Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain

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NICOTINE affects many aspects of behaviour including learning and memory[1,2] through its interaction with neuronal nicotinic acetylcholine receptors (nAChR). Functional nAChRs are pentameric proteins containing at least one type of α-subunit and one type of β-subunit[3–5]. The involvement of a particular neuronal nicotinic subunit in pharmacology and behaviour was examined using gene targeting to mutate β2, the most widely expressed nAChR subunit in the central nervous system[6–8]. We report here that high-affinity binding sites for nicotine are absent from the brains of mice homozygous for the β2-subunit mutation. Further, electrophysiological recording from brain slices reveals that thalamic neurons from these mice do not respond to nicotine application. Finally, behavioural tests demonstrate that nicotine no longer augments the performance of $\beta2^{-/-}$ mice on passive avoidance, a test of associative memory. Paradoxically, mutant mice are able to perform better than their non-mutant siblings on this task.

The $\beta2$-subunit of the nAChR was disrupted in embryonic stem (ES) cells, and mice deficient in this subunit were then generated (Fig. 1). $\beta2^{-/-}$ mice were viable, mated normally and showed no obvious physical deficits. Overall brain size and organization were normal (see for example Fig. 2*a*, *b*). Western blot analysis of total brain homogenates using anti-$\beta2$ monoclonal 270 (ref. 9; Fig. 1*d*) and immunocytochemistry throughout the brain using a polyclonal anti-$\beta2$ antibody[7] (not shown) demonstrated that the immunoreactivity detected in control mice was absent in $\beta2^{-/-}$ mice and was diminished in $\beta2^{+/-}$ mice. $\beta2$-encoding messenger RNA was undetectable in $\beta2^{-/-}$ mice by *in situ* hybridization using $\beta2$-antisense oligonucleotides (Fig. 2*a*).

The distributions of the $\alpha4$- and $\beta2$-subunits largely overlap in the brain, and these subunits are thought to combine to form the predominant nAChR isoform in the central nervous system (CNS)[10]. On the basis of oocyte expression experiments[11], $\beta4$ is the only subunit identified thus far that might also be able to form functional heteropentamers with the $\alpha4$-subunit. The $\beta4$-subunit was expressed in the medial habenula (MHb) and the interpeduncular nucleus (IPN) in the brain of wild-type, $\beta2^{+/-}$ and $\beta2^{-/-}$ mice (Fig. 2*a*). No $\beta4$ mRNA was seen elsewhere in the brain of mutant mice, indicating that its transcription was not upregulated to replace the $\beta2$-subunit. Nor was the expression of the $\alpha4$- (Fig. 2*a*), $\alpha5$- or $\beta3$-subunit mRNAs (not shown) significantly altered in mutant mice.

Equilibrium binding experiments have shown that nicotine binds to a population of high-affinity sites (dissociation constant K_D near 10 nM[12,13]) whose distribution tallies well with that of

