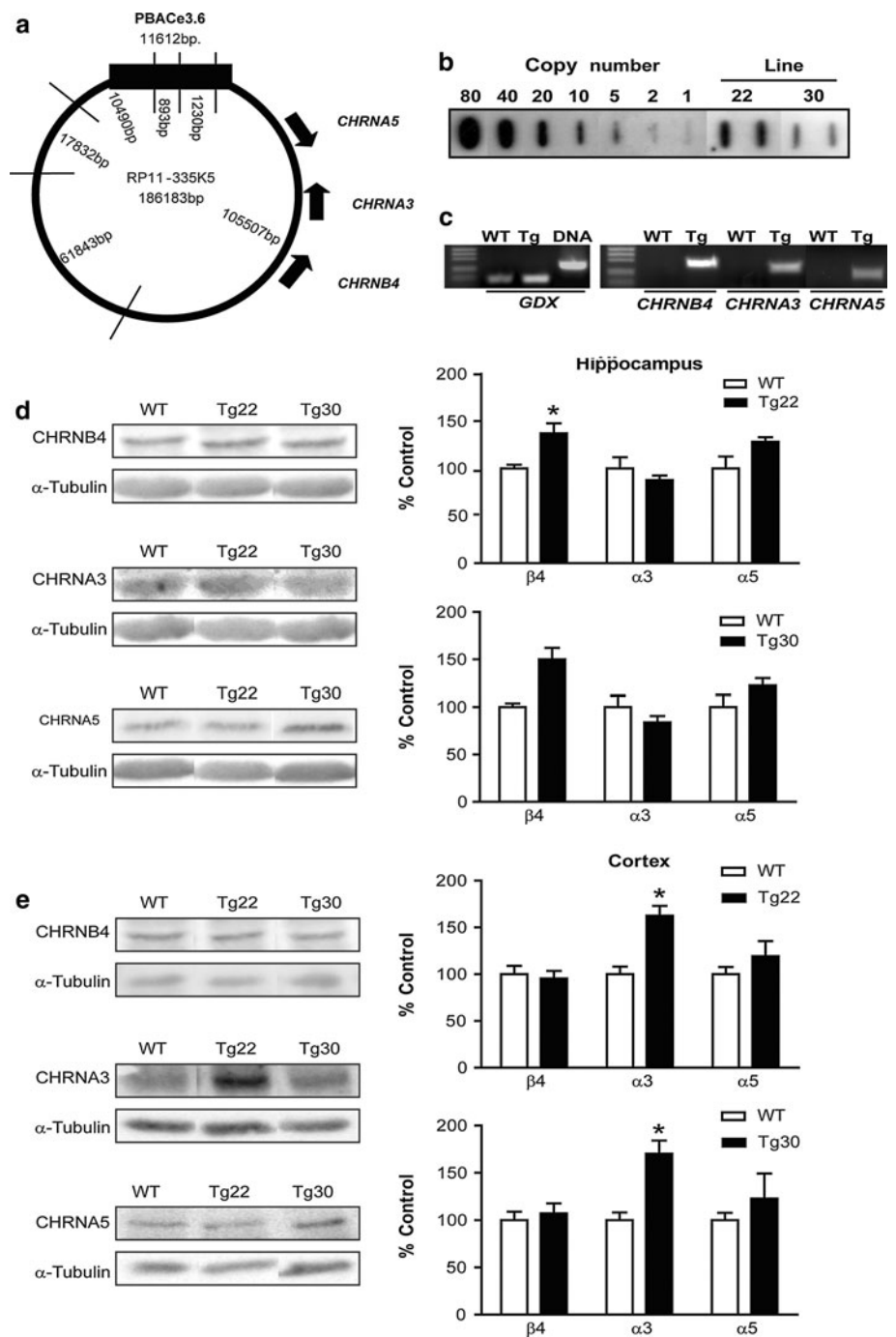
We investigated the regulation of the endogenous *Chrna5*, *Chrna3*, and *Chrb4* genes. qRT-PCR analysis (Table 1) with probes designed to detect mouse receptor subtypes did not show significant differences between genotypes in endogenous nAChR subunits expression in the cerebral cortex. However, *TgCHRNA5/A3/B4* mice showed a reduction in the *Chrna3* (−42.8%,  $p < 0.01$ ) expression in the hippocampus, without differences in the *Chrb4* and *Chrna5* mRNA levels with respect to WT.

