Mice lacking neuronal nicotinic acetylcholine receptor β4-subunit and mice lacking both α5- and β4-subunits are highly resistant to nicotine-induced seizures

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1Genetic Institute, Tel Aviv Sourasky Medical Center and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, and 2Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030 Submitted 8 December 2003; accepted in final form 23 February 2004

Kedmi, Merav, Arthur L. Beaudet, and Avi Orr-Urtreger. Mice lacking neuronal nicotinic acetylcholine receptor β4-subunit and mice lacking both α5- and β4-subunits are highly resistant to nicotine-induced seizures. Physiol Genomics 17: 221–229, 2004. First published March 2, 2004; 10.1152/physiolgenomics.00202.2003.—Nicotine, the main addictive component of tobacco, evokes a wide range of dose-dependent behaviors in rodents, and when administered in high doses, it can induce clonic-tonic seizures. Nicotine acts through the nicotinic acetylcholine receptors (nAChRs). Mutations in the human α4- and the β2-nAChR subunit genes cause autosomal dominant nocturnal frontal lobe epilepsy. Using transgenic mice with mutations in nAChR subunits, it was demonstrated previously that the α4-, α5-, and α7-subunits are involved in nicotine-induced seizures. To examine the possibility that the β4-subunit is also involved in this phenotype, we tested mice with homozygous β4-subunit deficiency. The β4 null mice were remarkably resistant to nicotine-induced seizures compared with wild-type and α5 null mice. We also generated mice with double deficiency of both α5- and β4-nAChR subunits and demonstrated that they were more resistant to nicotine’s convulsant effect than either the α5 or the β4 single mutant mice. In addition, the single α5 mutants and the double α5/β4-deficient mice exhibited a significantly shorter latency time to seizure than that of the wild-type mice. Our results thus show that β4-containing nAChRs have a crucial role in the pathogenesis of nicotine-induced seizures. Furthermore, by comparing multiple mutant mice with single and double subunit deficiency, we suggest that nicotinic receptors containing either α5- or β4-subunits are involved in nicotine-induced seizures and that receptors containing both subunits are likely to contribute to this phenomenon as well. However, the α5-subunit, but not the β4-subunit, regulates the rate of response to high doses of nicotine.

NICOTINE ACTS THROUGH THE nicotinic acetylcholine receptors (nAChRs) and can be toxic in high doses in mice, inducing tremors, seizures, and even death (41). The nAChRs are allosteric membrane proteins that belong to a large family of ligand-gated ion channels. Each receptor is composed of a combination of five subunits. To date, 12 neuronal subunits (α2–α10 and β2–β4) have been identified (reviewed in Ref. 29). They are widely distributed in the central and peripheral nervous systems and are also expressed in nonneuronal cells (11, 14, 23, 30, 37).

In humans, nAChRs are associated with a number of pathological conditions, including epilepsy (reviewed in Ref. 35). Mutations in the α4- and β2-subunit genes were associated with familial cases of autosomal dominant nocturnal frontal lobe epilepsy (15, 45, 52–54). In addition, genetic linkage was found between juvenile myoclonic epilepsy and the chromosomal region encompassing the α7-subunit gene (18).

In mice, a genetic linkage was shown between the α4-, α5-, α6-, and α7-nAChR subunit genes and sensitivity to nicotine-induced seizures (55, 56). Although this sensitivity was not changed in mice with homozygous α7 null mutation (25), mice heterozygous for the L250T “gain of function” mutation in this subunit showed increased sensitivity to the convulsant effect of nicotine (6, 28). Increased sensitivity to nicotine-induced seizures was also described in mice with “L9S’ knock-in” mutation in the α4-nAChR subunit (24). In contrast, reduced sensitivity to nicotine-induced seizures was found in mice with α5-nAChR subunit deficiency (48).

Based on the linkage studies results, the localization of the β4-nAChR subunit in a gene cluster with the α5- and α3-subunits (5, 19), and the coassembly of these subunits in Xenopus oocytes (27), we hypothesized that the β4-nAChR subunit may also play a role in nicotine-induced seizures. To test this possibility, the sensitivity to nicotine-induced seizures was compared between β4 null mice, wild-type mice, and mice lacking the α5-subunit. Furthermore, to determine whether there is an interaction between the α5- and the β4-subunits in nicotine-induced seizures phenomena, we generated double-mutant mice with deficiency in both the α5- and β4-subunits (α5–/–β4–/–) and analyzed the response of these double-mutant mice to nicotine. We show here that β4 null mice are more resistant to nicotine-induced seizures than wild-type and α5 null mice and that the α5–/–β4–/– double-knockout mice are extremely resistant to nicotine’s convulsant effect.

