

# The Nicotinic Acetylcholine Receptor Subunit $\alpha 5$ Mediates Short-Term Effects of Nicotine in Vivo

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## ABSTRACT

Nicotine, acting at pentameric neuronal nicotinic acetylcholine receptors (nAChRs), is the primary addictive component in tobacco. At low doses, it affects attention, learning, memory, anxiety, cardiovascular responses, thermoregulation, and nociception. At high doses, nicotine produces more drastic behaviors and eventually induces tonic-clonic seizures in rodents. In mammals, several subunits of the nAChRs have been cloned, including eight  $\alpha$  and three  $\beta$  subunits. To study the physiological role of the  $\alpha 5$  subunit, we have generated  $\alpha 5$ -deficient mice. These mice have a generally healthy appearance and are

normal in a standard battery of behavioral tests. However, the sensitivity of  $\alpha 5$  mutant mice to nicotine-induced behaviors and seizures is dramatically reduced compared with their wild-type littermates. These animals have a normal brain anatomy and normal levels of mRNA for other nAChR subunits, namely  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$ . In addition, [<sup>125</sup>I]-epibatidine and [<sup>125</sup>I]- $\alpha$ -bungarotoxin binding in the brains of  $\alpha 5$ -deficient mice is normal. Together, these results suggest a direct involvement of the  $\alpha 5$  subunit in the observed phenotypes.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that are expressed in both neuronal and non-neuronal tissues (Dani, 2001; Itier and Bertrand, 2001; De Biasi, 2002). To date, 11 nAChR subunits have been identified in mammals and designated as either  $\alpha$ -type ( $\alpha 2$ – $\alpha 7$ ,  $\alpha 9$ ,  $\alpha 10$ ) or  $\beta$ -type ( $\beta 2$ – $\beta 4$ ) based on their homology to the muscle  $\alpha 1$  subunit (Boulter et al., 1986, 1987). Expression studies in *Xenopus laevis* oocytes have demonstrated that the majority of functional neuronal nAChRs are composed of two  $\alpha$  and three  $\beta$  subunits, with “duplex” ( $\alpha/\beta$ ) or “triplex” combinations ( $\alpha_x\alpha_y\beta$  or  $\alpha_\beta\alpha_\beta\beta_y$ ; Anand et al., 1991; Cooper et al., 1991; Seguela et al., 1993; Boorman et al., 2000; Groot-Kormelink et al., 2001). The  $\alpha 5$  subunit participates in nAChR receptors with  $\alpha_x\alpha_y\beta$  combinations (Ramirez-Latorre et al., 1996; Gerzanich et al., 1998; Groot-Kormelink et al., 2001) but cannot yield functional receptors when expressed alone or in combination with  $\beta$  subunits only (Ramirez-Latorre et al., 1996). Although  $\alpha 5$  subunits are apparently unnecessary for the assembly of functional receptors, they can alter the pharmacology and

the biophysical properties of nAChRs, and these effects depend on the nature of the subunits coexpressed with  $\alpha 5$ . When expressed with  $\alpha 3$  and  $\beta 2$ ,  $\alpha 5$  increases the sensitivity to ACh, but this effect is not observed when  $\beta 4$  is present instead of  $\beta 2$  (Wang et al., 1996; Groot-Kormelink et al., 1998). Conversely, the presence of  $\alpha 5$  increases calcium permeability and rate of desensitization in both  $\alpha 3\beta 2$ - and  $\alpha 3\beta 4$ -containing nAChRs (Gerzanich et al., 1998). In chick sympathetic neurons, the deletion of  $\alpha 5$  alters the sensitivity of the native nAChR channels to both agonists and antagonists (Yu and Role, 1998a). Despite this molecular work, the relevance of  $\alpha 5$ -containing nAChRs for in vivo physiological processes remains elusive.

In the peripheral nervous system,  $\alpha 5$  is found in both sympathetic and parasympathetic ganglia (De Biasi, 2002) where  $\alpha 5$ -containing nAChRs might influence the autonomic control of several organ systems (Wang et al., 2002). In the central nervous system,  $\alpha 5$  is highly expressed in the CA1 area of the hippocampus, the interpeduncular nucleus (IPN), the ventral tegmental area (VTA), and the substantia nigra compacta (SNc) (Wada et al., 1990; Broide et al., 2002), areas in which nAChRs could potentially influence learning, memory, and drug-seeking behaviors. To study the role of the  $\alpha 5$  nAChR subunit in living animals, we generated  $\alpha 5$  knock-out mice by deleting most of exon 5, which contains three trans-

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**ABBREVIATIONS:** nAChR, nicotinic acetylcholine receptor; kb, kilobase(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; RT, reverse transcription; IPN, interpeduncular nucleus; Ctx, cortex; Hi, hippocampus; Hy, hypothalamus; MHb, medial habenula; SC, superior colliculus; SNc, substantia nigra compacta; Th, thalamus; VTA, ventral tegmental area.; Cpu, caudate putamen; BTX,  $\alpha$ -bungarotoxin.

membrane regions and the long intracellular loop. The  $\alpha 5$  null ( $-/-$ ) mice grow to adulthood with no visible phenotypic abnormalities and show normal behaviors in basal conditions. However,  $\alpha 5$   $-/-$  mice are less sensitive to nicotine-induced behaviors and seizures compared with their wild-type ( $+/+$ ) littermates. Our results demonstrate for the first time that  $\alpha 5$ -containing nAChRs are essential for the expression of nicotine-induced behaviors.

