

Heterozygous expression of a *Kcnt1* gain-of-function variant has differential effects on SST- and PV-expressing cortical GABAergic neurons

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Abstract

More than twenty recurrent missense gain-of-function (GOF) mutations have been identified in the sodium-activated potassium (K_{Na}) channel gene *KCNT1* in patients with severe developmental and epileptic encephalopathies (DEEs), most of which are resistant to current therapies. Defining the neuron types most vulnerable to *KCNT1* GOF will advance our understanding of disease mechanisms and provide refined targets for precision therapy efforts. Here, we assessed the effects of heterozygous expression of a *Kcnt1* GOF variant (Y777H) on K_{Na} currents and neuronal physiology among cortical glutamatergic and GABAergic neurons in mice, including those expressing vasoactive intestinal polypeptide (VIP), somatostatin (SST), and parvalbumin (PV), to identify and model the pathogenic mechanisms of autosomal dominant *KCNT1* GOF variants in DEEs. Although the *Kcnt1*-Y777H variant had no effects on glutamatergic or VIP neuron function, it increased subthreshold K_{Na} currents in both SST and PV neurons but with opposite effects on neuronal output; SST neurons became hypoexcitable with a higher rheobase current and lower action potential (AP) firing frequency, whereas PV neurons became hyperexcitable with a lower rheobase current and higher AP firing frequency. Further neurophysiological and computational modeling experiments showed that the differential effects of the Y777H variant on SST and PV neurons are not likely due to inherent differences in these neuron types, but to an increased persistent sodium current in PV, but not SST, neurons. The Y777H variant also increased excitatory input onto, and chemical and electrical synaptic connectivity between, SST neurons. Together, these data suggest differential pathogenic mechanisms, both direct and compensatory, contribute to disease phenotypes, and provide a salient example of how a pathogenic ion channel variant can cause opposite functional effects in closely related neuron subtypes due to interactions with other ionic conductances.

eLife assessment

Shore et al. report **important** findings on the impact of a gain-of-function mutation (Y777H) in the *Kcnt1* gene on ion currents and firing behavior in both excitatory and inhibitory neurons in the mouse cortex. The *KCNT1* gene encodes a subunit of the Na⁺-activated K⁺ (K_{Na}) channel, and the authors substantiate their claims with **solid** evidence from electrophysiological patch-clamp recordings of dissociated cortical neurons. Nevertheless, the majority of reviewers recommended additional studies to reinforce key findings, proposing the replication of experiments using a more physiologically intact preparation, such as an ex vivo slice.

Introduction

Heterozygous missense variants have been identified in the sodium-gated potassium channel gene *KCNT1* in more than 200 individuals exhibiting a wide spectrum of developmental and epileptic encephalopathies (DEEs), with the majority being classified as either epilepsy of infancy with migrating focal seizures (EIMFS) or autosomal dominant or sporadic sleep-related hypermotor epilepsy (ADSHE) (Barcia et al., 2012 [↗](#); Bonardi et al., 2021 [↗](#); Heron et al., 2012 [↗](#)). Each of these epilepsy syndromes result in early-onset, frequent seizures that are largely pharmacoresistant and often accompanied by a range of cognitive, psychiatric, and motor impairments. Thus, there is a critical need for a better understanding of how heterozygous expression of these *KCNT1* variants in the developing brain alters neuronal physiology and network behavior to lead to such devastating neurodevelopmental disorders.

KCNT1 encodes a tetrameric potassium channel that is widely expressed in both glutamatergic and GABAergic neurons of the brain, particularly those of the cerebellum, striatum, thalamus, hippocampus, and cortex (Berg et al., 2007 [↗](#); Bhattacharjee et al., 2002 [↗](#); Gertler et al., 2022 [↗](#); Rizzi et al., 2016 [↗](#)). Although its precise role in normal physiology is not well understood, at least in some neuronal types, *KCNT1* is activated by a persistent inward sodium leak (Na_p) current at rest, where it has a proposed role in fine tuning neuronal excitability by countering the effects of the Na_p current across subthreshold voltages (Budelli et al., 2009 [↗](#); Hage & Salkoff, 2012 [↗](#)). Consistent with this role, loss-of-function (LOF) studies using mouse models lacking *KCNT1*, and the associated sodium-activated potassium (K_{Na}) current, have shown enhanced AP firing across multiple neuron types (Evely et al., 2017 [↗](#); Liu et al., 2022 [↗](#); Lu et al., 2015 [↗](#); Martinez-Espinosa et al., 2015 [↗](#); Reijntjes et al., 2019 [↗](#); Zhang et al., 2022 [↗](#)). Characterizations of pathogenic DEE-associated *KCNT1* variants in heterologous cells found that nearly all cause gain-of-function (GOF) effects on the channel, increasing the associated K_{Na} current (Hinckley et al., 2023 [↗](#); Kim et al., 2014 [↗](#); McTague et al., 2018 [↗](#); Milligan et al., 2014 [↗](#); Tang et al., 2016 [↗](#)). Based on LOF studies, this would be expected to reduce neuronal excitability; however, it is difficult to predict the effects of these GOF variants on AP generation in neurons, particularly among neuronal subtypes, *a priori*.

To address this knowledge gap, we previously generated and characterized a mouse model expressing a human ADNFLE-associated *KCNT1* GOF variant (Y796H, or Y777H in mice) (Shore et al., 2020 [↗](#)). Although heterozygous expression of the *KCNT1*-Y796H variant is sufficient to cause severe childhood epilepsy in humans, we only observed rare behavioral seizures in heterozygous *Kcnt1*-Y777H mice; however, we identified hyperexcitable, hypersynchronous cortical networks and frequent, early-onset seizures in homozygous *Kcnt1*-Y777H mice. As a potential underlying mechanism of these network alterations, we demonstrated that homozygous *Kcnt1*-Y777H expression increases subthreshold K_{Na} currents and reduces excitability in GABAergic neuron

populations, particularly in those classified as non-fast spiking (NFS), but it does not alter glutamatergic neuron excitability. We further observed evidence of homeostatic compensation and network remodeling downstream of K_{Na} current increases during development, including increased excitatory input onto glutamatergic and NFS GABAergic neurons, and enhanced homotypic synaptic connectivity. Although these findings provide a strong mechanistic basis for understanding how KCNT1 GOF disrupts neuronal physiology and network behavior to lead to seizure disorders, key issues remain unresolved. First, considering the heterozygous nature of *KCNT1* GOF variants in the overwhelming majority of *KCNT1*-related DEE patients, it is crucial to determine whether heterozygous *Kcnt1*-Y777H expression results in similar neuronal impairments and network alterations to those with homozygous expression. Second, more recent studies, using both *in silico* modeling and additional construct-valid mouse models, have similarly identified impairments in GABAergic neuron excitability downstream of KCNT1 GOF (Gertler et al., 2022 [🔗](#); Kuchenbuch et al., 2021 [🔗](#); Wu et al., 2023 [🔗](#)), indicating impaired inhibition as a shared pathogenic mechanism in *KCNT1*-related DEEs; however, precisely which GABAergic subtypes are most impacted, and how, remains unknown.

Here, we assessed the effects of heterozygous *Kcnt1*-Y777H expression on K_{Na} currents and neuronal physiology among cortical glutamatergic and GABAergic neurons, including those expressing vasoactive intestinal polypeptide (VIP), somatostatin (SST), and parvalbumin (PV). Initial assessments of cortical neuron populations with heterozygous Y777H expression showed strikingly similar effects on K_{Na} currents and AP generation to those with homozygous expression, although not surprisingly, these effects were lesser in magnitude. Across all cortical neuron types examined, the heterozygous *Kcnt1*-Y777H variant caused a range of effects on neuronal excitability and action potential (AP) generation, from no change (glutamatergic and VIP GABAergic) to decreased excitability (SST GABAergic) to increased excitability (PV GABAergic). Neuron types that showed no change had K_{Na} currents that were only significantly increased at suprathreshold voltages and exhibited a steeper voltage dependence of activation. Interestingly, both SST and PV neurons showed similar increases in K_{Na} currents across subthreshold voltages, however, only PV neurons had additional increases in the persistent Na^+ current, which modeling experiments indicated was sufficient to overcome the effects of KCNT1 GOF and cause an overall increase in AP generation. SST neurons also showed an increase in excitatory input, and in homotypic electrical and chemical coupling. Taken together, these data provide further evidence of the enhanced vulnerability of GABAergic neurons, particularly those expressing SST and PV, to KCNT1 GOF. Moreover, these data show that heterozygous expression of a single *KCNT1* GOF variant can result in a complex array of neuron type-dependent effects, both direct and indirect, each potential contributors to the neural circuit pathology underlying *KCNT1*-related DEEs.

Results

Heterozygous *Kcnt1*-Y777H expression alters the shape and frequency of APs in NFS GABAergic neurons

We previously identified frequent, early-onset seizures in mice with homozygous expression of the *Kcnt1* GOF variant Y777H (hereafter referred to as YH-HOM), and as a potential underlying pathological mechanism, we demonstrated that homozygous *Kcnt1*-Y777H expression drastically impairs action potential (AP) shape and generation in non-fast spiking (NFS) GABAergic cortical neurons, with lesser effects on fast spiking (FS) GABAergic, and no significant effects on glutamatergic, cortical neurons (Shore et al., 2020 [🔗](#)). Considering that patients with *KCNT1*-associated epilepsy are predominantly heterozygous for *KCNT1* GOF variants, it is crucial to determine whether heterozygous expression of these variants, which likely leads to the formation of heteromeric channels consisting of WT and mutant subunits, results in similar neuronal impairments. To assess neuron subtype-specific effects of heterozygous KCNT1 GOF on membrane properties and AP firing, we isolated and cultured cortical neurons from pups with heterozygous

Kcnt1-Y777H expression (hereafter referred to as YH-HET), and their WT littermates, at postnatal day 0 (P0). After infecting the cultured neurons with AAV-*CaMKII*-GFP to facilitate glutamatergic neuron identification, we performed whole-cell, current-clamp analysis between 13 and 17 days *in vitro* (DIV). Moreover, to compare to homozygous *KCNT1* GOF effects observed previously, the recorded neurons were classified as glutamatergic, FS GABAergic, or NFS GABAergic, based on GFP expression, AP parameters, and evoked synaptic responses (see Methods).

Current-clamp recordings from YH-HET and WT glutamatergic neurons showed no significant differences in any membrane or AP shape property measured (**Fig. 1A** [1](#) and Table 1), similar to observations from the homozygous *KCNT1* GOF studies. Accordingly, AP firing frequencies across increasing current steps in YH-HET glutamatergic neurons were not altered compared with those of WT (**Fig. 1B** [1](#)). For FS GABAergic neurons, we previously showed that homozygous Y777H expression increases the rheobase—the minimal amount of current necessary to induce an AP—and reduces the AP firing frequency. Heterozygous Y777H expression did not alter the rheobase, or any other passive or active membrane property of FS GABAergic neurons (**Fig. 1A** [2](#) and Table 1). Although YH-HET FS GABAergic neurons appeared to fire fewer APs than their WT counterparts, particularly at higher current steps, this effect was not significant (**Fig. 1B** [2](#)). Lastly, we previously showed that homozygous Y777H expression has the strongest effects in NFS GABAergic neurons, showing a decrease in input resistance, accompanied by an increase in rheobase and a reduction in AP firing frequency. YH-HOM NFS GABAergic neurons also have narrower AP half-widths and larger afterhyperpolarizations (AHPs) than those of WT. Although heterozygous Y777H expression in NFS GABAergic neurons did not cause a significant decrease in input resistance (WT: 171 ± 16 ; YH-HET: 146 ± 14 , $p = 0.19$), it did increase the rheobase and reduce the AP firing frequency, particularly at lower current steps, relative to those of WT (**Fig. 1A** [3](#) and **1B**₃). YH-HET NFS GABAergic neurons also had narrower APs, larger AHPs, and more depolarized AP thresholds than those of WT (Table 1). Together, these data demonstrate that the neurophysiological effects on cortical neurons with monoallelic expression of the *Kcnt1*-Y777H variant, expressing channels with mutant and WT subunits, are similar to those with biallelic expression, expressing only mutant subunits, with both causing the strongest impairments in NFS GABAergic neurons.

Heterozygous *Kcnt1*-Y777H expression increases subthreshold K_{Na} currents in NFS GABAergic neurons

Previously, we showed that homozygous *Kcnt1*-Y777H expression in GABAergic cortical neurons increases the K_{Na} current across subthreshold voltages, an effect that is particularly evident in NFS GABAergic neurons; conversely, in glutamatergic cortical neurons with homozygous expression of the same variant, increases in K_{Na} currents are only apparent at depolarized voltages ($> +30$ mV) (Shore et al., 2020 [1](#)).

To assess the effects of heterozygous Y777H expression on *KCNT1* channel function, we measured the associated K_{Na} current in each cortical neuron subtype. We recorded K_{Na} currents by applying voltage steps to voltage-clamped neurons and comparing the delayed outward current before and after the addition of the voltage-gated sodium channel inhibitor TTX (**Fig. 2A** [1,3](#)). As reported previously, there were K_{Na} currents in all three WT neuron subtypes, beginning around -10 mV and increasing with depolarization (**Fig. 2A** [1,3](#) and **2B**_{1,3}), whereas at more negative potentials, the TTX-sensitive current was net inward (**Fig. 2C** [1,3](#)) due to the counteracting persistent Na^+ current.

In glutamatergic and NFS GABAergic YH-HET neurons, the overall K_{Na} current was increased relative to those of WT, as measured by a significant effect of genotype using a linear model (**Fig. 2B** [1,3](#)). Importantly, in each of these neuron subtypes, heterozygous Y777H expression increased K_{Na} currents with distinct, voltage-dependent patterns that appeared strikingly similar to those

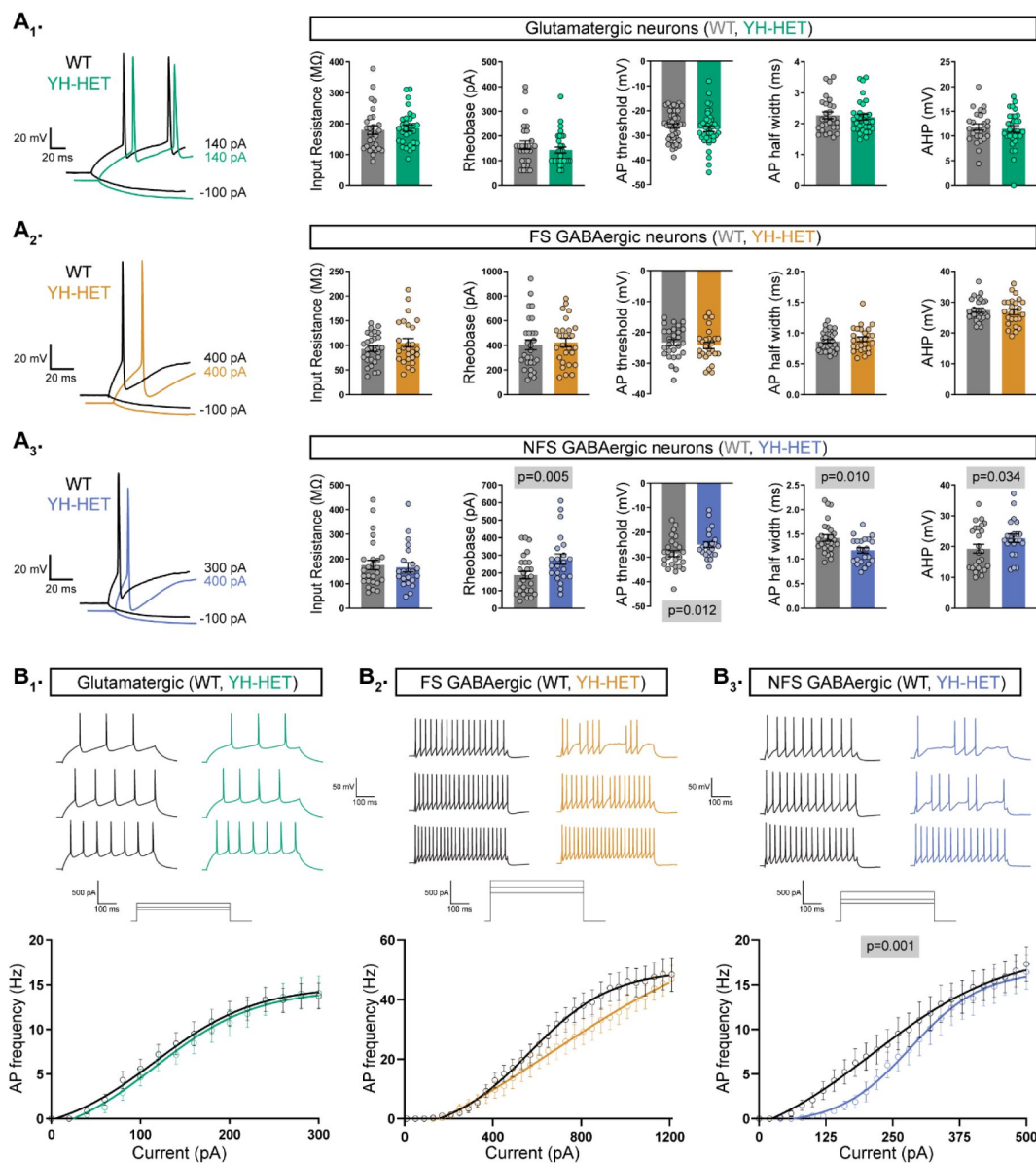


Figure 1.

