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Abstract

Objective

Recent reports have described single individuals with neurodevelopmental disability (NDD) harboring heterozygous *KCNQ3 de novo* variants (DNVs). We sought to assess whether pathogenic variants in *KCNQ3* cause NDD and to elucidate the associated phenotype and molecular mechanisms.

Methods

Patients with NDD and *KCNQ3* DNVs were identified through an international collaboration. Phenotypes were characterized by clinical assessment, review of charts and EEG recordings, and parental interview. Functional consequences of variants were analyzed *in vitro* by patch-clamp recording.

Results

Eleven patients were assessed. They had recurrent heterozygous DNVs in *KCNQ3* affecting residues R230 (R230C, R230H, R230S) and R227 (R227Q). All patients exhibited global developmental delay within the first two years of life. Most (8/11, 73%) were non-verbal or had a few words only. All patients had autistic features and autism spectrum disorder (ASD) was diagnosed in 5/11 (45%). EEGs performed before 10 years of age revealed frequent sleep-activated multifocal epileptiform discharges in 8/11 (73%). For 6/9 (67%) recorded between 1.5 and 6 years of age, spikes became near-continuous during sleep. Interestingly, most patients (9/11, 82%) did not have seizures and no patient had seizures in the neonatal period. Voltage-clamp recordings of the mutant KCNQ3 channels revealed gain-of-function (GoF) effects.

Interpretation

Specific GoF variants in *KCNQ3* cause NDD, ASD and abundant sleep-activated spikes. This new phenotype contrasts both with self-limited neonatal epilepsy due to *KCNQ3* partial loss-of-function, and with the neonatal or infantile-onset epileptic encephalopathies due to *KCNQ2* GoF.

Introduction

KCNQ2 and *KCNQ3* encode voltage-gated ion channel subunits mediating a subthreshold potassium current, called M-current (I_{KM}), important in limiting neuronal excitability.[1] Missense loss-of-function (LoF) variants in *KCNQ3* cause Benign Familial Neonatal Epilepsy (BFNE), characterized by seizures in the neonatal period with normal development,[2] though rare families with more severe epilepsy phenotypes have also been described.[3, 4] LoF variants in *KCNQ2* also cause BFNE, while *de novo* variants (DNVs) that result in more profound disruption of *KCNQ2* function (e.g., through dominant negative effects)[5] lead to KCNQ2 Encephalopathy, a severe developmental and epileptic encephalopathy (DEE) characterized by seizures with onset in the neonatal period and global neurodevelopmental disability (NDD).[6]

Voltage-gated potassium channel subunits contain six transmembrane segments (S1-S6) and cytoplasmic N- and C-termini. Within the S1-S4 voltage-sensing domain (VSD), the S4 transmembrane segment includes a series of positively-charged arginine residues that allows the channel to change its opening probability in response to changes in membrane potential.[7] Missense DNVs at the two outermost arginines of the KCNQ3 S4 segment (R1: R227Q; R2: R230C/S) have surfaced in heterogeneous cohorts studied by exome sequencing for DEE, NDD or ID [8-10] and cortical visual impairment.[11] Interestingly, DNVs in the corresponding residues in KCNQ2 (R1: R198; R2: R201), were shown to result in gain-of-function (GoF)[12] with distinct DEE phenotypes. Patients with the KCNQ2 R1 variant, R198Q, present in mid-infancy with West syndrome, without preceding seizures in the neonatal period,[13] whereas patients with the KCNQ2 R2 variants, R201C and R201H, present with neonatal-onset encephalopathy without seizures and later develop infantile spasms.[14] The phenotypic spectrum associated with KCNQ3 R227 and R230 variants has not yet been described.

Here, we delineate the novel electroclinical phenotype in 11 patients with 4 different heterozygous GoF DNVs at R227 and R230 in *KCNQ3*. In contrast to previously described patients with *KCNQ3* LoF, we find that these patients do not present with seizures in the neonatal period. Instead, within the first two years of life, they demonstrate global NDD and autism spectrum disorder (ASD) or autistic features. For 6/9 (67%) recorded between 1.5 and 6 years of age, spikes became near-continuous during sleep, raising concerns for epileptic encephalopathy. Sleep-activated spikes in two patients demonstrated a marked response to high-dose diazepam therapy, providing insight into a possible therapeutic intervention. Patch clamp analysis of each of the *KCNQ3* variants revealed GoF effects, including increased maximal current density and increased opening at membrane potentials where the channel would normally be inactive.

Materials and Methods

Patients

Patients with variants at R230 and R227 in KCNQ3 were identified by epilepsy gene panel or exome sequencing in clinical and research settings. All sites received prior approval by their human research ethics committee when indicated, and parental informed consent was obtained for each subject. Groups were connected through the Rational Intervention for KCNQ2/3 Epileptic Encephalopathy (RIKEE) database (www.rikee.org), which is curated at Baylor College of Medicine under an Institutional Review Board-approved research protocol.[15] One of the cases (patient 6) was previously reported with minimal clinical details as part of an Epi4K epileptic encephalopathy cohort;[16] the others have not been previously

reported. Pediatric epileptologists (TTS and MRC) reviewed the genetic test results and clinical reports, and evaluated the EEG recordings, where available. TTS, MRC, and ECC communicated with treating physicians and/or parents of all patients. Patients were considered to have sleep-activated spikes if the abundance of spikes increased by more than twice that of the awake state. Near-continuous was defined as present for more than 70 percent of the sleep record.