### [<sup>125</sup>I]-epibatidine binding sites

Images for [<sup>125</sup>I]-epibatidine binding to sections from WT and transgenic mice at five anatomical levels are shown in Fig. 2. This figure includes images from near adjacent section illustrating total [<sup>125</sup>I]-epibatidine binding and binding in the presence of 5I-A-85380, in which labeling of  $\beta$ 2\*-nAChR sites have been selectively inhibited. Both total binding and that in the presence of 5I-A-85380 indicated that there is no ectopic expression in the transgenic mice. Significant increases in total [<sup>125</sup>I]-epibatidine binding were detected in olfactory bulb, CA1 region of hippocampus, superficial gray area of the superior colliculus and pyriform cortex (Table 2). Subsequent analysis of the [<sup>125</sup>I]-epibatidine binding in the presence of 5I-A-85380, where labeling is restricted to  $\beta$ 4\*-nAChR binding sites, allowed the detection of additional sites. Significant increases in 5I-A-85380-resistant [<sup>125</sup>I]-epibatidine binding sites were noted in olfactory bulb, medial habenula, CA1 region of hippocampus, superior colliculus (both superficial gray and deeper layers), dorsal tegmental nucleus, and pyriform cortex. Although there was a tendency for increased binding in fasciculus retroflexus and interpeduncular nucleus, these changes were not statistically significant. In addition, lower levels of 5I-A-85380-resistant [<sup>125</sup>I]-epibatidine binding were found in the thalamus,

**Fig. 1** Generation and general characterization of TgCHRNA5/A3/B4 mice.

**a** Vector pBACe3.6 information carrying the Homo sapiens chromosome 15; clone RP11-335K5. **b** Slot-blot analysis of the transgene. Control of copy number is shown from 1 to 80 copies of the transgene. Line 22 and line 30 show 16–18 and 4–5 copies of the transgene, respectively. **c** Reverse transcriptase PCR of *CHRNA5*, *CHRNA3*, and *CHRNB4*. Note that cDNA control of all samples was performed hybridizing cDNA and DNA samples with Gdx primers. **d**, **e** Representative Western blot showing *CHRNA5*, *CHRNA3*, and *CHRNB4* immunolabeling of WT (*open bars*) and TgCHRNA5/A3/B4 (*filled bars*) mice hippocampus and cortex. Tubulin was used as loading control. Relative immunoreactivity was determined densitometrically. Ratios are expressed as mean percentage of control (WT)  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  comparison between genotypes, one-way ANOVA



cerebral cortex, hippocampus, and striatum, being slightly increased in transgenic mice but reaching statistical significance in the hippocampus and striatum.

#### General phenotypic characterization

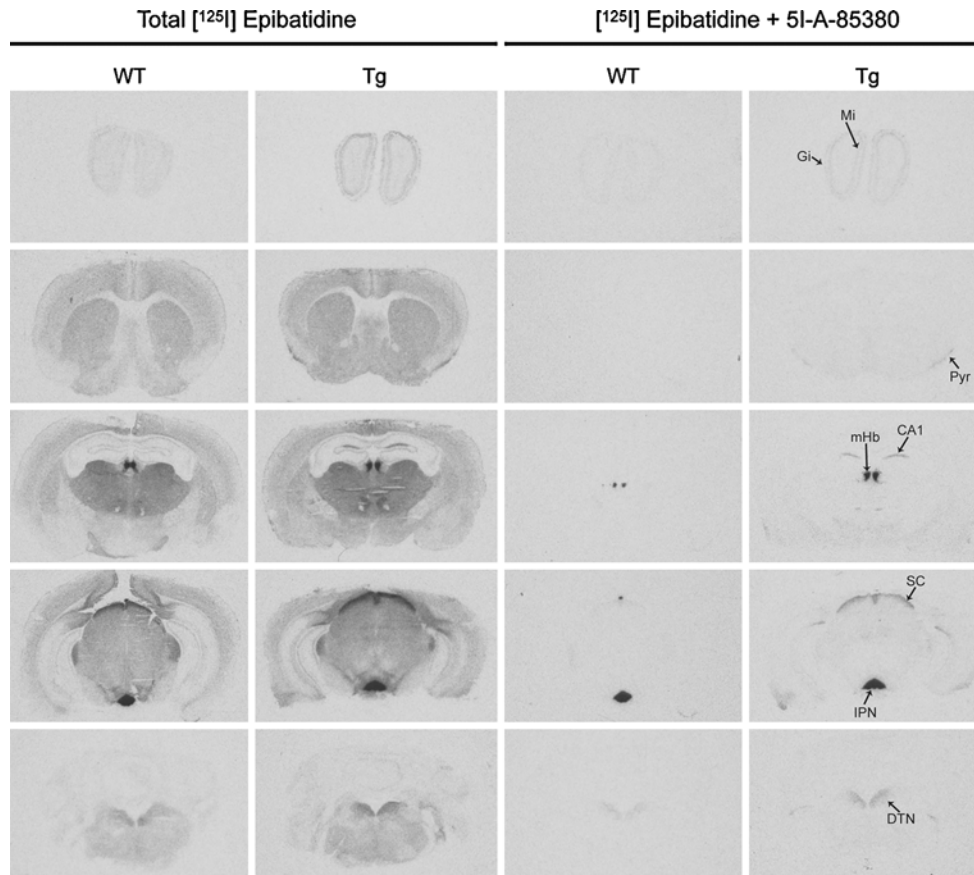
In the behavioral/neurological experiments we first ensured that the cluster overexpression had no consequences on the somatometric, neurologic or gross sensorimotor aspects (Table 3). TgCHRNA5/A3/B4 mice showed significantly

reduced sensitivity in the toe-pinch test ( $p = 0.01$ , Fisher Test) and performed better the balance test ( $p < 0.05$ , Fisher Test). In the open field, transgenic mice spent less time in the center ( $t_{(1,29)} = 2.52$ ,  $p < 0.05$ , Student's  $t$  test; Fig. 3a) and remained in the periphery ( $t_{(1,29)} = -2.52$ ,  $p < 0.05$ ; Fig. 3a). Besides, TgCHRNA5/A3/B4 mice showed increased speed when crossing the center of the apparatus compared with WT mice ( $t_{(1,29)} = -2.69$ ,  $p = 0.01$ ; Fig. 3b). Finally, the latency to enter the periphery was reduced in TgCHRNA5/A3/B4 mice