## Materials and Methods

**Targeted Deletion of the  $\alpha 5$  Gene.** The mouse gene for the nAChR  $\alpha 5$  subunit was isolated by screening a mouse 129/SvEv genomic library (a gift from Richard Behringer, M. D. Anderson Cancer Center, Houston, TX) with a rat cDNA probe, and a detailed restriction map was obtained. Most of exon 5, which contains three of the four transmembrane domains, was replaced with a neomycin resistance cassette (Neo), electroporated into AB2.2 embryonic stem cells, and transmitted into the germline as described previously (Orr-Urtreger et al., 1997). Chimeric mice were obtained and bred with C57BL/6J mice. The mutant allele (5.6-kb fragment) was differentiated from the wild-type (20.5-kb fragment) using Southern blot analysis with a flanking genomic probe. PCR with the following primers was designed to determine the genotype for the mutation:  $\alpha 5$  wild-type: forward, 5'-GTGAAAGAGAACGACGTCCGC-3'; reverse, 5'-GCCTCAGCCCCCTGAATGGTAG-3';  $\alpha 5$  mutant: forward 5'-CTTTTGTGCAAGACCGACCTGTCCG; reverse, 5'-CTCGATGC-GATGTTTCGCTTGGTG-3'. The wild-type product is 380 base pairs, and the mutant product is 290 base pairs.

**Animals.** All mice used in this study were back-crossed onto a C57BL/6 background for seven generations. Open-field, seizure, and histology experiments were done on 2- to 6-month-old mice, with male and female mice in an approximately 50/50 ratio. Mice were generated by crossing heterozygous male and female mice, weaned at 21 days of age, and housed in groups of two to five per cage under a 12-h/12-h light cycle, with food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use committee in accordance with federal guidelines.

**Basal Behavioral Battery.** To examine the role of  $\alpha 5$  nAChRs in basal behavior,  $\alpha 5$  homozygous mutant mice and their wild-type littermates were tested in a battery of behavioral experiments (for a description of the battery of behavioral tests, see Paylor et al., 1998). Mice were examined on the following tests: 1) a neurological screen for simple sensory and motor function; 2) open-field test for exploratory activity and anxiety-related responses; 3) light-dark exploration box for anxiety-related responses; 4) rotarod test for motor coordination and skill learning; 5) acoustic startle response and prepulse inhibition of the startle response; 6) startle habituation; 7) passive avoidance test; and 8) hotplate test for analgesia-related responses.

**Seizure Testing.** One day before seizure induction, mice were weighed, marked, and transferred to the testing room for acclimation. Nicotine tartrate (Sigma, St. Louis, MO), dissolved in phosphate-buffered saline (PBS) was administered i.p. in a volume of 10  $\mu$ l/g of body weight. The amounts of nicotine injected were 2, 3, 5, 7, 10, and 14 mg/kg. For each genotype, 5 to 14 mice were used at each nicotine concentration, except for very low doses (2 and 3 mg/kg) on  $\alpha 5$   $-/-$  mice and very high doses (10 and 14 mg/kg) on  $\alpha 5$   $+/+$  and  $\alpha 5$   $+/-$  mice, where less animals were used. On any given experimentation day, at least one mouse from each genotype received one high and one low dose of nicotine. Immediately after injection, mice were placed in a regular mouse cage with bedding, and behavioral responses were recorded by two investigators for 5 min. Experimenters were blind to the genotype of the mice. The effects of nicotine were dose-dependent. An arbitrary scale was created to assess sensitivity to nicotine as follows (Franceschini et al., 2002): 0, no obvious effects; 1, locomotor effects including sedation and increased explor-

atory activity; 2, tremors, tachypnea, and back arching; 3, rapid movements of the legs; 4, complete loss of righting reflex and seizures; and 5, death. Sensitivity to nicotine seizures was assessed by calculating the percentage of animals in each genotype group that had a score of 4 or 5. Data were fitted with a logistic curve to determine the  $EC_{50}$ .

**Effects of Nicotine on the Open Field.** Mice (9–22 per genotype per dose) were i.p. injected with either PBS alone or nicotine (0.1, 0.25, or 0.5 mg/kg) in PBS, in a volume of 10  $\mu$ l/g body weight. Immediately after injection, mice were placed in a clear Plexiglas box (40  $\times$  40  $\times$  40 cm) and their movements were monitored for 30 min using a computer-assisted Ethovision system (Noldus, the Netherlands). Total distance moved, average distance to the center, and the ratio of distance moved in a center square (20  $\times$  20 cm) to total distance moved were recorded.

**Histology.** Mice ( $n = 3$  per genotype) were decapitated under anesthesia, and their brains were removed and frozen in isopentane ( $-30^{\circ}\text{C}$ , 30 s). Fresh-frozen brains were cut (20- $\mu$ m sections) in a cryostat and sections were mounted onto either gelatin-coated slides (for receptor binding and histological staining) or slides with an additional coating of poly(L-lysine) kept at  $-20^{\circ}\text{C}$  (for in situ hybridization). Slide-mounted sections for receptor binding were stored at  $-20^{\circ}\text{C}$  until use. Sections for in situ hybridization and histological staining were postfixed in 4% paraformaldehyde (30 min, room temperature), washed three times in PBS, and stored desiccated at  $-20^{\circ}\text{C}$  until use. Slide-mounted brain sections for Nissl staining were stained with cresyl violet. Acetylcholinesterase histochemistry was performed as described previously (Orr-Urtreger et al., 2000).

**In Situ Hybridization.** Mouse DNA templates encoding the intracellular loop of various nAChR subunits were prepared by RT-PCR using RNA from the mouse septal neuroblastoma cell line SN56 as template. Primers for RT-PCR were designed with available rat nAChR cDNA sequences. The size and cDNA region of each nAChR subunit probe has been reported (Franceschini et al., 2002). In situ hybridization was performed as described previously (Broide et al., 1996). Briefly, sense and antisense  $^{35}\text{S}$ -UTP-labeled (PerkinElmer Life Sciences, Boston, MA) cRNA riboprobes were synthesized and hybridized to proteinase K-treated brain sections overnight at  $60^{\circ}\text{C}$ . Sections were washed and exposed to X-ray film for 3 to 7 days.

