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KCNQ2 and *KCNQ3* potassium channel genes in benign familial neonatal convulsions: expansion of the functional and mutation spectrum

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Summary

Benign familial neonatal convulsions (BFNC) is a rare autosomal dominant generalized epilepsy of the newborn infant. Seizures occur repeatedly in the first days of life and remit by approximately 4 months of age. Previously our laboratory cloned two novel potassium channel genes, *KCNQ2* and *KCNQ3*, and showed that they are mutated in patients with BFNC. In this report, we characterize the breakpoints of a previously reported interstitial deletion in the *KCNQ2* gene and show that only *KCNQ2* is deleted. We identify 11 novel mutations in *KCNQ2* and one novel mutation in the *KCNQ3* potassium channel genes. In one family, the phenotype extends beyond neonatal seizures and includes rolandic seizures, and a subset of families has onset of seizures in infancy. In the *Xenopus* oocyte expression system, we characterize five KCNQ2 and one KCNQ3 disease-causing mutations. These mutations cause a variable loss of function, and selective effects on the biophysical properties of KCNQ2/KCNQ3 heteromultimeric channels. We report here the first dominant negative mutation in *KCNQ2* that has a phenotype of neonatal seizures without permanent clinical CNS impairment.

Keywords: neonatal epilepsy; voltage-gated potassium channel; KCNQ2; KCNQ3; generalized seizures

Abbreviations: BFIC = benign familial infantile convulsions; BFNC = benign familial neonatal convulsions; CHRNA4 = α4 subunit of the nicotinic cholinergic receptor; FISH = fluorescence *in situ* hybridization; KCNQ2 = potassium channel subfamily Q member 2; KCNQ3 = potassium channel subfamily Q member 3; PCR = polymerase chain reaction; SNPs = single nucleotide polymorphisms; WT = wild-type.

Introduction

Benign familial neonatal convulsions (BFNC) (MIM 121200) is an autosomal dominantly inherited epilepsy disorder. Seizures usually begin in the first few days of life, and remit spontaneously at ~3–4 months of age (Ronen *et al*., 1993; Plouin, 1997). Although the spontaneous remission of seizures usually appears to be complete, ~16% of individuals with BFNC will experience one or more seizures later in life, often provoked by sudden unexpected stress (Ronen *et al*., 1993). This rate is much higher than the 1–2% seen in the general population. Genetic linkage studies have mapped two disease loci for BFNC, EBN1 on chromosome 20q and EBN2 on chromosome 8q (Leppert *et al*., 1989; Lewis *et al*., 1993). Characterization of an interstitial deletion on chromosome 20q in one family led us to clone the novel *KCNQ2* potassium channel gene and identify five additional mutations in families with BFNC (Singh *et al*., 1998). Using sequence databases and cDNA amplification, we cloned a homologous gene, *KCNQ3*, and discovered a single mutation in the only

known family that shows genetic linkage to chromosome 8q (Charlier *et al*., 1998). Many other laboratories have published findings that support the role of *KCNQ2* and *KCNQ3* in BFNC (Biervert *et al*., 1998; Lerche *et al*., 1999, 2001; Hirose *et al*., 2000; Lee *et al*., 2000; Miraglia del Giudice *et al*., 2000; Dedek *et al*., 2001; Moulard *et al*., 2001). From all studies to date, two families with *KCNQ2* mutations have clinical findings that are not specific to the neonatal period. In one BFNC family with a *KCNQ2* C-terminus mutation, the phenotype included benign familial infantile convulsions (BFIC), a seizure disorder which occurs immediately following the neonatal onset and remission period (Singh *et al*., 1998). Furthermore, Dedek *et al*. (2001) have demonstrated an S4 transmembrane domain *KCNQ2* mutation in a family with neonatal epilepsy and myokymia. Such findings indicate that *KCNQ2* has a significant role in both CNS and PNS function.

KCNQ2 and *KCNQ3* belong to a subfamily of potassium channels genes that have been implicated in other diseases. *KCNQ1* is mutated in the Romano Ward long QT syndrome of prolonged cardiac repolarization and the Jervell and Lange-Nielsen syndrome characterized by long QT and deafness (Neyroud *et al*., 1997; Sanguinetti, 1999; Splawski *et al*., 2000). *KCNQ4* is mutated in an autosomal dominant nonsyndromic hearing impairment (Coucke *et al*., 1999; Kubisch *et al*., 1999; Talebizadeh *et al*., 1999; Van Hauwe *et al*., 1999; Akita *et al*., 2001). The last member of this gene family, *KCNQ5*, which is widely expressed in brain and skeletal muscle, remains to be implicated in a disease (Kananura *et al*., 2000; Lerche *et al*., 2000; Schroeder *et al*., 2000).

The identification of mutations in the homologous *KCNQ2* and *KCNQ3* potassium channel genes in a single disorder support the hypothesis that these two potassium channels may make up a single functional entity. Consequently, co-expression of *KCNQ2* and *KCNQ3* forms a functional heteromeric potassium channel that yields currents that are 11-fold greater than either homomeric channel (Yang *et al*., 1998). Further evidence of co-localization and concerted function comes from immunohistochemical evidence that shows KCNQ2 and KCNQ3 co-immunoprecipitated from human brain lysates (Cooper *et al*., 2000). Importantly, Wang *et al*. (1998) demonstrated that the M-current, a tonic inhibitory potassium current, is made up of the KCNQ2 and KCNQ3 proteins (Brown and Adams, 1980). The M-current has a major role in controlling excitability because it regulates the ability of a neuron to fire an action potential (Marrion, 1997; McKinnon, 2000). Pharmacological reduction of the M-current results in the excessive firing of action potentials typical of an epileptic seizure (Brown and Adams, 1980).

Expression of a few of the mutations identified to date suggests that a partial loss of function in potassium current is sufficient to produce an epilepsy phenotype, and dominant negative mutations in either *KCNQ2* or *KCNQ3* may lead to a more severe phenotype (Jentsch, 2000). This is supported by the KCNQ2 homozygous knockout mouse, where animals die immediately after birth (Watanabe *et al*., 2000). The discovery of new BFNC mutations that alter the M-current by various and possibly novel mechanisms would lead to a greater understanding of the regulation of excitability in the mammalian nervous system. To this end, we have expanded the mutation and phenotypic spectrum of the *KCNQ2* and *KCNQ3* potassium channels in a large panel of BFNC cases and determined the functional consequences of a subset of these mutations in the *Xenopus* oocyte expression system.