**Table 1** Expression of the endogenous nAChR subunits by quantitative RT-PCR analysis in TgCHRNA5/A3/B4 versus WT mice

Gene	Region	Fold-change*	SE	p value	Sig.
Chrna3	Cortex	0.737	0.163	0.290	
	Hippocampus	0.572	0.098	0.009	**
Chrna5	Cortex	0.999	0.146	0.980	
	Hippocampus	0.791	0.317	0.379	
Chrb4	Cortex	0.839	0.059	0.061	
	Hippocampus	0.618	0.125	0.076	

\*  $p < 0.05$ , \*\*  $p < 0.01$ , Pair Wise Reallocation Randomisation Test© (Relative Expression Software Tool—384 (REST-384© vs.2)



**Fig. 2** Increased [<sup>125</sup>I]-epibatidine binding sites in TgCHRNA5/A3/B4 mice. [<sup>125</sup>I]-epibatidine binding in sections from WT and TgCHRNA5/A3/B4 mice at five anatomical levels. Columns on the left side show  $\alpha3\beta4^*$  and  $\alpha4\beta2^*$  binding sites. Columns on the right

side show  $\alpha3\beta4^*$  specific binding sites. *Mi* mitral cells, *Gi* granule layer, *Pyr* pyriform cortex, *mHb* medial habenulla, *CA1* CA1 region of the hippocampus, *SC* superior colliculus, *IPN* interpeduncular nucleus, *DTN* dorsal tegmental nucleus

( $t_{(1,29)} = 2.72$ ,  $p = 0.01$ ; Fig. 3c). In pharmacological studies, increasing doses of nicotine progressively decreased locomotion, with genotype-dependent differences (Two-way ANOVA genotype  $\times$  treatment  $F_{(4,153)} = 6.34$ ,  $p = 0.0001$ ; Fig. 3d). At 1 mg/kg, the effect of nicotine was significantly more marked in TgCHRNA5/A3/B4 mice ( $t_{(1,32)} = 3.26$ ,  $p < 0.01$ , Student's *t* test). For nicotine-induced seizure studies nicotine administration induced dose-dependent seizures in both genotypes, but more marked in TgCHRNA3/B4/A5 (two-way ANOVA

genotype  $\times$  treatment  $F_{(2,62)} = 9.59$ ,  $p = 0.00$ ). Dose-response curve was shifted to the left (Fig. 4a) and ED<sub>50</sub> was reduced ( $26.33 \pm 1.15$  in WT vs.  $6.5 \pm 1.23$  in TgCHRNA3/B4/A5  $F_{(1,4)} = 12.02$ ,  $p < 0.05$ , one-way ANOVA, Fig. 4b).

Nicotine self-administration

Repeated measures analysis showed main effects of active versus inactive nose-pokes in WT ( $F_{(1,22)} = 4.55$ ,

**Table 2** Brain regions of increased [<sup>125</sup>I]-epibatidine binding sites

Brain region	[ <sup>125</sup> I] epibatidine Fmol/mg (mean ± SEM)		[ <sup>125</sup> I] epibatidine + 5I-A-85380 Fmol/mg (mean ± SEM)	
	WT	Tg	WT	Tg
Olfactory bulb	1.61 ± 0.15	3.15 ± 0.30**	0.58 ± 0.05	1.12 ± 0.06**
Accessory olfactory bulb	17.91 ± 2.46	24.15 ± 3.07	8.28 ± 0.77	12.83 ± 2.98
Striatum	7.27 ± 0.53	8.69 ± 0.12	0.20 ± 0.02	0.56 ± 0.09*
Cerebral cortex	4.68 ± 0.09	5.91 ± 0.17*	0.09 ± 0.002	0.71 ± 0.36
Thalamus	21.89 ± 0.66	24.79 ± 0.48	0.29 ± 0.06	1.09 ± 0.21
Medial habenula	116.92 ± 2.50	123 ± 7.11	52.39 ± 5.62	83.67 ± 3.54**
Hippocampus, CA1	4.24 ± 0.36	10.11 ± 0.37*	0.89 ± 0.74	3.69 ± 0.32*
Hippocampus, other	2.65 ± 0.18	3.52 ± 0.49	0.17 ± 0.02	0.44 ± 0.03**
Fasciculus retroflexus	43.61 ± 3.95	34.96 ± 3.82	18.35 ± 1.85	23.81 ± 1.09
Interpeduncular nucleus	155.53 ± 28.55	143.06 ± 13.45	67.82 ± 9.80	86.62 ± 7.61
Superior colliculus, superficial gray	21.87 ± 1.67	37.90 ± 3.43*	0.75 ± 0.04	6.42 ± 0.77*
Superior colliculus, deeper layers	15.41 ± 0.53	19.11 ± 2.19	0.49 ± 0.05	3.37 ± 0.32**
Inferior colliculus	13.77 ± 0.54	17.43 ± 1.47	4.79 ± 0.89	5.42 ± 0.83
Dorsal tegmental nucleus	12.22 ± 1.19	14.27 ± 1.34	2.16 ± 0.35	4.72 ± 0.43**
Pyriiform cortex	1.33 ± 0.77	6.47 ± 0.94*	0.39 ± 0.03	2.00 ± 0.25**