Heterozygous *Kcnt1*-Y777H expression alters AP shape and generation in NFS GABAergic neurons.

(A₁-A₃) On the left, representative responses to step currents are shown for glutamatergic, and FS and NFS GABAergic, WT (black) and YH-HET (colors) neurons (top to bottom), illustrating the input resistance (in response to a depolarizing step) and the rheobase (the first trace with an AP in response to a hyperpolarizing step) for each neuron type. On the right, bar graphs show quantification and mean $\pm$ SEM of the membrane properties and AP parameters for each neuron type for WT (grey) and YH-HET (colors) groups, with individual neuron measurements overlaid in scatter plots. The p-values are shown on each graph where $p < 0.05$. **(B₁-B₃)** Representative traces are shown at low, medium, and high current steps for glutamatergic, and FS and NFS GABAergic, WT (black) and YH-HET (colors) neurons (left to right). The line graphs below show the number of APs (mean $\pm$ SEM) per current injection step in WT (black) and YH-HET (colors) neurons. Statistical significance was tested using Generalized Linear Mixed Models, and p-values are shown on each graph where $p < 0.05$.

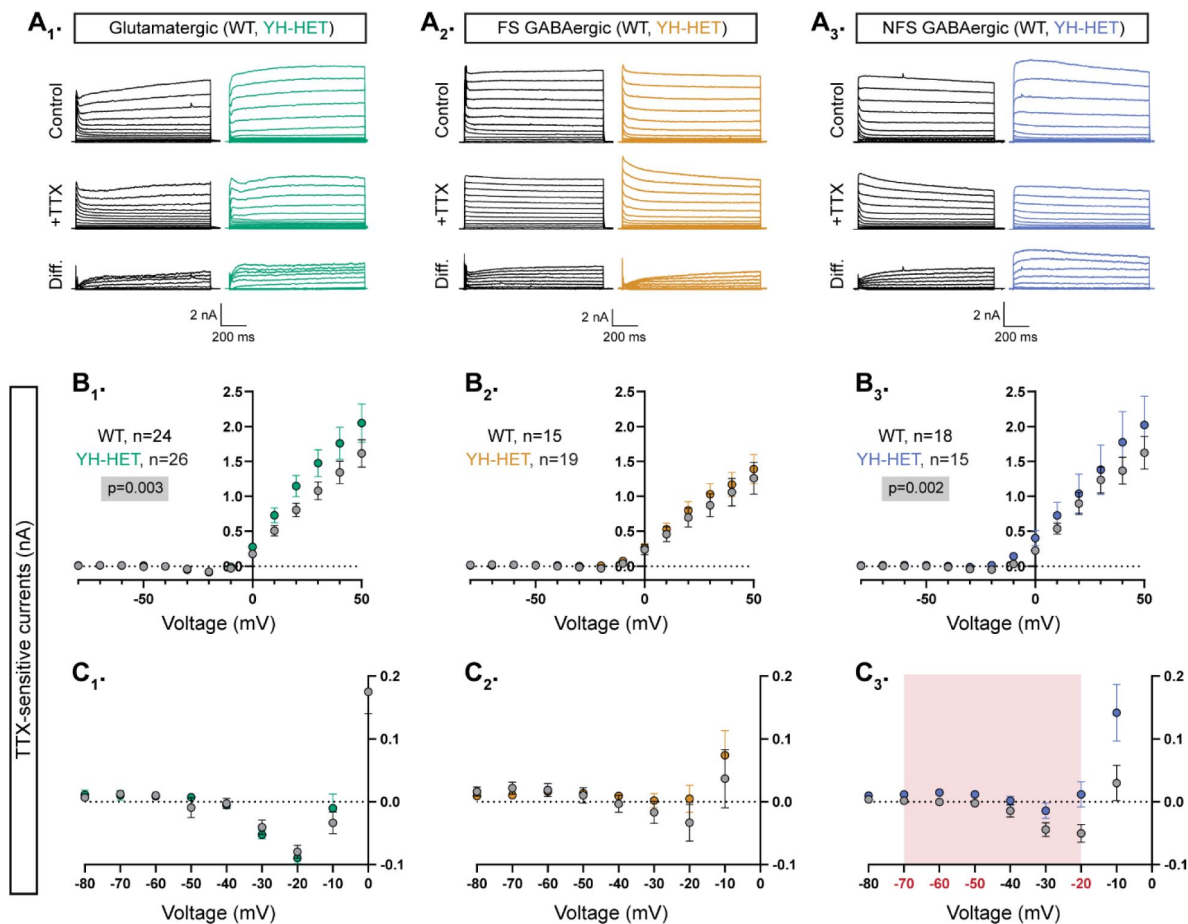


Figure 2.

Heterozygous *Kcnt1*-Y777H expression increases subthreshold K_{Na} currents in NFS GABAergic neurons.

(**A₁**-**A₃**) Representative traces in control (top), 0.5 μ M TTX (middle), and the difference current (bottom) calculated by subtracting the membrane current response to voltage steps (-80 to +50 mV) from a holding potential of -70 mV in TTX from the response in control external solution in glutamatergic, and FS and NFS GABAergic, WT (black) and YH-HET (colors) neurons. (**B₁**-**B₃**) Summary data shows the K_{Na} current (mean $\pm$ SEM) for each voltage step in glutamatergic, and FS and NFS GABAergic, WT (black and gray) and YH-HET (colors) neurons. The p-values are shown on each graph where $p < 0.05$, and the n values are the number of neurons recorded for each group. (**C₁**-**C₃**) Plots of the K_{Na} current (mean $\pm$ SEM) for each voltage step from -80 to 0 mV in WT (black and gray) and YH-HET (colors) neurons to illustrate the values that are too small to be seen on the graphs in **B₁**-**B₃**. The shaded red area in **C₃** indicates the subthreshold voltage range with significantly higher K_{Na} currents (red voltages along x-axis indicate steps where $p < 0.05$) in YH-HET relative to WT neurons. The p-values are shown on each graph where $p < 0.05$, and the n values are the number of neurons recorded for each group. Statistical significance for I-V plots was tested using Generalized Linear Mixed Models with genotype and current step as fixed effects followed by pairwise comparisons at each level.

reported with homozygous expression. For instance, we previously showed that, across negative potentials, YH-HOM and WT glutamatergic neuron K_{Na} currents are indistinguishable, whereas at more positive potentials ($> +30$ mV), the Y777H variant causes significant increases in K_{Na} currents. Similar voltage-dependent effects were observed in YH-HET glutamatergic neurons (**Fig. 2B** [1](#) and **2C** [1](#)), although pairwise comparisons showed that K_{Na} current increases at positive potentials in the YH-HET glutamatergic neurons were not significant. Conversely, we previously demonstrated broad increases in K_{Na} currents across negative potentials, with significant increases from -60 to $+10$ mV, in YH-HOM NFS GABAergic neurons compared with those of WT, with lesser effects across positive potentials. Similar voltage-dependent increases were observed in YH-HET NFS GABAergic neurons, with pairwise comparisons showing significant K_{Na} current increases at -70 , -60 , -50 , and -20 mV (**Fig. 2B** [3](#) and **2C** [3](#)). For both glutamatergic and NFS GABAergic neurons, the magnitudes of the K_{Na} current increases in YH-HET neurons were intermediate to those of WT and YH-HOM neurons (**Supplemental Fig. 2** [4](#)), demonstrating a gene dose-dependent effect of the *Kcnt1*-Y777H variant on K_{Na} current increases and validating a GOF effect of the heterozygous Y777H variant on channel function. Lastly, although previous studies showed that homozygous Y777H expression increases K_{Na} current at several negative voltage steps (-50 , -40 , and -10 mV) in FS GABAergic neurons, heterozygous expression of the same variant in FS GABAergic neurons caused no significant differences in K_{Na} currents compared with those of WT (**Fig. 2B** [2](#) and **2C** [2](#)).

Heterozygous *Kcnt1*-Y777H expression does not alter synaptic connectivity or the excitation-inhibition balance

In addition to alterations in the intrinsic passive and active membrane properties of cortical neurons with homozygous Y777H expression, we previously showed evidence of altered synaptic connectivity and activity, leading to hypersynchronous, hyperexcitable YH-HOM networks; more specifically, we found an increase in homotypic coupling between glutamatergic [excitatory-excitatory (E-E)] and GABAergic [inhibitory-inhibitory (I-I)] neuron pairs, and an increase in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs), accompanied by an increase in the E/I ratio, onto YH-HOM glutamatergic cortical neurons (Shore et al., 2020 [4](#)). First, to determine whether there are similar changes in synaptic connectivity in YH-HET networks, we performed paired recordings of glutamatergic (excitatory, E) and GABAergic (inhibitory, I) neurons and alternatively stimulated each neuron at 0.1 Hz to test baseline connection probability and strength at the four possible motifs (E-E, I-E, E-I, and I-I). Connection probability was not altered in YH-HET networks at any of the motifs tested (**Fig. 3A** [1-4](#), left bar graphs), and the amplitudes of the evoked postsynaptic currents (ePSCs) between connected neurons were not different between genotypes for any of the four connection types (**Fig. 3A** [1-4](#), right bar graphs), indicating grossly normal synaptic interactions among glutamatergic and GABAergic neurons in YH-HET networks. Next, to assess potential alterations in synaptic activity, we recorded spontaneous postsynaptic currents (sEPSCs and sIPSCs) onto voltage-clamped glutamatergic and GABAergic neurons. Unlike the observation of an increase in sEPSC frequency onto YH-HOM glutamatergic neurons, there was no difference in sEPSC, or sIPSC, frequency onto YH-HET glutamatergic neurons (**Fig. 3B** [1](#)). Furthermore, although we previously found no alterations in sPSC frequency onto YH-HOM GABAergic neurons, sEPSC and sIPSC frequencies were both slightly reduced onto YH-HET GABAergic neurons (**Fig. 3B** [2](#)). Finally, to assess the net effect of altered sPSC activity onto YH-HET neurons, we calculated the E/I ratio, based on the relative frequency and size of the sPSCs, and found no difference in the E/I ratio onto either YH-HET neuron type (**Fig. 3B** [3](#)). Thus, although heterozygous Y777H expression resulted in similar K_{Na} current increases and neuronal physiology effects to those found with homozygous Y777H expression, the broad effects on synaptic connectivity and activity found in YH-HOM networks were absent in YH-HET networks, which likely results in the observed reduction in seizure incidence in heterozygous, relative to homozygous, mice.

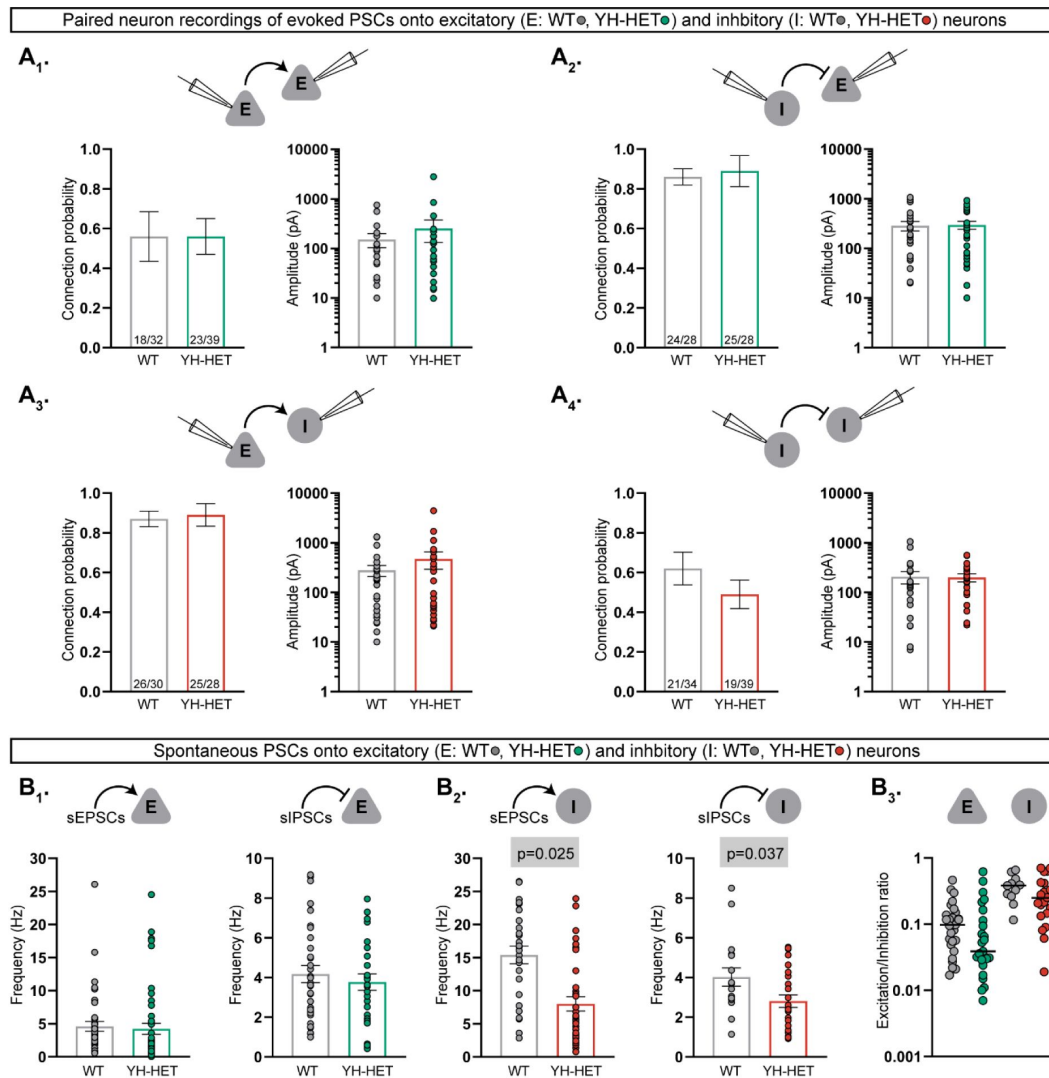


Figure 3.

Heterozygous *Kcnt1*-Y777H expression does not alter synaptic connectivity or the excitation- inhibition balance.

(**A₁**-**A₄**) Evoked postsynaptic currents (PSCs) were recorded from neuron pairs [glutamatergic (excitatory, E) and GABAergic (inhibitory, I)] by stimulating the neuron type indicated on the left and recording the response in the neuron type indicated on the right (WT, gray; YH-HET E, green; YH-HET I, red). Bar graphs below each recorded neuron pair schematic show summary data (mean $\pm$ SEM) of the connection probability (left graph; numbers on bars represent connected pair number/recorded pair number) and peak evoked PSC amplitude (right graph; dots represent individual evoked responses) between each motif. (**B₁**) Bar graphs with overlaid individual neuron measurements and mean $\pm$ SEM show the spontaneous EPSC (sEPSC) or IPSC (sIPSC) frequency onto E neurons (WT, gray; YH-HET, green). (**B₂**) Bar graphs with overlaid individual neuron measurements and mean $\pm$ SEM show the sEPSC or sIPSC frequency onto I neurons (WT, gray; YH-HET, red). (**B₃**) Scatter plots show individual E/I ratio measurements onto E neurons (WT, gray; YH-HET, green) and I neurons (WT, gray; YH-HET, red). The p-values are shown on each graph where $p < 0.05$.

Heterozygous *Kcnt1*-Y777H expression differentially affects the intrinsic excitability of SST- and PV-expressing GABAergic neurons

Next, we sought to determine which GABAergic subtypes are most impacted by heterozygous *Kcnt1*-Y777H expression. About 80-90% of cortical GABAergic neurons fall into three, largely non-overlapping populations that can be identified by their expression of unique markers: the Ca^{2+} -binding protein parvalbumin (PV; ~40%) and the neuropeptides somatostatin (SST; ~30%) and vasoactive intestinal polypeptide (VIP; ~15%) (Rudy et al., 2011; Tremblay et al., 2016). The majority of PV-expressing neurons have been characterized as FS, and VIP-expressing as NFS, whereas SST-expressing neurons, although largely thought to show NFS firing properties, also contain a population that exhibits a FS-like phenotype (Large et al., 2016; Ma et al., 2006). *KCNT1* is expressed at higher levels in both human and mouse cortical GABAergic neurons expressing PV and SST, than in those expressing VIP (Shore et al., 2020). Based on the expression profile of *KCNT1*, and our findings that homozygous and heterozygous expression of the *Kcnt1* GOF variant strongly reduced the excitability GABAergic neurons with NFS firing properties, we hypothesized that the SST-expressing neurons are the most vulnerable to the effects of *KCNT1* GOF. To test this hypothesis, we crossed PV-, SST-, and VIP-Cre mouse lines to the *Kcnt1*-Y777H mouse line and cultured neurons from the cortices of WT and YH-HET littermate progeny (Fig. 4A and Supplemental Fig. 4-1A). We infected the cultured neurons with AAV-*CaMKII*-GFP, to mark glutamatergic neurons, and AAV-*hSyn*-DIO-mCherry, to mark Cre recombinase-expressing GABAergic neurons, and performed whole-cell, patch-clamp electrophysiology at DIV 13-17 on GFP/mCherry⁺ neurons from each group.

