Conditional Deletions of Epilepsy-Associated KCNQ2 and KCNQ3 Channels from Cerebral Cortex Cause Differential Effects on Neuronal Excitability

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KCNQ2 and KCNQ3 potassium channels have emerged as central regulators of pyramidal neuron excitability and spiking behavior. However, despite an abundance of evidence demonstrating that KCNQ2/3 heteromers underlie critical potassium conductances, it is unknown whether KCNQ2, KCNQ3, or both are obligatory for maintaining normal pyramidal neuron excitability. Here, we demonstrate that conditional deletion of *Kcnq2* from cerebral cortical pyramidal neurons in mice results in abnormal electrocorticogram activity and early death, whereas similar deletion of *Kcnq3* does not. At the cellular level, *Kcnq2*-null, but not *Kcnq3*-null, CA1 pyramidal neurons show increased excitability manifested as a decreased medium afterhyperpolarization and a longer-lasting afterdepolarization. As a result, these *Kcnq2*-deficient neurons are hyperexcitable, responding to current injections with an increased number and frequency of action potentials. Biochemically, the *Kcnq2* deficiency secondarily results in a substantial loss of KCNQ3 and KCNQ5 protein levels, whereas loss of *Kcnq3* only leads to a modest reduction of other KCNQ channels. Consistent with this finding, KCNQ allosteric activators can still markedly dampen neuronal excitability in *Kcnq3*-null pyramidal neurons, but have only weak effects in *Kcnq2*-null pyramidal neurons. Together, our data reveal the indispensable function of KCNQ2 channels at both the cellular and systems levels, and demonstrate that pyramidal neurons have near normal excitability in the absence of KCNQ3 channels.

Key words: BFNC; epilepsy; KCNQ2; KCNQ3; M current

Introduction

The five members of the KCNQ family of potassium channels play important roles in neuronal physiology. Within the cerebral cortex, KCNQ channels have emerged as critical regulators of pyramidal neuron excitability and spiking behavior. In neurons, KCNQ channels are best known for their role in mediating the classical M current, a potassium conductance that increases as neurons approach action potential (AP) threshold (Wang et al., 1998; Jentsch, 2000). KCNQ channels, together with HCN and SK potassium channels, comprise the medium afterhyperpolarization (AHP), an inhibitory conductance elicited by a burst of APs (Gu et al., 2005, 2008). Consistent with these roles, experimentally blocking of KCNQ channels modulates spiking activity by converting simple spikes to high-frequency bursts and attenuating spike-frequency adaptation (Yue and Yaari, 2004; Otto et al., 2006). Accordingly, patients with loss-of-function and missense mutations in some KCNQ channels develop mild to very

severe pediatric epilepsy (Charlier et al., 1998; Singh et al., 1998; Ishii et al., 2012; Weckhuysen et al., 2012).

Of the five currently identified KCNQ family members, KCNQ2 and KCNQ3 are highly expressed in cerebral cortical pyramidal neurons and form the M current (Cooper et al., 2001). Multiple lines of evidence suggest that KCNQ2 and KCNQ3 exist and function primarily as heteromeric KCNQ2/3 channels, as the pharmacology of the M current best matches that of KCNQ2/3 heteromers coexpressed in heterologous cells (Wang et al., 1998). Furthermore, KCNQ2 and KCNQ3 channels express poorly in heterologous cells when alone, but robustly when they are coexpressed (Jentsch, 2000). *In vivo*, overexpression of a KCNQ2 dominant-negative subunit in transgenic mice significantly reduced the M current and caused elevated excitability and seizures (Peters et al., 2005). This dominant-negative subunit heteromerizes with KCNQ3 subunits and may eliminate all KCNQ2/3 function. Similarly, mice carrying missense *Kcnq2* or *Kcnq3* mutations identified in patients with benign familial neonatal convulsions (BFNCs) exhibited seizures (Singh et al., 2008).

These findings support a model in which both KCNQ2 and KCNQ3 are required for pyramidal neurons to control their excitability. However, the particularly high frequency of identified *Kcnq2* mutations in both mild and very severe forms of pediatric epilepsy raises the possibility that while KCNQ3 is involved in maintaining normal excitability in pyramidal neurons, KCNQ2 is obligatory. However, directly testing this hypothesis by com-

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paring neuronal excitability in *Kcnq2* and *Kcnq3* knock-out mice was until now not feasible, due to the perinatal lethality of *Kcnq2* knock-out mice (Watanabe et al., 2000).

Here, we generated transgenic mice with conditional deletion of *Kcnq2* or *Kcnq3* in cerebral cortical pyramidal neurons and report that these mice exhibit strikingly different phenotypes. Pyramidal neurons lacking *Kcnq2* are hyperexcitable and have a smaller medium afterhyperpolarization (mAHP) and a prolonged afterdepolarization (ADP). By contrast, those pyramidal neurons lacking *Kcnq3* are not hyperexcitable and have a near normal mAHP and ADP. Furthermore, conditional deletion of *Kcnq2* but not *Kcnq3* greatly reduces the protein levels of other KCNQ channels. These changes may explain why *Kcnq2* conditional knock-out mice uniquely exhibit aberrant cortical activity and death by the third week of life. Therefore, our work demonstrates that proper control of pyramidal neuron excitability requires the presence of KCNQ2 but not KCNQ3 channels.

Materials and Methods

All experiments were performed according to the guidelines of the University of Connecticut–Storrs Institutional Animal Care and Use Committee.