Data are expressed as mean ± SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$  comparison between genotypes, Student's  $t$  test,  $n = 3$  per genotype

**Table 3** General phenotypic characterization (SHIRPA)

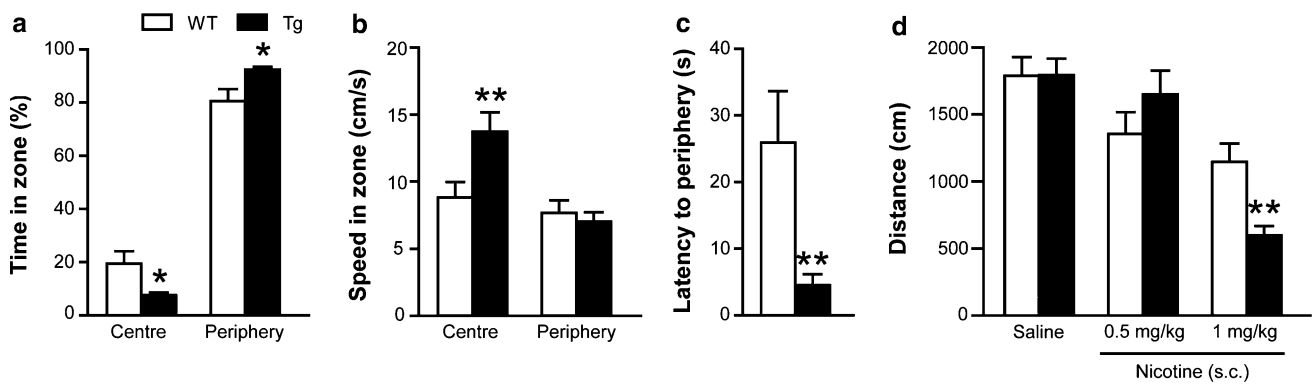
	WT (%)	TgCHRNA3/A5/B4 (%)	Fisher test
<b>Balance</b>			
1	13 (68.4)	6 (31.6)	0.0218*
2	0 (0.0)	5 (100.0)	
3	3 (42.9)	4 (57.1)	
<b>Prehensile reflex</b>			
1	15 (50.0)	15 (50.0)	0.9999
2	1 (100.0)	0 (0.0)	
<b>Traction</b>			
0	6 (85.7)	1 (14.3)	0.0631
1	5 (62.5)	3 (37.5)	
2	5 (35.7)	9 (64.3)	
3	0 (0.0)	2 (100.0)	
<b>Motor coordination</b>			
1	4 (0–15)	4 (0–5)	0.2692
2	17 (10–47)	16 (7–49)	0.8492
3	60 (8–60)	60 (12–60)	0.3824

\*  $p < 0.05$ , Fisher test

$p < 0.05$ ; Fig. 5a) and TgCHRNA5/A3/B4 mice ( $F_{(1,30)} = 9.018$ ,  $p < 0.01$ ; Fig. 5b) along days. WT mice discriminated the active and inactive holes on the seventh day of acquisition ( $t_{(1,27)} = 2.08$ ,  $p = 0.05$ , Student's  $t$  test), whereas TgCHRNA5/A3/B4 mice started to discriminate the two holes since the first day ( $t_{(1,30)} = 2.13$ ,  $p < 0.05$ , Student's  $t$  test). Four WT and five TgCHRNA5/A3/B4 mice (28.57 and 31.25%, respectively) achieved the