First, we measured the intrinsic membrane properties and AP shape parameters of the three GABAergic subtypes from the WT control groups to verify that they accurately reflected electrophysiological behaviors of these subtypes from previous *ex vivo* recordings (Taniguchi et al., 2011). As expected, the VIP neurons showed a relatively large input resistance, small rheobase, wide AP half-width, small AHP, and low firing rate (Supplemental Fig. 4-1B and 4-1C, purple); in contrast, PV neurons showed a small input resistance, large rheobase, narrow AP half-width, large AHP, and high firing rate (Supplemental Fig. 4-1B and 4-1C, orange). For SST neurons, the values measured for input resistance, rheobase, AP half-width, AHP, and firing rate were all intermediate to those of VIP and PV neurons (Supplemental Fig. 4-1B and 4-1C, blue). Moreover, as reported previously, SST neurons showed the most hyperpolarized AP threshold of the three groups. Together, these data demonstrate the ability of cortical GABAergic neuron subtypes to retain their characteristic *ex vivo* passive and active membrane properties *in vitro*.

Next, we assessed the effects of the Y777H variant on the passive and active membrane properties of each GABAergic subtype. Current-clamp recordings from YH-HET VIP neurons revealed no significant effect of the variant on any of the membrane or AP properties measured (Fig. 4B₁). Conversely, YH-HET SST neurons showed a strong decrease in input resistance, with an accompanying increase in rheobase current, relative to those of WT SST neurons (Fig. 4B₂), similar to the hypoexcitable membrane phenotype observed in the YH-HET NFS neurons, without altering AP shape parameters. YH-HET SST neurons also showed a reduction in the membrane time constant and an increase in membrane capacitance, which was accompanied by an increase in soma size (Table 4 and Supplemental Fig. 4-2), compared with those of WT SST neurons. Unexpectedly, YH-HET PV neurons exhibited a decrease in the rheobase current compared with that of WT (Fig. 4B₃), and an increase in AP amplitude (Table 4), suggesting the Y777H variant increases PV neuron excitability.

Next, we assessed AP firing frequency with incremental, 500-ms current steps in each of the three GABAergic subtypes. WT neurons showed AP firing frequencies similar to those reported previously from *ex vivo* recordings of neurons from the three Cre lines (Taniguchi et al., 2011), with VIP showing the lowest maximal firing frequency, PV the highest, and SST intermediate

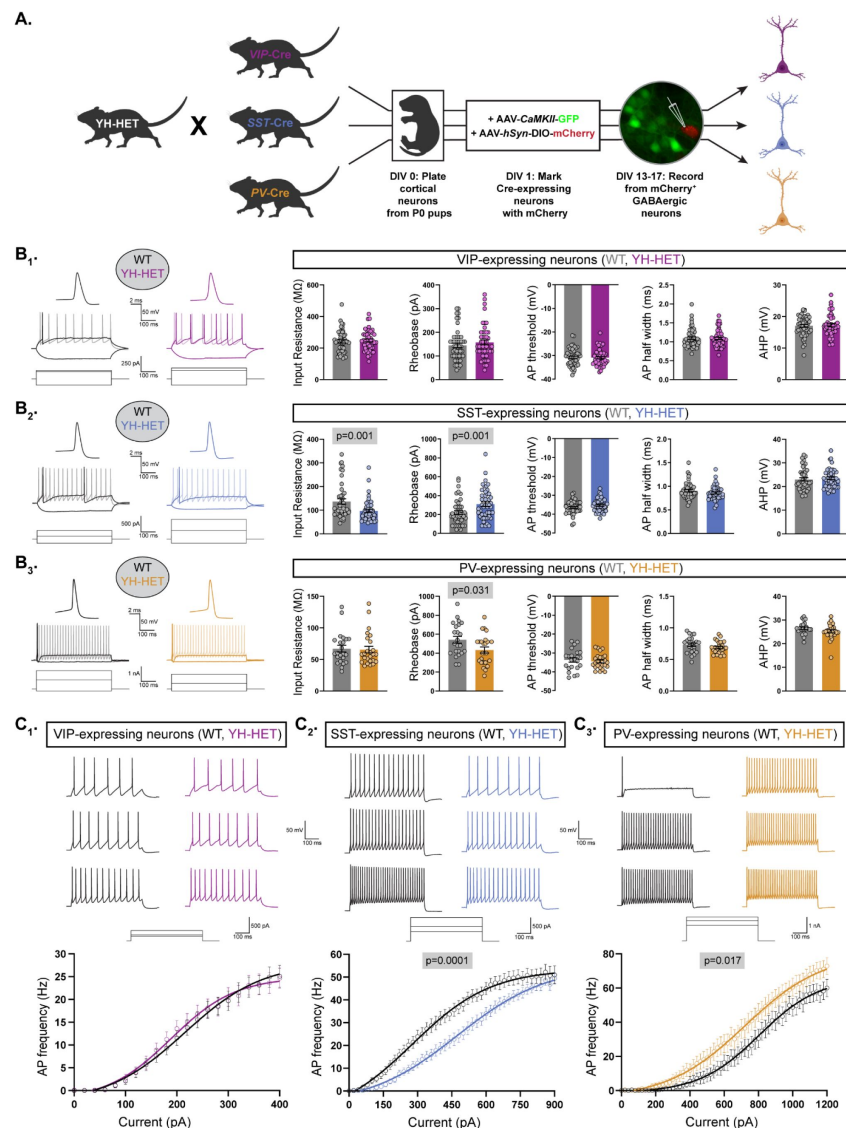


Figure 4.

Heterozygous *Kcnt1*-Y777H expression differentially affects the intrinsic excitability of SST- and PV-expressing GABAergic neurons.

(A) A schematic diagram illustrates the strategy for generating fluorescently labeled GABAergic subtype-specific neurons. YH-HET mice were crossed to VIP-, SST-, or PV- Cre mice, and the resulting P0 WT and YH-HET littermate pups were used to isolate and culture cortical neurons. At DIV 1, neurons were infected with AAV-*CamKII*-GFP to label glutamatergic neurons, and AAV-*hSyn*-DIO-mCherry to label Cre-expressing neurons. At DIV 13-17, whole-cell, patch-clamp electrophysiology was performed on mCherry⁺/GFP⁺ neurons. (B₁-B₃) On the left, representative responses to step currents are shown for VIP-, SST-, and PV-expressing WT (black) and YH-HET (colors) neurons (top to bottom). For each neuron type, the superimposed dark traces illustrate the input resistance (in response to a depolarizing step) and the rheobase (the first trace with an AP in response to a hyperpolarizing step), and the light trace shows the first step current response to induce repetitive AP firing across the step. Above the superimposed traces, the first AP of each rheobase trace is shown (same vertical scale, increased horizontal scale). On the right, bar graphs show quantification of the membrane properties and AP parameters for each neuron type (VIP, SST, and PV, top to bottom) for WT (grey) and YH-HET (colors) groups, with individual neuron measurements overlaid in scatter plots. The p-values are shown above each graph where $p < 0.05$. (C₁-C₃) For VIP-, SST-, and PV-expressing neurons (left to right), representative traces are shown at low, medium, and high current steps, and the line graphs below show the number of APs (mean $\pm$ SEM) per current injection step in WT (black) and YH-HET (colors) neurons. Statistical significance was tested using Generalized Linear Mixed Models, and p-values are shown above each graph where $p < 0.05$.

between VIP and PV (**Fig. 4C** [1-3](#) and Table 4). Consistent with a lack of any effects on membrane and AP shape properties, the AP firing frequencies across increasing current steps were indistinguishable between WT and YH-HET VIP neurons (**Fig. 4C** [1](#)). As expected, based on their hypoexcitable membrane properties, the YH-HET SST neurons fired fewer APs across all current steps relative to WT SST neurons (**Fig. 4C** [2](#)). In contrast to SST neurons, but consistent with their decreased rheobase current, YH-HET PV neurons fired more APs across all current steps relative to their WT counterparts (**Fig. 4C** [3](#)). Together, these data showed that, as hypothesized, KCNT1 GOF indeed strongly reduces the excitability of SST neurons, but unexpectedly, it also causes a hyperexcitable effect in PV neurons; thus, the same ion channel mutation can lead to opposite effects on excitability in the two largest GABAergic neuron subtypes.

The Y777H variant increases KCNT1-mediated currents across subthreshold voltages in SST- and PV- expressing GABAergic neurons

Are the observed differential effects of KCNT1 GOF on GABAergic neuronal physiology due to distinct patterns of K_{Na} current increases among GABAergic neuron subtypes? To answer this question, we first recorded K_{Na} currents from GABAergic neurons cultured and labeled as described above, by applying voltage steps to voltage-clamped neurons and comparing the delayed outward current before and after the addition of TTX (**Supplemental Fig. 5A** [1-3](#)). In all three subtypes, for both WT and YH-HET neurons, we observed K_{Na} currents beginning around -10 mV and increasing with depolarization (**Supplemental Fig. 5B** [1-3](#)). For YH-HET VIP neurons, pairwise comparisons to assess voltage-dependent differences showed that their K_{Na} currents were not different from those of WT at any voltage step (**Supplemental Fig. 5B** [1](#) and [5C1](#)). On the other hand, YH-HET SST neurons showed significant increases in K_{Na} currents compared with those of WT across multiple subthreshold voltage steps, including -60 , -50 , and -40 mV (**Supplemental Fig. 5B** [2](#) and [5C2](#)), similar to those observed in NFS GABAergic neurons with both heterozygous (**Fig. 2** [1](#)) and homozygous expression of the Y777H variant (Shore et al., 2020 [1](#)), indicating a direct current-to-phenotype relationship in YH-HET SST neurons. Somewhat unexpectedly, pairwise comparisons showed that K_{Na} currents were not different between YH-HET and WT PV neurons at any voltage step (**Supplemental Fig. 5B** [3](#) and [5C3](#)).

The lack of an increase in TTX-sensitive currents in PV neurons, despite alterations in rheobase, AP amplitude, and AP firing frequency, suggests that either a TTX-insensitive Na^+ source activates KCNT1, or that compensatory alterations in an opposing current mask an increase in K_{Na} and lead to the changes in AP firing. In past studies, the lack of selective KCNT1-specific inhibitors necessitated the use of indirect methods, such as TTX application or Na^+ replacement, to estimate the magnitude of K_{Na} . However, more recently, we identified and validated a small-molecule, selective KCNT1 channel inhibitor termed VU0606170, or VU170 (Spitznagel et al., 2020 [1](#)). To obtain a more accurate estimate of the KCNT1-mediated current in GABAergic subpopulations and disentangle potential confounds of using TTX, we applied the same voltage step protocol as in the TTX subtraction experiments but applied $10 \mu M$ VU170 instead.

In all three WT GABAergic neuron subtypes, subtraction of the VU170 trace from the control trace revealed nA-sized outward currents at depolarized potentials, and in YH-HET neurons, the overall VU170-sensitive current was increased relative to those of WT, as measured by a significant effect of genotype using a linear model (**Fig. 5A** [1-3](#) and [5B1-3](#)). Pairwise comparisons at each voltage step showed voltage-dependent differences in current increases among the YH-HET neuron subtypes. For YH-HET VIP neurons, a significant increase in the VU170 current was only observed at $+50$ mV (**Fig. 5B** [1](#)), whereas subthreshold currents were indistinguishable from those of WT (**Fig. 5C** [1](#)). Previously, we similarly observed K_{Na} current increases only at more positive potentials in YH-HOM glutamatergic neurons, and like YH-HET VIP neurons, their membrane and AP properties were unaltered by expression of the YH variant (Shore et al., 2020 [1](#)). Conversely, in YH-HET SST neurons, significant increases occurred at more negative voltage steps, from -60 to

–30 mV (**Fig. 5C** [2](#)). These changes were similar to those observed with TTX treatment, but slightly larger, possibly due to the lack of the counteracting effect of the persistent Na^+ current, which is also blocked by TTX. In contrast to the TTX results, YH-HET PV neurons showed an increase in VU170-sensitive currents, with significant increases from –70 to –50 mV (**Fig. 5C** [3](#)). Taken together, these data indeed identify distinct patterns of K_{Na} current increases among GABAergic subtypes, and for VIP and SST neurons, these current increases are consistent with the observed effects of KCNT1 GOF on neuronal physiology. On the other hand, PV neurons showed subthreshold-specific K_{Na} current increases that were highly similar to, and overlapping with, those of SST neurons; thus, differential K_{Na} current increases alone likely do not account for the opposite effects of KCNT1 GOF on physiology observed in these two neuron types.

Compartmental models of KCNT1 GOF in SST, but not PV, neurons are consistent with experimental data

The VU170-subtraction experiments showed that YH-HET SST and PV neurons have similar subthreshold increases in KCNT1-mediated currents, even though they exhibited opposing effects on neuron excitability and AP generation, and that YH-HET VIP neurons have suprathreshold increases in KCNT1-mediated currents with no effects on neuron physiology. Because GABAergic subtypes exhibit varying morphologies and express unique repertoires of ion channels, which are known to give rise to their characteristic membrane and AP firing properties, we hypothesized that at least some of the observed differential effects of KCNT1 GOF, in particular the opposite effects in SST and PV neurons, are due to these inherent, neuron-type-dependent differences. To test this hypothesis, we simulated the effect of KCNT1 GOF in compartmental models of these three cortical neuron types. We used a KCNT1 conductance with the Na^+ dependence constrained by prior studies in neurons ($\text{EC}_{50} = 40$ mM, slope 3.5; see Materials and Methods) and the voltage dependence based on the activation curves from our own experimental data of the VU170-sensitive current in each subtype (**Fig. 6A** [1](#)). The kinetics of the model current were set by measuring the onset time course of the VU170-sensitive current in outside-out membrane patches of each neuron type (**Fig. 6B** [1](#) and **6C** [1](#)). This conductance was inserted into compartmental models of 10 neurons per subtype, representing a variety of morphological and electrical properties of each subtype (Markram et al., 2015 [1](#)). Because the Y796H variant was previously shown to increase the Na^+ sensitivity of the channel, we modeled the GOF effect by reducing the EC_{50} for Na^+ activation to two levels (35 mM and 30 mM), which resulted in moderate K_{Na} current increases above WT levels (**Fig. 6D** [1](#)).

Consistent with our experimental data, introducing KCNT1 GOF at either level into model VIP neurons did not alter their AP firing rate, input resistance, or rheobase (**Fig. 6E** [1](#) and **6E** [2](#)). On the other hand, model SST neurons with KCNT1 GOF fired fewer APs per current step as the GOF was increased and had higher rheobases (**Fig. 6F** [1](#) and **6F** [2](#)), results that agree with our experimental observations.

Although the input resistance decreased on a per neuron basis with GOF, this effect was smaller than in the experimental data. In contrast to experimental data, but similar to model SST neurons, model PV neurons responded to KCNT1 GOF with decreased AP firing and an increased rheobase (**Fig. 6G** [1](#) and **6G** [2](#)), although the magnitude of these effects were smaller than those in model SST neurons. These data suggest that the increased excitability observed in YH-HET PV neurons is not simply due to an intrinsic property of this GABAergic subtype, but instead results from an indirect mechanism (e.g. a compensatory response like an increase in Na^+ channels) or depends on a feature of KCNT1 function not captured by the model.

Lastly, we hypothesized that the lack of GOF effects on VIP and glutamatergic neuron physiology is due to the altered kinetics of their KCNT1 channel activation curves, in particular their right shifted V_{50} and decreased slope, relative to those of SST or PV neurons (**Fig. 6A** [1](#)). To test this

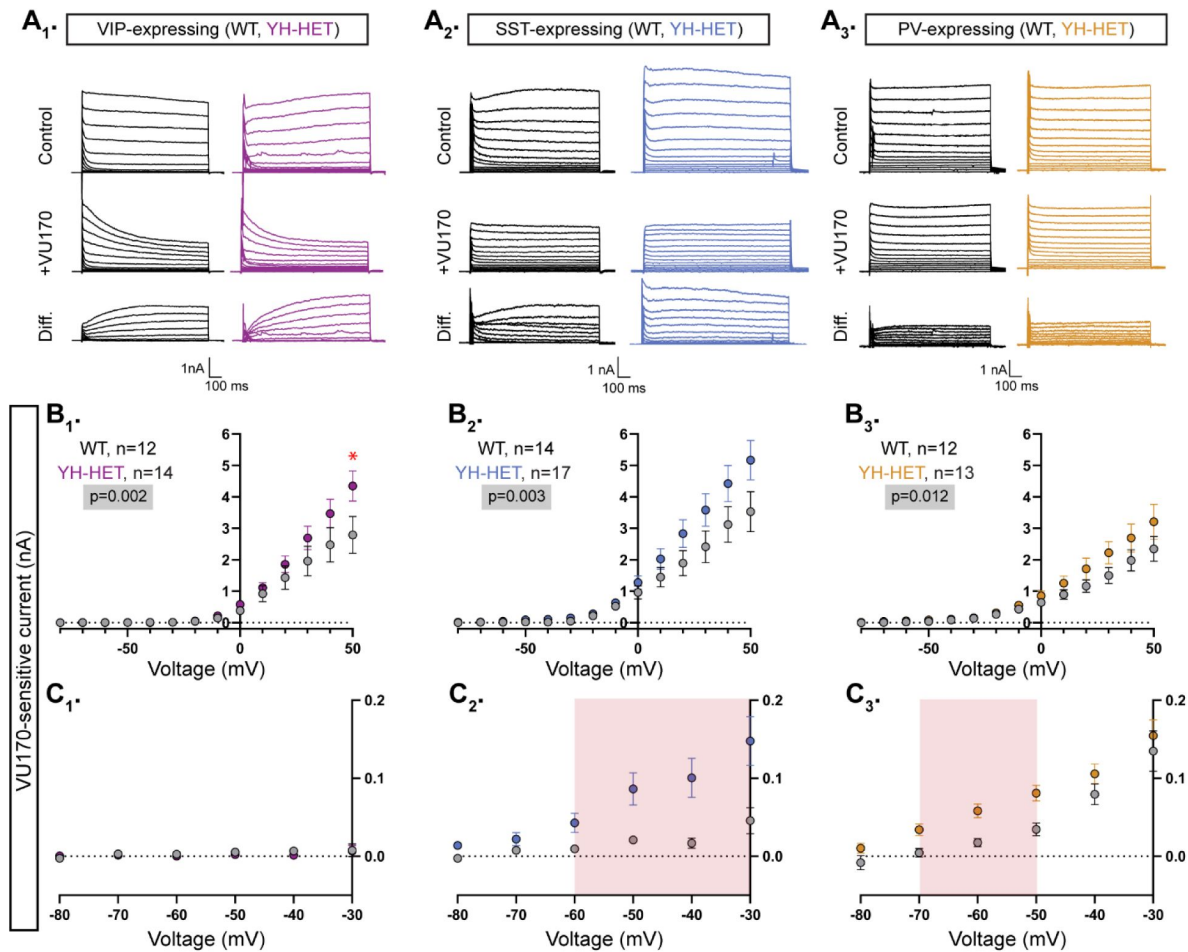


Figure 5.