Animals and genotyping. *Kcnq2* and *Kcnq3* conditional knock-out mice were generated using the Cre/loxP system by the Gene Targeting and Transgenic Facility of the University of Connecticut Health Center. Briefly, the targeting vector containing a neomycin cassette was electroporated into embryonic stem (ES) cells, and cells in which homologous recombination occurred were selected by neomycin resistance. These ES cells were injected into mouse embryos to obtain chimeric male mice, which were then used to generate *Kcnq2*^{fl/fl} and *Kcnq3*^{fl/fl} founder mice. The neomycin cassette, flanked by Frt sites, was then removed from all cells, including the germline, by FLP recombination, using the ROSA26-Flpe mice maintained in a C57BL/6 background. For our studies, we used the progeny of these mice, which lack the neomycin cassette in all somatic and germline cells. We refer to these as *Kcnq2*^{fl/+} and *Kcnq3*^{fl/+} mice. The floxed mice in a C57BL/6 background were then crossed with the *Emx1-ires-cre* recombinase strain, also in a C57BL/6 background (Jackson Laboratory), to obtain cerebral cortex-specific deletion of KCNQ channels. *Kcnq2*^{fl/+}; *Emx1*^{cre/+}, *Kcnq2*^{fl/+}; *Emx1*^{cre/cre}, or *Kcnq3*^{fl/fl}; *Emx1*^{cre/+} were crossed again to obtain the desired genotype. *Kcnq2*^{fl/fl}; *Emx1*^{+/+}, *Kcnq2*^{fl/+}; *Emx1*^{+/+}, *Kcnq2*^{+/+}; *Emx1*^{cre/+}, *Kcnq2*^{+/+}; *Emx1*^{cre/cre}, or *Kcnq3*^{fl/+}; *Emx1*^{+/+} were used as control mice. *Kcnq2*^{fl/fl}; *Emx1*^{cre/+} and *Kcnq3*^{fl/fl}; *Emx1*^{cre/cre} were considered conditional knock-out mice. For genotyping *Kcnq2*-floxed mice, two primers were included in each PCR: *Kcnq2* frt forward 5'-CCACTTGGTGATGGACTGTG-3', and *Kcnq2* frt reverse 5'-GCCTGTGTTTCCATTGCT-3'. The primers amplified a 483 bp fragment from the wild-type allele and a 581 bp product from the floxed allele. For *Kcnq3*-floxed mice, two primers were also included in each PCR: *Kcnq3* frt forward 5'-CAGCACTCCCATGACAAATG-3', and *Kcnq3* frt reverse 5'-TCTCCCATGGCAAGTATTC-3'. The primers amplified a 255 bp fragment from the wild-type allele and a 339 bp product from the floxed allele (Fig. 1A). To identify mice carrying *Emx1-ires-cre*, four primers were used in each PCR: cre forward 5'-GATCTCCGGTATTGAACTCCAGC-3'; cre-reverse 5'-GCTAAACATGCTTCATCGTCGG-3'; *Emx1-ires-cre* wild-type forward 5'-AAGGTGTGGTTCCAGAATCG-3'; and *Emx1-ires-cre* wild-type reverse 5'-CTCTCCACCAGAAGGCTGAG-3'. The primers amplified a 750 bp fragment in mice carrying the cre allele and a 378 bp fragment from the wild-type allele.

Western blotting. For Western blotting, mice [postnatal day (P) 15–P21] were anesthetized with isoflurane (Baxter Healthcare) and rapidly decapitated. Hippocampi were quickly removed and homogenized with a rotor-stator homogenizer in ice-cold 320 mM sucrose solution supplemented with protease inhibitor mixture and PMSF (1 mM) (Sigma-Aldrich). Hippocampi that were not used immediately were snap-frozen in liquid nitrogen and kept at –80°C until further use. Following homogenization and centrifugation (1000 × g, 10 min at 4°C) of the hippocampi, the supernatant was collected and further centrifuged

(16,100 × g, 45 min at 4°C) to isolate a crude membrane fraction. The resultant pellet was resuspended in 5% SDS RIPA buffer (Sigma-Aldrich). Protein concentration was then determined using the BCA assay (Thermo Fisher Scientific). A total of 200–400 μg of protein per lane was separated on reducing 7.5–10% SDS-polyacrylamide gels and transferred to PVDF membranes. Following transfer to PVDF membranes and before incubation with primary antibodies, we cut the membranes in half between the 75 and 50 kDa molecular protein standards (Liu and Fagotto, 2011). The higher molecular weight-containing membranes were probed with rabbit anti-KCNQ2 (1:200; Sigma-Aldrich), anti-KCNQ3 (1:200; Alomone Labs), or anti-KCNQ5 (1:200; Santa Cruz Biotechnology), whereas the lower molecular weight-containing membranes were probed with anti-β-actin (1:5000; Sigma-Aldrich). On occasion, PVDF membranes were stripped using the NewBlot PVDF stripping buffer (Li-Cor Biosciences) and reprobed. Protein was detected by infrared-labeled secondary antibodies (Thermo Fisher Scientific). Specifically, we visualized the mouse anti-β-actin with a goat anti-mouse fluorophore 680-conjugated secondary antibody, whereas the anti-KCNQs were visualized with an anti-rabbit fluorophore 800-conjugated secondary antibody. Both membranes were scanned simultaneously using the Odyssey imaging system (Li-Cor Biosciences; two-color Western blot detection with infrared fluorescence). KCNQ protein band intensities were normalized to the β-actin loading control.

Slice preparation and electrophysiology. Transverse slices of the hippocampus were prepared from P15–P20 mice. Briefly, mice were anesthetized using isoflurane (Baxter Healthcare) and rapidly killed by decapitation. The brain was quickly removed and placed in ice-cold cutting solution consisting of the following: 25 mM NaHCO₃, 200 mM sucrose, 10 mM glucose, 2.5 mM KCl, 1.3 mM NaH₂PO₄, 0.5 mM CaCl₂, and 7 mM MgCl₂. The cerebellum, prefrontal lobe, and temporal lobe were removed and 300 μm transverse hippocampal slices, including the surrounding structures, were cut using a vibratome (Microm HM 650 V, Thermo Fisher Scientific). Slices were then transferred to a holding chamber containing artificial CSF (ACSF) consisting of the following: 125 mM NaCl, 26 mM NaHCO₃, 2.5 mM KCl, 1 mM NaH₂PO₄, 1.3 mM MgCl₂, 2.5 mM CaCl₂, and 12 mM glucose. Slices were first equilibrated at 35°C for 30 min and maintained at room temperature (~22°C) for ≥1 h before any electrophysiological recordings. The cutting and holding solutions (ACSF) were saturated with 95% O₂ and 5% CO₂.

All experiments were performed at room temperature. Whole-cell recordings were obtained using electrodes pulled from thin-walled borosilicate glass capillaries having resistances between 2.5 and 4.5 MΩ when filled with recording solution as described below (World Precision Instruments). Pyramidal cells from the CA1 area of the hippocampus were visually identified with infrared differential interference contrast optics using a 60× water-immersion objective lens on an upright microscope (Olympus BX51, Olympus). The internal recording solution for whole-cell recording consisted of the following: 130 mM potassium methylsulfate, 10 mM KCl, 10 mM HEPES, 20 mM inositol, 4 mM NaCl, 4 mM Mg₂ATP, and 0.4 mM Na₄GTP (osmolality, 300–305 mOsm). The pH was adjusted to 7.25–7.3 with KOH. ACSF was used as an internal recording solution for the cell-attached recordings. For recordings of the M current, the extracellular solution also included TTX (500 nM; Alomone Labs), 4-AP (2 mM), CsCl (1 mM), CdCl₂ (100 μM), and apamin (100 nM) to block, respectively, voltage-gated sodium and calcium channels as well as calcium-activated SK channels, HCN channels, and A-type potassium channels (Sigma-Aldrich). CNQX (4 μM), APV (10 μM), and picrotoxin (100 μM) (Abcam) were present in experiments to block AMPA-mediated, NMDA-mediated, and GABA-mediated synaptic transmission, respectively (with the exception of the data presented in Figs. 2A, 5B). A calculated liquid junction potential of –6.5 mV was used when measuring the resting membrane potential (RMP) and AP threshold. RMP was measured in current clamp with zero holding current. AP threshold was defined as the membrane potential at which the AP rate of rise reached 50 mV/ms. All recordings were performed using a Multi-clamp 700B amplifier (Molecular Devices), low-pass-filtered at 2 kHz, and sampled at 10 kHz. Data were analyzed offline using Axograph X (Axograph), Clampfit (Molecular Devices), Origin (OriginLab), or Spike2 (Cambridge Electronic Design) software.

