

Increased Sensitivity of the Neuronal Nicotinic Receptor $\alpha 2$ Subunit Causes Familial Epilepsy with Nocturnal Wandering and Ictal Fear

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Sleep has traditionally been recognized as a precipitating factor for some forms of epilepsy, although differential diagnosis between some seizure types and parasomnias may be difficult. Autosomal dominant frontal lobe epilepsy is characterized by nocturnal seizures with hyperkinetic automatisms and poorly organized stereotyped movements and has been associated with mutations of the $\alpha 4$ and $\beta 2$ subunits of the neuronal nicotinic acetylcholine receptor. We performed a clinical and molecular genetic study of a large pedigree segregating sleep-related epilepsy in which seizures are associated with fear sensation, tongue movements, and nocturnal wandering, closely resembling nightmares and sleep walking. We identified a new genetic locus for familial sleep-related focal epilepsy on chromosome 8p12.3-8q12.3. By sequencing the positional candidate neuronal cholinergic receptor $\alpha 2$ subunit gene (*CHRNA2*), we detected a heterozygous missense mutation, I279N, in the first transmembrane domain that is crucial for receptor function. Whole-cell recordings of transiently transfected HEK293 cells expressing either the mutant or the wild-type receptor showed that the new *CHRNA2* mutation markedly increases the receptor sensitivity to acetylcholine, therefore indicating that the nicotinic $\alpha 2$ subunit alteration is the underlying cause. *CHRNA2* is the third neuronal cholinergic receptor gene to be associated with familial sleep-related epilepsies. Compared with the *CHRNA4* and *CHRNA2* mutations reported elsewhere, *CHRNA2* mutations cause a more complex and finalized ictal behavior.

Epilepsy is a common and heterogeneous neurological disorder, with an estimated prevalence in European populations that ranges from 3 to 8 per 1,000 inhabitants.^{1,2} The genetic etiology is known in many epilepsies, but specific gene mutations have been identified in only a few epilepsy syndromes. These Mendelian epilepsies are mainly channelopathies, and mutations in the genes encoding subunits of voltage-gated and ligand-gated ion channels are responsible for the clinical phenotypes.³ Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE [MIM 600513, 603204, and 605375]) is a clinically distinct childhood-onset focal epilepsy that displays clusters of sleep-related hypermotor seizures.⁴ ADNFLE is associated with mutations in the genes encoding the $\alpha 4$ and $\beta 2$ subunits of the neuronal acetylcholine receptor (*CHRNA4* and *CHRNA2*).^{5,6} The neuronal acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels distributed widely throughout the nervous system. So far, 12 nAChR subunits have been identified ($\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$).⁷ Each subunit spans the plasma membrane with four transmembrane domains (M1–M4) and gives rise to several different nAChR subtypes in the brain, based on the subunit composition, all of which have their own

unique biochemical, pharmacological, and biophysical characteristics.⁸ The nAChRs influence a wide range of physiological functions, such as sleep, arousal, anxiety, fatigue, and cognitive function. Furthermore, nAChR dysfunction is involved in a wide variety of diseases during development, adulthood, and aging.⁷ The M2 and M3 domains, which represent the ion-gating structure, are the specific target of ADNFLE mutations, both in the $\alpha 4$ and $\beta 2$ subunit genes.⁹ In contrast, the ligand-binding region—a complex and flexible structure that includes a short domain directly linked to M1¹⁰—localizes at the interface of α and the adjacent subunit.⁷

We performed a clinical and molecular genetic study of a family with seizures occurring during sleep and characterized by arousal followed by a prominent fear sensation, tongue movements, and nocturnal wanderings, suggesting frontolimbic involvement. We localized the genetic critical region and identified a missense mutation in the neuronal cholinergic receptor $\alpha 2$ subunit gene (*CHRNA2*) that affects the M1 domain of the receptor. Electrophysiological studies of the mutant receptor showed an increased sensitivity to the ligand.

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Received March 8, 2006; accepted for publication June 1, 2006; electronically published June 26, 2006.

