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Functional analysis of novel KCNQ2 and KCNQ3 gene variants found in a large pedigree with benign familial neonatal convulsions (BFNC)

Abstract Benign familial neonatal convulsion (BFNC) is a rare autosomal dominant disorder caused by mutations in KCNQ2 and KCNQ3, two genes encoding for potassium channel subunits. A large family with nine members affected by BFNC is described in the present study. All affected members of this family carry a novel deletion/insertion mutation in the KCNQ2 gene (c.761_770del10insA), which determines a premature truncation of the protein. In addition, in the family of the proposita's father, a novel sequence variant (c.2687A>G) in KCNQ3 leading to the p.N821S amino acid change was detected. When heterologously expressed in Chinese hamster ovary cells, KCNQ2 subunits carrying the mutation failed to form functional potassium channels in homomeric configuration and did not affect channels formed by KCNQ2 and/or KCNQ3 subunits. On the other hand, homomeric and heteromeric potassium channels formed by KCNQ3 subunits carrying the p.N821S variant were indistinguishable from those formed by wild-type KCNQ3 subunits. Finally, the current density of the cells mimicking the double heterozygotic condition for both KCNQ2 and KCNQ3 alleles of the proband was decreased by approximately 25% when compared to cells expressing only wild-type alleles. Collectively, these results suggest that, in the family investigated, the KCNQ2 mutation is responsible for the BFNC phenotype, possibly because of haplo-insufficiency, whereas the KCNQ3 variant is functionally silent, a result compatible with its lack of segregation with the BFNC phenotype.

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Introduction

Benign familial neonatal convulsions (BFNC; MIM 121200) is a rare autosomal-dominant idiopathic epilepsy of the newborn. This disease is characterized by the occurrence of multifocal or generalized tonic-clonic convulsions starting at around day 3 of postnatal life and spontaneously disappearing after a few weeks or months [1]. Although neurocognitive development is normal in most BFNC-affected individuals, 10–15% of them experience convulsive manifestations later in life [2].

Mutations in either KCNQ2 [3, 4] or KCNQ3 [5] genes have been causally linked to BFNC, although about 30% of BFNC-affected families fail to show KCNQ2 or KCNQ3 mutations or linkage to KCNQ2 or KCNQ3 loci [6]. KCNQ2 and KCNQ3 genes encode for voltage-gated K^+

channel subunits whose heteromeric assembly underlies the muscarinic-regulated K^+ current (I_{KM}) [7]. I_{KM} is a widespread regulator of neuronal excitability limiting repetitive firing and causing spike-frequency adaptation [8].

Around 30 KCNQ2 and 3 KCNQ3 mutations have been discovered so far in families affected by BFNC [6]. Those few mutations whose functional consequences have been investigated cause a variable reduction (usually less than 50%) in the maximal current carried by heteromeric KCNQ2/KCNQ3 channels [9–11]; a current reduction of more than 50% was found only in two cases, consistent with a dominant-negative effect [6, 12].

In the present study, we report clinical and genetic data on a large Italian BFNC family with nine affected members. In the index patient, we found heterozygous sequence variants in both KCNQ2 and KCNQ3 alleles; a c.761_770del 10insA in KCNQ2, determining a premature truncation of the protein, and a c.2687A>G substitution in KCNQ3, leading to the p.N821S amino acid change. In transfected mammalian cells, functional analysis by biochemical and electrophysiological means revealed that the KCNQ2 mutation failed to give rise to significant voltage-gated K^+ currents and did not affect channels formed by KCNQ2 and/or KCNQ3 subunits; on the other hand, KCNQ3 subunits carrying the c.2687A>G substitution displayed functional properties indistinguishable from those formed by wild-type KCNQ3 subunits.

Patients and methods

Clinical data

The pedigree of the family investigated is shown in Fig. 1a.

Subject IV-1(proband)

The index patient (Case IV-1, Fig. 1a) was the only daughter of two unrelated parents, delivered after a trouble-free pregnancy. At the age of 3 days, she had sub-continuous clonic seizures involving bilaterally upper and lower extremities with perioral cyanosis, lasting for 50–60 mins, and stopped by phenobarbital (PB; 20 mg kg^{-1}) i. v. in bolo. Despite immediately starting PB treatment (4.2 mg kg^{-1} day^{-1}) as chronic therapy, isolated, rare (two to three per day) and brief (5–10 s) seizures appeared during the next 3 days. At the age of 7 days, during a video-EEG recording, she suffered from a more prolonged clonic seizure (lasting 1 min), showing right head and eyes rotation, palpebral myoclonias and oro-alimentary automatisms. Cyanosis was evident. The EEG showed fast polispikes on the left centro-temporal areas; a secondary diffusion of spikes and spikes and waves complexes was evident on the left hemisphere and the centro-temporal right areas. Interictal EEG recording showed a normal organization of background activity with centro-temporal isolated spikes more evident on the left side. Neurological assessment between seizures and laboratory data (complete blood count, electrolytes, blood urea nitrogen, blood gas analysis, blood glucose) were normal. Cerebral ecography and cerebral MRI were normal. Starting from the age of 6 months, the EEG has been normal. After two isolated brief convulsive episodes occurring at the 13th and 15th day, no more seizures were noticed; PB was discontinued at the age of 12 months. She is now 18 months old; her neurological examination and psychomotor development are normal.

