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How Mutations in the nAChRs Can Cause ADNFLE Epilepsy

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Summary: *Purpose:* The linkage between autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and neuronal nicotinic acetylcholine receptor has been strongly reinforced by the report of five distinct mutations in the two genes coding for the major brain α4β2 nicotinic acetylcholine (ACh) receptors. As a first step toward understanding the basic mechanisms underlying this genetically transmissible neurologic disorder, we examined the similarities and differences of the functional properties displayed by naturally occurring mutant forms of this ligand-gated channel.

Methods: Functional studies of neuronal nicotinic ACh receptors reconstituted in *Xenopus* oocytes were designed to analyze the common traits displayed by the different mutations associated with ADNFLE.

Results: Coexpression of the control and mutated alleles harboring the α4S248F mutation obtained from patient DNAs yielded ACh-evoked currents of amplitude comparable to the control responses but with a higher sensitivity and desensitization to the natural agonist. Alternatively, the other mutants (α4L776ins3, α4S252L, and β2V287M) displayed an increased ACh sensitivity without pronounced desensitization. In addition, whereas a reduction of calcium permeability was observed for the mutants (α4S248F and α4L776ins3), no significant modification of ionic selectivity could be detected in the α4S252L mutation. Hence increase in ACh sensitivity is the only common characteristic so far observed between the four naturally occurring mutant receptors investigated.

Conclusions: Analyses of functional properties of four nAChR mutants associated with ADNFLE indicate that a gain of function of these mutant receptors may be at the origin of the neuronal network dysfunction that causes the epileptic seizures. These data are discussed in the context of our latest knowledge of the pyramidal cell function. **Key Words:** ADNFLE—Epilepsy—Nicotinic receptors—Genetic—Structure/function.

Whereas epilepsy is one of the most frequent neurologic disorders, its cause remains unclear. The recent identification of an idiopathic type of epilepsy that displays mendelian inheritance opened new perspective in the analyses of this important disorder. A mutation in *CHRNA4,* the gene coding for one of the neuronal nicotinic acetylcholine receptor subunit (nAChRs) linked with a nocturnal epilepsy originating in the frontal lobe that segregates as an autosomal dominant trait (autosomal dominant nocturnal frontal lobe epilepsy; ADNFLE) (1,2), stimulated new studies on these ligand-gated channels. The determinant role of nAChRs in ADNFLE was later confirmed with the identification of other mutations in *CHRNA4* (3,4) and *CHRNB2* (5,6). Because the major brain nAChRs result from the assembly of the α4 and β2 subunits (in a stoichiometry of 2 α and 3 β), it had been predicted that mutations in either of these subunits should cause similar forms of epilepsy.

Functional analyses carried out with recombinant receptors obtained by expression of the corresponding mutations revealed differences between mutant receptors. The first-identified α4S248F mutation caused a marked increase in receptor desensitization and reduction in mean ACh-evoked current amplitude (7,8). In contrast, the second-identified mutation (L776ins3), which corresponds to an insertion of an extra leucine in the second transmembrane domain, provoked no significant modification of either the current amplitude or desensitization (3). The common property reported at that time for these two mutant receptors was a reduction of the calcium permeability (3,7). It was concluded that ADNFLE was caused by a loss of nAChR function that may affect the release of other neurotransmitters (9).

The aim of this study was to examine and compare the effects caused by four of the identified ADNFLE mutations on the functional properties of the major brain α4β2 nAChRs. Placed in the perspective of finding the

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common determinants that could be at the origin of the epilepsy, reconstitution experiments were conducted with expression conditions that best resemble those of the patients.

METHODS

cDNA preparation for α4S248F, α4L776ins3, α4S252L, and β2V287M

Blood samples were taken from consenting individuals and genomic DNA extracted. Genomic amplification of exon 5 allowed us to obtain directly the α4S248F, α4L776ins3, and α4S252L mutations from the patients. A 1,460-bp fragment corresponding to *CHRNA4* exon 5 and flanking intronic regions was amplified by polymerase chain reaction (PCR) under the following standard conditions: primers 5′-GTGGACGTGGGCATGGATC-3′ and 5′-TACACACCAGGAAGAAAGGG-3′ Euro-Taq (Eurobio, Paris, France) with 1 m*M* MgCl2, and thermal cycling: 95°C for 3 min, followed by 30 cycles at 95°C for 1 min, 52°C for 30 s, and 72°C for 1 min. This fragment was double digested with 4 U of each *Bst*EII and *Bsr*DI restriction enzymes (New England, Biolabs, Basel, Switzerland) at 60°C overnight. The digested fragment (1,250 bp) containing the mutation to be investigated was purified (Qiagen Gel purification kit, Qiagen, Basel, Switzerland) from a 1.5% NuSieve GTG agarose gel (FMC). Meanwhile, the exon 5 from a wild-type *CHRNA4* gene, cloned by the Abbott laboratories and inserted in the pZeo/SV2 plasmid (Invitrogen, Groningen, The Netherlands), was removed from the plasmid using the same *Bst*EII and *Bsr*DI restriction enzymes (same conditions). The vector was then dephosphorylated using 2 U of calf alkaline phosphatase (Gibco BRL, Basel, Switzerland). The genomic *CHRNA4* exon 5 was then inserted into the plasmid using 40 U of T4 DNA ligase (New England, Biolabs) at 16°C overnight. This plasmid was then electroporated (BioRad apparatus and trays, Reinach BL, Switzerland) in ElectroComp TOP10F' cells (Invitrogen). To identify the different alleles, a 688-bp fragment corresponding to the 5′ half of CHRNA4 exon 5 and containing the α4S248F mutation was amplified using the primers: 5′-ACTTTCGCGGG TCACCCACCTG-3′ and 5′-GGCCGCTTGATGAG GAGCAG-3′ and the same standard conditions as described later. The alleles were identified by single-strand conformation analysis (SSCA) with an acrylamide gel gradient of 4–20%, migrated 3 h at 300 V, and stained with ethidium bromide. By comparison of the SSCA pattern of the clones with genomic DNA SSCA pattern, the two alleles were distinguished for all the heterozygous individuals. The selected clones were then grown and purified (Plasmid midi kit, Qiagen). All resulting cDNAs were checked for quality and fully sequenced. Exon 5 containing the α4S252L mutation was amplified and inserted into the expression vector using a comparable method.

Construction of the β2 subunit containing the V287M mutation was done by site-directed mutagenesis as previously described (6).

