The α2B-adrenergic receptor is mutant in cortical myoclonus and epilepsy

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The α_{2B} adrenergic receptor is mutant in cortical myoclonus and epilepsy

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Abstract

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 to chromosome 8q, 5p and 3q, but none of the causing genes has been identified. We aim at identifying the mutant gene responsible for this epileptic form.

Methods—Genes included in the ADCME critical region were prioritized and directly sequenced. Co-immunoprecipitation, immunofluorescence and electrophysiology approaches on transfected human cells have been utilized for testing the functional significance of the identified mutation.

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Results—Here we show that mutation in the α2-adrenergic receptor subtype B (α2B-AR) associates to ADCME by identifying a novel in-frame insertion/deletion in two Italian families. The mutation alters several conserved residues of the third intracellular (3i) loop, neither hampering the α2B-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 GPCRs with Regulators of G proteins Signaling proteins. Accordingly, the mutant α2B-AR increases the epinephrine-stimulated calcium signaling.

Interpretation—The identified mutation is responsible for ADCME, as the loss of α2B-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 for understanding the molecular pathway of epileptogenesis, widening the spectrum of possible therapeutic targets.

Introduction

Autosomal dominant cortical myoclonus and epilepsy (ADCME, OMIM 607876; also Cortical Myoclonic Tremor With Epilepsy, Familial, 2; FCMTE2) and benign adult myoclonic epilepsy (BAFME/FAME, OMIM 601068) are syndromes with high penetrance, characterized by rhythmic myoclonic jerks of cortical origin and focal or generalized tonic-clonic seizures, with non-progressive 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 intra-hemispheric and inter-hemispheric cortical spread of cortical myoclonic activity, indicating widespread cortical hyperexcitability with defective inhibitory cortical mechanisms [2]. The diseases-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 with ADCME in two unrelated families. The adrenergic system has been proposed since the late ‘80s to be implicated in epileptogenesis, as impaired activation of α_2 adrenergic receptors might contribute to epileptogenesis in the kindling model [7, 8]. The α_2 adrenergic receptors (α_2-ARs) belong to the G protein coupled receptors (GPCRs) family that binds the endogenous ligands epinephrine and norepinephrine. These seven 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, re-sensitizing 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 Regulators of G proteins Signaling (RGS) proteins to reduce signaling intensity [13] .

To date, three distinct α_2-adrenergic receptor (α_2-AR) subtypes (α_{2A}, α_{2B}, α_{2C}) have been described in humans [14], which are encoded by three intronless genes localized on chromosomes 10, 2 and 4. The α_2-ARs are distributed throughout the central nervous system (CNS) with no extensive overlap, indicating a probable role in discrete neuronal functions by coordinating independent neural signaling pathways. Although the three 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, Gi activation and binding of spinophilin and arrestin [16]. Interestingly, the α_{2B}-AR mutation reported here involves consistent changes of this crucial controller domain.

Patients and Methods

Patients' evaluation

All affected members underwent video-polygraphic study, jerk-locked back averaging (JLA), somatosensory evoked potentials (SEPs) and long latency reflex I (LLRI). Detailed methods have been described elsewhere [217], SEPs were judged as giant when the components N20-P25 and P25-N33 were larger than 8·6μV and 8·4μV. Neuropsychological evaluation included Wechsler Adult Intelligence Scale Revised.

Mutation detection

Mutation analysis of candidate genes was performed by amplifying coding sequences scanning by D-HPLC (Wave, Transgenomic) and direct sequencing. Screening of conrols for the ADCME mutation was performed by ARMS-PCR assay amplifying both the wild-type and the mutant alleles, together with a control fragment, in a single-tube PCR. The region flanking the mutation was amplified by two outer primers, producing a nonallele-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 wild-type and the mutant amplicons. The allele-specific primers specificity was conferred by the match of the 3' nucleotides with either the wild-type or the mutant allele. The tetra ARMS-PCR produced two allele-specific amplicons with different lengths (316bp for wild-type; 205bp for mutant) and one control amplicon that was always present (486bp). Primers sequences and PCR conditions are available upon request.

Targeted Capture and DNA Sequencing

gDNA were nebulized and the libraries prepared using a GS FLX Titanium Rapid Library Preparation Kit (Roche, Milan, Italy). In order to multiplex the two samples in a single sequencing run, two different MID identifiers have been used. The libraries were pooled and hybridized on a Titanium Optimized Sequence Capture 385K Array (Roche-Nimblegene, Madison, WI-USA). The array was designed to capture the coding and the UTRs regions of the UCSC genes localized in the extended linkage region (hg18/NCBI36; chr2:85,140,498-112,715,205). Additional 20 bp of flanking intronic sequence were 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 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 *P. troglodytes* (XP_003309176.1), *C. apella* (CAJ19284.1), *P. pithecia* (CAJ19290.1), *H. lar* (CAJ19281.1), *M. mulatta* (XP_001082230.1), *C. solatus* (CAJ19319.1), *P. abelii* (XP_002811692.1), *T. indicus* (AEP17928.1) and *C. porcellus* (XP_003471576.1).