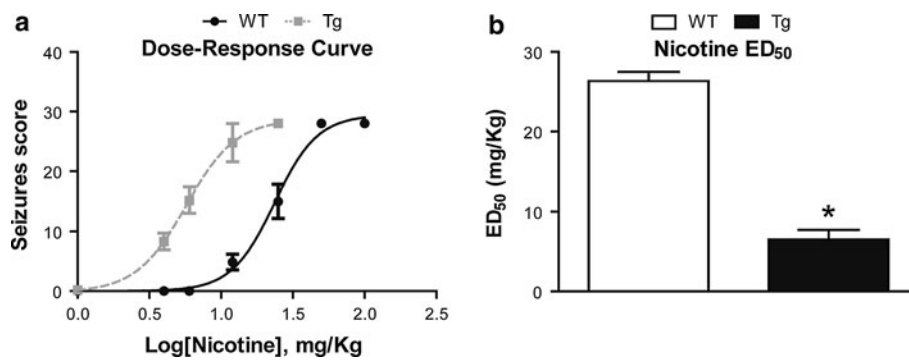
acquisition criteria and were moved to the progressive ratio test (Fig. 5c). TgCHRNA5/A3/B4 mice reached the FR1 stability criteria before WT littermates ( $6.60 \pm 1.29$  vs.  $9.50 \pm 0.87$ , respectively), but not significantly. Progressive ratio analysis revealed that TgCHRNA5/A3/B4 mice reached a higher breaking point than the WT ( $t_{(1,7)} = -3.11$ ,  $p < 0.05$ , Student's  $t$  test), indicating enhanced motivation of transgenic mice to seek for nicotine.





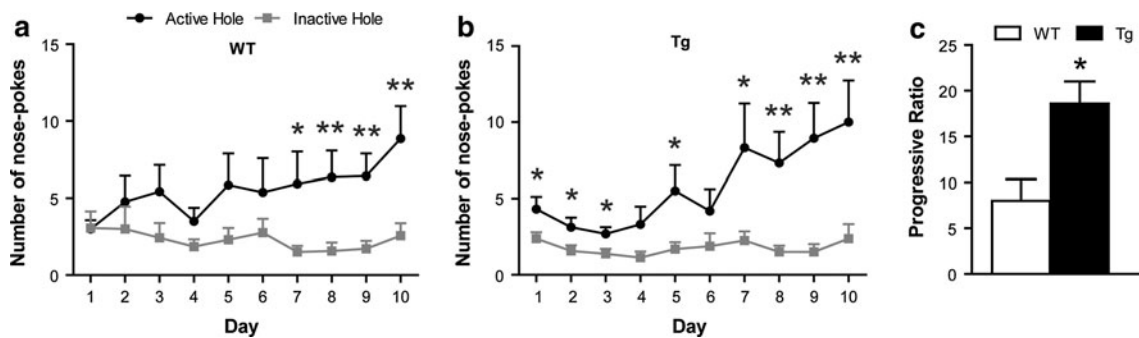
**Fig. 3** Behavioral characterization of TgCHRNA5/A3/B4 mice. **a–c** Open field test. Graphs show time spent (**a**) and speed (**b**) in the aversive (*centre*) and non-aversive (*periphery*) zones, and latency to periphery (**c**). Nicotine effects on locomotor activity (**d**). Distance

traveled was registered during 10 min after injection of saline, 0.5 and 1 mg/kg of nicotine. Data are expressed as mean ± SEM. \**p* < 0.05, \*\**p* < 0.01 comparison between genotypes, Student's *t* test



**Fig. 4** Nicotine-induced seizures. **a** Dose–response curve expressed as the seizures score in TgCHRNA3/B4/A5 (*gray square*) and WT (*black dot*) mice administered with increasing doses of nicotine (one dose per animal). Dose–response curve was shifted to the left in TgCHRNA3/B4/A5 as compared with WT mice denoting

hypersensitivity to nicotine-induced seizures. **b** Median effective dose (ED<sub>50</sub>) was reduced in TgCHRNA3/B4/A5 (*filled bars*) being three times more sensitive to nicotine than WT (*open bars*) mice. Data are expressed as mean ± SEM. \**p* < 0.05 comparison between genotypes and treatment, one-way ANOVA



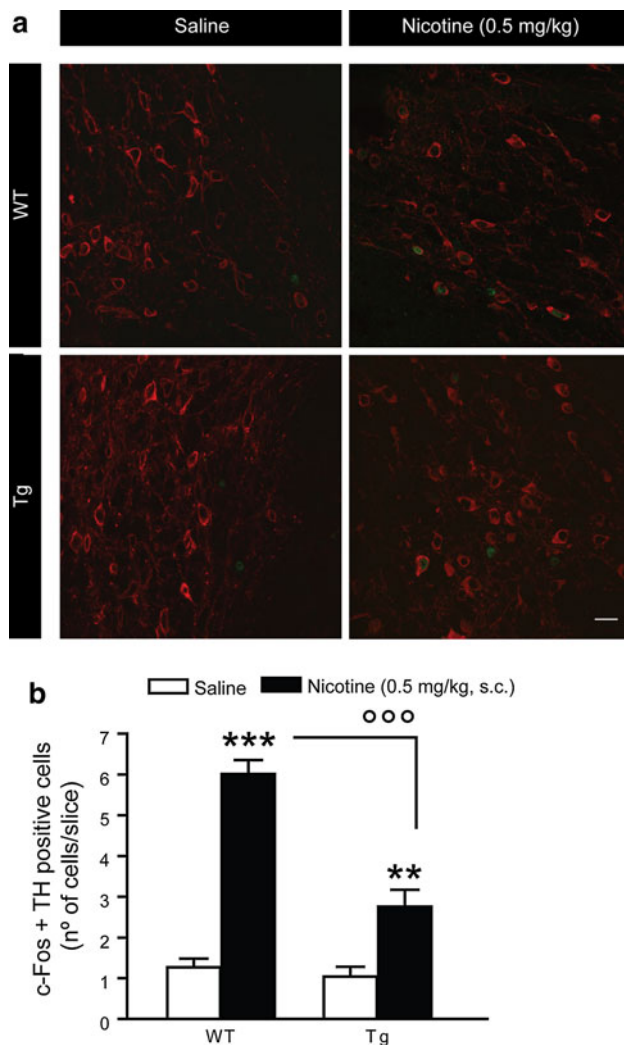
**Fig. 5** Nicotine self-administration in WT and TgCHRNA5/A3/B4 mice. Number of nose-pokes in the active (*black dots*) and inactive hole (*gray squares*) for WT (**a**) and TgCHRNA5/A3/B4 (**b**) mice. Mice were trained daily in 2-h sessions to obtain nicotine (0.015 mg/kg per infusion) for 10 days under a fixed ratio 1 schedule of

