The α_{2B}-Adrenergic Receptor Is Mutant in Cortical Myoclonus and Epilepsy

Maurizio De Fusco, BS,[1] Riccardo Vago, PhD,[1] Pasquale Striano, MD,[2] Carlo Di Bonaventura, MD,[3] Federico Zara, PhD,[4] Davide Mei, BS,[5] Min Seuk Kim, PhD,[6] Shmuel Muallem, PhD,[7] Yunjia Chen, PhD,[8] Qin Wang, PhD,[8] Renzo Guerrini, MD,[5,9] and Giorgio Casari, PhD[1]

Objective: Autosomal dominant cortical myoclonus and epilepsy (ADCME) is characterized by distal, fairly rhythmic myoclonus and epilepsy with variable severity. We have previously mapped the disease locus on chromosome 2p11.1-q12.2 by genome-wide linkage analysis. Additional pedigrees affected by similar forms of epilepsy have been associated with chromosomes 8q, 5p, and 3q, but none of the causing genes has been identified. We aim to identify the mutant gene responsible for this form of epilepsy.
Methods: Genes included in the ADCME critical region were directly sequenced. Coimmunoprecipitation, immunofluorescent, and electrophysiologic approaches to transfected human cells have been utilized for testing the functional significance of the identified mutation.
Results: Here we show that mutation in the α_2-adrenergic receptor subtype B (α_{2B}-AR) is associated with ADCME by identifying a novel in-frame insertion/deletion in 2 Italian families. The mutation alters several conserved residues of the third intracellular loop, hampering neither the α_{2B}-AR plasma membrane localization nor the arrestin-mediated internalization capacity, but altering the binding with the scaffolding protein spinophilin upon neurotransmitter activation. Spinophilin, in turn, regulates interaction of G protein coupled receptors with regulator of G protein signaling proteins. Accordingly, the mutant α_{2B}-AR increases the epinephrine-stimulated calcium signaling.
Interpretation: The identified mutation is responsible for ADCME, as the loss of α_{2B}-AR/spinophilin interaction causes a gain of function effect. This work implicates for the first time the α-adrenergic system in human epilepsy and opens new ways of understanding the molecular pathway of epileptogenesis, widening the spectrum of possible therapeutic targets.

ANN NEUROL 2014;75:77–87

Autosomal dominant cortical myoclonus and epilepsy (ADCME; Online Mendelian Inheritance in Man database [OMIM] 607876; also known as cortical myoclonic tremor with epilepsy, familial, 2 or FCMTE2) and benign adult myoclonic epilepsy (benign adult familial myoclonic epilepsy [BAFME]/familial adult myoclonic epilepsy [FAME], OMIM 601068) are syndromes with high penetrance, characterized by rhythmic myoclonic jerks of cortical origin and focal or generalized tonic–clonic seizures, with nonprogressive or slowly progressive course. Initially, these conditions were classified as separate entities, although they might exhibit considerable clinical overlap.[1] Clinical and neurophysiological features suggest a high propensity for intrahemispheric and

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.24028

Received Dec 31, 2012, and in revised form Jul 2, 2013. Accepted for publication Jul 12, 2013.

Address correspondence to Dr Casari, Vita-Salute San Raffaele University, School of Medicine and Center for Translational Genomics and Bioinformatics, Neurogenomics Unit, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy. E-mail casari.giorgio@hsr.it

From the [1]Center for Translational Genomics and Bioinformatics, San Raffaele Scientific Institute, and Vita-Salute San Raffaele University, Milan, Italy; [2]Pediatric Neurology and Muscular Diseases Unit, Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genoa, G. Gaslini Institute, Genoa, Italy; [3]Department of Neuroscience, Neurology Unit, La Sapienza University, Rome, Italy; [4]Laboratory of Neurogenetics, Department of Neurosciences, G. Gaslini Institute, Genoa, Italy; [5]Pediatric Neurology Unit and Laboratories, Anna Meyer Children's Hospital and University of Florence, Florence, Italy; [6]Department of Oral Physiology, School of Dentistry, Wonkwang University, Iksan, Jeonbuk, Republic of Korea; [7]Epithelial Signaling and Transport Section, Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institute of Health, Bethesda, MD; [8]Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL; and [9]Stella Maris, Pisa, Italy.

Additional Supporting Information may be found in the online version of this article.

interhemispheric cortical spread of cortical myoclonic activity, indicating widespread cortical hyperexcitability with defective inhibitory cortical mechanisms.[2] The disease-associated loci have been mapped to chromosomes 8q23.1-q24.11 and 2p11.1-q12.2 in Japanese and Italian families, respectively.[2–4] Additional loci have recently been identified on chromosomes 5p15.31-p15[5] and 3q26.32-3q28,[6] supporting genetic heterogeneity among pedigrees. Several candidates have been proposed but, to date, causative genes for this group of disorders have not yet been identified.

Here we report the association of the α_{2B}-adrenergic receptor (α_{2B}-AR) with ADCME in 2 unrelated families. The adrenergic system has been proposed since the late 1980s to be implicated in epileptogenesis, as impaired activation of α_2-ARs might contribute to epileptogenesis in the kindling model.[7,8] The α_2-ARs belong to the G protein coupled receptor (GPCR) family that binds the endogenous ligands epinephrine and norepinephrine. These 7 transmembrane-spanning receptors regulate their effector systems via coupling to heterotrimeric G proteins that mediate the physiological effects, such as sympathetic outflow and cardiovascular function.[9] The sympathetic nervous system activity is negatively regulated by α_2-adrenoreceptors that act as autoreceptors, suppressing release of catecholamines. Their inhibitory activities are mediated by inhibition of adenylyl cyclase and voltage-gated Ca^{2+} currents and activation of receptor-operated K^+ currents.[10] The α_2-ARs, like most GPCRs, are substrate of G protein coupled kinases (GRKs); GRK2 binds and phosphorylates the agonist-activated receptor, converting it into a target for high-affinity binding of arrestin to regulate the receptor signaling cascades. Bound arrestin shields the cytoplasmic surface of the receptor, precluding G protein binding and activation.[11] Desensitized receptor–arrestin complexes are endocytosed, and the receptors are dissociated, dephosphorylated, and recycled to the cell surface, resensitizing the cell for another round of signaling. Another important mode of regulation is by the effector spinophilin, which regulates multiple aspects of α_2-AR trafficking and signaling by antagonizing the interaction with GRK2 and subsequent arrestin binding.[12] Thence, the interaction of spinophilin with the α_2-AR decreases arrestin-dependent internalization of the receptor, thus stabilizing it at the cell surface, and slows the rate of both activation and resensitization of receptor-mediated signaling.[12] In addition, spinophilin mediates interaction of the α_{2B}-AR with regulator of G protein signaling (RGS) proteins to reduce signaling intensity.[13]

To date, 3 distinct α_2-AR subtypes (α_{2A}, α_{2B}, α_{2C}) have been described in humans,[14] which are encoded by 3 intronless genes localized on chromosomes 10, 2, and 4. The α_2-ARs are distributed throughout the central nervous system with no extensive overlap, indicating a probable role in discrete neuronal functions by coordinating independent neural signaling pathways. Although the 3 receptors have similar pharmacological properties, they show subtype-specific differences in susceptibility to regulatory phosphorylation and desensitization, as well as intracellular trafficking.[15] A key role in the signaling pathway is played by the third intracellular (3i) loop of the α_2-AR, which includes the sites for GRK phosphorylation, G_i activation, and binding of spinophilin and arrestin.[16] Interestingly, the α_{2B}-AR mutation reported here involves consistent changes of this crucial controller domain.

