Polarized axonal surface expression of neuronal KCNQ channels is mediated by multiple signals in the KCNQ2 and KCNQ3 C-terminal domains

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The M channels, important regulators of neuronal excitability, are voltage-gated potassium channels composed of KCNQ2–5 subunits. Mutations in KCNQ2 and KCNQ3 cause benign familial neonatal convulsions (BFNC), dominantly inherited epilepsy and myokymia. Crucial for their functions in controlling neuronal excitability, the M channels must be placed at specific regions of the neuronal membrane. However, the precise distribution of surface KCNQ channels is not known. Here, we show that KCNQ2/KCNQ3 channels are preferentially localized to the surface of axons both at the axonal initial segment and more distally. Whereas axonal initial segment targeting of surface KCNQ channels is mediated by ankyrin-G binding motifs of KCNQ2 and KCNQ3, sequences mediating targeting to more distal portion of the axon reside in the membrane proximal and A domains of the KCNQ2 C-terminal tail. We further show that several BFNC mutations of KCNQ2 and KCNQ3 disrupt surface expression or polarized surface distribution of KCNQ channels, thereby revealing impaired targeting of KCNQ channels to axonal surfaces as a BFNC etiology.

KCNQ Channels Localize Preferentially on the Axonal Surface. To examine surface and total expression of KCNQ channels in axons and dendrites, we expressed in cultured hippocampal neurons recombinant KCNQ proteins containing an extracellular HA tag (HA-KCNQ), whose surface expression and function in Xenopus oocytes have been demonstrated in ref. 28. Surface HA-KCNQ proteins were labeled with a mouse anti-HA antibody without permeabilization of neurons, whereas total HA-KCNQ proteins were visualized by immunostaining with a rat anti-HA antibody subsequent to neuronal permeabilization. Recapitulating the endogenous channel protein distribution (Fig. 7, which is published as supporting information on the PNAS web site), total HA-KCNQ2/KCNQ3 (Fig. 1 A–C) and HA-KCNQ3/KCNQ2 (Fig. 8 A–C), which is published as supporting information on the PNAS web site) channels resided in soma, dendrites, and axons. In contrast, the surface density of KCNQ channels was higher on axons than soma and dendrites, with the highest concentrations in proximal regions near the soma (Figs. 1 A–C and 8 A–C). In some transfected neurons, surface KCNQ channels were found on distal axons (Figs. 1D and 8D).

To quantify the surface and total expression, we measured the background-subtracted mean intensity of surface HA and total HA fluorescence in axons and dendrites. Axonal surface expression of HA-KCNQ2/KCNQ3 (Fig. 1E) and HA-KCNQ3/KCNQ2 (Fig. 8E) was much higher than HA-KCNQ2 or HA-KCNQ3 alone despite similar levels of the total protein expression in axons and dendrites. In contrast to the robust axonal surface expression, the dendritic surface HA fluorescence intensities of neurons expressing one or both channel subunits were not statistically different from the background fluorescence intensities of untransfected neuronal dendrites (Figs. 1E and 8E).

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Abbreviations: AIS, axonal initial segment; BFNC, benign familial neonatal convulsions; DIV, days in vitro; HA, hemagglutinin.

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Enrichment of Surface KCNQ Channels at the AIS. Because endogenous KCNQ channels are localized to the AIS (26, 27), we tested whether surface KCNQ channels are concentrated at the AIS by costaining with antibody that recognize epitopes common to voltage-gated sodium channels (Na), which are highly concentrated at the AIS (29). Our immunostaining revealed that surface HA-KCNQ2/KCNQ3 (Fig. 2A; see also Fig. 4A, which is published as supporting information on the PNAS web site) and HA-KCNQ3/KCNQ2 channels (Figs. 2B and 9B) are enriched at but not restricted to the AIS, where they colocalized with endogenous Na channels. Moreover, in 75% of the transfected neurons, the first 2- to 4-μm portion of the AIS stained positive for Na channels but not for surface HA-KCNQ channels (Fig. 2A and B).