Figure 1. Contrasting the effects of *Kcnq2* and *Kcnq3* conditional deletion on survival and ECoG activity. **A**, Targeting strategy for generation of *Kcnq2^{fl/fl}* and *Kcnq3^{fl/fl}* mice. Targeted exons (red, *Kcnq2*; blue, *Kcnq3*) were flanked by two loxP sites. Inset, PCR validating *Kcnq2^{fl/fl}* and *Kcnq3^{fl/fl}* genotypes. Cerebral cortex deletion was achieved by crossing *Kcnq2^{fl/fl}* and *Kcnq3^{fl/fl}* with *Emx1-ires-cre* (*Emx1*) mice. **B**, Immunoblot analysis of hippocampal membrane fractions of KCNQ2 or KCNQ3 from cerebral cortex-specific *Kcnq2 cKO* and *Kcnq3 cKO* mice. Levels of KCNQ2 and KCNQ3 are reduced by ~80% in the *Kcnq2 cKO* ($n = 8$) and *Kcnq3 cKO* ($n = 7$) mice, respectively. **C**, Survival curves of control (black, $n = 10$), *Kcnq2 cKO* (red, $n = 17$), and *Kcnq3 cKO* (blue, $n = 8$) mice. The survival graphs show that most *Kcnq2 cKO* mice die prematurely between P15 and P20. Control and *Kcnq3 cKO* mice did not die during the same time period. **D**, Left, Representative ECoG recordings from control, *Kcnq2 cKO*, and *Kcnq3 cKO* mice. Representative polyspike events were indicated by asterisk from *Kcnq2 cKO* and shown in extended time scale. Right, Polyspike events were counted every 30 s during the first 15 min of recordings from control (black, $n = 5$; P15–P19), *Kcnq2 cKO* (red, $n = 4$, P15–P19), and *Kcnq3 cKO* (blue, $n = 5$, P16–P19) mice.

Electrocorticography recordings. Subdural EEG electrodes were implanted in young mice (P15–P19). The recording electrodes used were modified with machine contact gold-plated miniature dip sockets 250 μ m in diameter (Newark Electronics). Two electrodes were implanted in each pup relative to bregma: one was placed anterior 2 mm, left 1 mm (approximately the level of frontal cortex olfactory bulb border), and the other was placed posterior 1 mm, right 1 mm, within the somatosensory cortex. The electrodes were implanted at a depth of ~0.7 mm from the skull surface. Electrodes were secured to the skull with cyanoacrylic (Vet-Bond) and dental cement. Differential voltage signals with the frontal left electrode as reference were amplified 1000 $\times$ with a DAM-50 differential amplifier (1 Hz low filter, 10 kHz high filter). Amplified signals were digitized at 5 kHz, acquired, and displayed continuously (Labchart 7, ADInstruments). EEGs were recorded immediately after pups recovered from anesthesia and recordings were made for 1–2 h. We analyzed data from recordings in which the investigator was blind to the genotype. We considered polyspike episodes events that exhibited ≥ 2 spikes followed by a wave.

Statistical analysis. Data are displayed as means $\pm$ SEM, and significance was determined with either one-way ANOVA or two-way repeated-measures ANOVA using either OriginLab or Prism (Graphpad). The n values indicate number of cells.

Results

Deletion of *Kcnq2*, but not of *Kcnq3*, from cerebral cortical pyramidal neurons leads to cortical hyperexcitability and early death

To overcome the perinatal lethality of constitutive *Kcnq2* knock-out mice and minimize any secondary effects of deleting KCNQ channels throughout the nervous system, we used the Cre/loxP system to generate conditional *Kcnq2* and *Kcnq3* knock-out mice. In the Cre/loxP system, controlled expression of Cre recombinase allows for recombination of two loxP sites, thus excising the intervening genomic sequence. We developed mice in which exons 2–5 of the *Kcnq2* gene and exons 2–4 of the *Kcnq3* gene are flanked by loxP sites and confirmed the floxed sequences by PCR (*Kcnq2^{fl/fl}* and *Kcnq3^{fl/fl}* mice; Fig. 1A). Cre excision of the floxed exons both removes the pore regions of the KCNQ2 and KCNQ3 channels and introduces a frame-shift mutation. If translation of the altered mRNAs occurs, only the very N-terminal regions of the channels would be translated.

To generate pyramidal neuron-specific *Kcnq2* conditional knock-out (*Kcnq2 cKO*) or *Kcnq3* conditional knock-out (*Kcnq3 cKO*) mice, we crossed the *Kcnq2^{fl/fl}* and *Kcnq3^{fl/fl}* mice to *Emx1-ires-cre* (*Emx1*) mice, which express Cre recombinase in neocortical and hippocampal pyramidal neurons during neurogenesis (embryonic day 10.5; Gorski et al., 2002). Conditional deletion of either *Kcnq2* or *Kcnq3* led to a significant reduction in KCNQ2 ($76 \pm 6.4\%$, $n = 8$) and KCNQ3 ($76 \pm 7.0\%$, $n = 7$) protein levels, respectively, in hippocampal membrane fractions of conditional knock-out compared with control mice (Fig. 1B). Previous work has shown that *Emx1*-targeted Cre recombinase leads to excision of floxed sequences in $\sim 88\%$ of pyramidal neurons, consistent with the *Emx1* expression pattern (Gorski et al., 2002; Ballester-Rosado et al., 2010). Considering that most KCNQ2 and KCNQ3 protein in the cerebral cortex is expressed by pyramidal neurons, but that these channels are also present in some parvalbumin-expressing and somatostatin-expressing interneurons (Cooper et al., 2001; Lawrence et al., 2006; Nieto-Gonzalez and Jensen, 2013), our observations are in agreement with predictions for the *Emx1-Cre* line.

Constitutive *Kcnq2* knock-out mice die soon after birth (Watanabe et al., 2000), while constitutive *Kcnq3* knock-out mice survive into adulthood (Tzingounis and Nicoll, 2008). In the case of *Kcnq3*, we found that, similar to the constitutive knock-out, *Kcnq3 cKO* mice also lived into adulthood (Fig. 1C). In contrast, conditional deletion of *Kcnq2* permitted survival until the third week of life (Fig. 1C). During the course of our studies, we witnessed a *Kcnq2 cKO* mouse that underwent a severe tonic-clonic seizure followed by premature death. Closer observation of other *Kcnq2 cKO* mice revealed unusual behaviors that could be consistent with seizures, such as rearing, jumping, and falling. No such behaviors were observed with either *Kcnq2* heterozygous or *Kcnq3 cKO* mice.