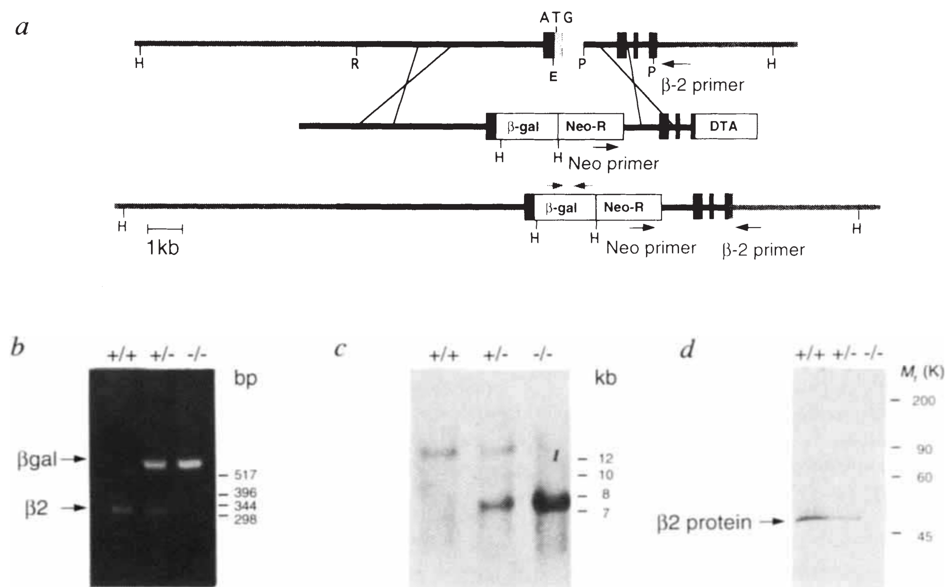


FIG. 1 Disruption of the gene encoding the $\beta2$-subunit of the neuronal nAChR. *a*, Top, Normal genomic structure of the mouse $\beta2$-subunit gene. Portion of exon one removed by the recombination event is shaded in light grey. ATG-initiator methionine. Boxes represent exons I–IV. Middle, Targeting replacement vector used to disrupt the endogenous $\beta2$-subunit gene. Initiator methionine and the rest of the first exon were replaced with the coding region of NLS-*lacZ* and the MC1 neo^R expression cassette[23]. The construct was able to direct *lacZ* expression after stable transfection of PC12 cells (not shown), but *lacZ* expression was never detected in recombinant animals, despite the lack of obvious recombination in the *lacZ* DNA. Diphtheria toxin-A gene (DTA)[24] was used to select against random integration. Bottom, Structure of the mutated $\beta2$-gene. Restriction sites: H, *Hin*dIII; R, *Eco*RI; E, *Eco*47III; P, *Pst*I. Black arrows, primers used to detect recombination events in ES cells. Grey arrows, primers used to detect the wild-type or mutated $\beta2$ genes. *b*, PCR analysis of tail DNA from +/+, +/− and −/− mouse. *c*, Southern blot analysis of tail DNA restricted with *Hin*dIII from the same mice analysed in *b*. *d*, Western blot analysis of total brain protein using a monoclonal antibody raised against the $\beta2$-subunit.

METHODS. *a*, The $\beta2$-targeting vector was constructed by inserting a multiple cloning site (MCS) into the MC1 *neo* cassette (GTCGACGGTACCGCCCGGGCAGGCCTGCTAGCTTAATTAAGCGGCCGCCTCGAGGGGCCCATGCATGGATCC). A 4.1 kb *Eco*RI–*Eco*47III $\beta2$-genomic fragment 5′ to the ATG and a 1.5 kb *Pst*I $\beta2$-genomic fragment starting within the first intron of the $\beta2$-gene were cloned into the MCS. HM1[25,26] embryonic stem cells (5×10^7) were transfected with the linearized targeting vector by electroporation as described[23]. Twenty-four surviving G418-resistant clones were screened by PCR ($\beta2$-primer GCCCAGACATAGGTCACATGATGGT; neo-primer GTTTATTGCAGCTTATAATGGTTACA). Four were positive and were later confirmed by Southern blot analysis (not shown). Clones were injected into 3.5-day-old blastocysts from non-agouti, C57BL/6 mice. All male chimaeric mice were mated to F1, C57BL/6 × DBA/2 non-agouti females. Of 15 chimaeras, one showed germline transmission. $\beta2^{+/-}$ heterozygotes were mated and offspring were evaluated by PCR analysis (*b*). *b*, PCR was 35 cycles of 94 °C for 1 min, 65 °C for 2 min and 72 °C for 1 min. *c*, Southern blotting was as described[27]. The 1.5 kb *Pst*I genomic fragment used for the targeting construct was labelled by random priming. *d*, Western blotting was as described[27] using monoclonal antibody 270[9].

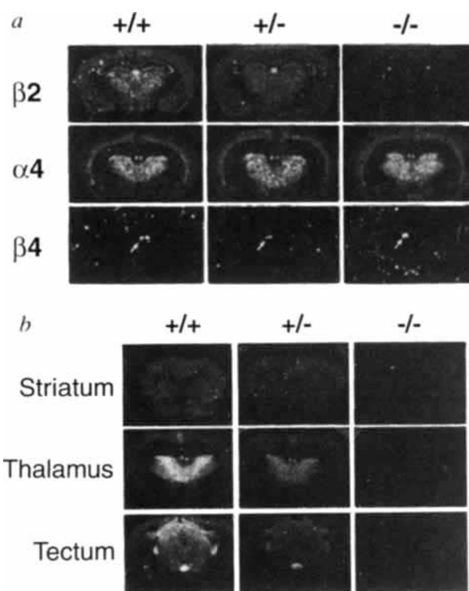


FIG. 2 Mapping of the neuronal nAChR in mouse brain using *in situ* hybridization and tritiated nicotine binding. *a*, *In situ* hybridization using antisense oligonucleotide probes based on the sequence of the cDNAs encoding the $\beta2$-, $\alpha4$- and $\beta4$-subunits of the nAChR to detect their respective mRNAs in serial sections from the brains of $\beta2^{+/+}$, $^{+/-}$ and $^{-/-}$ mice. Midthalamic sections are shown. White arrows indicate the MHb labelled by the $\beta4$-antisense oligonucleotide. *b*, Receptor autoradiography using tritiated nicotine revealing high-affinity binding sites in the brains of wild-type, heterozygous and $\beta2$-mutant mice. Representative sections at the level of the striatum, thalamus and tectum are shown.