reinforcement. Data are expressed as mean ± SEM. \**p* < 0.05, \*\**p* < 0.01 comparison between holes, Student's *t* test. **c** Progressive ratio of WT (*open bar*) and TgCHRNA5/A3/B4 (*filled bar*) mice that reached the criteria of acquisition. Data are expressed as mean ± SEM. \**p* < 0.05 comparison between genotypes, Student's *t* test

Nicotine-induced c-Fos expression

In the VTA the number of c-Fos/TH double-positive cells was quantified in mice receiving an acute injection of

either saline or nicotine (0.5 mg/kg s.c.; Fig. 6a, b). Both WT and TgCHRNA5/A3/B4 mice showed increased c-Fos/TH-positive cells after nicotine injection as compared with saline (WT; *t*<sub>(1,5)</sub> = 0.32, *p* < 0.001, and TgCHRNA5/A3/



**Fig. 6** c-Fos immunoreactivity in VTA DAergic neurons. **a** Photomicrograph illustrating c-Fos immunoreactive DAergic cells in VTA of WT and TgCHRNA5/A3/B4 mice treated with an acute injection of saline or nicotine (0.5 mg/kg). Slices are fluorescently double-labeled with anti-tyrosine hydroxylase (red) and anti c-Fos (green). Scale bar = 20  $\mu$ m. **b** Graph shows the number of TH/c-Fos positive cells per slice in VTA. Data are expressed as mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  comparison between treatment, and  $^{ooo}p < 0.001$  comparison between genotypes, Student's  $t$  test

B4;  $t_{(1,6)} = 1.55$ ,  $p = 0.01$ , Student's  $t$  test), but the effects of nicotine were significantly different between genotypes (genotype  $\times$  treatment  $F_{(3,15)} = 23.50$ ,  $p < 0.001$ , two-way ANOVA), hence nicotine-induced VTA c-Fos expression was significantly reduced in TgCHRNA5/A3/B4 mice as compared with WT littermates ( $t_{(1,5)} = 0.58$ ,  $p < 0.001$ , Student's  $t$  test). The same dose of nicotine of 0.5 mg/kg tended to increase c-Fos expression in the MHB of both genotypes (Fig. 7a) although it was not statistically significant [WT; ( $t_{(1,5)} = -1.91$ , NS, Student's  $t$  test), TgCHRNA5/A3/B4; ( $t_{(1,8)} = -1.94$ , NS, Student's  $t$  test)]. Nicotine produced the same effect in both