Molecular modeling

The three-dimensional structure of wild type and mutant α_{2B}-AR were produced using Swiss-Model server (http://swissmodel.expasy.org/)[1819], which performs sequence alignment and putative template protein selection for the generation of the 3d model of the query protein

Plasmid preparation

Full length wild type and mutant α_{2B}-AR was amplified from genomic DNA with primers encoding the KpnI-EcoRI sites and the 5'HA tag and subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA). Myc tagged spinophilin was prepared as described before [20]. FLAG-tagged arrestin3 were kindly provided by Dr Robert J. Lefkowitz (Duke University, Durham, NC, USA).

Immunofluorescence microscopy

HeLa cells were seeded at 10^4/well in 12-well plates on glass coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were transiently transfected with plasmid encoding wild type or mutant HA-tagged α_{2B}-AR alone or in combination with FLAG-tagged arrestin3 (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% BSA, 25mM HEPES and containing mouse anti-HA antibody (6μg/ml, Covance) for 1 hour at 4°C. After washing, cells were treated with 100 mM 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 (DAPI). Images were captured by using a ZEISS Imager A2 microscope and obtained under a 40x Plan-Apochromat M27 objective (Zeiss).

Co-immunoisolation of Spinophilin with the WT and mutant α_{2B}-AR

CosM6 cells were transfected with a plasmid encoding myc-tagged spinophilin (pCMV4-Myc-Sp) 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). 24 h post-transfection, cells were serum starved overnight, and then treated with 100 μM epinephrine (plus 1μM prazosin to block α1AR and 1μM propranolol to block βAR) or vehicle for 5 or 10 min. After stimulation, cells were lysed in buffer containing 20 mM HEPES (pH 7.4), 0.5% NP-40, 10% glycerol, 2 mM EDTA, 5 mM sodium fluoride and protease inhibitor cocktail (100 μM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor and 1μM pepstain), and centrifuged at 13,000 rpm for 30 min 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 co-immunoprecipation assay. Cell lysates were precleared with 35μl of protein G beads slurry for 45 min 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 times with lysis buffer and bound proteins were extracted with Laemmli sample buffer.

Current measurement in *Xenopus laevis* oocytes

The SPL 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 were used for cRNA synthesis in vitro using SP6 RNA polymerase (Ambion, Life Technologies, Paisley, UK). *X. laevis* oocytes were prepared as previously described [20]. Briefly, oocytes in stage V–VI were injected with 2–10 ng cRNA encoding wild type or mutant α_{2B}-AR alone or in combination with spinophilin and incubated at 18°C in 96 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM HEPES (pH 7·6) buffer. Current measurement was accomplished 48–96 h post-injection with the two-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 post-injection. To measure the Ca^{2+}-activated Cl^- current, membrane potential was held at –60 mV for continuous recording. To acquire epinephrine dose–response relationships, membrane potential was stepped from –60 mV to + 50 mV for 200 ms at 0.2 Hz. The results are shown as means ± s.e.m. of the peak current normalized on wild type.We used 2 oocyte preparations and did the 6 experiments over 4 days, starting 96 hrs after cRNA injection. In each experiment we performed the dose response in all conditions in the same day (wild type, mutant, wild type +SPL, mutant+SPL). The pick current at each concentration was used to calculate the response and for p we used all 6 experiments. The maximum current was similar for all conditions. The current was measured at a holding potential of −30 mV and the traces show the Ca2+ activated Cl− current.

Results

Identification of α_2-adrenergic receptor subtype B mutation in ADCME patients

We have previously mapped the ADCME locus on chromosome 2p1.1-q1.2 in a five-generation family from Tuscany with autosomal dominant pattern of inheritance [2] (Fig. 1; Family A). 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 (AIES) [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 result. Finally, we identified a novel in-frame insertion/deletion in the α_2-adrenergic receptor subtype B gene (α_{2B}-AR; *ADRA2B*), which substitutes five amino acids, HGGAL, with four new residues, QFGR, (indel; c.675_686delTGGTGGGGCTTTinsGTTTGGCAG; p.H225_L229delinsQ225_F_G_R228) (Fig. 2A).

This indel mutation completely co-segregates 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 (NHLBI GO Exome Sequencing Project, Seattle, WA; http://evs.gs.washington.edu/EVS/), and in the 1000 Genomes Project (http://www.1000genomes.org/), thus totaling 16080 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 factors for cardiovascular and metabolic diseases [2425] and to disregulate agonist-promoted receptor desensitization [26]. However, the identified mutation is syntenic to the more common variant showing 12 consecutive glutamic acid residues, variant that is not associated to the above mentioned effects. Other indels of the *ADRA2B* gene found in the control population are reported in a Supplementary Table 1.

In a second ADCME family from Tuscany (Fig. 1; Family B) we detected the same indel of the *ADRA2B* gene. Although no kinship between the two families was discovered by anamnestic analysis of members of the two 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 insertion and deletions by southern blot (data not shown).