METHODS

Generation of double-null mutant mice with deficiency in both the α5- and the β4-subunit genes. Targeting deletions of the individual α5- and β4-nAChR subunit genes in mice were previously described (48, 65). Since the α5- and the β4-nAChR subunit genes are localized within a gene cluster of about 70 kb length on mouse chromosome 9 (5, 19), it was not possible to generate double α5- and β4-deficient mice via breeding of the individual mutants. Therefore, mice with null mutations of both subunits were generated as follows. The β4-nAChR subunit replacement vector, which was described by Xu and coworkers (65), was electroporated into AB2.2 embryonic stem (ES) cells that were heterozygous for the α5 mutation. These ES cells were used to generate the α5-subunit knockout mice, as described by Salas and coworkers (48). The β4 replacement construct contained a 4.1 kb
deletion including most of exon 5, and the α5-subunit mutation also deleted most of exon 5. Both replacement vectors deleted three of the four transmembrane domains of the α5- and β4-subunits. ES cells with double α5β4-subunits deletions were then transmitted into the germ line as previously described (43). Chimeric mice were obtained and bred with C57BL/6J mice to obtain heterozygous double-null mutants.

Animals. All of the mice used in this study were from congenic lines that were backcrossed onto a C57BL/6J background for at least six generations after germ line transmission. Seizure experiments were done on a total of 213 2- to 6-mo-old mice, with a male-to-female ratio of ~50/50. Wild-type littermates from the three mutant strains were used as control mice. Prior to the experiments, the mice were housed in groups of 2–5 animals per cage under a 12:12-h light/dark cycle, with food and water ad libitum. All procedures were approved by the institutional animal and care committee, in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Each experimental mouse was genotyped twice, once before and once after the experiment. Three-way PCRs were performed to determine the mice’s genotypes with primers sequences as follows: for α5 genotype, α5 forward 5'-TCATGAAACTATCCAGACAGCA-3', α5 wild-type reverse 5'-TGGTACGGCGTCATTTAGGCGT-3'; for α5 mutant reverse 5'-TGGGAACACAATAGCAGGCA-3'; for β4 geno- type, β4 forward 5'-TGTAGGACCGACATCCGAAACA-3', β4 wild- type reverse 5'-TCTTACTACTGCTGCTGTC-3', β4 mutant reverse, 5'-AGTACCTTCTGAGGCGGAAAGA-3'. All PCR reactions were done in a total volume of 25 μl, using 1.25 U of Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO) and 500–1000 ng genomic DNA as a template, in a T3 Thermocycler (Biometra, Göttingen, Germany). The PCR conditions for α5 genotyping included an initial denaturation step of 95°C for 5 min followed by 35 cycles, each of 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s and a final extension step of 72°C for 5 min. The PCR conditions for β4 genotyping included an initial “hot-start” denaturation step of 95°C for 3 min followed by 35 cycles, each of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, and a final extension step of 72°C for 5 min. Seizure testing. A single dose of (~)-nicotine (Sigma-Aldrich) was injected intraperitoneally in a volume of 10 μl/g body wt. Since nicotine administration can have long-term effects by desensitizing the nAChRs, each mouse tested was injected only once with a single dose of nicotine. The amounts of nicotine injected were 3, 4, 4.5, and 6 mg/kg for wild-type mice; 3, 6, 9, 12, 15, and 18 mg/kg for α5−/− mice; and 6, 12, 15, 18, and 21 mg/kg for β4−/− mice. The α5−/− β4−/− mice were injected with the same doses as the β4−/− mice with an additional dose of 24 mg/kg. For each mouse strain 10–14 mice were tested for most nicotine concentrations. Four α5−/− mice were injected with low and high doses of nicotine (3 and 18 mg/kg, respectively), and six β4−/− mice were injected with low doses of nicotine (6 and 12 mg/kg). Immediately after injection, the mice were placed in a regular mouse cage and observed by two investigators for at least 5 min. One of the investigators was always blind to both the genotype of the mice and the dose of nicotine injected. Symptoms were scored on a scale of 1–6 (28) as follows: 1, no effect or immobility; 2, mild head tremors and straub tail; 3, more severe tremors and repetitive rapid movements of the forelimbs; 4, wild running and/or complete loss of righting response; 5, clonic seizures; 6, tonic seizures and death. Sensitivity to nicotine-induced seizures was determined by calculating the percentage of mice that reached a score of 5 or 6.

Multiplex RT-PCR. Total RNA was isolated from whole brain tissue using Tri-Reagent (Sigma-Aldrich) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg total RNA using 100 U of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 125 μM each dNTP (Pharmacia, Uppsala, Sweden), and 25 ng/μl oligo(dT)12–18 primer (Invitrogen) in a total volume of 10 μl. Multiplex RT-PCR of α3-, α5-, and β4-nAChR subunits and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) genes was performed. The primers’ sequences (Sigma-Genosys, Rehovot, Israel) are listed in Table 2. All primers are exons spanning. The PCR conditions included the following: an initial “hot-start” denaturation step of 95°C for 3 min followed by 45 cycles, each of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min, followed by 31 cycles with annealing temperature of 62°C, and a final extension step of 72°C for 10 min. Electrophoresis for amplified products was performed on 2% agarose gel stained with ethidium bromide (Sigma-Aldrich).