Subjects and Methods

Patients' Evaluation

All affected members underwent video-polygraphic study, jerk-locked back averaging (JLA), and recording of somatosensory evoked potentials (SEPs) and long latency reflex I. Detailed methods have been described elsewhere.[2,17] SEPs were judged as giant when the components N20-P25 and P25-N33 were larger than 8.6μV and 8.4μV, respectively. Neuropsychological evaluation included the Wechsler Adult Intelligence Scale Revised.

Mutation Detection

Mutation analysis of candidate genes was performed by amplifying coding sequences scanning by denaturing high-pressure liquid chromatography (WAVE; Transgenomic, Omaha, NE) and direct sequencing. Screening of controls for the ADCME mutation was performed by amplification refractory mutation system (ARMS)–polymerase chain reaction (PCR) assay amplifying both the wild-type (WT) and the mutant alleles, together with a control fragment, in a single-tube PCR. The region flanking the mutation was amplified by 2 outer primers, producing a non–allele-specific control amplicon. Two allele-specific inner primers were designed in opposite orientation and used in combination with the common outer primers to simultaneously amplify both the WT and the mutant amplicons. The allele-specific primers specificity was conferred by the match of the 3′ nucleotides with either the WT or the mutant allele. The tetra ARMS-PCR produced 2 allele-specific amplicons with different lengths (316bp for WT; 205bp for mutant) and 1 control amplicon that was always present (486bp). Primer sequences and PCR conditions are available upon request.

Targeted Capture and DNA Sequencing

gDNA was nebulized and the libraries were prepared using a GS FLX Titanium Rapid Library Preparation Kit (Roche, Milan, Italy). To multiplex the 2 samples in a single sequencing run, 2 different multiplex identifiers (MID) were been used. The libraries were pooled and hybridized on a Titanium

Optimized Sequence Capture 385K Array (Roche-Nimblegene, Madison, WI). The array was designed to capture the coding and the untranslated regions of the genes localized in the extended linkage region (hg18/NCBI36; chr2:85,140,498-112,715,205; http://genome.ucsc.edu/). An additional 20bp of flanking intronic sequence was added to each exon. Captured libraries were subjected to emulsion PCR, and DNA-carrying beads were enriched and used as template for sequencing according to the manufacturer's protocol. GS FLX sequence reads were aligned to the NCBI36/hg18 reference genome using the GS Reference Mapper v2.5.3. Variants were filtered and annotated using the ANNOVAR tool.

Sequence Alignments

We used ClustalW2 to compare α_{2B}-AR (NP_000673.2) with orthologs of *Pan troglodytes* (XP_003309176.1), *Cebus apella* (CAJ19284.1), *Pithecia pithecia* (CAJ19290.1), *Hylobates lar* (CAJ19281.1), *Macaca mulatta* (XP_001082230.1), *Cercopithecus solatus* (CAJ19319.1), *Pongo abelii* (XP_002811692.1), *Tapirus indicus* (AEP17928.1), and *Cavia porcellus* (XP_003471576.1).

Molecular Modeling

The 3-dimensional structure of WT and mutant α_{2B}-AR were produced using Swiss-Model server (http://swissmodel.expasy.org),[18,19] which performs sequence alignment and putative template protein selection for the generation of the third model of the query protein.

Plasmid Preparation

Full-length WT and mutant α_{2B}-AR was amplified from genomic DNA with primers encoding the KpnI-EcoRI sites and the 5′-hemagglutinin (HA) tag and subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Myc-tagged spinophilin was prepared as described before.[20] FLAG-tagged arrestin-3 was kindly provided by Dr Robert J. Lefkowitz (Duke University, Durham, NC).

Immunofluorescence Microscopy

HeLa cells were seeded at 10^4/well in 12-well plates on glass coverslips and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum. Cells were transiently transfected with plasmid encoding WT or mutant HA-tagged α_{2B}-AR alone or in combination with FLAG-tagged arrestin-3 (0.45μg and 0.15μg, respectively). After 36 hours, to visualize cell surface receptors, cells were washed and incubated with DMEM supplemented with 0.1% bovine serum albumin (BSA) and 25mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and containing mouse anti-HA antibody (6μg/ml; Covance, Princeton, NJ) for 1 hour at 4°C. After washing, cells were treated with 100 μM epinephrine for 10 minutes at 37°C, washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with Alexa 488 conjugated secondary antibody. Nuclei were stained by 4′,6-diamidino-2-phenylindole. Images were captured using a Zeiss Imager A2 microscope and obtained under a ×40 Plan-Apochromat M27 objective (Zeiss, Oberkochen, Germany).

Coimmunoisolation of Spinophilin with the WT and Mutant α_{2B}-AR

CosM6 cells were transfected with a plasmid encoding myc-tagged spinophilin (pCMV4-Myc-Spl) together with a plasmid encoding HA-tagged WT or mutant α2BAR (pcDNA3.1-HA-α2BAR or pcDNA3.1-HA-α2BAR-mut) using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, cells were serum starved overnight, and then treated with 100μM epinephrine (plus 1μM prazosin to block α1-AR and 1μM propranolol to block βAR) or vehicle for 5 or 10 minutes. After stimulation, cells were lysed in buffer containing 20mM HEPES (pH 7.4), 0.5% NP-40, 10% glycerol, 2mM ethylenediaminetetraacetic acid, 5mM sodium fluoride, and protease inhibitor cocktail (100μM phenylmethylsulfonyl fluoride, 10μg/ml leupeptin, 5μg/ml aprotinin, 1μg/ml soybean trypsin inhibitor, and 1μM pepstain), and centrifuged at 13,000rpm for 30 minutes at 4°C. The supernatant was collected as total cell lysates and measured for protein concentration. The equal amount of total proteins was used for coimmunoprecipitation assay. Cell lysates were precleared with 35μl of protein G beads slurry for 45 minutes at 4°C, and then incubated with HA.11 antibody (Covance, 1:100) overnight at 4°C. The HA immunocomplex was pull downed by adding 35μl of protein G beads slurry (pre-equilibrated with 0.25% BSA and washed) into the cell lysates and rotating for 2 hours at 4°C. Protein G beads were washed 3× with lysis buffer, and bound proteins were extracted with Laemmli sample buffer.