To directly inspect for aberrant cerebral cortical activity, we performed surface electrocorticography recordings (ECoGs) from control, *Kcnq2 cKO*, and *Kcnq3 cKO* mice. We then analyzed the initial 15 min of the recordings blind to the genotype. ECoGs from *Kcnq2 cKO* mice exhibited episodes of polyspike events (Fig. 1D) reaching to ~ 100 events within the first 15 min (92 ± 15 events, $n = 4$) compared with hardly any events in control and *Kcnq3 cKO* mice (control: 3.4 ± 3 events, $n = 5$; *Kcnq3 cKO*: 2.4 ± 1.2 events, $n = 5$). The polyspike events showed multiple repetitive spikes typically followed by a wave of variable duration (Fig. 1D). Episodes lasted between 100 and 300 ms and occurred with an average frequency of 6.1 ± 1 events per minute ($n = 4$ mice). These data indicate that deletion of KCNQ2 channels leads to elevated cortical excitability. Such hyperexcitability might be due to an imbalance in excitatory and inhibitory network activity caused by the selective removal of *Kcnq2* from

Figure 2. Contrasting the effects of *Kcnq2* and *Kcnq3* conditional deletion on the M current and mAHP. **A**, The M-current protocol and representative M-current traces. The M current was induced by a step hyperpolarization (1.5 s) to -55 mV from a holding potential of -20 mV in pyramidal neurons from either control, *Kcnq2 cKO*, or *Kcnq3 cKO* mice before (black) and after (gray) application of $20 \mu\text{M}$ XE991. Far right, Summary graph showing the effects of deleting either *Kcnq2* or *Kcnq3* on the M current ($n = 8-9$). **B**, A representative mAHP followed by a 50 ms current injection (1 nA) in pyramidal neurons from either control, *Kcnq2 cKO*, or *Kcnq3 cKO* mice. Far right, Summary graph showing the effects of deleting either *Kcnq2* or *Kcnq3* on the mAHP ($n = 12$). Membrane potential was kept at -60 mV by injecting a small DC current through the recording pipette. **C**, A representative ADP induced by a 2 ms 1 nA current injection in pyramidal neurons from either control, *Kcnq2 cKO*, or *Kcnq3 cKO* mice. Far right, Summary graph showing the effects of deleting either *Kcnq2* or *Kcnq3* and ADP ($n = 11-21$). Membrane potential was kept at -60 mV by injecting a small DC current through the recording pipette. Data shown are means $\pm$ SEM. Statistical significance was determined by one-way ANOVA (* $p < 0.05$; ** $p < 0.001$, *** $p < 0.0005$), using Tukey's *post hoc* test.

pyramidal neurons, and may contribute to the postnatal lethality of *Kcnq2 cKO* mice.

Differential dependence of the M current and mAHP on KCNQ2 and KCNQ3

We examined the effect of deleting KCNQ2 and KCNQ3 channels on pyramidal neuron excitability, focusing on hippocampal CA1 pyramidal neurons, because they are a good model system for cerebral cortex KCNQ channel activity (Peters et al., 2005; Otto et al., 2006; Singh et al., 2008). First, we determined whether the M current, a well established KCNQ2/3 current, was decreased in the conditional knock-out mice. The M current was obtained by first voltage-clamping neurons to -20 mV to open KCNQ channels and inactivate delayed rectifier potassium channels, followed by hyperpolarization to -55 mV to deactivate KCNQ channels. This standard M-current protocol was then repeated in the presence of $20 \mu\text{M}$ XE991, a concentration that leads to an almost complete block of KCNQ2/3 channels (Wang et al., 1998). The M current was then defined as the XE991-sensitive difference current (Fig. 2A). Deletion of either *Kcnq2* or *Kcnq3* led to a substantial loss of the M current (~ 85 and $\sim 50\%$ respectively; controls, 104 ± 15 pA, $n = 8$; ***Kcnq2 cKO*, 15 ± 9 pA, $n = 9$; **Kcnq3 cKO*, 49 ± 13 pA, $n = 9$; * $p < 0.05$ ** $p < 0.001$ vs controls, ANOVA-Tukey's *post hoc* test; Fig. 2A). This finding is consistent with

Figure 3. Elevated neuronal excitability of CA1 pyramidal neurons in *Kcnq2*-null but not *Kcnq3*-null neurons. **A**, Voltage responses to various current injection steps (1 s) in pyramidal neurons from either control ($n = 18$), *Kcnq2* cKO ($n = 18$), or *Kcnq3* cKO ($n = 18$) mice. Membrane potential was kept at -60 mV by injecting a small DC current through the recording pipette. Representative traces showing the effects of deleting either *Kcnq2* (red) or *Kcnq3* (blue) on pyramidal neuron excitability. **B**, Summary graphs showing the effect of *Kcnq2* or *Kcnq3* deletion on CA1 pyramidal neuron AP number, initial firing frequency, and final firing frequency. Data shown are means $\pm$ SEM. Statistical significance was determined by two-way repeated-measures ANOVA ($*p < 0.05$).

previous studies indicating that the M current is mediated by both KCNQ2 and KCNQ3 channels (Wang et al., 1998).

We next examined the contributions of KCNQ2 and KCNQ3 to the mAHP, a conductance that also requires the presence of KCNQ2/3 heteromers (Peters et al., 2005). We elicited the mAHP using a large depolarizing pulse (50 ms, 1 nA) in neurons current-clamped close to their RMP (Fig. 2B). To avoid any possible contamination from calcium-activated SK channels, the SK channel blocker apamin (100 nM) was included in the extracellular solution. An mAHP developed in control mice following the termination of the 50 ms depolarizing step, and *Kcnq3* cKO pyramidal neurons had an mAHP of similar peak amplitude (Fig. 2B). In contrast, deletion of *Kcnq2* led to a $>50\%$ reduction of the mAHP (Fig. 2B; control, 4.8 ± 0.6 mV, $n = 12$; *Kcnq2* cKO, 2.1 ± 0.3 mV, $n = 12$; *Kcnq3* cKO, 4.2 ± 0.6 mV, $n = 12$; $**p < 0.001$ vs control, ANOVA-Tukey's *post hoc* test). This differential contribution of KCNQ2 and KCNQ3 was unexpected given their similar role in the M current. Therefore, we also measured the

