

Coupling of Slack and Na_v1.6 sensitizes Slack to quinidine blockade and guides anti-seizure strategy development

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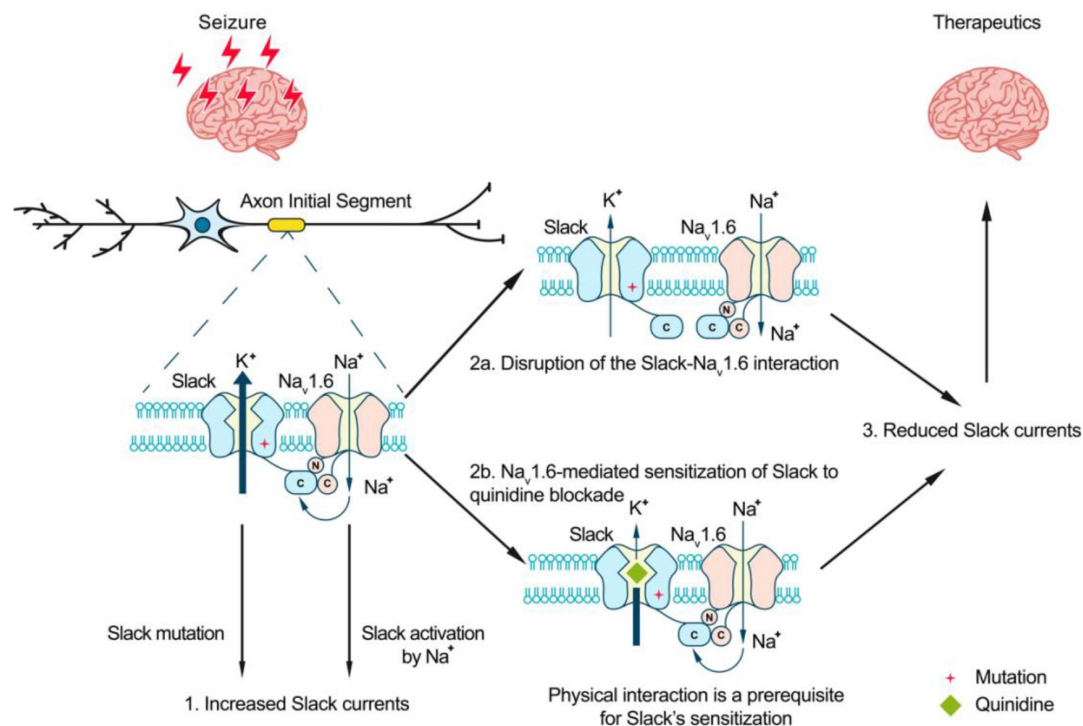
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Abstract

Quinidine has been used as an anticonvulsant to treat patients with KCNT1-related epilepsy by targeting gain-of-function KCNT1 pathogenic mutant variants. However, the detailed mechanism underlying quinidine's blockade against KCNT1 (Slack) remains elusive. Here, we report a functional and physical coupling of the voltage-gated sodium channel Na_v1.6 and Slack. Na_v1.6 binds to and highly sensitizes Slack to quinidine blockade. Homozygous knockout of Na_v1.6 reduces the sensitivity of native sodium-activated potassium currents to quinidine blockade. Na_v1.6-mediated sensitization requires the involvement of Na_v1.6's N- and C-termini binding to Slack's C-terminus, and is enhanced by transient sodium influx through Na_v1.6. Moreover, disrupting the Slack-Na_v1.6 interaction by viral expression of Slack's C-terminus can protect against Slack^{G269S}-induced seizures in mice. These insights about a Slack-Na_v1.6 complex challenge the traditional view of "Slack as an isolated target" for anti-epileptic drug discovery efforts, and can guide the development of innovative therapeutic strategies for KCNT1-related epilepsy.

Graphical abstract



eLife assessment

The authors report that an interaction between the sodium-activated potassium channel Slack and Nav1.6 sensitizes Slack to inhibition by quinidine. This is an **important** finding because it contributes to our understanding of how the antiseizure drug quinidine affects epilepsy syndromes arising from mutations in the Slack-encoding gene *KCNT1*. The results are largely **compelling**, although additional data would strengthen the claims of a direct channel-channel interaction in neuronal cell membranes.

Introduction

The sodium-activated potassium (K_{Na}) channels Slack and Slick were first identified in guinea pig cardiomyocytes, and were subsequently found to be encoded by two genes of the *Slo2* family^{1,2}. Slack channels encoded by the *Slo2.2* (*KCNT1*) gene are gated by Na^+ , while Slick channels encoded by the *Slo2.1* (*KCNT2*) gene are more sensitive to Cl^- than Na^+ . Slack channels are expressed at high levels in the central nervous system (CNS), especially the cortex and brainstem^{3–5}. Activation of Slack channels by intracellular sodium ions forms delayed outward currents in neurons, contributes to slow afterhyperpolarization (AHP) following repeated action potentials, and modulates the firing frequency of neurons^{6,7}.

Mutations in the *KCNT1* gene have been implicated in a wide spectrum of epileptic disorders, including early-onset epilepsy (e.g., epilepsy of infancy with migrating focal seizures (EIMFS), non-EIMFS developmental and epileptic encephalopathies, and autosomal dominant or sporadic sleep-related hypermotor epilepsy (ADSHE))^{8–11}. Over 50 mutations related to seizure disorders have been identified, typically displaying a gain-of-function (GOF) phenotype in heterologous expression systems^{11,12}. The prescribed antiarrhythmic drug, quinidine, has emerged as a

precision therapy for KCNT1-related epilepsy by blocking Slack mutant variants *in vitro* and conferring decreased seizure frequency and improved psychomotor development in clinical treatment^{13–16}. However, clinical quinidine therapy has shown limited success and contradictory therapeutic effects, probably due to poor blood–brain barrier penetration, dose-limiting off-target effects, phenotype-genotype associations, and rational therapeutic schedule^{16–19}.

Slack requires high intracellular free Na^+ concentrations ($[\text{Na}^+]_{\text{in}}$) for its activation in neurons (K_d of ~ 66 mM)²⁰. However, previous investigations have shown that the $[\text{Na}^+]_{\text{in}}$ at resting states (~ 10 mM) is much lower than the $[\text{Na}^+]_{\text{in}}$ needed for effective Slack activation (*e.g.*, K_d value)^{1,20,21}. Therefore, native Slack channels need to localize with Na^+ sources within a nanodomain to be activated and exert physiological function. Slack channels are known to be functionally coupled with sodium-permeable ion channels in neurons, such as voltage-gated sodium (Na_v) channels and AMPA receptors^{21,22}. A question arises as to whether these known Na^+ sources modulate Slack's sensitivity to quinidine blockade.

Here, we found that $\text{Na}_v1.6$ sensitizes Slack to quinidine blockade. Slack and $\text{Na}_v1.6$ form a complex that functions in $\text{Na}_v1.6$ -mediated transient sodium influx to sensitize Slack to quinidine blockade in HEK293 cells and in primary cortical neurons. The widespread expression of these channel proteins in cerebral cortex, hippocampus, and cerebellum supports that the $\text{Na}_v1.6$ -Slack complex is essential for the function of a wide range of electrically excitable neurons, and moreover, that this complex can be viewed as a vulnerable target for drug development to treat KCNT1-related disorders.

Results

$\text{Na}_v1.6$ sensitizes Slack to quinidine blockade

Slack currents are activated by sodium entry through voltage-gated sodium (Na_v) channels and ionotropic glutamate receptors (*e.g.* AMPA receptors)^{21–23}. To investigate potential modulators of Slack's sensitivity to quinidine blockade, we initially focused on the known Na^+ sources of Slack. Working in HEK293 cells, we co-expressed Slack with AMPA receptor subunits (GluA1, GluA2, GluA3, or GluA4) or Na_v channel α subunits ($\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$, or $\text{Na}_v1.6$), which are highly expressed in the central nervous system^{24–27}. The sensitivity of Slack to quinidine blockade was assessed based on the detected inhibitory effects of quinidine on delayed outward potassium currents^{13,28}. Interestingly, all neuronal Na_v channels significantly sensitized Slack to $30\text{ }\mu\text{M}$ quinidine blockade, whereas no effect was observed upon co-expression of Slack with GluA1, GluA2, GluA3, or GluA4 (Fig. S1).

When co-expressing Slack with $\text{Na}_v1.6$ in HEK293 cells, $\text{Na}_v1.6$ sensitized Slack to quinidine blockade by nearly 100-fold ($\text{IC}_{50} = 85.13\text{ }\mu\text{M}$ for Slack expressed alone and an $\text{IC}_{50} = 0.87\text{ }\mu\text{M}$ for Slack upon co-expression with $\text{Na}_v1.6$) (Fig. 1A–D,F). $\text{Na}_v1.6$ also exhibited >10 -fold selectivity in sensitizing Slack to quinidine blockade against $\text{Na}_v1.1$, $\text{Na}_v1.2$, and $\text{Na}_v1.3$ (Fig. 1C,F, and Supplementary Table 1). When we co-expressed the cardiac sodium channel $\text{Na}_v1.5$ with Slack, we observed only ~ 3 -fold sensitization of Slack to quinidine blockade (Fig. 1E,F). These results together indicate that the apparent functional coupling between Slack and Na_v channels is Na_v -channel-subtype-specific, with $\text{Na}_v1.6$ being particularly impactful in Slack's responsivity to quinidine blockade.

Slack and Slick are both K_{Na} channels (with 74% sequence identity) and adopt similar structures²⁹, and Slick is also blocked by quinidine³⁰. We next assessed whether $\text{Na}_v1.6$ sensitizes Slick to quinidine blockade and observed that, similar to Slack, co-expression of Slick and $\text{Na}_v1.6$ in HEK293 cells resulted in a sensitization of Slick to quinidine blockade (7-fold) (Fig.

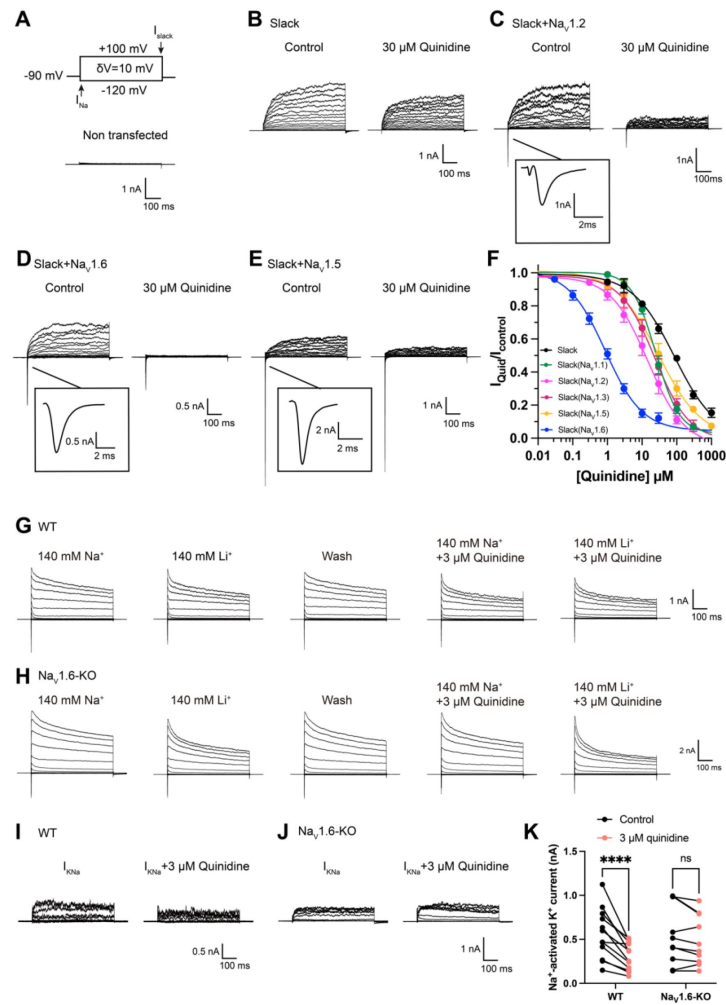


Figure 1.

NaV1.6 specifically sensitizes Slack to quinidine blockade.

(A) The voltage protocol and current traces from control (non-transfected) HEK293 cells. The arrows on the voltage protocol indicate the onset of inward sodium currents through Na_v channels and delayed outward potassium currents through Slack channels. The currents were evoked by applying 600-ms step pulses to voltages varying from -120 mV to +100 mV in 10 mV increments, with a holding potential of -90 mV and a stimulus frequency of 0.20 Hz. (B) Example current traces from HEK293 cells expressing Slack alone. The left traces show the family of control currents; the right traces show Slack currents remaining after application of 30 μM quinidine in the bath solution. (C-E) Example current traces from HEK293 cells co-expressing Slack with Na_v1.2 (C), Na_v1.6 (D), or Na_v1.5 (E) channels before and after application of 30 μM quinidine. (F) The concentration-response curves for blocking of Slack by quinidine at +100 mV upon expression of Slack alone (n = 6) and co-expression of Slack with Na_v1.1 (n = 7), Na_v1.2 (n = 10), Na_v1.3 (n = 13), Na_v1.5 (n = 9), or Na_v1.6 (n = 19). Please refer to Supplementary Table 1 for IC₅₀ values. (G-H) Delayed outward currents in primary cortical neurons from homozygous Na_v1.6 knockout C3HeB/FeJ mice (Na_v1.6-KO) (H) and the wild-type littermate controls (WT) (G). Current traces were elicited by 600-ms step pulses to voltages varying from -120 mV to +100 mV in 20 mV increments, with a holding potential of -70 mV, and recorded with different bath solutions in the following order: Na⁺-based bath solution (I_{Control}), replacement of external Na⁺ with Li⁺ in equivalent concentration (I_{Li}), washout of quinidine by Na⁺-based bath solution (I_{Wash}), Na⁺-based bath solution with 3 μM quinidine (I_{Quid}), Li⁺-based bath solution with 3 μM quinidine (I_{Li+Quid}). The removal and subsequent replacement of extracellular Na⁺ revealed the I_{KNa} in neurons. (I-J), The sensitivity of native sodium-activated potassium currents (I_{KNa}) to 3 μM quinidine blockade in WT (I) and Na_v1.6-KO (J) neurons. I_{KNa} before application of quinidine was obtained from the subtraction of I_{Control} and I_{Li}. Maintained I_{KNa} after application of 3 μM quinidine was obtained from the subtraction of I_{Quid} and I_{Li+Quid}. (K) Summarized amplitudes of I_{KNa} before and after application of 3 μM quinidine in the bath solution in WT (black, n = 12) and Na_v1.6-KO (red, n = 10) primary cortical neurons. ****p < 0.0001, Two-way repeated measures ANOVA followed by Bonferroni's post hoc test.