Mutagenesis of *KCNQ3* cDNA and heterologous expression

Variants were introduced in *KCNQ3* human cDNA cloned into pcDNA3.1 by QuikChange site-directed mutagenesis (Agilent Technologies, Milan, Italy), as previously described.[12] Channel subunits were expressed in Chinese Hamster Ovary (CHO) cells by transient transfection using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.[17] A plasmid encoding enhanced green fluorescent protein (Clontech) was used as a transfection marker; total cDNA in the transfection mixture was kept constant at 4 μg.

Whole-Cell Electrophysiology

Currents were recorded under whole-cell patch-clamp at room temperature (20-22°C) 1-2 days after transfection as reported.[12] Current densities (expressed in pA/pF) were calculated as peak K^+ currents at 0 mV divided by cell capacitance (C). To generate conductance-voltage curves, the cells were held at -80 mV, then depolarized for 1.5 s from - 120 to +20 mV in 10 mV increments, followed by an isopotential pulse at 0 mV of 300-ms duration. The current values recorded at the beginning of the 0 mV pulse were measured, normalized, and expressed as a function of the preceding voltages. The data were then fit to a Boltzmann distribution of the following form $y=max / [1 + exp(V_{1/2} - V) / k]$, where V is the test potential, $V_{1/2}$ the half-activation potential, and k the slope factor.

Multistate protein modeling

Three-dimensional models of KCNQ2 and KCNQ3 channels were generated using as templates the coordinates of six different states of Kv1.2/2.1 paddle chimera (PDB accession number 2R9R) by SWISS-MODEL. The models were optimized through all-atom energy minimization by the GROMOS96 implementation of Swiss-PDBViewer and analyzed using both the DeepView module of Swiss-PDBViewer (version 4.0.1; http://spdbv.vital-it.ch/) and PyMOL (http://www.pymol.org/), as described.[4, 12] Sequence alignment was performed using Clustal ω (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistics

The probability that a sequencing result reflected post-zygotic mosaicism was assessed by the binomial exact test, based on the expectation that heterozygous germline variants will be represented in approximately 50% of read observations. Electrophysiological data are expressed as mean±SEM. Statistically significant differences were evaluated with the Student t test or with ANOVA followed by the Student-Newman-Keuls test, with the threshold set at $p<0.05$.

Results

KCNQ3 DNVs are associated with a novel phenotype consisting of neurodevelopmental delay, autistic features and sleep-activated near-continuous multifocal spikes.

Index case (patient 1). A 30-month old boy with global developmental delay and ASD presented with episodes of head nodding and stumbling that raised concern for seizures. His development had been typical through the first year, but he did not walk until 18 months and he had no expressive language. He had poor eye contact, impaired joint attention, did not respond to his name and his behaviors were notable for stereotypies and echolalia. One week prior to presentation, his mother became concerned for worsening balance with increased falls and more impulsive and aggressive behavior. He was admitted for evaluation with a differential diagnosis that included subtle seizures as a cause of his exacerbated motor impairment. The events of concern were captured on long-term video-EEG monitoring and did not show an EEG correlate. His EEG background, however, was diffusely slow with frequent multifocal spike-and-wave discharges, most prominent in the posterior leads. These discharges increased in amplitude (to > 300 uV) and in abundance during sleep, becoming present for greater than 80% of the sleep record. Given these findings in the clinical context of worsened behaviors and motor performance, the treating physicians were concerned for an epileptic encephalopathy. Treatment with high-dose oral diazepam (1mg/kg) led to rapid resolution of the epileptiform abnormalities and improvements were subsequently noted across multiple developmental domains by his parents and therapists. Trio exome sequencing revealed a heterozygous *KCNQ3 de novo* variant predicted to result in the missense change R230H.

Cohort genotypes and phenotypes. We identified 10 other patients with NDD and variants in *KCNQ3* predicted to change R230 and R227 (Tables 1 & 2). These included two additional patients with R230H, five patients with R230C, one patient with R230S, and two patients with R227Q. Next generation sequencing revealed mosaicism in one parent and one proband. The asymptomatic mosaic mother of patient 3 carried the variant in 3 of 50 reads (6%, $p < 10^{-8}$, binomial exact test). Aside from patient 3, all variants were confirmed to be absent in parental samples. The DNA sequencing of patient 7 showed R230H in 22 of 121 reads (18%, $p < 10^{-8}$). R227Q, R230C, and R230S were absent from the population database gnomAD.[18] Interestingly, 1 of 122,950 individuals in the gnomAD dataset showed mosaic presence of R230H (45/145 reads, 31%, $p = 2.9 \times 10^{-6}$),[18, 19] similar to patient 7. Clinical information was not available regarding the gnomAD mosaic individual. *In silico* analysis predicted each of these variants to be deleterious with high probability (polyphen-2, > 0.999; SIFT, 0; CADD score > 30).[20-22]

For genome wide significance as an NDD gene, our 11 patients would need to have been observed from a cohort no larger than 47,000 individuals (p=2.40e-06, CCDS22).[23] Patient 6 was identified in an Epi4K cohort of 264 individuals,[16] but the method of ascertainment of most our other patients makes precise determination of the denominator impossible, precluding formal calculation.