The *Kcnt1*-Y777H variant increases KCNT1-mediated currents across subthreshold voltages in SST- and PV-expressing GABAergic neurons.

(**A₁-A₃**) Representative traces in control (top), 10 μ M VU170 (middle), and the difference current (bottom) calculated by subtracting the membrane current response to voltage steps (-80 to +50 mV) from a holding potential of -70 mV in VU170 from the response in control external solution in VIP-, SST-, and PV-expressing, WT (black) and YH-HET (colored) neurons. (**B₁-B₃**) Summary data shows the KCNT1 current (mean $\pm$ SEM) for each voltage step in VIP-, SST-, and PV-expressing, WT (black and gray) and YH-HET (colored) neurons. The p-values are shown on each graph where $p < 0.05$, and the n values are the number of neurons recorded for each group. (**C₁-C₃**) Plots of the K_{Na} current (mean $\pm$ SEM) for each voltage step from -80 to 0 mV in WT (black and gray) and YH-HET (colored) neurons to illustrate the values that are too small to be seen on the graphs in B₁-B₃. The shaded red areas in C₂ and C₃ indicate the subthreshold voltage range with significantly higher K_{Na} currents ($p < 0.05$) in YH-HET relative to WT neurons. Statistical significance for I-V plots was tested using Generalized Linear Mixed Models with genotype and current step as fixed effects followed by pairwise comparisons at each level.

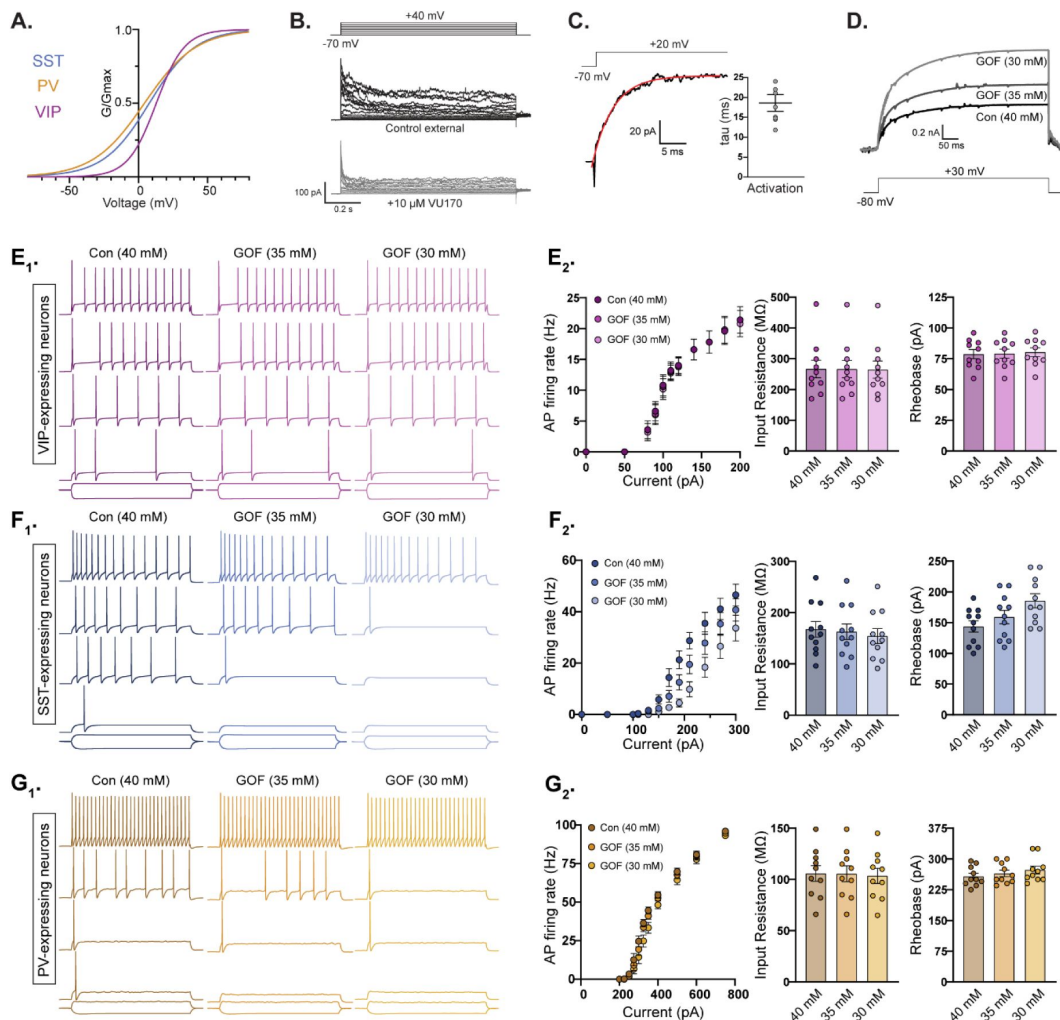


Figure 6.

Compartmental models of KCNT1 GOF in SST, but not PV, neurons are consistent with experimental data.

(A) Activation curves for VIP, SST, and PV neurons used to model the KCNT1 channel in each neuron type. **(B)** Representative traces of the response of an outside-out membrane patch pulled from an SST neuron in control external solution (top) and after application of 10 μ M VU170 (bottom). **(C)** Representative trace of the VU170-sensitive current in an outside-out patch pulled from an SST neuron with a single exponential fit (red curve) overlaid and summary data showing the time constant (mean $\pm$ SEM) obtained from these fits. **(D)** Simulated traces showing the KCNT1-mediated current in model neurons at three levels of Na⁺-sensitivity (Con-40 mM, GOF-35 mM, and GOF-30mM). **(E₁, F₁, and G₁)** Simulated traces from model VIP (E₁; purple), SST (F₁; blue), and PV (G₁; yellow) neurons in response to 500-ms current injections at three levels of Na⁺-sensitivity [control (Con), and two levels of KCNT1 GOF]. Representative traces are shown at increasing current steps from bottom to top for each level. **(E₂, F₂, and G₂)** Summary data from 10 model VIP (E₂; purple), SST (F₂; blue), and PV (G₂; yellow) neurons showing (from left to right) the number of APs at different current steps (F-I plot), the input resistance, and rheobase. The bar graphs (mean $\pm$ SEM) are overlaid with scatter plots of individual neuron measurements.

hypothesis, we performed simulations in which the activation curve parameters (V_{50} and slope) measured in SST neurons were inserted into VIP and glutamatergic neurons, with the Na EC_{50} set to 30 mM to simulate KCNT1 GOF (**Supplemental Fig. 6A**[1](#) and **6B**[1](#), blue traces). Under these conditions, VIP and glutamatergic neurons indeed fired fewer APs and had a significantly higher rheobase than those of their respective control neurons (**Supplemental Fig. 6A**[2](#) and **6B**[2](#)). This model-based evidence suggests that the relative sensitivity of SST and PV neurons, and resistance of VIP and glutamatergic neurons, to KCNT1 GOF is due to the differences in voltage dependence of the K_{Na} current in each subtype.

The persistent Na^+ current is increased by the *Kcnt1*-Y777H variant in PV, but not SST, neurons

The modeling results suggest that a subthreshold increase in the K_{Na} current alone is not sufficient to account for the alterations in PV neuron excitability. Moreover, the finding of an increase in the KCNT1-mediated current in YH-HET PV neurons when measured with VU170, but not TTX, isolation suggests there may be a compensatory upregulation of an opposing current that masks the increase in K_{Na} and leads to the unexplained increase in AP firing. The persistent Na^+ current (I_{NaP}) is active on the same time scale as K_{Na} and was previously shown to provide the Na^+ source for K_{Na} (Hage & Salkoff, 2012[1](#)); therefore, we hypothesized that KCNT1 GOF causes an increase in I_{NaP} in PV neurons that enhances their excitability. To test this hypothesis, we measured I_{NaP} in SST and PV neurons by applying TTX during slow voltage ramp protocols (20 mV/s) designed to isolate I_{NaP} from both the transient Na^+ current and the K_{Na} current.

In SST neurons, I_{NaP} did not differ between WT and YH-HET neurons, either in the shape of the response (**Fig. 7A**[1](#) and **7B**[1](#)), or the peak amplitude (**Fig. 7B**[2](#)). In PV neurons, however, the peak amplitude of I_{NaP} was significantly increased by the Y777H variant (**Fig. 7C**[1](#) and **7D**[1](#)). To examine whether this increase in I_{NaP} could account for the increase in AP firing observed in PV neurons, we again simulated the effect of KCNT1 GOF on PV neuron activity, but this time included an increase in I_{NaP} in the compartmental models. Indeed, an increase in I_{NaP} conductance, similar to what was seen in the experimental results (2-fold), was sufficient to increase the number of APs fired in response to increasing current steps (**Fig. 7E**[1](#) and **7F**[1](#)), even in the face of KCNT1 GOF (30 mM). Also, like the experimental data, the rheobase current was decreased (**Fig. 7F**[2](#)) and the AP height was increased (80.4 ± 0.7 vs. 82.1 ± 0.8 mV). These results suggest that the differential effects on AP firing in SST and PV neurons can be accounted for by the absence or presence of a secondary increase in I_{NaP} .

YH-HET SST neurons show increased chemical and electrical coupling and receive increased excitatory input

We previously showed that homozygous expression of the Y777H variant increased connections between GABAergic neurons (Shore et al., 2020[1](#)), but heterozygous expression did not (**Fig. 3**[1](#)). Because heterozygous Y777H variant expression caused the strongest excitability impairments in SST neurons, we hypothesized that alterations in synaptic connectivity and activity may be more likely to occur in this neuronal population. To test this hypothesis, we recorded from pairs of WT or YH-HET SST neurons as described above to measure connection probability and strength. Indeed, similar to the increase observed in I-I connections in YH-HOM networks, we found an increase in homotypic synaptic connections of SST neurons in YH-HET networks (30/40 connections) relative to those in WT (15/40 connections; **Fig. 8A**[1](#)). The amplitudes of the evoked postsynaptic currents (ePSCs) between connected SST neurons were not significantly different between the YH-HET and WT groups (**Fig. 8B**[1](#)).

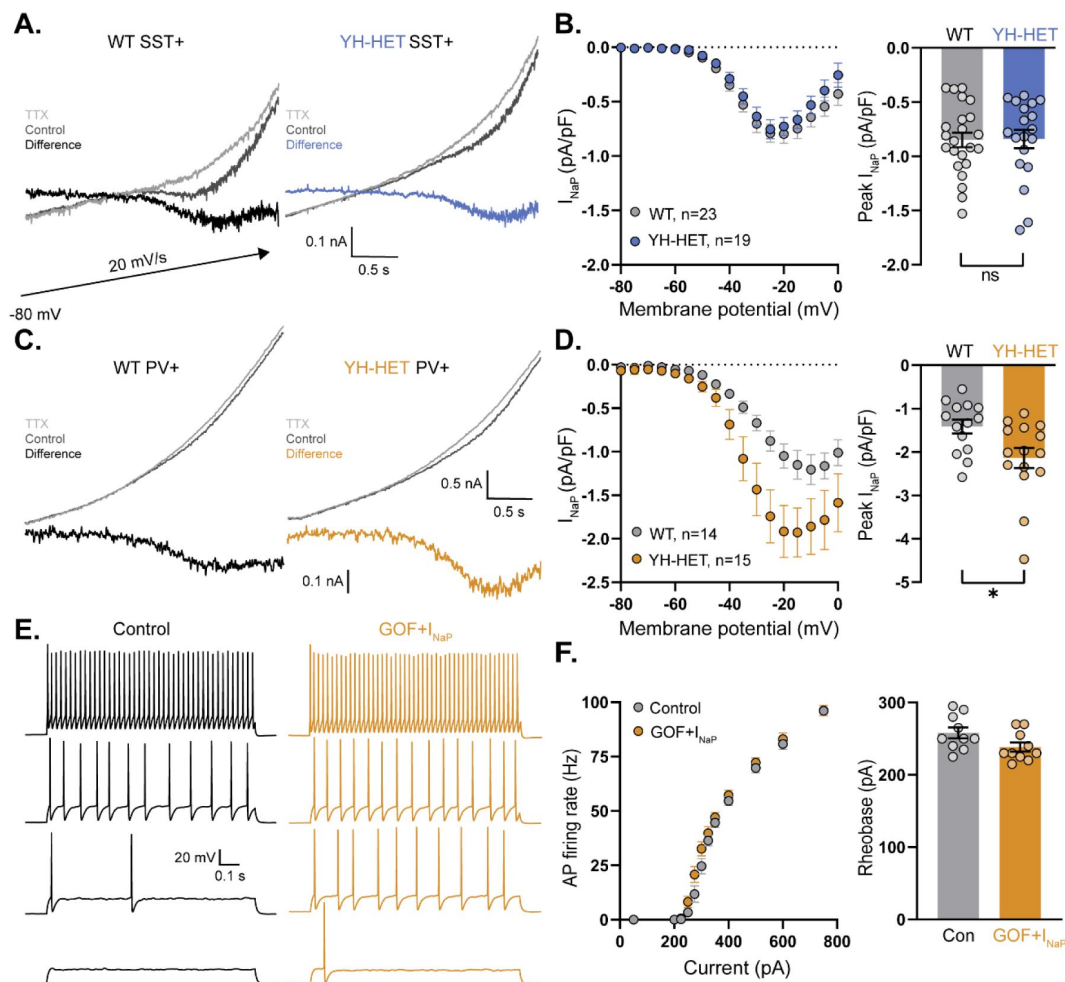


Figure 7.

The persistent Na^+ current is increased by the *Kcnt1*-Y777H variant in PV, but not SST, neurons.

(A) Representative traces of WT (left) and YH-HET (right) SST neurons in response to slow voltage ramp in control (dark gray) and 7 seconds after application of 0.5 nM TTX (light gray) with the difference current (I_{NaP}) plotted on the same scale. (B) I_{NaP} I-V curves of WT (gray) and YH-HET (blue) SST neurons constructed from averaging the ramp-evoked difference current (mean $\pm$ SEM) at 5-mV intervals (left), and a bar graph with overlaid scatter plot showing the peak negative value for each neuron (right). (C) Representative traces of WT (left) and YH-HET (right) PV neurons in response to slow voltage ramp in control (dark gray) and 7 seconds after application of 0.5 nM TTX (light gray) with the difference current (I_{NaP}) plotted on the same scale. The upper traces and difference currents are plotted on different scales. (D) I_{NaP} I-V curves of WT (gray) and YH-HET (orange) PV neurons constructed from averaging the ramp-evoked difference current (mean $\pm$ SEM) at 5-mV intervals (left), and a bar graph with overlaid scatter plot showing the peak negative value for each neuron (right). (E) Simulated traces from a model PV neuron in response to 500-ms current injections with control levels of KCNT1 (black traces) and KCNT1 GOF with a 2-fold increase in I_{NaP} (orange traces). Representative traces are shown at increasing current steps from bottom to top for each level. (F) F-I plot shows the increase in AP firing (left), and a bar graph with overlaid scatter plot of individual neuron values shows the decrease in rheobase associated with modeling KCNT1 GOF together with the increase in I_{NaP} . Asterisks indicate where $p < 0.05$.