S2A,B). These results support that Na_v1.6 regulates both Slack and Slick and that Na_v1.6 can sensitize K_{Na} channels to quinidine blockade *in vitro*. We also asked whether Na_v1.6 sensitizes native K_{Na} channels to quinidine blockade *in vivo*. We performed whole-cell patch-clamp recordings in primary cortical neurons from postnatal homozygous Na_v1.6 knockout C3HeB/FeJ mice and the wild-type littermate controls. K_{Na} currents (I_{KNa}) were isolated by replacing sodium ions with equivalent lithium ions in the bath solution (Fig. 1G,H). 3 μ M quinidine significantly blocked native I_{KNa} (44%) in wild-type neurons (Fig. 1I,K), while the same concentration of quinidine had no significant effect on I_{KNa} in Na_v1.6-knockout (Na_v1.6-KO) neurons (Fig. 1J,K). These results support that Na_v1.6 is required for the observed high sensitivity of native K_{Na} channels to quinidine blockade.

Transient sodium influx through Na_v1.6 enhances Na_v1.6-mediated sensitization of Slack to quinidine blockade

We next investigated the biomolecular mechanism underlying Na_v1.6-mediated sensitization of Slack to quinidine blockade. Considering that Slack currents are activated by sodium influx^{22,29}, we initially assessed the effects of Na_v1.6-mediated sodium influx on sensitizing Slack to quinidine blockade. We used 100 nM tetrodotoxin (TTX) to block Na_v1.6-mediated sodium influx (Fig. S3A,B)³¹. In HEK293 cells expressing Slack alone, 100 nM TTX did not affect Slack currents; nor did it affect Slack's sensitivity to quinidine blockade (IC_{50} = 83.27 μ M) (Fig. 2A,B and Fig. S3C,D). In contrast, upon co-expression of Slack and Na_v1.6 in HEK293 cells, bath-application of 100nM TTX significantly reduced the effects of Na_v1.6 in sensitizing Slack to quinidine blockade (IC_{50} = 25.04 μ M) (Fig. 2A,B). These findings support that sodium influx through Na_v1.6 contributes to Na_v1.6-mediated sensitization of Slack to quinidine blockade.

It is known that Na_v1.6-mediated sodium influx involves a transient inward flux that reaches a peak before subsequently decaying to the baseline within a few milliseconds; this is termed a transient sodium current (I_{NaT})³². A small fraction of Na_v1.6 currents are known to persist after the rapid decay of I_{NaT} , and these are termed persistent sodium currents (I_{NaP})³³. We isolated I_{NaT} and I_{NaP} to explore their potential contributions in sensitizing Slack to quinidine blockade. We selectively inactivated I_{NaT} using a depolarized prepulse of -40 mV (Fig. S4A) and selectively blocked I_{NaP} by bath-application of 20 μ M riluzole, which is a relatively specific I_{NaP} blocker that is known to stabilize inactivated-state Na_v channels and delay recovery from inactivation^{34,35}. Our findings ultimately confirmed that the 20 μ M riluzole selectively blocked I_{NaP} compared to I_{NaT} in HEK293 cells co-expressing Slack and Na_v1.6 (Fig. S4B) and that 20 μ M riluzole had no effect on Slack currents when expressed alone (Fig. S4C,D).

Consistent with previous investigations^{21,22}, inactivating I_{NaT} reduced whole-cell Slack currents by 20%, and blocking I_{NaP} reduced Slack currents by 40% at +100 mV (Fig. 2C,D,F,G), supporting that Na_v1.6-mediated sodium influx activates Slack. Interestingly, inactivating I_{NaT} resulted in a > 20-fold decrease in Slack's sensitization to quinidine blockade (IC_{50} = 22.26 μ M) (Fig. 2C,E). In contrast, blocking I_{NaP} had no effect on Slack's sensitization to quinidine blockade (IC_{50} = 1.60 μ M) (Fig. 2F,H). These findings indicate that Na_v1.6 sensitizes Slack to quinidine blockade via I_{NaT} but not I_{NaP} .

Given that Slack current amplitudes are sensitive to sodium influx, and considering that quinidine is a sodium channel blocker, we examined whether Na_v1.6 has higher sensitivity to quinidine blockade than other Na_v channel subtypes, which could plausibly explain the observed increased strength of sensitization. We used whole-cell patch-clamping to assess the sensitivity of Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.5, and Na_v1.6 to quinidine blockade. These sodium channels exhibited similar levels of quinidine sensitivity (IC_{50} values in the range of 35.61-129.84 μ M) (Fig. 2I,J and Supplementary Table 2), all of which were at least 40-fold lower than the Na_v1.6-mediated sensitization of Slack to quinidine blockade (Fig. 1F). Additionally, co-expressing Slack with Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.5, or Na_v1.6 in HEK293 cells did not change the sensitivity of these

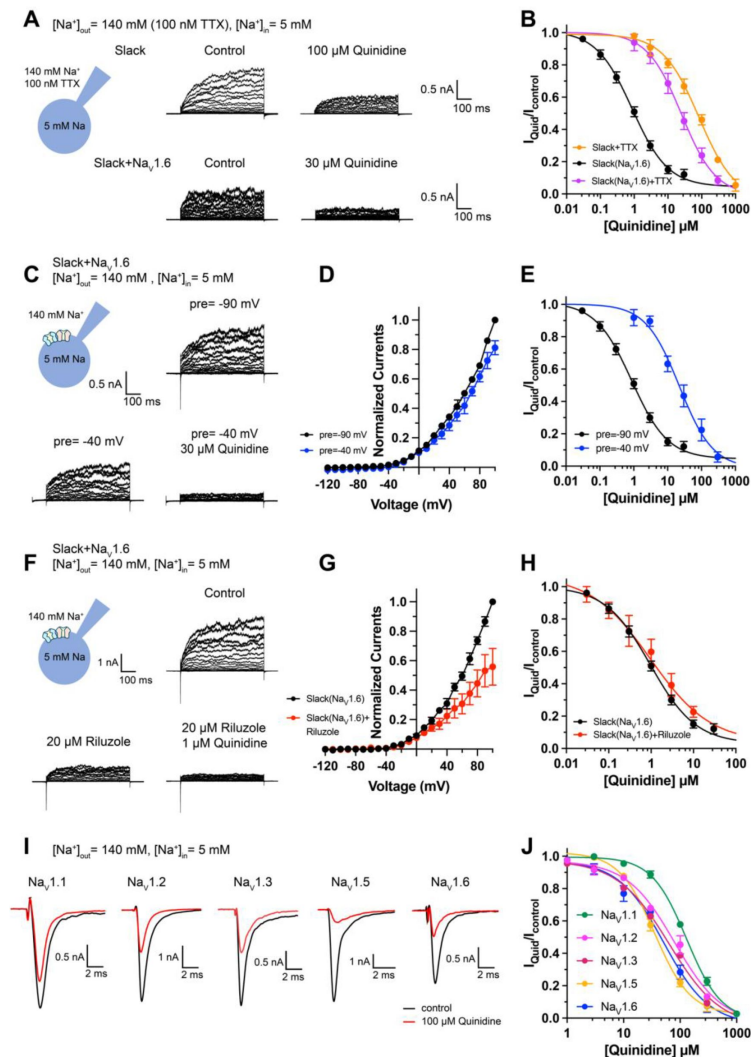


Figure 2.

Blocking transient sodium influx through $Na_V1.6$ reduces $Na_V1.6$ -mediated sensitization of Slack to quinidine blockade.

(A) Example current traces from HEK293 cells expressing Slack alone (top) and co-expressing Slack with $Na_V1.6$ (bottom), with 100nM TTX in the bath solution. The left traces show the family of control currents; the right traces show Slack currents remaining after application of quinidine. (B) The concentration-response curves for blocking of Slack by quinidine at +100 mV upon expression of Slack alone ($n = 3$) and co-expression of Slack with $Na_V1.6$ ($n = 7$), with 100 nM TTX in the bath solution. (C) Top, example current traces recorded from a HEK293 cell co-expressing Slack with $Na_V1.6$ and evoked from a 100-ms prepulse (pre) of -90 mV. Bottom, example current traces recorded from the same cell but evoked from a 100-ms prepulse of -40 mV, before and after application of quinidine. (D) I-V curves of Slack upon co-expression with $Na_V1.6$. The currents were evoked from a prepulse of -90 mV (black) or -40 mV (blue). (E) The concentration-response curves for blocking of Slack by quinidine at +100 mV with a prepulse of -90 mV (black, $n = 19$) or -40 mV (blue, $n = 5$). (F) Top, example current traces recorded from a HEK293 cell co-expressing Slack and $Na_V1.6$ without riluzole in the bath solution. Bottom, example current traces recorded from the same cell with 20 μ M riluzole in the bath solution, before and after application of quinidine. (G) I-V curves of Slack upon co-expression with $Na_V1.6$ before (black) and after (red) application of 20 μ M riluzole into bath solution. The concentration-response curves for blocking of Slack by quinidine upon co-expression of Slack with $Na_V1.6$, without ($n = 19$) or with ($n = 6$) 20 μ M riluzole in the bath solution. (I-J) The sensitivity of Na_V channel subtypes to quinidine blockade upon expression of Na_V alone in HEK293 cells. Example current traces (I) were evoked by a 50-ms step depolarization to 0 mV from a holding potential of -90 mV. The Concentration-response curves for blocking of Na_V channel subtypes by quinidine (J) were shown on the right panel ($n = 5$ for $Na_V1.1$, $n = 3$ for $Na_V1.2$, $n = 6$ for $Na_V1.3$, $n = 6$ for $Na_V1.5$, and $n = 4$ for $Na_V1.6$).

Na_v channel subtypes to quinidine blockade (Fig. S5 and Supplementary Table 2). Thus, differential quinidine affinity for specific Na_v channel subtypes cannot explain the large observed Na_v1.6-mediated sensitization of Slack to quinidine blockade. Moreover, it is clear that Na_v1.6-mediated sensitization of Slack to quinidine blockade is directly mediated by I_{NaT}, rather than through some secondary effects related to Na_v1.6's higher sensitivity to quinidine blockade.

Slack interacts with Na_v1.6 *in vitro* and *in vivo*

We found that the specific voltage-gated sodium channel blocker TTX did not completely abolish the effects of Na_v1.6 on sensitizing Slack to quinidine blockade (Fig. 2B), so it appears that a sodium-influx-independent mechanism is involved in the observed Na_v1.6-mediated sensitization of Slack to quinidine blockade. We therefore investigated a potential physical interaction between Slack and Na_v1.6. We initially assessed the cellular distribution of Na_v1.2, Na_v1.6, and Slack in the hippocampus and the neocortex of mouse. Consistent with previous studies^{36,37}, Na_v1.2 and Na_v1.6 were localized to the axonal initial segment (AIS) of neurons, evident as the co-localization of Na_v and AnkG, a sodium channel-associated protein known to accumulate at the AIS (Fig. 3A). Slack channels were also localized to the AIS of these neurons (Fig. 3A), indicating that Slack channels are located in close proximity to Na_v1.6 channels, and supporting their possible interaction *in vivo*. Moreover, Na_v1.6 was co-immunoprecipitated with Slack in homogenates from mouse cortical and hippocampal tissues and from HEK293T cells co-transfected with Slack and Na_v1.6 (Fig. 3B,C), supporting that a physical interaction between Slack and Na_v1.6 occurs *in vivo*.

To assess the interaction between Slack and Na_v1.6 inside living cells, we performed a FRET assay in transfected HEK293T cells³⁸. Briefly, we genetically fused mTFP1 and mVenus to the C-terminal regions of Slack and Na_v1.6, respectively (Fig. 3D). Upon imaging the emission spectra cells co-expressing Na_v1.6-mVenus and Slack-mTFP1 (measured at the plasma membrane region) (Fig. 3E), we detected positive FRET signals, indicating a Slack-Na_v1.6 interaction (Fig. 3F,H). The plasma membrane regions from HEK293T cells co-transfected with Na_v1.6 and Slack showed FRET efficiency values much larger than a negative control (in which standalone mVenus and mTFP1 proteins were co-expressed) (Fig. 3G,H), indicating that Slack channels reside in close spatial proximity (less than 10 nm) to Na_v1.6 channels in membranes of living cells.

We next characterized the consequences of the Slack-Na_v1.6 interaction in HEK293 cells using whole-cell recordings. Slack increased the rate of recovery from fast inactivation of Na_v1.6 (Fig. S6E), with no significant effects on the steady-state activation, steady-state fast inactivation, or ramp currents (Fig. S6C,D,F and Supplementary Table 3). Additionally, we found that Na_v1.6 had no significant effects on the activation rate or the current-voltage (I-V) relationship of Slack currents (Fig. S6A,B). These results indicate that the physical interaction between Slack and Na_v1.6 produces functional consequences. Taken together, these findings support functional and physical coupling of Slack and Na_v1.6 *in vitro* and *in vivo*.