**Receptor Autoradiography.** Slide-mounted brain sections were processed for  $^{125}\text{I}$ - $\alpha$ -BTX binding as described previously (Broide et al., 1996). Briefly, slides were incubated for 2 h at room temperature in binding buffer A (50 mM Tris base, pH 7.4, 120 mM NaCl, and 0.1% bovine serum albumin) containing 5 nM  $^{125}\text{I}$ - $\alpha$ -BTX (specific activity, 10–20  $\mu\text{Ci}/\mu\text{g}$ ; PerkinElmer Life Sciences). Nonspecific binding was defined on adjacent sections in the presence of 10  $\mu\text{M}$   $\alpha$ -cobratoxin. Slides were washed twice for 10 min in ice-cold binding buffer A, rinsed in water, dried, and exposed to  $\beta$ -Max (Amersham Biosciences, Piscataway, NJ) or BIOMAX (Eastman Kodak, Rochester, NY) film for 3 to 7 days.

Brain sections were processed for  $^{125}\text{I}$ -epibatidine binding by incubation in binding buffer B (50 mM Tris base, pH 7.4, 120 mM NaCl, 5 mM KCl, 2.5 mM  $\text{CaCl}_2$ , and 1 mM  $\text{MgCl}_2$ ) in the presence of 500 pM  $^{125}\text{I}$ -epibatidine (specific activity, 2200 Ci/mmol; PerkinElmer Life Sciences). Nonspecific binding was assessed on adjacent sections in the presence of 100  $\mu\text{M}$  nicotine. Slides were then washed twice in ice-cold binding buffer B, rinsed once in water, dried, and exposed to  $\beta$ -Max film for 3 to 12 h.

**Data Analysis and Statistics.** X-ray films were analyzed, and signals were quantified using computer-assisted densitometry (NIH Image program, <http://rsb.info.nih.gov/ni-image/>). Relative optical densities for discrete brain regions were measured and presented as a percentage of readings from wild-type brains in the same films. Care was taken to avoid overexposure and to make sure that the signal of interest was always within the linear range of the film. All data were examined by multivariate analysis of variance, followed by Newman-Keuls post hoc comparisons.

## Results

**Generation of  $\alpha 5$  nAChR Subunit Null Mice.** Mice deficient in the  $\alpha 5$  subunit were generated by replacing a 4-kb region containing most of exon 5 with a Neo-loxP-3'*hprt* cassette. This construct was then introduced into AB 2.2 embryonic stem cells from the 129/SvEv mouse strain, followed by transmission to the germline (Fig. 1A) (Orr-Urtreger et al., 1997). Southern blot analysis using a flanking genomic probe detected a new 5.6-kb mutant fragment in the heterozygote (+/-) and homozygote (-/-) mice (Fig. 1B) in addition to the 20.5-kb fragment in wild-type mice. The effect of the mutation on mRNA transcripts was examined using Northern blotting, and no detectable transcripts were found in homozygous mutant mice (Fig. 1C).

$\alpha 5$  (-/-) mice are viable and fertile, are born in the expected proportion from mating of heterozygote mice, grow to normal size, and show no obvious physical or neurological deficits. In a battery of behavioral tests (Paylor et al., 1998) the  $\alpha 5$  -/- mice behaved like their wild-type littermates (Table 1).

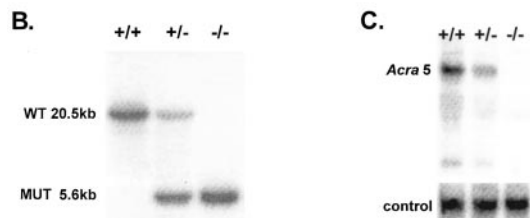
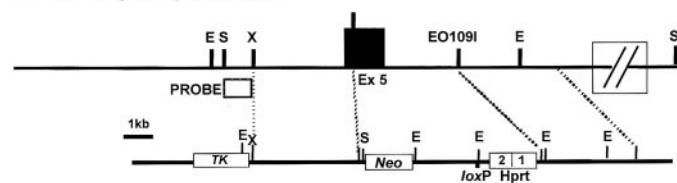
**$\alpha 5$  Null Mice Are Resistant to Nicotine-Induced Seizures.** Intraperitoneal injection of nicotine induced seizures in a dose-dependent manner in wild-type mice. However, only a small number of  $\alpha 5$  -/- mice suffered seizures at very high nicotine concentrations, making these animals almost refractory to nicotine-induced seizures (Fig. 2A). In +/+ and +/- mice, the EC<sub>50</sub> values of nicotine were  $4.1 \pm 0.1$  and  $4.3 \pm 0.05$  mg/kg, respectively, consistent with previous results (Broide et al., 2002; Franceschini et al., 2002). Only 35% of the  $\alpha 5$  -/- tested went into seizure, and for that group of animals, the EC<sub>50</sub> was 8.4 mg/kg. In addition, at every dose tested,  $\alpha 5$  mutant mice were less sensitive to the effects of nicotine (Fig. 2, B-D).

**Experimentally Naive  $\alpha 5$  Null Mice Are Resistant to the Hypolocomotive Effects of Nicotine.** In the open

field, nicotine initially produces a sedative effect in both mice and rats (Decker et al., 1995; Nagahara and Handa, 1999). We observed the locomotor effect of nicotine on  $\alpha 5$  -/- mice and their +/+ littermates, starting immediately after i.p. injection of nicotine or saline. In our hands, the effect of nicotine was largest during the first 5 min; at 30 min, the locomotion values returned to normal at every dose tested (not shown). To determine whether nicotine had different pharmacodynamics in the mutant mice, we ran the open-field test for 30 min and found that nicotine had no effect on  $\alpha 5$  -/- mice for the whole period of observation. In the  $\alpha 5$  -/- mice, the hypolocomotive effects of nicotine could be observed beginning at 1 mg/kg. Figure 3 shows data for the first 5 min in the open field after i.p. injection of nicotine. In  $\alpha 5$  +/+ animals, the lowest dose of nicotine (0.1 mg/kg) produced a small hyperlocomotive effect that failed to show statistical significance. At 0.25 mg/kg, nicotine had a sedative effect that was also not statistically significant. At 0.5 mg/kg, nicotine had a major effect on locomotion, decreasing it from  $1840 \pm 109$  to  $834 \pm 75$  cm ( $p < 0.0005$ ). At 1 mg/kg, locomotion was further decreased in the  $\alpha 5$  +/+ mice, but in some cases, the animals manifested the typical effects observed with high nicotine doses, such as rapid movements of the legs (wild run). In  $\alpha 5$  -/- mice, nicotine doses up to 0.5 mg/kg had no effect, but 1 and 3 mg/kg decreased locomotion from  $1814 \pm 117$  to  $942 \pm 151$  and  $452 \pm 86$  cm, respectively. The ratio of distance moved in a center square (20  $\times$  20 cm) to total distance moved, a measure of anxiety, was not statistically different between  $\alpha 5$  +/+ and  $\alpha 5$  -/- mice at any dose.

