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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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 administrated 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- and the β2-nAChR subunit deficiencies cause 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 “LyS’ 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

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).
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′-TCAGTAAAATCTTTACAGAGC-3′, α5 wild-type reverse 5′-TGTAGAGCGAGCATCCGAACA-3′, 4.5 mutant reverse 5′-GGTGGTGCTTACGCTTCATTG-3′; 5′-TGGGACACAAATACAGGAGC-3′; for β4 genotype, β4 forward 5′-TGTAGAGCGAGCATCCGAACA-3′, β4 wild-type reverse 5′-TCTCTATCTTGCCTGCTTCT-3′, β4 mutant reverse 5′-AGTACCTTCTGAGCGGAAAGA-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 50–1,000 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 3 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 μg/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/and 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 4 cycles, each of 94°C for 1 min, 67°C for 1 min, and 72°C for 1 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-, α6-, α7-, β2-, β3-, and β4-nAChR subunit genes in whole brain RNA of wild-type, α5/−/−, β4/−/−, and α5/−/− β4/−/− mice. Three different brain samples were tested for each genotype, 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 μM of MgCl2 and 0.5 μM each of 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 2. The expression of each nAChR subunit gene was normalized using Gapdh expression levels. The quantification procedure was as

<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>a2 (NM_144803)</td>
<td>GTCCGCGACAGCTCGGAAG7G</td>
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</tr>
<tr>
<td>a3 (AF495092)</td>
<td>TGTAGTCTGGTCCCTTCTTTG</td>
<td>AACGATGCGGAGGAGCATC</td>
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<tr>
<td>a5 (NM_015730)</td>
<td>AGGTCGAGTCTGAGAGGACGTC</td>
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</tr>
<tr>
<td>a6 (NM_021369)</td>
<td>AGTCATAGTCGTCAGGAG7C</td>
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</tr>
<tr>
<td>a7 (AF225980)</td>
<td>CGATCAGGCTGCAGGGAGGA</td>
<td>189</td>
</tr>
<tr>
<td>β2 (NM_009092)</td>
<td>TGAAGGCGAGTTGGCTCCTGGA</td>
<td>408</td>
</tr>
<tr>
<td>β3 (NM_173212)</td>
<td>CTTAGTGGGCGAAGAGATG</td>
<td>205</td>
</tr>
<tr>
<td>β4 (AF492840)</td>
<td>AGTACGACGATGCTTCCTTAC</td>
<td>1,452</td>
</tr>
<tr>
<td>Gapdh (M32599)</td>
<td>GTCGAGGCGAGTGTCGCTGGTAC</td>
<td>366</td>
</tr>
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</table>

*Some primers used for multiplex RT-PCR. *Some primers 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 then standardized (X/Y) and compared between the different groups of mice (WT, α5−/−, β4−/−, and α5−/−β4−/−). 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 Scheffé 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 α5- and β4-nAChR subunits deficiencies are viable. Double-knockout α5−/−β4−/− mice were born in the expected proportion of genotypes from heterogeneous breeding. The α5−/−β4−/− null mutants grew to normal size; they were viable and fertile and showed no obvious gross physical or neurological deficits. The average weight was 20.92 ± 4.11 g for the double α5β4-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 α5−/−β4−/− 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 α5β4 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 α5β4 null mutant allele and the wild-type allele (Fig. 1A). The 5.6-kb fragment represents the α5 mutant allele and is differentiated from the 20.5-kb wild-type fragment, whereas the 5.8-kb β4 mutant allele is identified instead of the 7.8-kb wild-type allele (Fig. 1A).

RT-PCR analysis of total brain tissues showed that α5−/−β4−/− mice did not express either the α5-nAChR subunit nor the β4-subunit. They did, however, express the α3-subunit gene, which is localized between the α5 and the β4 genes in the Chrnb4/Chrna3/Chrna5 gene cluster (5) on mouse chromosome 9 (19) (Fig. 1B).

The β4 null mice are less sensitive to nicotine-induced seizures than α5 null mice, and double α5β4 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 α5−/−, β4−/−, and α5−/−β4−/− mutant mice (Fig. 2). The α5−/− 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.
and P 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).