Na_v1.6's N-and C-termini bind to Slack's C-terminus and sensitize Slack to quinidine blockade

To explore whether the physical interaction between Slack and Na_v1.6 is required for sodium-influx-mediated sensitization of Slack to quinidine blockade, we performed inside-out patch-clamp recordings on HEK293 cells transfected with Slack alone or co-transfected with Slack and Na_v1.6. Note that in these experiments the intracellular sodium concentration ([Na⁺]_{in}) was raised to 140 mM (a concentration at which most Slack channels can be activated²⁰), seeking to mimic the increased intracellular sodium concentration upon sodium influx. When expressing Slack alone, increasing the sodium concentration did not sensitize Slack to quinidine blockade (IC₅₀ = 120.42 μM) (Fig. 4a). However, upon co-expression of Na_v1.6 and Slack, Na_v1.6 significantly sensitized

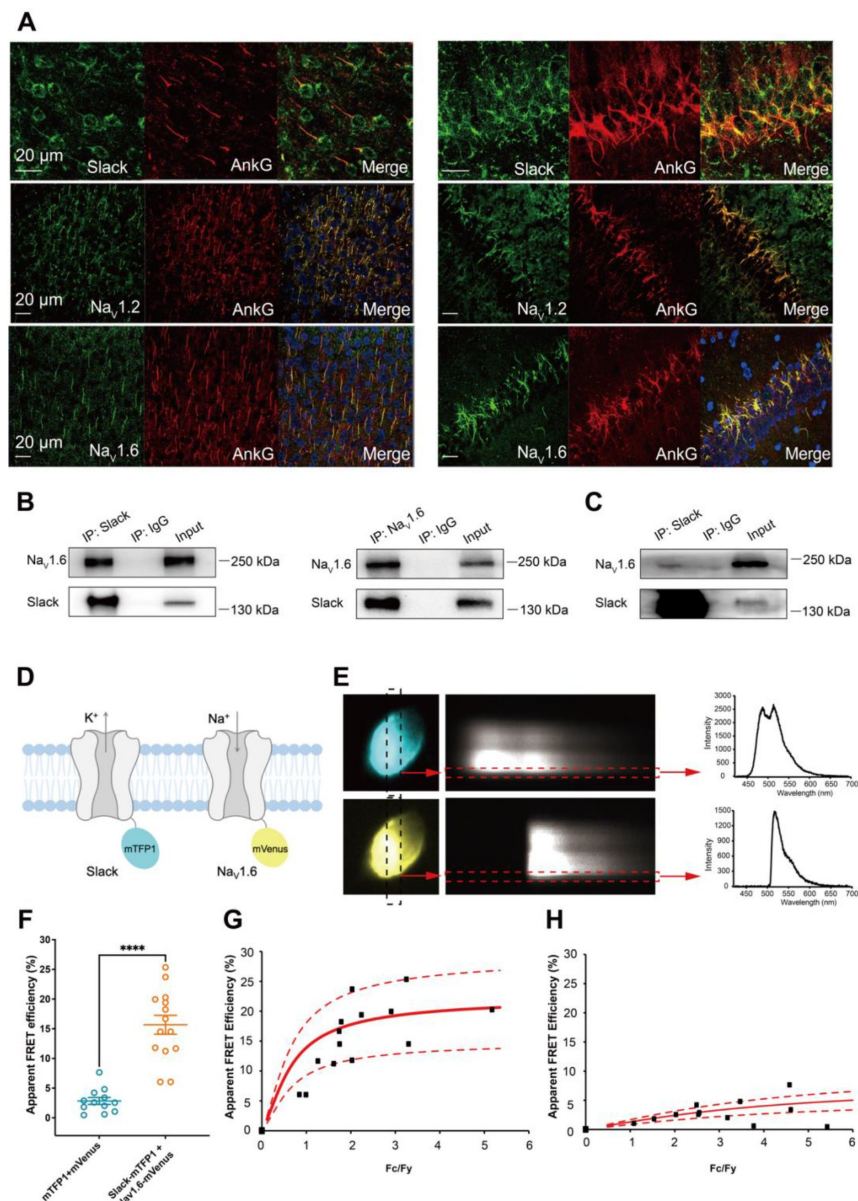


Figure 3.

Slack physically interacts with NaV1.6 *in vitro* and *in vivo*.

(A) Immunofluorescence of Slack, NaV1.2, NaV1.6 (green), and AnkG (red) in hippocampus CA1 (left) and neocortex (right). Confocal microscopy images were obtained from Coronal brain slices of C57BL/6 mice. The panels from top to bottom show the double staining of Slack with AnkG, NaV1.2 with AnkG, and NaV1.6 with AnkG, respectively. (B) Coimmunoprecipitation (Co-IP) of Slack and NaV1.6 in cell lysates from HEK293T cells co-transfected with Slack and NaV1.6. (C) Co-IP of Slack and NaV1.6 in mouse brain tissue lysates. Input volume corresponds to 10% of the total lysates for Co-IP. (D) A schematic diagram showing the fluorescence-labeled Slack and NaV1.6. mTFP1 and mVenus were fused to the C-terminal region of Slack (Slack-mTFP1) and NaV1.6 (NaV1.6-mVenus), respectively. (E) FRET imaging of Slack-mTFP1 and NaV1.6-mVenus co-expressed in HEK293 cells. The emission spectra measured from the edge of cell (dotted arrows in red) are used for FRET efficiency calculation. (F) The apparent FRET efficiency measured from cells co-expressing the fluorophore-tagged ion channels or co-expressing the fluorophores. **** $p < 0.0001$, Mann-Whitney test. (G-H) The FRET efficiency measured from cells co-expressing the fluorophore-tagged ion channels (G), or from cells co-expressing fluorophores (H). The efficiency value was plotted as a function of the fluorescence intensity ratio between mTFP1 and mVenus (Fc/Fy). Each symbol represents a single cell. The solid curve represents the FRET model that yields the best fit; dotted curves represent models with 5% higher or lower FRET efficiencies.

Slack to quinidine blockade ($IC_{50} = 2.91 \mu M$) (**Fig. 4A**). These results support that physical interaction between Slack and $Na_V1.6$ is a prerequisite for sodium-influx-mediated sensitization of Slack to quinidine blockade.

To investigate which interacting domains mediate $Na_V1.6$'s sensitization of Slack to quinidine blockade, we focused on $Na_V1.6$'s cytoplasmic fragments, including its N-terminus, inter domain linkers, and C-terminus (**Fig. 4B**). Whole-cell recordings from HEK293 cells co-expressing Slack with these $Na_V1.6$ fragments revealed that the N-terminus and C-terminus of $Na_V1.6$ significantly enhanced the sensitivity of Slack to quinidine blockade ($IC_{50} = 31.59 \mu M$ for Slack upon co-expression with $Na_V1.6$'s N-terminus and $IC_{50} = 43.70 \mu M$ for Slack upon co-expression with $Na_V1.6$'s C-terminus); note that the inter domain linkers had no effect (**Fig. 4C**).

Subsequent co-immunoprecipitation and glutathione S-transferase (GST) pull down assays of HEK293T cell lysates experimentally confirmed that $Na_V1.6$'s N- and C-termini each interact with Slack (**Fig. 4D** and Fig. S7). Additionally, whole-cell recordings using an $[Na^+]_{in}$ of 5 mM again showed that $Na_V1.6$'s N-terminus and $Na_V1.6$'s C-terminus sensitize Slack to quinidine blockade ($IC_{50} = 27.87 \mu M$) (**Fig. 4E**). And inside-out recordings using an $[Na^+]_{in}$ of 140 mM showed that co-expression of Slack, $Na_V1.6$'s N-terminus, and $Na_V1.6$'s C-terminus resulted in obvious sensitization of Slack to quinidine blockade, with an IC_{50} of $2.57 \mu M$ (**Fig. 4F**)—thus fully mimicking the aforementioned effects of full-length $Na_V1.6$ ($IC_{50} = 2.91 \mu M$) (**Fig. 4A**). These findings support that the binding of $Na_V1.6$'s N- and C-termini to Slack is required for $Na_V1.6$'s sensitization of Slack to quinidine blockade.

Recalling that $Na_V1.5$ had the least pronounced effect in sensitizing Slack to quinidine blockade among all examined Na_V channels ($IC_{50} = 29.46 \mu M$) (**Fig. 1F** and Supplementary Table 1). We constructed $Na_V1.5$ -1.6 chimeras to test the roles of $Na_V1.6$'s N- and C-termini in sensitizing Slack to quinidine blockade. The replacement of both the N-terminus [residues 1-131] and C-terminus [residues 1772-2016] of $Na_V1.5$ with $Na_V1.6$'s N-terminus [residues 1-132] and C-terminus [residues 1766-1980] (namely $Na_V1.5/6^{NC}$) fully mimicked effects of $Na_V1.6$ in sensitizing Slack to quinidine blockade ($IC_{50} = 1.13 \mu M$) (**Fig. 4G-I**). We also found that replacement of $Na_V1.5$'s N-terminus [residues 1-131] with $Na_V1.6$'s N-terminus [residues 1-132] (namely $Na_V1.5/6^N$) fully mimicked the effects of $Na_V1.6$ ($IC_{50} = 1.18 \mu M$) (**Fig. 4G-I**). Consistently, $Na_V1.5$'s C-terminus sensitized Slack to quinidine blockade ($IC_{50} = 37.59 \mu M$), whereas $Na_V1.5$'s N-terminus had no effect on Slack sensitization (**Fig. 4C**). These findings support that $Na_V1.6$'s N-terminus is essential for sensitizing Slack to quinidine blockade.

Having demonstrated that $Na_V1.6$ sensitizes Slack via $Na_V1.6$'s cytoplasmic N- and C-termini, we investigated which domains of Slack interact with $Na_V1.6$ and focused on Slack's cytoplasmic fragments, including Slack's N-terminus and C-terminus (**Fig. 5A**). In HEK293 cells co-expressing Na_V and Slack, Na_V -mediated sensitization of Slack to quinidine blockade was significantly attenuated upon the additional expression of Slack's C-terminus, but not of Slack's N-terminus (**Fig. 5B,C**), suggesting that Slack's C-terminus can disrupt the Slack- $Na_V1.6$ interaction by competing with Slack for binding to $Na_V1.6$. Consistently, Slack's C-terminus co-immunoprecipitated with $Na_V1.6$'s N- and C-termini in HEK293T cell lysates (**Fig. 5D**). Together, these results support that Slack's C-terminus physically interacts with $Na_V1.6$ and that this interaction is required for $Na_V1.6$'s sensitization of Slack to quinidine blockade.

Na_V1.6 binds to and sensitizes epilepsy-related

Slack mutant variants to quinidine blockade

Over 50 mutations in KCNT1 (Slack) have been identified to related to seizure disorders¹¹. Having established that $Na_V1.6$ can sensitize wild-type Slack to quinidine blockade, we next investigated whether $Na_V1.6$ also sensitizes epilepsy-related Slack mutant variants to quinidine

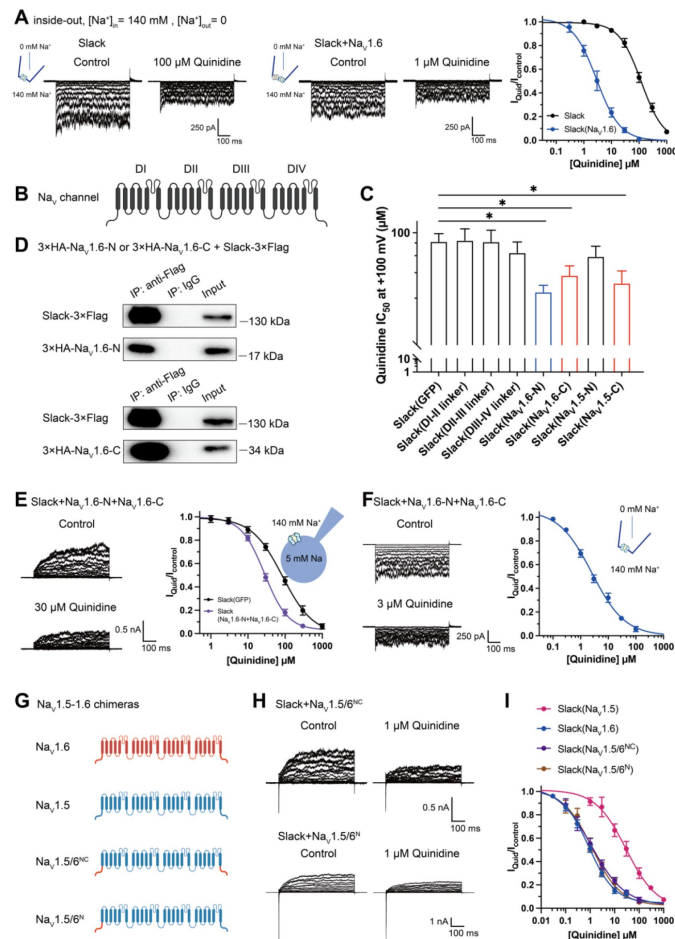


Figure 4.

Na_V1.6's N- and C-termini interacting with Slack is a prerequisite for Na_V1.6-mediated sensitization of Slack to quinidine blockade.

(A) The sensitivity of Slack to quinidine blockade upon expression of Slack alone ($n = 3$) and co-expression of Slack with Na_V1.6 ($n = 3$) from excised inside-out patches. The pipette solution contained (in mM) 130 KCl, 1 EDTA, 10 HEPES and 2 MgCl₂ (pH 7.3); the bath solution contained (in mM) 140 NaCl, 1 EDTA, 10 HEPES and 2 MgCl₂ (pH 7.4). The membrane voltage was held at 0 mV and stepped to voltages varying from -100 mV to 0 mV in 10 mV increments. Example current traces were shown on the left panel. The concentration-response curves for blocking of Slack by quinidine were shown on the right panel. (B) Domain architecture of the human Na_V channel pore-forming α subunit. Calculated IC₅₀ values at +100 mV of quinidine on Slack upon co-expression with indicated cytoplasmic fragments from Na_V channels. For Na_V1.6, cytoplasmic fragments used include N-terminus (Na_V1.6-N, residues 1-132), inter domain linkers (Domain I-II linker, residues 409-753; Domain II-III linker, residues 977-1199; Domain III-IV linker, residues 1461-1523), and C-terminus (Na_V1.6-C, residues 1766-1980). For Na_V1.5, cytoplasmic fragments used include N-terminus (Na_V1.5-N, residues 1-131) and C-terminus (Na_V1.5-C, residues 1772-2016). (D) Co-IP of Slack and terminal domains of Na_V1.6 in cell lysates from HEK293T cells co-expressing 3×Flag-tagged Slack (Slack-3×Flag) and 3×HA-tagged termini of Na_V1.6 (3×HA-Na_V1.6-N or 3×HA-Na_V1.6-C). The 3×Flag tag was fused to the C-terminal region of Slack and the 3×HA tag was fused to the N-terminal region of Na_V1.6's fragments. (E) The sensitivity of Slack to quinidine blockade upon co-expression of Slack with GFP ($n = 12$) or N- and C-termini of Na_V1.6 ($n = 11$), from whole-cell recordings. (F) The sensitivity of Slack to quinidine blockade upon co-expression of Slack with N- and C-termini of Na_V1.6, from excised inside-out recordings ($n = 10$, using the same protocols as in Fig. 4A). Example current traces before and after application of quinidine were shown on the left panel. The concentration-response curves were shown on the right panel. (G) A schematic diagram of the Na_V1.5-1.6 chimeric channels (Na_V1.5/6^{NC} and Na_V1.5/6^N) used in this study. (H) Example current traces recorded from HEK293 cells co-expressing Slack and Na_V1.5-1.6 chimeras before and after application of the indicated concentration of quinidine. (I) The concentration-response curves for blocking of Slack by quinidine upon co-expression of Slack with Na_V1.5 ($n = 9$), Na_V1.6 ($n = 19$), Na_V1.5/6^{NC} ($n = 9$), or Na_V1.5/6^N ($n = 9$).