**$\alpha 5$  Mutant Mice Show Normal Neuroanatomy.** The brains of  $\alpha 5$  +/- and -/- mice did not show any gross anatomical difference compared with wild-type littermates. For example, the hippocampus, one of the regions in which the  $\alpha 5$  subunit is expressed, displayed normal layering within all substructures, as assessed by Nissl (Fig. 4, A-C) and Acetylcholinesterase staining (Fig. 4, D-F). All other regions of the brain examined were also normal (data not

### A. $\alpha 5$ targeting construct



**Fig. 1.** Generation of  $\alpha 5$ -mutant mice. **A**, wild-type allele and targeting vector, depicted with restriction enzyme sites. Exon 5 is shown as a black box, and the probe used for Southern blotting as an open box underneath the wild-type allele. Restriction enzymes: E, *EcoRI*; E109I, *Eco109I*; S, *SacI*; X, *XhoI*. TK, thymidine kinase; Hprt, hypoxanthine phosphoribosyltransferase. **B**, Southern blot analysis of tail DNA from  $\alpha 5$  +/+,  $\alpha 5$  +/-, and  $\alpha 5$  -/- mice using the probe shown in **A**. **C**, Northern blot analysis for the expression of the  $\alpha 5$  subunit in the brains of  $\alpha 5$  +/+,  $\alpha 5$  +/-, and  $\alpha 5$  -/- mice. Rat cDNA was used as probe. As control, a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

**TABLE 1**

Behavioral responses of  $\alpha 5$  -/- and  $\alpha 5$  +/+ mice on a battery of behavioral tasks

There were no significant differences ( $p > 0.05$ ) on any of the measures between  $\alpha 5$  -/- and  $\alpha 5$  +/+ mice. A total of 17  $\alpha 5$  -/- mice (12 male and 5 female) and 18  $\alpha 5$  +/+ mice (13 male and 5 female) were studied. The numbers in parentheses represent the S.E.M. of the data presented.

Behavioral Measure	$\alpha 5$ +/+	$\alpha 5$ -/-
% with whiskers	88.9	100
% Abnormal response in cage	0	0
% Abnormal postural adjustments	0	0
% Abnormal touch reflex	0	0
Wire suspension (seconds)	43.3 (5.4)	40.5 (5.7)
Open-field total distance (centimeters)	5031 (454)	5651 (446)
Open-field rearing response (beam breaks)	331 (38.9)	342 (30.4)
Open-field center ratio	.295 (.027)	.307 (.018)
Light-dark. Total transitions	38.1 (2.8)	36.9 (3.4)
Time on rotarod (seconds)	128.5 (8.3)	114.1 (9.6)
Acoustic startle response	400 (56.8)	344.7 (60.8)
% Prepulse inhibition	45.8 (8.1)	50.3 (6.8)
Startle habituation score	268.5 (60.8)	133.2 (45.5)
Passive avoidance. Latency to enter (seconds)	109.8 (27)	92.5 (23.7)
Hotplate. Hind-limb response latency (seconds)	11.0 (.7)	9.5 (.9)

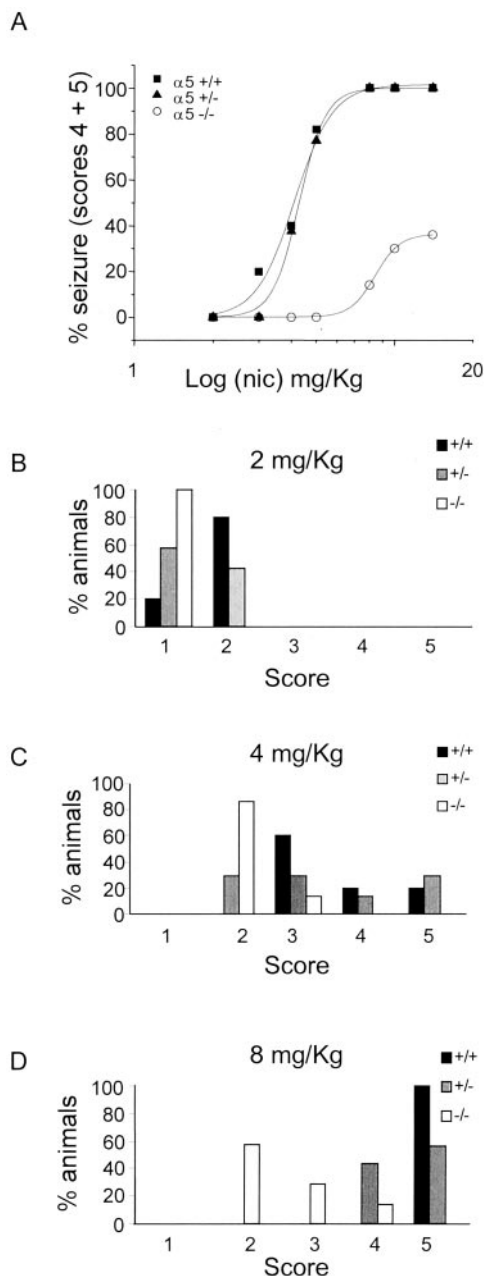


shown), including the IPN and VTA/SNc, which express high levels of  $\alpha 5$  mRNA.