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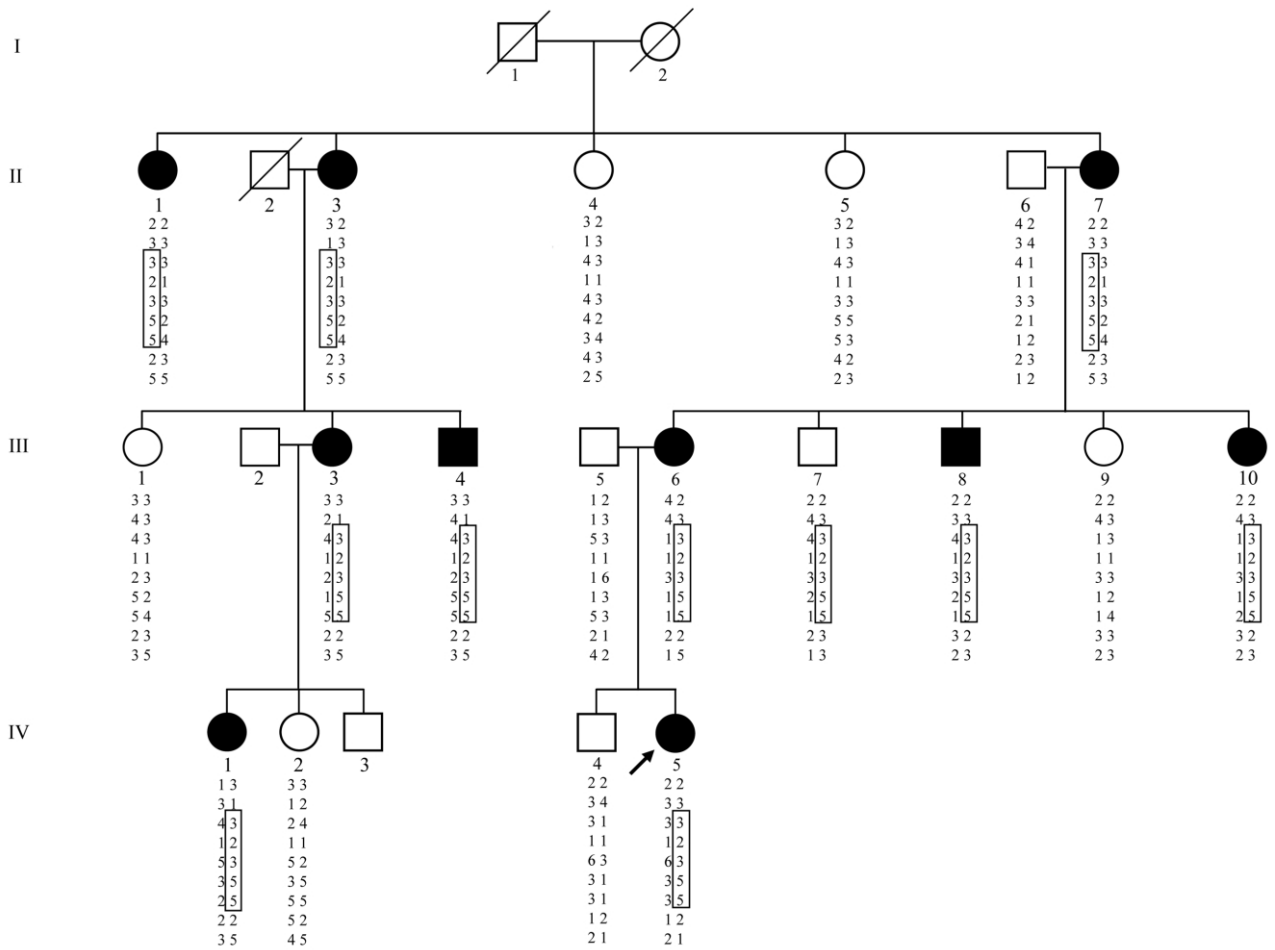


Figure 1. Pedigree structure and disease-haplotype segregation. Individuals available for typing are indicated by the generated genotype data. The arrow indicates the proband. Blackened symbols denote individuals affected by epilepsy with nocturnal wandering and ictal fear; unblackened symbols denote unaffected individuals. Circles and squares indicate females and males, respectively. Marker order and distances in cM are given in table 2. The disease haplotype is boxed.

Subjects and Methods

Patients

We obtained clinical and genealogical information through a validated questionnaire.^{11,12} All family members were personally examined (by C.M., T.P., and D.P.) Several seizures were recorded during electroencephalograms (EEGs), were videotaped in the proband, and were witnessed in the remaining affected family members. All individuals included in the study gave written informed consent. This study was approved by the ethical committees of the clinical institutions.

Genotyping and Linkage Analysis

Blood samples were obtained from 19 individuals (10 affected, 7 unaffected, and 2 spouses), and genomic DNA was isolated following standard procedures.¹³ We performed genomewide genotyping, using a set of 382 fluorescence-labeled polymorphic microsatellite markers (Linkage Mapping Dye Set 2 [Applied Biosystems]) spanning the 22 autosomes at an average reciprocal

distance of ~10 cM. An additional marker (*D8S283*) was added for fine mapping and haplotype analysis.

Data analysis was performed with the MLINK program (LINKAGE software package¹⁴) by assuming an autosomal dominant disease with incomplete penetrance (0.90) and a disease-gene frequency of 0.0001. The recombination frequencies (θ) were considered to be equal between males and females. Specific marker-allele frequencies were derived from the GDB Human Genome Database.

Mutational Analysis

We determined the 8-exon genomic structure of *CHRNA2* mRNA (GenBank accession number NM_000742) by comparing its 2,664-bp sequence with the corresponding genomic sequence. Exon-specific primers and PCR conditions are available on request. We performed mutation analysis, using direct DNA sequencing (DYEnamic ET Dye Terminator Kit [Amersham Biosciences]); DHPLC analysis (Wave [Transgenomic]) of exonic fragments, including intron-exon junctions; and direct DNA

Table 1. Clinical Characteristics of Italian Family with Epilepsy with Nocturnal Wandering and Ictal Fear

Patient	Sex	Age at Onset (years)	Age at Follow-up (years)	Seizure Semiology	Seizure Frequency	Precipitating Factors	EEG
II-1	F	5.5	80	Sudden awakening, vocalization or grunting, hypermotor behavior	NK, still present	NK	NA
II-3	F	29	82	Sudden awakening, sitting on the bed	NK	NK	NA
II-7	F	9	65	Sudden awakening, vocalization or grunting, hypermotor behavior	NK, still present	Coffee	NA
III-3	F	15	42	Sudden awakening, frightening and breathless sensations, tachycardia and tongue paresthesia	Daily clusters	NK	R frontotemporal SW
III-4	M	9	39	Sudden awakening, frightening and breathless sensation, wandering with complex motor behavior	Daily clusters	NK	NA
III-6	F	10	42	Sudden awakening, frightening and shivering sensations, tongue protrusion, wandering		Hot weather	Bilateral frontal SW
III-8	M	4.5	36	Sudden awakening followed by hypermotor behavior	Daily clusters	Smoking cigarettes	NA
III-10	F	6	25	Sudden awakening, vocalization or grunting, hypermotor behavior or wandering with complex behavior	Weekly clusters	Sudden noise, hot weather	NA
IV-1	F	6	14	Sudden awakening, sitting on the bed, hyperkinetic movements of lower limbs	Daily clusters	Sudden noise	L frontal SW
IV-5 ^a	F	6	13	Sudden awakening, vocalization or grunting, terrified expression and tongue protrusion	Daily clusters	Hot weather	L frontal spikes

NOTE.—L = left; NA = not available; NK = not known; R = right; SW = sharp waves.