mAHP with a different protocol, using a depolarization pulse (100 ms) of a magnitude adjusted to elicit 4–5 APs. Again we found that the mAHP in *Kcnq2* cKO mice was significantly reduced, unlike the mAHP in *Kcnq3* cKO mice (control, 4.4 ± 1 mV, $n = 8$; *Kcnq2* cKO, 1.4 ± 0.4 mV, $n = 9$; *Kcnq3* cKO, 3.7 ± 0.3 mV, $n = 8$; $**p < 0.001$ vs control, ANOVA-Tukey's *post hoc* test). Last, we repeated our mAHP experiments using a protocol that controlled for both the number and timing of APs. We induced five APs, each with a short 2 ms pulse in either 20 ms (50 Hz) or 100 ms intervals (100 Hz). As with our previous measurements, we found that the mAHP critically depends on the presence of KCNQ2 channels, but not of KCNQ3 channels. The reduction in the mAHP seen in *Kcnq2* cKO was independent of the stimulation frequency (50 Hz: control, 2.2 ± 0.1 mV, $n = 6$; *Kcnq2* cKO, 0.8 ± 0.3 mV, $n = 7$; *Kcnq3* cKO, 3.0 ± 0.4 mV, $n = 8$; $**p < 0.005$ vs control; 100 Hz: control, 2.4 ± 0.2 mV, $n = 6$; *Kcnq2* cKO, 1.2 ± 0.3 mV, $n = 7$; *Kcnq3* cKO, 2.9 ± 0.45 mV, $n = 8$; $**p < 0.01$ vs control, ANOVA-Tukey's *post hoc* test).

In CA1 pyramidal neurons, a prominent ADP develops following an AP elicited by a brief suprathreshold stimulus (Bean, 2007). Because the ADP is limited by the mAHP (Yue and Yaari, 2004, 2006), we predicted that the ADP would be of greater duration in *Kcnq2* cKO neurons compared with those lacking *Kcnq3*. We current-clamped CA1 pyramidal neurons close to their RMPs and injected a brief current pulse (2 ms, 1 nA) to elicit a single AP followed by an ADP. Indeed, there was an almost twofold increase in the ADP in *Kcnq2* cKO mice compared with control mice (Fig. 2C). In contrast, the ADP was not significantly increased in *Kcnq3* cKO mice; if anything, it tended to be smaller than that of controls (control, 225 ± 83 mV*ms, $n = 21$; **Kcnq2* cKO, 526 ± 70 mV*ms, $n = 19$; *Kcnq3* cKO, 33 ± 90 mV*ms, $n = 11$; * $p < 0.05$ vs control, ANOVA-Tukey's *post hoc* test). This is likely due to the faster repolarization time course of the ADP in *Kcnq3* cKO mice (ADP_{decay}: control, 17 ± 2.2 ms, $n = 18$; *Kcnq2* cKO, 25 ± 1.5 ms, $n = 19$; **Kcnq3* cKO, 12 ± 1.8 ms, $n = 11$; * $p < 0.05$ vs control, ANOVA-Tukey's *post hoc* test). These data demonstrate that the mAHP is highly dependent upon the presence of KCNQ2 channels, while the remaining KCNQ3 channels cannot fully compensate for the loss of KCNQ2 channels. They also demonstrate that, unlike the M current, the mAHP does not depend upon KCNQ3 channels.

Loss of *Kcnq2*, but not *Kcnq3*, leads to elevated neuronal excitability

The differential sensitivity of the mAHP to deletion of *Kcnq2* versus *Kcnq3* suggested that loss of KCNQ2 or KCNQ3 channels might have different effects on neuronal excitability. Therefore, we compared the excitability of CA1 pyramidal neurons from control, *Kcnq2* cKO, and *Kcnq3* cKO mice by examining the number of APs elicited by current injections of various amplitudes (1 s, 0 to +250 pA). Increasing the amplitude of the injected current led to a greater number of APs in all genotypes (Fig. 3). However, the number of APs was significantly greater in *Kcnq2*-null neurons compared with either control or *Kcnq3*-null neurons. As shown in Figure 3A, pyramidal neurons from *Kcnq2* cKO mice fired throughout the duration of the depolarizing pulse, signifying a decreased level of spike frequency adaptation. When quantified, these *Kcnq2*-deficient neurons were found to have both a greater initial firing frequency and a greater final firing frequency compared with either control or *Kcnq3*-null neurons (Fig. 3B).

Intrinsic membrane properties in *Kcnq2* cKO and *Kcnq3* cKO mice

In addition to the reduced mAHP, a change in the intrinsic membrane properties of *Kcnq2* cKO pyramidal neurons might also

Figure 4. Contrasting the effects of *Kcnq2* and *Kcnq3* deletion on CA1 pyramidal neuron intrinsic properties. **A**, Left, Voltage responses to various current injection steps (1 s from 0 to -100 pA) in pyramidal neurons from either control, *Kcnq2* cKO, or *Kcnq3* cKO mice. Membrane potential was kept at -60 mV by injecting a small DC current through the recording pipette. Right, Summary graphs showing the effect of deleting *Kcnq2* or *Kcnq3* on the R_N ($n = 21$ – 26). R_N was determined by the slope of the line fitted to voltage versus current relationship. **B**, Left, Voltage responses to a current injection ramp (2.5 s ramp from -100 to $+200$ pA; 0.12 nA/s) recorded in pyramidal neurons from either control, *Kcnq2* cKO, or *Kcnq3* cKO mice. Dashed boxed area is shown in extended time scale to show a burst of APs from *Kcnq2* cKO mice. Far right, Summary graph showing the effects of deleting *Kcnq2* or *Kcnq3* in the R_N ($n = 7$ – 9). R_N was measured from the slope between -63 mV and the voltage before the first AP. **C**, Representative single APs recorded for each genotype of CA1 pyramidal neurons elicited by 1 nA 2 ms current injection. Inset shows the first derivative of the AP from the different genotypes. Right, Summary graphs showing the effects of deleting *Kcnq2* or *Kcnq3* on the AP threshold ($n = 12$ – 15). For the box plots, the central line represents the median value, the boundaries of the box represent the SD, the square symbol represents the mean value, and whiskers represent the minimum and maximum data value. Statistical significance was determined by one-way ANOVA (* $p < 0.05$) using Tukey's *post hoc* test.

contribute to their increased excitability. Indeed, pharmacologically blocking KCNQ channels in wild-type neurons increases their input resistance (R_N), which in turn increases neuronal excitability (Yue and Yaari, 2004; Shah et al., 2008). We determined the R_N of *Kcnq*-null neurons by giving a series of hyperpolarizing current injections to pyramidal neurons from control, *Kcnq2* cKO, and *Kcnq3* cKO mice (Fig. 4A). No significant changes in the R_N were observed between the different genotypes (R_N : control, 427 ± 18 M Ω , $n = 26$; *Kcnq2* cKO, 462 ± 17 M Ω , $n = 23$; *Kcnq3* cKO, 462 ± 23 M Ω , $n = 21$; $p > 0.05$, ANOVA; Fig. 4A). Given that KCNQ channels are primarily active close to spike threshold, we also measured R_N using a slow depolarizing ramp protocol (0.12 nA/s; Hu et al., 2007). As seen in Figure 4B, neurons from control, *Kcnq2* cKO, and *Kcnq3* cKO mice had similar R_N 's up to AP threshold (R_N : control, 215 ± 4 M Ω , $n = 7$; *Kcnq2* cKO, 185 ± 10 M Ω , $n = 9$; *Kcnq3* cKO, 156 ± 4 M Ω , $n = 7$; $p > 0.05$, ANOVA). Consistent with their increased excitability, loss of KCNQ2 channels led pyramidal neurons to fire first in bursts when the depolarization ramp surpassed spike threshold (2.7 ± 0.2 APs/burst; $n = 9$; Fig. 4B). This is likely due to the loss of the mAHP and associated increased ADP in *Kcnq2*-null neurons.