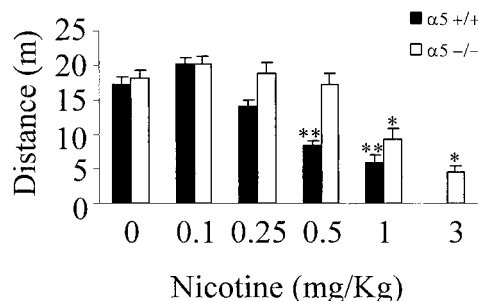
**$\alpha 5$  Mutant Mice Have Normal Levels of  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$  nAChR Subunits.** To determine whether other nAChR subunits that are potentially relevant to nicotine-induced seizures might be differentially regulated in the absence of the  $\alpha 5$  subunit, we performed *in situ* hybridization experiments to examine the patterns and levels of mRNA distribution for the  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$  nAChR subunits in brains of  $\alpha 5$   $+/+$ ,  $+/+$ , and  $-/-$  mice (Fig. 5). As described previously (Broide et al., 2002; Franceschini et al., 2002)  $\alpha 4$

mRNA levels were high in the thalamus (Th), medial habenula (MHb), SN, and VTA, and moderate in cortex (Ctx), hippocampus (Hi), and hypothalamus (Hy).  $\alpha 6$  mRNA was high in SN and VTA, and moderate in the superior colliculus (SC).  $\alpha 7$  signal was high in Hi, Hy, amygdala, SC, and inferior colliculus, with lower levels in the Ctx and caudate putamen (Cpu). Strong signal for  $\beta 2$  was found in the Th, Hi, and MHb, with lower levels in the Ctx, Cpu, SN, and olfactory bulb.  $\beta 4$  mRNA signal was restricted to the olfactory bulb, MHb, IPN, and pineal gland. There were no statistically significant differences between  $\alpha 5$   $+/+$ ,  $+/+$ , and  $-/-$  mice in the levels of  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$  transcripts for all brain regions examined (Table 2).

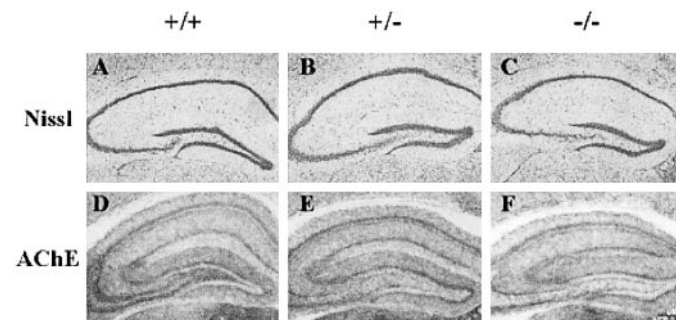
To study the levels of nAChR receptor subtypes expressed in  $\alpha 5$  mutant and wild-type mice, we performed receptor binding experiments on brain sections from  $\alpha 5$   $+/+$ ,  $+/+$ , and  $-/-$  mice. First, we used 500 pM  $^{125}$ I-epibatidine, which binds, at this concentration, to at least two subtypes of nicotinic receptors, probably containing  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\beta 2$ , and  $\beta 4$  subunits (Zoli et al., 1998; Whiteaker et al., 2000b; Champiaux et al., 2002). In  $+/+$  littermates, high levels of  $^{125}$ I-epibatidine binding were observed in the Th, SC, MHb, and IPN. More modest levels were found in the Ctx and Cpu (Fig. 6). A similar pattern of  $^{125}$ I-epibatidine binding site distribution was observed in both  $\alpha 5$   $+/+$  and  $-/-$  mouse brains (Fig. 6). In addition, we used  $^{125}$ I- $\alpha$ -BTX to study  $\alpha 7$ -containing nAChR levels in  $\alpha 5$   $+/+$ ,  $+/+$ , and  $-/-$  brains. High levels of  $^{125}$ I- $\alpha$ -BTX binding were found in the Hi, Hy, amygdala, SC, and inferior colliculus of  $+/+$  mouse brains. Lower levels of



**Fig. 2.** Nicotine-induced seizures and behavior. A, dose-response curves for the convulsant effects of nicotine in  $\alpha 5$   $+/+$ ,  $\alpha 5$   $+/+$ , and  $\alpha 5$   $-/-$  mice. Data show the percentage of mice undergoing seizure that either survived (score 4) or died (score 5). B–D, decreased sensitivity to the effects of nicotine in  $\alpha 5$   $-/-$  at different doses. The percentage of animals that obtained each score is depicted for three doses of nicotine: 2 (B), 4 (C), and 8 mg/kg (D).



**Fig. 3.** Reduced effect of low doses of nicotine in the open field in  $\alpha 5$   $-/-$  mice. Dose-dependent effects of nicotine on the total distance moved in the open field during 5 min for  $\alpha 5$   $+/+$  and  $\alpha 5$   $-/-$  mice, immediately after i.p. injection of nicotine or vehicle. \*,  $p < 0.0005$ ; \*\*,  $p < 0.0001$  compared with 0 mg/kg in the corresponding genotype (analysis of variance and Newman-Keuls post hoc comparison).



**Fig. 4.** Normal anatomy in  $\alpha 5$   $-/-$  mouse brains. A–F, transverse sections of mouse brains at the hippocampus level. A–C, sections stained with cresyl violet. D and F, sections stained for acetylcholinesterase. Normal hippocampal formations with correct layers are seen in  $\alpha 5$   $-/-$  mice. Scale bar, 200  $\mu$ m.