To quantify the polarized surface expression of KCNQ channels, we calculated the ratio of the mean surface fluorescence intensity for major axonal and dendritic branches. The axon/dendrite ratios for HA-KCNQ2/KCNQ3, and HA-KCNQ3/KCNQ2 were significantly larger than that for CD4 with equal distribution in axons and dendrites (Fig. 2C). To quantify the enrichment of surface KCNQ channels at the AIS, we calculated the ratio of the mean surface fluorescence intensity of the proximal axon including the AIS (0–30 μm from soma) to the distal axon (50–80 μm from soma). The AIS/distal axon ratios for surface HA-KCNQ2/KCNQ3, surface HA-KCNQ3/KCNQ2, and Na channels were 2- to 3-fold larger than that for surface HA-Kv1.2 (Fig. 2D), which is not enriched at the AIS and is uniformly distributed along the axon.

Mutation of the Ankyrin-G Binding Motifs Reduces but Does Not Abolish Preferential Targeting of KCNQ Channels to Axonal Membranes. Because AIS localization of endogenous KCNQ2 and KCNQ3 requires their binding to ankyrin-G (27), we next tested whether ankyrin-G binding motifs in the distal C-termini of KCNQ2 and KCNQ3 (27) are necessary for the enrichment of HA-tagged KCNQ channels at the AIS surface by replacing the critical acidic residues in these motifs with alamines (E810A, D812A of KCNQ2 and E837A, D839A of KCNQ3). Similar mutations abolish AIS retention of HA tagged-neurofascin fused to the KCNQ2 or KCNQ3 C-terminal domain (27). Mutation of ankyrin-G binding motifs of both subunits abolished the enrichment of HA-KCNQ2/KCNQ3 (Fig. 3A) and HA-KCNQ3/KCNQ2 channels (Fig. 10A, which is published as supporting information on the PNAS web site) at the AIS surface and reduced their surface AIS/distal axon ratio to 1 (Figs. 3C and 10C), confirming that these motifs are AIS-targeting signals. Interestingly, mutating these motifs in both subunits reduced but did not abolish the surface expression of the channels on the more distal axon (Figs. 3C and 10C), suggesting that additional signals exist for axon targeting.

Fusion of KCNQ2 C-Terminal Sequences Targets CD4 to Axons and the AIS. Whereas Fusion of KCNQ3 C-Terminal Sequences Concentrates CD4 only to the AIS. To search for axon-targeting signals, we fused the C-terminal cytoplasmic domains (C-tails) of KCNQ2 and KCNQ3 to the cytoplasmic C terminus of CD4. Compared with the uniform distribution of CD4 on axonal and dendritic surfaces (Fig. 4A), fusion of the KCNQ2 C-tail preferentially targeted CD4 to axon and enriched it at the AIS (Fig. 4A). In contrast, fusion of KCNQ3 C-tail concentrated CD4 only to the AIS without causing other changes in the more distal axonal and dendritic surface expression (Fig. 4A). The surface axon/dendrite ratio was 3.9 ± 1.3 for CD4-KCNQ2 C-tail, and 1.0 ± 0.1 for CD4-KCNQ3 C-tail (Fig. 4C), suggesting that the C-terminal domain of KCNQ2 but not KCNQ3 contains signals for axonal targeting.

Because axonal surface expression of heteromeric KCNQ2/KCNQ3 channels was impaired partially by mutation of ankyrin-G binding motifs of KCNQ2 and KCNQ3 (Fig. 3), we next tested whether these motifs may act as axonal targeting signals. Mutating the ankyrin-G binding motif of either KCNQ2...
KCNQ2 is not required for axon targeting. (Fig. 4) KCNQ2 had no effect on axon targeting of CD4-KCNQ2 C-tail (Fig. 4). The enrichment of the CD4 fusion proteins on the AIS surface completely abolished AIS enrichment (Fig. 4). Both subunits (E810A and D812A) of HA-KCNQ2 or KCNQ3 subunits to minimize any possible complication in channel folding induced by both HA tag and these mutations.

The C-terminal frameshift mutations of KCNQ2 (P681-FS and G838-FS) caused by 1-bp deletion replace the distal C-terminal sequences with unrelated amino acid sequences starting from residue P681 (31) or G838 (32). Whereas both frameshift mutations had no effect on the total protein expression in axons, the P681-FS, but not the G838-FS, mutation abolished axonal surface expression and slightly elevated the dendritic total protein level of HA-KCNQ3/KCNQ2 (Fig. 6C).