Electrophysiology

Expression of recombinant nAChRs in *Xenopus* oocytes was carried out as previously described (10). In brief, stage 5–6 oocytes harvested from female *Xenopus* were enzymatically and mechanically dissociated and, on the next day, 2 ng cDNA was injected into the nucleus. Equal amount of plasmids containing the α and β cDNAs were injected. To simulate the expression of a "heterozygote," 0.5 ng of α4 control + 0.5 ng α4 mutated and 1 ng β2 was injected. After 2–3 days, oocytes were recorded using a two-electrode voltage clamp (Geneclamp; Axon Instrument, Foster City, CA, U.S.A.). Unless indicated, cells were held at –100 mV, and their response to agonists or to carbamazepine determined. Oocytes were continuously superfused with an artificial extracellular medium containing in m*M:* 82.5 NaCl; 2.5 KCl; 2.5 CaCl2; 1 MgCl2; 5 HEPES, at pH 7.4 (adjusted with NaOH).

Data analyses

Dose–response activation relations were fitted with a sum of two empirical Hill equations:

$$y = I_{max}\{a/[1 + EC_{50}\,H/x)^{nH1}] + (1 - a)/[1 + (EC_{50}\,L/x)^{nH2}]\} \quad (1)$$

where I_{max} is the maximal current amplitude and x is the agonist concentration. EC_{50}H, nH1, and a are the half-effective concentration, the Hill coefficient, and the percentage of receptors in the high-affinity state, whereas EC_{50}L and nH2 are the half-effective concentration and the Hill coefficient in the low-affinity state.

Dose–response inhibition curves were fitted by a single Hill equation in the form:

$$Y = 1/(1 + x/IC_{50})^{nH} \quad (2)$$

where y is the fraction of remaining current, x is the antagonist concentration, IC_{50} is the concentration producing half-inhibition, and nH is the empirical Hill coefficient.

Calcium-reversal potential was fitted by the Goldman–Hodgkin–Katz (GHK) constant field equation (11). Sodium and calcium permeabilities were computed relative to potassium that was assumed to be unity. Thus pNa/pK and pCa/pK were adjusted to obtain the best fits.

Unless specified standard error of means were computed and values indicated.

RESULTS

To date, five distinct mutations of the nAChR subunits linked with ADNFLE have been found from a variety of ethnic groups (Fig. 1A). Observed independent (12) and

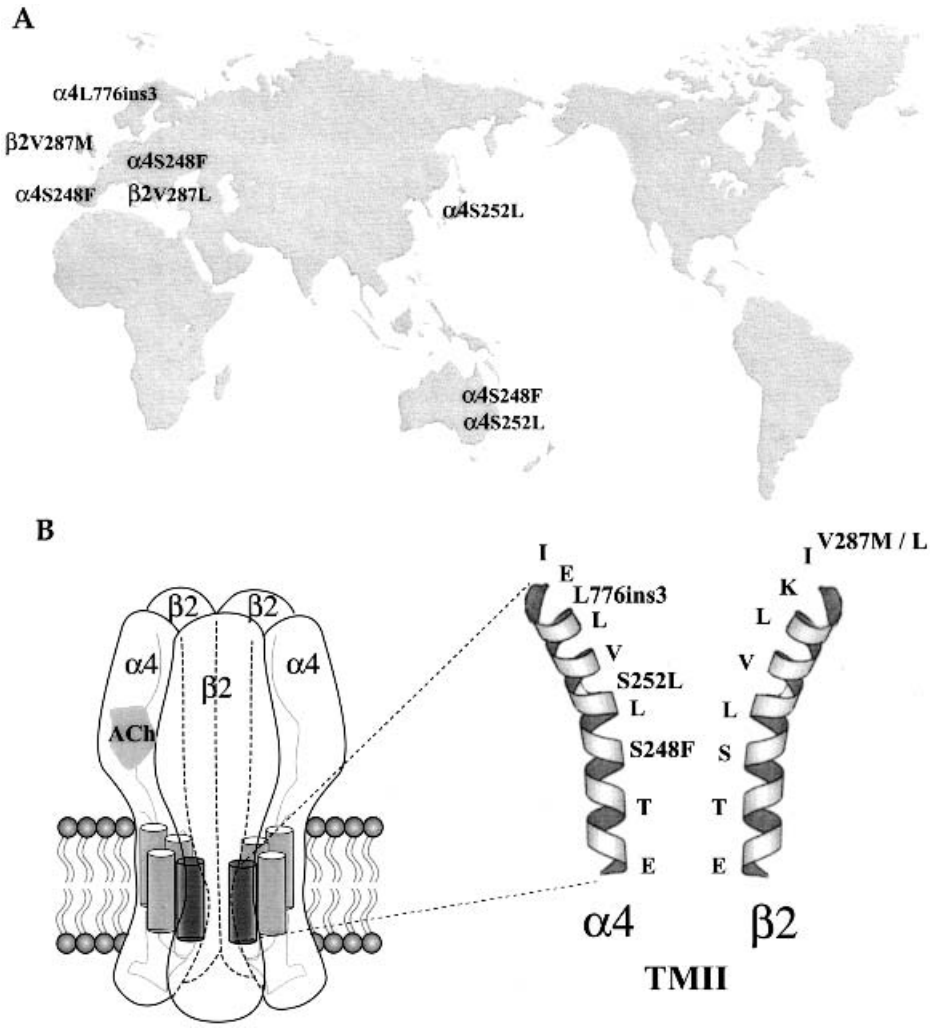

FIG. 1. Geographic and physical distribution of the different mutations associated with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). **A:** Worldwide map with the localization of the families in which the different ADNFLE mutations were identified. **B:** Schematic representation of a neuronal α4β2 nicotinic acetylcholine receptor (nAChR) with, in inset, a blowup of the second transmembrane (TMII) domain and the location of the different mutations identified in **A.**

spontaneous (13) nature of these mutations explained their widespread geographic occurrence. All mutations identified to date are located within the second transmembrane segment (TMII; Fig. 1B).

Previous characterizations of the first identified α4S248F mutation in the *CHRNA4* gene have shown that this single amino acid substitution causes marked alteration of the receptor function (7,8,14–16). Although providing important information, receptors reconstituted in these experiments may differ from those of ADNFLE patients in two respects. First, cDNAs used in these approaches have been obtained by genetic engineering and may therefore differ from those of the patients. Second, it is known that ADNFLE patients are heterozygotes for either the *CHRNA4* or *CHRNB2* genes. This indicates that a single individual carries two alleles coding for the *CHRNA4* or *CHRNB2* and that only one of the two alleles contains the mutation (Fig. 2A). Hence, unless gene promotors differ between the two alleles, it is expected that both alleles should be equally expressed in a given cell. Considering this genetic element, it follows that care must be taken when attempting to deduce pathophysiologic issues from the physiologic experiments carried out in oocytes.