Subjects and methods

Ascertainment of individuals with BFNC

All BFNC cases were seen or questioned by local neurologists and report a family history of seizures occurring in the first days of life. A total of 30 unrelated families or probands were recruited into our study. All participants or guardians signed University of Utah Institutional Review Board approved informed consent forms. DNA was isolated from peripheral blood using the Purgene isolation kit (Gentra).

Detection of large intragenic deletion in K1547

In the chromosome 20q deleted region established by fluorescence *in situ* hybridization (FISH) (Singh *et al*., 1998), individuals in K1547 were genotyped for polymorphic microsatellites using standard techniques and sequenced for single nucleotide polymorphisms identified from dbSNP. Using standard techniques of Southern blotting, probes flanking the region containing mis-inherited single nucleotide polymorphisms (SNPs) were used to detect band-shifts in genomic DNA cut with the following restriction enzymes: *Bgl*II, *Kpn*I, *Xba*I, *Bam*HI, *Hind*III and *Xmn*I. Long polymerase chain reaction (PCR) was performed using Expand PCR (Roche) on DNA from a K1547 patient using the primer sequences: forward, CAAACCTTGGCAAAGG-TGCCAC; and reverse, GCTGGCCCAGAGTCCTGAGTG. Long PCR did not amplify the wild-type fragment of ~50 kb in unaffected individuals, but yielded a single PCR product of 4.6 kb in affected individuals. This PCR product was sequenced as described below.

Mutation detection in KCNQ2 and KCNQ3

DNA was amplified and purified as described previously (Singh *et al*., 1998). Sequencing was performed on an ABI 3700 according to the manufacturer's protocols (Perkin Elmer). Seventeen primer pairs were used to sequence *KCNQ2* and 15 primer pairs were used to sequence *KCNQ3*. For sequencing frameshift mutations, PCR products were first subjected to TA cloning according to the manufacturer's specifications (Invitrogen). For single-stranded conformational polymorphism (SSCP) on population control samples, PCR products were electrophoresed on 4–12%,

4–20% or 20% acrylamide TBE (Tris/borate/EDTA) gels at 4° or 25°C (Novex).

Construction of KCNQ2 and KCNQ3 mutations and in vitro *transcription of cRNA*

Four BFNC-associated mutations (Q323X, R333Q, R448X, 867ins) located in the C-terminal segment of *KCNQ2* and one mutation located in the S4 transmembrane domain (M208V) were chosen for electrophysiology analysis. In addition, we characterized one *KCNQ3* pore mutation (D305G) and a putative *KCNQ3* mutation (N468S). We cloned wild-type (WT) *KCNQ2* in the pKS+ expression vector, and WT *KCNQ3* cDNA in pTLN was kindly supplied by Dr T. Jentsch. Mutations were introduced into WT cDNAs by the megaprimer method (Sarkar and Sommer, 1990). *KCNQ2* cRNA for injection into oocytes was prepared with Capscribe (Roche) T3 polymerase following linearization of the plasmid with *Pvu*1. *KCNQ3* cDNA was linearized with *Hpa*1 and cRNA transcribed with SP6 polymerase. RNA quality was checked by gel electrophoresis and concentrations were quantified by the Ribogreen fluorescent assay (Molecular Probes, Eugene, Oregon, USA).

Isolation of oocytes and injection of RNA

Xenopus laevis frogs were anaesthetized by immersion in 0.2% tricaine for 10–15 min. Ovarian lobes were digested with 2 mg/ml Type 2A collagenase (Worthington, Lakewood, New Jersey, USA) in Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) for 1.5 h to remove follicle cells. Stage IV and V oocytes were injected with cRNA, then cultured in Barth's solution (88 mM NaCl, 1 mM KCl, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 2.4 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4) supplemented with 50 μg/ml gentamicin and 1 mM pyruvate at 18°C.

Control oocytes were injected with 5 ng of WT *KCNQ2* plus 5 ng of WT *KCNQ3* cRNA. Other oocytes from the same isolation were injected with 2.5 ng mutant *KCNQ2* plus 2.5 ng WT *KCNQ2* plus 5 ng WT *KCNQ3* cRNA to study the *KCNQ2* mutants. *KCNQ3* mutants were characterized by injecting oocytes with 2.5 ng mutant *KCNQ3* plus 2.5 ng WT *KCNQ3* plus 5 ng WT *KCNQ2* cRNA. Currents are referred to by the mutant subunit such that oocytes injected with D305G *KCNQ3* + WT *KCNQ3* + WT *KCNQ2* cRNA are labelled as 'D305G KCNQ3'. Currents were recorded from oocytes 2 days after injection with cRNA.

Voltage clamp and data analysis

Oocytes were bathed in a chloride-free [replaced with 2-(N-morpholino) ethane sulphonic acid (MES)] solution containing 96 mM NaMES, 2 mM KMES, 2 mM $CaMES_2$, 5 mM HEPES and 1 mM $MgCl_2$ that was adjusted to pH 7.6 with methane sulphonic acid. Currents were recorded at room temperature (21–23°C) using standard two-microelectrode voltage clamp techniques (Stuhmer, 1992). Glass microelectrodes were filled with 3M KCl and broken to obtain tip resistances of 0.5 – 1.0MΩ. Oocytes were voltage-clamped with a Geneclamp 500 amplifier (Axon Instruments, Foster City, California, USA). Voltage commands were generated using pCLAMP software (Axon Instruments), a personal computer and a TL-1 D/A interface (Axon Instruments). Currents were recorded in response to 2 s pulses applied every 12 s to potentials ranging from +30 to –70 mV from a holding potential of –80 mV. After each test pulse, deactivating ('tail') currents were recorded at a potential of –60 mV. Currents were measured at the end of each 2 s pulse to determine the current-voltage relationship for each cell. The tail currents were normalized to the largest current and plotted as a function of test potential to obtain the voltage dependence of current activation. Normalized tail current amplitude (I_n) was plotted versus test potential (V_t) and fitted to a Boltzmann function, $I_n = 1/(1 + \exp[(V_{1/2} - V_t)/k])$. $V_{1/2}$ isthe voltage at which the current is half-activated and k is the slope factor. The kinetics of current activation could not be measured accurately because of the confounding effects of large currents (external K^+ accumulation and reduced voltage control) on the apparent rate of current activation. Current deactivation is less affected by artefact, so we measured the time constant for decay of tail currents by fitting records with a single exponential function. Data analyses were performed using pCLAMP and ORIGIN (Northampton, Massachusetts, USA) software. Data are expressed as the mean ± SEM (n = number of oocytes). Differences between WT and mutant channel current–voltage relationships and time constants for deactivation were evaluated by two-way ANOVA (analysis of variance). A value of $P < 0.05$ was considered significant.