Of the C-terminal truncation mutations of KCNQ2, the Q323X missense mutation deletes the entire KCNQ2 C-terminal domain (33), whereas the Y534X mutation caused by a 5-bp insertion at
meric channels are primarily expressed in axon (Fig. 1) and surface KCNQ2 (G310V; ref. 35) caused an 
reduction in axonal surface expression of HA-KCNQ3 (K526N) (Fig. 6C) and diminished the surface axon/
dendrite ratio (Fig. 6B).

Interestingly, the missense mutations in the pore and the sixth transmembrane segment of KCNQ3 (P681-FS and Y534X) dramatically decreased axonal surface expression (by \( \approx 50\% \) for Y284C and \( \approx 75\% \) for A306T) and significantly reduced the surface axon/dendrite ratio but not AIS concentration (Fig. 6C). Total channel protein levels in axons and dendrites were decreased by A306T but not Y284C mutation (Fig. 6C). Finally, the BFNC pore mutation of KCNQ3 (G310V; ref. 35) caused an \( \approx 50\% \) reduction in axonal surface expression and total protein expression in axons and dendrites (Fig. 6E) but had no effect on the AIS localization (Fig. 6F). No significant surface expression of wild-type and mutant channels was detected in dendrites (Fig. 6 C and E).

Discussion

In this study, we show that surface KCNQ2/KCNQ3 heteromeric channels are primarily expressed in axon (Fig. 1) and

![Figure 5](image)

Fig. 5. Fusion of A domain or membrane proximal-domain of KCNQ2 targets CD4 to axonal surface. (A) Surface immunostaining of hippocampal neurons (DIV 10) transfected with CD4 fused to different regions of KCNQ2 C-tail (Upper). Camera lucida drawings (A Lower) of the neurons from A Upper show MAP2-positive dendrites (red) and MAP2-negative axons (blue). Arrows show the main axonal branches. (Scale bars: 40 \( \mu m \).) (B) Schematic drawing (not to scale) of KCNQ2 subunit showing four distinct regions of C-tail: membrane proximal domain (MP; amino acids 323–500), A domain (A; amino acids 501–579), subunit interaction domain (Sid, amino acids 580–623), and ankyrin-G binding domain (AIS; amino acids 624–844). (C) The axon/dendrite ratio revealing the conversion of nonpolarized CD4 distribution (\( n = 15 \)) to polarized axonal surface expression of CD4 fused to KCNQ2 MP + A + Sid (\( n = 14; *** P \leq 0.005 \)), A + Sid (\( n = 15; *** P \leq 0.001 \)), MP (\( n = 22; ** P \leq 0.01 \)), and A (\( n = 28; * P \leq 0.05 \)) but not to the Sid domain (\( n = 19; P > 0.05 \)).

![Figure 6](image)

Fig. 6. Certain BFNC mutations disrupt polarized axonal surface expression of heteromeric KCNQ2/3 channels. (A) Schematic drawing (not to scale) of KCNQ2 with various BFNC mutations: C-terminal frameshift mutations replacing distal C-termini with completely different peptide sequences (P681-FS and G838-FS), C-terminal truncation (Q323X and Y534X), a missense mutation in C-terminal A-domain (K526N), and missense mutations in the pore and the sixth transmembrane segment (Y284C and A306T). (B) The axon/dendrite ratio was decreased by K526N (\( * P \leq 0.05 \)), Y284C (*** \( P \leq 0.001 \)), A306T (** \( P \leq 0.01 \)), and G838-FS mutation (*** \( P \leq 0.001 \)) but not to the AIS. The axon/dendrite and AIS/distal axon ratios were decreased by K526N (** \( P \leq 0.01 \)) and Y534X mutation. (C) Axonal surface expression of HA-KCNQ3/KCNQ2 channels was abolished by P681-FS (\( n = 22 \)), Q323X (\( n = 10 \)), and Y534X mutation (\( n = 9; *** P \leq 0.001 \)). It is reduced by Y284C (\( n = 12; ** P \leq 0.01 \)), A306T (\( n = 16; *** P \leq 0.001 \)), K526N (\( n = 25; ** P \leq 0.01 \)), and G838-FS mutation (\( n = 8; * P \leq 0.05 \)) compared with wild-type (\( n = 44 \)). The total expression in axon was decreased by K526N (*** \( P < 0.001 \)), Y534X (** \( P \leq 0.01 \)), and A306T mutation (*** \( P \leq 0.001 \)). The total expression in dendrite was decreased by A306T mutation (\( P \leq 0.05 \)) but increased by P681-FS mutation (*** \( P \leq 0.01 \)). Dendritic surface expression was unaffected by these mutations (\( P > 0.05 \)). (D) Schematic drawing (not to scale) of KCNQ3 with a missense mutation in the pore region (G310V). (E) The KCNQ3 G310V mutation (\( n = 13 \)) reduced surface (*** \( P \leq 0.001 \)) and total expression (*** \( P \leq 0.001 \)) of HA-KCNQ2/KCNQ3 in axons, and dendritic total expression (*** \( P \leq 0.001 \)) compared with wild type (\( n = 24 \)). (F) The axon/dendrite ratio but not the AIS/distal axon ratio was decreased by G310V mutation (*** \( P \leq 0.01 \)), arbitrary unit.
and the AIS (Fig. 6).