Figure 5. Contrasting the effects of deleting *Kcnq2* and *Kcnq3* on KCNQ channel levels and activity. **A**, Left, Hippocampal membrane fractions from the indicated mouse lines were probed with antibodies against KCNQ2, KCNQ3, or KCNQ5 channels. Western blots were also probed with a β -actin antibody as a protein loading control (detected between the 37 and 50 kDa molecular weight standards; predicted molecular weight is 42 kDa). Right, Summary graph showing the change in KCNQ protein level in *Kcnq2* cKO or *Kcnq3* cKO mice in relation to control mice ($n = 3-8$). **B**, Sample traces of tonic firing activity in CA1 pyramidal neurons from the indicated mouse lines in the background presence of 8.5 mM $[K^+]_o$, and following application of either retigabine (Ret; 20 μ M), ICA-27243 (ICA; 25 μ M), or XE991 (20 μ M). Left, Summary graph of firing rate changes induced by retigabine or ICA-27243 in either control, *Kcnq2* cKO, or *Kcnq3* cKO mice ($n = 7-12$). Cell-attached recordings took place in the voltage-clamp configuration. Data shown are means $\pm$ SEM. Statistical significance was determined by one-way ANOVA ($*p < 0.05$) using Dunnett's *post hoc* test.

However, we were surprised to find that the threshold voltage for AP generation was shifted to more depolarized values in *Kcnq2* cKO mice (control, -53 ± 1.2 mV, $n = 15$; *Kcnq2* cKO, -49 ± 1.1 mV, $n = 12$; *Kcnq3* cKO, -54 ± 0.6 mV, $n = 11$; $*p < 0.05$, ANOVA-Tukey's *post hoc* test; Fig. 4C), an effect opposite of that predicted for the loss of a potassium channel. This shift was not accompanied by a decrease in AP amplitude (control, 105 ± 1.0 mV, $n = 15$; *Kcnq2* cKO, 103 ± 1.3 mV, $n = 12$; *Kcnq3* cKO, 103 ± 1.2 mV, $n = 11$; $p > 0.05$, ANOVA) or in a decrease in the maximum rate of AP activation (control, 265 ± 9 mV/ms, $n = 15$; *Kcnq2* cKO, 258 ± 10 mV/ms, $n = 12$; *Kcnq3* cKO, 255 ± 8 mV/ms, $n = 11$; $p > 0.05$, ANOVA), suggesting that altered properties of voltage-gated sodium channels did not underlie the shift.

Two other intrinsic membrane properties were unchanged: (1) the RMP (control, -63 ± 1.0 mV, $n = 28$; *Kcnq2* cKO, $-63 \pm$

0.6 mV, $n = 24$; $p = 0.83$; *Kcnq3* cKO, -65 ± 0.8 , $n = 23$; $p > 0.05$, ANOVA-Tukey's *post hoc* test), and (2) hyperpolarization activation current activity, as measured by the voltage sag that develops during a large hyperpolarizing step (sag ratio for -100 pA: control, 0.62 ± 0.01 , $n = 26$; *Kcnq2* cKO, 0.60 ± 0.01 , $n = 23$; *Kcnq3* cKO, 0.63 ± 1.3 , $n = 21$; $p > 0.05$, ANOVA). These data indicate that intrinsic membrane properties are largely unchanged by the loss of either *Kcnq2* or *Kcnq3*.

Differential dependence of global KCNQ channel activity on *Kcnq2* and *Kcnq3*

Although KCNQ2 and KCNQ3 are primarily expressed as KCNQ2/3 heteromers in neurons, they can also form homomers in heterologous cells to varying extents (Wang et al., 1998; Tatulian et al., 2001). The distinctive cellular phenotypes of *Kcnq2*-null and *Kcnq3*-null neurons raised the issue of the extent to which loss of one channel impairs expression of the other. To explore this question, we assayed the remaining KCNQ subunit protein levels in *Kcnq2* cKO and *Kcnq3* cKO mice by quantitative fluorescent Western blotting. We found that in hippocampal membrane fractions from *Kcnq2* cKO mice, both KCNQ2 and KCNQ3 protein levels were greatly reduced by ~ 75 and $\sim 60\%$, respectively (Fig. 5A). Importantly, despite the presence of $\sim 40\%$ of the normal KCNQ3 protein levels in *Kcnq2* cKO mice, KCNQ3 seems to contribute little to the M current, as the M current is reduced by $\sim 85\%$ in *Kcnq2* cKO mice. We should also note that our membrane isolation protocol does not distinguish between plasma membrane fractions and endoplasmic reticulum (ER) membrane fractions. Therefore, the remaining KCNQ3 protein might reflect KCNQ3 residing in the ER, and explain why there is a greater loss of the M current.

In addition to KCNQ2 and KCNQ3 channels, pyramidal neurons also express KCNQ5 channels, which can either function as homomers or as heteromers with KCNQ3 channels (Lerche et al., 2000; Schroeder et al., 2000). Unexpectedly, we also observed a reduction of KCNQ5 protein levels in *Kcnq2* cKO mice ($\sim 40\%$ reduction; Fig. 5A). In contrast, we only observed a modest decrease in KCNQ2 and KCNQ5 protein levels (~ 15 and $\sim 20\%$ reduction, respectively) in hippocampi from *Kcnq3* cKO mice, despite $\sim 75\%$ loss of KCNQ3. These results indicate that loss of KCNQ2 channels leads to a substantial reduction of all KCNQ channels in the hippocampus, while loss of KCNQ3 channels does not.

To corroborate the biochemical results, we determined the effects of KCNQ-specific allosteric activators on the spontaneous firing of CA1 pyramidal neurons. The use of allosteric activators was necessary as no KCNQ subtype-selective inhibitors are avail-

able. If there is indeed a substantial decrease in KCNQ channels in *Kcnq2 cKO* and *Kcnq3 cKO* mice, these activators should be less able to inhibit tonic firing. For these experiments, we first used the cell-attached recording configuration to prevent any rundown of KCNQ-channel activity during prolonged recordings. Furthermore, the extracellular potassium concentration was increased from 2.5 to 8.5 mM to shift the potassium equilibrium potential to more depolarized values, leading to a higher tonic AP firing rate and enhancing the role of all KCNQ channels in regulating the pyramidal neuron firing rate (Vervaeke et al., 2006). These conditions increased the sensitivity of our measurements.