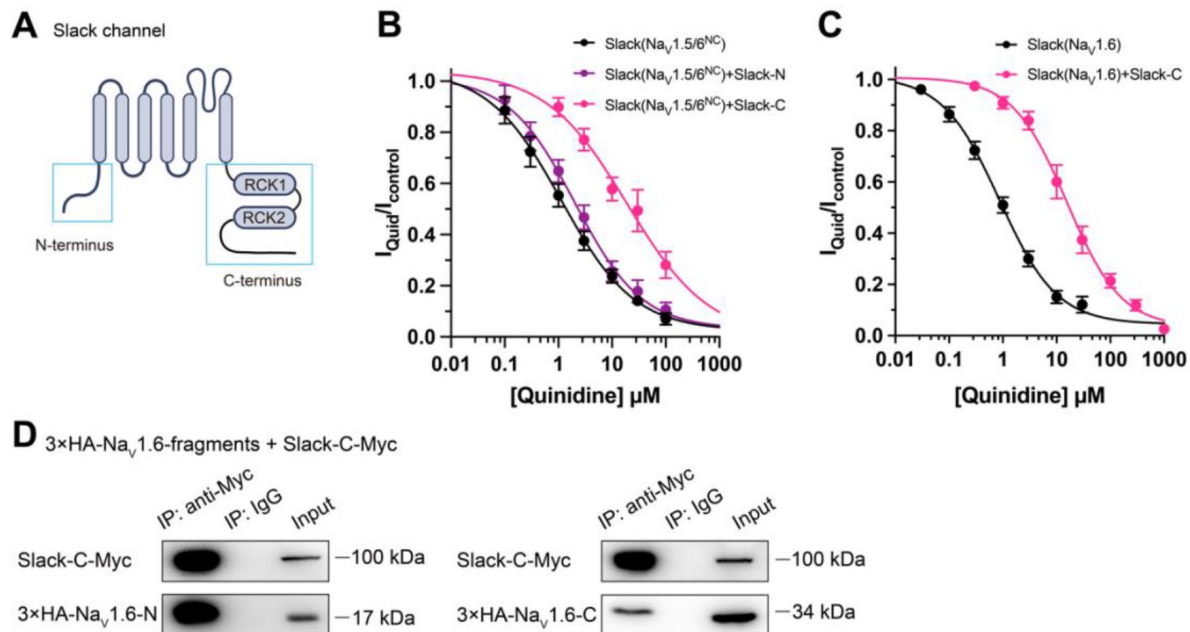


Figure 5.

Slack's C-terminus is required for Na_v1.6-mediated sensitization of Slack to quinidine blockade.

(A) Domain architecture of the human Slack channel subunit. Slack's N-terminus (Slack-N, residues 1-116) and C-terminus (Slack-C, residues 345-1235) were shown in the blue boxes. (B) The concentration-response curves for blocking of Slack by quinidine upon additional expression of Slack's N- or C-terminus in HEK293T cells co-expressing Slack and Na_v1.5/6^{NC}. (C) The concentration-response curves for blocking Slack by quinidine upon additional expression of Slack's C-terminus in HEK293 cells co-expressing Slack and Na_v1.6. (D) Co-IP of Myc-tagged Slack's C-terminus (Slack-C-Myc) with 3xHA-tagged Na_v1.6's termini (3xHA-Na_v1.6-N or 3xHA-Na_v1.6-C) in HEK293T cell lysates. The 3xHA tag was fused to the N-terminal region of Na_v1.6's fragments, and the Myc tag was fused to the C-terminal region of Slack's fragment.

blockade. We chose 3 Slack pathogenic mutant variants (K629N, R950Q, and K985N) initially detected in patients with KCNT1-related epilepsy.^{15,39,40} Considering that these 3 mutations are located in Slack's C-terminus, and recalling that Slack's C-terminus interacts with Na_v1.6 (Fig. 5D), we first used co-immunoprecipitation assays and successfully confirmed that each of these Slack mutant variants interacts with Na_v1.6 in HEK293T cell lysates (Fig. 6A).

Subsequently, whole-cell recordings revealed that Na_v1.6 significantly sensitized all of the examined Slack mutant variants to quinidine blockade, with IC₅₀ values ranging from 0.26 to 2.41 μM (Fig. 6B-D and Supplementary Table 4). These results support that Na_v1.6 interacts with examined Slack mutant variants and sensitizes them to quinidine blockade. It is plausible that the Slack-Na_v1.6 interaction contributes to the therapeutic role of quinidine in the treatment of KCNT1-related epilepsy.

Viral expression of Slack's C-terminus

prevents Slack^{G269S}-induced seizures

Having established that blocking Na_v1.6-mediated sodium influx significantly reduced Slack current amplitudes (Fig. 2D,G), we found that the heterozygous knockout of Na_v1.6 significantly reduced the afterhyperpolarization amplitude in murine hippocampal neurons (Fig. S8), together indicating that Na_v1.6 activates native Slack through providing Na⁺. We therefore assumed that disruption of the Slack-Na_v1.6 interaction should reduce the amount of Na⁺ in the close vicinity of epilepsy-related Slack mutant variants and thereby counter the increased current amplitudes of these Slack mutant variants. Pursuing this, we disrupted the Slack-Na_v interaction by overexpressing Slack's C-terminus (to compete with Slack) and measured whole-cell current densities. In HEK293 cells co-expressing epilepsy-related Slack mutant variants (G288S, R398Q)^{41,42} and Na_v1.5/6^{NC}, expression of Slack's C-terminus significantly reduced whole-cell current densities of Slack^{G269S} and Slack^{R398Q} (Fig. 7A,B), supporting that disrupting the Slack-Na_v1.6 interaction can indeed reduce current amplitudes of Slack mutant variants, which may protect against seizures induced by Slack mutant variants.

We next induced an *in vivo* epilepsy model by introducing a Slack G269S variant into C57BL/6N mice using adeno-associated virus (AAV) injection to mimic the human Slack mutation G288S.⁴¹ Specifically, we delivered stereotactic injections of AAV9 containing expression cassettes for Slack^{G269S} (or GFP negative controls) into the hippocampal CA1 region of 3-week-old C57BL/6N mice (Fig. 7C,D). At 3-5 week intervals after AAV injection, we quantified the seizure susceptibility of mice upon the induction of a classic kainic acid (KA) model of temporal lobe epilepsy.^{43,44} In this model, seizures with stage IV or higher (as defined by a modified Racine, Pinal, and Rovner scale⁴⁵) are induced in rodents by intraperitoneal administration of 28 mg/kg KA.

We assessed a time course of KA-induced seizure stages at 10-min intervals and found that viral expression of Slack^{G269S} resulted in faster seizure progression in mice compared to control GFP expression (Fig. 7E). We calculated the total seizure score per mouse to assess seizure severity.⁴⁶ Slack^{G269S}-expressing mice showed significantly higher seizure severity than GFP-expressing mice (Fig. 7F). The percentage of mice with stage VI-IX seizures also increased, from 9.1% in GFP-expressing control mice to 58.3% in the Slack^{G269S}-expressing mice (Fig. 7G). These results support that viral expression of Slack^{G269S} significantly increases seizure susceptibility in mice.

To evaluate the potential therapeutic effects of disrupting the Slack-Na_v1.6 interaction, we delivered two AAV9s (one for Slack^{G269S} and one for Slack's C-terminus [residues 326-1238]) into the CA1 region of mice (Fig. 7D). Viral expression of Slack's C-terminus in Slack^{G269S}-expressing mice significantly decreased seizure progression, seizure severity, and the percentage of mice experiencing stage VI-IX seizures (Fig. 7E-G). These results support that viral expression of

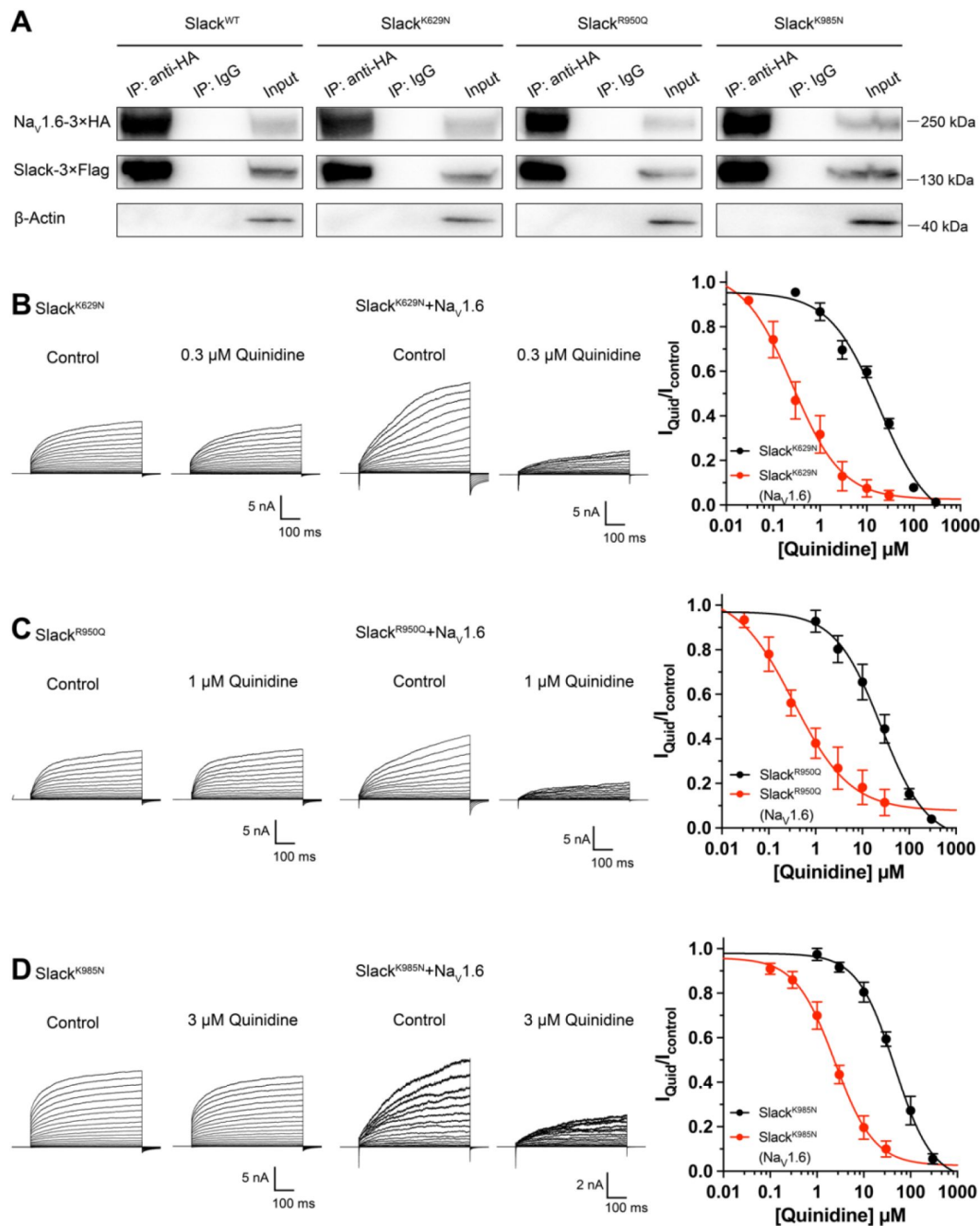


Figure 6.

Na_v1.6 sensitizes epilepsy-related Slack mutant variants to quinidine blockade.

(A) Co-IP of 3×Flag-tagged Slack or its mutations (Slack-3×Flag) with 3×HA-tagged Na_v1.6 (Na_v1.6-3×HA) in HEK293T cell lysates. The tags were all fused to the C-terminal region of wild-type or mutant ion channels. **(B–D)** The sensitivity of Slack mutant variants (Slack^{K629N} **[B]**, Slack^{R950Q} **[C]**, and Slack^{K985N} **[D]**) to quinidine blockade upon expression of Slack mutant variants alone and co-expression of Slack mutant variants with Na_v1.6. Left, example current traces recorded from HEK293 cells expressing Slack mutant variants alone and co-expressing Slack mutant variants with Na_v1.6, before and after application of the indicated concentrations of quinidine. Right, the concentration-response curves for blocking of Slack mutant variants by quinidine upon expression of Slack mutant variants alone ($n = 8$ for Slack^{K629N}, $n = 7$ for Slack^{R950Q}, and $n = 5$ for Slack^{K985N}) and co-expression of Slack mutant variants with Na_v1.6 ($n = 8$ for Slack^{K629N} upon co-expression with Na_v1.6, $n = 5$ for Slack^{R950Q} upon co-expression with Na_v1.6, and $n = 7$ for Slack^{K985N} upon co-expression with Na_v1.6). Please refer to Supplementary Table 4 for IC₅₀ values.