In an attempt to mimic the heterozygous nature of the *CHRNA4* gene, co-injection of both the normal and mutated cDNAs were done (17). Moreover, to be as close as possible to the patient condition, exon 5 from the α4 subunit patient DNA was amplified by polymerase chain reaction (PCR) and inserted into the expression vector. More detailed analyses of these reconstituted heterozygous receptors showed important aspects. Whereas α4S248F-containing receptors exhibited acetylcholine (ACh)-evoked currents of significantly lower amplitude than the controls (8), heterozygous receptors could not be distinguished on the basis of their ACh-evoked currents (Fig. 2D). In contrast, the pronounced desensitization of the α4S248F mutant was conserved in these heterozygous type receptors (Fig. 2B). Recent functional experiments on transfected cells expressing the human α4β2 receptors have shown that dose–response relations of these nAChRs display a dual sensitivity profile with a

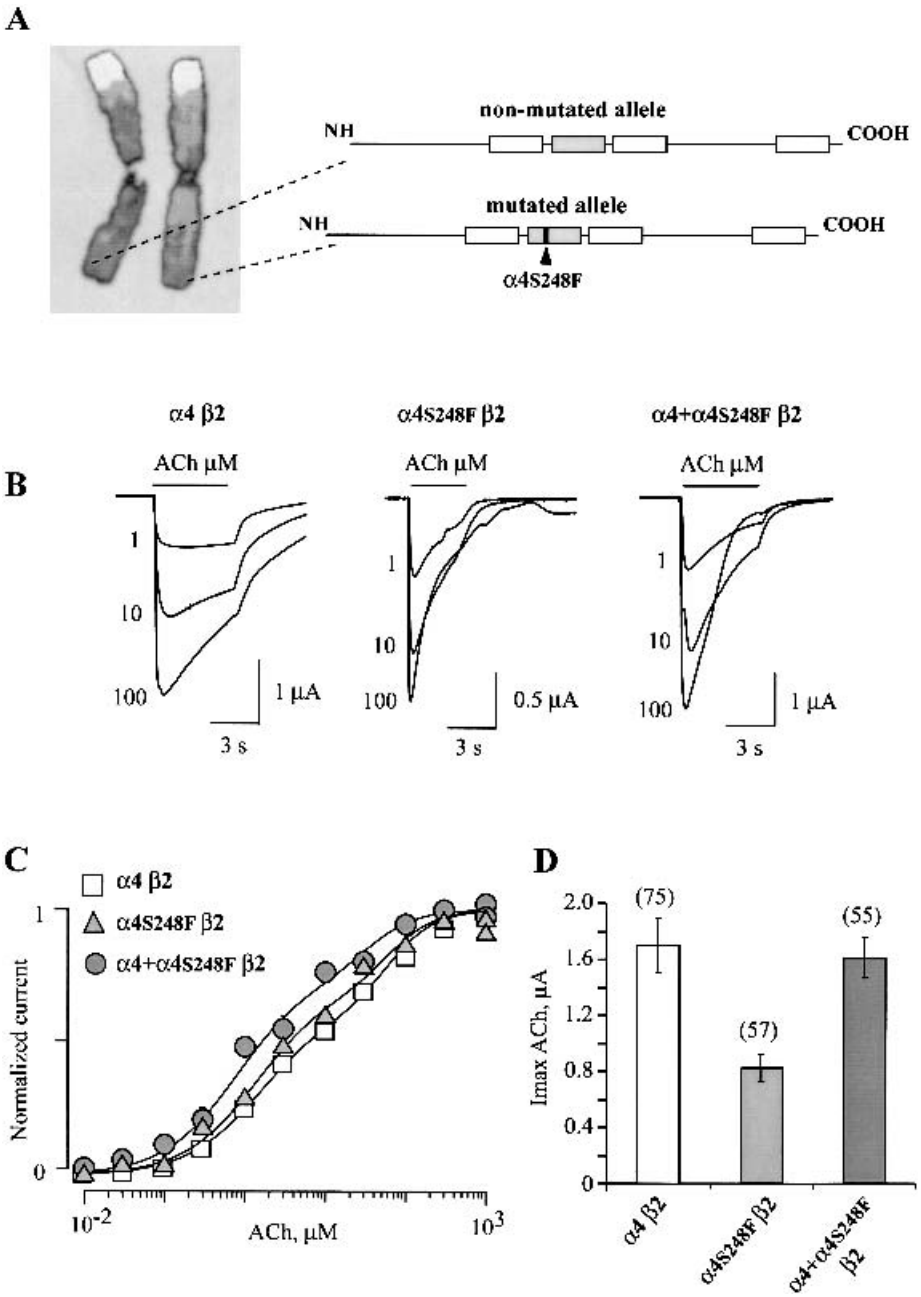

FIG. 2. Dominant phenotype of the α4S248F mutant is revealed in heterozygous expression. **A:** Schematic representation of a chromosome, its two alleles, and the α4S248F mutation. **B:** Acetylcholine (ACh)-evoked currents recorded in control, homozygous, and heterozygous expression of the α4S248F mutant. Currents evoked by three ACh concentrations are superimposed. Cells were held at –100 mV throughout the experiment. **C:** ACh dose–response relations of the control (squares), homozygous (triangles), and heterozygous (circles) α4S248F mutant. Lines through the data points are the best fits obtained with Eq. 1 (see Methods). Parameters of the fits are given in Table 1. **D:** Histograms of the maximal ACh-evoked currents of the three combinations.

high- and a low-affinity component (18). Performing the same measurements on control allele, α4S248F mutated allele, and heterozygous type (control + S248F) revealed that the latter present a significantly higher sensitivity to ACh than do controls (Fig. 2C, Table 1).

The identification of different mutations raises the question of a possible common alteration of the receptor properties that would be at the origin of ADNFLE. To examine this question, experiments were carried out with the recently identified α4S252L mutant (4). Injection of α4S252L with β2 in *Xenopus* oocytes yielded functional receptors that displayed ACh-evoked currents that could not be distinguished on the basis of their time course from those of the control (data not shown). Measurement of the ACh-evoked currents in a large number of cells and over a broad range of concentrations allowed further characterization of these receptors. As described earlier, both homozygous and heterozygous types of expression were carried out. As seen in Fig. 3A (and Table 1) receptors carrying the α4S252L mutation displayed a significantly higher sensitivity to ACh than did cells expressing only the control allele. Determination of the maximal evoked currents revealed significant lower ACh-evoked currents in the α4S252L homozygous expression, whereas heterozygous were indistinguishable from the control (Fig. 3B).