All 11 patients had some degree of intellectual disability (ID) and delays across multiple developmental domains, coming to clinical attention between the ages of 4 and 18 months. Delayed language was universal but patients often presented with concurrent or preceding gross motor delays. Patient 3 did not develop head control until after 6 months. Four patients were late to sit and all but two individuals (patients 7 and 11) were delayed in walking. While all patients ultimately walked, walking was often characterized as broad-based and unsteady with poor balance, variably reported as ataxic.

Language development was abnormal in all cases. Three patients were nonverbal. Five developed single words, but two of these subsequently regressed to become nonverbal. Patient 7, mosaic for R230H, and the two patients (10 and 11) who carried the R227Q variant had language delay with first words at 2 or 3 years, but were ultimately able to speak in sentences.

ASD was diagnosed in 5/11 (45%) patients and autistic features were reported in the remaining six. Stereotypies, mouthing non-food objects, and aggressive, impulsive and self-injurious behaviors were common features. Hypotonia and strabismus were each reported in 7/11 (64%) individuals. Brain MRI studies were normal or showed non-specific abnormalities. The MRI of patient 5 showed diminished white matter and abnormal frontal sulcation not consistent with acquired injury, though he had a history of preterm delivery at 34 weeks of gestation.

Two patients (4 and 6) were diagnosed with generalized tonic-clonic seizures from 13 years and from 10 months of age, respectively. Atonic seizures were also reported for these patients, as well as absence seizures for patient 6. The remaining patients were not diagnosed with seizures (9/11, 82%). No patients had seizures in the neonatal period.

All 11 patients had EEGs recorded at some point between 1 and 10 years of age, and 8 of them (73%) had focal or multifocal spikes that were markedly activated by sleep. In 6/9 patients (67%) with sleep EEGs reviewed between 18 months and 6 years of age, epileptiform discharges became near-continuous during sleep. For four of these children (patients 1, 2, 3 and 8), parents noticed recurrent episodes of unresponsive staring or deteriorating motor function with subtle jerks or loss of tone that led to assessment with prolonged video-EEG recording. While the events of concern could not always be captured, the spikes observed were not time-locked with jerks, loss of tone or unresponsive staring. In 5 cases (patients 1, 2, 3, 8 and 9), the discovery of the markedly abnormal sleep EEG given the clinical context raised concern for epileptic encephalopathy, leading physicians to treat with anti-seizure medications including high dose diazepam with the goal of reducing or eliminating the epileptiform abnormalities. The clinical response to treatments varied; some benefits were reported although no worsening was seen when the anti-seizure medications were discontinued. Treatment with high-dose oral diazepam (Patients 1 and 3) or corticosteroids (patients 8 and 9) was followed by reduction of the sleep-activated spikes on EEG but with inconsistent effects on behavior.

KCNQ3 R227 and R230 variants exhibit gain-of-function with increased current density and hyperpolarized activation voltage-dependence

KCNQ3 R227 (R1) and R230 (R2) are the outermost of the positively-charged residues of the S4 voltage-sensor (**Figure 1A**); in KCNQ2, R1 and R2 correspond to R198 and R201, respectively (**Figure 1B**). The functional properties of channels formed by KCNQ3 R227Q or R230C/H/S variants were characterized as homomers and as heteromers with KCNQ2 subunits.

Wild-type homomeric KCNQ3 channels generated small K^+-selective and voltage-dependent currents that activated around -60 mV and displayed a $V_{1/2}$ of -38 mV (**Figure 1C, D; Table 3**). At a holding voltage of -80 mV, the vast majority of KCNQ3 channels were closed; therefore, the ratio between the currents

measured at the beginning of the depolarization step ($I_{Instant}$) and those at the end of the 0 mV depolarization ($I_{steady\text{-}state}$) was close to zero (**Table 3**). By contrast, homomeric KCNQ3 channels in which the charged side chain at R230 was substituted by cysteine, serine, or histidine residues (R2C, R2S, and R2H, respectively) showed an almost complete loss of time-dependent current activation kinetics; as a result, the $I_{Instant}/I_{steady\text{-}state}$ ratio was close to unity (**Table 3**). Similar, although quantitatively smaller, effects were observed upon neutralization of the R227 residue with glutamine (R1Q); in fact, KCNQ3 R227Q channels retained voltage-dependent gating, although with a drastic (>70 mV) hyperpolarization of the voltage requirement for activation. Notably, this functional change is qualitatively similar but quantitatively larger than that produced by the corresponding substitution (R198Q) in KCNQ2 (~ 30 mV).[24]

In addition, the amplitude of K^+ current carried by each of the four mutant channels at depolarized membrane potentials was increased approximately ten-fold, compared to wild-type KCNQ3 channels (**Table 3**). In contrast to the dramatic changes in voltage-sensitivity and current size described in all four mutant channels, other important properties, such as the sensitivity to blockade by tetraethylammonium (TEA), a pharmacological feature discriminating between KCNQ3 and KCNQ2 channels, or the K^+ reversal potential, indicative of channel selectivity for K^+ ions, were unchanged from the wild type (**Table 3**).