Results

In a large Swedish BFNC kindred 1547, an interstitial deletion previously identified by FISH was characterized to determine the exact location of the breakpoints. Two overlapping bac (bacterial artificial chromosome) clones, AL121827 and AL353658, which encompassed the *KCNQ2* gene were identified from the Human Genome Project Working Draft. Novel, informative microsatellites and SNPs within these two bac clones are considered to be inside the deleted region because they were hemizygous and misinherited between affected parent-child pairs in K1547. Using this strategy in addition to Southern blotting, we reduced the location of the deleted region to within ~5 kb on each side of the breakpoint. A pair of primers located in exon 8 and distal to the 3′UTR (untranslated region) amplified a 4.6 kb product that, when sequenced, identified the exact location of the breakpoint in K1547 (Fig. 1A–C). The deletion extends from intron 8 through the 3′UTR of *KCNQ2* and removes 48.7 kb close to the telomere of chromosome 20q. The gene directly

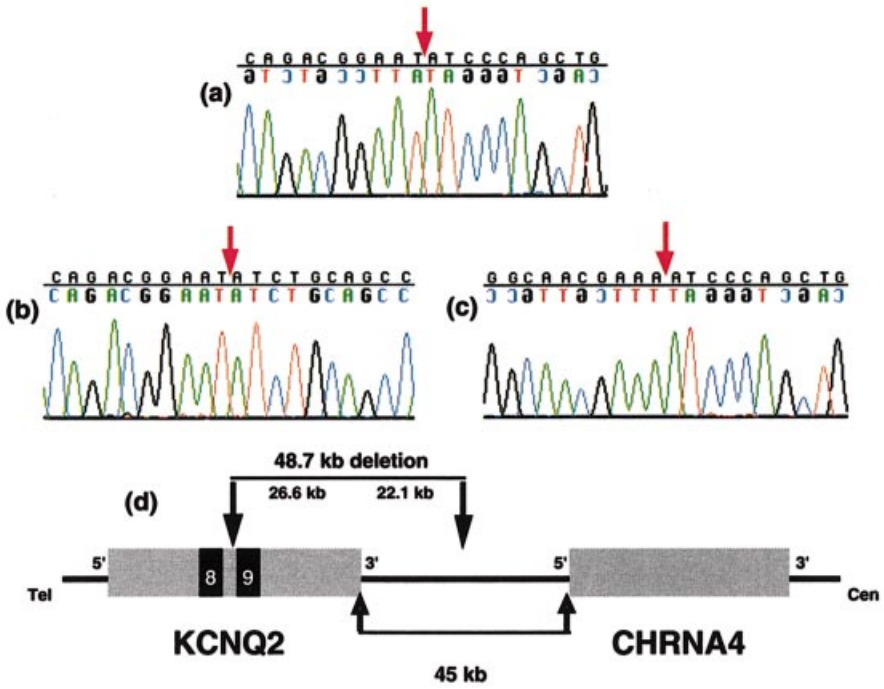


Fig. 1 Identification of the deletion breakpoints in BFNC kindred 1547. (**A**) Sequence of the deletion chromosome. (**B,C**) Sequence of the WT chromosomes. (**D**) Location of the 48.7 kb deletion relative to the adjacent nicotinic cholinergic receptor gene, *CHRNA4*. The deletion removes exons 9 through 17 of *KCNQ2* and 22.1 kb past the WT stop codon.

proximal to *KCNQ2* is the nicotinic cholinergic receptor *CHRNA4* previously shown to be mutated in autosomal dominant nocturnal frontal lobe epilepsy (Steinlein *et al*., 1995). The *CHRNA4* gene as well as 22 kB 5′ is still preserved in K1547 BFNC patients deleted for part of the *KCNQ2* gene (Fig. 1D).

Direct sequencing of *KCNQ2* and *KCNQ3* yielded novel mutations in 11 additional BFNC families not found in a panel of 70 control individuals. These include missense, nonsense and frameshift mutations in the S2, S4 and S5 transmembrane domains, the S4-S5 intracellular loop, the pore and the C-terminus of the *KCNQ2* gene (Table 1). In the *KCNQ3* gene, a single pore missense mutation was identified in a proband with a family history of neonatal seizures (Table 1). In the remaining 11 families with no mutations identified by sequencing, we failed to amplify the deletion product that was discovered in K1547. There were no significant phenotypic differences between the cases where disease-causing mutations were identified and cases where no *KCNQ2* or *KCNQ3* mutations were identified by sequencing.

The age of seizure onset listed in Table 1 is not corrected for the occurrence of premature births in BFNC individuals. Data for premature births were available for 10 out of 17 pedigrees with *KCNQ2* mutations. Of these, two premature births for K1705 and K1547, respectively, were noted. A K1705 individual was born 1 month prematurely and had convulsions beginning at 1 month of age. A K1547 individual was born 1 month prematurely and had convulsions beginning at 3–4 weeks of age. Surprisingly, phenotypic information, including age of onset and later seizure type obtained from all Utah study families with mutations in *KCNQ2* does not support any specific genotype-phenotype correlation (Table 1). The age of seizure remission has been a defining phenotypic criteria of the BFNC diagnosis. A genotype–phenotype correlation cannot be surmised from the age of seizure remission because these dates are confounded in many cases by the age at which antiepileptic drugs were stopped, with no further seizures. However, the age of seizure remission is well documented for K1705 and accurately represents the natural course of the BFNC disorder. In this family, the seizures in 31 affected individuals (68%) ceased before the sixth week of life. In the literature and in the cases cited here, 29 mutations have been identified in *KCNQ2*. All three mutations identified in *KCNQ3* are present in the pore region of the molecule (Fig. 2).