Subjects in the maternal lineage

At the age of 3 days, the mother of the proband (case III-5) had the same type of seizures described in her daughter (with the exception of the duration, which was shorter), but these seizures spontaneously remitted after 1–2 min. Seizures (one to two per week) continued for months and disappeared before the sixth month of life without any pharmacological treatment. Analogously, many subjects in the maternal lineage (subjects II-4, II-5, II-6, III-7) showed seizures starting at the age of 3–5 days and disappearing after 5 months without any anti-epileptic drug (AED) therapy. On the contrary, although subjects III-10 and III-11 had seizures at the same age, they immediately underwent PB treatment, and seizures disappeared in 1 week. At the time of writing, they showed no neurological abnormalities and normal IQ.

Subject III-4, the maternal aunt of the proband, was born after a trouble-free pregnancy at 40 weeks. Birth clinical documents do not report relevant perinatal events. Starting from the age of 3 days, she had rare and brief clonic seizures (one or two per week). No therapy was introduced. At the age of 6 months, she had a prolonged tonic seizure with cyanosis, for which hospitalization in intensive care unit was necessary. EEG recording showed multifocal epileptiform abnormalities asynchronous over both cerebral hemispheres. Screening laboratory data (complete blood count, electrolytes, blood urea nitrogen, blood gas analysis, blood glucose, cerebrospinal fluid cytology) and CT scan were normal. Despite PB treatment, she continued to have seizures; ACTH treatment was started, and complete seizures control was immediately achieved. No more seizures were noticed after this time; PB was discontinued at the age of 36 months. A mild developmental delay in the first years of life and mild learning disabilities during school age were observed. She is now 37 years old, without major neurological deficit but with mild mental retardation (full IQ=65, by WAIS-R).

Neurological history was unremarkable for individuals II-3, II-7, III-6, III-8 and III-9.

Subjects in the paternal lineage

The father of the proposita (case III-1) was born at term by a spontaneous vaginal delivery following an uncomplicated gestation. He had a normal neurological and psychological development and normal schooling. Seizures have never been described in his life. At the age of 10, he suffered from migraine with typical aura. Crises were

Fig. 1 Pedigree and genomic DNA analysis of the BFNC-affected family. **a** Pedigree of the family; *solid black symbols* are BFNC-affected subjects, while *open symbols* represent healthy individuals; *horizontal hatched circle* represents a subject with BFNC who later developed West syndrome; the *arrow* indicates the proband; *NA* blood sample not available. *Empty dots* mark subjects of the family carrying the KCNQ2 mutation, while *stars* indicate those carrying KCNQ3 gene variant. **b** Electropherograms of genomic DNA fragments containing the sequence variants identified in KCNQ2 (*left panels*) and KCNQ3 (*right panels*). In the *top left panel*, the *arrow* indicates the site of insertion/deletion. The *boxed sequence* in the control (*middle left panel*) represents the deleted sequence. In the *bottom left panel*, the sequence of the isolated and subcloned mutated allele is shown; the *boxed nucleotide* shows the inserted adenine nucleotide at the insertion/deletion site. In the *top right panel*, the *arrow* indicates the nucleotide change detected in the proband (*top*, IV-1) and in the father (*bottom*, III-1), while the mother (*middle*, III-V) shows a wild-type sequence
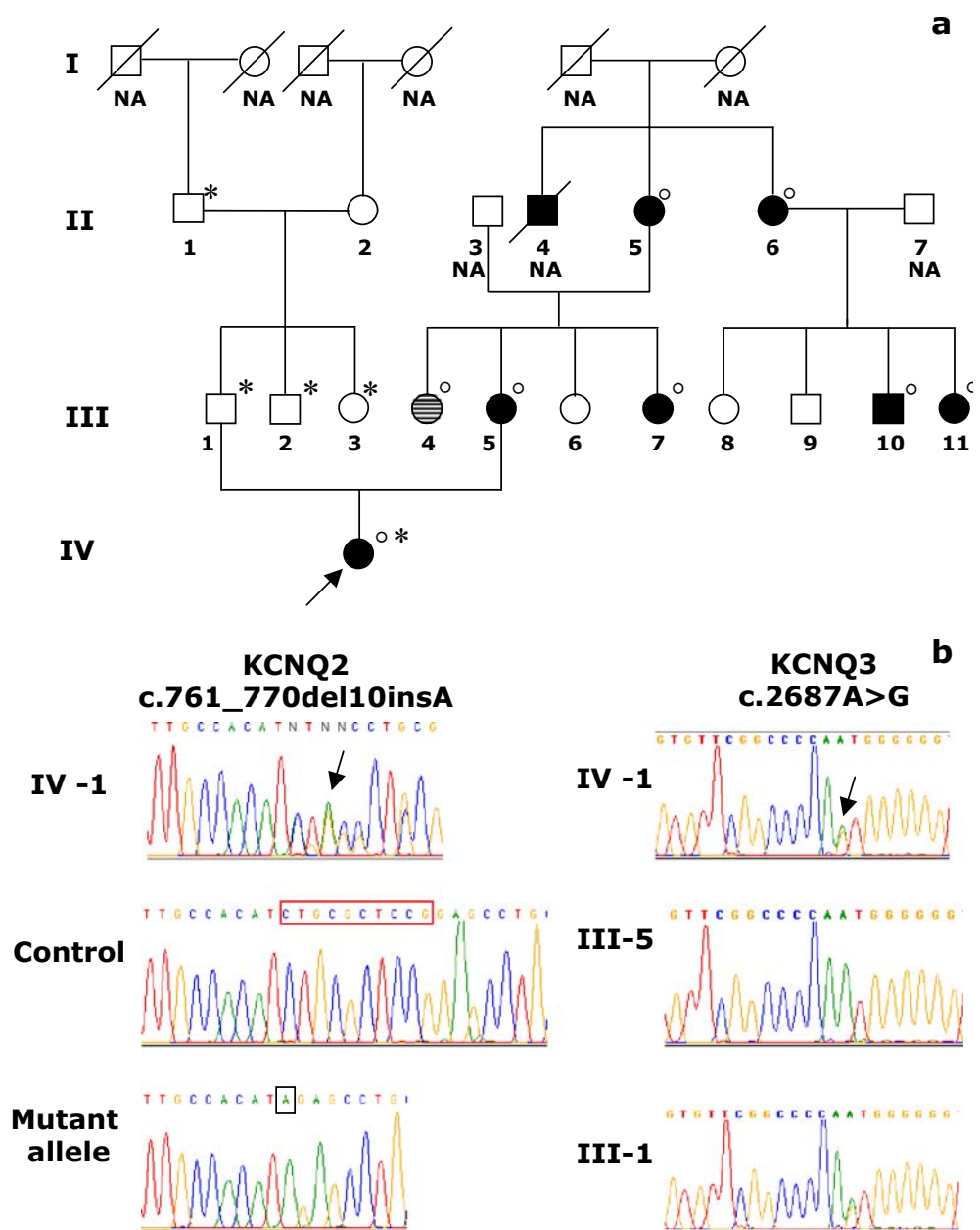
frequent in adolescence, but are now less numerous (one or two episodes per year). Symptoms started with right arm paraesthesiae, followed by aphasia and visual negative scotomas. Neurological exam, EEG and MRI are normal. At the age of 20 months, his sister (case III-3) presented a single febrile tonic-clonic convulsion lasting 3–5 min. No therapy was started, and no seizures relapsed during the following years. At the age of 11, she suffered from migraine with typical aura, with the same characteristics described for the brother's attacks. She is now a 30-year-old woman without any neurological problem. Neurological history was unremarkable for individuals II-2 and III-2.