TABLE 1. *Pharmacologic properties of control and ADNFLE mutated nAChRs*

cDNA type	%(H)	EC_{50} (H) ACh (μ*M*)	nH1	%(L)	EC_{50}(L) ACh (μ*M*)	nH2	EC_{50} Choline (m*M*)	nH	IC_{50} ACh (n*M*)	nH	IC_{50} CBZ (μ*M*)	nH	I_{max}ACh (μA)
α4β2	62 ± 3	1.6 ± 0.1 (n = 75)	0.95 ± 0.07	38 ± 3	62 ± 8.3	1.5 ± 0.2	0 (n = 3)	0	111 ± 2 (n = 4)	1.4 ± 0.15	140 ± 85 (n = 9)	0.85 ± 0.09	1.7 ± 0.2 (n = 75)
α4S248Fβ2	70 ± 2	1.4 ± 0.1 (n = 57)	0.95 ± 0.06	30 ± 2	62 ± 2.3	1.5 ± 0.1	—	—	0.028 (n = 2)	0.8	51 (n = 2)	0.8	0.8 ± 0.1 (n = 57)
α4+ α4S248Fβ2	61 ± 4	0.89 ± 0.2 (n = 55)	1.33 ± 0.06	39 ± 4	33.6 ± 5.7	1.43 ± 0.05	1.5 ± 0.2 (n = 6)	1.72 ± 0.14	—	—	—	—	1.6 ± 0.14 (n = 55)
α4β2	65 ± 5	0.55 ± 0.05 (n = 8)	1.1 ± 0.03	35 ± 5	51 ± 3.5	1.28 ± 0	0 (n = 3)	0	—	—	—	—	1.8 ± 0.11 (n = 15)
α4L (776ins3)β2	74 ± 3	0.14 ± 0.04 (n = 6)	1.33 ± 0.06	26 ± 3	10.2 ± 2.3	1.28 ± 0.1	—	—	38 ± 16 (n = 5)	1.2 ± 0.11	66 (n = 2)	0.8	1.62 ± 0.11 (n = 22)
α4+α4L (776ins3)β2	—	—	—	—	—	—	0 (n = 3)	0	—	—	—	—	—
α4β2	23 ± 7	4.5 ± 0.32 (n = 7)	1.04 ± 0.04	77 ± 7	75 ± 12	1.53 ± 0.08	0 (n = 3)	0	28 ± 3 (n = 6)	1.2 ± 0.1	171 ± 104 (n = 5)	1.32 ± 0.2	20.7 ± 3 (n = 15)
α4S252Lβ2	66 ± 10	0.13 ± 0.04 (n = 13)	1.06 ± 0.05	34 ± 10	6.18 ± 1.15	1.33 ± 0.06	—	—	2 ± 0.2 (n = 6)	0.55 ± 0.02	177 ± 48 (n = 5)	1.47 ± 0.2	11.6 ± 2 (n = 18)
α4+ α4S252Lβ2	72 ± 5	0.75 ± 0.17 (n = 7)	1.27 ± 0.08	28 ± 5	30 ± 4	1.47 ± 0.16	0.49 ± 0.1 (n = 7)	2 ± 0	22 ± 2 (n = 6)	0.8 ± 0.06	296 ± 6 (n = 5)	1.38 ± 0.15	20.8 ± 2 (n = 17)
α4β2	26 ± 5	3.9 ± 1.3 (n = 8)	1.2 ± 0.2	74 ± 5	47.6 ± 18.8	1.3 ± 0.2	0 (n = 3)	0	—	—	—	—	21.8 ± 3.2 (n = 5)
α4β2V287M	77 ± 2	0.25 ± 0.04 (n = 7)	1.6 ± 0.1	23 ± 2	2.9 ± 0.9	1.9 ± 0.1	—	—	—	—	—	—	7.8 ± 3.2 (n = 4)
α4β2+ β2V287M	65 ± 3	0.42 ± 0.14 (n = 7)	1.6 ± 0.1	35 ± 3	5.3 ± 2.3	1 ± 0.34	0.43 ± 0.07 (n = 4)	1.73 ± 0.05	—	—	71 ± 8 (n = 6)	1.16 ± 0.06	18.9 ± 4 (n = 7)

ACh, choline, and carbamazepine sensitivities were determined for the four different mutations expressed either in homozygous or heterozygous combination.
Values and standard error of means are indicated; number of cells tested in each condition is given in parenthesis. (H) and (L) refer, respectively, to the high- and low-affinity components.

Because it had been previously shown that receptors containing the α4S248F or α4L776ins3 mutants are less permeable to calcium than are the controls, ionic selectivity of the α4S252L was determined. Best assessments of the ionic selectivity are obtained by measuring the response-reversal potential in different ionic conditions. As shown in Fig. 3C and D, the control and the heterozygote containing α4S252L mutant present a comparable dependence of their reversal potential versus the extracellular calcium concentration. In addition, ACh-evoked currents recorded in three calcium concentrations were of similar time course and amplitude for both the control and heterozygous mutant nAChRs (right panel, Fig. 3C and D).

Receptor desensitization, that is, the reduction of response on sustained agonist exposure, is one of the important properties of ligand-gated channels. As described earlier, α4S248F mutant receptors are more prone to desensitization than are control receptors (7,8,15). Measurements of successive currents evoked by brief ACh test pulses first in control condition and during a sustained exposure to a low-ACh concentration reveal desensitization properties (Fig. 4). As seen in this figure, homozygote control, α4S248F, and α4S252L can be recognized on the basis of their desensitization profile. However, when the same experiments were carried out with the heterozygous expression, both α4S248F- and α4S252L-containing receptors displayed properties close to those of control receptors.