Current Measurement in Xenopus laevis Oocytes

The spinophilin cDNA was cloned in pCMV-myc vector (Clontech, Palo Alto, CA). For the synthesis of cRNA, the desired cDNAs constructs were linearized and the T7 promoter upstream of the sequence of interest was used for cRNA synthesis in vitro using SP6 RNA polymerase (Ambion, Life Technologies, Paisley, UK). *Xenopus laevis* oocytes were prepared as previously described.[20] Briefly, oocytes in stage V–VI were injected with 2 to 10ng cRNA encoding WT or mutant α_{2B}-AR alone or in combination with spinophilin and incubated at 18°C in 96mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, and 5mM HEPES (pH 7.6) buffer. Current measurement was accomplished 48 to 96 hours postinjection with the 2-electrode voltage-clamp procedure. When performing dose response, the effect of each α_{2B}-AR and spinophilin concentration on Ca^{2+} signaling was measured after the same incubation time postinjection. To measure the Ca^{2+}-activated Cl^- current, membrane potential was held at −60mV for continuous recording. To acquire epinephrine dose–response relationships, membrane potential was stepped from −60mV to +50mV for 200 milliseconds at 0.2Hz. The results are shown as means ± standard error of the mean of the peak current normalized on WT. We used 2 oocyte preparations and did the 6 experiments over 4 days, starting 96 hours after cRNA injection. In each

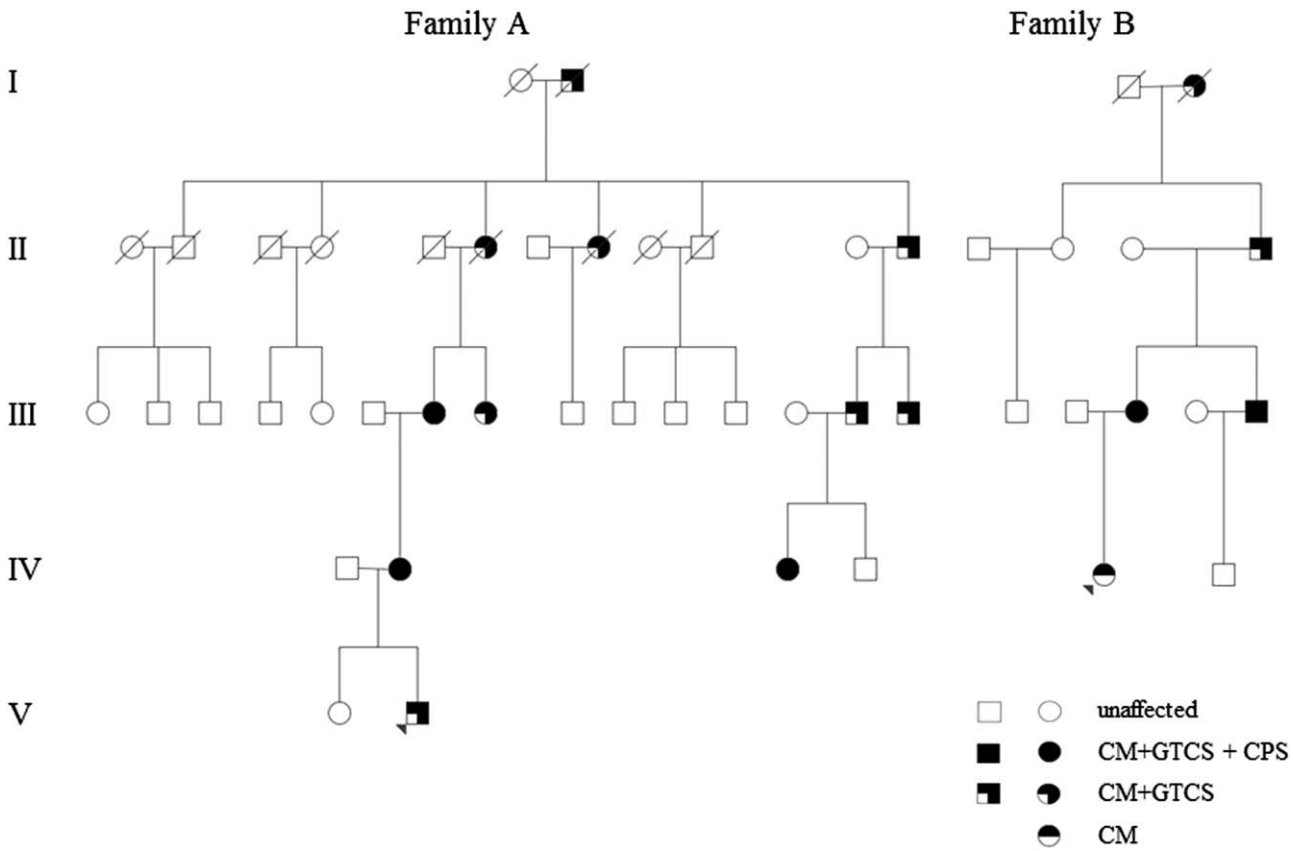

FIGURE 1: Pedigrees of autosomal dominant cortical myoclonus and epilepsy families. CM = cortical myoclonus; CPS = complex partial seizure; GTCS = generalized tonic-clonic seizure. Probands are indicated by arrowheads.

experiment, we performed the dose response in all conditions on the same day (WT, mutant, WT + spinophilin, mutant + spinophilin). The pick current at each concentration was used to calculate the response, and for probability we used all 6 experiments. The maximum current was similar for all conditions. The current was measured at a holding potential of −30mV, and the traces show the Ca^{2+} activated Cl^{-} current.

Results

Identification of α_{2B}-AR Mutation in ADCME Patients

We have previously mapped the ADCME locus on chromosome 2p1.1-q1.2 in a 5-generation family from Tuscany with autosomal dominant pattern of inheritance (Fig 1; Family A).[2]

Among the several genes included in the critical region, we prioritized as possible candidates *SIAT9*, demonstrated to be involved in the autosomal recessive Amish infantile epilepsy syndrome[21]; *KCNIP3*, coding for a calcium-binding protein that regulates voltage-gated potassium current and hence neuron excitability[22]; *REEP1*, involved in the spastic paraplegia autosomal dominant type 31[23]; *VAMP5* and *VAMP8*, required for vesicle fusion and neurotransmitter release; and the neuronal transcription factor *NPAS2*. Sequence analysis of the coding regions of these genes gave negative results. Finally, we identified a novel in-frame insertion/deletion in the α_{2B}-AR gene (*ADRA2B*), which substitutes 5 amino acids, HGGAL, with 4 new residues, QFGR (indel; c.675_686delTGGTGGGGCTTTinsGTTTGGCAG; p.H225_L229delinsQ225_F_G_R228; Fig 2A).

This indel mutation completely cosegregates with the disease phenotype and is absent in 575 unrelated controls from Tuscany (data not shown), in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), in the Exome Variant Server (National Heart, Lung, and Blood Institute GO Exome Sequencing Project, Seattle, WA; http://evs.gs.washington.edu/EVS/), and in the 1000 Genomes Project (http://www.1000genomes.org/), thus totaling 16,080 human chromosomes. It is worth noting that a frequent *ADRA2B* polymorphism resulting in a different number of the encoded glutamic acid residues in the monotonous stretch p.E297_E309 is known to represent a risk factor for cardiovascular and metabolic diseases[24,25] and to dysregulate agonist-promoted receptor desensitization.[26] However, the identified mutation is syntenic to the more common variant, showing 12 consecutive glutamic acid residues; this variant is not

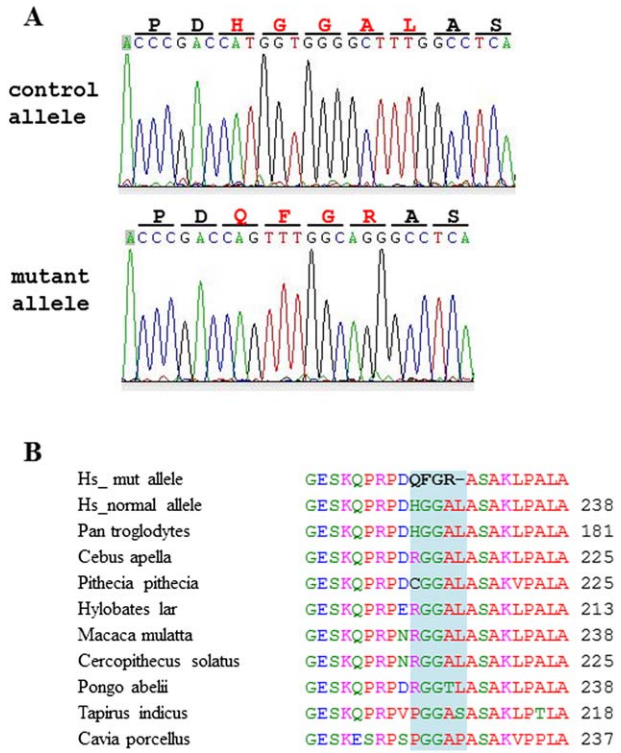

FIGURE 2: The autosomal dominant cortical myoclonus and epilepsy mutation of the α_{2B}-adrenergic receptor. (A) Control and mutant alleles. (B) Partial sequence alignment of adrenergic receptor in multiple species. The mutant sequence is highlighted. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

associated with the abovementioned effects. Other indels of the *ADRA2B* gene found in the control population are reported in the Supplementary Table.