Quantitative real-time PCR assay. Real-time PCR analyses were performed to determine the expression of α2-, α3-, α4-, α5-, α7-, β2-, β3-, and β4-nAChR subunit genes in whole brain RNA of 4-nAChR subunit genes in whole brain RNA of both genotypes, and the quantitative RT-PCRs were done in triplicate. cDNA was synthesized as described above except for the use of 75 ng/μl random primers (Invitrogen). Quantitative RT-PCRs were performed using LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany). The PCR reactions were performed at a total volume of 10 μl using 3 mM of MgCl2 and 0.5 μM of each primer (except for Gapdh and α4 where primer concentrations of 0.2 μM and 0.3 μM were used, respectively). All PCR conditions included a preincubation step of 10 min at 95°C followed by 45 cycles. Each cycle consisted of a denaturation step of 10 s at 95°C, an annealing step at various temperatures (60–67°C) and times (5–10 s; specific annealing conditions are available upon request), an elongation step of 10 s at 72°C for all primers, except for Gapdh and α7, where an 18-s elongation step was used, and fluorescence measurement at 83°C for 5 s. The primer pair sequences (Sigma-Genosys) and product sizes are described in Table 1. The expression of each nAChR subunit gene was normalized using Gapdh expression levels. The quantification procedure was as follows.

### Table 1. Primer pairs used for RT-PCR analyses

<table>
<thead>
<tr>
<th>Gene (GenBank Accession No.)</th>
<th>Primer Sequence (5’ → 3’)</th>
<th>Fragment Size, bp</th>
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<tr>
<td>α2 (NM_144803)</td>
<td>GTGGCAAGACCTGCGAGTG</td>
<td>127</td>
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<tr>
<td>α3 (AF49902)</td>
<td>GTGAGGATTCGAGGATCCCGCTG</td>
<td>150</td>
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<tr>
<td>α4 (NM_015730)</td>
<td>AGGCGGCTCTGAGAAACACCTG</td>
<td>150</td>
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<tr>
<td>α5 (AF204689)</td>
<td>AGTGGCAAGACCTGCGAGTG</td>
<td>259</td>
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<tr>
<td>α6 (NM_021369)</td>
<td>CGTGGTACGGCGTCATTTAGGCG</td>
<td>257</td>
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<tr>
<td>α7 (AF225900)</td>
<td>AGTGGCAAGACCTGCGAGTG</td>
<td>408</td>
</tr>
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<td>β2 (NM_009602)</td>
<td>GTGGCAAGACCTGCGAGTG</td>
<td>189</td>
</tr>
<tr>
<td>β3 (NM_173212)</td>
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<td>151</td>
</tr>
<tr>
<td>β4 (AF492840)</td>
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<td>205</td>
</tr>
<tr>
<td>Gapdh (M32599)</td>
<td>AGTGGCAAGACCTGCGAGTG</td>
<td>1,452</td>
</tr>
</tbody>
</table>

*Primer pairs used for multiplex RT-PCR. **Primer pairs used for LightCycler real-time RT-PCR."
follows. A standard curve was made for each nAChR subunit gene and for the housekeeping gene (Gapdh) using dilution series of cDNAs. The log concentrations of the nAChR subunit gene (X) and the housekeeping gene (Y) were calculated from a standard curve using LightCycler 5.1 software (Roche Applied Science). The expression levels were then standardized (X/Y) and compared between the different groups of mice (WT, $\alpha_5^{-/-}$, $\beta_4^{-/-}$, and $\alpha_5^{-/-}/\beta_4^{-/-}$). Amplified products were checked by electrophoresis on 2% agarose gel stained with ethidium bromide (Sigma-Aldrich).

Statistical analysis. The results are expressed as means ± SD. Statistical significance was determined by analysis of variance and the Scheffe’s post hoc, Student’s $t$-test, or Mann-Whitney tests (SPSS Inc.). The level of statistically significant differences was defined as $P < 0.05$. The dose response to nicotine was fitted by nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA) to obtain ED$_{50}$ values. Graphs were constructed with Prism software.

RESULTS

Double-null mutants with $\alpha_5$- and $\beta_4$-nAChR subunits deficiencies are viable. Double-knockout $\alpha_5^{-/-}/\beta_4^{-/-}$ mice were born in the expected proportion of genotypes from heterozygous breeding. All $\alpha_5^{-/-}/\beta_4^{-/-}$ mutant mice grew to normal size; they were viable and fertile and showed no obvious gross physical or neurological defects. The average weight was 20.92 ± 4.11 g for the double $\alpha_5\beta_4$-nAChR subunits knockout mice ($n = 67$, age = 11.10 ± 4.16 wk) compared with 20.88 ± 4.4 g for wild-type control mice ($n = 45$, age = 12.84 ± 6.4 wk). All $\alpha_5^{-/-}/\beta_4^{-/-}$ breeding pairs were fertile, with average litter size of 7.25 ± 2.66 ($n = 24$ litters). The male-to-female ratio of all offspring from both heterozygous and double $\alpha_5\beta_4$ null breeding pairs was 1.02 (total of 48 breeding pairs and 339 offspring).