^a Proband.

sequencing (DYEnamic ET Dye Terminator Kit [Amersham Biosciences]).

Constructs and Site-Directed Mutagenesis

We isolated human $\alpha 2$ (*CHRNA2*) and $\beta 4$ (*CHRN4* [GenBank accession number NM_000750]) nicotinic receptor cDNAs by PCR amplification of commercially available brain cDNA panels (BD Biosciences Clontech cDNA Panels) and cloned them into the expression vectors pcDNA3.1 (Invitrogen) and pcDNA3 (Invitrogen), respectively. To obtain an $\alpha 2^{1279N}$ pcDNA3.1 construct, we introduced the 836T→A (I279N) mutation into the *CHRNA2*-pcDNA3 ($\alpha 2$ pcDNA3.1), using PCR-based site-directed mutagenesis (QuickChange site-directed mutagenesis kit [Stratagene])

with the following primers: 5'-TCCCCTGCCTGCTCAACTCCTG-CCTCACTGTG-3' (forward) and 5'-CACCGTGAGGCAGGAGTTG-AGCAGGCAGGGGA-3' (reverse). All constructs were sequence verified.

Electrophysiology of the Mutant Channel

Cell culture and transfection procedure.—Either wild-type or mutant $\alpha 2$ constructs were transiently cotransfected with the $\beta 4$ subunit (cDNA ratios were $\alpha 2$: $\beta 4$ of 1:1, for wild-type receptors; $\alpha 2^{1279N}$: $\beta 4$ of 1:1, for homozygous mutant receptor; and $\alpha 2$: $\alpha 2^{1279N}$: $\beta 4$ of 1:1:2, for the simulated heterozygous state) into HEK293 cells. For subsequent detection of cells expressing nAChRs, an expression vector for the enhanced green fluorescent protein (E-GFP pcDNA3 [Clontech Laboratories]) was added to the transfection mixture. The cell culture method and transfection procedure were reported elsewhere.^{15,16}

Patch-clamp recording and data analysis.—At 36–48 h after transfection, currents were recorded using the whole-cell configuration of the patch-clamp method. Cells were voltage clamped at –60 mV with an Axopatch 200B amplifier (Molecular Devices); cell capacitance and series resistance were always compensated (75%–85%) before each experiment. Pipette resistances were 2–3 M Ω . Currents were low-pass filtered at 2 kHz and were acquired online at 5–10 kHz with pClamp hardware and software (Molecular Devices). The extracellular solution contained 130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 5 mM D-glucose (pH 7.3). Stock solutions for nicotine and acetylcholine were prepared fresh each week. The different ligand pipettes con-

Table 2. Two-Point LOD Scores for 8p21.2-8q12 Markers

Marker	LOD Score at $\theta =$						
	.00	.01	.05	.10	.20	.30	.40
<i>D8S549</i>	–5.90	–1.84	–1.03	–.64	–.25	–.08	–.01
<i>D8S258</i>	–3.33	.50	1.03	1.11	.94	.63	.29
<i>D8S1771</i>	1.56	1.53	1.41	1.25	.92	.58	.26
836T→A	3.26	3.21	3.01	2.73	2.09	1.35	.55
<i>D8S283</i>	.61	.61	.62	.59	.48	.33	.16
<i>D8S505</i>	.94	1.03	1.24	1.33	1.20	.84	.36
<i>D8S285</i>	2.01	2.00	1.94	1.81	1.42	.92	.37
<i>D8S260</i>	1.84	1.81	1.66	1.47	1.06	.61	.20
<i>D8S270</i>	–9.56	–3.86	–1.80	–.99	–.34	–.13	–.06

tained 10 mM NaCl, 1.3 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 130 mM DL-aspartic acid potassium salt, 10 mM ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 1 mM Na-ATP (pH 7.3).

Currents were elicited by applying the ligand for 1–2 s to wild-type, mutant, or simulated heterozygous receptors with an SF-77B Perfusion Fast-Step system (Warner Instruments). During the experiment, the maximal response (at 100 μM agonist) was always tested at regular intervals, to exclude artifacts due to channel rundown. Each ligand concentration was usually applied at least 2–3 times, and ~120 s were left between subsequent applications, to allow full receptor recovery from desensitization.

Data were analyzed offline by use of pClamp8 and Origin6 (Microcal) software. We generated dose-response curves to nicotine and acetylcholine by plotting average peak currents obtained at the indicated ligand concentrations and normalized to the current obtained in the presence of 100 μM agonist ($n = 6$

for each concentration point). The experimental points were fitted with a single-term empirical Hill equation:

$$\frac{I_L}{I_{\max}} = \left[1 + \left[\frac{EC_{50}}{(L)^n} \right] \right]^{-1}$$

where L is the ligand, I_L is the whole-cell current activated at a certain L , I_{\max} is the maximal current (i.e., at $L = 100 \mu\text{M}$), EC_{50} is the L producing half activation, and n is the Hill coefficient.