In order to exclude other possible variations that could explain or contribute to the onset of the ADCME phenotype, we extensively sequenced two 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 family B phenotype (clinical findings are summarized in Table 1). Briefly, family B is a four-generation kindred including 5 affected members (4 living). All patients exhibited postural hand and upper limbs myoclonus. Epilepsy occurred in all but one (IV:1) individuals 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: 2 and III:5. Individual III:2 exhibited borderline cognitive level, while all remaining affected individuals had normal intelligence. Cortical tremor was the presenting symptom in all affected individuals, appearing between the ages of 18 to 50 years. Generalized or focal paroxysmal activity was identified in all patients. Jerk-locked back averaged EEG, somatosensory evoked potentials, and long-loop reflex, were consistent with the cortical reflex myoclonus.

We took into consideration a possible co-morbidity of epilepsy with cardiovascular diseases as the *ADRA2B* gene is also expressed in the heart and vascular smooth muscles. A careful anamnesis was performed on all ADCME patients giving negative evidence for cardiovascular diseases; two of the oldest *ADRA2B* mutation carriers developed age-related dementia.

Mutant α_{2B}-adrenergic receptor internalization is unaffected

The indel localizes in the third intracellular (3i) loop, a crucial domain for receptor localization and signal transduction, conserved in primates [16] (Fig 2B). The mutant amino acid stretch Gln225_Phe_Gly_Arg228 is expected to result in different physical-chemical properties of the domain in comparison to the wild type, due to the insertion of a very hydrophobic and bulky residue (Phe226) and a positive charge (Arg228). Since no crystal structure of the α_{2B}-AR is yet available, we generated a three-dimensional configuration model in order 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 Fig. 3, the mutant is predicted to prominently alter the conformation of the specific region of the 3i loop. We therefore tested if these changes can affect the α_{2B}-AR subcellular distribution and its ability to interact with other proteins.

Since the 3i loop has been demonstrated to be critical for intramembrane stability [30] we first determined the wild type and mutant α_{2B}-AR subcellular localization by immunofluorescence. Both wild type 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 ER-Golgi compartments and its delivery to the plasma membrane (Fig. 4A and D). When cells were stimulated with epinephrine, little or no redistribution of α_{2B}-ARs was detected (Fig. 4B and E), while in the presence of arrestin-3, wild type α_{2B}-AR relocalizes to endosomes as expected [31] as well as the mutant receptor (Fig. 4C and F). These results suggest that both wild type 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 that indeed is apart from the arrestin-3- α_{2B}-AR interaction domain .

Mutant α_{2B}-adrenergic receptor 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 initially investigated by GST-pull down; both wild type 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 co-immunoisolation assay. As shown in the Fig. 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 co-isolated with the α_{2B}-AR, thus indicating that spinophilin preferentially interacts with the agonist-activated α_{2B}-AR, in line with previous findings[35]. After 5-min treatment of epinephrine, spinophilin was co-immunoisolated with comparable amount of wild type and mutant α_{2B}-AR. However, the amount of spinophilin co-isolated with the mutant α_{2B}-AR dropped back to the basal level after epinephrine treatment for 10 min, whereas the amount of spinophilin co-isolated with the wild type α_{2B}-AR remained elevated with the same treatment (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 attenuate the signaling by recruiting RGS proteins to the receptors-G protein complex, which accelerates the Gα-GTPase activity to terminate the signal [13]. To test the effect of the mutation on the role of spinophilin in Ca^{2+} signaling, *Xenopus laevis* oocytes were injected with cRNA encoding wild type 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 wild type ($p=0.055$), but the presence of spinophilin does not result in an increase in EC_{50} (α_{2B}-AR + SPN versus α_{2B}-AR mutant + SPN: $p<0.001$; 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, in the neurotransmitter-gated ion channels, the nicotinic acetylcholine receptor and the γ-aminobutyric acid receptor subtype A ($GABA_A$), in

transcription factors (ARX), in proteins involved in synaptic vesicle release, and in cerebral cortex development and plasticity [33]. 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 two distinct clinical entities. However, we cannot exclude the presence of variations in gene regulatory regions or other non-coding 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}, 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[35]. The less frequent shorter allele has been associated with undetectable agonist-induced down-regulation of the receptor [36], though 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 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 the α_{2B}-AR with spinophilin in the agonist-bound, active state. Since spinophilin regulates α_{2B}-AR signaling by binding the 3i loop to recruit RGS proteins and thus 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 on epileptic neuronal firing depending on the specific neuronal host. Norepinephrine is unique among the monoamine transmitters, in that it exerts anti-epileptogenic actions in the kindling model, where selective depletion of norepinephrine in the bundle from the locus coeruleus to the forebrain markedly facilitates kindling development [37]. The anti-epileptogenic 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 [38]. This mechanism is possibly mediated by α_2 presynaptic autoreceptor responsible for auto-inhibition 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[39].

On the other hand, the same effect of attenuating neuronal excitation may be exerted on inhibitory interneuron by postsynaptic α_2 receptors. Indeed, 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 cAMP formation through G_i inhibition of adenylyl cyclase [40]. Low cAMP signalling reduces the activity of the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and enhances neuronal network firing propensity [41].