Southern blot analysis using SacI restriction enzyme digest and flanking genomic probes differentiated between the double $\alpha_5\beta_4$ null mutant allele and the wild-type allele (Fig. 1A). The 5.6-kb fragment represents the $\alpha_5$-null allele and is differentiable from the 20.5-kb wild-type fragment, whereas the 5.8-kb $\beta_4$ mutant allele is identified instead of the 7.8-kb wild-type allele (Fig. 1A).

RT-PCR analysis of total brain tissues showed that $\alpha_5^{-/-}/\beta_4^{-/-}$ mice did not express either the $\alpha_5$-nAChR subunit nor the $\beta_4$-subunit. They did, however, express the $\alpha_3$-subunit gene, which is localized between the $\alpha_5$ and the $\beta_4$ genes in the Chrnb4/Chrna3/Chrna5 gene cluster (5) on mouse chromosome 9 (19) (Fig. 1B).

The $\beta_4$ null mice are less sensitive to nicotine-induced seizures than $\alpha_5$ null mice, and double $\alpha_5\beta_4$ null mutants are highly resistant to nicotine-induced seizures. The intraperitoneal injection of nicotine evoked seizure activity in a dose-dependent manner in wild-type mice as well as in $\alpha_5^{-/-}$, $\beta_4^{-/-}$, and $\alpha_5^{-/-}/\beta_4^{-/-}$ mutant mice (Fig. 2). The $\alpha_5^{-/-}$ mice were less sensitive to nicotine-induced seizures than wild-type mice: their ED$_{50}$ value of nicotine was 8.89 ± 0.23 mg/kg.

Fig. 1: A: Southern blot analysis of wild-type, homozygous, and heterozygous $\alpha_5\beta_4$ double-mutant mice. Southern blot analysis of tail DNAs using SacI restriction enzyme digest and flanking genomic probes. The genotypes of the mice are shown at the top, and the wild-type and mutant alleles of $\alpha_5$- and $\beta_4$-nAChR subunit genes are indicated on the left. The two uppermost alleles shown in lane 1 on the left demonstrate the wild-type alleles of $\alpha_5$ (top allele) and $\beta_4$-nAChR subunit genes. These allele sizes are 20.5 kb and 7.8 kb, respectively. The two lower alleles shown in lane 2 demonstrate the mutant $\alpha_5$ and $\beta_4$ alleles, with allele sizes of 5.6 and 5.8 kb, respectively. Note that only the mutant $\alpha_5$ and $\beta_4$ alleles are present in the homozygous double $\alpha_5^{-/-}/\beta_4^{-/-}$ mutant mouse (lane 2). All four normal and mutant alleles are present in DNA obtained from heterozygous double-mutant mice (lanes 3 and 4). B: expression of the $\alpha_3$, $\alpha_5$, and $\beta_4$-nAChR subunit genes in wild-type, $\alpha_5^{-/-}$, $\beta_4^{-/-}$, and $\alpha_5^{-/-}/\beta_4^{-/-}$ mice. Multiplex RT-PCR amplification of $\beta_4$, $\alpha_5$, and $\alpha_3$-nAChR subunits and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) genes using exon-spanning primers resulted in amplicons of 1,452, 259, and 366 bp in length, respectively. RT-PCR was performed using whole brain RNA from wild-type (lane 2 from left), $\alpha_5$ null (lane 3), $\beta_4$ null (lane 4), and double $\alpha_5\beta_4$ null (lane 5) mice. RT-PCR without cDNA was used as a negative control (lane 6). Lanes 1 and 7 contain a 100-bp ladder. Note that $\alpha_5^{-/-}$ null mice do not express the $\alpha_3$-subunit, and the $\beta_4^{-/-}$ mice do not express the $\beta_4$-subunit. The double $\alpha_5^{-/-}/\beta_4^{-/-}$ mice do not express both $\alpha_5$- and $\beta_4$-nAChR subunits. The expression of $\alpha_3$-nAChR subunit gene is observed in the brains of all three knockout strains tested.
and P the markably resistant to nicotine-induced seizures. Only 50% of 12, 15, and 18 mg/kg nicotine to average behavioral scores induced by intraperitoneal injections indicated in Table 2.

...post hoc test). fi cant (one-way analysis of variance and the Sheffe difference was nearly signi ﬀ.