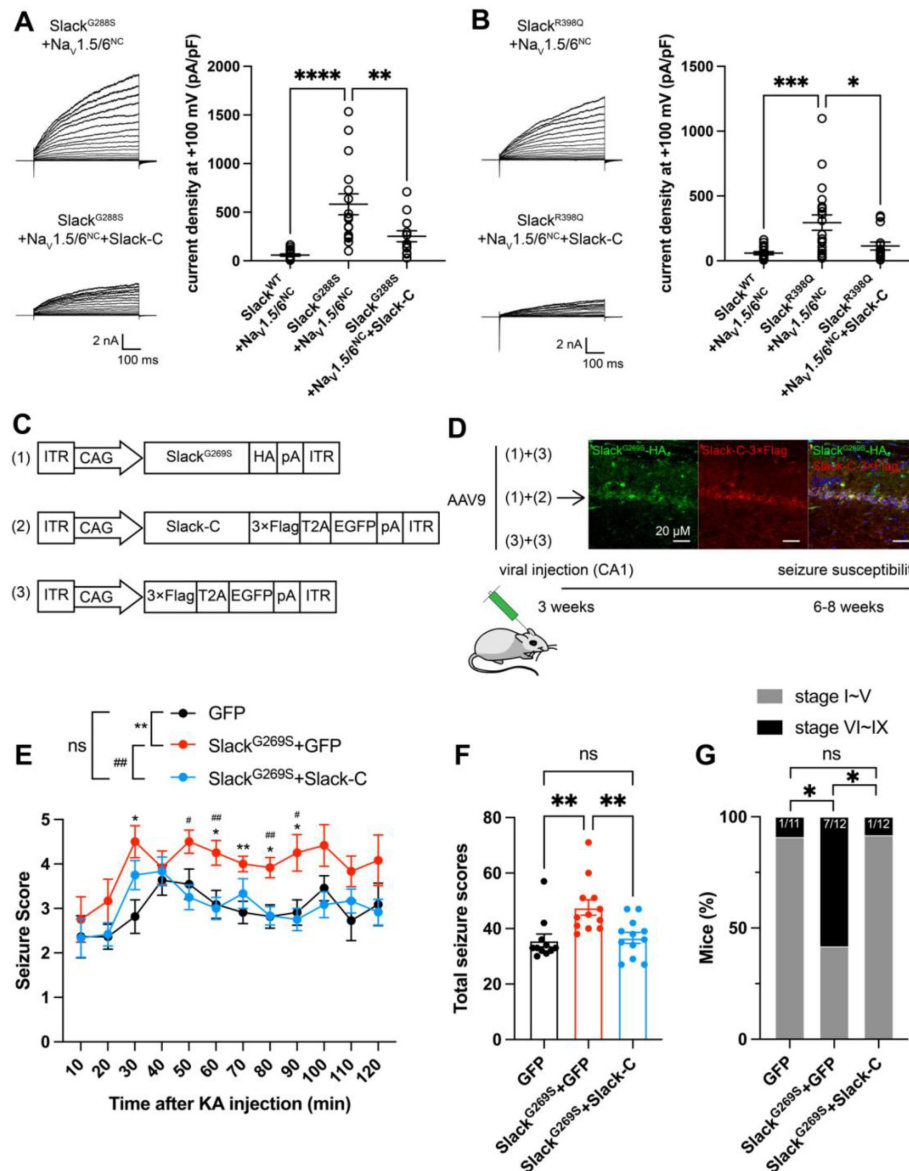


Figure 7.

Viral expression of Slack's C-terminus prevents Slack^{G269S}-induced seizures.

(A-B) The current densities of Slack mutant variants (Slack^{G288S} [A] and Slack^{R398Q} [B]) upon co-expression with Na_v1.5/6^{NC} in HEK293T cells were reduced by additional expression of Slack's C-terminus. Left, example current traces from HEK293T cells co-expressing Slack mutant variants and Na_v1.5/6^{NC} or co-expressing Slack mutant variants, Na_v1.5/6^{NC}, and Slack's C-terminus. Right, summarized current densities at +100 mV. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA followed by Bonferroni's post hoc test. (C) Architecture for expression cassettes of AAVs. (D) Top, Immunofluorescence of HA-tagged Slack^{G269S} (green), 3×Flag-tagged Slack's C-terminus (red), and DAPI (blue) at 5 weeks after viral injection of Slack^{G269S} with Slack's C-terminus into CA1 of mice. Bottom, study design and timeline for the stereotactic injection model. (E) Time-course of KA-induced seizure stage changes at 10-min intervals based on a modified Racine, Pinal, and Rovner scale (please refer to Methods for further details). The number of mice used: "GFP" control group ($n = 11$), "Slack^{G269S}+GFP" group ($n = 12$), "Slack^{G269S}+Slack-C" group ($n = 12$). "GFP" vs. "Slack^{G269S}+GFP": $F_{(1,21)} = 10.48$, $p = 0.0040$, * $p < 0.05$, ** $p < 0.01$; "Slack^{G269S}+GFP" vs. "Slack^{G269S}+Slack-C": $F_{(1,22)} = 10.30$, $p = 0.0040$, # $p < 0.05$, ## $p < 0.01$. "GFP" vs. "Slack^{G269S}+Slack-C": $F_{(1,21)} = 0.09574$, $p = 0.7600$. Repeated two-way ANOVA followed by Bonferroni's post hoc test. (F) Total seizure score per mouse over the 2 h after KA injection of these three groups. * $p < 0.05$, ** $p < 0.01$; one-way ANOVA followed by Bonferroni's post hoc test. (G) The percentage of mice with stage VI-IX seizures over the 2 h after KA injection in each group. * $p < 0.05$; Fisher's exact test.

Slack's C-terminus can prevent Slack^{G269S}-induced seizures in mice, thus showcasing that using Slack's C-terminus to disrupt the Slack-Na_v1.6 interaction is a promising therapeutic intervention to treat KCNT1-related epilepsy.

Discussion

We here found that Na_v1.6's N- and C-termini bind to Slack's C-terminus and sensitize Slack to quinidine blockade via Na_v1.6-mediated transient sodium currents. These results suggest that the pharmacological blocking effects of a channel blocker are not exclusively mediated by the channel *per se.*, but modulated by channel's interacting proteins. Moreover, we show that viral expression of Slack's C-terminus can rescue the increased seizure susceptibility and confer protection against Slack^{G269S}-induced seizures in mice.

At resting membrane potential, the intracellular sodium concentration ($[Na^+]_{in}$) in neurons (equal to ~10 mM) is too low to effectively activate Slack (K_d of 66 mM)^{1,20}. Slack is functionally coupled to sodium influx, which is known to be mediated by ion channels and receptors, including Na_v, AMPARs, and NMDARs^{21–23,47}. Such Na⁺ sources can provide both the membrane depolarization and the Na⁺ entry known to be required for Slack activation, enabling Slack to contribute both to action potential repolarization during neuronal high-frequency firing^{7,48} and to regulating excitatory postsynaptic potential (EPSP) at post-synaptic neurons²³. Our results support that Slack and Na_v1.6 form a channel complex, while also implying that Na_v1.6-mediated sodium influx increases the Na⁺ concentration in the close vicinity of Slack to activate Slack. As a low threshold Na_v channel subtype, Na_v1.6 has been reported to dominate the initiation and propagation of action potentials in axon initial segments (AIS) in excitatory neurons^{36,49–51}. The activation of Slack by Na_v1.6 at AIS has multiple impacts, including ensuring the timing of the fast-activated component of Slack currents, regulating the action potential amplitude, and apparently contributing to intrinsic neuronal excitability²⁹. The use of HEK cells and cultured primary cortical neurons in this study may not fully capture the complexity of native Slack-Na_v1.6 interaction. Future studies employing more intact systems, such as *in vivo* models, could provide a more comprehensive understanding of the physiological relevance of the native Slack-Na_v1.6 interaction.

An interesting question arises from our observation that Slack's sensitivity to quinidine blockade is enhanced by I_{NaT} but not I_{NaP} : what can explain the distinct I_{NaT} and I_{NaP} contributions? We speculate that with physical modulation by Na_v1.6, I_{NaT} may elicit a specific open conformation of Slack that brings its quinidine binding pocket into a high affinity state, which could lead to a substantial increase in Slack's sensitivity to quinidine blockade. The possibility of this hypothetical open conformation is supported by previous reports of the presence of subconductance states detected in single-channel recordings of *Xenopus* oocytes expressing Slack channels⁵², which implies that there are multiple open conformations of Slack. Although only one open conformation of Slack has been observed in cryo-EM, the gap between maximum conformational open probability (~1.0) and maximum functional open probability (~0.7) implies a subclass within this class of open channels⁵³. Additionally, it's worth noting that blocking of I_{NaP} by application of riluzole has its limitation on selectivity. Riluzole has been reported to bind to Slack channels with low affinity⁵⁴. Therefore, the dual binding of riluzole to both Slack and Na_v1.6 may stabilize or inhibit the Slack-Na_v1.6 interaction, consequently influencing the sensitization of Slack to quinidine blockade.

As previously mentioned, gain-of-function Slack mutant variants have been linked to a broad spectrum of epileptic disorders that are accompanied by intellectual disabilities and both psychomotor and developmental defects^{18,55}. Given that many patients are refractory or non-responsive to conventional anticonvulsants^{12,55,56}, and considering the limited success of quinidine in clinical treatment of KCNT1-related epilepsy, inhibitors targeting Slack are needed

urgently.¹⁸ Several small-molecule inhibitors against Slack have been reported, providing informative starting points for drug development efforts with KCNT1-related epilepsy.^{28,57,58} However, our discovery of the Slack-Nav1.6 complex challenges the traditional view that Slack acts as an isolated target in KCNT1-related epilepsy.¹⁸ Indeed, our study supports that co-expression of Slack and Nav1.6 in heterologous cell models should be performed when analyzing clinically relevant Slack mutations and when screening anti-epileptic drugs for use in treating KCNT1-related disorders.

Genotype-phenotype analysis has shown that ADSHE-related mutations are clustered in the regulator of conductance of K⁺ (RCK2) domain; while EIMFS-related mutations do not show a particular pattern of distribution.¹¹ All functionally tested Slack mutant variants show gain-of-function phenotypes, with increased Slack currents.^{9,11,18} Further, the epilepsy-related Slack mutant variants confer their gain-of-function phenotypes through two molecular mechanisms: increasing maximal channel open probability (P_{max}) or increasing sodium sensitivity (K_d) of Slack.⁵⁹ Both P_{max} and K_d are highly sensitive to $[Na^+]_{in}$; these are respectively analogous to the efficacy and potency of $[Na^+]_{in}$ on Slack currents.⁵⁹ Notably, several Slack mutant variants show gain-of-function phenotypes only at high $[Na^+]_{in}$ (e.g. 80 mM).⁵⁹ These results indicate that the gain-of-function phenotype of epilepsy-related Slack mutant variants is aggravated by high $[Na^+]_{in}$. Our discovery of functional coupling between Nav1.6 and Slack presents a plausible basis for how Nav1.6-mediated sodium influx can increase $[Na^+]_{in}$ and thus apparently aggravate the gain-of-function phenotype of Slack mutant variants. Therefore, it makes sense that disruption of the Slack-Nav1.6 interaction by overexpressing Slack's C-terminus reduces the current amplitudes of gain-of-function Slack mutant variants (**Fig. 7A,B**). Our successful demonstration that viral expression of Slack's C-terminus prevents epilepsy-related Slack^{G269S}-induced seizures in mice (**Fig. 7E-G**) warrants further translational evaluation for developing therapeutic interventions to treat KCNT1-related epilepsy.

Methods

Animals

C57BL/6 mice were purchased from Charles River Laboratories. Nav1.6 knockout C3HeB/FeJ mice were generous gifts from Professor Yousheng Shu at Fudan University. All animals were housed on a 12-hour light/dark cycle with *ad libitum* access to food and water. All procedures related to animal care and treatment were approved by the Peking University Institutional Animal Care and Use Committee and met the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Each effort was made to minimize animal suffering and the number of animals used. The experiments were blind to viral treatment condition during behavioral testing.

Antibodies and Reagents

Commercial antibodies used were: anti-AnkG (Santa Cruz), anti-Slack (NeuroMab), anti-Nav1.2 (Alomone), anti-Nav1.6 (Alomone), anti-HA (Abbkine), anti-Flag (Abbkine), anti- β -Actin (Biodragon), HRP Goat Anti-Mouse IgG LCS (Abbkine), HRP Mouse Anti-Rabbit IgG LCS (Abbkine), Alexa Fluor 488-AffinityPure Fab Fragment Donkey anti-rabbit IgG (Jackson), Alexa Fluor 594 Donkey anti-mouse IgG (Yeaston). GPCR Extraction Reagent was from Pierce, NP40 lysis buffer was from Beyotime, protease inhibitor mixture cocktail was from Roche Applied Science, rabbit IgG and mouse IgG was from Santa Cruz, and Protein G Dynabeads were from Invitrogen. Tetrodotoxin was from Absin Bioscience. Quinidine was from Macklin, and riluzole was from Meilunbio. All other reagents were purchased from Sigma-Aldrich.

Molecular cloning

Human Slack-B (Ref Seq: NM_020822.3) and Na_v1.6 (Ref Seq: NM_014191.4) were subcloned into the modified pcDNA3.1(+) vector using Gibson assembly. All mutations and chimeras of ion channels were also constructed using Gibson assembly. For GST pull down assay, the segments of Slack and Na_v1.6 were subcloned into pCDNA3.1(+) and pGEX-4T-1 vector, respectively. For FRET experiments, mVenus-tag was fused to the C-terminus of Na_v1.6 sequence, and mTFP1-tag was also fused to the C-terminus of Slack sequence.

Immunoprecipitation

The brain tissues or HEK293T cells co-expressing full-length or fragments of Slack and Na_v1.6 were homogenized and lysed in GPCR Extraction Reagent (Pierce) with cocktail for 30 min at 4 °C. The homogenate was centrifuged for 20 min at 16 000 g and 4 °C to remove cell debris and then supernatant was incubated with 5 µg Slack antibody (NeuroMab) or Nav1.6 antibody (Alomone) for 12 h at 4 °C with constant rotation. 40 µl of protein G Dynabeads (Invitrogen) was then added and the incubation was continued until the next day. Beads were then washed three times with NP40 lysis. Between washes, the beads were collected by DynaMag. The remaining proteins were eluted from the beads by re-suspending the beads in 1×SDS-PAGE loading buffer and incubating for 30 min at 37 °C. The resultant materials from immunoprecipitation or lysates were then subjected to western blot analysis.

Western blot analysis

Proteins suspended in 1×SDS-PAGE loading buffer were denatured for 30 min at 37 °C. Then proteins were loaded on 6% or 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose filter membrane (PALL). Non-specific binding sites were blocked with Tris-buffered saline-Tween (0.02 M Tris, 0.137 M NaCl and 0.1% Tween 20) containing 5% non-fat dried milk. Subsequently, proteins of interest were probed with primary antibodies for overnight at 4 °C. After incubation with a secondary antibody, immunoreactive bands were visualized using HRP Substrate Peroxide Solution (Millipore) according to the manufacturer's recommendation.

GST pull down assay

Plasmids encoding GST-fused Na_v1.6 segments were transformed into BL21(DE3). After expressing recombinant proteins induced by overnight application of isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.1mM) at 25°C, the bacteria were collected, lysed and incubated with GSH beads using BeaverBeads GSH kit (Beaver) according to the manufacturer's instruction.

Plasmids encoding KCNT1 channel or HA-tagged KCNT1 segments were transfected into HEK293T cells using lipofectamine 2000 (Introvigen). 40 h after transfection, cells were lysed in NP40 lysis buffer with inhibitor cocktail, and then centrifugated at 4 °C, 15000 g for 20 min. The supernatants were incubated with protein-bound beads. The protein-bound beads were washed by washing buffer (50 mM Tris-HCl pH = 7.4, 120 mM NaCl, 2 mM EGTA, 2 mM DTT, 0.1% triton X-100, and 0.2% Tween-20) for 5 min 3 times and then denatured with 1×SDS-PAGE loading buffer and incubating for 30 min at 37 °C. The resultant materials were subjected to western blot analysis.