To mimic the genetic condition of patients, who carry a single mutant allele, and considering that I_{KM} in adult neurons is mainly formed by tetrameric co-assembly of KCNQ2 and KCNQ3 subunits, we transfected CHO cells with *KCNQ2* and *KCNQ3* cDNAs in a 1:1 ratio (to mimic the genetic balance of normal individuals), and *KCNQ2*+*KCNQ3*+mutant *KCNQ3* 1:0.5:0.5 ratio (to mimic the genetic balance of affected individuals). Co-expression of KCNQ3 R227Q, R230C, R230H, or R230S variants with KCNQ2 and KCNQ3 subunits caused a statistically significant hyperpolarization in activation voltage-dependence of about 6 mV, without affecting current density or TEA sensitivity when compared to KCNQ2+KCNQ3 channel controls (**Table 3**).

Mechanistic basis for the gain-of-function by *KCNQ3* R227 and R230 variants

We used a model based on the atomic structure of Kv1.2/2.1 channels to analyze the mechanistic basis for the functional effects observed. In the resting state, the positively-charged side chains of R227 (R1) and R230 (R2) in the KCNQ3 VSD establish ionized hydrogen bonds with nearby polar or charged residues: R227 with C136 in S1, and R230 with E170 and D202 in S2 and S3, respectively (**Figure 2A**). These interactions are all lost when the S4 moves toward the extracellular space during activation;[25] therefore, the R227Q or the R230C/S/H substitutions are predicted to selectively destabilize the resting (closed) conformation of the VSD, possibly explaining the observed GoF effects. Noteworthy, R198 in KCNQ2 (R1, corresponding to KCNQ3 R227) in addition to C106 (corresponding to KCNQ3 C136), also establishes a strong hydrogen bond with S110 (**Figure 2B**, top right panel); in KCNQ3, this position is occupied by a non-polar residue (A140) that is unable to interact with R227 (R1; **Figure 2B**, top left panel). The fact that the R227 residue in KCNQ3 only establishes a weak hydrogen bond with the nearby C residue, whereas the corresponding R198 residue in KCNQ2 is also engaged in a stronger hydrogen bond with S110 renders the VSD resting state less stable in KCNQ3 when compared to KCNQ2, likely contributing to the lower activation midpoint of the former,[12] and, possibly, to the more dramatic $V_{1/2}$ hyperpolarizing effect of the

KCNQ3 R227Q substitution (Q1; **Figure 2B**, bottom left panel) when compared to the R198Q substitution in KCNQ2[24] (Q1; **Figure 2B**, bottom right panel).

Discussion

Inherited variants in *KCNQ3* are known to be associated with BFNE. Our series describes the novel phenotype in patients with *de novo KCNQ3* missense variants at R227 and R230, characterized by NDD, ASD, and sleep-activated near-continuous multifocal spikes, and increases the number of reported patients with this mutational hotspot to 16. Substitutions at R230C, R230H and R230S variants all resulted in strong GoF effects, while similar but smaller effects were exhibited by R227Q.

While formal calculation of genome-wide significance was not possible, given our inability to know the total number of individuals sequenced for NDD, we calculated an upper limit of 47,000. Our collaboration is highly unlikely to have drawn from such a large population. Supporting this, the largest NDD cohort from which cases have been identified to date, the Deciphering Developmental Disorders Study, was smaller than this limit by an order of magnitude and identified two such patients.[10] The similarity of clinical presentation and the complementary functional work we present provide additional support for *KCNQ3* as an NDD gene.

Patients with *KCNQ3* GoF variants at R227 and R230 presented with developmental delay within the first 2 years with over a third of the cohort presenting before 12 months. Patients with R230C/H/S variants were usually ambulatory by 2 years of age, but were either non-verbal or had single words and were cognitively impaired with ASD or autistic features. Patient 7, whose testing revealed mosaicism for R230H, had a relatively milder phenotype, and the mother of patient 3, with low-level mosaicism for R230H, was unaffected. The NDD of the two patients with R227Q was also less severe, consistent with our findings of milder alteration of in vitro functional properties of channels carrying this variant compared to those carrying R230C/H/S variants. While these findings are suggestive of a positive correlation between the extent of gain-of-function and severity, there is currently insufficient data for proper statistical assessment, which will have to wait for larger numbers of patients.

Previously published studies sequencing cohorts of patients with DEE, NDD, ID and cortical visual impairment have identified one patient with R227Q, three with R230C, and two with R230S DNVs in *KCNQ3*.[8-11, 16] Although limited, the clinical features reported in those studies (**Table 4**) seem consistent with our phenotype.

Multifocal status epilepticus during sleep

EEG recordings showed sleep-activated spikes in all but two patients monitored during sleep. In six patients who had EEGs performed between 1.5 and 6.5 years of age, spikes became near-continuous during sleep raising concerns for epileptic encephalopathy in the clinical setting. Continuous spike and wave during slow wave sleep (CSWS) is an epilepsy syndrome characterized by neurocognitive regression or stagnation associated with near-continuous diffuse spike-waves occurring during sleep, an electrographic pattern referred to as electrical status epilepticus during slow sleep (ESES). When we analyzed the EEGs for the purpose of this study, we found that the spikes were multifocal with a posterior

predominance which suggested the term "multifocal status epilepticus during sleep" (MSES). Some of our patients had language regression, but we do not have longitudinal testing to determine the timing and extent of regression or developmental plateauing or correlate it with the appearance of MSES. In most patients in whom MSES was detected, EEG monitoring was prompted by concern for seizures. Although these patients were not diagnosed with seizures, the presence of near-continuous spikes during sleep led to treatment based on the concept that reducing the abundance of epileptiform abnormalities may prevent or reverse developmental stagnation or regression.[26, 27] Our numbers are too small to draw conclusions about electrographic responses to standard therapies, such as diazepam,[28] and more recently described treatments, such as amantadine, were not used.[29]