METHODS. *a*, *In situ* hybridization was as described in ref. 8. Oligonucleotides: $\beta2$, 5'-TCGCATGTGGTCCGCAATGAAGCGTACGCCATCCACTGCTTCCCG-3'; $\alpha4$, 5'-ACCTTCTCAACCTCTGATGTCTTCAAGTCAGGGACCTCAAGGGGGG-3'; $\beta4$, 5'-ACCAGGCTGACTTCAAGACCGGGACGCTTCATGAAGAGGAAGGTG-3'. *b*, ^{3}H-nicotine binding was as described in ref. 28. Coronal sections (14 μm) were incubated at room temperature for 30 min in 50 mM Tris pH 7.4, 8 mM $CaCl_2$, 4 nM ^{3}H-L-nicotine. Non-specific binding was evaluated in the presence of 10 μM L-nicotine bitartrate. After incubation, sections were rinsed 2 × 2 min in ice-cold PBS and briefly rinsed in ice-cold water. Slides were exposed for 60 days to Hyperfilm ^{3}H.

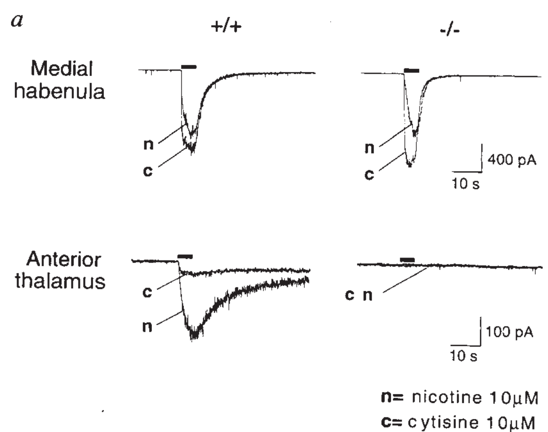
the $\alpha4$- and $\beta2$-subunits[12-14]. Quantitative receptor autoradiography was done using ^{3}H-nicotine (4 nM) to visualize high-affinity nAChR in brain sections from $\beta2^{+/+}$, $^{+/-}$ and $^{-/-}$ mice (Fig. 2*b*). Nicotine binding *in situ* was completely abolished in $\beta2^{-/-}$ animals, and was reduced by roughly 50% in all brain areas in $\beta2^{+/-}$ animals, demonstrating that the $\beta2$-subunit mediates this high-affinity binding.

Neurons of the anterior thalamus express very high levels of $\beta2$- and $\alpha4$-subunit mRNAs and responded consistently to 10 μM nicotine in a slice preparation from wild-type animals with an average inward current of 155 ± 73 pA (Fig. 3*a*) which was blocked by 1 μM dihydro-β-erythroidine. The agonist order of the response was compatible with that seen for $\alpha4/\beta2$-containing nicotine receptors *in vitro*[11] (nicotine > DMPP > cytisine) (Fig. 3*a*).

In $\beta2^{-/-}$ mice the response of anterior thalamic neurons to nicotine was completely abolished in all neurons tested (Fig. 3*b*). As a control, neurons in the MHb, where both $\alpha3$ and $\beta4$ are strongly expressed, were also tested. Nicotine caused an average inward current of 505 ± 132 pA in wild-type mice, and the agonist potency of this response followed the rank order for the $\alpha3/\beta4$-containing receptor (cytisine = nicotine > DMPP) (Fig. 3*a*) and the response was maintained in mutant mice.

The $\beta2$-subunit is expressed in the ganglia of wild-type animals[6-8], but there was no apparent difference in heart rate or basal body temperature in mutant mice. Spontaneous locomotor activity, which is sensitive to high doses of nicotine and is not modified by drugs selective for the $\beta2/\alpha4$ isoform of the nAChR[15], was not significantly different in $\beta2^{-/-}$, $\beta2^{+/-}$ and $\beta2^{+/+}$ mice.