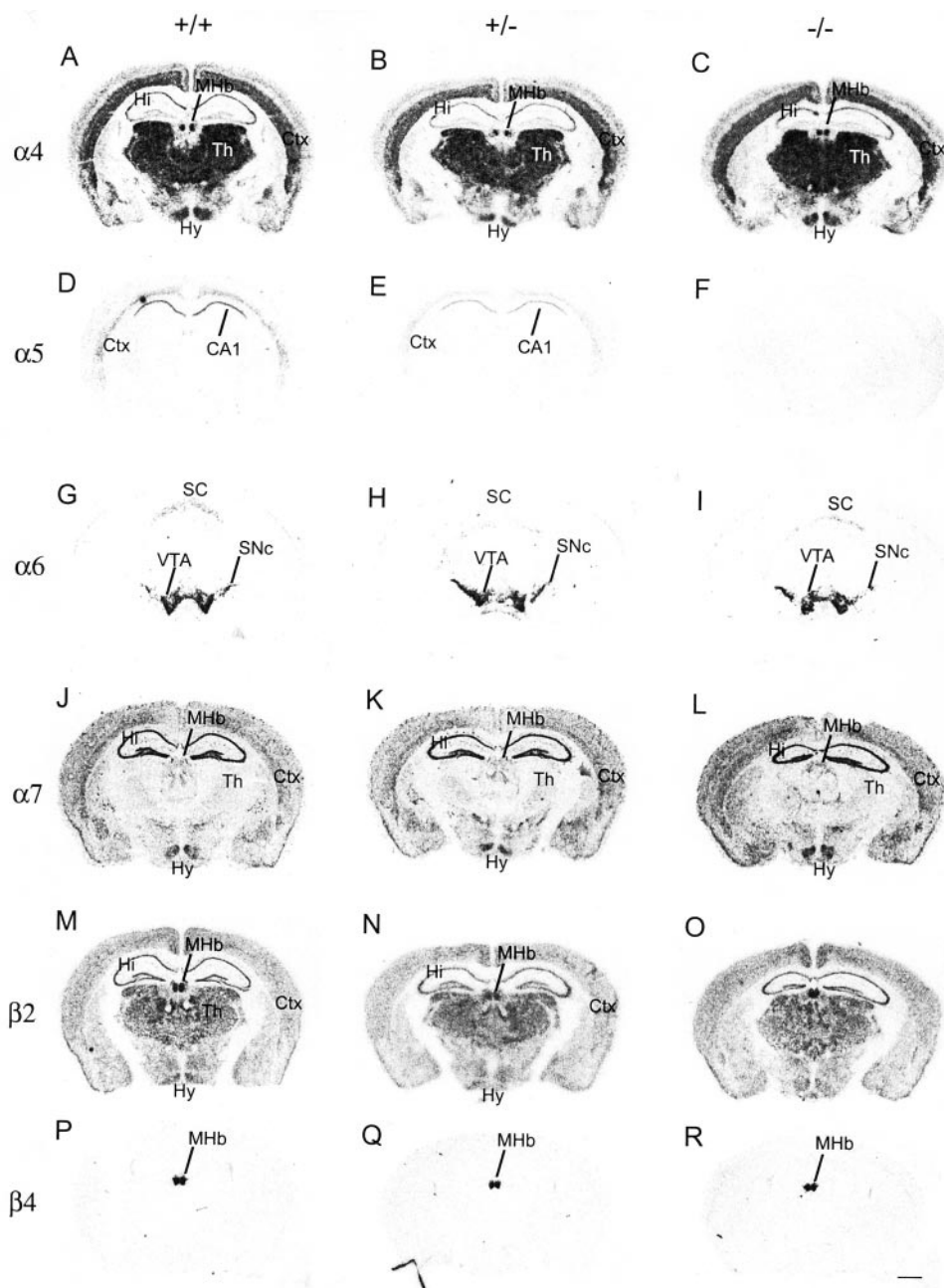
$^{125}\text{I}$ - $\alpha$ -BTX binding were found in the Ctx and Cpu. The same pattern of expression was observed in both  $\alpha 5$   $+/+$ , and  $-/-$  brains.  $^{125}\text{I}$ -Epibatidine and  $^{125}\text{I}$ -BTX binding signals were quantified by measuring relative optical densities from three brains per genotype. No statistically significant differences were found among  $\alpha 5$   $+/+$ ,  $+/-$ , and  $-/-$  mice in any of the brain regions analyzed (Table 2).

## Discussion

We have shown in the present study that mice lacking the  $\alpha 5$  nAChR subunit survive to adulthood and have no readily detectable abnormalities. In a battery of behavioral tests,  $\alpha 5$   $-/-$  mice showed no significant difference from their wild-type littermates. Because each of the tests performed measures a behavior that is influenced by multiple genes (Flint,

2003), our data suggest that  $\alpha 5$  does not have a major effect on the behavioral traits studied. Although  $\alpha 5$   $-/-$  mice display normal behavior in basal conditions, they are significantly less sensitive to the effects of nicotine than the wild-type littermates. This resistance to nicotine treatment was observed at both low and high nicotine doses and affected not only seizure sensitivity but also other behavioral effects, particularly those related to locomotor activity. Twice as much nicotine was needed in  $\alpha 5$   $-/-$  mice to elicit the effects observed in their wild-type littermates. Thus, although  $\alpha 5$ -containing nAChRs may not be essential for the expression of certain behaviors in basal conditions, they might be important mediators of the effects of nicotine.