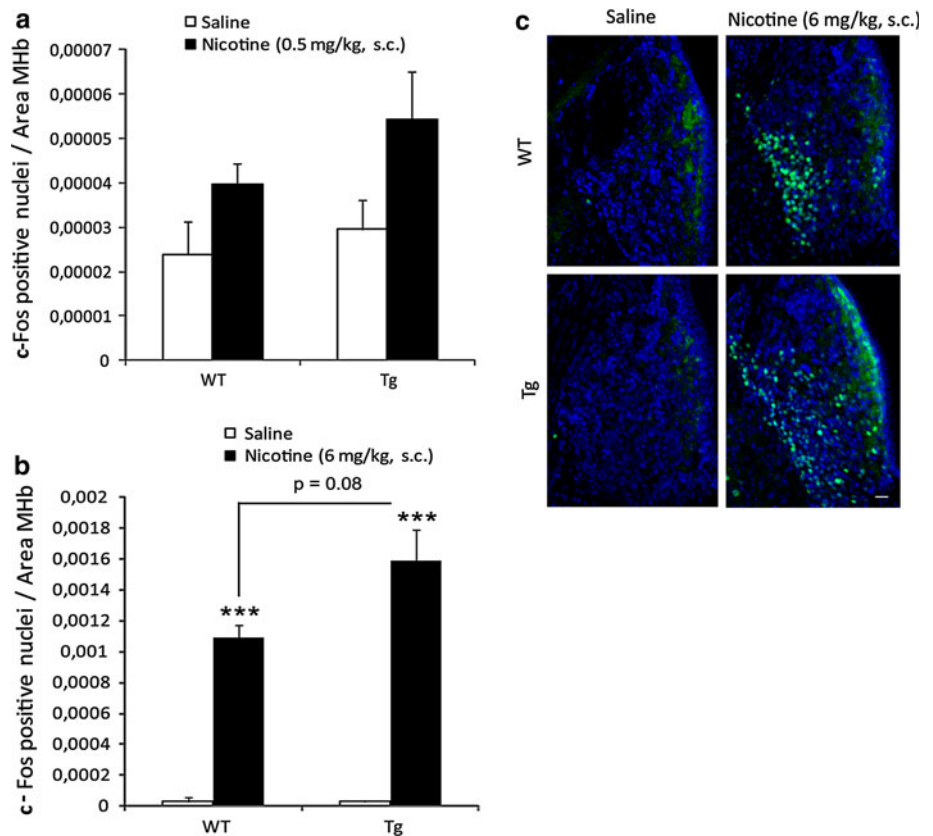
genotypes (genotype  $\times$  treatment  $F_{(1,17)} = 0.28$ , NS, two-way ANOVA); however, there was a tendency to be higher c-Fos expression in transgenic mice. To further examine the activation of the MHB after an acute administration of nicotine we used a dose of 6 mg/kg. c-Fos expression was increased in mice receiving nicotine compared with the saline group both in WT ( $t_{(1,4)} = -12.11$ ,  $p < 0.001$ , Student's  $t$  test; Fig. 7b, c) and TgCHRNA5/A3/B4 mice ( $t_{(1,4)} = -7.72$ ,  $p < 0.001$ , Student's  $t$  test). The effects of nicotine were significantly different between genotypes (genotype  $\times$  treatment  $F_{(1,12)} = 5.12$ ,  $p = 0.05$ , two-way ANOVA) although the comparison among nicotine-treated groups did not reach statistical significance ( $t_{(1,4)} = -2.27$ , NS, Student's  $t$  test).

## Discussion

Human genetic studies have demonstrated that polymorphisms in the CHRNA5/A3/B4 gene cluster located on chromosome 15 may influence smoking-related behaviors. Furthermore, some of these variants give rise to increased mRNA levels of the subunits (Doyle et al. 2011; Falvella et al. 2009; Schlaepfer et al. 2008; Wang et al. 2009; Xu et al. 2006). Even though overexpression of these genes has been associated with cigarette smoking, previous studies have only explored the role of single receptor subunits by knocking out or overexpressing individual mouse nAChR subunits. Thus, we explored the effects of overexpression of the whole human CHRNA5/A3/B4 cluster containing all three subunits using a bacterial artificial chromosome (BAC) transgenic mouse model. Our study demonstrates that overexpression of the human CHRNA5/A3/B4 genomic region in mice increases the reinforcing properties of nicotine (0.015 mg/kg per infusion) as revealed by an increased break point for intravenous self-administration. In addition, we observed reduced activation of VTA dopaminergic neurons upon an acute nicotine injection, which may reflect alterations in the balance between the rewarding and the aversive effects of nicotine. This change in balance between the rewarding and aversive properties may prompt the animals to initially consume the drug. We also found increased sensitivity to the pharmacological effects of nicotine, including higher activation of the MHB after acute nicotine administration together with an enhanced anxiety-like phenotype.

Multiple lines of evidence suggest coordinate regulation of the CHRNA5, CHRNA3, and CHRNB4 genes. In our study western blot analysis showed increased expression of the  $\alpha 3$  and  $\beta 4$  subunits in our Tg mice. Consistently, TgCHRNA5/A3/B4 mice showed increased 5I-A-85380-resistant [125I]-epibatidine binding sites in brain regions where  $\alpha 3\beta 4^*$ -nAChRs are known to be normally expressed

**Fig. 7** c-Fos immunoreactivity in the MHb. **a, b** Number of c-Fos positive nuclei per area of the MHb of WT and TgCHRNA5/A3/B4 mice treated with an acute injection of saline or nicotine [0.5 (**a**) or 6 (**b**) mg/kg, s.c.]. **c** Photomicrograph illustrating c-Fos immunoreactive neurons in the MHb of WT and TgCHRNA5/A3/B4 mice treated with an acute injection of saline or nicotine (6 mg/kg, s.c.). Slices are fluorescently labeled with anti c-Fos (green) and Hoechst (blue). Scale bar = 20  $\mu$ m. Data are expressed as mean  $\pm$  SEM. \*\*\* $p < 0.001$  comparison between treatment, Student's *t* test



(Soria et al. 2005), including regions implicated in the control of nicotine addiction, such as the MHb and the interpeduncular nucleus (IPN) (Fowler et al. 2011; Frahm et al. 2011). Interestingly, our Tg mice showed a substantial increase in the expression of  $\beta 4^*$ -nAChRs in the CA1 region of hippocampus, also described previously in Tabac mice (Frahm et al. 2011), thus implicating the hippocampus as a putative region mediating the reinforcing effects of nicotine. The observed increase in binding sites in Tg mice was accompanied by increased sensitivity to nicotine-induced hypolocomotion and seizures. The present data are in accordance with previous studies reported in which deletion of the  $\alpha 3$ ,  $\beta 4$  or  $\alpha 5$  subunits individually, or in combination, result in a phenotype resistant to the acute effects of nicotine, specifically hypolocomotion and seizures (Salas et al. 2003a, b).