Two patients in our cohort were diagnosed with generalized tonic-clonic seizures, atonic seizures, as well as absence seizures, although their events were never captured on EEG. Absence epilepsy/seizures was intriguingly also noted in the limited clinical details for two patients with *KCNQ3* variants previously reported cohorts (Table 4).[10, 11] However, the full spectrum of epileptic disorders in patients with *KCNQ3* gain-of-function variants awaits further characterization with ictal video-EEG recordings and classification of the events. Our study has the limitations of being retrospective: evaluation (e.g., cognitive/behavioral testing, timing and length of EEG recordings) and treatment (including medication selection and duration of treatment) were determined at the discretion of the treating physicians and did not follow a research protocol.

KCNQ3 and *KCNQ2* genotypes and phenotypes

Brain KCNQ2 and KCNQ3 subunits co-assemble as heteromeric channels,[30] and inherited LoF missense variants in these genes cause an autosomal dominant phenotype, Benign Familial Neonatal Epilepsy (BFNE).[31-33] Most *de novo KCNQ2* LoF variants result in a severe DEE with seizures onset in the neonatal period.[6, 15, 17, 34] However, *de novo KCNQ2* GoF variants R201C and R201H are associated with a distinct neonatal syndrome characterized by non-epileptic myoclonus, pathological breathing, and a suppression-burst EEG pattern in the absence of seizures.[14] We now report that *de novo* GoF variants at the *KCNQ3* R230 position, homologous to *KCNQ2* R201, cause NDD associated with ASD/autistic features and MSES without neonatal seizures. While the *KCNQ2* R198Q variant has been found recurrently in patients with West syndrome without prior neonatal seizures,[24] we found the homologous *KCNQ3* variant, R227Q, in two patients with less severe NDD without any history of seizures. These findings further extend the phenotypes associated with *KCNQ2* and *KCNQ3* GoF variants, which have in common the absence of neonatal seizures, the main characteristic of LoF variants (Table 5).

Our understanding of the mechanism by which GoF changes in KCNQ3 result in the described clinical phenotype with NDD and without neonatal seizures is limited by the lack of *in vivo* studies. In particular, it is unclear why the LoF condition presents in the neonatal period, whereas the GoF condition results in cognitive and behavioral disturbances that only become apparent later. Interestingly, a parallel but reverse genotype-phenotype correlation has been reported for *SCN2A-related disorders*, where GoF results in early epilepsy and LoF imparts neurodevelopmental disability with autistic features and more variable epilepsy phenotypes with later onset.[35] This similarity may not be coincidental, as both channels are localized at the axon initial segment and seizures in early epilepsy caused by *KCNQ3* LoF variant, like

those caused by *SCN2A* GoF variants, are responsive to sodium channel blockers, such as carbamazepine.[36, 37]

The reason for the differences in phenotypes between *KCNQ2* and *KCNQ3* variants at homologous positions is unknown and fuller investigation of this will likely require *in vivo* developmental studies. In rodents, the ratio of *KCNQ3* to *KCNQ2* expression is low at birth and increases during postnatal development.[38] Similar findings have been shown in the human brain,[39] and may explain the earlier onset and more severe disability of *KCNQ2* GoF pathogenic variants compared to *KCNQ3*.

While the features of neonatal-onset *KCNQ2* and *KCNQ3* related epilepsy are distinctive,[36, 40] enabling early recognition of the phenotype and genetic testing, global NDD is clinically and genetically heterogeneous. The prevalence of *KCNQ3* R227 and R230 variants in the general population of children with NDDs is unknown, but is likely under-recognized, as neither exome sequencing nor sleep EEG is currently routinely included in the evaluation of children with NDD and autism.

A monogenic cause of neurodevelopmental disability and autism

Monogenic subtypes of autism are increasingly being identified, particularly when comorbid with ID.[41, 42] Epilepsy, ID and autism often co-occur and share genetic causes and perhaps underlying mechanisms.[43] As near-continuous epileptiform activity during sleep may interfere with development and treatment with benzodiazepines may be successful at abolishing the electrographic pattern, sleep EEG recording for patients with NDD/ID with autism may have clinical utility.

Limitations of this study arise from the rarity of the disorder, and include differences in patient evaluation between sites, and the potential for ascertainment bias, as parents of severely affected children may be more likely to seek clinical genetic evaluation and participate in research. Additional work, including standardized assessment of a larger patient group, will enable further characterization of *KCNQ3* GoF pathogenic variants.

Conclusion

Our findings show that GoF missense variants at R230 and R227 in *KCNQ3* do not cause neonatal epilepsy, and instead result in a novel phenotype characterized by NDD with ASD and MSES. Our work provides another example of the delineation of distinct phenotypes associated with different classes of variants in ion channel genes, expands the phenotypic spectrum associated with pathogenic variants in *KCNQ3*, complements the GoF phenotypes reported for *KCNQ2*, and adds *KCNQ3* to genetic causes of autism.

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The authors report no potential conflicts.