KCNQ2/KCNQ3 genes mutation analysis

Blood samples were taken with informed consent from all available affected and non-affected members of the family. Total DNA was extracted from 5 ml of blood with the IsoQuick Nucleic Acid Extraction kit (ORCA Research, Bothell, WA, USA). KCNQ2 and KCNQ3 genomic sequences were available at the Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway). All nucleotide numbers refer to the published cDNA sequences: NM_004518 (KCNQ2) and NM_004519 (KCNQ3). All exons of KCNQ2 and KCNQ3 were amplified with 17 and

15 sets of primers, respectively. Amplification was performed in a Master cycler Gradient (Eppendorf, Hamburg, Germany) using 25 pmol of each primer and 0.5 U *Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) in 35 cycles according to the manufacturer's instructions. Mutation analysis was performed by direct sequencing of all exons with the same primers used for amplification (primer sequences are available on request). PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and then directly sequenced with Big Dye Terminator Cycle Sequencing Kit in an automated sequencer (ABI 3100 PE, Applied Biosystems). Control subjects were recruited from the same Italian regions where the family comes from and were checked by direct sequencing for the presence of the nucleotide variations under examination. All mutations are described according to the mutation nomenclature ([13] and at the http://www.hgvs.org/mutnomen). The ins/del mutation in KCNQ2 gene was first characterized by subcloning and sequencing of two independent PCR products. In addition to that, since this mutation produces the loss of an *Hpa*II restriction enzyme site, the segregation of this mutation in the family was verified both by restriction enzyme analysis and by sequencing. NetStart program (version1.0) is available at http://www.cbs.dtu.dk/services/NetStart/.

Mutagenesis of KCNQ2 and KCNQ3

Mutations were engineered on human KCNQ2 and KCNQ3 cDNAs cloned into pcDNA3.1 (Invitrogen, Milan, Italy) by sequence overlap extension PCR with the *Pfu* DNA polymerase, as described in [14]. For some experiments, the KCNQ2 mutation was incorporated in a cDNA construct encoding for a fusion protein (EGFP-Q2) between KCNQ2 and the Enhanced Green Fluorescent Protein (EGFP), in which the EGFP was covalently linked at the N terminus of the KCNQ2 subunit by means of a 7 glutamine (Q) residues spacer to increase protein flexibility at the junction site (Soldovieri et al., manuscript in preparation).

Heterologous expression of wild-type and mutant KCNQ subunits

Channel subunits were expressed in Chinese hamster ovary (CHO) cells by transient transfection. CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), non-essential amino acids (0.1 mM), penicillin (50 U ml^{-1}) and streptomycin (50 μg ml^{-1}) in a humidified atmosphere at 37°C with 5% CO_2 in 100-mm plastic Petri dishes. For electrophysiological experiments, the cells were seeded on glass coverslips (Carolina Biological Supply Company, Burlington, NC, USA) and transfected on the next day with the appropriate cDNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, always using a total amount of 4 μg of cDNA. When needed, a

plasmid encoding for enhanced green fluorescent protein (EGFP) (Clontech, Palo Alto, CA, USA) was used as transfection marker. All the experiments were performed 1–2 days after transfection.