Thus, cortical hyperexcitability in ADCME might result from impaired GABAergic inhibition that controls neuronal excitability [42] and modulates oscillatory activities in the central motor networks [43]. We propose that the ADCME mutation exerts a gain of function effect by reducing the interaction with spinophilin, and 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 cerebello-thalamo-cortical loop [17]. α_2-AR stimulation can induce a switch from tonic to burst pattern without changing the neuronal firing rate [44], 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 neurones [45]. Agonists of α_2-AR affect length and density of dendritic spines in cultured cortical neurones 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 [45]. Increased mutant adrenoceptor function might therefore promote anatomo-pathological 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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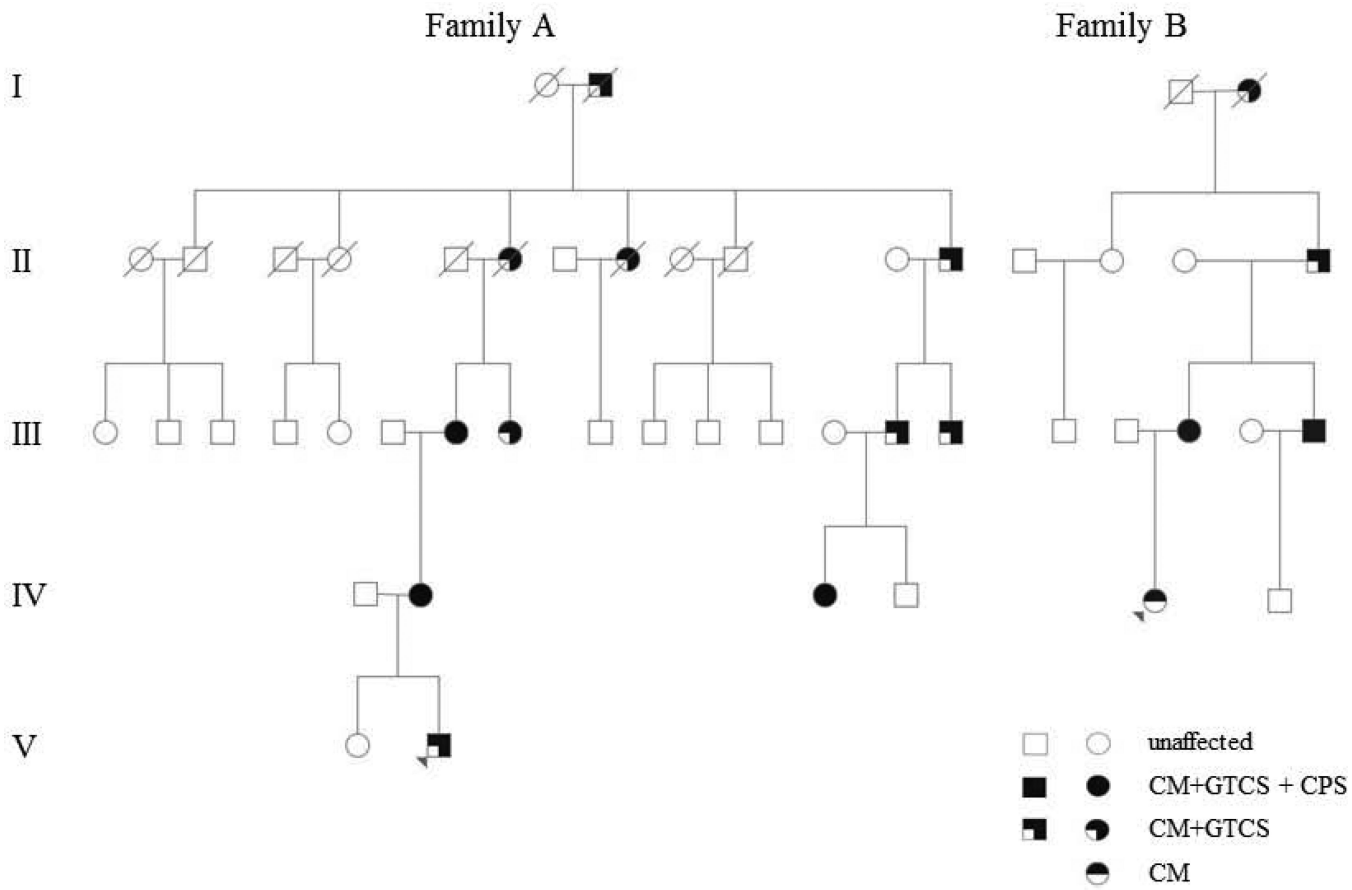

Figure 1. Pedigrees of ADCME families

CM, cortical myoclonus; GTCS, generalized tonic-clonic seizure; CPS, complex partial seizure. Probands are indicated by arrowheads.