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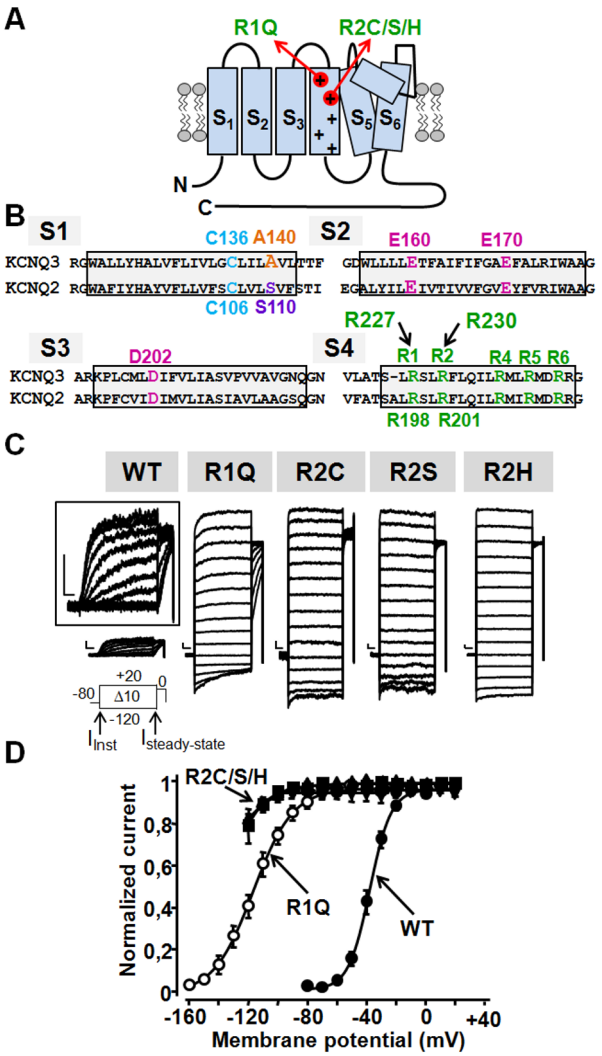
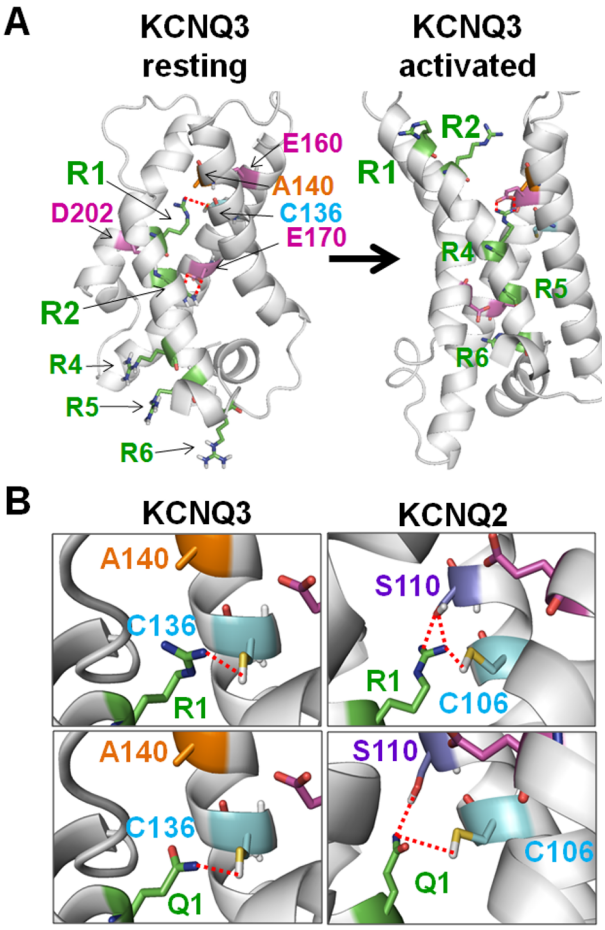
Figure 1. Functional consequences of the R227Q and R230C/S/H variants in KCNQ3. (A) Topological representation of a single KCNQ subunit; the red arrows highlight the position of the first two arginines (R1 and R2) along the S4 primary sequence, where variants of interest in the present study are located. (B) Sequence alignment of the four transmembrane regions (S1, S2, S3, and S4) of the VSD of KCNQ3 and KCNQ2 subunits. Residues relevant to the present study are colored as follows: green for positively-charged, pink for negatively-charged, orange for non-polar, and blue for polar; among polar amino acids, C is in light blue, whereas S is in violet. R1, R2, R4, R5, and R6 refer to the positively charges arginines numbered according to their postion along the S4 primary sequence. (C) Macroscopic currents from KCNQ3 (WT), KCNQ3 R227Q (R1Q), KCNQ3 R230C (R2C), KCNQ3 R230S (R2S), or KCNQ3 R230H (R2H) homomeric channels, in response to the indicated voltage protocol. Inset shows an enlarged view of KCNQ3 traces. The arrows on the voltage protocol indicate the time chosen for current analysis, as explained in the text. Current scale, 100 pA; time scale, 0.2 s. (D) Conductance/voltage curves for KCNQ3 (WT, filled circles), KCNQ3 R227Q (R1Q, empty circles), KCNQ3 R230C (R2C, filled inverted triangles), KCNQ3 R230S (R2S, triangles), or KCNQ3 R230H (R2H, filled squares) homomeric channels, as indicated. Continuous lines are Boltzmann fits to the experimental data. Each data point is the mean-SEM of 9-21 cells recorded in at least three separate experimental sessions.