### Physiological Implications of Polarized Surface Distribution of KCNQ Channels in Axons and the AIS

The main objective of our study was to determine surface distribution of KCNQ channels. Because there are no available antibodies that recognize the extracellular regions of these proteins, we determined surface distribution of the recombinant KCNQ2 and KCNQ3 proteins with an extracellular HA tag. HA-KCNQ channels are preferentially expressed on the surface of axons (Fig. 1), consistent with recent studies of endogenous KCNQ2 and KCNQ3 (24–27). Importantly, surface HA-KCNQ channels are enriched at the AIS via their interaction with ankyrin-G (Figs. 2 and 3), as are endogenous KCNQ2 and KCNQ3 (26, 27). These similarities strongly support the notion that surface distribution of HA-KCNQ channels likely reflects that of endogenous channels.

The AIS is a strategic site for KCNQ channels to shape the waveform of the spike after depolarization (16, 21), modulate spike frequency adaptation (16, 18–20), and control action potential firing threshold in response to neurotransmitter actions (2). Surface distribution in distal axons and presynaptic terminals may allow KCNQ channels to regulate action potential propagation along the axon (22, 26) and neurotransmitter release from the nerve terminal (23). Surface KCNQ channels in the AIS and axon also may be important for generating hippocampal theta oscillations (17, 18), which are strongly implicated in sensory-motor behavior, learning and memory, and synaptic plasticity (36). These strategic placements of KCNQ channels are likely important for their ability to prevent intrinsic bursting and epileptiform activity (16, 18), although it remains possible that some KCNQ channels reside in somatodendritic regions as suggested (21, 37). Our study further suggests that reduced KCNQ channel densities in the AIS and axon may contribute to pathological conditions such as BFNC (1), epileptic seizures (12, 13), various pain states (14, 15, 38), myokemia (9), cognitive impairment (39, 40), and, possibly, mental retardation (34).

### Some BFNC Mutations Affect Surface Expression and Localization of KCNQ Channels

Our study highlights the fact that different BFNC mutations disrupt KCNQ channel activity in different ways, by altering channel properties, surface trafficking, or targeting to the axon and AIS. Several BFNC mutations in KCNQ2 C-terminal domain impaired axonal surface expression, underscoring the importance of this cytoplasmic domain in channel folding, assembly, and axonal targeting. Certain BFNC missense mutations of KCNQ2 and KCNQ3 that cause a modest reduction in current or biophysical properties (7, 34, 41) had additional effects in reducing axonal surface expression. Because many genetic and acquired disorders result from altered channel function and impaired channel trafficking (42), it would be important to develop therapies that also correct trafficking defects.

### Possible Molecular Mechanisms for Axonal Targeting of KCNQ Channels

Our findings are compatible with two possible scenarios for targeting KCNQ channels to the surface of axons and the AIS. In the first model, KCNQ channels are directly sorted to the axonal but not somatodendritic plasma membranes, similar to the neuronal glial cell adhesion molecules (43, 44). Here, the axon-targeting signals of KCNQ2 may recruit specific proteins implicated in anterograde axonal transport of cargo along microtubules such as axonal kinesins (45–47). Alternatively, the axon targeting signals of KCNQ2 may mediate selective endocytosis of surface KCNQ channels from somatodendritic regions but not axons, as proposed for Na+-, L-2, (48, 49) and VAMP, a presynaptic vesicle protein (43). Axonal KCNQ channels then are concentrated at the AIS and stabilized at the proximal axons through their interaction with ankyrin-G, an interaction that appears to facilitate channel targeting to distal axons. The channel sequences identified in this study to be important for axonal targeting will be useful in future studies of the axonal-targeting mechanism in both vertebrates and invertebrates, considering that ankyrin-G binding domain is exclusive to vertebrates, whereas MP and A domains are highly conserved between vertebrates and invertebrates (27).