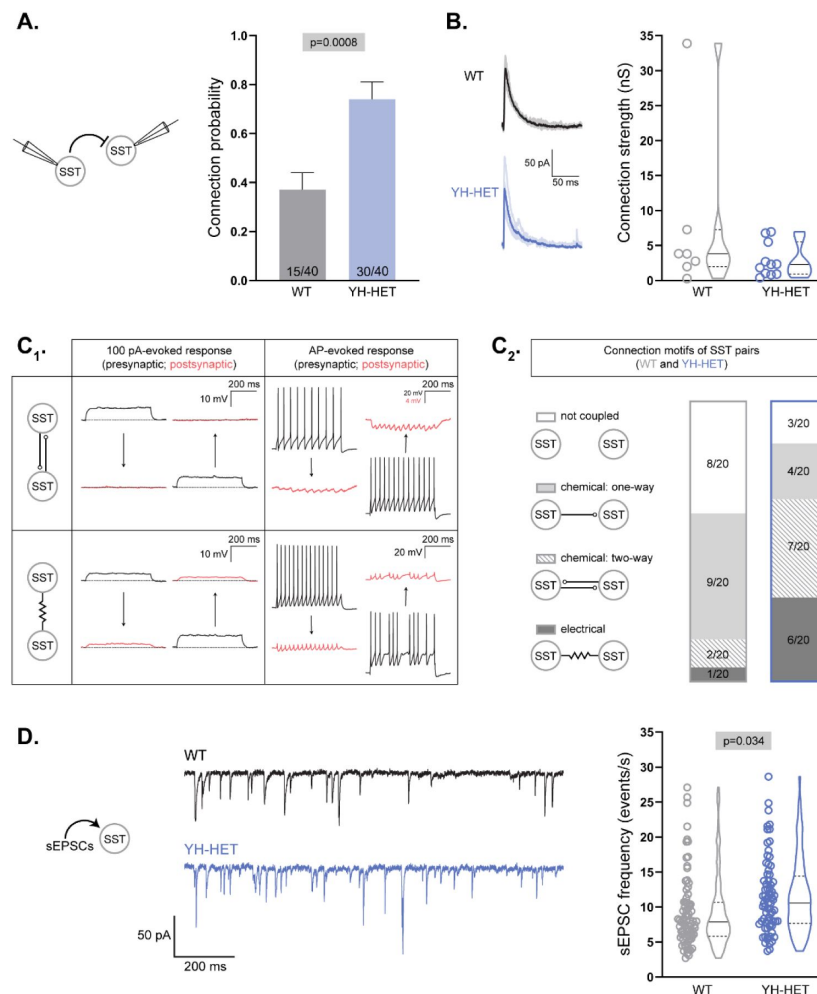


Figure 8.

YH-HET SST neurons show increased chemical and electrical coupling and receive increased excitatory input.

(A and B) Evoked postsynaptic currents (PSCs) were recorded from SST neuron pairs by stimulating each neuron at 0.1 Hz and recording the response onto the partner. **(A)** A bar graph shows the connection probability (mean + SEM) between SST neurons (WT, gray; YH-HET, blue; numbers on bars represent connected pair number/recorded pair number). **(B)** On the left, example traces of evoked IPSCs [individual IPSCs (light) overlaid by averaged IPSCs (dark)], and on the right, a graph of connection strength between SST neurons (WT, gray; YH-HET, blue; individual measurements and summary violin plots). **(C₁)** A schematic illustrates representative responses (presynaptic, black; postsynaptic, red) between chemically coupled (upper two panels) and electrically coupled (lower two panels) SST neurons following 100-pA (left two panels) and AP (right two panels) stimulation. **(C₂)** Summary data of four possible connection motifs (not coupled, chemical: one-way, chemical: two-way, and electrical) tested among WT (gray box) and YH-HET (blue box) SST neuron pairs (20 pairs/group). **(D)** On the left, example traces of spontaneous EPSCs (sEPSCs) recorded onto SST neurons (WT, black; YH-HET, blue). On the right, a graph shows individual neuron measurements and summary violin plots of the sEPSC frequency onto SST neurons (WT, gray; YH-HET, blue). All significant p -values are displayed at the top of each graph.

There are two main types of synaptic coupling between neurons: chemical, which is mediated by neurotransmitter release, and electrical, which is mediated by gap junctions. To test for alterations in each type of synaptic coupling due to expression of the *Kcnt1* variant, we recorded from 20 pairs of WT or YH- HET SST neurons in close proximity (< 100 μm apart). Under current-clamp conditions, we injected a series of hyperpolarizing and depolarizing current steps into one neuron and assessed whether the current induced a simultaneous voltage deflection in the paired, non-injected neuron (Fig. 8C₁; 100-pA-evoked response). We then evoked a train of APs in one neuron and assessed the AP-induced voltage changes in the paired, non-injected neuron (Fig. 8C₁; AP-evoked response). SST pairs were considered to be chemically coupled if the AP trains in one neuron induced corresponding IPSCs, without inducing voltage deflections in response to the current steps, onto the paired neuron (Fig. 8C₁; top panels). SST pairs were considered to be electrically coupled if the current steps injected into one neuron induced simultaneous voltage deflections in the paired neuron (Fig. 8C₁; bottom panel, left). Electrically coupled SST neurons also frequently showed AP-evoked spikelets onto paired neurons due to low-pass filtering (Fig. 8C₁; bottom panel, right), which results in a greater attenuation of the high frequency portion (spike), than the low frequency portion (AHP), of an action potential as it passes through an electrical synapse.

Of the 20 WT SST neuron pairs tested, 12 were coupled, and the majority (9/12) of those were one-way chemical connections (Fig. 8C₂). Of the 20 YH-HET SST neuron pairs tested, 17 were coupled, and the majority (13/17) of those were two-way, or bidirectionally, connected (Fig. 8C₂). Of the 3 bidirectionally connected WT SST neuron pairs, 2 were chemical and 1 was electrical, whereas of the 13 bidirectionally connected YH-HET SST neuron pairs, 7 were chemical and 6 were electrical (Fig. 8C₂). Thus, these data suggest that heterozygous Y777H expression increases reciprocal connectivity among SST neurons, of both the chemical and electrical sort.

Finally, although we previously found no alterations in the sEPSC frequency onto the total population of YH-HOM GABAergic neurons, there was an increase in the sEPSC frequency onto those with an NFS phenotype (Shore et al., 2020), suggesting there may be a compensatory increase in excitatory drive onto NFS YH-HOM GABAergic neurons to offset the effects of their decreased membrane excitability. To assess whether there is a similar increase in sEPSC frequency onto SST- expressing YH-HET neurons, which showed a similar decrease in membrane excitability to that of NFS YH-HOM GABAergic neurons, we recorded sEPSCs onto voltage-clamped WT and YH-HET SST neurons. Indeed, there was an increase in sEPSC frequency onto YH-HET SST neurons compared with that of WT (Fig. 8D), suggesting a compensatory increase in excitatory drive onto the most impaired GABAergic subtype.

Discussion

More than a decade ago, autosomal dominant mutations were identified in the sodium-gated potassium channel *KCNT1* in multiple patients with severe, childhood epilepsy syndromes (Barcia et al., 2012; Heron et al., 2012). *KCNT1*-related epilepsy patients suffer not only with frequent, early-onset seizures, but also with cognitive impairments, movement disorders, and sometimes additional behavioral and/or psychiatric problems (Bonardi et al., 2021). Unfortunately, after over 10 years of research efforts, *KCNT1*-related epilepsies remain highly refractory to current anti-seizure medications (Bonardi et al., 2021); thus, a better understanding of the pathological mechanisms underlying *KCNT1*- related epilepsies—from mutated gene to altered K^+ current to altered neuronal physiology to altered neural network to seizure—is imperative for advancing therapeutic strategies to improve seizure control and enhance patient quality of life.

Heterozygous vs. homozygous *KCNT1* GOF variant effects

Despite the heterozygous nature of *KCNT1* GOF variants in the overwhelming majority of *KCNT1*-related DEE patients, research efforts, including our own, have largely focused on homozygous GOF variant effects on channel function and neuronal physiology (Gertler et al., 2019 [DOI](#); Kim et al., 2014 [DOI](#); McTague et al., 2018 [DOI](#); Mikati et al., 2015 [DOI](#); Milligan et al., 2014 [DOI](#); Quraishi et al., 2019 [DOI](#); Shore et al., 2020 [DOI](#); Tang et al., 2016 [DOI](#)). Heterozygous *KCNT1* GOF variant expression likely results in the formation of heteromeric KCNT1 channels, consisting of both WT and mutant subunits, whose characteristics and kinetics may differ from those of homomeric KCNT1 channels; thus, it is critical to distinguish between GOF variant effects in heteromeric and homomeric KCNT1 channels to identify the pathogenic mechanisms of *KCNT1* GOF variants in DEEs. Recent work in heterologous cells has shown that KCNT1 GOF heteromeric channels, with WT and mutant subunits, exhibit K_{Na} current increases that lie between those of WT and KCNT1 GOF homomeric channels (Cole et al., 2021 [DOI](#); Dilella et al., 2018 [DOI](#); Rizzo et al., 2016 [DOI](#)). Furthermore, other recent studies have similarly shown that heterozygous *Kcnt1* GOF variant expression in neurons causes effects on K_{Na} current and excitability that are intermediate to those of WT and homozygous variant-expressing neurons (Gertler et al., 2022 [DOI](#); Wu et al., 2023 [DOI](#)).

Here, we assessed the effects of heterozygous expression of *Kcnt1*-Y777H on K_{Na} currents, neuronal physiology, and synaptic connectivity among cortical neuron subtypes in mice, and compared the effects to those observed previously with homozygous expression of the same variant (Shore et al., 2020 [DOI](#)). We found that the heterozygous YH variant caused similar effects on K_{Na} currents and neuronal physiology to those caused by the homozygous YH variant, including subthreshold-specific increases in K_{Na} current, altered AP shape, and impaired AP firing of NFS GABAergic neurons, but with magnitudes intermediate to those of WT and YH-HOM neurons. At the network and whole-animal level, we previously showed that homozygous expression of the YH variant in mice increases homotypic synaptic connectivity (E-E and I-I) and the E/I ratio, resulting in hypersynchronous, hyperexcitable YH-HOM cortical networks and frequent seizures. Here, we found no evidence of similar network alterations in YH-HET cortical networks, which likely explains the infrequent seizures observed in the heterozygous YH mouse model, and together with the YH-HOM data, indicate a strong relationship between network abnormalities and seizure propensity downstream of the YH variant. Furthermore, these data suggest that heterozygous and homozygous *KCNT1* variant expression cause similar, gene-dose-dependent GOF effects at the current and neuron level, but not at the network or whole-animal level.

Neuron-type-dependent *KCNT1* GOF variant effects

Prior work has illustrated the importance of cell context in delineating the functional effects of ion channel variants on neuronal physiology. For instance, several studies using LOF channelopathy models of both sodium and potassium channels have identified excitability effects on one neuron type without effects on another (Hedrich et al., 2014 [DOI](#); Soh et al., 2018 [DOI](#); Tai et al., 2014 [DOI](#); Yu et al., 2006 [DOI](#)). Other studies have shown that the same sodium channel variant can even cause opposite effects on excitability in different neuron types (Makinson et al., 2016 [DOI](#); Rush et al., 2006 [DOI](#); Studtmann et al., 2022 [DOI](#)). Thus, understanding how KCNT GOF alters networks to lead to hyperexcitability and seizures requires a thorough evaluation of how the variant impacts each neuron type that participates in the network. With this goal in mind, we assessed the effects of heterozygous *Kcnt1* GOF on K_{Na} currents and AP generation among cortical glutamatergic neurons and all three major GABAergic neuron subtypes, including those expressing VIP, SST, and PV.

Using a new, more selective inhibitor of KCNT1 to isolate and measure the KCNT1-mediated current, we observed voltage-dependent differences in current increases among the YH-HET neuron types. YH-HET glutamatergic and VIP neurons showed KCNT1-mediated current increases across suprathreshold voltages, whereas YH-HET SST and PV neurons had current increases across subthreshold voltages. These neuron-type dependent effects of KCNT1 GOF on K_{Na} current increases are similar to those observed previously in YH-HET and YH-HOM, glutamatergic and

GABAergic neuron subpopulations using TTX (Shore et al., 2020). Moreover, these results are similar to those reported in a recent study using a different KCNT1 GOF mouse model (human R474H, mouse R455H), which showed K_{Na} current increases selectively across positive potentials in glutamatergic neurons but across positive and negative potentials in FS and NFS GABAergic neurons (Wu et al., 2023). The mechanism underlying these neuron-type-dependent KCNT1 GOF effects on K_{Na} current increases is unknown, however, we have observed differences even in WT KCNT1 channel activation curves between glutamatergic and VIP neurons (steeper slopes of activation) and SST and PV neurons (shallower slopes of activation), suggesting there are factors affecting KCNT1 channel kinetics that are inherent to each neuron type (Fig. 6A). Future studies should investigate these potential factors including (1) differential expression of alternative KCNT1 splice forms, some of which are known to have different activation kinetics, (2) differential expression of other channels such as KCNT2, which can form heteromers with KCNT1 and alter its biophysical properties, and (3) differential expression and/or localization of, and/or coupling to, sodium channels that act as the source of KCNT1 channel activation (Chen et al., 2009; Hage & Salkoff, 2012; Joiner et al., 1998).

Consistent with the differential KCNT1 GOF effects on K_{Na} currents, we observed a range of effects on membrane properties and AP generation among the cortical neuron populations. We observed no effects on YH-HET glutamatergic and VIP neurons, whereas YH-HET SST neurons showed reduced AP firing, with decreased input resistance and increased rheobase, and YH-HET PV neurons showed increased AP firing, with decreased rheobase. As a complementary approach, we performed *in silico* electrophysiology using our measured neuron-type-specific KCNT1 channel activation kinetics, with and without GOF, for each neuron type. For glutamatergic and VIP neurons, the steeper slopes of activation limited the effects of GOF on intrinsic excitability, whereas for SST neurons, the shallower slope of activation decreased the input resistance, increased the rheobase, and impaired AP generation downstream of KCNT1 GOF. Each of these modeling results are similar to those observed using *in vitro* electrophysiology, indicating direct K_{Na} current-to-KCNT1 GOF phenotype relationships in these neuron types. Conversely, for PV neurons, unlike the experimental observation of increased YH-HET PV neuron excitability, *in silico* electrophysiology predicted a slight reduction in PV neuron excitability downstream of KCNT1 GOF, suggesting that the observed GOF effects on PV physiology are due to an indirect or compensatory mechanism.

Evidence of potential neuron and network compensatory responses to KCNT1 GOF

In a healthy brain, there are multiple homeostatic compensation mechanisms to maintain neuronal and network activity at their proper physiological levels (Davis, 2013; Debanne et al., 2019; Debanne & Russier, 2019; Marder & Goaillard, 2006; Turrigiano, 2011, 2012; Yang & Prescott, 2023), whereas in a brain with epilepsy-causing genetic mutations or injury, it is thought that failure of homeostatic compensation can result in hypersynchronous, hyperexcitable networks and, ultimately, seizures (Issa et al., 2023; Lignani et al., 2020; Staley, 2015). As YH-HET mice have only been observed to have infrequent seizures and YH-HET networks appear largely intact, it is plausible that there are adaptive or homeostatic mechanisms downstream of the YH variant that limit KCNT1 GOF effects largely to those observed at the current and neuron level. These regulatory mechanisms may include alterations in ion currents and/or synaptic input to restore proper AP shape or neuronal excitability, or alterations in chemical and/or electrical connectivity to stabilize neural networks.

Neuronal excitability is regulated by the inward and outward flow of opposing currents through sodium and potassium channels; thus, it is not surprising that mutations affecting these channels can lead to compensatory up- or down-regulation of other ion channels or currents to maintain excitability and proper neuron function. In this study, our *in silico* electrophysiology predictions of YH-HET PV neuron excitability were opposite to those of our *in vitro* electrophysiology data; thus,

we hypothesized that there must be a compensatory increase in an ion current that counteracts the increased K_{Na} current selectively in YH-HET PV neurons. Accordingly, we identified a subthreshold increase in the persistent Na^+ current, across a similar voltage range to the increased K_{Na} current, in YH-HET PV, but not SST, neurons. Furthermore, *in silico* approaches indicated that this increased I_{NaP} was sufficient to overcome the effects of KCNT1 GOF and cause an overall increase in AP generation in YH-HET PV neurons, which aligned with our experimental data. Similar compensatory mechanisms have been identified in other epilepsy models such as the downregulation of multiple potassium channels in a sodium channel (*Scn2a*) LOF mouse model, resulting in neuronal hyperexcitability (Zhang et al., 2021 [DOI](#)). Conversely, another KCNT1 GOF model (*Kcnt1*-R455H) showed upregulation of a sodium channel ($Na_v1.6$), accompanied by an increase in sodium currents, in *Kcnt1*-R455H cortical neurons, but the effects and cell-type specificity of $Na_v1.6$ upregulation were not investigated (Wu et al., 2023 [DOI](#)). Future studies should be performed to understand how and why I_{NaP} is increased selectively in YH-HET PV neurons, and to determine whether the increased I_{NaP} ultimately contributes to network hyperexcitability and seizures.