RT-PCR and Southern Blot Analysis

Human tissue was obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore. Brains from one male (16 years old, 16 h post mortem) and one female (35 years old, 6 h post mortem) who had no history of primary neurological or psychiatric disorders were pro-

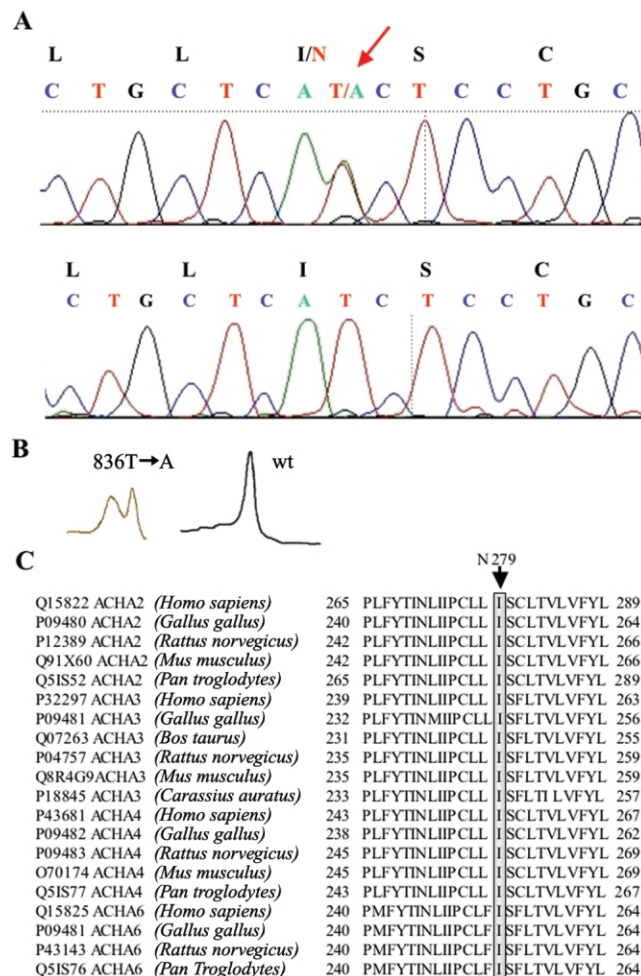


Figure 2. *CHRNA2* mutation in familial epilepsy with nocturnal wandering and ictal fear. *A*, Electropherograms of *CHRNA2* gene sequence spanning the missense mutation in the proband (top) and a control (bottom). The arrow indicates the 836T→A transversion. *B*, DHPLC elution profile of the heterozygous proband (left) and a wild-type (wt) control (right). *C*, Evolutionary conservation of different α-subunit I279 residues among different species (UniProtKB/Swiss-Prot).