The functional consequences of coexpression of WT and seven mutant *KCNQ2* or *KCNQ3* subunits were evaluated in the *Xenopus* oocyte expression system in two separate experiments. In each case, the mutant channels were compared with WT channels in oocytes obtained from the same frog. Three *KCNQ2* variants (Q323X, R333Q and 867ins) were characterized in the first batch of oocytes along with the controls (Fig. 3A–D). Two *KCNQ2* variants (M208V and R448X), and two *KCNQ3* variants (N468S and D305G) were characterized in the second batch of oocytes along with the controls (Fig. 3E–H).

Table 1 *Genotype–phenotype*

Mutations[a]	Kindred (number assessed/number affected)	Clinician	Age of onset (median)	Number of individuals and later seizure phenotype (onset)
KCNQ2				
129+1 G to T	K4594 (3/7)	Thompson	1–5 days	None
M208V	K4629 (2/2)	Ronen	1–2 days	2/2 generalized (4–7 years)
H228Q	K2519[b] (6/6)	Kelly	1–6 days	None
L243F	K2670 (2/4)	Van Orman/Filloux	3–5 days	None
W269X	K5150 (7/7)	Prince	1–3 days, 8 months	2/7 febrile, generalized in adulthood
283insGT[i]	K1504[c] (19/19)	Quattlebaum	1 days– 3 months (3 days)	5/19 generalized seizures (21–45 years)
Y284C[i]	K3904 (2/2)	Murphy	1–6 days (1 day)	None
A306T[i]	K1705[d] (69/69)	Ronen/Rosales	1–30 days[k] (3 days)	11/69 febrile, generalized (1–16 years)
Q323X	K4443[e] (6/6)	Hattori	2–7 days	2/6 BECTS (2 years, 4 years)
R333Q	K4516 (2/2)	Novak	3–7 days	None
De1 382–3′ utr[i]	K1547[f] (11/11)	Bjerre	2–4 days, 1 month[k] (3 days)	4/11 febrile, generalized until 10 years
R448X	K1525[g] (6/6)	Zonana	2–9 days	None
522 de113[i]	K3369 (6/6)	McHarg/Schreiber	2–15 days, 3–5 months	1/6 generalized (2 years)
544–1 G to A[i]	K3933 (6/6)	Gagnon	2–7 days, 4 months (3 days)	None
R581X	K3450 (4/4)	Shevell	2–6 days	None
653de11	K-SR2 (1)	Ryan/Sigurdardottir	5 days	None
867insGGGCC	K3963 (12/12)	Lewis/Rioux	3–7 days	3/12 seizures continuous until age 2,3,7 years
KCNQ3				
D305G	K-SR3 (1)	Ryan	2 days	None
G310V[j]	K-SR1[h] (14/14)	Ryan	2–14 days	None

[a]Mutations in bold are new; [b]Crispen and Kelly, 1985; [c]Quattlebaum, 1979; [d]Ronen *et al*., 1993; [e]Maihara *et al*., 1999; [f]Bjerre and Corelius, 1968; [g]Zonana *et al*., 1984; [h]Ryan *et al*., 1991; [i]Singh *et al*., 1998; [j]Charlier *et al*., 1998; [k]Premature birth. BECTS = benign epilepsy with centrotemporal splices

Oocytes injected with 5 ng each of WT *KCNQ2* and *KCNQ3* cRNA expressed a robust delayed rectifier K^+ current with properties similar to M-current of neurons (Wang *et al*., 1998). Representative examples of currents recorded from oocytes expressing mutant KCNQ channel subunits are compared with WT channel currents in Fig. 3A and E. KCNQ2/KCNQ3 current activated with a bi-exponential time course to reach a near steady state magnitude within 2 s. Repolarization of the membrane potential to –60 mV elicited a deactivating (tail) current that decayed to baseline within 2 s. Oocytes injected with mutant plus WT KCNQ subunits had reduced current compared with WT channels, but had similar biophysical properties. The peak current was plotted as a function of test potential to obtain the current-voltage relationships shown in Fig. 3B and F. All mutant subunits, except N468S KCNQ3 and M208V KCNQ2, caused a reduction in KCNQ2/KCNQ3 current independent of test voltage.

With the exception of 867ins KCNQ2, the voltage dependence of current activation was similar for WT channels and all mutant channels (Fig. 3C and G). The $V_{1/2}$ for activation of 867ins KCNQ2 current was shifted by +3.8 mV (Fig. 3C). The rate of current deactivation can also affect the magnitude of K^+ current during repolarization of an action potential. The rate of deactivation was similar at potentials negative to –80 mV for all mutant channels, but was faster at less negative potentials for oocytes expressing the *KCNQ2* subunits R333Q or 867ins (Fig. 3D) or M208V KCNQ2 (Fig. 3H). The change in deactivation kinetics was not simply related to current magnitude, indicating that these mutant subunits accelerated the rate of closure of hetero-multimeric channels. M208V KCNQ2 had the greatest effect on channel deactivation, but only slightly reduced current magnitude (Fig. 3F). To accentuate the possible effects of M208V on current magnitude, we performed an additional experiment where oocytes were injected with 5 ng of WT KCNQ3 and 5 ng of M208V KCNQ2 cRNA, rather than equal amounts of WT and mutant *KCNQ2* cRNA as was done for the experiments presented in Fig. 3A–H. In the absence of WT *KCNQ2* subunits, M208V KCNQ2 subunits significantly reduced M-channel current function (Fig. 3I and J). In addition, the acceleration of current deactivation caused by M208V KCNQ2 was much more obvious (Fig. 3K) and biexponential at membrane potentials positive to –90 mV (Fig. 3L).