Electrophysiology

Currents from CHO cells were recorded at room temperature (20–22°C) using a commercially available amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA) and the whole-cell configuration of the patch-clamp technique, using glass micropipettes of 3–5 MΩ resistance. The extracellular solution contained (in millimolar): 138 NaCl, 2 $CaCl_2$, 5.4 KCl, 1 $MgCl_2$, 10 glucose and 10 HEPES, pH 7.4 with NaOH; the pipette (intracellular) solution contained (in millimolar): 140 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 5 Mg-ATP, 0.25 cAMP, pH 7.3–7.4 with KOH. The pCLAMP (version 6.0.4, Axon Instruments) software was used for data acquisition and analysis. Data are expressed as the mean±SEM. When necessary, statistical differences were evaluated with the Student's *t* test ($p < 0.05$).

Western blots

Twenty-four hours post-transfection, CHO cells were lysed, and KCNQ2 subunit expression was investigated by Western blotting on 6% SDS-PAGE gels using three different antibodies: one against an epitope in the KCNQ2 N-terminal region (sc-7793; Santa Cruz, CA, USA; 1:200 dilution), another against a C-terminal epitope in KCNQ2 (sc-7792; Santa Cruz, CA, USA; 1:200 dilution) and a third one against an epitope in the EGFP sequence (Clontech; 1:1,000 dilution), followed by chemiluminescence detection. The same blots were stripped and reprobed with an anti-α-tubulin antibody (Sigma, Milan, Italy; dilution 1:2,000) to check for equal protein loading. Images were captured and stored on a ChemiDoc station and analysed with the Quantity One analysis software (BioRad, Segrate, Italy).

Statistical analysis

Statistical comparisons between experimental groups were performed using the Student's *t* test. Differences were considered to be statistically significant when *p* values were less than 0.05.

Results

Molecular genetic analysis

Mutation analysis of the KCNQ2 and KCNQ3 genes was performed on the DNA of the index case of the family. The entire coding regions and the exon–intron junctions of KCNQ2 and KCNQ3 were analysed by direct sequencing. In this subject, KCNQ2 showed a heterozygous deletion of

10 bp and an insertion of a single adenine nucleotide in position 761 of the cDNA sequence (c.761_770del10insA) (Fig. 1b). This mutation generated a stop codon at the deletion/insertion site, thus producing a truncated N-terminal peptide of 194 residues. Twenty-seven base pairs downstream the stop codon, a second open reading frame is maintained, starting at the ATG corresponding to the methionine in position 208 of the wild-type KCNQ2 sequence. Bioinformatic analysis using the NetStart program (version 1.0) indicates a low score, 0.383, for this ATG, 0.5 being the minimal value required for an ATG to represent a probable translation start site. The presence of the mutation generates a loss of one *Hpa*II restriction enzyme site, which was used to follow the segregation of the mutation in the family together with the direct sequencing of the PCR product. Thus, the c.761_770del10insA mutation was found in the proband (IV-1), in her mother (III-5), in two affected maternal aunts (III-4 and III-7) and in the maternal grandmother (II-5). The grandmother's sister (II-6) and two of her four children (III-10 and III-11) are also carrying the same mutation.

Subsequent analysis of the coding region of KCNQ3 in the proband led to the identification of a heterozygous base change (c.2687A>G), leading to the p.N821S amino acid substitution. This KCNQ3 gene variant is inherited from the father (III-1) and is also present in his brother and sister (III-2 and III-3) and in the paternal grandfather (II-1), while it was not detected in 300 control chromosomes from healthy individuals. Thus, the proband is a double heterozygous for the insertion/deletion in KCNQ2 inherited from the mother and the single base substitution in KCNQ3 inherited from the father.

Biochemical analysis of wild-type and mutant
KCNQ2 subunits heterologously expressed
in CHO cells

Since the KCNQ2 mutation generated a stop codon at position 194 followed by an inframe ATG, the possibility existed that the KCNQ2 mutant allele generated in vivo two putative peptides corresponding to partial KCNQ2 sequences: the first corresponding to the N-terminal 194 amino acids and the second containing the last 665 amino acids of the C-terminus.

To investigate the biochemical consequences of this mutation and particularly whether the downstream C-terminal fragment was being synthesized in vivo, we performed Western blot experiments in CHO cells transfected with plasmids encoding for wild-type or mutant KCNQ2 subunits using three different antibodies. The first antibody recognized an epitope natively present in the N-terminus of KCNQ2 (anti-Q2N Ab). Using this antibody, wild-type KCNQ2 subunit expression was revealed by a single immunoreactive band of an approximate molecular weight (MW) of 95 kDa, as previously shown with other antibodies [15] (Fig. 2a, lane 2). By contrast, in cells transfected with the plasmid encoding for KCNQ2 subunits carrying the c.761_770del10insA mutation, a single immunoreactive

band of an approximate MW of 21 kDa was revealed (Fig. 2a, lane 3), which closely matched that expected for the N-terminal fragment of 194 residues. Interestingly, in cells transfected with the cDNA encoding for wild-type EGFP-KCNQ2 fusion proteins, this antibody revealed a single band of a MW of approximately 130 kDa, consistent with the MW of the EGFP-KCNQ2 fusion subunit. By contrast, in lysates from cells transfected with the EGFP-KCNQ2 plasmid carrying the c.761_770del10insA mutation, the antibody revealed a predominant band of about 55 kDa in size, presumably corresponding to the EGFP covalently linked to the 194 amino acid N-terminal fragment generated by the mutation found in our family. Similar results were also observed when a monoclonal antibody directed against EGFP was used (Fig. 2b). This antibody failed to recognize any band in cells transfected with non-EGFP fusion constructs (lanes 2 and 3), whereas it recognized a single band of approximately 130 kDa in wild-type EGFP-KCNQ2-transfected cells (lane 4) and a prominent band of about 55 kDa in size in cells transfected with the EGFP-KCNQ2 plasmid carrying the c.761_770del10insA mutation (lane 5).