In a second ADCME family from Tuscany (see Fig 1; Family B), we detected the same indel of the *ADRA2B* gene. Although no kinship between the 2 families was discovered by anamnestic analysis of members of the 2 pedigrees, a common ancestor is to be expected, as they share the same disease haplotype spanning the entire critical region on chromosome 2 (data not shown).

BAFME pedigrees originating from southern Italy have been shown to map in the ADCME locus.[27,28] However, we failed to detect any *ADRA2B* mutations in these pedigrees by direct sequence analysis and excluded potential large insertions and deletions by Southern blot (data not shown).

To exclude other possible variations that could explain or contribute to the onset of the ADCME phenotype, we extensively sequenced 2 affected members of Family A by target capturing all the coding regions included in the ADCME critical region followed by highly redundant next generation sequencing. The *ADRA2B* mutation was confirmed as the unique relevant chance (data not shown).

Clinical Features of the ADCME Families

The clinical features of Family A have been previously described[2] and closely resemble the Family B phenotype (clinical findings are summarized in the Table). Briefly, Family B is a 4-generation kindred including 5 affected members (4 living). All patients exhibited postural hand and upper limbs myoclonus. Epilepsy occurred in all but 1 (IV:1) individual and was characterized by rare generalized tonic–clonic seizures, at times precipitated by visual stimuli, occurring in all patients, and by drug-resistant focal motor or complex partial seizures occurring in Patients III:3 and III:5. Individual III:3 exhibited a borderline cognitive level, whereas all remaining affected individuals had normal intelligence. Cortical tremor was the presenting symptom in all affected individuals, appearing between the ages of 18 and 50 years. Generalized or focal paroxysmal activity was identified in all patients. Jerk-locked back-averaged electroencephalography, somatosensory evoked potentials, and long-loop reflex were consistent with the cortical reflex myoclonus.

We took into consideration a possible comorbidity of epilepsy with cardiovascular diseases, as the *ADRA2B* gene is also expressed in the heart and vascular smooth

TABLE 1. Clinical Features of Affected Members of Family B

Patient ID/ Sex/Age, yr	Age at Onset, yr	Mental Status	Seizure Types	Brain MRI	Anticonvulsants
I:2/F/70	NA	Normal	GTCS	NA	No
II:4/M/84	49	Normal	GTCS	Normal	PB
III:3/F/55	28	Borderline	GTCS, focal seizures	Normal	PB, VPA, CNZ
III:5/M/45	27	Normal	GTCS, focal seizures	Normal	VPA, LEV, CNZ
IV:1/F/29	18	Normal	No	ND	No

CNZ = clonazepam; F = female; GTCS = generalized tonic–clonic seizures; LEV = levetiracetam; M = male; MRI = magnetic resonance imaging; NA = not available; ND = not done; PB = phenobarbital; VPA = valproate.

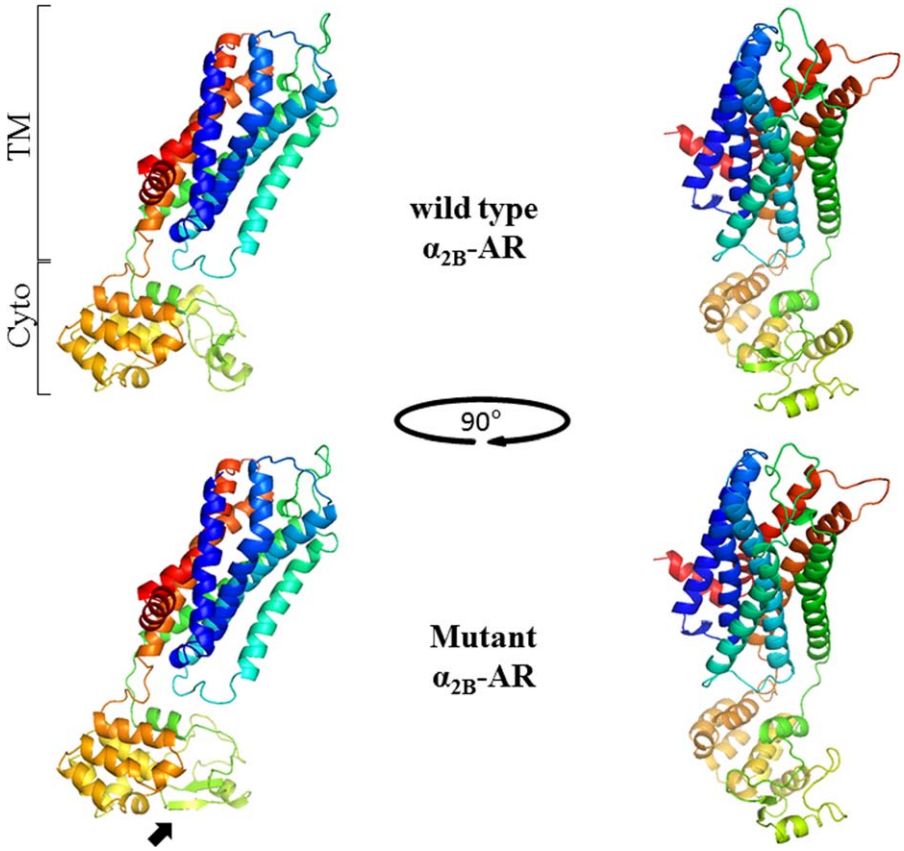

FIGURE 3: Molecular modeling of wild-type and mutant α_{2B}-adrenergic receptor (α_{2B}-AR). Ribbon diagram of the α_{2B}-AR are rainbow gradient-colored (N terminus [blue] to C terminus [red]). Arrow points to the mutant stretch. Cyto = cytosolic domain; TM = transmembrane domain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

muscles. A careful anamnesis was performed on all ADCME patients, giving negative evidence for cardiovascular diseases; 2 of the oldest *ADRA2B* mutation carriers developed age-related dementia.

Mutant α_{2B}-AR Internalization Is Unaffected

The indel localizes in the 3i loop, a crucial domain for receptor localization and signal transduction, which is conserved in primates (see Fig 2B).[16] The mutant amino acid stretch Gln225_Phe_Gly_Arg228 is expected to result in different physicochemical properties of the domain in comparison to the WT, due to the insertion of a very hydrophobic and bulky residue (Phe226) and a positive charge (Arg228). Because no crystal structure of the α_{2B}-AR is yet available, we generated a 3-dimensional configuration model to investigate the impact of the mutation on the structure of the receptor by using the dopamine D3 receptor as a template.[29] As shown in Figure 3, the mutant is predicted to prominently alter the conformation of the specific region of the 3i loop. We therefore tested whether these changes can affect the α_{2B}-AR subcellular distribution and its ability to interact with other proteins.