Nicotine-induced seizures have been examined in different strains of mice, and using different pharmacological techniques. Previous studies pointed to the  $\alpha 7$  subunit as the



**Fig. 5.** Normal levels of expression of other nAChR subunits in  $\alpha 5$   $-/-$  mouse brains. Autoradiographic images of brain sections at the levels of the hippocampus and substantia nigra from  $\alpha 5$   $+/+$ ,  $\alpha 5$   $+/-$ , and  $\alpha 5$   $-/-$  mice. Sections show the distribution of mRNA for the  $\alpha 4$  (A–C),  $\alpha 5$  (D–F),  $\alpha 6$  (G–I),  $\alpha 7$  (J–L),  $\beta 2$  (M–O), and  $\beta 4$  (P–R) subunits. Scale bar, 1 mm.

main candidate responsible for nicotine-induced seizures (Miner et al., 1984, 1985; Miner and Collins, 1989), but  $\alpha 7$   $-/-$  mice in a C57BL background display normal sensitivity to high doses of nicotine (Franceschini et al., 2002). In contrast, mice engineered to have a partial gain of function of  $\alpha 7$ -containing receptors display increased sensitivity to nicotine-induced seizures (Broide et al., 2002; Gil et al., 2002). These results suggest that the role of  $\alpha 7$  subunits in nicotine-induced seizures is complex and is probably influenced by the genetic background of the animals tested (Miner et al., 1984, 1985; Miner and Collins, 1989). Studies with strain-specific variants of different nAChR subunits have also implicated  $\alpha 4$ -,  $\alpha 5$ -, and  $\alpha 6$ -containing receptors as possible mediators of nicotine-induced seizures (Stitzel et al., 1998, 2000), and our experiments confirm the role of  $\alpha 5$  in mediating the convulsant effects of nicotine.

There is abundant evidence that tonic and clonic seizures can originate in the hippocampus (Stitzel et al., 2000; McCormick and Contreras, 2001). Because the expression of  $\alpha 5$  is restricted to the hippocampal CA1 region, it is tempting to speculate that nicotine-induced seizures are mediated by the activation of neuronal circuits within this area. The majority of hippocampal neurons display a rapidly activating and desensitizing current that is mediated by  $\alpha 7$ -containing nAChRs (Alkondon and Albuquerque, 1993; Orr-Urtreger et al., 1997; Zarei et al., 1999). A smaller proportion of neurons expresses nAChR currents with slower kinetics, and these

cells are thought to express  $\beta 2$ -containing nAChRs (Sudweeks and Yakel, 2000; Khiroug et al., 2002). Because the CA1 region is the only place in the central nervous system where  $\alpha 5$  and  $\alpha 7$  subunits are coexpressed (Figs. 5 and 6), and because there is evidence that the  $\alpha 7$  nAChR subunit might form both homomeric and heteromeric channels (Cuevas and Berg, 1998; Yu and Role, 1998b; Khiroug et al., 2002), one possibility is that  $\alpha 5$  and  $\alpha 7$  subunits coassemble, probably with  $\beta 2$ , to form functional nAChRs in CA1. Alternatively,  $\alpha 5$  could participate in receptors containing the  $\alpha 4$  and  $\beta 2$  subunits, because those subunits are also expressed in this hippocampal region, and 25% of brain  $\alpha 4\beta 2$ -containing nAChRs might include the  $\alpha 5$  subunit (Gerzanich et al., 1998).

Although our data would agree with the hypothesis of nicotine-induced seizures originating in the hippocampus, there is evidence that the IPN is able to mediate seizure activity in rodents and humans (Myers and Shapiro, 1979; Olsen et al., 1985; Chiba and Wada, 1995). Hence,  $\alpha 5$ -containing receptors in the IPN might also mediate the effects of nicotine. This hypothesis is supported by the fact that partial kainic acid-induced lesions in the IPN of the rat suppress the hypolocomotive effect of nicotine in the open field (Hentall and Gollapudi, 1995). Expression of  $\alpha 5$  is also high in the VTA/SNc area. There are numerous reports of seizures originated in the substantia nigra, but the pars reticulata (which does not express the  $\alpha 5$  subunit), not the pars compacta

TABLE 2

Density of  $^{125}\text{I}$ -bungarotoxin and  $^{125}\text{I}$ -epibatidine binding, and  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$  mRNA expression in various regions of wild-type (+/+), heterozygous (+/-) and homozygous (-/-)  $\alpha 5$  null mouse brains

Data represent the mean  $\pm$  S.E.M. for three to five animals.

Label	Cortex	Hippocampus		Caudate Putamen	Thalamus	Medial Habenula	IPN	Substantia Nigra	Superior Colliculus
		CA1	DG						
%									
<sup>125</sup> I-α-BTX									
+/+	100 ± 17	100 ± 8	100 ± 4	100 ± 5					100 ± 7
+/-	104 ± 3	131 ± 6	102 ± 2	128 ± 23					94 ± 5
-/-	115 ± 12	117 ± 3	98 ± 9	152 ± 5					107 ± 2
<sup>125</sup> I-Epibatidine									
+/+	100 ± 6.1			100 ± 9	100 ± 4	100 ± 5	100 ± 2		100 ± 4
+/-	92 ± 7.7			93 ± 10	105 ± 3	93 ± 0.1	95 ± 9		85 ± 5
-/-	85 ± 11.6			97 ± 12	97 ± 5	103 ± 5	94 ± 2		92 ± 2
α4 mRNA									
+/+	100 ± 2	100 ± 8			100 ± 4	100 ± 3		100 ± 1	
+/-	93 ± 1	123 ± 10			96 ± 1.3	95 ± 2		102 ± 1	
-/-	92 ± 5	93 ± 15			93 ± 6	98 ± 4		102 ± 1	
α5 mRNA									
+/+	100 ± 18	100 ± 6				100 ± 2	100 ± 7	100 ± 4	
+/-	57 ± 10*	57 ± 2*				53 ± 2*	62 ± 8*	55 ± 18*	
-/-	0 ± 1*	0 ± 3*				0 ± 4*	0 ± 3*	0.8 ± 0.5*	
α6 mRNA									
+/+								100 ± 4	100 ± 4
+/-								97 ± 4	84 ± 7
-/-								94 ± 3	84 ± 8
α7 mRNA									
+/+	100 ± 12	100 ± 4	100 ± 1	100 ± 8					100 ± 12
+/-	103 ± 20	119 ± 14	106 ± 11	108 ± 19					89 ± 14
-/-	105 ± 26	110 ± 11	114 ± 10	118 ± 19					83 ± 13
β2 mRNA									
+/+	100 ± 9	100 ± 10	100 ± 6		100 ± 8	100 ± 3		100 ± 8	100 ± 13
+/-	106 ± 22	84 ± 8	86 ± 9		107 ± 14	96 ± 4		102 ± 13	89 ± 9
-/-	101 ± 29	87 ± 14	97 ± 16		98 ± 12	99 ± 5		91 ± 20	94 ± 14
β4 mRNA									
+/+						100 ± 4			
+/-						92 ± 3			
-/-						93 ± 2			

\*  $p < 0.05$ .