Interestingly, no band was observed in cells transfected with plasmids encoding for the c.761_770del10insA mutation, either in the non-fusion protein (Fig. 2c; lane 3) or in EGFP-KCNQ2 fusion constructs (Fig. 2c; lane 5), when an antibody recognizing an epitope natively present in the C-terminus of KCNQ2 (anti-Q2C Ab) was used; by contrast, the usual 95 or 130 kDa bands were clearly detected in KCNQ2- (Fig. 2c; lane 2) or EGFP-KCNQ2-transfected cells (Fig. 2c; lane 4), respectively.

Electrophysiological properties of wild-type
and mutant subunits

Heterologous expression of wild-type KCNQ2 subunits led to the appearance of voltage-dependent K^+-selective currents characterized by a rather slow time-course of activation and deactivation and a threshold for current activation of around -50 mV (Figs. 3a and b). Interestingly, macroscopic K^+ currents formed by EGFP-KCNQ2 subunits were indistinguishable in size and functional properties from those formed by non-fusion KCNQ2 subunits. In fact, the density of the macroscopic K^+ currents recorded at +20 mV in EGFP-KCNQ2-transfected cells was 36.4 ± 6.3 pA/pF ($n=30$), whereas that of KCNQ2-transfected cells was 51.5 ± 7.1 pA/pF ($n=38$; $p>0.05$). In addition, the gating properties of homomeric channels formed by KCNQ2 or EGFP-KCNQ2 subunits were indistinguishable (Soldovieri et al., manuscript in preparation).

By contrast, the density of the macroscopic K^+ currents recorded at +20 mV in cells transfected with the plasmids encoding for KCNQ2 subunits carrying the c.761_770del10insA mutation (1.1 ± 0.2 pA/pF; $n=11$) was indistinguishable from that found in cells transfected with EGFP alone (0.6 ± 0.1 pA/pF; $n=7$) (Fig. 2b), suggesting that mutant subunits failed to give rise to measurable currents. Similar results were also observed in cells expressing EGFP-

a Anti-Q2N Ab

b Anti-EGFP Ab

c Anti-Q2C Ab

1. CHO
2. Q2
3. Q2 c.761_770del10insA
4. EGFP-Q2
5. EGFP-Q2 c.761_770del10insA
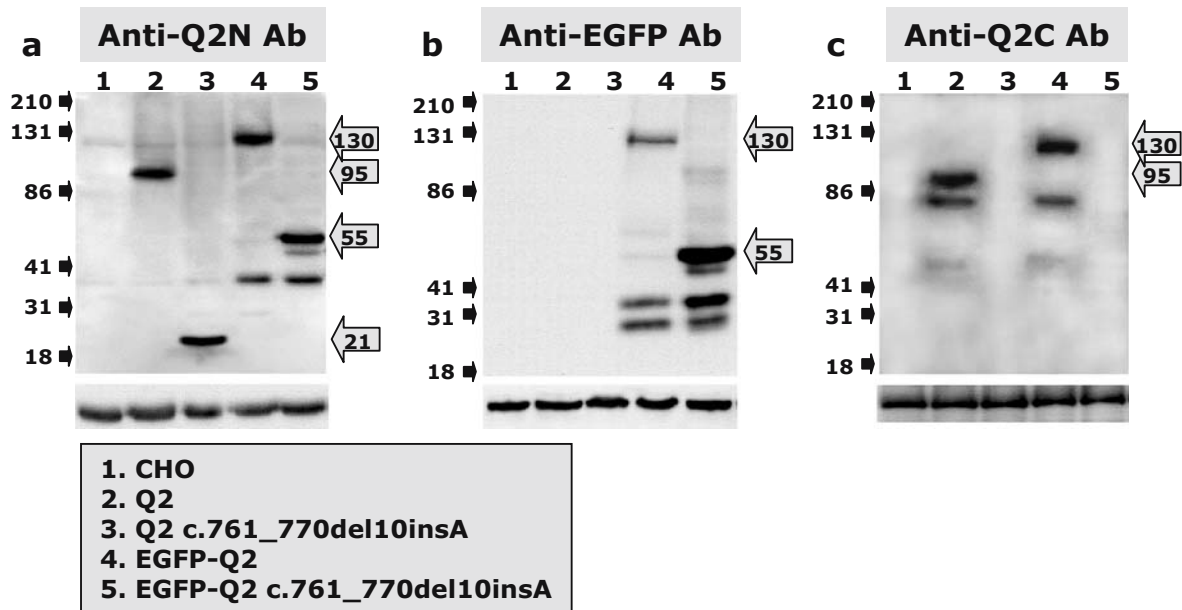
Fig. 2 Biochemical analysis of wild-type and mutant KCNQ2 subunits heterologously expressed in CHO cells. Western blot analysis on total cell lysates from control CHO cells (*lanes 1*) or from cells transfected with the plasmids encoding for KCNQ2 (*lanes 2*), KCNQ2 c.761_770del10insA (*lanes 3*), EGFP-KCNQ2 (*lanes 4*) or EGFP-KCNQ2 c.761_770del10insA (*lanes 5*). Protein expression in the blots of the *upper part* of the figure was revealed by the following primary antibodies: **a** a polyclonal antibody recognizing an epitope natively present in the N-terminus of KCNQ2 (anti-Q2N Ab), **b** a monoclonal antibody directed against EGFP (anti-EGFP) and **c** a polyclonal antibody recognizing an epitope natively present in the C-terminus of KCNQ2 (anti-Q2C Ab). The *lower blots* in each panel show the expression, on the same filters, of α-tubulin, used as an internal standard for equal protein loading. In the *upper blots* of each panel, the *arrows on the right* indicate the MW of the major bands revealed: the band of an approximate MW of 130 kDa corresponds to full-length EGFP-KCNQ2 subunits, the band of an approximate MW of 95 kDa corresponds to full-length KCNQ2 subunits, the band of approximate MW of 55 kDa corresponds to EGFP-KCNQ2 subunits truncated by the c.761_770del10insA mutation, the band of an approximate MW of 21 kDa corresponds to KCNQ2 subunits truncated by the c.761_770del10insA mutation. On the *left* of each panel, the positions and MW of known proteins used as standards is shown. In *lanes 4* and *5* of both panels **a** and **b**, other bands in the 30–40 kDa MW range were evident; these bands are likely to represent various forms of EGFP or EGFP-KCNQ2 fusion proteins. An additional band of approximately 80 kDa in *lanes 2* and *4* of panel **c** was consistently observed; the nature of this band is unknown at the moment