Carbamazepine (CBZ) was found to be one of the most efficacious antiepileptic treatments (AEDs) for the members of the Australian family with ADNFLE (19). Interestingly, it was reported that this compound acts as a noncompetitive inhibitor at the α4β2 nAChRs (16). Furthermore, it was shown that both α4S248F and α4L776ins3 mutants are blocked by CBZ at concentrations comparable to those observed in patient blood (16). To complement these measurements, CBZ sensitivity of several mutants was compared. CBZ dose–response inhibition was determined for two of the recently identified mutations (Fig. 5A and B, Table 1). Although incubation with 320 μ*M* CBZ caused a significant inhibition of the ACh-evoked currents (right panels, Fig. 5A and B), blockade was more pronounced at the β2V287M mutant than at the α4S252L. Differences in CBZ sensitivities are best detected when observing the concentration that produces half inhibition (Fig. 5C, Table 1). Indeed, the three mutations α4S248F, α4L776ins3, and β2V287M present a significantly higher CBZ sensitivity than either control or the α4S252L mutant.

In the search of an association between functional modification of the receptor properties and epilepsy, it is important to recall the mechanisms involved at the synaptic level. After depolarization of the synaptic bouton, calcium rapidly enters the cell and provokes the release of ACh contained in clear vesicles. This causes a very rapid and massive increase of ACh concentration in the extracellular space. ACh is then cleaved by ACh-esterase

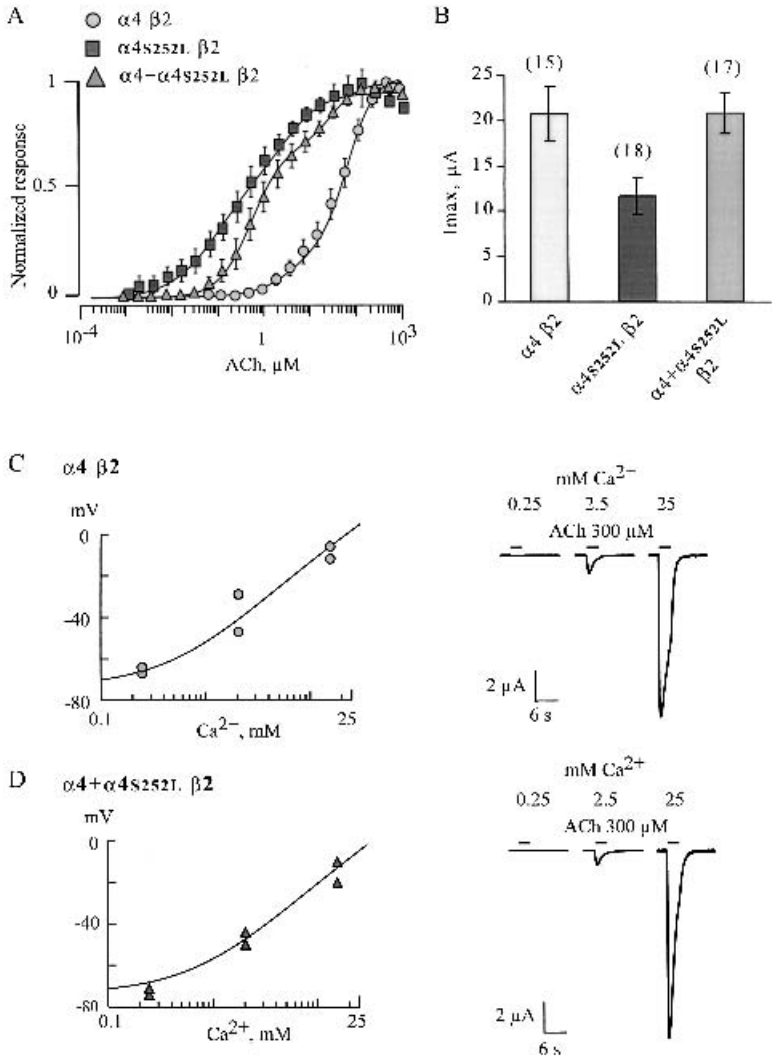

FIG. 3. Properties of receptors containing the α4S252L mutant. **A:** Acetylcholine (ACh) dose–response relations of the control α4β2 receptors (circles, n = 7), α4S252L β2 (homozygous, filled squares, n = 13) and co-injection of α4 and α4S252L together with β2 (heterozygous, triangles, n = 7). Lines through the data points are the best fits obtained with two empiric equations; values are indicated in Table 1. **B:** Mean amplitude of the ACh-evoked currents recorded in α4β2 control, homozygous, and heterozygous α4S252L-containing receptors. Currents evoked by saturating ACh pulses (300 μM, 3 s) were recorded in 15, 18, and 17 cells, respectively. **C, D:** Calcium permeability of α4β2- and α4S252L-containing receptors. Reversal potentials of the ACh-evoked currents were measured in three external calcium concentrations. Typical currents recorded in these three conditions are represented in the right panels. Lines through the data points are the best fit obtained with the GHK equation (11). PCa/pK were of 1.5 and 1 for the control and α4S252L-containing receptors, respectively.

with the production of choline (Fig. 6A). It was reported that choline acts as agonist at some nAChR subtypes (20,21). In view of this observation, it was necessary to examine whether choline that is present in micromolar concentration in the cerebrospinal fluid and likely reaches high concentration values after ACh release can activate the mutant receptors. Applications of brief choline pulses caused no detectable current at the control α4β2 receptor, even at 5 m*M* (Fig. 6B). Similarly, choline failed to activate receptors containing the α4L776ins3 mutant. In contrast, choline evoked a small but significant inward current in α4S248F-, α4S252L-, and β2V287M-containing receptors (Fig. 6D–F). Respective EC_{50} values for these three mutant receptors ranged, however, between 0.4 and 1.5 m*M* (Table 1).

DISCUSSION

Early results obtained in reconstitution studies correlate only partially with the natural condition. One of these important limitations was that reconstitution was obtained by expression of the mutated α4 and control β2 cDNAs. Whereas in a scientific approach, this constitutes the first step, it should be recalled that patients with ADNFLE are heterozygotes (2,3). Thus unless the promotor sequences of the two alleles profoundly differ, it is expected that both mutated and nonmutated alleles should be equally expressed in a given neuron. Therefore, experiments should include reconstitution with the mutated cDNA alone and by coexpression of the mutated and nonmutated alleles. The main differences between homozygous and heterozygous expression of the α4S248F mutant reside in the maximal amplitude of the ACh-evoked current (Fig. 2B) and reduction of the desensitization caused by prolonged agonist exposure (Fig. 4). Interestingly, coexpression experiments reveal a dominant effect in which the fast desensitization observed during the ACh response persists, as observed in homozygous receptors, but is accompanied by an increase in the ACh sensitivity (Fig. 2B and C). Although