Figure 2. Structural modeling of KCNQ3 VSD in resting and activated states, and comparison with KCNQ2. (A) Structural model of the resting (left panel) and activated (right panel) gating states of the VSD from a single KCNQ3 subunit, as indicated. Residues relevant to the present study are colored as follows: green for positively-charged, pink for negatively-charged, orange for non-polar, and blue for polar; among polar amino acids, C is in light blue, whereas S is in violet. R1, R2, R4, R5, and R6 refer to the positively charges arginines numbered according to their position along the S_4 primary sequence. (B) An enlarged view of the resting state of the VSD of KCNQ3 (top left panel) and KCNQ2 (top right panel). Lower panels highlight the ionic interactions established when the R1 residues are substituted with Q (Q1) in KCNQ3 (left) or KCNQ2 (right) subunits. In all panels, dashed red lines indicate ionic interactions among residues.



ANA_25522_KCNQ3 GoF Figure 1.tif



ANA_25522_KCNQ3 GoF Figure 2.tif

Table 1: Clinical features of *KCNQ3* gain-of-function variants

Case	Variant	Age Sex	Neurodevelopment	Other features	Brain MRI
1	c.689G>A p.R230H	4y M	Walked at 18m, ataxic gait Few words; ASD diagnosis at 21m, ID, echolalia, Impulsive, aggressive behavior; stereotypies	Hypotonia Esotropia	Normal at 37m
2	c.688C>A p.R230S	23y M	Walked at 23m; ataxic gait Non-verbal, autistic features	Hypotonia	Mild hypoplasia of corpus callosum, mild cerebellar atrophy at 19m
3	c.689G>A p.R230H*	5y M	Head lag at 6m; sat at 13m; walked at 25m; ataxic gait Non-verbal (few words, then regressed), Impulsive, repetitive behaviors, poor eye contact	Hypotonia Exotropia	Mild T2 hyperintensities in the bilateral periatrial white matter at 15m & 3.5y
4	c.688C>T p.R230C	20y F	Sat at 12m, walked at 24m 4-5 words, moderate ID Stereotypies; aggressive behavior	Exotropia, Possible CVI	Normal at 4y, 6y & 15y
5	c.688C>T p.R230C	4y F	Sat at 13m, walked with assistance at 34m; 2 words at 34m; poor eye contact	Birth at 34 wks Hypotonia Strabismus	Diminished white matter, right > left, and abnormal frontal sulcation at 13m & 32m
6	c.688C>T p.R230C	11y M	Walked at 23m; ASD diagnosis at 3y; Non-verbal (few words then regressed); impulsive; self-injurious behaviors	Strabismus	Normal at 10m
7	c.689G>A p.R230H 18% mosaic	5y M	Walked at 14m, ataxic gait; Fine motor impairment; Words by 2y, sentences by 3y; ASD diagnosis at 3y	Hypotonia Strabismus	Normal at 4y
8	c.688C>T p.R230C	21y M	Walked by 18m; Non-verbal; ASD, severe ID	Left esotropia	Normal at 3y
9	c.688C>T p.R230C	8y M	Sat at 12m; walked at 26m; Non-verbal; anxiety, aggressive behavior; Autistic features (stereotypies, poor eye contact)	Hypotonia	Nonspecific white matter lesions at 18m
10	c.680G>A p.R227Q	9y F	Walked at 22m; Speaks in 2-3 word sentences ASD diagnosis at 2y; stereotypies, echolalia	Hypotonia	Normal at 9y & 12y
11	c.680G>A p.R227Q	18y F	Walked at 12m; words at 3y, sentences by 6y; Echolalia, stereotypies, sensory issues; dysarthria; FSIQ 42; assistance to brush teeth, comb hair		Normal at 6y

Legend: *ASD* autism spectrum disorder, *CVI* cortical visual impairment, *FSIQ* full scale intelligence quotient, *ID* intellectual disability, * unaffected mother with low-level mosaicism (5-6%);

Table 2: Electroclinical features of patients with *KCNQ3* gain-of-function variants

Patient Variant	EEG	Seizures	AEDs
1 R230H	Diffusely slow with posterior spikes in wakefulness MSES in sleep (posterior predominant EDs) at 30m	No (Staring and jerks recorded at 30m)	For MSES at 30m: DZP (++), CLB (++)
2 R230S	Spikes (L) at 12m MSES at 18m & 4y (R>L) Spikes (R>L) at 8y; No spikes (awake) at 12y & 19y	Staring spells at 3y	VPA
3 R230H	Diffusely slow with posterior spikes in wakefulness MSES at 3.5y & 4.5y (posterior predominant EDs)	No (Staring spells recorded at 3y)	LEV at 3.5y DZP (++) for MSES at 4.5y
4 R230C	Normal at 4.5y Diffusely slow electrical activity at 16y	GTC from 13y Atonic seizures at 15y	VPA, CLB, LCM (all for seizures)
5 R230C	Frequent sleep-activated L posterior > R central EDs at 3y	No	none
6 R230C	MSES at 6y (L>R central & temporal EDs)	GTC from 10m Atonic seizures Absence seizures	VPA, LEV, OXC, RUF, KD (all for seizures)
7 R230H mosaic	Normal at 4.5y	No	none
8 R230C	Diffusely slow with posterior spikes in wakefulness MSES at 30m, 3.5y, 4 y, 4.5y & 5y (posterior predominant EDs)	Staring spells reported at 2y	VPA for staring spells For MSES: LTG, CS (+), CLB
9 R230C	Diffusely slow in wakefulness MSES at 3.5y, 4y, 4.5y, 5.5y, 6.5y	No	For MSES: CS (+), ETX, CLB
10 R227Q	Frequent sleep-activated L frontotemporal EDs at 9y	No	none
11 R227Q	Normal at 2.5y (awake only) Normal at 18y (awake only)	Staring spells reported at 2.5y	none