KCNQ2 c.761_770del10insA mutant subunits (the K^+ current density at +20 mV in these cells was 0.7 ± 0.2 pA/ pF; $n=6$). Interestingly, transfection with plasmids encoding for c.761_770del10insA mutant KCNQ2 subunits did not affect the current density recorded from wild-type KCNQ2-transfected cells (Fig. 3b).

In CHO cells, expression of KCNQ3 subunits in homomeric configuration generates functional voltage-dependent K^+ currents, although the current density recorded from KCNQ3-transfected cells is lower than that measured in cells expressing KCNQ2 subunits homomerically (Figs. 3a and b). K^+ current density generated upon CHO cells transfection with KCNQ3 p.N821S subunits was identical to that found in KCNQ3-transfected cells (Figs. 3a and b). Interestingly, the kinetics and the voltage-dependence of these mutant KCNQ3 currents also were indistinguishable from those carried by homomeric wild-type KCNQ3 channels (data not shown). Furthermore, mutant KCNQ2 subunits failed to affect homomeric channels composed of wild-type or mutant KCNQ3 subunits (Fig. 3b).

Heteromeric assembly KCNQ2 and KCNQ3 subunits seems to represent the main molecular substrate of the M-current [7]. Co-expression of KCNQ3 with KCNQ2 subunits generates macroscopic currents much larger than those expected from simple summation of homomeric KCNQ3 or KCNQ2 currents (Fig. 3c). K^+ current densities recorded in KCNQ3 p.N821S plus KCNQ2 transfected cells were similar to those observed upon KCNQ3 plus KCNQ2 subunits co-expression (Fig. 3c), suggesting that mutant KCNQ3 subunits were fully able to substitute for wild-type KCNQ3 subunits in forming heteromeric channels with KCNQ2 subunits. To verify this hypothesis, we compared the TEA sensitivity of the currents recorded from cells co-expressing KCNQ3 p.N821S and KCNQ2 subunits with that of cells co-expressing wild-type KCNQ3 and KCNQ2 subunits, taking advantage of the tenfold lower sensitivity to pharmacological blockade by TEA of heteromeric channels when compared to homomeric KCNQ2 channels. As shown in Fig. 3d, the percentage of current blockade by 0.3 and 3 mM TEA was identical between cells expressing KCNQ2 plus KCNQ3 and KCNQ2 plus KCNQ3 p.N821S subunits, confirming the ability of mutant KCNQ3 subunits to form heteromultimers with wild-type KCNQ2 subunits. Furthermore, expression of KCNQ2 subunits carrying the c.761_770del10insA mutation failed to modify TEA sensitivity of homomeric channels composed of wild-type KCNQ2 subunits (Fig. 3d).
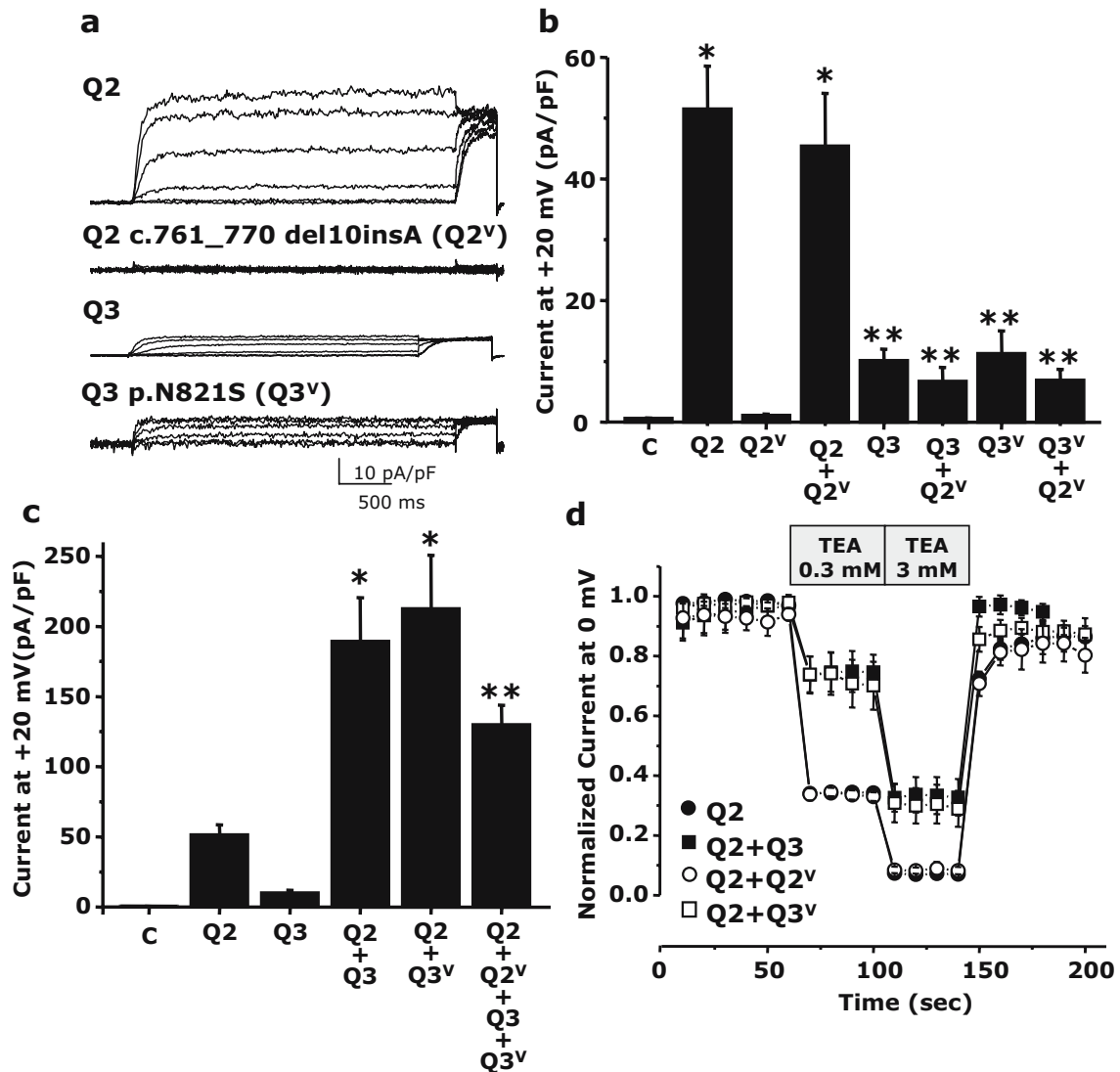