The ED50 value was higher than the ED 50 values of all other groups other group of mice, except between 5 and 4 mice; and 5.36 ± 1.15, 6.00 ± 0.00, and 6.00 ± 0.00, respectively) in α5−/− mice (P < 0.005, P < 0.005, and P < 0.05, respectively, Fig. 3).

Mice with the α5−/β4−/− double-null mutation were remarkably resistant to nicotine-induced seizures. Only 50% of the α5−/β4−/− mice injected with a very high dose of nicotine (24 mg/kg) exhibited seizure behavior (Fig. 2). Their ED50 value was higher than the ED50 values of all other groups of mice tested (Fig. 2, Table 2). The average behavioral scores induced by intraperitoneal injections of 18 mg/kg nicotine to α5−/β4−/− mice were significantly lower than the scores induced by the same dose in β4−/− and α5−/− mice (P < 0.05 and P < 0.01, respectively, Fig. 3).

Table 2. Nicotine-induced seizures in mice

<table>
<thead>
<tr>
<th>Total No. of Mice Tested</th>
<th>ED50, mg/kg</th>
<th>Mean Dose that Induced Seizures, mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>45</td>
<td>4.38 ± 0.21</td>
</tr>
<tr>
<td>α5−/−</td>
<td>57</td>
<td>8.89 ± 0.23</td>
</tr>
<tr>
<td>β4−/−</td>
<td>44</td>
<td>13.58 ± 2.34</td>
</tr>
<tr>
<td>α5−/−β4−/−</td>
<td>67</td>
<td>14.75 ± 0.49</td>
</tr>
</tbody>
</table>

Data are means ± SD; numbers in parentheses represent the number of mice that developed seizures. *P < 0.0005 for each genotype compared with each other group of mice, except between β4−/− and α5−/β4−/− mice, where the difference was nearly significant (one-way analysis of variance and the Sheffe post hoc test).

Fig. 2. Comparison of nicotine-induced sensitivity phenotype between wild-type mice and mice deficient in α5, β4, and α5β4-nAChR subunit genes. Dose response curves for the convulsant effect of nicotine after intraperitoneal injection of a single dose are presented. The percentage of mice that underwent clonic or tonic seizures (scores 5 or 6, respectively) is shown on the y-axis, and the dose of nicotine injected (mg/kg) is shown on the x-axis. A total of 213 mice were tested. The number of mice tested for each dose of nicotine is described in the text. The ED50 of nicotine for each strain of mice tested is indicated in Table 2.

...knockout mice showed signiﬁcantly reduced sensitivity to the behavioral effects of nicotine compared with α5−/− mice at every dose tested. The mean behavioral scores for 12, 15, and 18 mg/kg injections of nicotine were 2.71 ± 0.95, 4.40 ± 0.84, and 5.00 ± 0.78, respectively, for β4−/− mice; and 5.36 ± 1.15, 6.00 ± 0.00, and 6.00 ± 0.00, respectively, for α5−/− mice. The mean scores of α5−/β4−/− mice were 2.92 ± 0.67, 3.75 ± 1.06, and 4.00 ± 0.99 for intraperitoneal nicotine of 12, 15, and 18 mg/kg, respectively. At the highest dose shown here (18 mg/kg), the double α5β4 null mice were significantly (P < 0.05) more resistant to nicotine effects than β4−/− mice.

Fig. 3. Reduced sensitivity of α5−/−, β4−/−, and double α5−/−β4−/− mice to high doses of nicotine. Histograms represent a comparison between the three mutant strains after an intraperitoneal injection of 12, 15, or 18 mg/kg of nicotine (A, B, and C, respectively). The nicotine-induced sensitivity scores are indicated on the x-axis. Column heights indicate the percentage of mice in each score (y-axis). Note, that β4−/− knockout mice showed significantly reduced sensitivity to the behavioral effects of nicotine compared with α5−/− mice at every dose tested. The mean behavioral scores for 12, 15, and 18 mg/kg injections of nicotine were 2.71 ± 0.95, 4.40 ± 0.84, and 5.00 ± 0.78, respectively, for β4−/− mice; and 5.36 ± 1.15, 6.00 ± 0.00, and 6.00 ± 0.00, respectively, for α5−/− mice. The mean scores of α5−/β4−/− mice were 2.92 ± 0.67, 3.75 ± 1.06, and 4.00 ± 0.99 for intraperitoneal nicotine of 12, 15, and 18 mg/kg, respectively. At the highest dose shown here (18 mg/kg), the double α5β4 null mice were significantly (P < 0.05) more resistant to nicotine effects than β4−/− mice.
Determination of the mean dose of nicotine that induces seizures is an additional indication of the relative sensitivity of mice to an intraperitoneal injection of nicotine. This value was determined by calculating the average dose of nicotine injected to those mice that underwent seizures (scores 5 or 6 only). The highest mean dose of nicotine that induces seizures was detected in the α5−/−β4−/− mice, and the lowest was in the wild-type mice (Table 2). The differences were highly significant when each group of mice (wild-type, α5−/−, β4−/−, and α5−/−β4−/−) was compared with the other group (P < 0.005), but nearly significant (P = 0.081) when β4−/− mice were compared with α5−/−β4−/− mice (Table 2).