The main finding of our study was that Tg mice showed increased nicotine-reinforcing effects, as suggested by improved acquisition of nicotine self-administration and better performance in the progressive ratio schedule. TgCHRNA5/A3/B4 mice showed a preference for the active hole over the inactive hole on day one, whereas WT mice started to discriminate after the seventh day. Under the progressive-ratio TgCHRNA5/A3/B4 reached a higher break point to obtain a nicotine infusion than WT animals. This reveals an enhanced motivation of TgCHRNA5/A3/B4 mice

to obtain the drug and suggests higher sensitivity to the reinforcing effects at this dose (0.015 mg/kg per infusion).

The habenula-interpeduncular system has been proposed as a pathway putatively involved in nicotine-induced locomotion, seizures, and consumption. Thus, we checked if the higher nicotine sensitivity observed in our TgCHRNA5/A3/B4 mice was correlated with higher MHb activation. We used a high dose of nicotine (6 mg/kg) that induced more pronounced seizures in our Tg mice compared with WT animals. As anticipated, the analysis of c-Fos expression in the MHb after an acute injection of nicotine revealed increased activation in Tg mice. Interestingly, 0.5 mg/kg of nicotine induced less activation of DAergic VTA neurons in TgCHRNA5/A3/B4 mice compared with their WT littermates. This dose activated the MHb in both genotypes, being higher (though not reaching statistical significance) in Tg mice. Since recent work demonstrates a critical role for the MHb in the circuitry controlling nicotine consumption (Fowler et al. 2011; Frahm et al. 2011), these data suggest that overexpression of the CHRNA5/A3/B4 cluster could alter the rewarding and aversive properties of nicotine. According to the study of Frahm et al. (2011) overexpression of the  $\beta 4$ -nAChR subunit regulates the aversive effects of nicotine through increasing MHb activity. We hypothesize that the decreased activation of the DAergic neurons found in our

Tg mice could be mediated by activation of the habenula-interpeduncular pathway through increased activity of  $\alpha 3\beta 4^*$ -nAChRs. Although this effect is translated to decreased nicotine consumption (Frahm et al. 2011), contrary to what we observed in our Tg mice, this could be due to the lower dose of nicotine that we used in the self-administration paradigm. Indeed, similar results were found between  $\alpha 5$  null mutant and WT mice in the study of Fowler et al. (2011) when low doses of nicotine were used. The study by Fowler et al. (2011) demonstrated that nicotine activates the habenula-interpeduncular pathway through  $\alpha 5$ -containing nAChRs, triggering an inhibitory motivational signal that limits nicotine intake. Moreover, the same study showed that  $\alpha 5$  subunit knockdown in the MHB does not alter the rewarding effects of nicotine, suggesting that  $\alpha 5$ -containing nAChRs located in the VTA may have a role in the rewarding effects of nicotine. Given that our Tg mice overexpress the  $\alpha 5$  subunit we hypothesize that augmented  $\alpha 5$ -containing nAChRs in VTA may enhance the sensitivity to low doses of nicotine and thus increase the rewarding effects of the drug. The high-affinity  $\alpha 4\beta 2$ -nAChR is the most abundant receptor in the brain and the principal mediator of nicotine dependence (Maskos et al. 2005; Mineur and Picciotto 2008; Tapper et al. 2004). Both  $\alpha 4\beta 2$  and  $\alpha 4\alpha 5\beta 2$  subtypes are present on the soma of dopaminergic neurons (Drenan et al. 2008; Klink et al. 2001; Marubio et al. 2003). Since the introduction of  $\alpha 5$  subunit in 293 cells expressing  $\alpha 4$  and  $\beta 2$  strongly favors assembly of  $\alpha 4\alpha 5\beta 2$  receptors and increases constitutive binding density (Gahring and Rogers 2010), overexpressing the  $\alpha 5$  subunit in our Tg mice could lead to increased levels of  $\alpha 4\alpha 5\beta 2$ -nAChRs on the cell surface of dopaminergic neurons, thus enhancing the rewarding effects of nicotine when low doses of the drug are used.

In summary, overexpression of the CHRNA5/A3/B4 cluster increases nicotine preference and sensitivity to its pharmacological effects due to augmented nicotine-binding sites that may enhance liability to consume nicotine through alterations in its reinforcing pathways. The present results suggest that the CHRNA5/A3/B4 genomic cluster is directly via the VTA or indirectly via the habenula-interpeduncular pathway an important determinant of tobacco consumption. Our study provides the first in vivo evidence that increased expression of these nicotinic receptor subunits enhances the motivational effects of low doses of nicotine and leads to nicotine addiction-related behaviors. However, further behavioral studies will be needed to clarify the aversive effects of high doses of nicotine in this Tg mouse model.

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