Because the 3i loop has been demonstrated to be critical for intramembrane stability,[30] we first determined the WT and mutant α_{2B}-AR subcellular localization by immunofluorescence. Both WT and mutant α_{2B}-AR show the typical plasma membrane localization in the absence of agonist, indicating that the mutation does not affect the maturation of the protein in the endoplasmic reticulum–Golgi compartments and its delivery to the plasma membrane (Fig 4). When cells were stimulated with epinephrine, little or no redistribution of α_{2B}-ARs was detected, whereas in the presence of arrestin-3, WT α_{2B}-AR relocalizes to endosomes as expected[31] as well as the mutant receptor. These results suggest that both WT and mutant α_{2B}-AR internalize in an arrestin-3–dependent manner and that the arrestin-3 interaction with the 3i loop is not precluded by the mutation, which is apart from the arrestin-3–α_{2B}-AR interaction domain.

Mutant α_{2B}-AR Alters Spinophilin Interaction

We studied the possible destabilizing effect of the mutant isoform on the interaction between the α_{2B}-AR and spinophilin, as the latter has been demonstrated to specifically bind the α_{2A}-AR 3i loop.[32] The interaction was

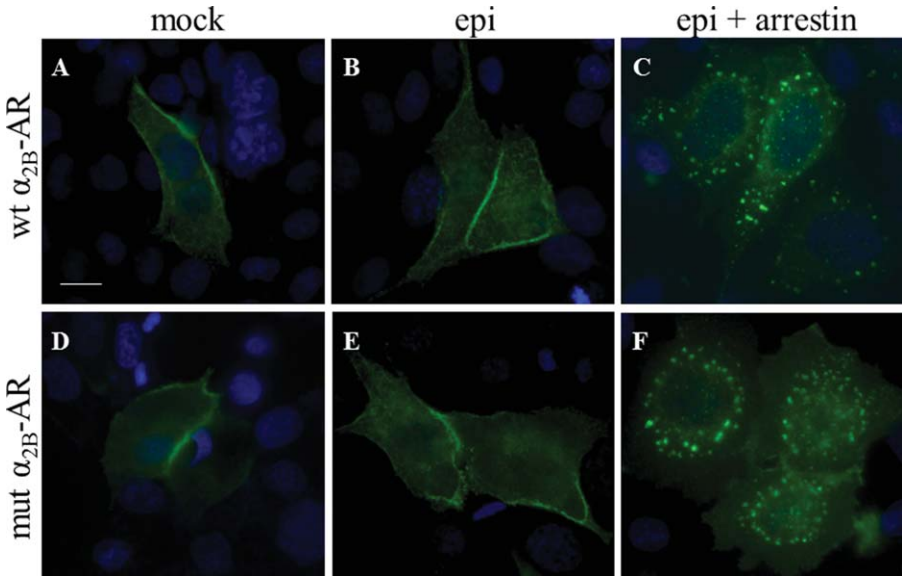

FIGURE 4: Wild-type and mutant α_{2B}-adrenergic receptor (α_{2B}-AR) internalization. Representative images are shown of HeLa cells transfected with wild-type (wt α_{2B}-AR) or mutant (mut α_{2B}-AR) hemagglutinin-tagged α_{2B}-AR alone or in combination with arrestin-3 and stimulated with epinephrine (epi), where indicated. Scale bar = 30μm.

initially investigated by glutathione S-transferase pull-down; both WT and mutant α_{2B}-AR were found to bind spinophilin, indicating that the mutant retains the ability to bind spinophilin in vitro (data not shown).

We confirmed this interaction by coimmunoisolation assay. As shown in Figure 5A and B, spinophilin was present in the immunocomplex with α_{2B}-AR. Activation of the receptor by epinephrine significantly enhanced the amount of spinophilin coisolated with the α_{2B}-AR, thus indicating that spinophilin preferentially interacts with the agonist-activated α_{2B}-AR, in line with previous findings.[32] After 5-minute treatment with epinephrine, spinophilin was coimmunoisolated with comparable amounts of WT and mutant α_{2B}-AR. However, the amount of spinophilin coisolated with the mutant α_{2B}-AR dropped back to the basal level after epinephrine treatment for 10 minutes, whereas the amount of spinophilin coisolated with the WT α_{2B}-AR remained elevated with the same treatment (see Fig 5C). These data suggest that the mutant α_{2B}-AR cannot form a stable complex with spinophilin, which would result in lack of spinophilin regulation on responses mediated by this mutant α_{2B}-AR.

Mutation in α_{2B}-AR Induces an Alteration in the Ca^{2+} Signaling

We therefore tested the effect of the α_{2B}-AR mutation on epinephrine-activated Ca^{2+} signaling. Epinephrine stimulation activates a Gq-mediated Ca^{2+} signaling as revealed by activation of the native oocytes Ca^{2+}-activated Cl^- current. Spinophilin attenuates the signaling by recruiting RGS proteins to the receptors–G protein complex, which accelerates the Gα-guanosine triphosphatase activity to terminate the signal.[13] To test the effect of the mutation on the role of spinophilin in Ca^{2+} signaling, *X. laevis* oocytes were injected with cRNA encoding WT or mutant α_{2B}-AR alone or in combination with spinophilin, and Ca^{2+}-activated Cl^- current was measured upon stimulation with increasing concentrations of epinephrine. The dose–response measurement showed that spinophilin significantly increased the EC_{50} for epinephrine ($p < 0.01$; Fig 6), as previously demonstrated for α_{1B}-AR.[13] The expression of the mutant α_{2B}-AR exhibits per se a trend toward significant reduction of the EC_{50} compared to WT ($p = 0.055$), but the presence of spinophilin does not result in an increase in EC_{50} (α_{2B}-AR + spinophilin vs α_{2B}-AR mutant + spinophilin: $p < 0.001$; see Fig 6). Therefore, we conclude that the mutant α_{2B}-AR significantly increases the intensity of Ca^{2+} signaling by the receptors.

Discussion

Although Mendelian inherited epilepsies represent a small share of epilepsy at large, the study of mutant proteins has greatly improved our understanding of the disease mechanisms. Genetic forms of epilepsy have been associated with mutations in the voltage-gated sodium, potassium, and calcium channels, the neurotransmitter-gated ion channels, the nicotinic acetylcholine receptor, the γ-aminobutyric acid (GABA) receptor subtype A, transcription factors (ARX), proteins involved in synaptic vesicle release, and cerebral cortex development and plasticity.[33]