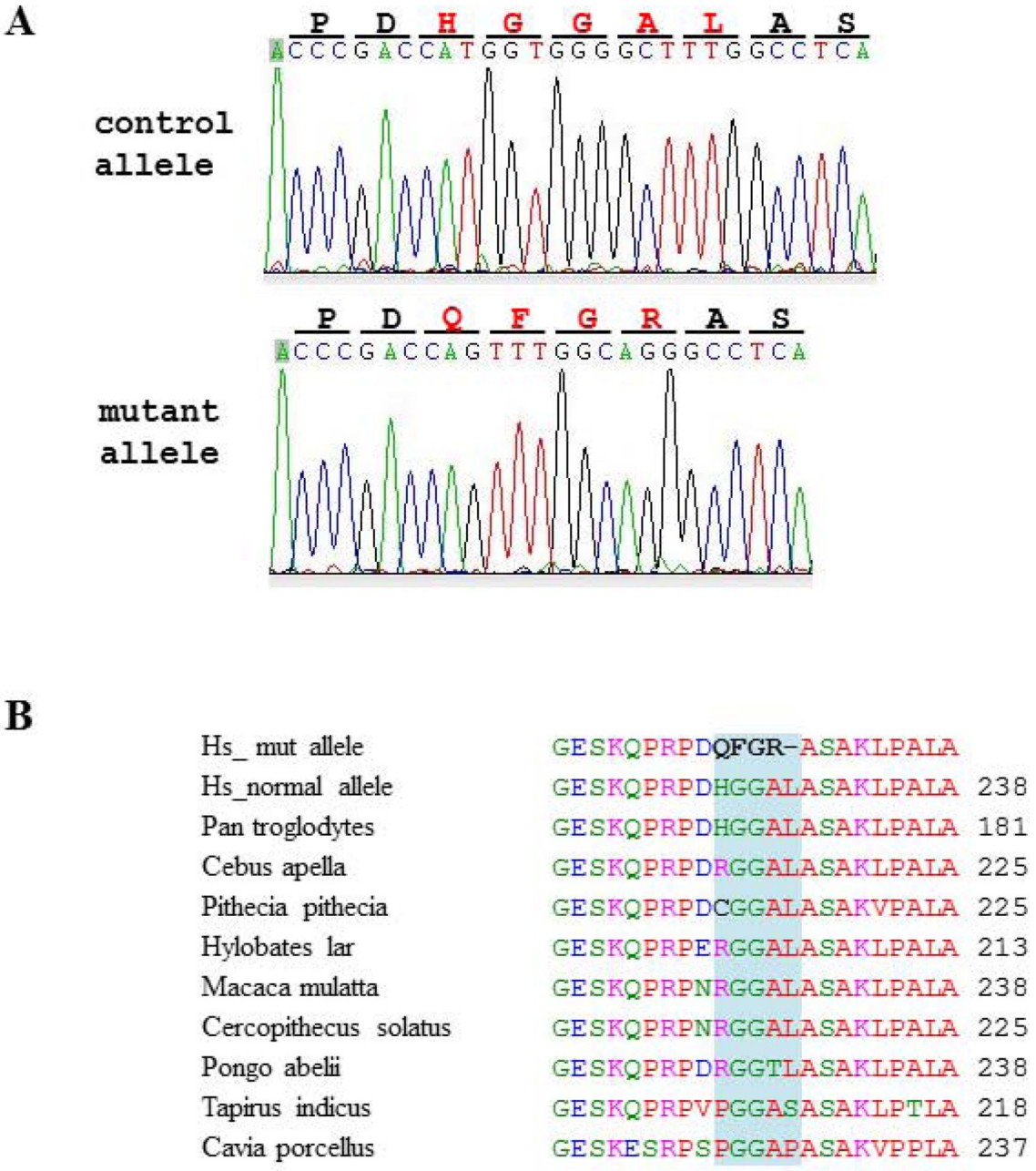

Figure 2. The ADCME mutation of the α_{2B}-AR

(A) Control and mutant alleles. (B) Partial sequence alignment of AR in multiple species. The mutant sequence is highlighted.