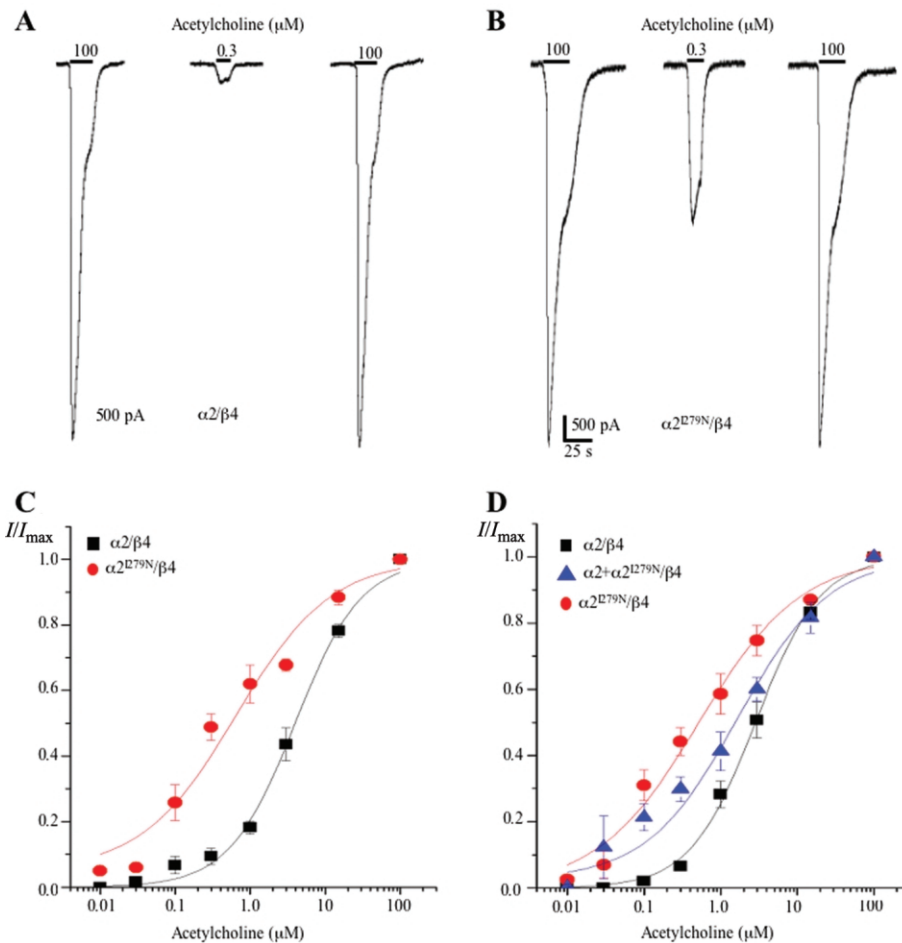


Figure 3. Electrophysiological properties of wild-type and mutant $\alpha 2/\beta 4$ nicotinic receptors. Representative current traces evoked by pulses of the indicated acetylcholine concentration for either $\alpha 2/\beta 4$ (A) or $\alpha 2^{1279N}/\beta 4$ (B) nAChRs. Note the larger response in $\alpha 2^{1279N}/\beta 4$ receptors at nonsaturating doses of agonist. The maximal response was always tested at regular intervals during the experiment, to exclude artifacts due to channel rundown. Similar results were obtained with nicotine. No significant difference was observed in the current density of wild-type ($\alpha 2/\beta 4$), mutant ($\alpha 2^{1279N}/\beta 4$), and heterozygous ($\alpha 2 + \alpha 2^{1279N}/\beta 4$) receptors, tested in the presence of 100 μM agonist. C and D, Dose-response curves to acetylcholine (C) and nicotine (D). Data points are average peak currents obtained at the indicated ligand concentration and normalized to the current obtained in the presence of 100 μM agonist ($n = 6$ for each concentration point). The experimental points were fitted with a single-term empirical Hill equation, providing the following estimates for EC_{50} : acetylcholine, $3.94 \pm 0.31 \mu\text{M}$ (for $\alpha 2/\beta 4$) and $0.64 \pm 0.14 \mu\text{M}$ (for $\alpha 2^{1279N}/\beta 4$); nicotine, $2.84 \pm 0.16 \mu\text{M}$ (for $\alpha 2/\beta 4$), $0.52 \pm 0.07 \mu\text{M}$ (for $\alpha 2^{1279N}/\beta 4$), and $1.66 \pm 0.25 \mu\text{M}$ (for $\alpha 2 + \alpha 2^{1279N}/\beta 4$).

cessed as described elsewhere (Brain and Tissue Bank for Developmental Disorders Web site). Samples from the two brains were dissected from exactly the same areas (brain cortex regions are indicated by Brodmann areas): prefrontal (area 10), ventral prefrontal (area 11), anterior cingulate (area 32), insular (area 43), temporal (area 21), parietal (area 4), occipital cortices (area 18), putamen, caudate, amygdala, hippocampus, and thalamus.

Total RNA was purified from dissected tissues (Trizol [Invitrogen]) and was retrotranscribed with M-MLV reverse transcriptase (Promega) in the presence of oligo-dT. PCR reactions were performed for 22 cycles by use of AmpliTaq Gold polymerase (Perkin Elmer) and gene-specific oligonucleotides designed across exons (sequences available on request). Control reactions were performed with samples without reverse transcription.

PCR products were electrophoresed on 1% agarose gels, were

blotted onto nylon filters, and were hybridized with ^{32}P -labeled *CHRNA2*-, *CHRNA4*-, *CHRN2*-, and *GAPDH*-specific probes. Filters were exposed to BioMax films (Kodak) for 5 min (*GAPDH*) or 3 h (*CHRNA2*, *CHRNA4*, and *CHRN2*).

Results

Patients and Pedigree

Clinical information was obtained from 24 individuals across four generations of a Sardinian family (fig. 1). Ten family members had clusters of seizures exclusively during sleep. Mean age at seizure onset was 10 years (median 7.5 years; range 4–29 years). The proband (IV-5) had episodes with sudden awakening since age 6 years, vocalization

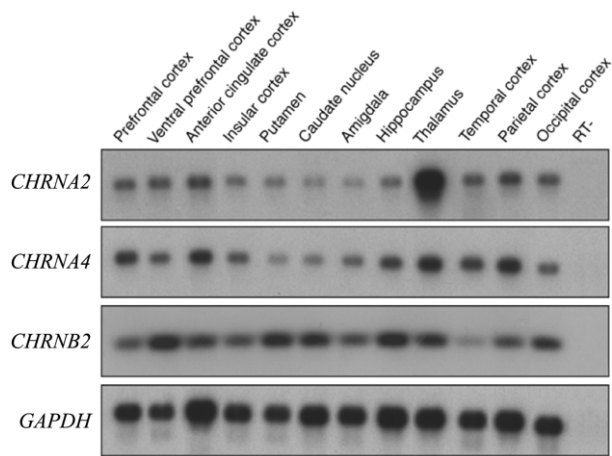


Figure 4. Expression pattern of *CHRNA2*, *CHRNA4*, and *CHRN2* nicotinic subunits in human brain. For quantitative analysis of different α and β subunits' expression in the human brain, we optimized a semiquantitative RT-PCR method coupled with Southern blot analysis. With this analysis, a relatively stronger expression was observed in the thalamus for *CHRNA2* mRNA, whereas mild expression was seen in the other brain regions analyzed for both *CHRNA2* and *CHRNA4*. *CHRN2* showed a homogeneous level of expression throughout the different brain areas, with the exception of the temporal cortex. *GAPDH* was used as positive control. RT- = negative control.

or grunting, fearful expression, and peculiar protrusive movements of the tongue. Seizures lasted 20–30 s and occurred in clusters of 2–4 attacks, usually while she was falling asleep. The remaining affected family members had nocturnal clusters of similar episodes including frightening, tingling, or shiver sensations; tongue movements; and complex motor behaviors, such as walking outside the room and descending the stairs (table 1). Clinical diagnosis in the older family members had been delayed because nocturnal attacks had been interpreted as nightmares and sleep walking.

Seizure frequency and severity varied within the family, having a tendency to be more severe at onset, with occurrence of clusters of seizures every night in most patients. Complex motor behaviors and nocturnal wanderings were more frequent during childhood and adolescence, whereas, from late adolescence through adulthood, seizures occurred with weekly-to-monthly frequency and were characterized by milder motor manifestations. Only the two youngest affected individuals (IV-1 and IV-5) were seizure free while taking antiepileptic drugs.