Latency to seizure is significantly shorter in α5−/− and α5−/−β4−/− mice. Latency to seizure was measured from intraperitoneal injection of nicotine to the first appearance of clonic or tonic seizures. Latency to seizure was significantly shorter in α5−/− and in α5−/−β4−/− mice (61.13 ± 26.95 s and 53.40 ± 30.50 s, respectively) than in wild-type mice (102.54 ± 25.09 s; P < 0.005, Fig. 4A). The time to seizure in β4−/− mice was longer (86.00 ± 60.14 s) than in α5−/− and α5−/−β4−/− mice (Fig. 4A).

Since latency to seizure may be influenced by the different doses of nicotine injected in each group of mutants, we also determined specific latency times for the mean doses of nicotine that induce seizures (Table 2) in each group. The specific latency to seizure was calculated only in those mice that developed clonic or tonic seizures after an intraperitoneal injection of nicotine in mean doses ± SD (4–6, 12–15, 18 mg/kg, and 18–21 mg/kg for wild-type, α5−/−, β4−/−, and α5−/−β4−/− mice, respectively; Fig. 4B). Here, too, α5−/− and α5−/−β4−/− mice had a significantly shorter latency to seizure than wild-type mice (56.52 ± 29.48, 61.00 ± 32.29, and 103.35 ± 25.33 s, respectively, P < 0.05), whereas the latency of β4−/− mice (95.25 ± 55.68 s) was similar to that of wild-type mice (Fig. 4B).

Reduced α3-nAChR subunit gene expression in brains of α5−/−, β4−/−, and α5−/−β4−/− mice. Using quantitative real time PCR, we examined the possibility that deletions of α5- and/or β4-subunits can affect the expression of other nAChR subunits that might mediate seizure activity. The expression levels of α2-, α4-, α5-, α6-, α7-, β2-, β3-, and β4-nAChR subunits were analyzed in whole brains of naive wild-type and knockout mice. The expression of the α3-subunit gene, which is localized in the Chrnb4/Chrna3/Chrna5 gene cluster (5) between the α5 and the β4 genes, was also tested. The α2-, α4-, α6-, α7-, β2-, and β3-subunit expression did not differ between wild-type, α5−/−, β4−/−, and α5−/−β4−/− mice. The α5- and β4-subunit expression was also not changed in the brains of β4 and α5 null mice, respectively (Table 3). The level of expression of the α3-subunit gene, however, was significantly lower (P < 0.0005) in brains of α5−/−, β4−/−, and α5−/−β4−/− mice compared with the level of α3-subunit expression in brains of wild-type mice (Table 3).

**DISCUSSION**

We demonstrate here that the β4-nAChR subunit is involved in the phenomenon of nicotine-induced seizures in mice. Several other nAChR subunits have thus far been shown to be associated with sensitivity to nicotine-induced seizures. Genetic studies comparing inbred strains of mice suggested that the α4-, α5-, α6-, and α7-nAChR subunits are linked to nicotine-induced seizures (55, 56), and further studies in animal models with mutations in nAChR subunits confirmed the involvement of the α7−, α4−, and α5-subunits in this murine phenotype. Whereas heterozygous mice with α7 L250T and α4 L9′S knock-in mutations are more sensitive to nicotine-induced seizures than wild-type mice (6, 24, 28), α5 null mutant mice are less sensitive to nicotine-induced seizures (48). Since the β4- and the α5-subunits are localized within the same gene locus on mouse chromosome 9 and are also coexpressed in several brain areas that are associated with seizures in rodents (49, 59, 62), we hypothesized that the β4-nAChR subunit can also mediate nicotine-induced seizures. The data presented here suggest lines of evidence to substantiate this hypothesis.

The β4 null mice were significantly more resistant to nicotine-induced seizures than α5 null mice. This difference be-
between the two mutant strains suggested that the presence of β4-subunits in nicotinic receptors generates significantly more sensitive channels to nicotine-induced seizures than the presence of α5-subunits. To test the possibility that receptors containing both β4- and α5-nAChR subunits might also be important in mediating seizure activity, we generated a third type of mutant mice lacking both α5- and β4-subunits. The double α5<sup>-/-</sup>β4<sup>-/-</sup> nulls were highly resistant to the convulsant effect of nicotine: about six times as much nicotine was needed to produce seizures in 50% of the double-mutant mice than in 50% of the wild-type controls. When the mean dose of nicotine that produced seizures and the ED<sub>50</sub> values were compared with wild-type controls, the expression levels of all other subunits remained unchanged. NT, not tested. *P < 0.0005 compared with wild type (one-way analysis of variance and Sheffe post hoc test). The expression levels of all other subunits were not significantly changed.