Legend: *AEDs* anti-epileptic drugs, *CLB* clobazam, *CS* corticosteroids, *DZP* diazepam, *EDs* epileptiform discharges, *EEG* electroencephalogram, *ETX* ethosuximide, *GTC* generalized tonic-clonic seizure, *KD* ketogenic diet, *L* left, *LEV* levetiracetam, *MSES* multifocal status epilepticus during sleep, *OXC* oxcarbazepine, *R* right, *RUF* rufinamide, *VPA* valproic acid, *++* Response; *+* Partial response

Table 3. Biophysical and pharmacological properties of channels carrying KCNQ3 variants.

	n	V½ (mV)	k (mV/efold)	$I_{Instant}$/ $I_{steady-state}$	Current density (pA/pF)	E_k (mV)	Blockade by TEA (%)		
							0.3 mM	3 mM	30 mM
KCNQ3	21	-38.4±1.0	7.1±0.4	0.04±0.02	10.6±1.3	-79.0±0.1	6.4±1.8	13.0±3.4	61.7±5.7
KCNQ3 R1Q	9	-112.0±2.4[a]	10.8±0.9[a]	0.91±0.02[a]	89.6±17.5[a]	-79.9±0.3	--	--	61.1±6.8
KCNQ3 R2C	12	--	--	1.00±0.01[a]	121.0±21.0[a]	-79.9±0.3	--	--	58.6±13
KCNQ3 R2S	16	--	--	0.98±0.03[a]	89.7±12.2[a]	-80.1±0.1	--	--	66.1±6.1
KCNQ3 R2H	12	--	--	0.98±0.02[a]	132.2±20.0[a]	-79.3±0.4	--	--	70.9±7.3
KCNQ2 + KCNQ3	16	-33.6±1.2	13.6±0.4	0.04±0.02	133.5±19.0	--	15.6±3.1	50.5±3.1	78.8±5.6
KCNQ2 + KCNQ3 + KCNQ3 R1Q	9	-39.5±3.0[b]	14.7±0.8	0.04±0.01	101.3±20.2	--	19.3±2.0	44.1±4.3	85.3±2.1
KCNQ2 + KCNQ3 + KCNQ3 R2C	9	-39.9±3.7[b]	15.3±0.7	0.10±0.03[b]	108.8±16.9	--	14.0±6.2	47.1±9.9	77.0±7.3
KCNQ2 + KCNQ3 + KCNQ3 R2S	14	-39.0±1.5[b]	15.0±0.6	0.07±0.02[b]	116.7±12.0	--	12.9±2.3	43.6±8.2	80.1±6.5
KCNQ2 + KCNQ3 + KCNQ3 R2H	14	-39.5±1.5[b]	14.2±0.4	0.08±0.02[b]	123.0±15.5	--	20.8±3.1	47.4±2.5	78.8±3.2

[a] $p<0.05$ versus KCNQ3; [b] $p<0.05$ versus KCNQ2+KCNQ3

Table 4. Previously published patients with *KCNQ3* R227 & R230 variants

Publication Case ID	Variant	Sex	Neurodevelopment	Other Features	EEG	Seizures	Brain MRI
Rauch *et al*, 2012 TUTLN	c.688C>T p.R230C	F	Sat at 12 m, walked at 24 m Nonverbal at 42 m Moderate ID; "autistic, aggressive, anxious"	Strabismus	"Multifocal sharp waves, sharp slow waves"	No	6 m MRI: "Hypointensity in the left ventricle"
Grozeva *et al*, 2015 5410783	c.688C>A p.R230S*	F	Non-syndromic ID				
Bosch *et al*, 2016 24	c.688C>T p.R230C	F	ID at 4 y	Cortical visual impairment		Absence epilepsy	
DDD, 2017 261649	c.688C>A p.R230S	F	Broad-based gait Delayed speech & language Severe ID; Recurrent hand flapping	Strabismus Microcephaly		Absence seizures	
DDD, 2017 272471	c.680G>A p.R227Q	M	Global developmental delay				

* Inheritance unknown

Table 5. Gain-of-function variants in the voltage sensor domain S4 segments of *KCNQ2* and *KCNQ3* have diverse electroclinical phenotypes.

S4 Arginine	*KCNQ2*: Known Variants	Phenotypes	*KCNQ3*: Known Variants	Phenotypes
R1	R198Q	West syndrome (hypsarrhythmia, infantile spasms, emergence of developmental delay) without preceding neonatal seizures or encephalopathy	R227Q	Neurodevelopmental disability: verbal, with Autism spectrum disorder or autistic features and sleep-activated spikes
R2	R201C R201H	Profound neonatal onset encephalopathy with non-epileptic myoclonus, burst-suppression EEG and apnea with West syndrome later in infancy	R230C R230H R230S	Neurodevelopmental disability: non-verbal with Autism spectrum disorder or autistic features and Multifocal status-epilepticus during sleep

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