Cell culture

The human embryonic kidney cells (HEK293 and HEK293T) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 15% Fetal Bovine Serum (FBS, PAN-Biotech) at 37°C and 5% CO₂. Primary cortical neurons were prepared from either sex of postnatal homozygous Na_v1.6 knockout C3HeB/FeJ mice and the wild-type littermate controls. After the mice were decapitated, the cortices were removed and separated from the meninges and surrounding tissue. Tissues were digested in 2 mg/mL Papain (Aladdin) containing 2 µg/mL DNase I (Psaitiong)

for 30 min followed by centrifugation and resuspension. Subsequently, the cells were plated on poly-D-lysine (0.05 mg/mL) pre-coated glass coverslips in plating medium (DMEM containing 15% FBS), at a density of 4×10^5 cells/ml, and cultivated at 37°C and 5% CO₂ in a humidified incubator. 5 h after plating, the medium was replaced with Neurobasal Plus medium (Invitrogen) containing 2% v/v B-27 supplement (Invitrogen), 2 mM Glutamax (Invitrogen), 50 U/mL penicillin and streptomycin (Life Technologies). The primary neurons were grown 6-10 days before electrophysiological recordings with half of the media replaced every three days.

Voltage-clamp recordings

The plasmids expressing full-length or fragments of Slack and Na_v channels (excluding full-length Na_v1.6) were co-transfected into HEK293 cells using lipofectamine 2000 (Introvigen). To co-express Slack with Na_v1.6, the plasmid expressing Slack was transfected into a stable HEK293 cell line expressing Na_v1.6. 18-36 h after transfection, voltage-clamp recordings were obtained using a HEKA EPC-10 patch-clamp amplifier (HEKA Electronic) and PatchMaster software (HEKA Electronic). For all whole-cell patch clamp experiments in HEK cells except for data presented in Fig. S2d, the extracellular recording solution contained (in mM): 140 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 5 glucose, 10 HEPES, 1 Tetraethylammonium chloride (310 mOsm/L, pH 7.30 with NaOH). The recording pipette intracellular solution (5 mM Na) contained (in mM): 100 K-gluconate, 30 KCl, 15 Choline-Cl, 5 NaCl, 10 glucose, 5 EGTA, 10 HEPES (300 mOsm/L, pH 7.30 with KOH). For data presented in Fig. S6D, the extracellular recording solution remains the same as above, the pipette intracellular solution contained (in mM): 140 CsF, 10 NaCl, 5 EGTA, 10 HEPES (pH 7.30 with NaOH), indicating that the unusual right shifts (15–20 mV) in voltage dependence of Na_v1.6 were induced by components in pipette solution, not recording system errors. For primary cortical neurons, intracellular solution (0 mM Na) was used to prevent the activation of sodium-activated potassium channels by basal intracellular sodium ions. NaCl in the intracellular solution was replaced with choline chloride in an equimolar concentration. For inside out patch-clamps, the bath solution contained (in mM): 140 NaCl, 1 EDTA, 10 HEPES and 2 MgCl₂ (310 mOsm/L, pH 7.30 with NaOH). Pipette solution contained (in mM): 130 KCl, 1 EDTA, 10 HEPES and 2 MgCl₂ (300 mOsm/L, pH 7.30 with KOH). The pipettes were fabricated by a DMZ Universal Electrode puller (Zeitz Instruments) using borosilicate glass, with a resistance of 1.5-3.5 MΩ for whole-cell patch clamp recordings and 8.0-10.0 MΩ for inside-out patch clamp recordings. All experiments were performed at room temperature. The concentration-response curves were fitted to four-parameter Hill equation:

$$Y = Bottom + (Top - Bottom)/(1 + 10^{(lgIC_{50} - X) * H}) \quad (1)$$

where Y is the value of $I_{Quinidine}/I_{Control}$, Top is the maximum response, Bottom is the minimum response, X is the lg of concentration, IC₅₀ is the drug concentration producing the half-maximum response, and H is the Hill coefficient. Significance of fitted IC₅₀ values compared to control was analyzed using extra sum-of-squares F test.

For data presented in fig. S3, cells were excluded from analysis if series resistance > 5 MΩ and series resistance compensation was set to 70%–90%.

The time constants (τ) of activation were fitted with a single exponential equation:

$$I(t) = Offset + A * \exp(-\frac{t}{\tau}) \quad (2)$$

where I is the current amplitude, t is time, offset represents the asymptote of the fit, and A represents the amplitude for the activation or inactivation.

Steady-state fast inactivation (I-V) and conductance-voltage (G-V) relationships were fitting with Boltzmann equations:

$$I/I_{max} = 1/(1 + \exp(\frac{V_m - V_{1/2}}{k})) \quad (3)$$

$$G/G_{max} = 1/(1 + \exp(\frac{V_m - V_{1/2}}{-k})) \quad (4)$$

$$G = I/(V_m - E_{Na}) \quad (5)$$

where I is the peak current, G is conductance, V_m is the stimulus potential, $V_{1/2}$ is the midpoint voltage, E_{Na} is the equilibrium potential, and k is the slope factor. Significance of fitted $V_{1/2}$ compared to control was analyzed using extra sum-of-squares F test.

Recovery from fast inactivation data were fitted with a single exponential equation:

$$I/I_{max} = A * (1 - \exp(-\frac{t}{\tau})) \quad (6)$$

Where I is the peak current of test pulse, I_{max} is the peak current of first pulse, A is the proportional coefficient, t is the delay time between the two pulses, and τ is the time constant of recovery from fast inactivation.

Acute slice preparation and current-clamp recordings

Horizontal slices containing hippocampus were obtained from 6-8 weeks old heterozygous $Na_v1.6$ knockout ($Scn8a^{+/-}$) C3HeB/FeJ mice and the wild-type littermate controls. In brief, animals were anesthetized and perfused intracardially with ice-cold modified “cutting solution” containing (in mM): 110 choline chloride, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 , 10 glucose; bubbled continuously with 95% O_2 /5% CO_2 to maintain PH at 7.2. The brain was then removed and submerged in ice-cold “cutting solution”. Next, the brain was cut into 300 μm slices with a vibratome (WPI). Slices were incubated in oxygenated (95% O_2 and 5% CO_2) “recording solution” containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 , 10 glucose (315 mOsm/L, PH 7.4, 37°C) for 30 min, and stored at room temperature.

Slices were subsequently transferred to a submerged chamber containing “recording solution” maintained at 34-36 °C. Whole-cell recordings were obtained from hippocampal CA1 neurons under a $\times 60$ water-immersion objective of an Olympus BX51WI microscope (Olympus). Pipettes had resistances of 5-8 M Ω . For current-clamp recordings, the external solution (unless otherwise noted) was supplemented with 0.05 mM (2R)-amino-5-phosphonovaleric acid (APV), 0.01 mM 6-cyano-7-nitro-quinoxaline-2,3-dione, 0.01 mM bicuculline and 0.001 mM CGP 55845, and internal pipette solution containing (in mM): 118 KMeSO₄, 15 KCl, 10 HEPES, 2 $MgCl_2$, 0.2 EGTA, and 4 Na_2ATP , 0.3 Tris-GTP, 14 Tris-phosphocreatinin (pH 7.3 with KOH).

In our whole-cell current-clamp recordings with an Axon 700B amplifier (Molecular Devices), we initially applied a 100 ms, 20 pA test pulse to the recording neurons right after breaking into the whole-cell configuration. A fast-rising component and a slow-rising component of voltage response were clearly visible. Then we zoomed into the fast-rising component of the voltage responses and turned up the pipette capacitance neutralization slowly to shorten the rise time of the fast-rising component until the oscillations of voltage responses appeared. Subsequently, we decreased the capacitance compensation just until the oscillations disappeared. For bridge balance of current-clamp recordings, we increased the value of bridge balance slowly until the fast component of the voltage response disappeared, and the slow-rising component appeared to rise directly from the baseline. Series resistance and pipette capacitance were compensated using the bridge balance and pipette capacitance neutralization options in the Multiclamp 700B command

software (Molecular Devices). The bridge balance value was between 20 and 30 MΩ and the pipette capacitance neutralization value was between 3 and 5 pF. For in vitro experiments, the cells were selected by criteria based on hippocampal CA1 cell morphology and electrophysiological properties in the slices. Electrophysiological recordings were made using a Multiclamp 700B amplifier (Molecular Devices). Recordings were filtered at 10 kHz and sampled at 50 kHz. Data were acquired and analyzed using pClamp10.0 (Molecular Devices). Series resistance was in the order of 10–30 MΩ and was approximately 60–80% compensated. Recordings were discarded if the series resistance increased by more than 20% during the time course of the recordings.

Fluorescence Imaging and FRET Quantification

The spectroscopic imaging was built upon a Nikon TE2000-U microscope. The excitation light was generated by an Ar laser. The fluorescent protein mVenus fused to Na_v1.6 and mTFP1 fused to Slack were excited by laser line at 500 and 400–440 nm, respectively. The duration of light exposure was controlled by a computer-driven mechanical shutter (Uniblitz). A spectrograph (Acton SpectraPro 2150i) was used in conjunction with a charge coupled device (CCD) camera (Roper Cascade 128B). In this recording mode two filter cubes (Chroma) were used to collect spectroscopic images from each cell (excitation, dichroic): cube I, D436/20, 455dclp; cube II, HQ500/20, Q515lp. No emission filter was used in these cubes. Under the experimental conditions, auto fluorescence from untransfected cells was negligible. Fluorescence imaging and analysis were done using the MetaMorph software (Universal Imaging). User-designed macros were used for automatic collection of the bright field cell image, the fluorescence cell image, and the spectroscopic image. Emission spectra were collected from the plasma membrane of the cell by positioning the spectrograph slit across a cell and recording the fluorescence intensity at the position corresponding to the membrane region (**Fig. 2e**, dotted lines in red); the same slit position applied to both the spectrum taken with the mTFP1 excitation and the spectrum taken with the mVenus excitation. Using this approach, the spectral and positional information are well preserved, thus allowing reliable quantification of FRET efficiency specifically from the cell membrane. Spectra were corrected for background light, which was estimated from the blank region of the same image.

FRET data was quantified in two ways. First, the FRET ratio was calculated from the increase in mVenus emission due to energy transfer as described in the previous study.⁶⁰ Briefly, mTFP1 emission was separated from mVenus emission by fitting of standard spectra acquired from cells expressing only mVenus or mTFP1. The fraction of mVenus-tagged molecules that are associated with mTFP1-tagged molecules, Ab , is calculated as

$$Ab = 1/(1 + K_D/[D_{free}]) \quad (7)$$

where K_D is the dissociation constant and $[D_{free}]$ is the concentration of free donor molecules. Note that

$$FRET\ Ratio = 1 + Ab * (FRET\ Ratio_{max} - 1) \quad (8)$$

Regression analysis was used to estimate Ab in individual cells. From each cell, the FRET ratio_{exp} was experimentally determined. The predicted Ab value was then computed by adjusting two parameters, FRET Ratio_{max} and apparent K_D . Ab was in turn used to give a predicted FRET ratio_{predicted}. By minimizing the squared errors (FRET ratio_{exp} – FRET ratio_{predicted})², K_D was determined.

Second, apparent FRET efficiency was also calculated from the enhancement of mVenus fluorescence emission due to energy transfer^{60–63} using a method as previously described.⁶⁴ Briefly, Ratio A_0 and Ratio A were measured to calculate FRET efficiency. Ratio A_0

represents the ratio between tetramethylrhodamine maleimide emission intensities (in the absence of fluorescein maleimide) upon excitation at the donor and acceptor excitation wavelengths^{63–65}, and was calculated in the present study at the mVenus peak emission wavelength. A particular advantage of quantifying Ratio A_0 for FRET measurement is that changes in fluorescence intensity caused by many experimental factors can be cancelled out by the ratiometric measurement. A similar ratio, termed Ratio A, was determined in the presence of mTFP1 in the same way as Ratio A_0 . If FRET occurred, the Ratio A value should be higher than Ratio A_0 ; the difference between Ratio A and Ratio A_0 was directly proportional to the FRET efficiency by the factor of extinction coefficient ratio of mTFP1 and mVenus.^{63–65}

Immunostaining

After deep anesthesia with sodium pentobarbital, mice were sacrificed by perfusion with 0.5% paraformaldehyde and 0.5% sucrose (wt/vol) in 0.1 M phosphate buffer (pH 7.4). The brain was removed and post-fixed in the same fixative for 2 h, and subsequently immersed in 30% sucrose in 0.1 M phosphate buffer for 48 h. Cryostat coronal sections (20 μ m) were obtained using a freezing microtome (Leica). The sections were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.4), permeabilized in 0.5% Triton X-100 in PBS for 30 min, and incubated in a blocking solution (5% BSA, 0.1% Triton X-100 in PBS, vol/vol) at 20–25 °C for 2 h, followed by overnight incubation at 4 °C with primary antibody to AnkG (1:100, Santa Cruz, sc-31778), Slack (1:100, NeuroMab, 73-051), Na_v1.2 (1:200, Alomone, ASC-002), Na_v1.6 (1:200, Alomone, ASC-006), Flag (1:500, Abbkine, ABT2010), and HA (1:500, Abbkine, ABT2040) in blocking solution. After a complete wash in PBS, the sections were incubated in Alexa 488-conjugated donkey anti-rabbit IgG and Alexa 594 donkey anti-mouse IgG in blocking solution at 20–25 °C for 2 h. The sections were subsequently washed and rinsed in Dapi solution. Images were taken in the linear range of the photomultiplier with a laser scanning confocal microscope (ZEISS LSM 510 META NLO).

Adeno-associated virus construction and injection

The adeno-associated viruses (AAVs) and the negative GFP control were from Shanghai GeneChem. Co., Ltd. The full-length Slack_{G269S} sequence (1-1238aa) was ligated into modified CV232 (CAG-MCS-HA-Poly A) adeno-associated viral vector.

The Slack's C-terminus sequence (326-1238aa) and the negative control were ligated into GV634 (CAG-MCS-3×Flag-T2A-EGFP-SV40-Poly A) adeno-associated viral vector. The viruses (>10¹¹ TU/ml) were used in the present study.