Table 3. Expression levels of α2, α3, α4, α5, α6, α7, β2, β3, and β4 neuronal nAChR subunits in brains of wild-type and null mutant mice

<table>
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<th>Wild Type</th>
<th>α5&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>β4&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<td>α3</td>
<td>1.81±0.06</td>
<td>0.74±0.10*</td>
<td>0.90±0.25*</td>
</tr>
<tr>
<td>α4</td>
<td>0.98±0.14</td>
<td>0.84±0.06</td>
<td>1.25±0.30</td>
</tr>
<tr>
<td>α5</td>
<td>1.62±0.38</td>
<td>NT</td>
<td>1.41±0.21</td>
</tr>
<tr>
<td>α6</td>
<td>1.31±0.07</td>
<td>0.93±0.06</td>
<td>0.99±0.01</td>
</tr>
<tr>
<td>α7</td>
<td>0.93±0.08</td>
<td>0.89±0.02</td>
<td>1.23±0.12</td>
</tr>
<tr>
<td>β2</td>
<td>1.00±0.12</td>
<td>0.82±0.05</td>
<td>1.05±0.14</td>
</tr>
<tr>
<td>β3</td>
<td>1.17±0.11</td>
<td>1.24±0.11</td>
<td>1.11±0.22</td>
</tr>
<tr>
<td>β4</td>
<td>0.60±0.07</td>
<td>0.60±0.12</td>
<td>NT</td>
</tr>
</tbody>
</table>

The expression level of each nicotinic acetylcholine receptor (nAChR) subunit in the brain was determined relatively to Gapdh gene expression. Each value represents the mean ± SD for three brains. Only α3-subunit expression was significantly changed (reduced) in α5, β4, and α5β4 null mutant mice compared with wild-type controls. The expression levels of all other subunits remain unchanged. NT, not tested. *P < 0.0005 compared with wild type (one-way analysis of variance and Sheffe post hoc test). The expression levels of all other subunits were not significantly changed.

The β4-nAChR subunit is expressed in certain brain areas that are known to mediate seizure activity in rodents and humans. Moderate expression levels of β4 were demonstrated in the hippocampus (17) where tonic-clonic seizures can originate (reviewed in Ref. 38). High levels of β4 mRNA expression were detected in the pineal gland and in the medial habenula (17, 49). The pineal gland produces melatonin, a hormone with anticonvulsant properties in rodents (9) and humans (7, 20, 42). Melatonin was shown to interact with nicotinic receptors; it inhibited fast excitatory postsynaptic potentials in guinea pig submucosal neurons by blocking nAChR (2) and also inhibited nicotine-stimulated dopamine release in PC12 cells (50). Low β4-subunit expression was found in the interpeduncular nucleus (49) where seizures might be initiated (10). The interpeduncular nucleus is a target for the medial habenula where β4 expression, along with α3-, α4-, and α5-subunit expression, were detected in nicotine-responsive cells and where receptors containing β4- and α3-subunits likely contribute to the major types of nAChRs (17, 46, 51). Low mRNA expression levels of β4 were also detected in the inferior colliculus (49), another target of the medial habenula, which plays an important role in audiogenic seizures (reviewed in Ref. 26). It is noteworthy that microinjection of nAChR antagonists to the inferior colliculus affected seizure initiation threshold (39), further suggesting that nAChRs might modulate seizure activity there.

Our findings that mice lacking both α5- and β4-subunits are more resistant to nicotine-induced seizures than mice lacking individual α5- or β4-subunits suggested that brain areas expressing both subunits might be prime sites for seizure activity in mice. Such coexpression was detected in the hippocampal CA1 region (17). Sudweeks and Yakel (57), however, did not find significant coexpression of α5 and β4 in individual CA1 neurons. Therefore, it is possible that at least two types of nAChRs, one that contains β4 and the other that contains α5, mediate nicotine-induced seizures in the hippocampal CA1 region. Several other expression studies using either in situ hybridization or RT-PCR of α5- and β4-subunits suggest that additional brain areas, such as the interpeduncular nucleus (48, 49), substantia nigra, and ventral tegmental area (1, 34), may coexpress both subunits and, therefore, may also contribute to seizure activity in mice.