Linkage and Mutational Analysis

To map the genetic locus associated with this form of sleep-related partial epilepsy, we performed a genomewide linkage analysis. Markers on chromosome 8p21.2-8q12 showed significant suggestive evidence of linkage to this

form (table 2). This wide region spans a genomic area of 60 cM between markers *D8S258* and *D8S270* and harbors a cluster of neuronal cholinergic receptor genes (*CHRNA2*, *CHRN3*, and *CHRNA6*), encoding the $\alpha2$, $\beta3$, and $\alpha6$ subunits (UCSC Genome Browser).

Sequence analysis of the entire coding region of the $\alpha2$ subunit revealed a heterozygous single-nucleotide substitution (836T→A) in exon 6 of the proband (IV-5) (fig. 2A). This transversion leads to the substitution of a hydrophobic isoleucine at residue 279 with a polar asparagine (I279N).

Denaturing high-performance liquid chromatography (DHPLC) (fig. 2B) and sequence analysis detected the presence of this mutation in all the affected family members and in a phenotypically healthy individual (III-7), thus suggesting incomplete penetrance for this disease. The mutation was not found in 340 control chromosomes. The I279 residue is invariant in nicotinic α subunits from different species (fig. 2C), localizes to the first transmembrane domain (M1) of the $\alpha2$ nAChR, and apparently does not directly contribute to the structure of the ion gate.¹⁷

Electrophysiology of the Mutant Receptor

To test the electrophysiological behavior of the mutant $\alpha2^{I279N}$ nAChR subunit, we patch-clamped HEK 293 cells transiently coexpressing either $\alpha2/\beta4$, $\alpha2^{I279N}/\beta4$, or $\alpha2 + \alpha2^{I279N}/\beta4$ receptor subunits. We first analyzed the $\alpha2/\beta4$ and $\alpha2^{I279N}/\beta4$ receptors (fig. 3A and 3B), and we observed differences between these two types of receptors at low concentrations of acetylcholine. $\alpha2^{I279N}/\beta4$ mutant receptor was activated by 0.3 μM acetylcholine challenge to ~50% of the current evoked with saturating acetylcholine (100 μM). On the contrary, the $\alpha2/\beta4$ wild-type response was fivefold lower and displayed currents barely reaching 10% of maximal amplitude.

Determination of the dose-response curves to acetylcholine and nicotine over a broad range of agonist concentrations (fig. 3C and 3D, respectively) showed that $\alpha2^{I279N}/\beta4$ receptor has a significantly higher sensitivity to the natural agonist. Moreover, cells expressing both wild-type and mutant receptors—thus mimicking the heterozygous state—showed an intermediate response, as demonstrated by the mean (\pm SD) EC_{50} values for nicotinic activation: 1.66 \pm 0.25 μM (for $\alpha2 + \alpha2^{I279N}/\beta4$), 2.84 \pm 0.16 μM (for $\alpha2/\beta4$), and 0.52 \pm 0.07 μM (for $\alpha2^{I279N}/\beta4$) (fig. 3D).

The desensitization properties and channel permeability were scarcely affected by the $\alpha2^{I279N}$ mutation. The monoexponential time constants of current decay in the presence of 100 μM nicotine were 3.73 \pm 0.9 s for wild-type, 4.72 \pm 0.97 s for heterozygous, and 5.14 \pm 0.98 s for mutant receptors. In addition, the $\alpha2^{I279N}$ mutation produced minor variations in the receptor current response to substitution of external sodium with potassium and to the partial substitution of external sodium with 20 mM calcium (data not shown).

Wide expression of nAChRs has been reported in primate brain,¹⁸ including the $\alpha 2$ subunit.¹⁹ With the aim of confirming these data in humans, we examined the expression patterns of the $\alpha 2$, $\alpha 4$, and $\beta 2$ focal epilepsy-causing genes in distinct areas of post mortem human brain by semiquantitative RT-PCR. $\alpha 2$ mRNA exhibited the highest level of expression in the thalamus, but a diffuse expression was detected in the entire investigated sample, although quantitative differences were identified among cortical and subcortical areas. In particular, levels of expression of $\alpha 2$ transcripts in the isocortex (prefrontal, ventral prefrontal, anterior cingulate, insular, temporal, superior parietal, inferior parietal, and occipital cortex) were rather homogeneous and of intermediate intensity between the thalamus, which had the highest signal, and the hippocampus and subcortical nuclei, where $\alpha 2$ expression was moderate to weak (fig. 4). $\alpha 4$ Subunit expression was similar to the $\alpha 2$ pattern, with the exception of the thalamic sample where $\alpha 2$ transcript is considerably more represented. In contrast, $\beta 2$ mRNA was quite uniformly distributed throughout the different brain regions, with the exception of temporal cortex, where the transcript was barely detectable.

Discussion

This study identifies a new genetic locus on chromosome 8p12.3-8q12.3 for familial sleep-related focal epilepsy with nocturnal wandering and ictal fear and establishes an association with the nicotinic $\alpha 2$ subunit gene as the underlying cause. Moreover, our results provide insight into the pathological role of M1 domain mutation and of the nicotinic $\alpha 2$ subunit in the CNS, confirming that mutations in nAChRs genes are involved in the epileptogenesis related to sleep and arousal mechanisms.