For dorsal CA1 viral injection, C57BL/6J mice aged 3 weeks were anesthetized with isoflurane and placed in a stereotaxic apparatus (RWD Life Science Co., Ltd.). Using a 5 μ L micro syringe (Hamilton) with a 30 gauge needle (RWD Life Science Co., Ltd.), 600 nL of the viruses was delivered at 10 nL/min by a micro-syringe pump (RWD Life Science Co., Ltd.) at the following site in each of the bilateral CA1 regions, using the stereotaxic coordinates: 2.5 mm (anterior-posterior) from bregma, 2 mm (medio-lateral), $\pm$ 1.5 mm (dorsal-ventral)⁶⁶. The syringe was left in place for 5 min after each injection and withdrawn slowly. The exposed skin was closed by surgical sutures and returned to home cage for recovery. All the experiments were conducted after at least 3 weeks of recovery. All the mice were sacrificed after experiments to confirm the injection sites and the viral trans-infection effects by checking EGFP under a fluorescence microscope (ZEISS LSM 510 META NLO).

Kainic acid-induced status epilepticus

KA (Sigma-Aldrich) was intraperitoneally administered to produce seizures with stage IV or higher. The dose of kainic acid used was 28 mg/kg for mice (6–8 weeks)^{67,68}. To assess epilepsy susceptibility, seizures were rated using a modified Racine, Pinal, and Rovner scale^{45,69}: (1) Facial movements; (2) head nodding; (3) forelimb clonus; (4) dorsal extension (rearing); (5) Loss of

balance and falling; (6) Repeated rearing and failing; (7) Violent jumping and running; (8) Stage 7 with periods of tonus; (9) Dead. Seizures was terminated 2 h after onset with the use of sodium pentobarbital (30 mg/kg; Sigma-Aldrich).

Statistical analysis

For in vitro experiments, the cells were evenly suspended and then randomly distributed in each well tested. For in vivo experiments, the animals were distributed into various treatment groups randomly. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software) and SPSS 26.0 software (SPSS Inc.). Before statistical analysis, variation within each group of data and the assumptions of the tests were checked. Comparisons between two independent groups were made using unpaired Student's two-tailed t test. Comparisons among nonlinear fitted values were made using extra sum-of-squares F test. Comparisons among three or more groups were made using one- or two-way analysis of variance followed by Bonferroni's post hoc test. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported previously in the field^{37,70}. All experiments and analysis of data were performed in a blinded manner by investigators who were unaware of the genotype or manipulation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data are presented as mean $\pm$ SEM.

Author contributions

T.Y. and Y.J. performed and analyzed voltage-clamp recordings. Y.W. performed and analyzed Western blotting, immunoprecipitation. S.X., C.P., and G.D. performed and analyzed pull down assay. Q.C. performed the immunostaining. H.Z. and F.Y. performed and analyzed the FRET imaging. H.S., N.L., and X.M. performed and analyzed the current clamp recordings. T.Y., H.Y., Z.G., and J.D. performed the molecular cloning. Z.H., T.Y., and Y.W. designed the experiments. Z.H., T.Y., and Y.J. wrote the manuscript. Z.H., F.Y., Y.Y., and Q.S. reviewed the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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69. Racine RJ (1972) **Modification of seizure activity by electrical stimulation II. Motor seizure.** *Electroencephalogr Clin Neurophysiol* **32**:281–94 [https://doi.org/10.1016/0013-4694\(72\)90177-0](https://doi.org/10.1016/0013-4694(72)90177-0)
70. Huang Z, Lujan R, Martinez-Hernandez J, Lewis AS, Chetkovich DM, Shah MM. (2012) **TRIP8b-independent trafficking and plasticity of adult cortical presynaptic HCN1 channels** *J Neurosci* **32**:14835–48 <https://doi.org/10.1523/JNEUROSCI.1544-12.2012>

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Editors

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Senior Editor

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National Institute of Neurological Disorders and Stroke, National Institutes of Health, United States of America

Reviewer #1 (Public Review):

Despite numerous studies on quinidine therapies for epilepsies associated with GOF mutant variants of Slack, there is no consensus on its utility due to contradictory results. In this study Yuan et al. investigated the role of different sodium selective ion channels on the sensitization of Slack to quinidine block. The study employed electrophysiological approaches, FRET studies, genetically modified proteins and biochemistry to demonstrate that Nav1.6 N- and C-tail interacts with Slack's C-terminus and significantly increases Slack sensitivity to quinidine blockade in vitro and in vivo. This finding inspired the authors to investigate whether they could rescue Slack GOF mutant variants by simply disrupting the interaction between Slack and Nav1.6. They find that the isolated C-terminus of Slack can reduce the current amplitude of Slack GOF mutant variants co-expressed with Nav1.6 in HEK cells and prevent Slack induced seizures in mouse models of epilepsy. This study adds to the growing list of channels that are modulated by protein-protein interactions, and is of great value for future therapeutic strategies.

Reviewer #2 (Public Review):

This is a very interesting paper about the coupling of Slack and Nav1.6 and the insight this brings to the effects of quinidine to treat some epilepsy syndromes.

Slack is a sodium-activated potassium channel that is important to hyperpolarization of neurons after an action potential. Slack is encoded by KNCT1 which has mutations in some epilepsy syndromes. These types of epilepsy are treated with quinidine but this is an atypical antiseizure drug, not used for other types of epilepsy. For sufficient sodium to activate Slack, Slack needs to be close to a channel that allows robust sodium entry, like Nav channels or AMPA receptors. but more mechanistic information is not available. Of particular interest to the authors is what allows quinidine to be effective in reducing Slack.

In the manuscript, the authors show that Nav, not AMPA receptors, are responsible for Slack's sensitization to quinidine blockade, at least in cultured neurons (HeK293, primary cortical neurons). Most of the paper focuses on the evidence that Nav1.6 promotes Slack sensitivity to quinidine.

Reviewer #3 (Public Review):

Yuan et al., set out to examine the role of functional and structural interaction between Slack and Navs on the Slack sensitivity to quinidine. Through pharmacological and genetic means they identify Nav1.6 as the privileged Nav isoform in sensitizing Slack to quinidine. Through

biochemical assays, they then determine that the C-terminus of Slack physically interacts with the N- and C-termini of NaV1.6. Using the information gleaned from the in vitro experiments the authors then show that virally-mediated transduction of Slack's C-terminus lessens the extent of SlackG269S-induced seizures. These data uncover a previously unrecognized interaction between a sodium and a potassium channel, which contributes to the latter's sensitivity to quinidine.

Author Response

The following is the authors' response to the original reviews.

We would like to thank the editor and all the reviewers for their time and thoughtful consideration of our manuscript. We appreciate the valuable comments. Our provisional response to the “public review” has been published and now we have corrected factual errors and enhanced the clarity of writings based on the “recommendations for the authors.” We believe these corrections will improve the quality and accuracy of our manuscript.

Specific responses to the reviewers' recommendations for the authors are as follows:

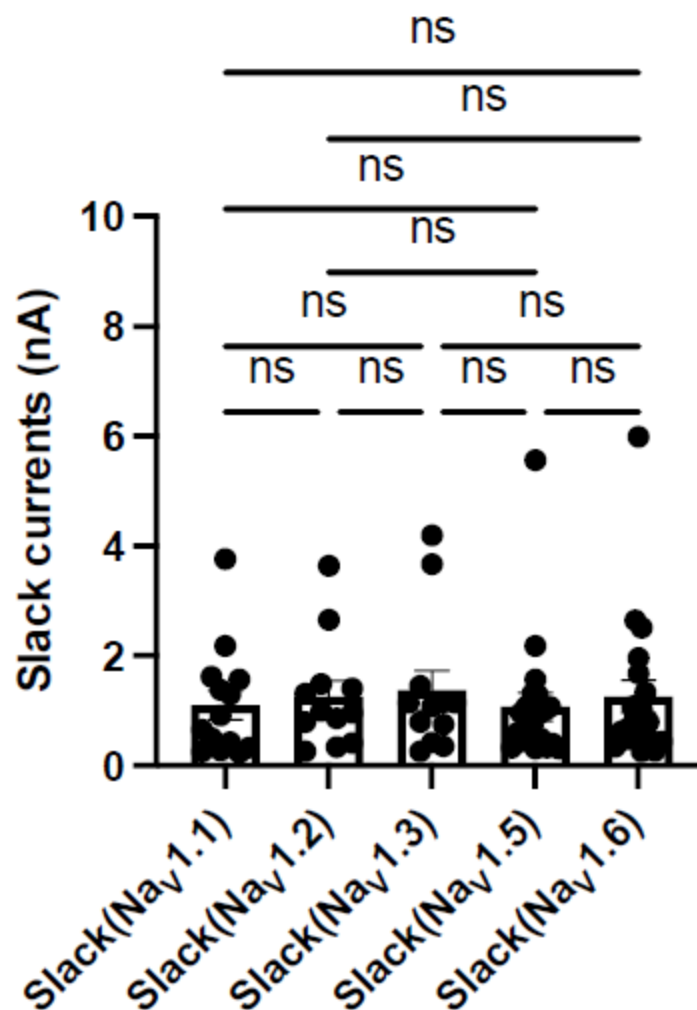
Reviewer #1 (Recommendations For The Authors):

- 1. Is the Slack current amplitude dependent on the Nav subtype? Differences in Slack current amplitude might explain the sensitization of Slack to quinidine.*

We appreciate the reviewer for raising this point. We examined Slack current amplitudes upon co-expression of Slack with specific NaV subtypes in HEK293 cells. The results have shown that there are no significant differences in Slack current amplitudes upon co-expression of Slack with different NaV channel subtypes (Author response image 1), suggesting whole-cell Slack current amplitudes cannot explain the varied ability of NaV subtypes to sensitize Slack to quinidine blockade.

Author response image 1.

The amplitudes of Slack currents upon co-expression of Slack with specific NaV subtypes in HEK293 cells. ns, $p > 0.05$, one-way ANOVA followed by Bonferroni's post hoc test.



1. Is the open probability changed by the presence of Nav1.6 and/or by the other Nav subtypes? Changes in open probability might explain the Nav1.6 induced sensitization of Slack to quinidine block.

We appreciate the reviewer for raising this point. To investigate the effect of different Nav channel subtypes on Slack open probability, we will perform the single-channel recordings in future studies.

1. Could the authors elaborate more on the coupling between INaT mediated sensitization of Slack to block by quinidine and the Nav1.6 N- and C-tail induced sensitization?

We appreciate the reviewer for raising this point. We fully agree the importance of investigating the detailed mechanism underlying the sensitization of Slack to quinidine blockade. To address the questions, we plan to employ structural biological methods, such as cryo-electron microscopy (cryo-EM).

1. Line 85: The authors use an outdated nomenclature of AMPAR subtypes. I would suggest changing to GluA1, GluA2, GluA3 and GluA4.

We appreciate the reviewer's suggestion. We have changed the term "GluR" to "GluA" in the revised manuscript.

The authors do not explain the rationale by using the different homomeric AMPAR subtypes. Most often the AMPARs express as heteromeric receptors decorated by auxiliary subunits. Also, is the GluA2 the edited version?

We thank the reviewer for raising this point. While AMPARs are often expressed as heteromeric receptors with auxiliary subunits, we focused on the homomeric AMPAR subtypes for initial screening. Through our investigation, we found no significant effects on sensitizing Slack to quinidine blockade. Additionally, the GluA2 used in our study is unedited.

1. Line 144: I expect a reduction in current amplitude caused by blocking INaT and INaP is tested at +100mV?

We thank the reviewer for raising this point. The reduction in current amplitude was indeed tested at +100 mV and we have included this information in the revised manuscript.

1. Line 157 and line 162: Reference to Supplementary table S3 should be Table S2.

We thank the reviewer for pointing this out. The reference to "Table S3" has been corrected to "Table S2" in the revised manuscript.

1. How many times did the authors repeat the co-immunoprecipitation? Some of the bands are very weak, and repeats are necessary for all blots.

We thank the reviewer for raising this concern. We performed the co-immunoprecipitation experiments three times independently.

1. Line 288: The authors are showing the chimeric construct in Figures 7A and B but are referring to the full length Nav1.6 in the main text line 288.

We apologize for the confusion. We have clarified in the revised manuscript that we used Nav1.5/6NC in our study.

1. Figure 1 line 23: 1 uM quinidine must be 30 uM quinidine?

We thank the reviewer for catching this error. We have corrected the concentration value in the caption of Figure 1 from "1 μ M" to "30 μ M" in the revised manuscript.

1. Figure 2 line 53: I expect IC50 is measured at +100mV? Same question for line 60 in same figure text.

We thank the reviewer for pointing this out. We have now included this information in the revised manuscript.

1. Figure 4B color coding is confusing.

We apologize for the confusion. We would like to clarify that Fig. 4B illustrates the domain architecture of the human NaV channel pore-forming α subunit, and we have changed the color from dark blue to black in the revised figure.

1. *Figure S6: Text for figure S6E and S6F has been swapped (line 96 to 106).*

We thank the reviewer for raising this point. We have rectified the swapped captions for Fig. S6E and Fig. S6F in the revised manuscript.

1. *Methods section line 652: Kainite acid should be changed to kainic acid*

We thank the reviewer for catching this typo. The term “kainite acid” has been corrected to “kainic acid” in the revised manuscript.

Reviewer #2 (Recommendations For The Authors):

1. *Discuss limitations about the use of non-neuronal cells or cultured primary neurons rather than a more intact system.*

We thank the reviewer for raising this point. We have discussed the limitations about the use of non-neuronal cells or cultured primary neurons rather than a more intact system (line 344 to line 348).

1. *Riluzole is not a selective drug, so the limitations of this drug should be discussed.*

We thank the reviewer for raising this point. We have discussed the limitations of riluzole in the revised manuscript (line 360 to line 364).

1. *Remove the term in vivo.*

We thank the reviewer for raising this point. In our experiments, although we did not conduct experiments directly in living organisms, our results demonstrated the coimmunoprecipitation of Nav1.6 with Slack in homogenates from mouse cortical and hippocampal tissues (Fig. 3C). This result may support that the interaction between Slack and Nav1.6 occurs in vivo.

1. *Figure 1*

①C *Why does Nav1.2 have a small inward current before the large inward current in the inset? The slope of the rising phase of the larger sodium current seems greater than Nav1.6 or Nav1.5. Was this examined?*

We apologize for the confusion. We would like to clarify that the small inward current can be attributed to the current of membrane capacitance (slow capacitance or C-slow). The larger inward current is mediated by Nav1.2. Additionally, we did not compare the slope of the rising phase of Nav subtypes sodium currents but primarily focused on the current amplitudes.