The ED50 value was higher than the ED50 values of all other groups other group of mice, except between α5−/− mice and mice de fi

The nicotine-induced sensitivity phenotype between wild-type mice and mice de fi

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 12, 15, and 18 mg/kg nicotine to β4−/− mice (2.71 ± 0.95, 4.40 ± 0.84, and 5.00 ± 0.78, respectively) were significantly lower compared with the scores induced by the same doses (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>Genotype</th>
<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>
<td>5.13 ± 1.01* (24)</td>
</tr>
<tr>
<td>α5−/−</td>
<td>57</td>
<td>8.89 ± 0.23</td>
<td>12.66 ± 3.02* (32)</td>
</tr>
<tr>
<td>β4−/−</td>
<td>44</td>
<td>13.58 ± 2.34</td>
<td>17.84 ± 2.34* (17)</td>
</tr>
<tr>
<td>α5−/−β4−/−</td>
<td>67</td>
<td>14.75 ± 0.49</td>
<td>20.20 ± 3.49* (15)</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 Sheffé 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.

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 y-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 time to seizure is significantly shorter in α5−/− and α5−/−β4−/− mice. Latency time to seizure was measured from the intraperitoneal injection of nicotine to the appearance of seizure. The time to seizure in all the mice that developed seizures (scores 5 or 6) for all nicotine doses is localized in the Chrnb4 gene cluster (5). 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-, 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/Chra3/Chrad5 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 support these findings 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-

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**Fig. 4.** Latency time to seizure in wild-type, α5−/−, β4−/−, and α5−/−β4−/− knockout mice. Histograms represent the time in seconds ± SD from the intraperitoneal injection of nicotine to the appearance of seizure. A: the latency time to seizure in all the mice that developed seizures (scores 5 or 6) for all nicotine doses. B: the latency time to seizure only in those mice that underwent seizures when injected with the “mean doses that induced seizures” for each genotype (see also Table 2). The numbers below the bars (n) represent the number of mice that underwent seizures in each category. The latency time to nicotine-induced seizures is significantly shorter in all knockout strains of mice with α5-nAChR subunit deficiency: the α5−/− and the α5−/−β4−/− null mutant mice. *P < 0.005, **P < 0.05 compared with wild-type mice (one-way analysis of variance and the Scheffé post hoc).

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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−/−β4−/− 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 ED50 values were compared between the strains, the α5−/−β4−/− mutants were more resistant to nicotine than the β4 null mice. These results demonstrate that each subunit contributes to nicotine-induced seizures by itself and that nicotinic receptors which contain either the α5 or the β4 are involved in seizure activity in murine brains. Our data may also suggest that channels, which assemble both α5- and β4-subunits, are involved in nicotine-induced seizures as well.

In addition to properties such as ED50 and mean dose of nicotine that produce seizures, the latency time to seizure can also serve as an indicator for the level of sensitivity to nicotine-induced seizures (6, 56). Our data, obtained from comparisons between single and double-knockout mice, suggested, however, that latency time to seizure is a distinct feature of the seizure phenotype in mice. Although β4−/− and wild-type mice showed a similar latency, α5−/− mice, which were less sensitive to nicotine-induced seizures than wild-type mice, exhibited a significantly shorter latency time to seizure. The α5−/−β4−/− mice, which were most resistant to seizures, also exhibited a significantly shorter latency. These results demonstrate, therefore, that in vivo studies that use multiple genetic animal models help to dissect and identify the pharmacological properties of nAChR subunits that are involved in nicotine-induced seizures. They imply that the α5-subunit, and not β4, regulates the rate of response to high doses of nicotine and that receptors that do not contain α5 respond to nicotine more quickly. An α5-subunit may, therefore, exert an inhibitory effect on the time to seizures.