The heteropentameric nicotinic receptor hosts two ligand-binding sites located in an extracellular domain directly linked to the amino-terminus of the M1 domain, at the interface of α and β subunits.²⁰ Agonist binding at these sites leads to the conformational changes operated by M1 domain to the channel gate (M2 segment).²¹ In fact, studies on the protein structure showed that most of the M2 helix is probably separated from M1 and M3 by water-filled space.¹⁷ However, few interhelices hydrophobic residues are strictly juxtaposed and crucial for the transmission of the conformational change. Among these, the F225 residue of the M1 segment of the *Torpedo californica* α subunit closely contacts the L253 of the M2 segment.¹⁷ It is worth noting that the corresponding residue in the $\alpha 2$ subunit of the human receptor is the evolutionarily conserved I279 residue, which is mutated in our patients. Recently, the first M3 domain mutation of the $\beta 2$ subunit (I312M) was associated with epilepsy.⁹ It is intriguing that I312 of the human $\beta 2$ subunit cor-

responds to I289 in the *T. californica* α subunit, a residue in contact with L257 of the M2 domain¹⁷ and probably interfering with the gating process. We propose that the $\alpha 2$ subunit I279N mutation, which cosegregates with the disease in our family, alters the relative movements of the inner helices and outer shell during gating of the receptor.

Therefore, the M1 mutation would influence the transition between the closed and open state of nAChR, rather than altering agonist-binding sites, and would promote the increased sensitivity of the mutant receptor. As a consequence, neuronal cells harboring the mutant receptors would respond to lower cholinergic stimuli.

While hypermotor manifestations of classical ADNFLE result from activation of frontomesial and orbitofrontal cortical areas,²² in the family described here, seizures are also related to sleep but are manifested with prominent fear and more-organized motor behaviors, such as nocturnal wanderings. These features are more in keeping with frontolimbic and anterior cingulate involvement.²³ Ictal single-photon emission CT studies suggest the role of cingulated gyrus in sleep-arousal-related seizures with prominent motor manifestations.²³⁻²⁵

Cholinergic neurons are known to exert a modulating effect on sleep and arousal oscillations at thalamic and cortical levels.²⁶ Activation of the ascending cholinergic system is associated with the abolition of spindle and slow waves in the EEG and with enhancement of higher-frequency activities, like natural waking.²⁶ Neuronal nAChRs seems to be expressed preferentially in the presynaptic surface,^{27,28} thus regulating the release of acetylcholine or other neurotransmitters, such as gamma-amino butyric acid (GABA) and glutamate.^{29,30} In this way, they can produce an aut synaptic or heterosynaptic regulation. We therefore hypothesize that an unbalanced excitation/inhibition circuitry, due to the mutant nicotinic receptor on GABAergic reticular thalamic neurons, generates seizures by facilitating synchronizing spontaneous oscillations in thalamo-cortical connections.

Actually, the highest expression of *CHRNA2* in the thalamic area would further support this hypothesis and could be consistent with epileptogenesis arising from networks that regulate arousal from sleep. However, it is difficult to argue to what extent variations in the topographic distributions of $\alpha 4$ and $\alpha 2$ might account for the peculiarity of ictal symptoms in this family, with respect to the prominent afamilial hypermotor behavior that is most often observed in ADNFLE.

Our results, besides increasing the number of genes implicated in inheritable forms of epilepsy, strongly suggest a pathogenetic role for the nicotinic $\alpha 2$ subunit in epilepsy with nocturnal seizures characterized by fear and complex motor behaviors. However, further studies are needed to investigate whether mutations or common polymorphisms of the *CHRNA2* gene represent a risk factor for sleep-related epilepsies with similar semiology.

Acknowledgments

We thank the Lega Italiana contro l'Epilessia and acknowledge the support of the Italian Minister of Health (grant RBNE01NR34_009 [to G. Casari and R.G.]) and the Téléthon Foundation (grants GP0023y01, GTF03007, and PRIN 2006 [to A.B.]). C.M. is supported by a grant from the Italian Ministry of Education, University and Research (MIUR). M.P. is a young investigator financed by MIUR under the program "Rientro dei cervelli." Human tissue was obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders, under contracts N01-HD-4-3368 and N01-4-3383. We thank Luca Rampoldi for critical discussion.

Web Resources

Accession numbers and URLs for data presented herein are as follows:

Brain and Tissue Bank for Developmental Disorders, <http://medschool.umaryland.edu/BTBank/>
GDB Human Genome Database, <http://www.gdb.org/> (for allele frequencies)
GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *CHRNA2* [accession number NM_000742] and *CHRN4* [accession number NM_000750])
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for ADNFLE)
UCSC Genome Browser, <http://genome.ucsc.edu/> (for selecting critical region candidate genes)
UniProtKB/Swiss-Prot, <http://au.expasy.org/uniprot> (for nicotinic receptor subunit proteins alignment)