Since a *KCNQ2* mutation has previously been reported in a family with seizures beginning in the neonatal and infantile period, we screened eight probands with the typical BFIC phenotype. In a family of Chinese origin, a missense *KCNQ3* variant, N468S, was identified in all of the three siblings affected with BFIC. This amino acid change may be a rare polymorphism because it occurs in a region that is not conserved in this potassium channel gene subfamily. Because ethnically matched controls were unavailable, we examined this *KCNQ3* amino acid change for its effects on the heteromeric potassium current. N468S has no statistically significant effect on the current or biophysical properties of the heteromeric channel ($P < 0.074$) and is, therefore,

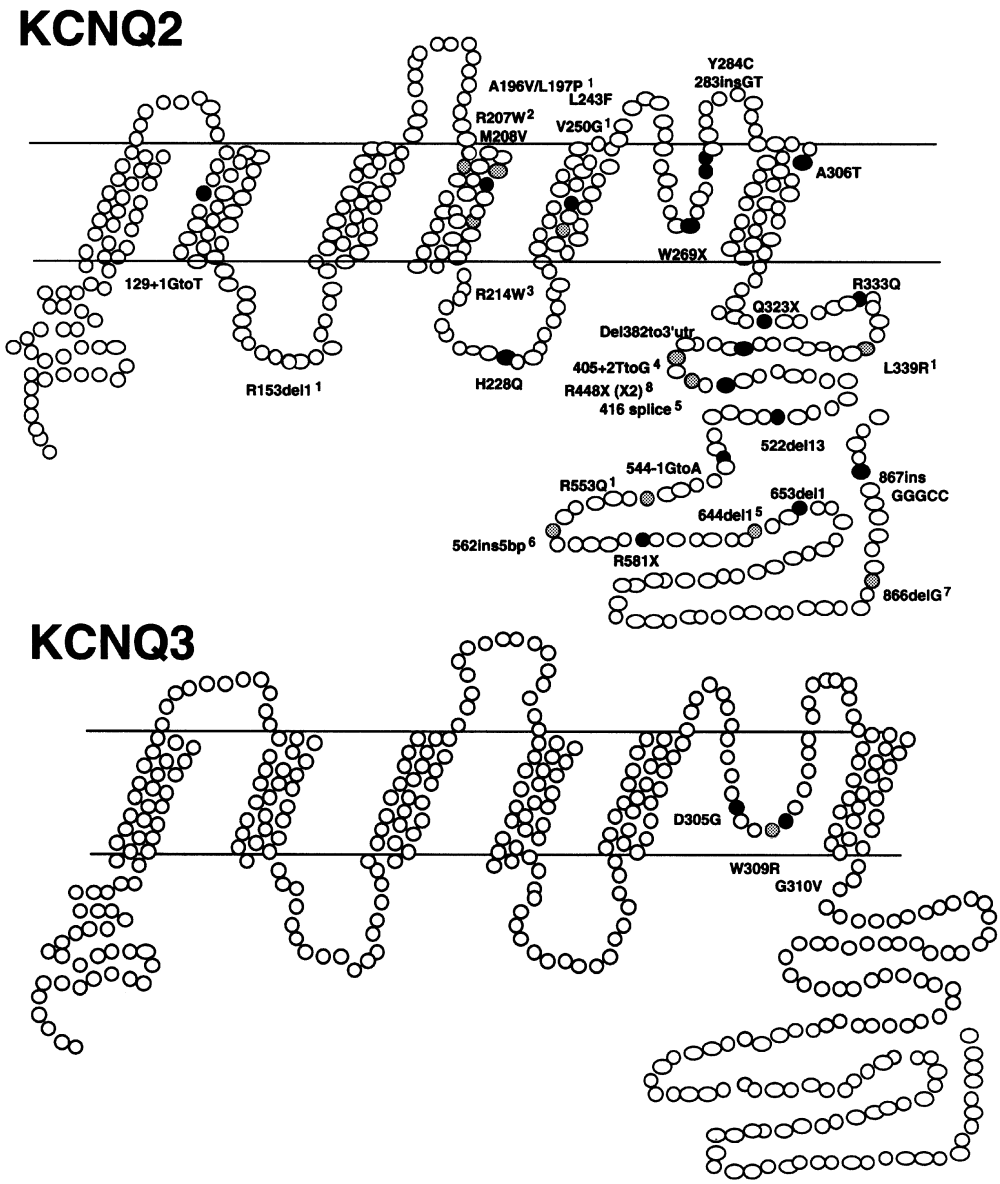


Fig. 2 Location of 29 mutations in the *KCNQ2* gene and three mutations in the *KCNQ3* gene from all studies. The predicted structure for *KCNQ2* and *KCNQ3* is six transmembrane domains interrupted by a pore region and intracellular N- and C-termini. Mutations are missense, splice site, insertions, deletions and nonsense for *KCNQ2*, and missense only for *KCNQ3*. [1]Moulard *et al*., 2001; [2]Dedek *et al*., 2001; [3]Miraglia del Giudice *et al*., 2000; [4]Lee *et al*., 2000; [5]Lerche *et al*., 2001; [6]Biervert *et al*., 1998; [7]Lerche *et al*., 1999; [8]Mutations observed in two presumably unrelated families [Moulard *et al*. (2001) and this study (K1525)]; *KCNQ3* W309R: Hirose *et al*., 2000. Black: mutations from two Utah studies; grey: mutations from other laboratories.

considered a polymorphic amino acid variation (Fig. 3F–H). The small size of the family precludes linkage analysis from yielding a significant result that implicates a disease locus.

The rank order for reduction of current recorded at a test potential of 0 mV for all the mutants is summarized in Fig. 4. All mutants except N468S KCNQ3 caused a statistically significant reduction in current, but only 867ins KCNQ2 caused a reduction of current that exceeded 50% (70% at 0 mV).