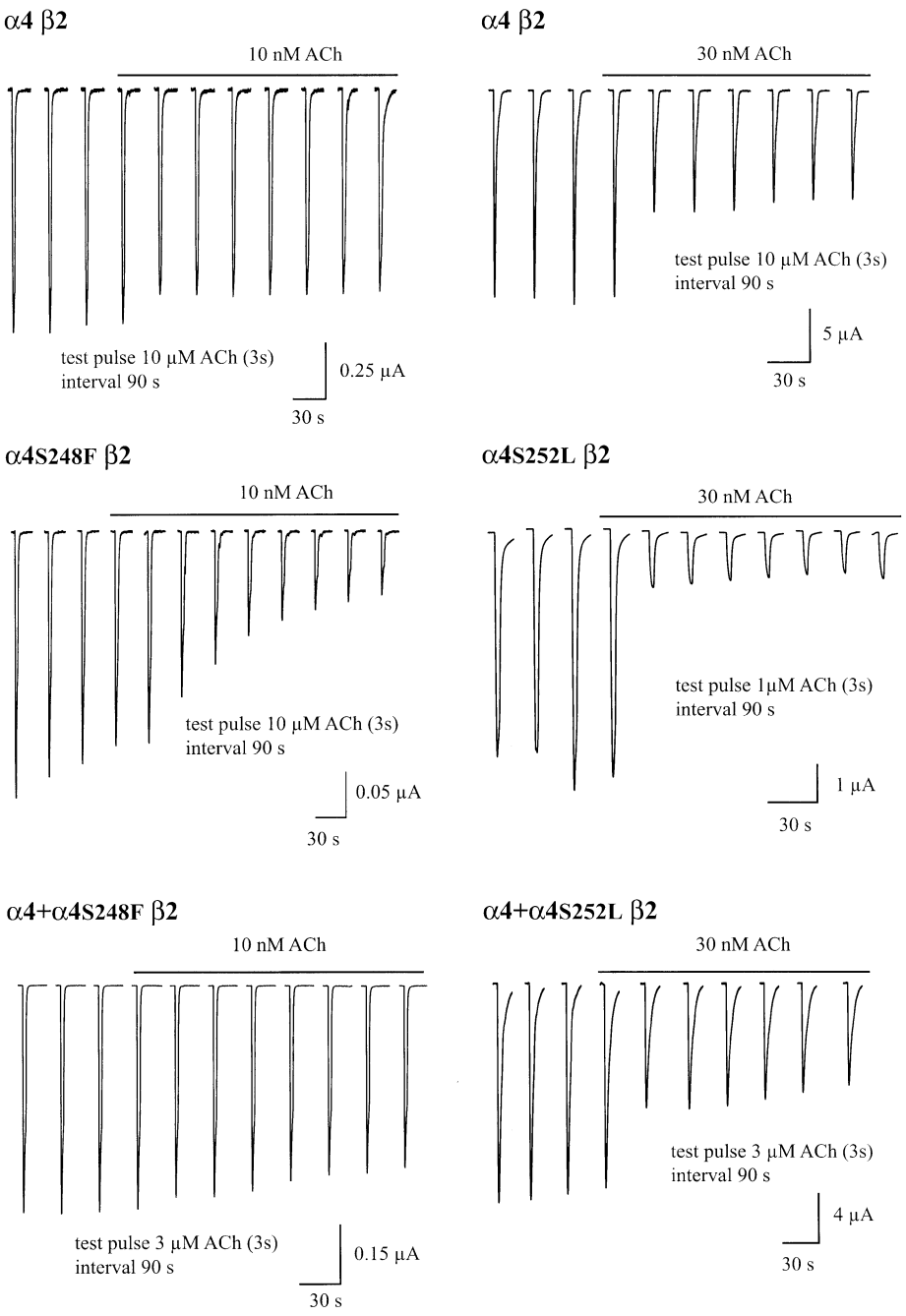

FIG. 4. Receptor desensitization caused by prolonged agonist exposure. Currents evoked by brief acetylcholine (ACh) test pulses were recorded first in control solution and then in presence of a low but sustained agonist concentration. Left panels illustrate the effects of 10 n*M* ACh on the control allele of a patient **(top),** the mutated α4S248F allele from the same patient **(middle),** and the heterozygous expression of the control and mutated alleles **(bottom).** Right panels illustrate a comparable experimental paradigm realized on the two alleles from a patient of another family with the α4S252L mutation. Bars above the traces indicate the beginning and duration of prolonged ACh exposures.

these experiments do not allow us to conclude that the majority of receptors expressed at the oocyte surface contain equal amounts of mutated and nonmutated α4 subunit, they suggest that a new receptor phenotype emerges in the heterozygous expression.

First identified in a Japanese family, the α4S252L mutation (4) was later observed in an Australian family (13). Functional properties of this mutant can be summarized as follows for heterozygous expression: no alteration of the maximal amplitude of the ACh-evoked current, absence of increased fast or slow desensitization, and normal calcium permeability. The only and major difference was in the sensitivity to ACh as observed for the EC_{50} values (Table 1). Similar results were obtained when comparing data obtained for the V287M *CHRNB2* mutant.

Additional characterizations of all the mutants were therefore pursued both for their sensitivity to the AED CBZ and their putative sensitivity to choline. While it was previously shown that α4S248F and α4L776ins3 mutants displayed an increased sensitivity to CBZ (16), a feature that is conserved in the β2V287M mutant, this signature was absent from the α4S252L mutant (Fig. 5 and Table 1). Although it is tempting to conclude that