First, we used retigabine. Recently approved by the Food and Drug Administration, retigabine is a KCNQ channel agonist that activates KCNQ2, KCNQ3, and KCNQ5 channels. As with previous studies (Yue and Yaari, 2006; Hu et al., 2007), application of retigabine (20 μ M) completely inhibited the tonic firing of pyramidal neurons in control mice (Fig. 5B). In contrast, retigabine was much less effective in blocking the pyramidal neuron tonic firing activity in *Kcnq2 cKO* mice, and slightly less effective in *Kcnq3 cKO* mice (percentage inhibition: control, $99.0 \pm 1\%$, $n = 8$; **Kcnq2 cKO*, $39 \pm 8\%$, $n = 12$; **Kcnq3 cKO*, $71 \pm 9\%$, $n = 9$; * $p < 0.05$ vs control, ANOVA-Dunnett's *post hoc* test). This finding is consistent with our immunoblot data that showed a greater reduction of all KCNQ channels in *Kcnq2 cKO* mice compared with *Kcnq3 cKO* mice.

Next, we used the KCNQ2-selective agonist ICA-27243 (25 μ M), which activates KCNQ2 homomeric and heteromeric channels (Wickenden et al., 2008; Blom et al., 2010). As with retigabine, ICA-27243 silenced pyramidal neurons in control mice (Fig. 5B). In contrast, ICA-27243 did not inhibit tonic AP firing in *Kcnq2*-null neurons, but instead increased firing (Fig. 5B). This confirms that the inhibitory effects of ICA-27243 are due to the presence of KCNQ2 channels and indicates that ICA-27243 acts on a different target to depolarize neurons. We then used ICA-27243 as a probe for the presence of KCNQ2 channels in *Kcnq3 cKO* mice. Consistent with our biochemical data showing retention of KCNQ2 channels, ICA-27243 substantially reduced tonic firing rate in *Kcnq3 cKO* mice (percentage inhibition: control, $99.5 \pm 0.2\%$, $n = 8$; *Kcnq3 cKO*, $74 \pm 6\%$, $n = 7$; Fig. 5B).

To confirm the presence of KCNQ2 channels in the *Kcnq3*-null neurons using ICA-27243 free from any complications arising from nonspecific changes to membrane potential, we assessed its effect in current-clamp whole-cell configuration. We compared the firing properties of CA1 pyramidal neurons from control, *Kcnq2 cKO*, and *Kcnq3 cKO* mice by examining the number of APs elicited by 1 s current injections of various amplitudes and

Figure 6. The KCNQ2 allosteric activator ICA-27243 inhibits AP firing in *Kcnq3 cKO* but not *Kcnq2 cKO* mice. **A**, Voltage responses to various current injection steps (1 s) in pyramidal neurons from either control, *Kcnq2 cKO*, or *Kcnq3 cKO* mice. Membrane potential was kept at -60 mV by injecting a small DC current through the recording pipette. Representative traces showing the effects of deleting either *Kcnq2* (red) or *Kcnq3* (blue) on pyramidal neuron excitability and in the presence or absence of ICA-27243 (25 μ M). **B**, Summary graphs showing the effect of ICA-27243 on pyramidal neuron AP number in control ($n = 6$), *Kcnq2 cKO* ($n = 7$), or *Kcnq3 cKO* ($n = 6$) mice. Data shown are means $\pm$ SEM. Statistical significance was determined by two-way repeated-measures ANOVA (* $p < 0.05$).

by holding the membrane potential at -60 mV. As shown in Figure 6, the number of APs before and after application of ICA-27243 did not change in *Kcnq2*-null neurons, unlike ICA-27243-treated pyramidal neurons in control and *Kcnq3 cKO* mice. This inhibition suggests that homomeric KCNQ2 channels are present and are readily engaged by KCNQ2 allosteric activators in the absence of KCNQ3 channels.

Discussion

The present study shows that KCNQ2 channels are indispensable for pyramidal neurons to control their excitability and can function independently of KCNQ3. Our work shows that loss of KCNQ2 channels leads to premature postnatal death and cortical hyperexcitability. The elevated CA1 pyramidal neuronal activity in *Kcnq2 cKO* mice is likely due to the severe concomitant reduction of KCNQ3 and KCNQ5 protein levels, smaller mAHP, and prolonged ADP. In contrast, *Kcnq3 cKO* mice are viable and show little evidence of elevated neuronal activity in CA1 pyramidal neurons. Loss of KCNQ3 channels leads to a modest loss of KCNQ2 and

KCNQ5 levels, and the use of KCNQ2 allosteric activators demonstrates that functional KCNQ2 channels remain in these mice. These findings clarify the role of KCNQ2 and KCNQ3 channels in controlling pyramidal neuron excitability, and may thereby give insight into their relative contributions to human pediatric epilepsies.

Homeostatic adaptations in the absence of KCNQ2 or KCNQ3 channels

We did not find any significant changes to either the RMP or R_N in neurons lacking KCNQ2 or KCNQ3, which is consistent with previous findings in mice with loss-of-function mutations in *Kcnq2* or *Kcnq3* and mice expressing dominant-negative KCNQ subunits (Peters et al., 2005; Singh et al., 2008). However, multiple pharmacological studies have shown that XE991, a selective KCNQ channel inhibitor, depolarizes neurons, allowing them to reach AP threshold quicker and more frequently (Yue and Yaari, 2004; Hu et al., 2007; Shah et al., 2008). The absence of changes to RMP and R_N in KCNQ-mutant mice therefore suggests that there may be homeostatic adaptations of intrinsic membrane properties in the absence of normally functioning KCNQ channels. This is supported by our observation that AP threshold was shifted to more depolarized membrane potentials in KCNQ2-null neurons, a direction counter to what one would expect for reducing a resting potassium conductance.

Homeostatic adaptation might also explain the near-normal mAHP and firing properties in the absence of KCNQ3 channels. KCNQ2/3 channels mediate the mAHP, a potassium-mediated hyperpolarization that typically activates following a brief surge of spiking activity (Storm, 1989; Peters et al., 2005). The mAHP is the primary limit on the ADP that follows AP firing, and it also contributes to spike frequency adaptation. Similar to the study of Peters et al. (2005) on the effect of KCNQ2 dominant-negative channels, we find that deletion of *Kcnq2* from pyramidal neurons significantly reduces the mAHP, and thus prolongs the ADP and results in less spike frequency adaptation. In contrast, loss of KCNQ3 does not decrease the mAHP and, if anything, enhances the mAHP based on the significant acceleration of the ADP repolarization. These data indicate that although the mAHP is mediated by KCNQ2/3 heteromers in wild-type mice, homeostatic adaptations can support near-normal levels of the mAHP in the absence of KCNQ3, but not KCNQ2.