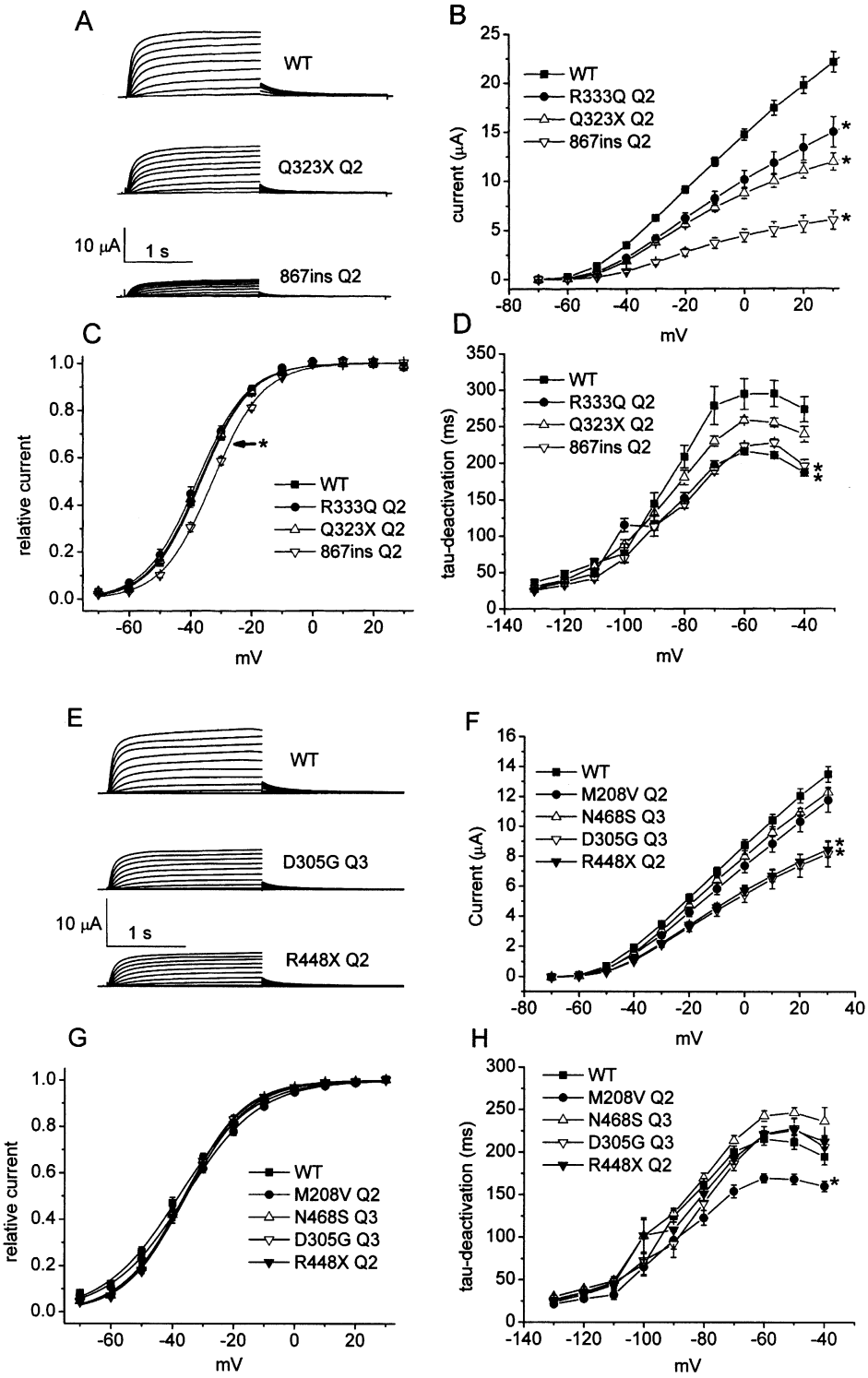
Discussion

Our present findings expand the spectrum of mutations to novel regions of the KCNQ2 protein. Eleven *KCNQ2* mutations reported in this study bring the total number of *KCNQ2* mutations in the Utah cohort to 17 out of a total 30 families and probands. Also from this sample set, a second *KCNQ3* mutation was identified in the pore, the only region of the *KCNQ3* gene in which mutations have been identified.

The specific breakpoints of a large deletion previously found in K1547 extend from intron 8 of *KCNQ2* through

22.1 kb past the *KCNQ2* stop codon, deleting the last nine exons of *KCNQ2 (*Singh *et al*., 1998). The likely disease-causing mechanism is haploinsufficiency of the KCNQ2 protein, because mRNA produced from the deleted *KCNQ2* allele lacks the poly A tail and is potentially degraded rapidly.

The failure to identify more mutations may result from the inability of the present methods to detect novel large interstitial deletions or duplications, inversions, mutations in regulatory regions of *KCNQ2* and *KCNQ3*, or possibly other genes. The recent report of mutations in the α2 subunit of the voltage gated sodium channel in two families with benign familial neonatal-infantile seizures supports this last hypothesis (Heron *et al*., 2002). In 11 probands where no mutations were identified in *KCNQ2* and *KCNQ3*, we



sequenced all 16 exons that encode the fifth homolog of the KCNQ gene family, *KCNQ5*. No mutations were identified in the coding region of *KCNQ5* for the BFNC probands (data not shown).

From this and other published studies, we know that 57% of mutations in *KCNQ2* are found in the C-terminus. Of note is the absence of mutations in the S1 and S3 transmembrane domains of both KCNQ2 and KCNQ3 proteins. This is also the case with KCNQ1 (KvLQT1), which is mutated in the Romano-Ward or Jervell and Lange-Nielsen syndromes (Neyroud *et al*., 1997; Splawski *et al*., 2000). Of the 85 different *KCNQ1* mutations found in 151 families, no mutations have been found in the S1 and S3 transmembrane domains (Splawski *et al*., 2000). Both *KCNQ1* and *KCNQ2* have disease-causing mutations in the S2/S3 and S4/S5 intracellular loops whereas mutations have not been identified in the extracellular domains of *KCNQ1*, *KCNQ2* or *KCNQ3*. The most common type of mutation in *KCNQ2* identified to date is a truncation of the C-terminus caused either by nonsense mutations or frameshift mutations. The C-terminal region is likely to be important in subunit co-assembly as indicated by the study of chimeric channels constructed by exchange of this region between *KCNQ1*, *KCNQ2* and *KCNQ3* subunits (Lerche *et al*., 2001; Schwake *et al*., 2003). Two domains of the *KCNQ2* C-terminal region have been shown to interact with calmodulin and loss of