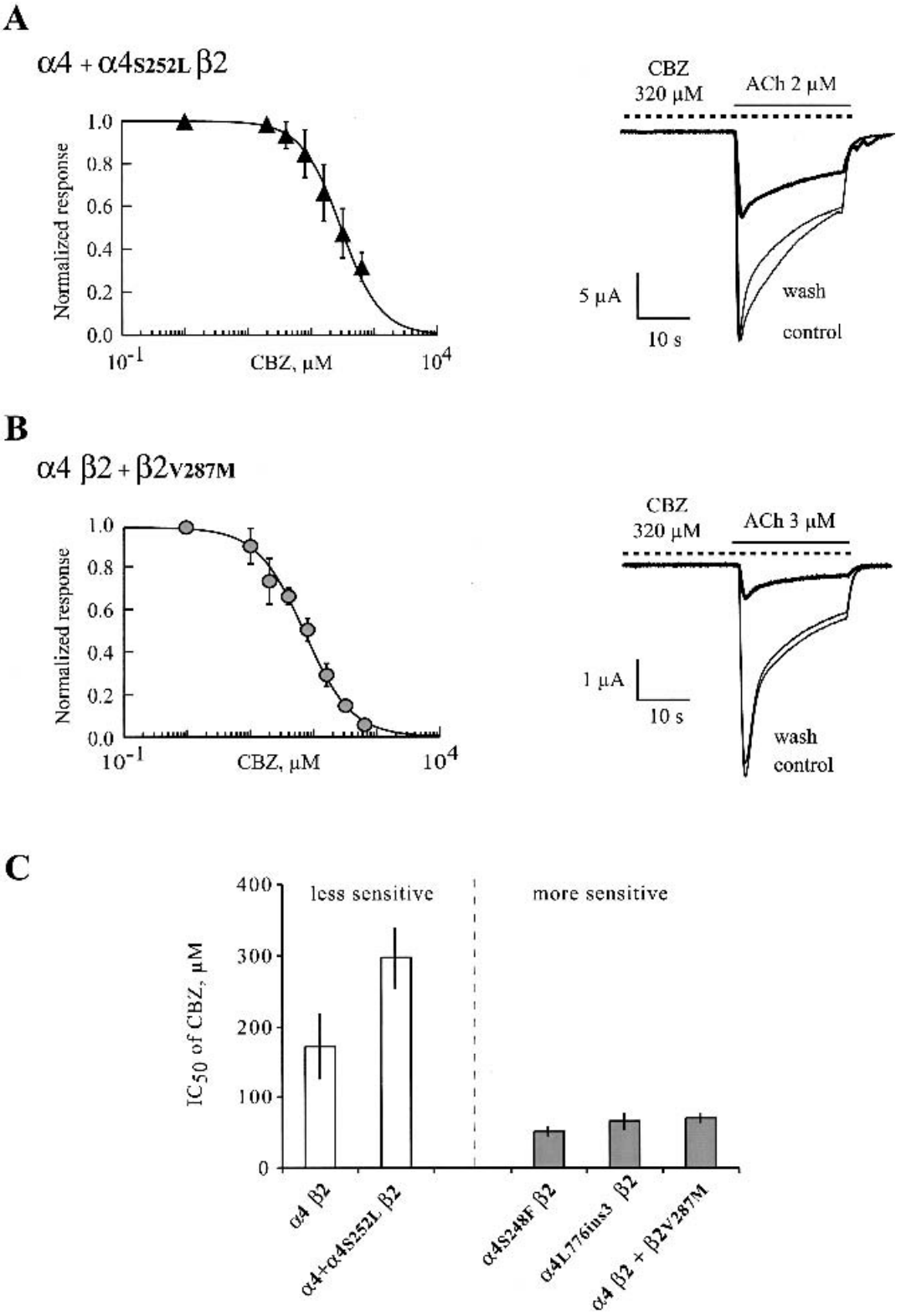

FIG. 5. Carbamazepine (CBZ) inhibits the acetylcholine (ACh)-evoked currents. **A, B:** Dose–response inhibition profiles were determined over a broad range of CBZ concentrations. ACh-evoked currents were normalized to unity and plotted as a function of CBZ concentration. Typical experimental protocols are illustrated by the traces in the right panel. Percentage of inhibition was obtained by measuring the current at the end of the ACh test pulse. Lines through the data points are the best fits obtained with the empirical Hill equation (Eq. 2). **C:** CBZ IC_{50} values of control $\alpha4\beta2$ nicotinic acetylcholine receptor nAChR and four of the known mutants are represented. Dark histograms indicate the receptors that are more sensitive to CBZ than control.