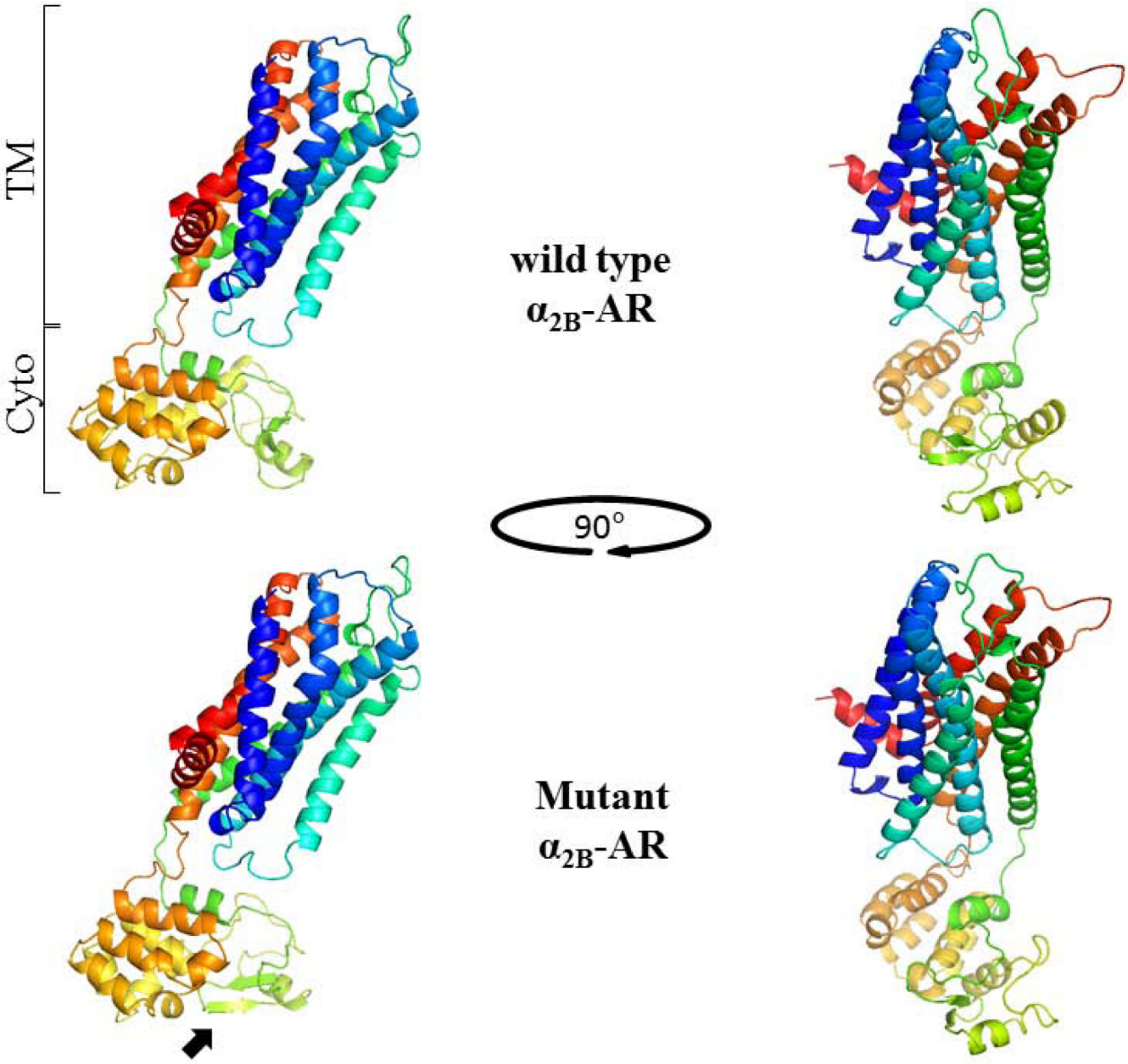

Figure 3. Molecular modeling of wild type and mutant α_{2B}-AR

Ribbon diagram of the α_{2B}-AR are rainbow gradient-colored (N terminus, blue, to C terminus, red). Arrow points to the mutant stretch. TM, transmembrane domain; Cyto, cytosolic domain.