Lastly, in addition to the KCNT1 GOF current-mediated impairments in SST neuron excitability, we identified several potential compensatory YH-HET SST synaptic and network alterations. For instance, there was an increased frequency of sEPSCs onto YH-HET SST neurons, suggestive of a compensatory increase in excitatory input to counteract their reduced intrinsic excitability and increase their AP firing, as has been observed previously in hypoexcitable SST neurons in another epilepsy model (Halabisky et al., 2010 [DOI](#)). There was also an increase in chemical and electrical coupling between YH-HET SST neurons. Although SST neurons normally show a relatively low rate of homotypic chemical coupling, particularly in the adult cortex, more than half of nearby SST neuron pairs are coupled electrically (Amitai et al., 2002 [DOI](#); Gibson et al., 1999 [DOI](#), 2005 [DOI](#); Hu & Agmon, 2015 [DOI](#); Urban-Ciecko & Barth, 2016 [DOI](#)). This coupling is thought to be important for fine tuning neural circuits by regulating such processes as synchronous AP firing, synaptic integration, and network rhythmicity (Alcami & Pereda, 2019 [DOI](#); Connors, 2017 [DOI](#)). The observed increase in electrical coupling should not only contribute to the reduced intrinsic excitability properties of individual YH-HET SST neurons, increasing their capacitance and decreasing their input resistance, but may also act as a homeostatic mechanism to restore synchronicity and stability to YH-HET SST networks (Lane et al., 2016 [DOI](#); Parker et al., 2009 [DOI](#)).

The *in vitro* and *in silico* nature of this study are limitations. Although the cultured VIP, SST, and PV neurons showed characteristic electrophysiological properties, it is unknown how much overlap there is between the *in vitro* and *in vivo* populations. For instance, it is possible that our culture conditions select for more mature neurons of each type or preferentially support the survival of subpopulations; thus, we may be assessing KCNT1 GOF effects on a small portion of a given subtype. However, an advantage of the *in vitro* prep is that the compensatory alterations we observe are not likely consequences of seizure activity, which has been shown to alter interneuron properties in epilepsy models. Furthermore, the *in silico* neurons we used to model each neuron type were designed to accurately reflect the morphological and electrophysiological properties of each subtype, but they may lack more detailed features of ion channels, such as post-translational modifications and subcellular localizations, that can have important functional effects. Our KCNT1 model conductance is also hampered by an incomplete understanding of the relationship between Na^+ influx, membrane voltage, and channel gating in neurons.

Despite the potential limitations, this study provides three major findings that advance our understanding of the relationship between ion channel function and disease. First, heterozygous *KCNT1* expression causes changes in current flow and neuron excitability that are qualitatively similar to homozygous expression, but of a lower magnitude; however, the synaptic activity and connectivity changes are different, which likely leads to the discrepancy in seizure incidence. This finding suggests a non-linear relationship between GOF, at the level of the ion channel current, and disease severity. Second, the same *KCNT1* variant can produce opposite effects on neuron

excitability in closely related GABAergic neuron subtypes, and these opposite effects are likely due to compensation in one neuron type (PV) that is absent in the other (SST). Previous studies have observed opposite effects on neuronal excitability due to Na⁺ channel variants, but the underlying mechanism, when explored, was proposed to be intrinsic differences in ion channel expression, not compensation. Third, we observed increased synaptic and gap junction connectivity among SST neurons, demonstrating that the effects of KCNT1 GOF extend to structural alterations as well. The effects of this increased connectivity, especially by gap junctions, on neuronal excitability and network behavior offers an exciting avenue for future research. Finally, it will be critical to determine whether these alterations downstream of KCNT1 GOF potentiate or attenuate network pathology and seizure activity, thus providing a better understanding of disease mechanisms and prompting novel therapeutic design.

Materials and methods

Mice

Mice were bred, and mouse procedures were conducted, in compliance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Vermont (animal protocol numbers: 16-001, 19-034, and X9-022). Mice were maintained in ventilated cages at controlled temperature (22–23°C), humidity ~60%, and 12-h light: 12-h dark cycles (lights on at 7:00 AM, off 7:00 PM). Mice had access to regular chow and water, *ad libitum*. For all experiments, male and female littermates were used for each genotype. The ages of the mice for each experiment are indicated in the following relevant sections.

Mouse lines and strains used for these studies include: *Kcnt1*^{Y777H} knockin in the C57BL/6NJ (B6NJ) strain (formal gene and allele symbol: *Kcnt1*^{em1(Y777H)Frk}), *Sst*-IRES-Cre (*Sst*^{tm2.1(cre)Zjh}/J; Jackson Labs stock: 013044), *Vip*-IRES-Cre (*Vip*^{tm1(cre)Zjh}/J; Jackson Labs stock: 010908), *Pvalb*-IRES-Cre (B6.129P2-*Pvalb*^{tm1(cre)Arbr}/J; Jackson Lab stock: 017320), and C57BL/6J (Jackson Labs stock: 000664) mice.

Kcnt1^{Y777H} mice were genotyped using PCR amplification primers (*Kcnt1* forward primer: 5'-CTAGGGCTGCAAACACAACA-3'; *Kcnt1* reverse primer: 5'-TCAAGCAGCAACACGATAGG-3') with standard thermocycler amplification conditions, and the annealing temperature set at 58°C. Following amplification, a restriction cut was performed with the enzyme *NlaIII* to distinguish mutant (127 and 44 bp products after cut) from wildtype (WT) alleles (171 bp product). Progeny of the *Kcnt1*^{Y777H} mice crossed to each Cre mouse line were genotyped for the presence of a Cre transgene using a forward primer (5'-TCGCGATTATCT TCTATA TCTTCAG-3') and reverse primer (5'-GCTCGACCAGTTTAGTTACCC-3'), resulting in a 455 bp product.

Primary Astrocyte Feeder Layer Culture

Astrocyte feeder layers were generated to support the growth and maintenance of primary neurons. Briefly, cortices were dissected from P0-1 WT C57BL/6J mice (Jackson Labs stock: 000664) of either sex. The cortices were incubated in 0.05% trypsin-EDTA (Gibco) for 15 min at 37°C in a Thermomixer (Eppendorf) with gentle agitation (800 rpm). Then, the cortices were mechanically dissociated with a 1- mL pipette tip, and the cells were plated into T-75 flasks containing filter-sterilized astrocyte media [DMEM media supplemented with glutamine (Gibco), 10% fetal bovine serum (FBS, GE Healthcare), 1X MITO+ Serum Extender (Corning), and 0.2X penicillin/streptomycin (Gibco)]. After the astrocytes reached confluency, they were washed with PBS (Gibco) and incubated for 5 min in 0.05% trypsin-EDTA at 37°C, washed, and then resuspended in astrocyte media. Astrocytes were added to 6-well plates containing 25-mm coverslips precoated with coating mixture [0.7 mg/ml collagen I (Corning) and 0.1 mg/ml poly-D-lysine (Sigma) in 10 mM acetic acid].

Primary Cortical Neuron Culture

For the primary neuron culture, the dorsomedial cortices from P0-1 WT and *Kcnt1*^{m/+} mice of both sexes were dissected in cold HBSS (Gibco). The tissue was then digested with papain (Worthington) for 60-75 min and treated with inactivating solution (Worthington) for 10 min, both while shaking at 800 rpm at 37°C in a Thermomixer. The cells were then mechanically dissociated and counted. The dissociated cells were added at 200,000 cells/well to 6-well plates containing astrocyte-coated coverslips in filter-sterilized NBA plus [Neurobasal-A medium (Gibco) supplemented with 1X Glutamax (Gibco), 1X B27 (Invitrogen), and 0.2X penicillin/streptomycin (Gibco)]. After plating (12-24 h), approximately 4×10^{10} genome copies (GC) of AAV8-*CaMKII*-GFP (UNC Vector Core) were added to each well. For experiments to assess KCNT1 effects on GABAergic subtypes, approximately 4×10^{10} genome copies (GC) of AAV9-*hSyn*-DIO-mCherry (addgene) were also added to each well to mark Cre-expressing neurons. Every 3-4 days, 20-40% of the media was replaced with fresh NBA plus.

Electrophysiology

Whole-cell recordings were performed with patch-clamp amplifiers (MultiClamp 700B; Molecular Devices) under the control of Clampex 10.3 or 10.5 (Molecular Devices, pClamp, RRID:SCR_011323). Data were acquired at 20 kHz and low-pass filtered at 6 kHz. The series resistance was compensated at 70%, and only cells with series resistances maintained at less than 15 MΩ were analyzed. Patch electrodes were pulled from 1.5-mm o.d. thin-walled glass capillaries (Sutter Instruments) in five stages on a micropipette puller (model P-97; Sutter Instruments). Internal solution contained the following: 136 mM K-gluconate, 17.8 mM HEPES, 1 mM EGTA, 0.6 mM MgCl₂, 4 mM ATP, 0.3 mM GTP, 12 mM creatine phosphate, and 50 U/ml phosphocreatine kinase. Alternatively, internal solution contained: 136 mM KCl, 17.8 mM HEPES, 1 mM EGTA, 0.6 mM MgCl₂, 4 mM ATP, 0.3 mM GTP, 12 mM creatine phosphate, and 50 U/ml phosphocreatine kinase. The pipette resistance was between 2 and 4 MΩ. Standard extracellular solution contained the following (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 MgCl₂, and 2 CaCl₂ (pH 7.3, 305 mOsm). All experiments were performed at room temperature (22–23°C). Whole-cell recordings were performed on cortical neurons from control and mutant groups in parallel on the same day (day 13–17 *in vitro*). All experiments were performed by two independent investigators blinded to the genotypes.

For current-clamp experiments, the intrinsic electrophysiological properties of neurons were tested by injecting 500-ms square current pulses incrementing in 20 pA steps, starting at –100 pA. Resting membrane potential (V_m) was calculated from a 50 ms average before current injection. The membrane time constant (τ) was calculated from an exponential fit of current stimulus offset. Input resistance (R_{in}) was calculated from the steady state of the voltage responses to the hyperpolarizing current steps. Membrane capacitance was calculated by dividing the time constant by the input resistance. Action potentials (APs) were evoked with 0.5 s, 20 pA depolarizing current steps. Rheobase was defined as the minimum current required to evoke an AP during the 500 ms of sustained somatic current injections. AP threshold was defined as the membrane potential at the inflection point of the rising phase of the AP. AP amplitude was defined as the difference in membrane potential between the AP peak and threshold, and the afterhyperpolarization (AHP) was the difference between the AP threshold and the lowest V_m value within 50 ms. The AP half-width was defined as the width of the AP at half-maximal amplitude. To obtain the neuron's maximum firing frequency, depolarizing currents in 20-pA steps were injected until the number of APs per stimulus reached a plateau phase. The membrane potential values were not corrected for the liquid junction potential. GABAergic neurons were classified as fast spiking (FS) if their maximum mean firing rate reached above 60 Hz and their AP half-widths increased by less than 25% during 1 s of sustained firing (Avermann et al., 2012 [DOI](#); Casale et al., 2015 [DOI](#)). All others were considered non-fast spiking (NFS).

For voltage-clamp experiments to measure synaptic currents, neurons were held at -70 mV, except for evoked IPSC measurements, for which neurons were held at 0 mV. AP-evoked EPSCs were triggered by a 2 ms somatic depolarization to 0 mV. The shape of the evoked response, the reversal potential and the effect of receptor antagonists [10 μ M NBQX (Tocris Bioscience) or 20 μ M bicuculline (BIC, Tocris Bioscience)] were analyzed to verify the glutamatergic or GABAergic identities of the currents. Neurons were stimulated at 0.1 Hz in standard external solution to measure basal-evoked synaptic responses. Electrophysiology data were analyzed offline with AxoGraph X software (AxoGraph Scientific, RRID:SCR_014284). Spontaneous synaptic potentials were recorded in control solution with either NBQX or bicuculline to isolate EPSCs or IPSCs, respectively. Data were filtered at 1 kHz and analyzed using template-based miniature event detection algorithms implemented in the AxoGraph X. The threshold for detection was set at three times the baseline SD from a template of 0.5 ms rise time and 3 ms decay. The E/I ratio was calculated as the product of the sEPSC frequency and charge over the sum of the sEPSC frequency and charge and the product of the sIPSC frequency and charge.

K_{Na} and KCNT1 Current Measurements

For voltage-clamp experiments to measure the sodium-activated K⁺ current or KCNT1-mediated current, neurons were held at -70 mV and given 1 s voltage pulses in 10 mV steps over a range of -80 to $+50$ mV. Recordings were obtained for each cell in standard extracellular solution or extracellular solution containing 0.5 μ M tetrodotoxin (TTX) or 10 μ M VU170. TTX or VU170 was applied directly on the recorded neuron with a custom-built fast flow perfusion system capable of complete solution exchange in less than 1 s to minimize the time interval between control and drug recordings and current rundown. Current traces from the TTX or VU170 solution were subtracted from the current traces obtained from the standard solution. The difference current over the 100 ms at the end of the voltage pulse was considered the steady state K_{Na} current. The same protocol was used for outside-out patch experiments.

I_{NaP} Current Measurements

For voltage-clamp experiments to measure the persistent Na⁺ current, neurons were held at -80 mV and given a 5 s voltage ramp at 20 mV/s. The ramp was repeated every 10 s. Recordings were obtained for each cell in standard extracellular solution or extracellular solution containing 0.5 μ M TTX. TTX was applied directly on the recorded neuron with a custom-built fast flow perfusion system capable of complete solution exchange in less than 1 s to minimize the time interval between control and TTX recordings. The first current trace after TTX application was subtracted from the averaged current traces obtained from the standard solution. At this time point, the effect of TTX on the Na⁺-activated K⁺ current is minimal, while the persistent Na⁺ current is maximally inhibited. This allowed us to isolate the persistent Na⁺ current from K_{Na} and obtain a more accurate estimate of changes in I_{NaP}.

Neuron Modeling

Model neurons (~ 10 per class) representing excitatory (Pyramidal Cell, PC), SST (Martinotti Cell, MC), PV (Large Basket Cell, LBC), and (Bipolar Cell, BP) VIP neurons were downloaded from the EPFL/Blue Brain project (<https://bbp.epfl.ch/nmc-portal/downloads.html> [↗](#)), and implemented in the NEURON environment. Because no KCNT1-like conductance was present in the original models, a Hodgkin-Huxley KCNT1 model conductance was created with both Na⁺- and voltage-dependent gates and inserted into the dendritic, somatic, and axonal compartments of model neurons (0.03 S/cm²). The Na⁺ dependence was modeled after Bischoff et al. (EC₅₀ = 40 mM, slope 3.5), and the voltage dependence was modeled after our own experimental data (**Fig. 5** [↗](#)), corrected to account for the calculated liquid junction potential in the experimental data. To verify that this approach reproduced experimental data, voltage step protocols were run in NEURON with 10 mM and 0 mM internal Na⁺. “K_{Na}” currents were obtained by subtraction, and the amplitude and kinetics of the resulting currents were verified to be similar to experimental values. To model the effects of

KCNT1 GOF, the EC_{50} for Na^+ was decreased to either 35 or 30 mM, because the Y796H variant was previously shown to increase the Na^+ sensitivity of the channel. These changes resulted in moderate increases in the current above WT levels (**Fig. 6B**), consistent with the experimental data (**Fig. 4** and **Fig. 5**). Modeling data was generated by running current clamp protocols in NEURON for each of the model neurons and analyzed in the same way as experimental data. For modeling the increase in I_{NaP} , the control I_{NaP} conductance was set to 0.001 times the conductance of the transient Na^+ current in each compartment. This produced I_{NaP} currents approximately 1% of the peak of the transient Na^+ current. KCNT1 GOF was modeled by decreasing the Na^+ EC_{50} to 30 mM and the I_{NaP} increase was modeled by increasing its conductance 2-fold, in accordance with experimental data.

Immunostaining, Imaging, and Quantification

For live imaging of GABAergic subtypes *in vitro*, cortical neurons isolated and cultured from VIP-Cre, SST-Cre, and PV-Cre pups, were infected with AAV-*CamKII*-GFP and AAV-*hSyn*-DIO-mCherry viruses at DIV 1 to mark glutamatergic and Cre-expressing, GABAergic neurons, respectively. Images were captured from live cultures at DIV 14 (VIP-Cre and SST-Cre) or DIV 16 (PV-Cre) using an inverted Olympus IX73 epifluorescent microscope with an Olympus PlanFluor 10× objective lens and an Andor Zyla sCMOS 4.2 camera controlled by µManager 2.0-β software (Edelstein et al., 2014). The same acquisition parameters were used for each image and each group.

For soma size measurements, DIV 16 cultured SST-Cre neurons on coverslips were washed three times with prewarmed PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and then washed again three times with PBS. The fixed neurons were then incubated in blocking buffer (10% normal goat serum and 0.1% Triton-100 in 1X PBS) for 1 h at room temperature, followed by incubation with a Neu-N primary antibody (Synaptic Systems 266-004) diluted in blocking buffer (1:500) overnight at 4°C. The next day, the neurons were washed three times with PBS for 10–15 min, incubated with goat anti-guinea pig, Alexa Fluor 647 secondary antibody (Invitrogen A21450) in blocking buffer (1:1000) for 1 h at room temperature, and washed again three times with PBS for 10–15 min. The coverslips were then flipped and mounted on glass slides using Fluoromount G with DAPI. Images (1024 × 1024 pixels) of Neu-N to mark all neurons, *CamKII*-GFP to mark glutamatergic neurons, and *hSyn*-DIO-mCherry to mark Cre-expressing neurons, were obtained using a DeltaVision Restoration Microscopy System (Applied Precision/GE Life Sciences) with an inverted Olympus IX70 microscope with a 20 × oil objective, SoftWoRx software, and a CoolSNAP-HQ charge-coupled device digital camera (Photometrics). Image exposure times and settings were kept the same between groups in a culture and were optimized to ensure that there were no saturated pixels. To analyze soma size, regions of interest (ROIs) were drawn around cell bodies using the mCherry channel with Fiji software (Schindelin et al., 2012), and then the cell body areas were measured for each mCherry⁺ neuron imaged.