It is worth noting that sympathetic and parasympathetic ganglia, including enteric and some sensory peripheral ganglionic neurons, express the α3-, α5-, α7-, β2-, and β4-nAChR subunits (reviewed in Ref. 61). Most nAChR in the autonomic ganglia are composed of combinations of α3-subunit with β2- or β4-subunits (reviewed in Ref. 61). Since nicotine was injected intraperitoneally, one should consider the possibility that in addition to the effects of nicotine on central neuronal nicotinic receptors, peripheral nAChRs were also activated and played a role in nicotine-induced seizures. Important evidence for the role of the peripheral nervous system in seizure activity is the use of vagus nerve stimulation as a mode of treatment in human epilepsy (reviewed in Ref. 4), as well as to dramatically reduce seizure activity in dogs, cats, and rodents models of epilepsy (22, 58, 63, 67). However, other experiments have suggested that the peripheral nAChR subunits do not play a key role in nicotine-induced seizures. Hexamethonium, a nicotinic antagonist that poorly penetrates the blood-brain barrier, showed little blockade of the convulsive effect of peripherally
administered nicotine (13). Furthermore, when hexamethonium was administered through intracerebroventricular injections it abolished the nicotine-induced seizures response (3). Although these studies emphasized that nicotine-induced seizures are centrally mediated, the possibility that intraperitoneal nicotine exerts some of its seizure activity via peripheral neuronal nicotinic receptors cannot be excluded.

When interpreting results of experiments with knockout mice, it is critical to consider the possibility of overexpression or of downregulation of related genes. We therefore evaluated the possibility that other nAChR subunit genes that may modulate sensitivity to nicotine-induced seizures, such as α2, α3, α4, α5, α6, α7, β2, β3, and β4, are underexpressed or overexpressed in the mutant mice. Of these subunits, only α3 expression was significantly reduced in brains derived from α5−/−, β4−/−, and α5−/−β4−/− mice. It is noteworthy that even low levels of α3-subunit expression are sufficient to maintain a grossly normal phenotype in the α5 (48), β4 (65), and double α5β4 null mice, as in heterozygous α3 null mice, unlike the lethal phenotype of the homozygous α3-deficient mutants (64). It was recently demonstrated that mice heterozygous for the α3 null mutation are less sensitive to nicotine-induced seizures than wild-type mice and that certain brain areas of β4−/− mice have decreased α3 expression (47), thereby suggesting that the reduced α3 expression observed in the brains of the three mutant strains tested (α5−/−, β4−/−, and double α5−/−β4−/) contributed to nicotine-induced seizure resistance. However, since the α3 gene expression was 2−3 times lower in all of the three mutant strains, we concluded that the downregulation of α3 was not responsible for the major differences in sensitivity to nicotine-induced seizures and that these differences are likely to be the result of individual or double subunit deficiencies.

The α3-, α5-, and β4-nAChR subunit genes are localized in a cluster designated as Chrnb4/Chrna3/Chrna5 on mouse chromosome 9 (5, 19). The evolutionary conserved organization of this cluster, the ability of α3-, α5-, and β4-subunits to form a functional receptor, and their overlapping expression in the brain (17, 49, 60, 62) suggested that these three genes share common temporal and spatial transcriptional regulatory mechanisms. One such element was detected in the rat Chrnb4/Chrna3/Chrna5 cluster, which harbors an enhancer located in the 3′-untranslated exon of the β4 gene (16, 40, 66). A possible explanation for our results is that other transcriptional regulatory elements are located in the genomic sequences that were deleted while constructing the α5- and the β4-nAChR subunit replacement mutants.

Another possible explanation for the decreased α3-subunit expression in individual α5 or β4 and double α5β4 null mutants is that the absence of these subunits affects the assembly of α3-containing receptors. It was previously shown that α3-, α5-, and β4-nAChR subunits are coexpressed in sympathetic neurons, adrenal chromaffin cells, and in some central neurons (8, 62), suggesting that α3-subunits can be incorporated with β4- and/or α5-subunits. Furthermore, when expressed in Xenopus oocytes the α3-, α5-, and β4-subunits can form a functional receptor (31).

Death of α3-expressing neurons is yet another mechanism that may explain the reduced α3 expression in brains of α5, β4, and α5β4 knockout mice. The involvement of nAChRs in apoptosis was demonstrated in brains of homozygous α7 L250T−“gain of function” mutant mice (44) and in neuron-like cells that express the mutant chick α7 V251T subunit (36). Additionally, it was shown that nicotine could exert a neuroprotective effect via α7-nAChRs and α4β2-nAChRs (12, 21, 32). Although there is no published data, thus far, linking receptors containing α5- or β4-subunits to neuronal death or neuroprotection, it might be possible that absence of these subunits may lead to neuronal cell death and to decreased α3 expression.

In summary, we have demonstrated here that the β4-nAChR subunit plays an important role in nicotine-induced seizure activity in mice and that mice lacking this subunit are highly resistant to increasing doses of nicotine. Furthermore, by studying double α5β4 knockout mice, we showed that these subunits interact to modulate the severity of the nicotine-induced seizures phenotype. In contrast, the latency to seizure, which is a distinct component of this phenotype, is preferentially determined by the α5 subunit. We therefore suggest that both α5- and β4-nAChR subunits should be considered as candidate genes that may be involved in human epilepsies.

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GRANTS

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REFERENCES