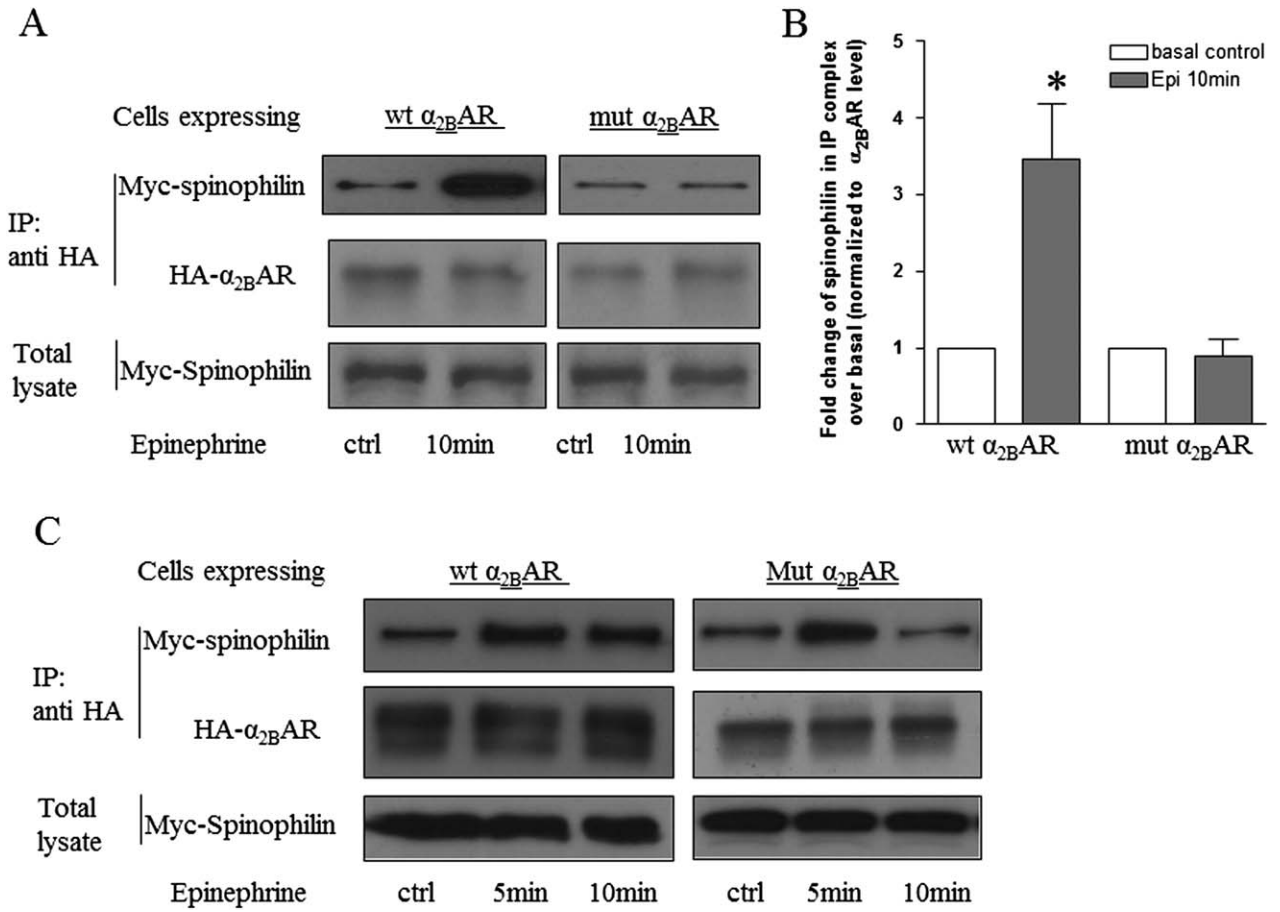

FIGURE 5: The mutant α_{2B}-adrenergic receptor (α_{2B}-AR) fails to form a stable complex with spinophilin. COS M6 cells coexpressing spinophilin with wild-type (wt) or mutant (mut) α_{2B}-AR are stimulated with epinephrine, and the α_{2B}-AR–spinophilin complex is isolated with an hemagglutinin (HA) antibody. (A) Representative blot showing spinophilin interaction with the wild-type and mutant α_{2B}-AR after 10-minute stimulation of epinephrine. (B) Quantitation of the α_{2B}-AR–spinophilin interaction representing 3 independent coimmunoisolation experiments. Data are expressed as the fold change of spinophilin complexed with wild-type or mutant α_{2B}-AR over no-stimulation control. Values are given as the mean ± standard error of the mean; *$p < 0.05$, epinephrine (Epi) stimulated versus control. (C) Representative blots showing interaction between spinophilin and wild-type (left) or mutant (right) α_{2B}-AR at indicated time points. ctrl = control; IP = immunoprecipitation.

Here we provide evidence that α_{2B}-AR is directly involved in human epilepsy such as ADCME. We did not detect any mutation in *ADRA2B* in BAFME/FAME patients, suggesting the possibility of classifying ADCME and BAFME/FAME as 2 distinct clinical entities. However, we cannot exclude the presence of variations in gene regulatory regions or other noncoding genomic elements potentially affecting gene function.

ADCME is characterized by familial occurrence of cortical reflex myoclonus manifested as action-induced shivering movement of the hands and upper limb jerking variably associated with focal and generalized tonic–clonic seizures of variable severity and, in a minority of patients, with borderline or moderately impaired cognitive skills. Worsening of myoclonus is often observed in advanced age.[34]

The indel of the α_{2B}-AR does not affect the protein localization, but our model predicts a change in folding of the 3i loop, the largest cytoplasmic domain that mediates agonist-dependent binding and activation of heterotrimeric G proteins. Although the 3i loop exerts the same function in all α_{2A}, α_{2B}, and α_{2C}-AR subtypes, it shows constitutive difference, as its amino acid sequence is poorly conserved among subtypes, suggesting a possible subtype-specific sensitivity to regulation. The α_{2B}-AR 3i loop is bigger and contains a long stretch of glutamic acids that counterbalances the diffuse positive charge of the domain, which is instead predominant in the α_{2A}-AR and α_{2C}-AR 3i loops. This glutamic acid repeat is polymorphic for the presence of long and short alleles with 12 or 9 residue repetitions, respectively. The less frequent shorter allele has been associated with undetectable agonist-induced downregulation of the receptor,[35] although no association with epilepsy was reported so far. However, the α_{2B}-AR ADCME mutation reported here engages the most common longer allele, and therefore it