Data Presentation and Statistical Analysis

Prism 10 (GraphPad Prism, RRID:SCR_002798) was used to create all graphs. To test for statistical significance for all whole cell electrophysiology experiments, we used generalized linear mixed models (GLMM) in SPSS [28.0 Chicago, III (IBM, RRID:SCR_002865)], which allows for within-subject correlations and the specification of the most appropriate distribution for the data. Because neurons and animals from the same culture or animal are not independent measurements, culture or litter was used as the subject variable, and animals, neurons, and voltage steps were considered within-subject measurements. All data distributions were assessed with the Shapiro-Wilk test. Datasets that were significantly different from the normal distribution ($p < 0.05$) were fit with models using the gamma distribution and a log link function, except for synaptic connection probability, which was fit with the binomial distribution and probit link. Normal datasets were fit with models using a linear distribution and identity link. We used the model-based estimator for

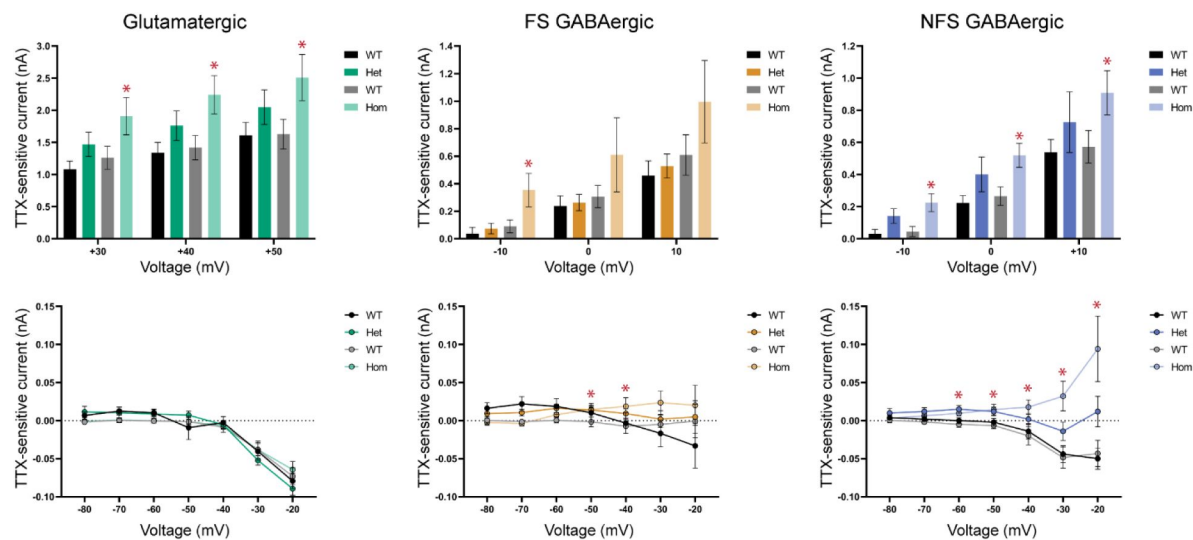
the covariance matrix and goodness of fit was determined using the corrected quasi likelihood under independence model criterion and by the visual assessment of residuals. All values reported in the text, figures, and tables are estimated marginal means $\pm$ standard error.

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Author Contributions

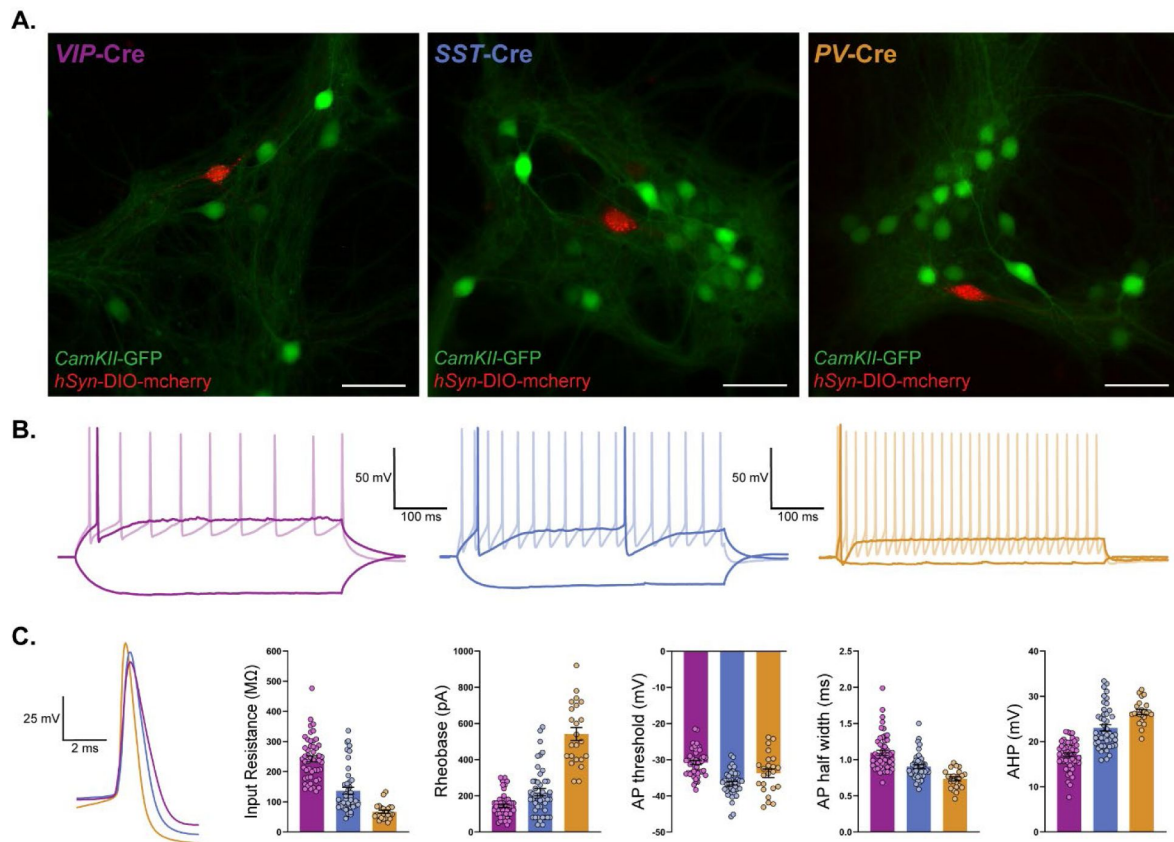
Conceptualization, A.N.S., W.N.F., M.C.W.; Methodology, A.M.Q., B.D.S., K.A.E., C.D.W., Formal Analysis, A.N.S., M.C.W.; Investigation, A.N.S., M.C.W.; Writing-Original Draft, A.N.S., M.C.W., Revising and Editing, A.N.S., M.C.W.; Supervision, K.A.E., C.D.W., W.N.F., M.C.W.; Funding Acquisition, W.N.F., M.C.W.



Supplemental Figure 2-1.

The magnitudes of the K_{Na} current increases in YH-HET glutamatergic and NFS GABAergic neurons are intermediate to those of WT and YH-HOM neurons.

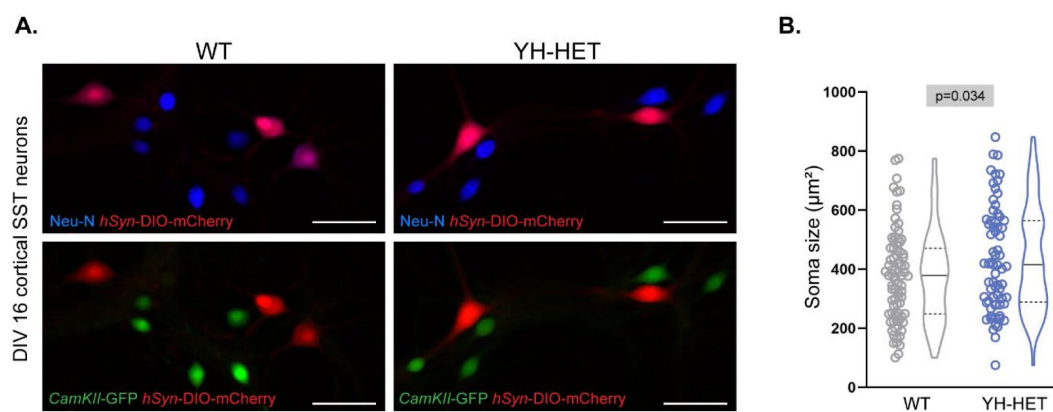
Bar graphs (top three) illustrate differences in K_{Na} current (mean $\pm$ SEM) between YH-HET [dark color; WT littermates (black)] and YH-HOM [light color; WT littermates (gray)] neurons for voltage steps where significant increases were previously observed (red asterisks where $p < 0.05$) in glutamatergic, and FS and NFS GABAergic, YH- HOM neurons. Line graphs (bottom three) illustrate differences in K_{Na} current (mean $\pm$ SEM) between YH- HET [dark color; WT littermates (black)] and YH-HOM [light color; WT littermates (gray)] neurons across negative voltage steps (-80 to -20 mV; red asterisks where $p < 0.05$ in YH-HOM compared with WT) in glutamatergic, and FS and NFS GABAergic, neurons.



Supplemental Figure 4-1.

Cre recombinase-expressing, cortical GABAergic neuron subpopulations retain characteristic electrophysiological features in culture.

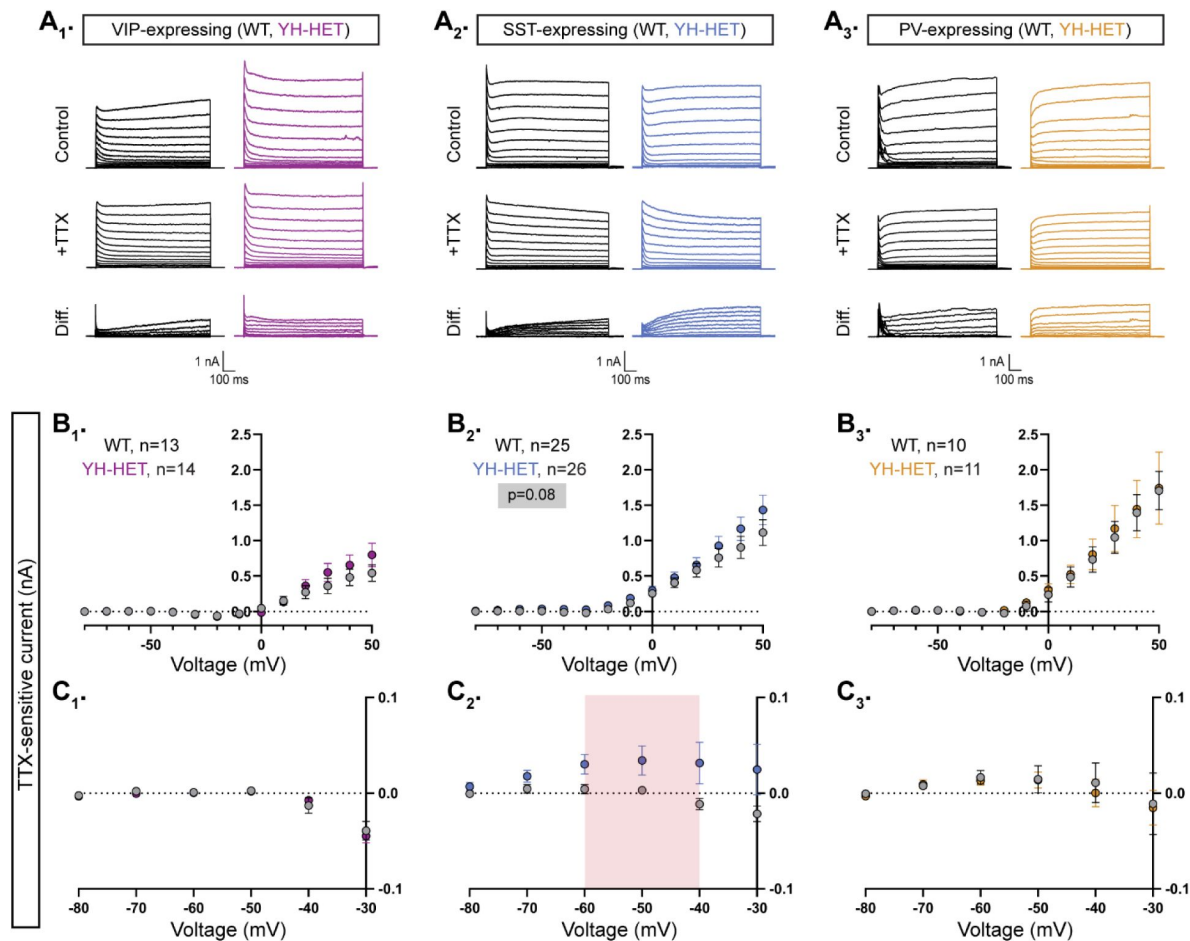
Cortical neurons isolated and cultured from P0 pups (*Kcnt1*^{+/+}) from VIP-, SST-, or PV-Cre mouse lines were infected with AAV-*CamKII*-GFP to label glutamatergic neurons, and AAV-*hSyn*-DIO-mCherry to label Cre-expressing neurons, at DIV 1. **(A)** Images show GFP⁺ glutamatergic neurons and mCherry⁺ VIP-, SST-, or PV-expressing neurons (left to right) at DIV 14 (VIP and SST) or DIV 16 (PV). The scale bars represent 50 μ m. **(B)** Representative responses to step currents are shown for VIP (purple), SST (blue), and PV (orange) neurons (left to right). For each neuron type, the superimposed dark traces illustrate the input resistance (in response to a depolarizing step) and the rheobase (the first trace with an AP in response to a hyperpolarizing step), and the light trace shows the first step current response to induce repetitive AP firing across the step. **(C)** On the left, the first AP of each rheobase trace is shown for VIP (purple), SST (blue), and PV (orange) neurons. On the right, bar graphs show quantification of the membrane properties and AP parameters for each neuron type (VIP, SST, and PV, left to right), with individual neuron measurements overlaid in scatter plots.



Supplemental Figure 4-2.

YH-HET SST neurons have increased soma size compared with those of WT.

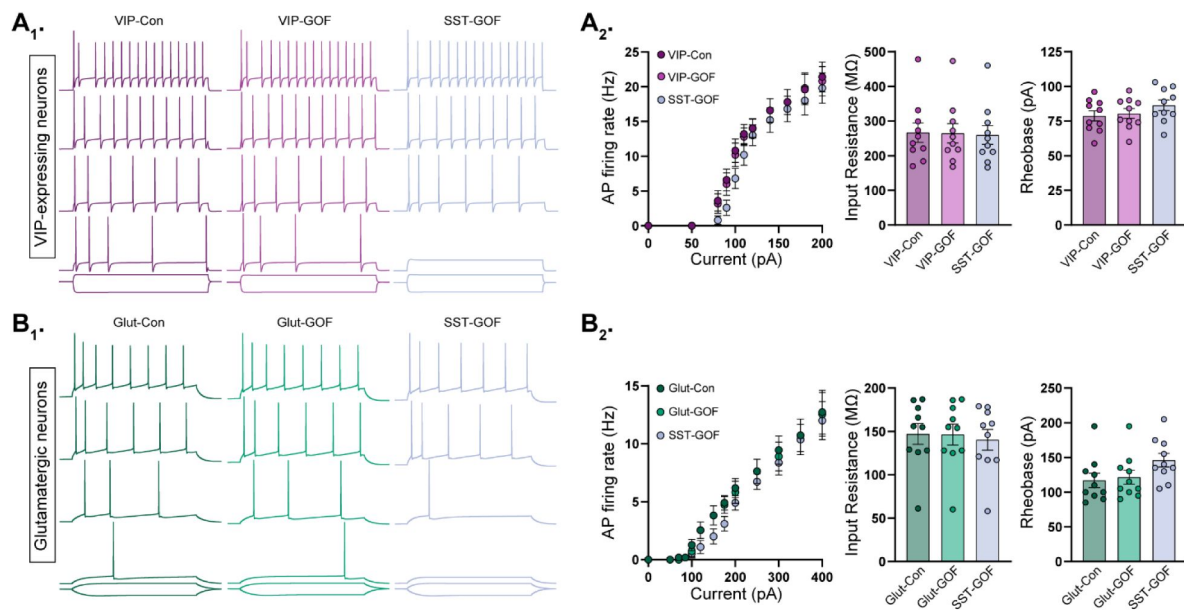
Cortical neurons were isolated and cultured from P0 pup progeny (WT and YH-HET) of YH-HET mice crossed to SST-Cre mice. Neurons were infected with AAV-*CamKII-GFP* to label glutamatergic neurons, and AAV-*hSyn-DIO-mCherry* to label Cre-expressing neurons, at DIV 1, and immunostained using anti-Neu- N antibodies at DIV 16. **(A)** Top two images show mCherry⁺ SST-expressing neurons as a subpopulation of Neu-N⁺ cortical neurons, and bottom two images show GFP⁺, mCherry⁺ SST-expressing neurons intermingled with GFP⁺ glutamatergic neurons. The scale bars represent 50 μm . **(B)** A graph shows individual neuron measurements and summary violin plots of the mCherry⁺ neuron soma size (WT, gray; YH-HET, blue) with the p-value displayed at the top.



Supplemental Figure 5.