Learning and memory were examined in mutant and wild-type mice using two procedures. The Morris water maze[16,17] evaluates spatial orientation learning. The performance of $\beta2^{-/-}$ mice on this test did not differ from that of wild-type mice when tested on the visible platform task (not shown), or on the hidden platform task (minimum swim-time reached after 5 days of training: $\beta2^{-/-}$ mice ($n=8$), 7.4 ± 1.4 s; wild-type mice ($n=8$), 8.2 ± 2.0 s). In the transfer test both groups of animals spent roughly 35% of the time in the platform quadrant, with the same



b Responses to nicotinic agonists

Nucleus	Genotype	No. of cells tested	Responses	Responses (%)
Antero-dorsal thalamus	+/+	22	22	100
	−/−	15	0	0
Latero-dorsal thalamus	+/+	14	14	100
	−/−	9	0	0
Antero-ventral thalamus	+/+	36	36	100
	−/−	10	0	0
Medial habenula	+/+	8	8	100
	−/−	9	8	89

FIG. 3 Patch-clamp recording of nicotine-evoked currents in the MHb and anterior thalamus of $\beta2^{+/+}$ and $^{-/-}$ mice. *a*, Representative recordings from cells in the MHb and the anterior thalamus of wild-type and $\beta2^{-/-}$ mice. The off-rate of the agonist is significantly greater in the MHb than in the anterior thalamus, resulting in a different kinetics of response in the two structures. The response to nicotinic agonists of the MHb is maintained in $\beta2^{-/-}$ animals, whereas the response to nicotinic agonists of the anterior thalamus is completely abolished in $\beta2^{-/-}$ mice. *b*, Table of responses to nicotinic agonists in various nuclei of $\beta2^{+/+}$ and $^{-/-}$ mice.

METHODS. Coronal slices were obtained from the thalamus of 8–12-day-old mice using a Dosaka slicer in ice-cold ACSF medium (125 mM NaCl, 26 mM $NaHCO_3$, 25 mM glucose, 1.25 mM NaH_2PO_4, 2.5 mM KCl 2.5, 2 mM $CaCl_2$, 1 mM $MgCl_2$ pH 7.3). Slices were maintained in the same medium for 1–8 h. Cells in slices were visualized through a Zeiss microscope. Whole-cell recordings were obtained with 2–4 MOhm hard-glass pipettes containing 150 mM CsCl, 10 mM EGTA, 10 mM HEPES, 4 mM di-sodium-ATP, 4 mM $MgCl_2$, pH adjusted to 7.3 with KOH. Pulses (5–10 s) of drug were applied rapidly to the cell through a 50 μm diameter pipette above the slice, fed by gravity with a solution containing 150 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$. Recordings were made in the presence of CNQX (5 μM) and of the $GABA_A$ antagonist SR-95531 (10 μM) (R.B.I.). Currents were recorded with an Axopatch 1D (Axon Instrument) patch amplifier, digitized on a Compaq PC and further analysed with the PClamp program (Axon Instrument).