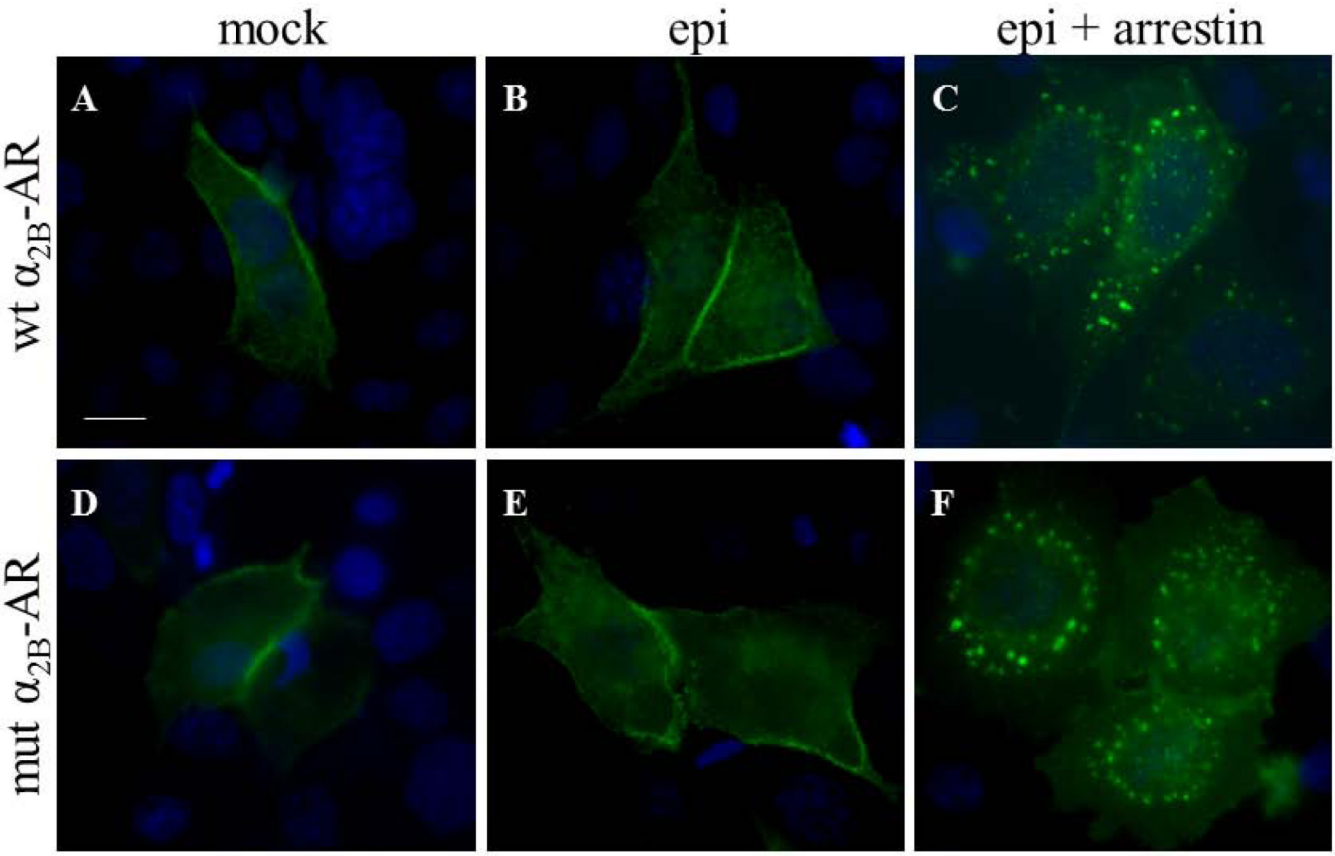

Figure 4. Wild type and mutant α_{2B}-AR internalization

Representative images of HeLa cells transfected with wild type (α_{2B}-AR WT) or mutant (α_{2B}-AR mut) HA-tagged α_{2B}-AR alone or in combination with arrestin3 and stimulated with epinephrine (epi), where indicated. Scale bar is 30 μm.

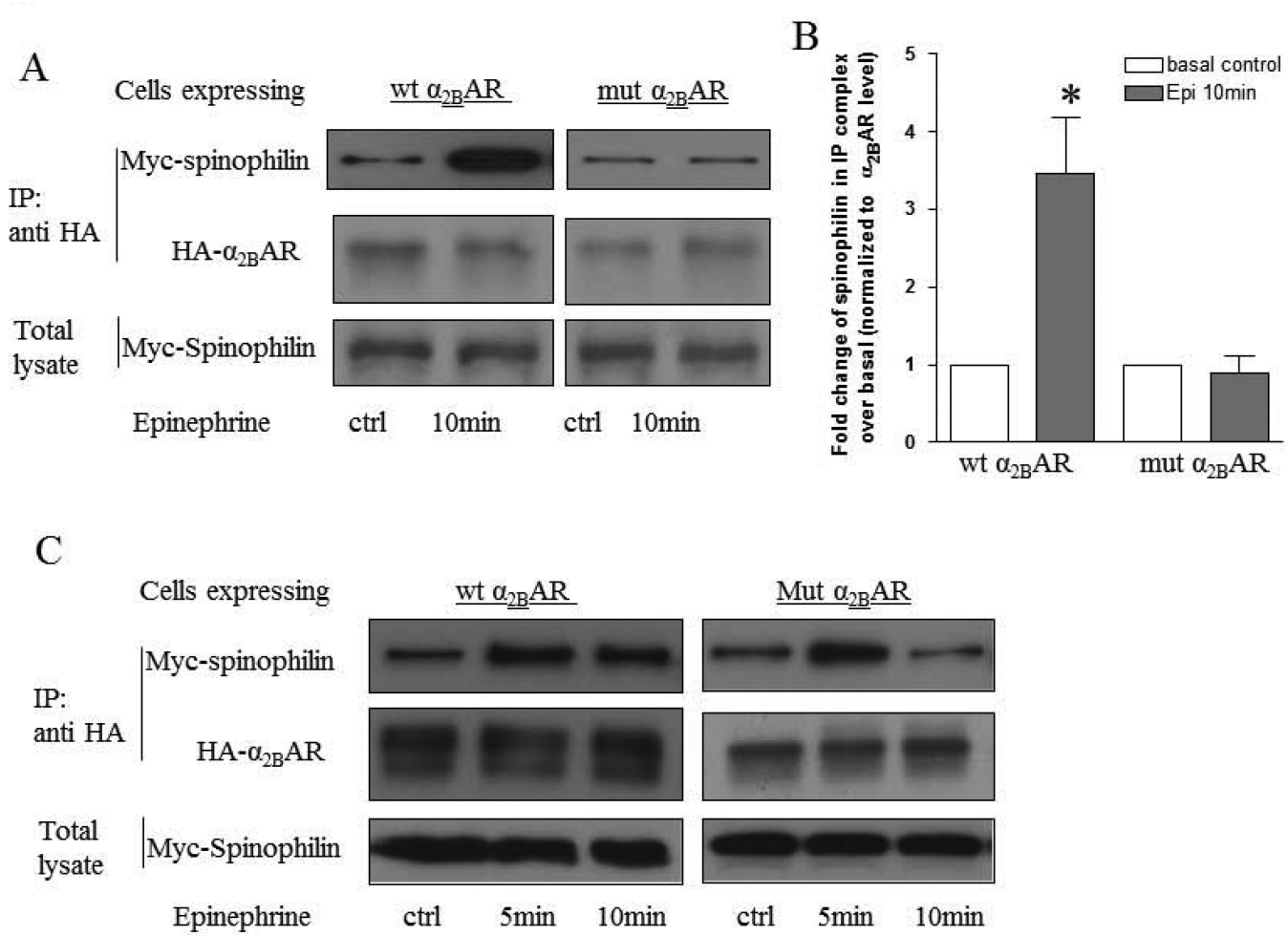

Figure 5. The mutant α_{2B}-AR fails to form a stable complex with spinophilin

COS M6 cells co-expressing spinophilin with wild type or mutant α_{2B}-AR are stimulated with epinephrine and the α_{2B}-AR-spinophilin complex is isolated with an HA antibody. (A) Representative blot showing spinophilin interaction with the wild type and mutant α_{2B}-AR after 10 min stimulation of epinephrine. (B) Quantitation of α_{2B}-AR-spinophilin interaction representing three 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 ± SEM; *$p < 0.05$, epinephrine stimulated versus control. (C) Representative blots showing interaction between spinophilin and wild type (left) or mutant (right) α_{2B}-AR at indicated time points.