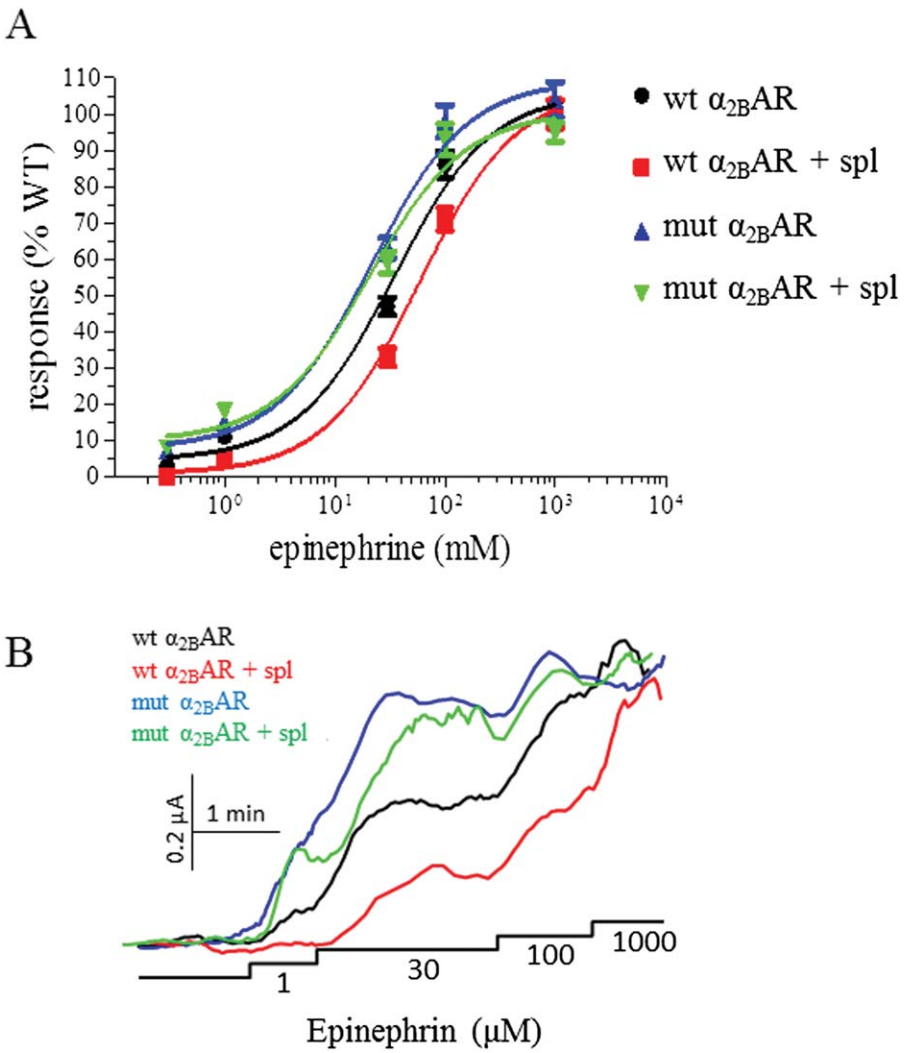

FIGURE 6: Effect of α_{2B}-adrenergic receptor (α_{2B}-AR)–spinophilin binding on Ca^{2+} signaling in *Xenopus oocytes*. (A) Oocytes expressing wild-type or mutant α_{2B}-AR alone (wt α_{2B}-AR and mut α_{2B}-AR, respectively) or with spinophilin (wt α_{2B}-AR + spl and mut α_{2B}-AR + spl, respectively) were stimulated with increasing concentrations of epinephrine while measuring the Ca^{2+}-activated Cl^- current. The results are shown as mean ± standard error of the mean of the peak current from at least 6 replicates in 4 independent experiments (wt α_{2B}-AR vs wt α_{2B}-AR + spl, $p < 0.01$; wt α_{2B}-AR + spl vs mut α_{2B}-AR + spl, $p < 0.001$). (B) A raw data trace is shown. Oocytes injected with the indicated cRNA was used 96 hours postinjection to measure activation of the Ca^{2+}-activated Cl^- current by the indicated concentrations of epinephrine.

was the isoform used for building both the control and ADCME mutant transfection constructs.

The mutation does not impair the receptor internalization triggered by the binding with arrestin-3, but alters the interaction capacity of α_{2B}-AR with spinophilin in the agonist-bound, active state. Because spinophilin regulates α_{2B}-AR signaling by binding the 3i loop to recruit RGS proteins, resulting in signal attenuation,[12] the ADCME mutation, by reducing this binding, increases the intensity of receptor activation. Accordingly, mutant α_{2B}-AR shows an increased apparent affinity to epinephrine-stimulated calcium signaling, in line with the increased potency of epinephrine in stimulating calcium signaling after spinophilin depletion.[13]

Adrenergic stimulation seems to have a dual role in epileptic neuronal firing depending on the specific neuronal host. Norepinephrine is unique among the monoamine transmitters, in that it exerts antiepileptogenic actions in the kindling model, where selective depletion of norepinephrine in the bundle from the locus coeruleus to the forebrain markedly facilitates kindling development.[36] The antiepileptogenic actions of norepinephrine are mediated by the α_2 subtype receptors, and kindled animal models indicate that impaired activation of α_2 receptors may contribute to epileptogenesis.[37] This mechanism is possibly mediated by the α_2 presynaptic autoreceptor responsible for autoinhibition of norepinephrine release, as suggested for locus coeruleus neurons, where

α_2-AR activates the inwardly rectifying K^+ currents, resulting in decreased spontaneous firing activity.[38]

Conversely, the same effect of attenuating neuronal excitation may be exerted on inhibitory interneurons by postsynaptic α_2 receptors. Dysfunction of GABAergic signaling plays a critical role in the pathogenesis of epilepsy; in particular, norepinephrine suppression of the GABA response has been demonstrated to be mediated by α_2-AR that decreases intracellular cyclic adenosine monophosphate (cAMP) formation through G_i inhibition of adenylyl cyclase.[39] Low cAMP signaling reduces the activity of the hyperpolarization-activated cyclic nucleotide-gated channels and enhances neuronal network firing propensity.[40]

Thus, cortical hyperexcitability in ADCME might result from impaired GABAergic inhibition that controls neuronal excitability[41] and modulates oscillatory activities in the central motor networks.[42] We propose that the ADCME mutation exerts a gain of function effect by reducing the interaction with spinophilin, thus increasing receptor activity.

Clinical and electrophysiological features in these families suggest cortical hyperexcitability, which can be the result of enhanced intrinsic rhythmic activity of cortical generators[2] or decreased cortical inhibition caused by dysfunction of the cerebellothalamocortical loop.[17] α_2-AR stimulation can induce a switch from tonic to burst pattern without changing the neuronal firing rate,[43] which may be at the origin of chronic motor disturbance of this condition. α_2-AR have also been shown to regulate dendrite development in mammalian cortical neurons. Agonists of α_2-AR affect length and density of dendritic spines in cultured cortical neurons, and these effects are blocked by α_2-AR antagonists. These changes in the density and length of dendritic spines correlate with increased expression of spinophilin and a decreased phosphorylation of spinophilin.[44] Increased mutant adrenoceptor function might therefore promote anatomopathological changes in the brain underlying the mild age-dependent progression of the syndrome.

This new association of α_{2B}-AR with a genetic form of epilepsy has remarkable potential pharmacological relevance by posing the AR agonists and antagonists in a new light and encouraging the design of subtype-specific antagonists to treat at least some forms of the disease.

Acknowledgment

This work was supported by the Italian Minister of Health (grant RC2011 to R.G.) and by National Institutes of Health (NIH) grant MH081917 (Q.W.). We thank Drs M. R. Vannutelli and S. Anand for statistical assistance.

Potential Conflicts of Interest

Nothing to report.