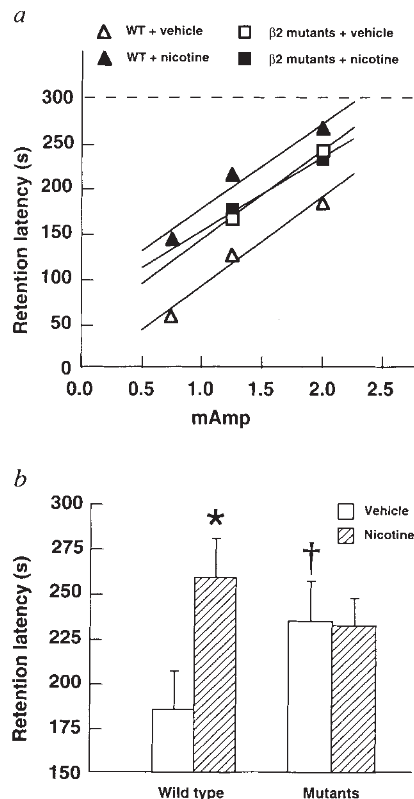


FIG. 4 Performance of $\beta2^{-/-}$ mice and their wild-type siblings on the passive avoidance test. *a*, Response to various levels of foot shock in retention test following a post-training injection of either vehicle or nicotine (10 μg kg^{-1}). Time to first-time entry of the dark chamber (average step-through latency) during the training trial was 17.0 ± 3.6 s for mutant mice and 15.0 ± 3.5 s for their non-mutant siblings. WT, wild-type mice. *b*, Bar graph showing the difference in time spent in the light chamber (retention latency) between wild-type and homozygous $\beta2$ mutant mice injected with either vehicle or nicotine (10 μg kg^{-1}) at foot shock intensity of 2.00 mA. Data are represented as means±s.e.m. of the following groups: wild type + vehicle ($n = 27$); wild type + nicotine ($n = 23$); $\beta2$-mutant mice + vehicle ($n = 17$); $\beta2$-mutant mice + nicotine ($n = 17$). Statistical analysis was performed on the data shown in *a* (obtained at 1.25 and 2.00 mA shock intensity) using a factorial analysis design with 3 between-subject variables and 2 levels for each variable. The analysis indicated a significant interaction between mutant/wild type and vehicle/nicotine factors ($F(1.82) = 5.728$, $P < 0.05$). The analysis of the interaction of simple effects showed a significant difference between mutant mice and wild-type mice injected with vehicle († $P < 0.05$), and between vehicle and nicotine treatment of wild-type mice (* $P < 0.01$).

METHODS. Passive avoidance test was as described in the text[18,19]. In all behavioural tests, each mutant mouse was paired with a control, wild-type animal of the same sex and the same litter (animals were near seven months old for the test). Nicotine bitartrate (Sigma) was freshly dissolved in PBS before each experiment. Intraperitoneal injection of the same volume of either nicotine or vehicle immediately followed foot shock during the training trial.

number of platform crossings ($\beta2^{-/-}$ mice, 4 ± 0.4; wild-type mice, 3.9 ± 0.6).

Retention of an inhibitory avoidance response was assessed using the passive avoidance test, which was also chosen for its pharmacological sensitivity to nicotine administration[18,19]. This test consisted of a training trial in which the mouse was placed in a well lit chamber of a shuttle box, and the latency to enter the adjacent dark chamber was measured. Upon entry to the dark chamber, a mild, inescapable foot shock was delivered, and vehicle or nicotine (10 μg kg^{-1}) was injected into the mouse. After 24 h, retention was assessed by measuring the latency to enter the dark chamber. Time spent in the light chamber (retention latency) increased proportionally to the applied foot shock in both mutant and wild-type mice. Nicotine alone without a shock has no effect on this test[18]. However, treatment with nicotine after foot shock consistently aided retention ($P < 0.01$) by shifting the curve upwards by about 80 s only in wild-type mice (Fig. 4*a*). Nicotine administration was completely ineffective in $\beta2^{-/-}$ mice. Interestingly, retention latency was significantly higher for mutant mice than for their non-mutant, vehicle-injected siblings ($P < 0.05$) (Fig. 4*b*).

Increased retention in the passive avoidance test can be observed in animals with a decreased pain threshold or increased emotionality. Therefore, further behavioural testing was done on all mice included in this experiment. Mutant mice did not differ from their non-mutant siblings for flinch, vocalization or jump response to foot shock (not shown). Possible changes in aversive motivation were studied by measuring exploratory activity in the two-compartment test, which has been pharmacologically validated as an assay of emotionality[20,21]. In a 15 min test, average time spent and locomotor activity in the dark compartment, as well as transitions between compartments, did not differ between the mutant and wild-type mice (not shown). Although this test does not completely rule out changes in motivation in mutant animals, it is an appropriate control for passive avoidance as both tests use dark/lit compartments.

Studies using low doses of nicotine[22] or specific nicotine agonists[15] suggest that high-affinity nAChRs in the brain mediate the effects of nicotine on passive avoidance. This study demonstrates that the $\beta2$-containing nAChRs make up the high-affinity binding sites for nicotine, and also that nicotine cannot change the performance of $\beta2^{-/-}$ mice on passive avoidance, providing the first molecular evidence that nAChRs mediate this effect. The enhanced performance of mutant mice versus wild-type mice is quite surprising, however, and further experiments using these mutant mice will help elucidate the mechanism underlying this behavioural difference. These mice provide a model system for studying the pharmacological effects of nicotine in the CNS, and may be useful in elucidating the role of high-affinity nAChRs in cognitive processes, in nicotine addiction and in dementias involving deficits of the nicotinic system. □

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