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>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>α5−/−</th>
<th>β4−/−</th>
<th>α5−/−β4−/−</th>
</tr>
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<tbody>
<tr>
<td>α2</td>
<td>0.98±0.16</td>
<td>1.32±0.32</td>
<td>1.10±0.06</td>
<td>1.19±0.08</td>
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<tr>
<td>α3</td>
<td>1.81±0.06</td>
<td>0.74±0.10*</td>
<td>0.90±0.25*</td>
<td>0.61±0.01*</td>
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<tr>
<td>α4</td>
<td>0.98±0.14</td>
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<td>α5</td>
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<td>α6</td>
<td>1.31±0.07</td>
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<td>α7</td>
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<tr>
<td>β4</td>
<td>0.60±0.07</td>
<td>0.60±0.12</td>
<td>NT</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 nAChR channels (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 seizure, such as \( \alpha_2, \alpha_3, \alpha_4, \alpha_5, \alpha_6, \beta_2, \beta_3, \) and \( \beta_4, \) are underexpressed or overexpressed in the mutant mice. Of these subunits, only \( \alpha_3 \) expression was significantly reduced in brains derived from \( \alpha_5^{−/−}, \beta_4^{−/−}, \) and \( \alpha_5^{−/−}\beta_4^{−/−} \) mice. It is noteworthy that even low levels of \( \alpha_3 \)-subunit expression are sufficient to maintain a grossly normal phenotype in the \( \alpha_5 \) (48), \( \beta_4 \) (65), and double \( \alpha_5\beta_4 \) null mice, as in heterozygous \( \alpha_3 \) null mice, unlike the lethal phenotype of the homozygous \( \alpha_3 \)-deficient mutants (64). It was recently demonstrated that mice heterozygous for the \( \alpha_3 \) null mutation are less sensitive to nicotine-induced seizures than wild-type mice and that certain brain areas of \( \beta_4^{−/−} \) mice have decreased \( \alpha_3 \) expression (47), thereby suggesting that the reduced \( \alpha_3 \) expression observed in the brains of the three mutant strains tested (\( \alpha_5^{−/−}, \beta_4^{−/−}, \) and double \( \alpha_5^{−/−}\beta_4^{−/−} \)) contributed to nicotine-induced seizure resistivity. However, since the \( \alpha_3 \) gene expression was 2–3 times lower in all of the three mutant strains, we concluded that the downregulation of \( \alpha_3 \) 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 \( \alpha_3, \alpha_5, \) and \( \beta_4 \)-nAChR subunit genes are localized in a cluster designated as \( \text{Chrnb4/Chrna3/Chrna5} \) on mouse chromosome 9 (5, 19). The evolutionary conserved organization of this cluster, the ability of \( \alpha_3, \alpha_5, \) and \( \beta_4 \)-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 \( \text{Chrnb4/Chrna3/Chrna5} \) cluster, which harbors an enhancer located in the 3' untranslated exon of the \( \beta_4 \) 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 \( \alpha_5 \)- and the \( \beta_4 \)-nAChR subunit replacement mutants.

Another possible explanation for the decreased \( \alpha_3 \)-subunit expression in individual \( \alpha_5 \) or \( \beta_4 \) and double \( \alpha_5\beta_4 \) null mutants is that the absence of these subunits affects the assembly of \( \alpha_3 \)-containing receptors. It was previously shown that \( \alpha_3, \alpha_5, \) and \( \beta_4 \)-nAChR subunits are coexpressed in sympathetic neurons, adrenal chromaffin cells, and in some central neurons (8, 62), suggesting that \( \alpha_3 \)-subunits can be incorporated with \( \beta_4 \)- and/or \( \alpha_5 \)-subunits. Furthermore, when expressed in \( \text{Xenopus} \) oocytes the \( \alpha_3, \alpha_5, \) and \( \beta_4 \)-subunits can form a functional receptor (31).

Death of \( \alpha_3 \)-expressing neurons is yet another mechanism that may explain the reduced \( \alpha_3 \) expression in brains of \( \alpha_5, \beta_4, \) and \( \alpha_5\beta_4 \) knockout mice. The involvement of nAChRs in apoptosis was demonstrated in brains of homozygous \( \alpha_7 \) L250T-"gain of function" mutant mice (44) and in neuron-like cells that express the mutant chick \( \alpha_7 \) V251T subunit (36). Additionally, it was shown that nicotine could exert a neuroprotective effect via \( \alpha_7 \)-nAChRs and \( \alpha_4\beta_2 \)-nAChRs (12, 21, 32). Although there is no published data, thus far, linking receptors containing \( \alpha_5 \) or \( \beta_4 \)-subunits to neuronal death or neuroprotection, it might be possible that absence of these subunits may lead to neuronal cell death and to decreased \( \alpha_3 \) expression.

In summary, we have demonstrated here that the \( \beta_4 \)-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 \( \alpha_5\beta_4 \) knockout mice, we showed that these subunits interact to modulate the severity of the nicotine-induced seizures phenotype. In contrast, the latency time to seizure, which is a distinct component of this phenotype, is preferentially determined by the \( \alpha_5 \) subunit. We therefore suggest that both \( \alpha_5 \) - and \( \beta_4 \)-nAChR subunits should be considered as candidate genes that may be involved in human epilepsies.

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Preliminary accounts of some of this work have been previously presented (33).

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REFERENCES