②D-E

For Nav1.5 the sodium current is very large compared to Nav1.6. Is it possible the greater effect of quinidine for Nav1.6 is due to the lesser sodium current of Nav1.6?

We thank the reviewer for raising this point. We would like to clarify that our results indicate that transient sodium currents contribute to the sensitization of Slack to quinidine blockade

(Fig. 2C,E). Therefore, it is unlikely that the greater effect observed for NaV1.6 in sensitizing Slack is due to its lower sodium currents.

③The differences between WT and KO in G-H are hard to appreciate. Could quantification be shown? The text uses words like "block" but this is not clear from the figure. It seems that the replacement of Na⁺ with Li⁺ did not block the outward current or effect of quinidine.

We apologize for the confusion. We would like to clarify the methods used in this experiment. The lithium ion (Li⁺) is a much weaker activator of sodium-activated potassium channel Slack than sodium ion (Na⁺)^{1,2}.

1. Zhang Z, Rosenhouse-Dantsker A, Tang QY, Noskov S, Logothetis DE. The RCK2 domain uses a coordination site present in Kir channels to confer sodium sensitivity to Slo2.2 channels. J Neurosci. Jun 2 2010;30(22):7554-62. doi:10.1523/JNEUROSCI.0525-10.2010
2. Kaczmarek LK. Slack, Slick and Sodium-Activated Potassium Channels. ISRN Neurosci. Apr 18 2013;2013(2013)doi:10.1155/2013/354262

Therefore, we replaced Na⁺ with Li⁺ in the bath solution to measure the current amplitudes of sodium-activated potassium currents (IKNa)³.

1. Budelli G, Hage TA, Wei A, et al. Na⁺-activated K⁺ channels express a large delayed outward current in neurons during normal physiology. Nat Neurosci. Jun 2009;12(6):745-50. doi:10.1038/nn.2313

The following equation was used for quantification:

$$I_{KNa} = \text{Outward current amplitudes}_{Na^+ \text{ based bath solution}} - \text{Outward current amplitudes}_{Li^+ \text{ based bath solution}}$$

Furthermore, the remaining IKNa after application of 3 μM quinidine in the bath solution was measured as the following:

$$I_{KNa} \text{ with } 3 \mu\text{M quinidine} = \text{Outward current amplitudes}_{Na^+ \text{ based bath solution with } 3 \mu\text{M quinidine}} - \text{Outward current amplitudes}_{Li^+ \text{ based bath solution with } 3 \mu\text{M quinidine}}$$

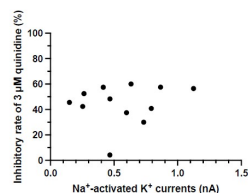
The quantification results were presented in Fig. 1K. The term "block" used in the text referred to the inhibitory effect of quinidine on IKNa.

④In K, for the WT, why is the effect of quinidine only striking for the largest currents?

We thank the reviewer for raising this point. After conducting an analysis, we found no correlation between the inhibitory effect of quinidine and the amplitudes of baseline IKNa in WT neurons (p = 0.6294) (Author response image 2). Therefore, the effect of quinidine is not solely limited to targeting the larger currents.

Author response image 2.

The correlation between the inhibitory effect of quinidine and the amplitudes of baseline IKNa in WT neurons (data from manuscript Fig. 1K). r = 0.1555, p=0.6294, Pearson correlation analysis.



1. Figure 2

①A. The argument could be better made if the same concentration of quinidine were used for Slack and Slack + Nav1.6. It is recognized a greater sensitivity to quinidine is to be shown but as presented the figure is a bit confusing.

We apologize for the confusion. We would like to clarify that the presented concentrations of quinidine were chosen to be near the IC₅₀ values for Slack and Slack+Nav1.6.

②C. Can the authors add the effect of quinidine to the condition where the prepulse potential was -90?

We apologize for the confusion. We would like to clarify that the condition of prepulse potential at -90 mV is the same as the condition in Fig. 1. We only changed one experiment condition where the prepulse potential was changed to -40 mV from -90 mV.

1. Figure 3.

①line 80 should be coronal not coronary

We thank the reviewer for catching this error. We have corrected the term “coronary” to “coronal” in the caption of Figure 3.

②A. Clarify these 6 panels.

We thank the reviewer for raising this point. We have clarified the captions of Fig. 3A in the revised manuscript.

③Please enlarge fonts in D.

We thank the reviewer’s suggestion. We’ve enlarged the fonts in Fig. 3D in the revised manuscript.

④F. The variances should be checked with a test to determine if they are significantly different because they look different - if so, data can be transformed and if transformed data have variances that are equivalent a t-test can be used on the transformed data. Otherwise, Mann-Whitney should be used.

We thank the reviewer for pointing this out. We have reanalyzed the data in Fig. 3F using Mann Whitney test after identifying the different variances in the two groups.

1. Figure 7. The images need more clarity. They are very hard to see. Text is also hard to see.

We apologize for the lack of clarity in the images and text. we would like to provide a concise summary of the key findings shown in this figure.

Figure 7 illustrates an innovative intervention for treating SlackG269S-induced seizures in mice by disrupting the Slack-Nav1.6 interaction. Our results showed that blocking Nav1.6-mediated sodium influx significantly reduced Slack current amplitudes (Fig. 2D,G), suggesting that the Slack-Nav1.6 interaction contributes to the current amplitudes of epilepsy-related Slack mutant variants, aggravating the gain-of-function phenotype. Additionally, Slack's C-terminus is involved in the Slack-Nav1.6 interaction (Fig. 5D). We assumed that overexpressing Slack's C-terminus can disrupt the Slack-Nav1.6 interaction (compete with Slack) and thereby encounter the current amplitudes of epilepsy-related Slack mutant variants.

In HEK293 cells, overexpression of Slack's C-terminus indeed significantly reduced the current amplitudes of epilepsy-related SlackG288S and SlackR398Q upon co-expression with Nav1.5/6NC (Fig. 7A,B). Subsequently, we evaluated this intervention in an in vivo epilepsy model by introducing the Slack G269S variant into C57BL/6N mice using AAV injection, mimicking the human Slack mutation G288S that we previously identified (Fig. 7C-G).

②It is not clear how data were obtained because injection of kainic acid does not lead to a convulsive seizure every 10 min for several hours, which is what appears to be shown. Individual seizures are just at the beginning and then they merge at the start of status epilepticus. After the onset of status epilepticus the animals twitch, have varied movements, sometime rear and fall, but there is not a return to normal behavior. Therefore one can not call them individual seizures. In some strains of mice, however, individual convulsive seizures do occur (even if the EEG shows status epilepticus is occurring) but there are rarely more than 5 over several hours and the graph has many more. Please explain.

We apologize for the confusion. Regarding the data acquisition in relation to kainic acid injection, we initiated the timing following intraperitoneal injection of kainic acid and recorded the seizure scores of per mouse at ten-minute intervals, following the methodology described in previous studies⁴.

1. Huang Z, Walker MC, Shah MM. Loss of dendritic HCN1 subunits enhances cortical excitability and epileptogenesis. J Neurosci. Sep 2 2009;29(35):10979-88. doi:10.1523/JNEUROSCI.1531-09.2009

The seizure scores were determined using a modified Racine, Pinal, and Rovner scale^{5,6}: (1) Facial movements; (2) head nodding; (3) forelimb clonus; (4) dorsal extension (rearing); (5) Loss of balance and falling; (6) Repeated rearing and failing; (7) Violent jumping and running; (8) Stage 7 with periods of tonus; (9) Dead.

1. Pinel JP, Rovner LI. Electrode placement and kindling-induced experimental epilepsy. Exp Neurol. Jan 15 1978;58(2):335-46. doi:10.1016/0014-4886(78)90145-0
2. Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol. Mar 1972;32(3):281-94. doi:10.1016/0013-4694(72)90177-0

1. The graphical abstract is quite complicated and somewhat hard to follow. Please simplify and clarify. One aspect of the abstract to clarify is the direction of what is first and second and third (etc.) because arrows point to many directions.

We thank the review for raising this point. In the revised manuscript, we have included numbering of three components within the graphical abstract:

1. Pathological phenotype: Increased Slack currents.
2. Two types of interventions:
 - 2a. Disruption of the Slack-Nav1.6 interaction.
 - 2b. Nav1.6-mediated sensitization of Slack to quinidine blockade.
1. Therapeutic effects: Reduced Slack currents.

Reviewer #3 (Recommendations For The Authors):

1. *A reference to homozygous knockout is made in the abstract; however, only heterozygous mice are mentioned in the methods section. The genotype of the mice needs to be made clear in the manuscript. Furthermore, at what age were these mice used in the study. Since homozygous knockout of Nav1.6 is lethal at a very young age (<4 wks), it would be important to clarify that point as well.*

We thank the reviewer for pointing this out. In the revised manuscript, we have included information about the source of the primary cortical neurons used in our study. These neurons were obtained from postnatal homozygous Nav1.6 knockout C3HeB/FeJ mice and their wild-type littermate controls.

1. *Coimmunoprecipitation studies in Fig. 3C are not convincing. There appears to be a signal in the control lane. Furthermore, it appears that brightness levels were adjusted of that image, thereby removing completely the background.*

We thank the reviewer for pointing this out. We have replaced Fig. 3C with an unadjusted version in the revised manuscript.

1. *In Fig. 1B, the authors indicate that 30 microM of quinidine was used, while the corresponding figure legend suggest that 1 microM. Please clarify.*

We apologize for this error. We have corrected the concentration value in the caption of Figure 1 from "1 μ M" to "30 μ M" in the revised manuscript.

1. *How long were the cells exposed to quinidine before the functional measurement were performed?*

We thank the reviewer for pointing this out. The cells were exposed to the bath solution with quinidine for about one minute before applying step pulses.

1. *In Fig. 6B-D, it is not clear to what extent co-expression of Slack mutants and Nav1.6 increases sodium-activated potassium current.*

We thank the reviewer for pointing this out. We notice that the current amplitudes of Slack mutants exhibit a considerable degree of variation, ranging from less than 1 nA to over 20 nA (n = 5-8). To accurately measure the effects of Nav1.6 on increasing current amplitudes of

Slack mutants, we plan to apply tetrodotoxin in the bath solution to block NaV1.6 sodium currents upon coexpression of Slack mutants with NaV1.6.

1. In Fig.7A and B, it appears that some recordings had no sodium-activated potassium currents. Why were these included in analysis? How was transfection efficacy assessed?

We apologize for the confusion. We would like to clarify that all recordings included in analysis indeed exhibited outward sodium-activated potassium currents. The current density data in Fig. 7A-B are listed in Author response table 1 (in pA/pF):

Author response table 1.

Slack ^{WT} +Nav1.5/6 ^{NC}	Slack ^{G288S} +Nav1.5/6 ^{NC}	Slack ^{G288S} +Nav1.5/6 ^{NC} + Slack-C	Slack ^{R398Q} +Nav1.5/6 ^{NC}	Slack ^{R398Q} +Nav1.5/6 ^{NC} + Slack-C
45.7062698	1343.0427	146.625544	1097.53966	122.010728
30.7140853	100.195545	708.842096	106.979589	137.667904
34.4261155	234.543173	175.227216	26.9605994	43.0093997
163.431187	350.710883	132.744072	142.696986	71.4532872
141.803904	432.673971	76.266757	413.042558	13.2912269
118.632034	726.993992	294.93293	472.86561	65.8624425
112.282335	1534.00324	28.7317243	20.9398649	6.78008203
78.7868707	448.570294	385.820065	169.129103	40.8888059
22.7697719	637.444699	81.6508777	58.2735467	301.448341
25.2011022	264.343228	69.5166761	151.568144	335.735711
13.4787027	247.186348	523.8495	561.561133	346.715622
103.175163	343.493192	268.178503	41.1991032	57.663085
17.5164398	193.574748	385.111276	358.11085	28.218072
12.676606	1131.67671		30.9273288	88.1375255
14.4606515	483.821067		310.904435	48.1832061
5.44739492	835.724237		404.011252	
28.2582741			746.11088	
45.8459095			77.7351747	
103.298424			203.509686	
			380.939323	
			419.377419	

Regarding the assessment of transfection efficacy, we estimated it approximately by using fluorescence proteins as reporters, which were co-expressed with the relevant proteins via

the selfcleaving 2A peptide.

1. Greater detail needs to be provided for the generation of NaV1.5 and NaV1.6 chimeras. Specifically, what AA residues were changed between sodium channel isoforms?

We thank reviewer for pointing this out. In the revised manuscript, we have included the specific amino acid residues that were changed between NaV1.5 and NaV1.6 to generate the chimeric constructs.

1. In line 481, the authors refer to Fig. S2d instead of Fig. S6D. This should be corrected. Furthermore, the unusual shift in sodium current kinetics that the authors observe might be due in part to junction potential. Did the authors take that into consideration?

We apologize for this error. The reference to "Fig. S2d" has been corrected to "Fig. S6D" in the revised manuscript.

Regarding the unusual shift observed in the sodium current kinetics, we agree with the reviewer's suggestion that the junction potential may contribute to this phenomenon. During patch-clamp recordings, we ensure that the junction potential was properly compensated by the amplifier. Additionally, the replacement of CsF in pipette solution may have contributed to the observed unusual shift, as CsF in pipette solution has been reported to shift the voltage dependence of activation and fast/slow inactivation of NaV channels towards more negative potentials⁷.

1. Korngreen A. Advanced patch-clamp analysis for neuroscientists. Neuromethods. Humana Press; 2016:xii, 350 pages.

1. Legends for Fig.S6E and S6F are flipped. Please correct.

We apologize for this error. We have rectified the flipped captions for figure S6E and S6F in the revised manuscript.

1. Variance should be provided for the IC50 values and kinetic parameters of the sodium channels in the supplemental tables.

We thank the reviewer for raising this point. We have included the 95% confidence interval (95%CI) for the IC50 values and kinetic parameters in the revised supplementary tables.

Additionally, we have corrected some equations in the methods section:

1. Line 500 and line 503: We have corrected equation (1) by adding the parameter hill coefficient.
2. Line 514: We have revised equation (4) from " $G/G_{max} = 1/(1 + \exp(\frac{V_m - V_{1/2}}{k}))$ " to

$$G/G_{max} = 1 / \left(1 + \exp \left(\frac{V_m - V_{1/2}}{-k} \right) \right)$$