Fig. 3 Electrophysiological properties of wild-type and variant KCNQ2 and KCNQ3 subunits heterologously expressed in CHO cells. **a** Representative patch-clamp recordings from CHO cells transfected with the plasmids encoding for KCNQ2 (*Q2*), KCNQ2 c.761_770del10insA (*$Q2^V$*), KCNQ3 (*Q3*) or KCNQ3 p.N821S (*$Q3^V$*), as indicated. Holding potential: −80 mV; step potentials from −80 to +20 mV, in 20-mV steps, followed by a pulse to a constant value of 0 mV of 375–600 ms. **b**, **c** Quantification of the current densities recorded from the experimental groups indicated at the *bottom of the panels*. Current densities were calculated by dividing the peak current at the end of the +20-mV pulse by the membrane capacitance in each recorded cell. Each *bar* is the mean±SEM of 11–32 cells recorded from at least two different transfections. In panel **b**, * indicates values statistically different from control (*C*) values but not between each other; ** indicates values statistically different from control (*C*) and from *Q2* and *$Q2+Q2^V$* values, but not among each other. In panel **c**, * indicates values statistically different from *Q2* and *Q3* values, but not between each other; ** indicates a value that is statistically different from control (*C*) and from *Q2* and *Q3* values, as well as from that of the *Q2+Q3* group. **d** Time-course of the effects of the indicated concentrations of TEA on the currents recorded from cells transfected with the plasmids encoding for KCNQ2 (*filled circles*), KCNQ2 plus KCNQ2 c.761_770del10insA (*empty circles*), KCNQ2 plus KCNQ3 (*filled squares*) and KCNQ2 plus KCNQ3 p.N821S (*empty squares*). K^+ currents in these cells were activated with voltage pulses at 0 mV, each lasting 2 s, delivered at a frequency of 0.1 Hz; solution changes were performed via a computer-controlled fast-perfusion apparatus. For each cell, the currents measured at the end of the 0-mV pulse were normalized to the maximal value; the results from four cells per experimental group were averaged and expressed as a function of time. The *boxes on top of the panel* correspond to the duration of the TEA exposures

Finally, the simultaneous expression of all four cDNAs encoding for each subunit type (Q2, $Q2^V$, Q3 and $Q3^V$) in a 0.5:0.5:0.5:0.5 ratio caused a decrease in the K^+ current density of approximately 25% when compared to that recorded in cells expressing wild-type KCNQ2 and KCNQ3 subunits in a 1:1 ratio (Fig. 3c).

Discussion

Clinical and genetic data from a large BFNC family with nine affected members are reported in the present study. Eight of the affected members exhibit typical BFNC, with seizure occurrence on day 3 and remission before 4–5

months of age. During this period of time, seizure frequency was quite variable among different members of the family. Nevertheless, direct comparison among these individuals cannot be made because in some cases, PB therapy was started early (at the time the first seizure occurred, as in subjects III-10 and III-11), while in others, no AED therapy was administered (subjects IV-1, III-7, II-4, II-5, II-6).