References

1. Rocca WA, Savettieri G, Anderson DW, Meneghini F, Grigoletto F, Morgante L, Reggio A, Salemi G, Patti F, Di Perri R (2001) Door-to-door prevalence survey of epilepsy in three Sicilian municipalities. *Neuroepidemiology* 20:237–241
2. Forsgren L, Beghi E, Oun A, Sillanpaa M (2005) The epidemiology of epilepsy in Europe—a systematic review. *Eur J Neurol* 12:245–253
3. Gourfinkel-An I, Baulac S, Nabbout R, Ruberg M, Baulac M, Brice A, LeGuern E (2004) Monogenic idiopathic epilepsies. *Lancet Neurol* 3:209–218
4. Callenbach PM, van den Maagdenberg AM, Frants RR, Brouwer OF (2005) Clinical and genetic aspects of idiopathic epilepsies in childhood. *Eur J Paediatr Neurol* 9:91–103
5. Steinlein O, Sander T, Stoodt J, Kretz R, Janz D, Propping P (1997) Possible association of a silent polymorphism in the neuronal nicotinic acetylcholine receptor subunit $\alpha 4$ with common idiopathic generalized epilepsies. *Am J Med Genet* 74:445–449
6. De Fusco M, Becchetti A, Patrignani A, Annesi G, Gambardella A, Quattrone A, Ballabio A, Wanke E, Casari G (2000) The nicotinic receptor $\beta 2$ subunit is mutant in nocturnal frontal lobe epilepsy. *Nat Genet* 26:275–276
7. Gotti C, Clementi F (2004) Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol* 74:363–396
8. Bertrand D, Picard F, Le Hellard S, Weiland S, Favre I, Phillips H, Bertrand S, Berkovic SF, Malafosse A, Mulley J (2002) How mutations in the nAChRs can cause ADNFLE epilepsy. *Epilepsia Suppl* 5 43:112–122
9. Bertrand D, Elmslie F, Hughes E, Trounce J, Sander T, Bertrand S, Steinlein OK (2005) The *CHRN2* mutation I312M is associated with epilepsy and distinct memory deficits. *Neurobiol Dis* 20:799–804
10. Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. *J Mol Biol* 346:967–989
11. Reutens DC, Howell RA, Gebert KE, Berkovic SF (1992) Validation of a questionnaire for clinical seizure diagnosis. *Epilepsia* 33:1065–1071
12. Bonanni P, Malcarne M, Moro F, Veggiotti P, Buti D, Ferrari AR, Parrini E, Mei D, Volzone A, Zara F, Heron SE, Bordo L, Marini C, Guerrini R (2004) Generalized epilepsy with febrile seizures plus (GEFS⁺): clinical spectrum in seven Italian families unrelated to *SCN1A*, *SCN1B*, and *GABRG2* gene mutations. *Epilepsia* 45:149–158
13. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Lathrop GM, Lalouel JM, Julier C, Ott J (1985) Multilocus linkage analysis in humans: detection of linkage and estimation of recombination. *Am J Hum Genet* 37:482–498
15. Schonherr R, Rosati B, Hehl S, Rao VG, Arcangeli A, Olivetto M, Heinemann SH, Wanke E (1999) Functional role of the slow activation property of ERG K⁺ channels. *Eur J Neurosci* 11:753–760
16. Becchetti A, De Fusco M, Crociani O, Cherubini A, Restano-Cassulini R, Lecchi M, Masi A, Arcangeli A, Casari G, Wanke E (2002) The functional properties of the human *ether-à-go-go*-like (HELK2) K⁺ channel. *Eur J Neurosci* 16:415–428
17. Miyazawa A, Fujiyoshi Y, Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* 423:949–955
18. Quik M, Polonskaya Y, Gillespie A, Jakowec M, Lloyd GK, Langston JW (2000) Localization of nicotinic receptor subunit mRNAs in monkey brain by in situ hybridization. *J Comp Neurol* 425:58–69
19. Han ZY, Le Novère N, Zoli M, Hill JA Jr, Champiaux N, Changeux JP (2000) Localization of nAChR subunit mRNAs in the brain of *Macaca mulatta*. *Eur J Neurosci* 12:3664–3674
20. Karlin A (2002) Emerging structure of the nicotinic acetylcholine receptors. *Nat Rev Neurosci* 3:102–114
21. Wilson G, Karlin A (2001) Acetylcholine receptor channel structure in the resting, open, and desensitized states probed with the substituted-cysteine-accessibility method. *Proc Natl Acad Sci USA* 98:1241–1248
22. Hayman M, Scheffer IE, Chinvarun Y, Berlangieri SU, Berkovic SF (1997) Autosomal dominant nocturnal frontal lobe epilepsy: demonstration of focal frontal onset and intrafamilial variation. *Neurology* 49:969–975
23. Nobili L, Cossu M, Mai R, Tassi L, Cardinale F, Castana L, Citterio A, Sartori I, Lo Russo G, Francione S (2004) Sleep-related hyperkinetic seizures of temporal lobe origin. *Neurology* 62:482–485
24. Vetrugno R, Mascalchi M, Vella A, Della Nave R, Provini F, Plazzi G, Volterrani D, Bertelli P, Vattimo A, Lugaresi E, Montagna P (2005) Paroxysmal arousal in epilepsy associated with cingulate hyperperfusion. *Neurology* 64:356–358
25. Schindler K, Gast H, Bassetti C, Wiest R, Fritschi J, Meyer K, Kollar M, Wissmeyer M, Lovblad K, Weder B, Donati F (2001)

- Hyperperfusion of anterior cingulate gyrus in a case of paroxysmal nocturnal dystonia. *Neurology* 57:917–920
26. Steriade M, McCormick DA, Sejnowski TJ (1993) Thalamocortical oscillations in the sleeping and aroused brain. *Science* 262:679–685
 27. Lubin M, Erisir A, Aoki C (1999) Ultrastructural immunocalization of the $\alpha 7$ nAChR subunit in guinea pig medial prefrontal cortex. *Ann N Y Acad Sci* 868:628–632
 28. Clarke PB, Pert A (1985) Autoradiographic evidence for nicotinic receptors on nigrostriatal and mesolimbic dopaminergic neurons. *Brain Res* 348:355–358
 29. Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX (2000) Nicotinic receptor activation in human cerebral cortical interneurons: a mechanism for inhibition and disinhibition of neuronal networks. *J Neurosci* 20:66–75
 30. Radcliffe KA, Dani JA (1998) Nicotinic stimulation produces multiple forms of increased glutamatergic synaptic transmission. *J Neurosci* 18:7075–7083