Like the mAHP, the M current is a potassium conductance primarily mediated by KCNQ2/3 heteromeric channels and is extremely sensitive to the loss of KCNQ2. However, in contrast to the mAHP, we find that the M current is also sensitive to the loss of KCNQ3. For this conductance, other channels seem to only partially compensate for the loss of KCNQ3. Further studies will be necessary to fully understand the differential compensatory changes enacted to maintain the mAHP and M current after loss of KCNQ subunits.

In contrast to our current findings, we previously reported that loss of KCNQ3 channels does not impair M-current levels in CA1 pyramidal neurons (Tzingounis and Nicoll, 2008). One major difference between the two studies is the use of conditional versus constitutive *Kcnq3* knock-out mice. In constitutive *Kcnq3* knock-out mice, *Kcnq3* deletion occurs throughout the nervous system and development, unlike the conditional knock-out mice used in the current study. Consequently, global versus conditional *Kcnq3* deletion may engage different compensatory programs at the network level, leading to different effects on the M current.

Trafficking differences may explain differential sensitivity to loss of KCNQ2 versus KCNQ3

Nearly all of our experiments suggest that KCNQ2 channels, but not KCNQ3 channels, are required for pyramidal neurons to control their excitability. Although some of these differences may be due to divergent compensatory mechanisms at either the cellular or network level, differences in KCNQ2 versus KCNQ3 trafficking may explain some or all of the phenotypic disparities.

Our Western blotting results indicate that KCNQ3 protein levels critically depend on KCNQ2 channels, whereas KCNQ2 levels only modestly rely on KCNQ3 channels in hippocampal neurons. This suggests that KCNQ3 channels may be unable to exist as homomers, whereas KCNQ2 homomeric channels may functionally compensate for the loss of KCNQ2/3 channels. This interpretation is consistent with our pharmacological experiments demonstrating functional KCNQ2-containing channels in *Kcnq3*-null neurons. Prior studies on the ability of KCNQ2 channels to traffic and function as homomers have given mixed results. A report in heterologous cells showed that coexpression of KCNQ2 and KCNQ3 channels primarily leads to increased surface expression of KCNQ3 channels, but not of KCNQ2 channels (Surti et al., 2005). However, several others have reported that robust surface expression of exogenous KCNQ2 required coexpression with KCNQ3 channels in heterologous systems and in cultured neurons (Schwake et al., 2000; Etzeberria et al., 2004; Rasmussen et al., 2007). Our work clarifies this issue by showing that pyramidal neurons sustain the majority of native KCNQ2 channels in the absence of KCNQ3 channels.

Although the mechanism that leads to KCNQ3 protein loss in the absence of KCNQ2 is unknown, it was suggested that KCNQ3 channels have an ER retention motif that is masked by the presence of KCNQ2 channels (Nakajo and Kubo, 2008). It is possible that in the absence of KCNQ2 channels, accumulation of KCNQ3 channels in the ER activates the ER-associated degradation response and directs KCNQ3 channels to degradation pathways. KCNQ2 channels have not been shown to have such an ER retention motif; instead, several studies suggest that exiting of KCNQ2 from the ER relies on calmodulin (Gamper et al., 2005; Etzeberria et al., 2008; Alaimo et al., 2009; Kosenko et al., 2012). Thus, KCNQ2 channels may not strictly require KCNQ3 channels for ER exit, and this could explain why we see only modest KCNQ2 protein loss in the absence of KCNQ3. Future work is needed to identify the complete mechanism by which KCNQ2 and KCNQ3 levels are regulated in neurons.

Unexpectedly, there was also a ~40% loss of KCNQ5 protein in *Kcnq2* cKO mice. KCNQ5 and KCNQ2 do not form heteromeric channels (Lerche et al., 2000; Schroeder et al., 2000), and therefore, any effects of KCNQ2 on KCNQ5 should be indirect. KCNQ5 channels have been proposed to function as homomers and KCNQ3/5 heteromers. However, *Kcnq3* cKO mice had only a modest loss of KCNQ5 protein, and thus the larger loss in *Kcnq2* cKO mice is unlikely to be solely due to the reduction of KCNQ3 protein levels. One possibility is that the elevated cortical network activity in *Kcnq2* cKO mice downregulates KCNQ3 and KCNQ5 channels; recent work has shown that seizure activity can decrease KCNQ protein levels in the brain (Maslarova et al., 2013). However, another study found that elevated excitability increases KCNQ2/3 mRNA transcripts (Zhang and Shapiro, 2012). Future studies are needed to clarify how KCNQ protein levels are affected by seizure activity, and whether this mechanism explains the loss of KCNQ5 in *Kcnq2* cKO mice.

Possible clinical implications

It is now well documented that mutations of *Kcnq2* can lead to neurological disorders of varying severity, from BFNCs to neonatal epileptic encephalopathy, Ohtahara syndrome, and infantile spasms (Singh et al., 2003; Millichap and Cooper, 2012; Saitsu et al., 2012; Weckhuysen et al., 2012; Allen et al., 2013). In contrast, disease-causing mutations in *Kcnq3* are much less frequent and have been predominantly associated with BFNCs (Maljevic et al., 2008). Although our conditional *Kcnq2* and *Kcnq3* mice are not exact models of KCNQ channelopathies, they might still provide insight for the prevalence of *Kcnq2*-related pediatric epilepsy disorders.

Our work suggests that *Kcnq2* truncation or trafficking mutations also impair the activity of KCNQ2/3, and possibly of KCNQ5 channels. Such a broad loss of KCNQ channel function is likely to lead to hyperexcitability. Indeed, some epilepsy-associated mutations in *Kcnq2* prevent normal KCNQ2 surface expression (Schwake et al., 2000; Maljevic et al., 2010; Orhan et al., 2013). On the other hand, our work suggests that *Kcnq3* mutations might not significantly reduce KCNQ2 and KCNQ5 channel levels in pyramidal neurons, allowing the remaining KCNQ channels to function and dampen neuronal excitability. Consistent with this hypothesis, truncation or trafficking mutations in *Kcnq3* have not been identified in BFNC patients (Maljevic et al., 2010). Instead, the handful of identified *Kcnq3* mutations in BFNC patients (Maljevic et al., 2010) and in a patient with epileptic encephalopathy (Allen et al., 2013) involve domains important for gating and ion conduction. Such mutations will alter the biophysical properties of KCNQ2/3 channels.

In summary, our work demonstrates that KCNQ2 channels are obligatory components of KCNQ channels in pyramidal neurons and are likely sufficient to maintain pyramidal neuron firing properties at near-normal levels in the absence of KCNQ2/3 channels.

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