In one BFNC-affected member of the family (individual III-4), who did not undergo early AED treatment, a severe type of epilepsy (West Syndrome) occurred at 6 months of age, and a mild mental retardation and neurological problems relapsed in adult life, consistent with the fact that 10–15% of BFNC individuals experience convulsive manifestations later in life [2]. At this stage, it is hard to establish whether the different outcome of these patients is due to a partly different genetic background or whether it is a consequence of the severe type of epilepsy that occurred early in life.

As a matter of fact, all family members suffering from BFNC carry an identical novel deletion/insertion mutation in the KCNQ2 gene (c.761_770del10insA). This is in agreement with what has already been reported in the literature [6, 16, 17], where several pedigrees show a wide range of severity in the clinical phenotype apparently associated to the same KCNQ2 mutation without excluding the possible involvement of other environmental or genetic factors.

KCNQ2 subunits display a putative secondary structure with six transmembrane domains (from S_1 to S_6); therefore, this mutation led to KCNQ2 truncation at position 194, immediately before the S_4 domain, the main structural element for voltage sensing [18]. When heterologously expressed in mammalian cells by transient transfection procedures, KCNQ2 subunits carrying the c.761_770del10insA mutation were synthesized in significant amount, as revealed by the ability of antibodies directed against native or artificially engineered N-terminal epitopes to detect truncated fragments of the KCNQ2 subunit of appropriate molecular sizes in Western blot experiments. However, electrophysiological experiments carried out in the same cells revealed that mutant KCNQ2 subunits were unable to form functional homomeric K^+ channels. This result is consistent with the notion that the mutation generated truncated KCNQ2 subunits lacking most of the voltage-dependent gating determinants (the S_4 segment in particular), as well as the S_5–S_6 region, where the molecular determinants for K^+ selectivity are located [19]. We also checked the possibility that, in addition to the 194-amino-acid N-terminal fragment, the KCNQ2 mutant allele might have generated a 665-amino-acid C-terminal fragment encompassing a large part of the KCNQ2 subunit, including the critical regions needed for regulation by protein kinase C, A-kinase anchoring proteins [20] and calmodulin [21, 22]. In fact, in other ion channels including K^+ channels, it has been reported that splitting the subunits and expressing the two domains separately or in isolation may generate ion channels with novel functional properties

[23]. However, Western blot experiments performed using antibodies targeting an epitope in the C-terminal region of KCNQ2 subunits failed to detect significant amounts of C-terminal fragments in cells transfected with the mutant KCNQ2, arguing against the possibility that this C-terminal fragment might have been responsible for the mutation-induced suppression of the K^+ channel function revealed by the present electrophysiological experiments. Furthermore, mutant KCNQ2 subunits did not exert dominant-negative effects since they failed to interfere with the ability of wild-type KCNQ2 or KCNQ3 subunits to form functional channels; as a matter of fact, truncated subunits generated by the mutation lacked the C-terminal subunit interaction domain (or *sid*) required for homomeric and heteromeric subunit assembly [24, 25]. This result also supports the notion that the KCNQ2 C-terminal fragment encompassing the *sid* domain, which might have prompted dominant-negative effects, was not synthesized upon transfection with KCNQ2 c.761_770del10insA subunits.

Interestingly, in addition to the described KCNQ2 mutation, in the family of the proposita's father, the p.N821S amino acid substitution was detected. N821 is not particularly conserved among the other members of the KCNQ potassium channel family, and the substituting serine residue is present in the rat, mouse and bos taurus orthologs of KCNQ3. When heterologously expressed in CHO cells, homomeric potassium channels formed by KCNQ3 subunits carrying the p.N821S replacement were indistinguishable from those formed by wild-type KCNQ3 subunits. Furthermore, the p.N821S variant did not interfere with the ability of KCNQ3 subunits to form heteromeric channels with KCNQ2 subunits. These results suggested that the p.N821S amino acid change in KCNQ3 was not responsible for BFNC in the family here investigated. Indeed the KCNQ3 c.2687A>G substitution did not segregate with the BFNC phenotype. Furthermore, the observation that the c.2687A>G nucleotide change was not found in 300 alleles from control healthy individuals suggests that this gene variant is likely to represent a rare KCNQ3 gene polymorphism. Indeed the presence of KCNQ3 variant was observed in four subjects within the paternal lineage, two of them showing migraine with typical aura and one of them associated also to one event of febrile convulsion. In spite of the fact that functional data obtained do not support a clear role for KCNQ3 variant in the migraine phenotype here observed, we cannot exclude that this variant, maybe in association with some other genetic factor, could have some role in the pathogenesis of these paroxysms.

Finally, we attempted to mimic the double heterozygous condition of the proband by simultaneously co-expressing in our heterologous system all four cDNAs reproducing each of the two alleles present at the KCNQ2 and KCNQ3 loci of the proband at a 0.5:0.5:0.5:0.5 ratio. The fact that the K^+ current density in these cells was reduced by approximately 25% when compared to cells expressing only the two wild-type KCNQ2 and KNCQ3 subunits at a 1:1 ratio argues against possible dominant-negative effects

caused by variant alleles and is in line with the hypothesis that a rather small I_{KM} deficit is sufficient to cause BFNC [3–5].

Collectively, the present results suggest that, in the family investigated, the KCNQ2 mutation segregates with the BFNC phenotype and is likely to be responsible for the BFNC phenotype. Haplo-insufficiency caused by the mutant KCNQ2 allele, rather than dominant-negative effects, seems to represent the primary mechanism for I_{KM} deficit and BFNC pathogenesis in the affected family. On the other hand, the KCNQ3 gene variant is functionally silent, a result compatible with its lack of segregation with BFNC.

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