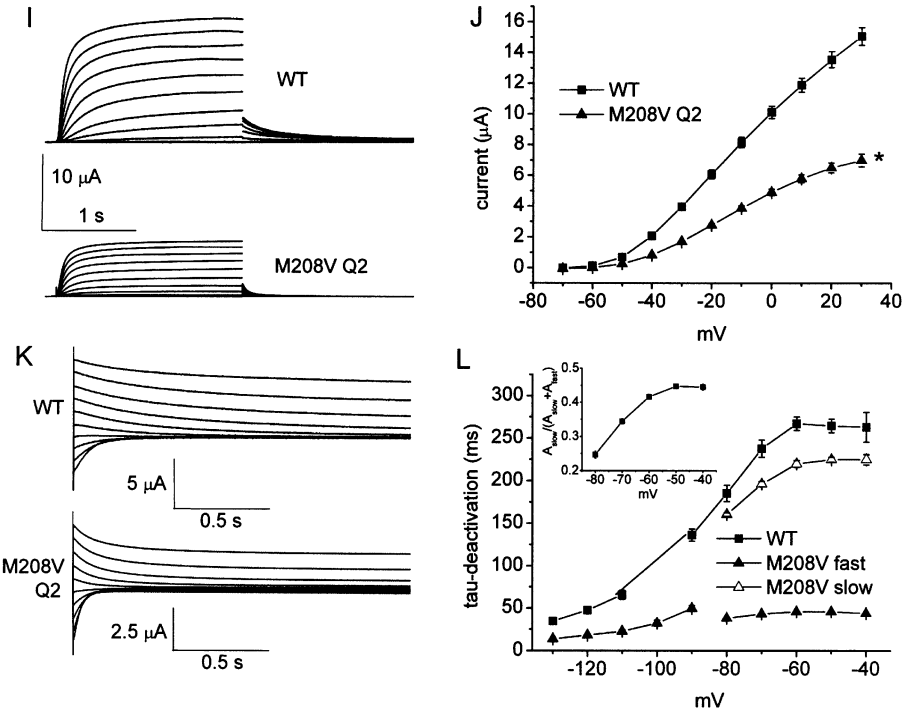


Fig. 3 Biophysical properties of WT and mutant KCNQ2/KCNQ3 channel currents. (**A**–**H**) Oocytes were injected with 2.5 ng mutant *KCNQ2* plus 2.5 ng WT *KCNQ2* plus 5 ng WT *KCNQ3* or 2.5 ng mutant *KCNQ3* plus 2.5 ng WT *KCNQ3* plus 5 ng WT *KCNQ2* cRNA. (**I**–**L**) Oocytes were injected with 5 ng M208V *KCNQ2* plus 5 ng WT *KCNQ3* cRNA. (**A**, **E**, **I**) Example of currents measured in response to test potentials ranging from +30 to –50 mV, applied in 10 mV increments. (**B**, **F**, **J**) Current voltage relationships for oocytes expressing WT or mutant channels (n = 10–15 for each group, $^*P < 0.001$). Voltage dependence of KCNQ2/KCNQ3 current activation determined by tail current analysis; smooth curves represent best fits of averaged data to a Boltzmann function (n = 10 for each group) in (**C**), WT ($V_{1/2}$ = –36.6 + 0.7 mV; k = 8.1 + 0.1 mV); R333Q ($V_{1/2}$ = –38.0 + 1.1 mV; k = 8.2 + 0.3 mV); Q323X ($V_{1/2}$ = –37.1 + 0.7 mV; k = 8.2 + 0.1 mV); 867ins ($V_{1/2}$ = –32.8 + 0.7 mV; k = 8.2 + 0.2 mV) and (**G**), WT ($V_{1/2}$ = –35.7 + 1.8 mV; k = 12 + 0.6 mV); N468S KCNQ3 ($V_{1/2}$ = –36.0 + 1.0 mV; k = 10.0 + 0.5 mV); D305G KCNQ3 ($V_{1/2}$ = –35.8 + 0.6 mV; k = 9.8 + 0.5 mV); M208V KCNQ2 ($V_{1/2}$ = –35.8 + 1.1 mV; k = 12.1 + 0.3 mV); R448X KCNQ2 ($V_{1/2}$ = –35.3 + 1.1 mV; k = 10.2 + 0.4 mV). Voltage dependence of deactivation time constants in (**D**) (n = 7 in each group, $^*P < 0.001$) and (**H**) (n = 5 in each group, $^*P < 0.01$). (**K**) Tail currents measured at 10 mV increments for potentials ranging from –130 mV to –40 mV after an activating pulse to +20 mV. (**L**) Voltage dependence of deactivation time constants (n = 5 each group). Inset in (**L**) is a plot of the relative amplitude of the slow component of current deactivation for potentials where deactivation was best fit with a two-exponential function.

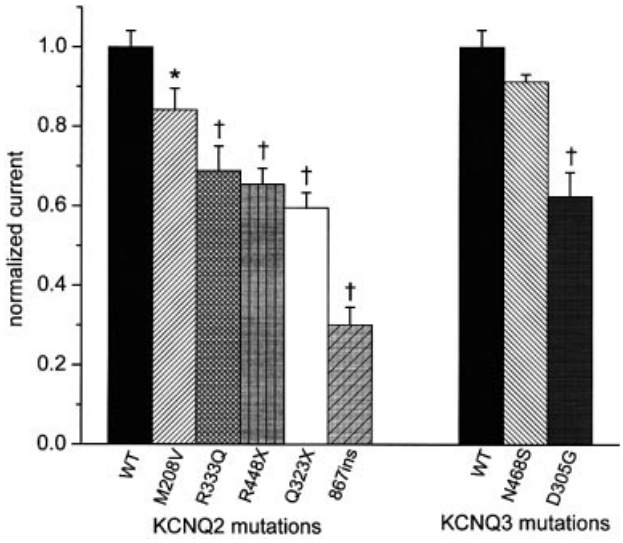


Fig. 4 Relative KCNQ2/KCNQ3 currents at a test potential of 0 mV. Current magnitude measured at the end of a 2 s pulse to 0 mV were normalized to WT channel currents recorded from the same batch of oocytes. * $P < 0.03$, † $P < 0.001$.

calmodulin binding reduces channel activity (Wen and Levitan, 2002; Yus-Najera *et al*., 2002). We identified only one mutation, R333Q, which resides in the first interaction site (amino acids 321–358).