(SNc), seems to be responsible for these effects. Furthermore, seizures originated in the pars reticulata are mainly clonic, whereas nicotine-induced seizures are clearly tonic-clonic (Gale, 1985; Fan et al., 2000; Deransart et al., 2001). Instead of mediating the convulsant effects of nicotine,  $\alpha 5$ -containing nAChRs in the VTA/SNc might be important for the locomotor effects elicited by nicotine. In experimentally naive rats, nicotine decreases locomotion, but in a familiar environment, it enhances locomotion (Museo and Wise, 1990; Stolerman et al., 1995; Louis and Clarke, 1998). The locomotor alterations produced by nicotine's activation of dopaminergic neurons in the mesencephalon might be one of the effects that reinforce the use of tobacco (Di Chiara, 2000). Dopaminergic neurons in the VTA/SN area express mRNA encoding for the  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  nAChR subunits (Wada et al., 1989, 1990; Klink et al., 2001). A series of studies points to the  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ , and  $\beta 2$  subunits as important for nicotine's effects on DA release and locomotor responses (Pidoplichko et al., 1997; le Novère et al., 1999; Ross et al., 2000; Broide et al., 2002; Champiaux et al., 2002). Klink et al. (2001) recently proposed the existence of four main nAChR subtypes in VTA/SN neurons, two of which might incorporate  $\alpha 5$  in  $(\alpha 4\alpha 6\alpha 5(\beta 2))_2$  and  $(\alpha 4)2\alpha 5(\beta 2)_2$  receptors. Therefore, it is possible that the nicotine-induced locomotor effects are mediated by channels located in the VTA/SN area that contain both  $\alpha 4$  and  $\alpha 5$  subunits.

A latent possibility in most knock-out mice experiments is that of compensation by up-regulation of genes with functions similar to the one ablated. Alternatively, it is possible that the lack of a particular gene creates a general defect in some tissue, creating an indirect phenotype. To assess these possibilities, we studied the brain anatomy, the mRNA expression of other nAChR subunits, and the binding of nicotinic drugs in  $\alpha 5$   $-/-$  mice. None of these experiments revealed any differences between  $\alpha 5$   $+/+$ ,  $\alpha 5$   $+/-$ , and  $\alpha 5$   $-/-$  mouse brains. These results indicate that the  $\alpha 5$  null mutation does not result in the total loss of any binding site. However, it is possible that, although there is no difference in mRNA expression and toxin binding, the functionality of nAChRs is changed in  $\alpha 5$   $-/-$  mice by post-translational modifications, receptor clustering, or other alternative mechanisms.

Possible changes in affinity for nAChR ligands were not addressed but will have to be examined in future studies. Overall, our data argue that the reduced sensitivity to nicotine observed in the  $\alpha 5$   $-/-$  mice is a direct consequence of the lack of  $\alpha 5$ -containing receptors.

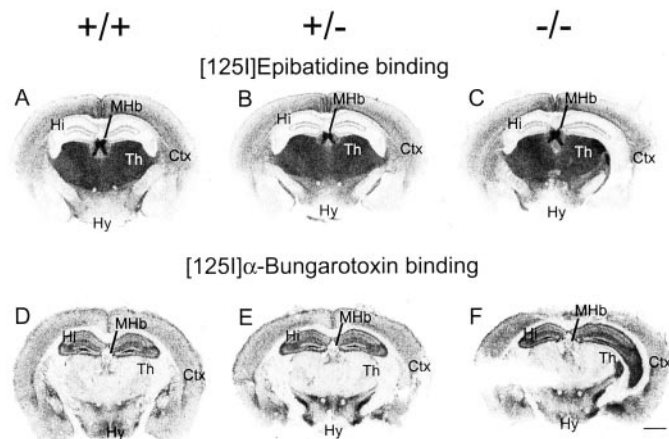
In conclusion, our data demonstrate that  $\alpha 5$ -containing nAChRs influence the expression of nicotine-induced seizures and other behavioral manifestations after short-term administration of nicotine. Our data could be relevant for the study of certain human pathologies such as idiopathic epilepsies, in which mutations on nAChR subunits have been reported to be the genetic cause (Itier and Bertrand, 2002). In addition, our results demonstrated that  $\alpha 5$ -containing nAChRs are critical mediators of behavioral effects that might be relevant for the mechanisms underlying nicotine addiction. We have studied a range of doses that covers from the very low doses, which are similar to those obtained from smoked tobacco and are enough to produce dependence in animals (Corrigall, 1999), to the high doses that are necessary to produce seizures and death. At every dose tested, the effect of short-term nicotine administration is significantly reduced in  $\alpha 5$   $-/-$  mice. Although short-term nicotine administration might not be a perfect model for smokers, the first cigarette of the day, which could be considered "short-term", is usually reported as the most pleasurable one. Therefore, although the long-term effects of cigarette smoking may include many receptors and brain regions (Buisson and Bertrand, 2002), we have shown that  $\alpha 5$ -containing nAChRs participate in the short-term effects of nicotine, which are important for the emergence and maintenance of the smoking habit.

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**Fig. 6.** Normal levels of  $^{125}\text{I}$ -epibatidine and  $^{125}\text{I}$ - $\alpha$ -BTX in  $\alpha 5$   $-/-$  brains. Autoradiographic images of brain sections at the levels of the hippocampus from  $\alpha 5$   $+/+$ ,  $\alpha 5$   $+/-$ , and  $\alpha 5$   $-/-$  mice. Sections show the distribution of  $^{125}\text{I}$ -epibatidine (A–C) and  $^{125}\text{I}$ - $\alpha$ -BTX (D–F) binding sites. Scale bar, 1 mm.

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