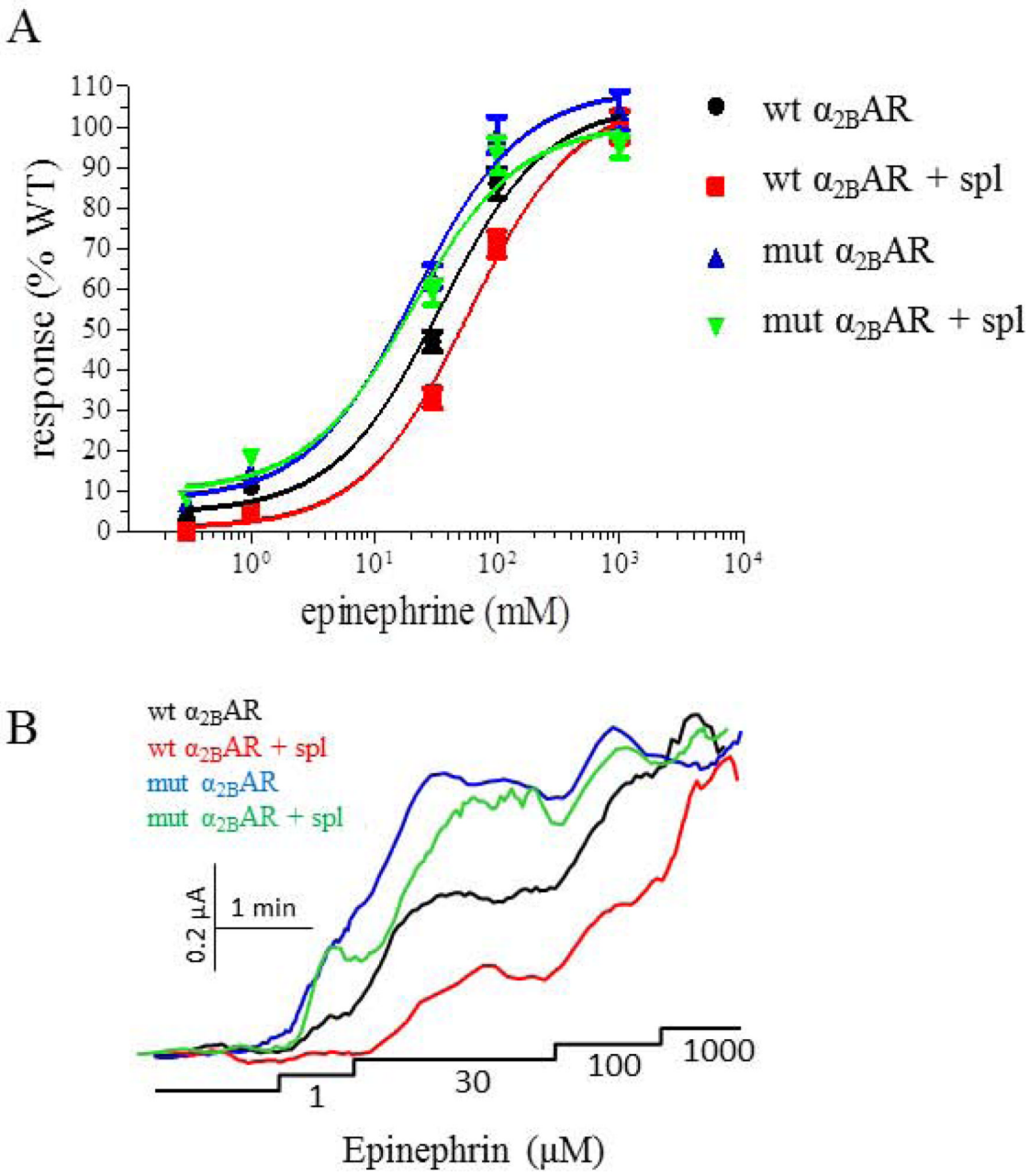

Figure 6. Effect of α_{2B}-AR-spinophilin binding on Ca^{2+} signaling in *Xenopus oocytes*

(A) Oocytes expressing wild type or mutant α_{2B}-AR alone (wt α_{2B}-AR and mut α_{2B}-AR) 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 means ± s.e.m. 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 hrs post injection to measure activation of the Ca2+-activated Cl− current by the indicated concentrations of epinephrine.

Table 1

Clinical features of affected members of family B.

Patient ID/Sex/Age, y	Age at onset	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:2/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

NA. not available; IQ: intelligence quotient; GTCS: generalized tonic-clonic seizures; PB: Phenobarbital; VPA: valproate; CNZ: clonazepam, LEV: levetiracetam