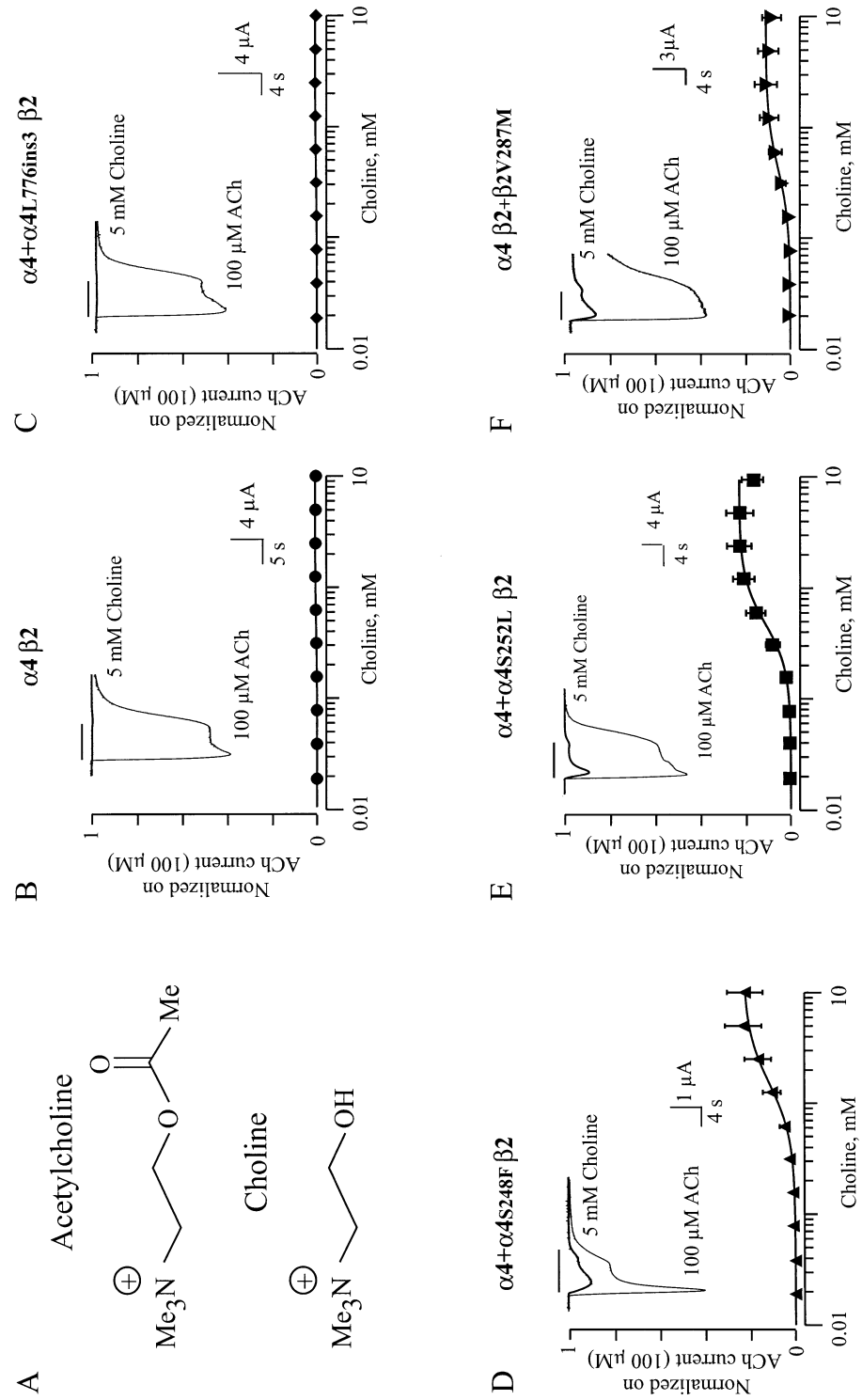

FIG. 6. Choline activates some receptor combinations. **A:** Chemical structure of acetylcholine (ACh) and choline. **B–F:** Dose–response activation of five different receptor combinations. ACh-responding cells were first challenged with an ACh pulse (100 μM, 5 s) to determine their maximal currents. Pulses of choline (5 s) of several concentrations were then applied, and current recorded. Typical ACh and choline responses are represented in the insets. Choline-evoked currents were normalized with respect to ACh and plotted as a function of the choline concentration. Lines through the data points are the best fits obtained with a single classic empiric Hill equation.

some families may respond best to certain AEDs, no such correlation was observed so far. The observation that choline acts as an agonist at some nAChRs subtypes (20,21) led to the hypothesis that *CHRNA4* and/or *CHRNB2* mutations could affect the receptor pharmacologic profile. Indeed, we found that α4 mutations (α4S248F, α4S252L) and the β2 mutation (V287M) cause a significant modification of the receptor profile, with choline acting as a partial agonist with a sensitivity in the millimolar range (Fig. 6 and Table 1).

At present, no clinical differences have been observed between ADNFLE patients and their familial origin. This suggests either that there is a common path for the different mutations or that, although present, differences cannot be detected. Assuming a common cause of ADNFLE implies that the four mutations characterized herein must result in the same alteration of the neuronal network function. Data obtained from all experiments suggest that the only common trait is an increase in the receptor ACh sensitivity. In an attempt to link the observation of an increased ACh sensitivity, or receptor gain of function, and epilepsy, several parameters must be taken into account.

Before a conclusive picture emerges on the role of nAChRs in the human brain, several issues must be considered. First, macroscopic distribution of the subunits between rodent and human differ (22,23). Second, knowledge of the subcellular localization in the human brain is rather limited. Despite these difficulties, hypotheses concerning the linkage between nAChR dysfunction and seizure triggering can still be formulated. Because seizures in ADNFLE originate in the frontal lobe and exclusively during sleep, these clinical data provide further information on the neuronal circuits involved in the epileptic discharges. Its is widely documented that thalamocortical loops play a major role during the sleep and that partial firing synchrony can be observed in neuron ensembles. Both thalamus and cortex receive cholinergic afferents and, therefore, influence of nAChRs at these two levels may influence the thalamocortical loop function.

Investigations of cortical pyramidal cells using multiple patch-clamp recordings have revealed that the action potential generated in the cell soma can backpropagate into the main dendrite (24–27). In addition, it was shown that apical dendrites produce a calcium-mediated action potential that propagates toward the cell soma. Thought to be at the origin of the computational power of pyramidal cells, this dual firing mode is illustrated in Fig. 7. Interestingly, as shown on this schema, cholinergic fibers spread around the dendrite of cortical pyramidal cells, whereas axons from pyramidal cells project to the thalamus, where they make synaptic contact. Although our knowledge of the precise circuit is incomplete, it is important to note that pyramidal cells express different receptor subtypes including α4β2 (28,29).

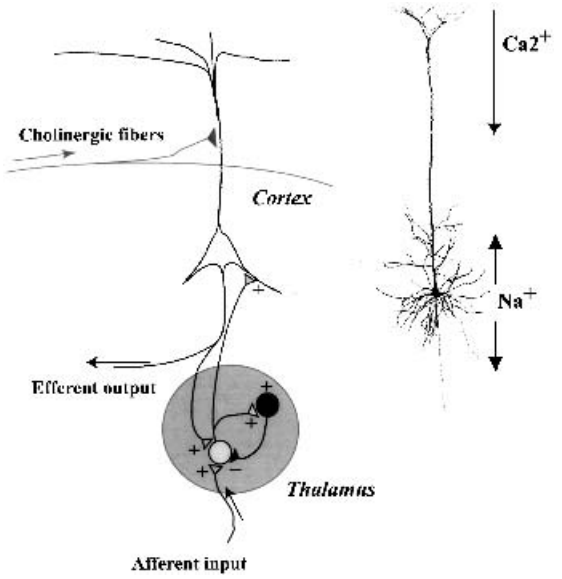

FIG. 7. Thalamocortical loop and the dual action potential in pyramidal cells. Left panel illustrates a schematic diagram of the putative thalamocortical loop with the cholinergic fibers innervating the apical dendrite. Right panel, image of a single pyramidal cell identified by intracellular labeling. Origin and direction of propagation of the sodium and calcium–mediated action potential are represented.

Assuming that cholinergic fibers release ACh in the vicinity of the main dendrite of pyramidal cells, it is possible to formulate a new hypothesis for epileptic seizures triggered by a gain of function of the α4β2. According to this schema, activation of nAChRs on the main dendrite will strongly attenuate signals coming from the apical dendrites but less than the active backpropagation from the cell soma. This perturbation corresponds to a decrease of the synaptic input from the most external cortical layers and a reinforcement of the input from somatic dendrites. In addition, this should strengthen the thalamocortical loops favoring the risk of oscillation.

In conclusion, the identification of an increasing number of mutations in the *CHRNA* and *CHRNB* genes linked with a particular type of genetically transmissible epilepsy (ADNFLE) strongly establishes the major role played by these receptors in a neurologic disease. Functional characterization of four different mutations of these nAChRs yielded particular physiologic and pharmacologic signatures for each of the four mutants. At present, the sole common trait between these mutants is an increased ACh sensitivity, which suggests that mutations causing a gain of the receptor function are at the origin of the epileptic seizures. Although discovery of novel mutations will further support this hypothesis, these data highlight the critical role of the nAChRs in the frontal thalamocortical loops and provide a first substrate

for the understanding of the contribution of these receptors in cognitive tasks.

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