References

1. Striano P, Zara F, Striano S. Autosomal dominant cortical tremor, myoclonus and epilepsy: many syndromes, one phenotype. Acta Neurol Scand 2005;111:211–217.
2. Guerrini R, Bonanni P, Patrignani A, et al. Autosomal dominant cortical myoclonus and epilepsy (ADCME) with complex partial and generalized seizures: a newly recognized epilepsy syndrome with linkage to chromosome 2p11.1-q12.2. Brain 2001;124(pt 12): 2459–2475.
3. Mikami M, Yasuda T, Terao A, et al. Localization of a gene for benign adult familial myoclonic epilepsy to chromosome 8q23.3-q24.1. Am J Hum Genet 1999;65:745–751.
4. de Falco FA, Striano P, de Falco A, et al. Benign adult familial myoclonic epilepsy: genetic heterogeneity and allelism with ADCME. Neurology 2003;60:1381–1385.
5. Depienne C, Magnin E, Bouteiller D, et al. Familial cortical myoclonic tremor with epilepsy: the third locus (FCMTE3) maps to 5p. Neurology 2010;74:2000–2003.
6. Yeetong P, Ausavarat S, Bhidayasiri R, et al. A newly identified locus for benign adult familial myoclonic epilepsy on chromosome 3q26.32-3q28. Eur J Hum Genet 2013;21:225–228.
7. McIntyre DC, Wong RK. Cellular and synaptic properties of amygdala-kindled pyriform cortex in vitro. J Neurophysiol 1986; 55:1295–1307.
8. Platt K, Butler LS, Bonhaus DW, McNamara JO. Evidence implicating alpha-2 adrenergic receptors in the anticonvulsant action of intranigral muscimol. J Pharmacol Exp Ther 1987;241:751–754.
9. Altman JD, Trendelenburg AU, MacMillan L, et al. Abnormal regulation of the sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. Mol Pharmacol 1999;56:154–161.
10. Limbird LE. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. FASEB J 1988;2:2686–2695.
11. Krupnick JG, Gurevich VV, Benovic JL. Mechanism of quenching of phototransduction. Binding competition between arrestin and transducin for phosphorhodopsin. J Biol Chem 1997;272:18125–18131.
12. Wang Q, Zhao J, Brady AE, et al. Spinophilin blocks arrestin actions in vitro and in vivo at G protein-coupled receptors. Science 2004;304:1940–1944.
13. Wang X, Zeng W, Kim MS, et al. Spinophilin/neurabin reciprocally regulate signaling intensity by G protein-coupled receptors. EMBO J 2007;26:2768–2776.
14. Bylund DB, Eikenberg DC, Hieble JP, et al. International Union of Pharmacology nomenclature of adrenoceptors. Pharmacol Rev 1994;46:121–136.
15. Daunt DA, Hurt C, Hein L, et al. Subtype-specific intracellular trafficking of alpha2-adrenergic receptors. Mol Pharmacol 1997;51: 711–720.
16. Wang Q, Limbird LE. Regulation of alpha2AR trafficking and signaling by interacting proteins. Biochem Pharmacol 2007;73:1135–1145.
17. Striano P, Madia F, Minetti C, et al. Electroclinical and genetic findings in a family with cortical tremor, myoclonus, and epilepsy. Epilepsia 2005;46:1993–1995.
18. Kopp J, Schwede T. The SWISS-MODEL repository of annotated three-dimensional protein structure homology models. Nucleic Acids Res 2004;32(Database issue):D230–D234.

19. Kiefer F, Arnold K, Kunzli M, et al. The SWISS-MODEL repository and associated resources. Nucleic Acids Res 2009;37(Database issue):D387–D392.

20. Wang X, Zeng W, Soyombo AA, et al. Spinophilin regulates Ca2+ signalling by binding the N-terminal domain of RGS2 and the third intracellular loop of G-protein-coupled receptors. Nat Cell Biol 2005;7:405–411.

21. Simpson MA, Cross H, Proukakis C, et al. Infantile-onset symptomatic epilepsy syndrome caused by a homozygous loss-of-function mutation of GM3 synthase. Nat Genet 2004;36:1225–1229.

22. An WF, Bowlby MR, Betty M, et al. Modulation of A-type potassium channels by a family of calcium sensors. Nature 2000;403: 553–556.

23. Zuchner S, Wang G, Tran-Viet KN, et al. Mutations in the novel mitochondrial protein REEP1 cause hereditary spastic paraplegia type 31. Am J Hum Genet 2006;79:365–369.

24. Laukkanen JA, Makikallio TH, Kauhanen J, Kurl S. Insertion/deletion polymorphism in alpha2-adrenergic receptor gene is a genetic risk factor for sudden cardiac death. Am Heart J 2009; 158:615–621.

25. Heinonen P, Koulu M, Pesonen U, et al. Identification of a three-amino acid deletion in the alpha2B-adrenergic receptor that is associated with reduced basal metabolic rate in obese subjects. J Clin Endocrinol Metab 1999;84:2429–2433.

26. Small KM, Brown KM, Forbes SL, Liggett SB. Polymorphic deletion of three intracellular acidic residues of the alpha 2B-adrenergic receptor decreases G protein-coupled receptor kinase-mediated phosphorylation and desensitization. J Biol Chem 2001;276:4917–4922.

27. Madia F, Striano P, Di Bonaventura C, et al. Benign adult familial myoclonic epilepsy (BAFME): evidence of an extended founder haplotype on chromosome 2p11.1-q12.2 in five Italian families. Neurogenetics 2008;9:139–142.

28. Striano P, Chifari R, Striano S, et al. A new benign adult familial myoclonic epilepsy (BAFME) pedigree suggesting linkage to chromosome 2p11.1-q12.2. Epilepsia 2004;45:190–192.

29. Chien EY, Liu W, Zhao Q, et al. Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. Science 2010;330:1091–1095.

30. Brady AE, Wang Q, Colbran RJ, et al. Spinophilin stabilizes cell surface expression of alpha 2B-adrenergic receptors. J Biol Chem 2003;278:32405–32412.

31. DeGraff JL, Gagnon AW, Benovic JL, Orsini MJ. Role of arrestins in endocytosis and signaling of alpha2-adrenergic receptor subtypes. J Biol Chem 1999;274:11253–11259.

32. Richman JG, Brady AE, Wang Q, et al. Agonist-regulated interaction between alpha2-adrenergic receptors and spinophilin. J Biol Chem 2001;276:15003–15008.

33. Poduri A, Lowenstein D. Epilepsy genetics—past, present, and future. Curr Opin Genet Dev 2011;21:325–332.

34. Coppola A, Santulli L, Del Gaudio L, et al. Natural history and long-term evolution in families with autosomal dominant cortical tremor, myoclonus, and epilepsy. Epilepsia 2011;52:1245–1250.

35. Salim S, Desai AN, Taneja M, Eikenburg DC. Chronic adrenaline treatment fails to down-regulate the Del301–303-alpha2B-adrenoceptor in neuronal cells. Br J Pharmacol 2009;158:314–327.

36. Corcoran ME, Mason ST. Role of forebrain catecholamines in amygdaloid kindling. Brain Res 1980;190:473–484.

37. Chen LS, Weingart JB, McNamara JO. Biochemical and radiohistochemical analyses of alpha-2 adrenergic receptors in the kindling model of epilepsy. J Pharmacol Exp Ther 1990;253:1272–1277.

38. Arima J, Kubo C, Ishibashi H, Akaike N. Alpha2-Adrenoceptor-mediated potassium currents in acutely dissociated rat locus coeruleus neurones. J Physiol 1998;508(pt 1):57–66.

39. Zha DJ, Wang ZM, Lin Y, et al. Effects of noradrenaline on the GABA response in rat isolated spiral ganglion neurons in culture. J Neurochem 2007;103:57–66.

40. Wang M, Ramos BP, Paspalas CD, et al. Alpha2A-adrenoceptors strengthen working memory networks by inhibiting cAMP-HCN channel signaling in prefrontal cortex. Cell 2007;129:397–410.

41. Marco P, Sola RG, Pulido P, et al. Inhibitory neurons in the human epileptogenic temporal neocortex. An immunocytochemical study. Brain 1996;119(pt 4):1327–1347.

42. Valzania F, Strafella AP, Tropeani A, et al. Facilitation of rhythmic events in progressive myoclonus epilepsy: a transcranial magnetic stimulation study. Clin Neurophysiol 1999;110:152–157.

43. Delaville C, Zapata J, Cardoit L, Benazzouz A. Activation of subthalamic alpha 2 noradrenergic receptors induces motor deficits as a consequence of neuronal burst firing. Neurobiol Dis 2012;47: 322–330.

44. Hu J, Vidovic M, Chen MM, et al. Activation of alpha 2A adrenoceptors alters dendritic spine development and the expression of spinophilin in cultured cortical neurones. Brain Res 2008;1199:37–45.