Expression of the C-terminal missense mutant R333Q caused <50% reduction in current compared with WT heteromeric channels. The moderate effect of R333Q was similar to the Y284C pore and A306T S6 transmembrane missense mutations originally identified by our laboratory (Singh *et al*., 1998). The Y284C mutation reduced the maximum WT heteromeric potassium current by 20–30%, while the A306T mutation produces a 20–40% reduction in the current with no alterations in biophysical properties, i.e. kinetics of channel activation and deactivation (Schroeder *et al*., 1998). Interestingly, experiments that examine the surface expression of these BFNC mutants have shown that neither A306T nor Y284C significantly alters the membrane surface expression of the KCNQ2 protein (Schwake *et al*., 2000). R333Q appears to retain the ability to co-assemble with WT subunits to form functional channels *in vitro*, but may function less efficiently due to a decrease in the probability of opening or a reduced single channel conductance. In contrast, the S4 transmembrane domain missense mutation M208V causes a minor effect on maximal current, but clearly exhibits a faster rate of deactivation. Recently, Castaldo *et al*. (2002) examined the S4 transmembrane domain BFNC mutation R214W and observed slower opening and faster closing kinetics, and a decreased voltage sensitivity with no concomitant changes in maximal current or plasma membrane expression.

In an effort to understand the functional consequence of *KCNQ1* mutations that cause LQT syndrome, Seebohm *et al*. (2001) examined the disease-causing *KCNQ1* L273F mutation and also introduced the homologous mutation in *KCNQ2* at amino acid position 243 (Seebohm *et al*., 2001). This *KCNQ2* L243F experimental mutant, subsequently identified in our panel of BFNC families, exhibits fast inactivation—a striking difference from WT heteromeric channels that do not inactivate.

The *KCNQ2* C-terminal truncation mutants Q323X and R448X reduce current by <50%. Neither mutation causes any major changes in the biophysical properties, suggesting that the presence of a mutant subunit did not significantly alter the gating properties in a heteromultimeric channel. The previously published *KCNQ2* truncation mutant 562ins does not reach the membrane surface in oocytes and fails to facilitate the surface expression of *KCNQ3*, the effect seen reciprocally with WT KCNQ2 and KCNQ3 proteins (Schwake *et al*., 2000).

The C-terminus 5-bp insertion that occurs five amino acids before the WT stop codon (867insGGGCC) is predicted to produce a 99% normal KCNQ2 protein, with a new C-terminus that is 57 codons longer than the WT protein. In the *Xenopus* oocyte expression system, this mutation appears to have a dominant-negative effect on KCNQ2/KCNQ3 channel function as indicated by the >50% reduction in current magnitude. In addition, this mutation caused a slight positive shift in the voltage dependence of channel activation and an enhanced rate of deactivation, indicating altered gating of the heteromultimeric channel complex. A closely related mutation identified by Lerche *et al*. (1999), 866delG, did not cause a dominant negative effect possibly due to the examination of this mutant in homomeric KCNQ2 channels rather than heteromeric KCNQ2/KCNQ3 channels. Another dominant negative mutation described in the literature is R207W, which is found in the voltage-sensing pore region of *KCNQ2* (Dedek *et al*., 2001). Interestingly, individuals with R207W who have BFNC also experience the peripheral nerve disorder of myokymia, suggesting that dominant negative mutations would cause BFNC with concomitant neurological effects. Epilepsy is the only hyperexcitable neurological phenotype observed in eight individuals who carry the 867insGGGCC *KCNQ2* mutation.

The pore G310V missense mutation in the *KCNQ3* potassium channel gene caused a 20% reduction in the maximum heteromeric channel current, while having no effect on the surface expression of the protein (Charlier *et al*., 1998; Schroeder *et al*., 1998). A second *KCNQ3* pore mutation from our BFNC panel, D305G, reduced the maximal heteromeric current by ~40% with no alterations in voltage dependence of activation or deactivation kinetics. *KCNQ2* and *KCNQ3* mutations are likely to exert significant effects in a wide variety of *in vivo* systems, resulting, for example, from altered regulation by intracellular second messenger cascades. Clearly, the sequelae of events beginning with *KCNQ2* and *KCNQ3* mutations and resulting in the phenotype of BFNC is complex, and only one aspect is examined in the *Xenopus* oocyte expession system.

The 10-fold higher prevalence of *KCNQ2* disease-causing mutations compared with *KCNQ3* found over all studies suggests that *KCNQ3* may have a greater functional role. The *in vitro* study that demonstrates co-assembly of *KCNQ3*, not *KCNQ2*, with *KCNQ5* to produce the M-current supports this hypothesis (Lerche *et al*., 2000; Schroeder *et al*., 2000).

We have previously observed that *KCNQ2* mutations occur in both neonatal-onset seizures and infantile-onset seizures (beyond 4 months) in a single family K3369 (Table 1; Singh *et al*., 1998). In the present study, three families with *KCNQ2* mutations have a subset of cases with infantile-onset seizures. Importantly, at least one individual in every family with a *KCNQ2* or *KCNQ3* mutation has an onset of seizures during the first week of life—a hallmark of the BFNC disorder.

A variable number of individuals in eight out of 16 families with *KCNQ2* mutations have a later seizure phenotype beyond the 1–4 month age of seizures with BFNC. This supports the original observations of an increased risk of subsequent epilepsy in families with BFNC (Ronen *et al*., 1993; Berkovic *et al*., 1994). A single family in the study reported here (K4443) has neonatal seizures that remit at ~4 months followed, in two patients, by progression to rolandic seizures at ~4 years (Maihara *et al*., 1999). The rolandic seizures remit in adolescence with no impairment of mental function. Other families have febrile seizures, absence seizures and generalized seizures beginning as early as 1 year of age and show considerable intrafamilial variability in age and type of seizure. Interestingly, clinical reports of K1705 and K4629 clearly record that the later seizures are easily provoked by stressors such as auditory stimuli or emotional tension (Ronen *et al*., 1993). A propensity for adolescent, juvenile or adulthood seizures following an early childhood seizure disorder is also seen in cases of febrile seizures and generalized epilepsy with febrile seizures plus (Peiffer *et al*., 1999; Singh *et al*., 1999). From our present study, it appears that *KCNQ2* mutations with comparable effects on K^+ current have variable effects on the type and frequency of the later seizures seen in BFNC families.

In summary, we have expanded the naturally occurring mutation spectrum of *KCNQ2* to include the S2 transmembrane domain and the S4–S5 intracellular loop. Furthermore, this study supports our previous findings that the C-terminus is the region that contains the most mutations. From all studies, *KCNQ2* remains the predominant gene for BFNC because 29 mutations have been identified whereas three mutations have been identified in *KCNQ3*. Most BFNC mutations appear to cause loss of function and we document here the first dominant negative mutation in a pure epilepsy phenotype.

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