### Materials and Methods

#### Materials

Reagents used include rabbit anti-KCNQ2 and KCNQ3 antibodies (kind gifts from Ed Cooper, University of Pennsylvania, Philadelphia), mouse anti-HA monoclonal antibody (Covance Research Products, Berkeley, CA), rat anti-HA-monoclonal antibody (Roche Applied Sciences), mouse anti-human CD4 antibody (Caltag), rabbit anti-MAP2 antibody (Chemicon), Alexa660-conjugated strepavidin, Alexa350- and Alexa488-conjugated secondary antibodies (Amersham Pharmacia Life Science), and Cy2, Cy5, and biotin-conjugated secondary antibodies (The Jackson Laboratory).

#### DNA Constructs and Mutagenesis

Plasmids pcDNA3-KCNQ2 and -KCNQ3 with and without extracellular HA tag (HA-KCNQ2 or HA-KCNQ3) were kind gifts of Thomas Jentsch, Hamburg University, Hamburg, Germany. CD4 fusion to KCNQ2 and KCNQ3 C-tails were constructed by inserting into the C-terminal domain of CD4 between engineered NotI and XhoI sites in pcDNA3-CD4 the following PCR fragments: full C-tail (amino acids 323–844), MP + A + Sid (amino acids 323–623), MP (amino acids 323–500), A domain (amino acids 501–579), and Sid (amino acids 580–623) of KCNQ2 and full C-tail (amino acids 362–872) of KCNQ3. Mutations in the ankyrin-G binding motifs of KCNQ2 (E810A/D812A) and KCNQ3 (E837A/D839A), and BFNC mutations were generated by the QuikChange Site-Directed Mutagenesis Kit (Stratagene) as previously described in refs. 7, 8, and 31–35 and verified by sequencing the entire construct. The P681-FS and G838-FS mutations of KCNQ2 were introduced to pcDNA3-KCNQ2 containing the 3′ untranslated region of KCNQ2 subcloned into SacII and XbaI.

#### Immunocytochemistry

Primary hippocampal cultures from 18-day embryonic rats were prepared and transfected at 7 days in vitro (DIV) as described in ref. 50. Surface immunostaining for HA-KCNQ channels at 10 DIV was performed as described in ref. 50 with the following modifications: Surface HA-KCNQ proteins were labeled with mouse anti-HA antibody (1:500 dilution) at 4°C overnight and visualized with biotin-conjugated secondary antibodies for 2 h, followed by Alexa 660-conjugated strepavidin. Neurons were then permeabilized and incubated with rat anti-HA antibody to label total HA-KCNQ proteins (1:1,000 dilution) and rabbit anti-MAP2 antibody (1:1,000 dilution), followed by Alexa488- and Alexa350-conjugated secondary antibodies, respectively. Immunostaining of surface CD4 fusion proteins or endogenous KCNQ2 and KCNQ3 proteins with rabbit anti-KCNQ2 and KCNQ3 antibodies (1:200 dilution) was performed as described in ref. 50.
Image Acquisition and Quantification. Fluorescence images of pyramidal neurons were acquired and the fluorescence intensity profiles of the major dendritic and axonal processes were quantified as described in ref. 50 (see Fig. 8 legend). Grayscale-inverted images and camera lucida drawing were generated in PHOTOSHOP (Adobe Systems, San Jose) as described in ref. 50 (see Fig. 7 legend). Background-subtracted mean fluorescence intensity of the axon within 30 μm of the soma (AIS) or at 50–80 μm from the soma (distal dendrite) and axon dendrite ratios were determined for the distal dendrite and axon/dendrite ratios. All fluorescence intensity quantification was reported as mean ± SEM. ANOVA and post-ANOVA Tukey’s multiple comparison tests were performed to identify the statistically significant difference between groups of three or more, whereas the Student t test was performed for groups of two by using PRISM4 (GraphPad, San Diego).

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