The *Kcnt1*-Y777H variant increases K_{Na} currents measured with TTX subtraction across subthreshold voltages in SST-expressing GABAergic neurons.

(**A₁-A₃**) Representative traces in control (top), 0.5 μ M TTX (middle), and the difference current (bottom) calculated by subtracting the membrane current response to voltage steps (-80 to +50 mV) from a holding potential of -70 mV in TTX from the response in control external solution in VIP-, SST-, and PV-expressing, WT (black) and YH-HET (colors) neurons. (**B₁-B₃**) Summary data shows the K_{Na} current (mean $\pm$ SEM) for each voltage step in VIP-, SST-, and PV-expressing, WT (black and gray) and YH-HET (colors) neurons. The p-values are shown on each graph where $p < 0.05$, and the n values are the number of neurons recorded for each group. (**C₁-C₃**) Plots of the K_{Na} current (mean $\pm$ SEM) for each voltage step from -80 to 0 mV in WT (black and gray) and YH-HET (colors) neurons to illustrate the values that are too small to be seen on the graphs in B₁-B₃. The shaded red area in C₂ indicates the subthreshold voltage range with significantly higher K_{Na} currents in YH-HET relative to WT neurons. Statistical significance for I-V plots was tested using Generalized Linear Mixed Models with genotype and current step as fixed effects followed by pairwise comparisons at each level.



Supplemental Figure 6.

Model VIP and glutamatergic neurons with insertion of SST-like, KCNT1 GOF kinetics show reduced excitability.

(A₁) Simulated traces from model VIP neurons, using VIP KCNT1 activation curve parameters (purple) or SST KCNT activation curve parameters (blue), in response to 500- ms current injections at two levels of Na⁺-sensitivity (Con-40 mM and GOF-30mM). Representative traces are shown at increasing current steps from bottom to top for each level. **(A₂)** Summary data from 10 model VIP neurons, with VIP KCNT1 parameters (VIP-Con and VIP-GOF) or SST KCNT1 parameters (SST-GOF) showing (from left to right) the number of APs at different current steps (F-I plot), the input resistance, and rheobase. The bar graphs (mean ± SEM) are overlaid with scatter plots of individual neuron measurements. **(B₁)** Simulated traces from model glutamatergic neurons, using glutamatergic KCNT1 activation curve parameters (green) or SST KCNT activation curve parameters (blue), in response to 500- ms current injections at two levels of Na⁺-sensitivity (Con-40 mM and GOF-30mM). Representative traces are shown at increasing current steps from bottom to top for each level. **(B₂)** Summary data from 10 model glutamatergic neurons, with glutamatergic KCNT1 parameters (Glut-Con and Glut-GOF) or SST KCNT1 parameters (SST-GOF) showing (from left to right) the number of APs at different current steps (F-I plot), the input resistance, and rheobase. The bar graphs (mean ± SEM) are overlaid with scatter plots of individual neuron measurements.

	Glutamatergic			FS GABAergic			NFS GABAergic		
	WT	YH-HET		WT	YH-HET		WT	YH-HET	
	N = 9	N = 9		N = 9	N = 9		N = 9	N = 9	
	n = 27	n = 30	p-value	n = 27	n = 29	p-value	n = 26	n = 22	p-value
V _{rest} (mV)	-57.9±1.6	-57.0±1.7	0.61	-53.8±1.8	-52.8±1.8	0.60	-56.3±1.8	-54.4±1.9	0.34
R _{in} (MΩ)	177±17	182±16	0.80	95±9	105±10	0.40	171±16	146±14	0.19
Tau (ms)	32.9±3.8	39.3±4.3	0.14	17.3±2.0	16.7±1.9	0.75	24.9±2.9	22.2±2.7	0.37
C _m (pF)	198±15	215±16	0.23	183±14	163±12	0.15	143±11	164±13	0.09
Rheo (pA)	171±19	148±16	0.25	400±45	415±47	0.78	196±22	297±36	0.005
AP _{thresh} (mV)	-28.2±1.5	28.3±1.5	0.93	-25.7±1.6	-25.8±1.6	0.95	-28.2±1.6	-24.4±1.7	0.012
AP _{amp} (mV)	77.8±2.0	77.5±1.5	0.51	70.9±1.8	67.8±1.7	0.15	71.5±1.8	68.7±1.9	0.28
AP _{hw} (ms)	2.19±0.12	2.20±0.12	0.95	0.89±0.05	0.91±0.05	0.85	1.36±0.08	1.11±0.07	0.01
AHP (mV)	11.8±0.6	11.8±0.6	0.96	27.5±1.3	26.9±1.4	0.78	19.3±1.0	22.6±1.2	0.034
AP _{mfr} (Hz)	22.4±2.3	22.4±2.4	0.99	68.8±6.2	71.4±6.6	0.53	35.0±3.8	31.8±3.5	0.25

For an explanation of the parameters, see Methods. R_{in} = input resistance, Tau = membrane time constant, Rheo = rheobase current, C_m = membrane capacitance, AP = action potential, thresh = threshold, amp = amplitude, hw = half width, AHP = afterhyperpolarization, and mfr = maximum firing rate. Values shown are estimated marginal means ± the standard error as determined by implementing a Generalized Linear Mixed Model. P values less than 0.05 are in red, bold type. For each subgroup, N values are the number of mouse pups, and n values are the number of neurons.

Table S1.

Electrophysiological parameters of current clamp recordings from neuronal cultures.

	SST ⁺ GABAergic			VIP ⁺ GABAergic			PV ⁺ GABAergic		
	WT	YH-HET		WT	YH-HET		WT	YH-HET	
	N = 4	N = 5		N = 3	N = 4		N = 3	N = 4	
	n = 45	n = 46	p-value	n = 50	n = 48	p-value	n = 23	n = 25	p-value
R _{in} (MΩ)	133±18	95±13	0.001	243±10	249±11	0.645	67±4	66±3	0.850
Tau (ms)	16.9±1.1	13.5±0.8	0.012	35.1±2.4	33.1±2.3	0.384	6.7±0.5	6.0±0.4	0.302
C _m (pF)	135±13	152±15	0.032	148±6	134±6	0.055	103±5	90±4	0.050
Rheo (pA)	232±41	319±56	0.001	149±12	157±13	0.344	546±33	441±24	0.031
AP _{thresh} (mV)	-36.4±0.8	-35.2±0.8	0.113	-30.9±0.4	-31.0±0.4	0.850	-34.2±1.8	-35.5±1.9	0.308
AP _{amp} (mV)	79.9±1.5	80.2±1.5	0.850	75.6±0.9	75.5±0.9	0.943	77.6±1.0	82.7±1.0	0.002
AP _{hw} (ms)	0.91±0.02	0.86±0.02	0.142	1.09±0.04	1.09±0.04	0.916	0.73±0.03	0.69±0.02	0.180
AHP (mV)	23.0±0.7	23.5±0.7	0.554	17.2±0.9	17.4±0.9	0.860	26.6±0.6	25.3±0.5	0.150
AP _{mfr} (Hz)	57.6±5.1	55.7±4.9	0.432	35.5±2.8	35.9±2.8	0.857	69.4±4.9	79.0±5.3	0.068

For an explanation of the parameters, see Methods. R_{in} = input resistance, Tau = membrane time constant, Rheo = rheobase current, C_m = membrane capacitance, AP = action potential, thresh = threshold, amp = amplitude, hw = half width, AHP = afterhyperpolarization, and mfr = maximum firing rate. Values shown are estimated marginal means ± the standard error as determined by implementing a Generalized Linear Mixed Model. P values less than 0.05 are in red, bold type. For each subgroup, N values are the number of mouse pups, and n values are the number of neurons.

Table S2.

Electrophysiological parameters of current clamp recordings from neuronal cultures of three major GABAergic subtypes.

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Editors

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Reviewer #1 (Public Review):

Summary:

This manuscript reports the effects of a heterozygous mutation in the KCNT1 potassium channels on the properties of ion currents and the firing behavior of excitatory and inhibitory neurons in the cortex of mice expressing KCNT1-Y777H. In humans, this mutation as well as multiple other heterozygotic mutations produce very severe early-onset seizures and produce a major disruption of all intellectual function. In contrast, in mice, this heterozygous mutation appears to have no behavioral phenotype or any increased propensity to seizures. A relevant phenotype is, however, evident in mice with the homozygous mutation, and the authors have previously published the results of similar experiments with the homozygotes. As perhaps expected, the neuronal effects of the heterozygous mutation presented in this manuscript are generally similar but markedly smaller than the previously published findings on homozygotes. There are, however, some interesting differences, particularly on PV+ interneurons, which appear to be more excitable than wild type in the heterozygotes but more excitable in the homozygotes. This raises the interesting question (which could be more explicitly discussed by the authors) as to whether the reported changes represent homeostatic events that suppress the seizure phenotype in the mouse heterozygotes or simply changes in excitability that do not reach the threshold for behavioral outcomes.

Strengths and Weaknesses:

1. The authors find that the heterozygous mutation in PV+ interneurons increases their excitability, a result that is opposite from their previous observation in neurons with the corresponding homozygous mutation. They propose that this results from the selective upregulation of a persistent sodium current INaP in the PV+ interneurons. While the observations are very interesting, there are three issues concerning this interpretation that should be addressed:
 - A) The protocol for measuring the INaP current could potentially lead to results that could be (mis)interpreted in different ways in different cells. First, neither K currents nor Ca currents are blocked in these experiments. Instead, TTX is applied to the cells relatively rapidly (within 1 second) and the ramp protocol is applied immediately thereafter. It is stated that, at this time, Na currents and INaP are fully blocked but that any effects on Na-activated K currents are minimal. In theory, this would allow the pre- to post-difference current to represent a relatively uncontaminated INaP. This would, however, only work if activation of KNa currents following Na entry is very slow, taking many seconds. A good deal of literature has suggested that the kinetics of activation of KNa currents by Na influx vary substantially between cell types, such that single action potentials and single excitatory synaptic events rapidly evoke KNa currents in some cell types. This is, of course, much faster than the time of TTX application. Most importantly, the kinetics of KNa activation may be different in different neuronal types, which would lead to errors that could produce different estimates of INaP in PV+ interneurons vs other cell types.
 - B) As the authors recognize, INaP current provides a major source of cytoplasmic sodium ions for the activation. An expected outcome of increased INaP is, therefore, further activation of KNa currents, rather than a compensatory increase in an inward current that counteracts the increase in KNa currents, as is suggested in the discussion.
 - C) Numerical simulations, in general, provide a very useful way to evaluate the significance of experimental findings. Nevertheless, while the in-silico modeling suggests that increases in INaP can increase firing rate in models of PV+ neurons, there is as yet insufficient information on the relative locations of the INaP channels and the kinetics of sodium transfer to KNa channels to evaluate the validity of this specific model.
2. The greatest effect of TTX application would be expected to be the elimination of large transient inward sodium currents. Why are no such currents visible in the control (pre-TTX) or the difference currents (Fig. 2)? Is it possible I missed something in the methods?
3. As expected, the changes in many of the measured parameters are smaller in the present study with heterozygotes than those previously reported for the homozygous mutation. Some of the statements on the significance of some of the present findings need to be stated more clearly. For example, in the results section describing Fig. 2, it is stated that "In glutamatergic and NFS GABAergic YH-HET neurons, the overall KNa current was increased ...as measured by a significant effect of genotype" Later in the same paragraph it is stated that the increases in KNa current are not significant. Apparently, different tests lead to different conclusions. Both for the purpose of understanding the pathophysiological effects of changes in KNa current and for making further numerical simulations, more explicit clarifying statements should be made.

4. The effects of the KCNT1 channel blocker VU170 on potassium currents are somewhat larger and different from those of TTX, suggesting that additional sources of sodium may contribute to activating KCNT1, as suggested by the authors. Because VU170 is, however, a novel pharmacological agent, it may be appropriate to make more careful statements on this. While the original published description of this compound reported no effect on a variety of other channels, there are many that were not tested, including Na and cation channels that are known to activate KCNT1, raising the possibility of off-target effects.
5. The experiments were carried out at room temperature. Is it possible that different effects on firing patterns in heterozygotes and homozygotes would be observed at more physiological temperatures?

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Reviewer #2 (Public Review):

Summary:

In this manuscript, Shore et al. investigate the consequent changes in excitability and synaptic efficacy of diverse neuronal populations in an animal model of juvenile epilepsy. Using electrophysiological patch-clamp recordings from dissociated neuronal cultures, the authors find diverging changes in two major populations of inhibitory cell types, namely somatostatin (SST)- and parvalbumin (PV)-positive interneurons, in mice expressing a variant of the KCNT1 potassium channel. They further suggest that the differential effects are due to a compensatory increase in the persistent sodium current in PV interneurons in pharmacological and in silico experiments.

Strengths:

1. Heterozygous KCNT1 gain of function variant was used which more accurately models the human disorder.
2. The manuscript is clearly written, and the flow is easy to follow. The authors explicitly state the similarities and differences between the current findings and the previously published results in the homozygous KCNT1 gain of function variant.
3. This study uses a variety of approaches including patch clamp recording, in silico modeling, and pharmacology that together make the claims stronger.
4. Pharmacological experiments are fraught with off-target effects and thus it bolsters the authors' claims when multiple channel blockers (TTX and VU170) are used to reconstruct the sodium-activated potassium current. Having said that, it would be helpful to see the two drug manipulations be used in the same experiment. Notably, does the more selective blocker VU170 mimic the results of TTX for NFS GABAergic cells in Figure 2? And does it unmask a genotype difference for FS GABAergic cells like the one seen in PV interneurons in Figure 5C3.

Weaknesses:

1. This study relies on recordings in dissociated cortical neurons. Although specific WT interneurons showed intrinsic membrane properties like those reported for acute brain slices, it is unclear whether the same will be true for those cells expressing KCNT1 variants. This reviewer highly recommends confirming some of the key findings using an ex vivo slice preparation. This is especially important given the discrepant result of reduced excitability of PV cells reported by Gertler et al., 2022 (cited here in the manuscript but not discussed in this context) in acute hippocampal slices for a different KCTN1 gain of function variant.
2. It is unclear how different pieces of results fit together to form a story about the disease pathophysiology. For example, hyperexcitability of PV cells would suggest more inhibition which would counter seizure propensity. However, spontaneous inhibitory postsynaptic currents show no change in pyramidal neurons. Moreover, how do the authors reconcile that the reductions in synaptic inputs onto interneurons in Figure 3B with the increases in Figure 8? This should be discussed.
3. Similarly, the results in this work are not entirely internally consistent. For example, given the good correspondence between FS and NFS GABAergic cells with PV and SST expression, why are FS GABAergic cells hyperexcitable in Figure 1? If anything, there is a tendency to show reduced excitability like the NFS GABAergic cells. Also, why do the WT I-V curves look so different between Figures 2 and 5? This reviewer suggests at least a brief explanation in the discussion.
4. Given the authors' claim that the KCNT1 activation curve is a major contributor to the observed excitability differences in specific GABA cell subtypes, it would be helpful to directly measure the activation curve in the variants experimentally as was done for WT KCNT1 in Figure 6A and use the derived kinetics in the compartmental model.

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Reviewer #3 (Public Review):

Summary:

The present manuscript by Shore et al. entitled "Reduced GABAergic Neuron Excitability, Altered Synaptic Connectivity, and Seizures in a KCNT1 Gain-of-Function Mouse Model of Childhood Epilepsy" describes in vitro and in silico results obtained in cortical neurons from mice carrying the KCNT1-Y777H gain-of-function (GOF) variant in the KCNT1 gene encoding for a subunit of the Na⁺-activated K⁺ (KNa) channel. This variant corresponds to the human Y796H variant found in a family with Autosomal Dominant Nocturnal Frontal lobe epilepsy. The occurrence of GOF variants in potassium channel encoding genes is well known, and among potential pathophysiological mechanisms, impaired inhibition has been documented as responsible for KCNT1-related DEEs. Therefore, building on a previous study by the same group performed in homozygous KI animals, and considering that the largest majority of pathogenic KCNT1 variants in humans occur in heterozygosis, the Authors have investigated the effects of heterozygous Kcnt1-Y777H expression on KNa currents and neuronal physiology among cortical glutamatergic and the 3 main classes of GABAergic neurons, namely those expressing vasoactive intestinal polypeptide (VIP), somatostatin (SST), and parvalbumin (PV), crossing KCNT1-Y777H mice with PV⁻, SST⁻ and PV-cre mouse lines, and recording from GABAergic neurons identified by their expression of mCherry (but negative for GFP used to mark excitatory neurons).

The results obtained revealed heterogeneous effects of the variant on KNa and action potential firing rates in distinct neuronal subpopulations, ranging from no change (glutamatergic and VIP GABAergic) to decreased excitability (SST GABAergic) to increased excitability (PV GABAergic). In particular, modelling and in vitro data revealed that an

increase in persistent Na current occurring in PV neurons was sufficient to overcome the effects of KCNT1 GOF and cause an overall increase in AP generation.

Strengths:

The paper is very well written, the results clearly presented and interpreted, and the discussion focuses on the most relevant points.

The recordings performed in distinct neuronal subpopulations are a clear strength of the paper. The finding that the same variant can cause opposite effects and trigger specific homeostatic mechanisms in distinct neuronal populations is very relevant for the field, as it narrows the existing gap between experimental models and clinical evidence.

Weaknesses:

My main concern is in the epileptic phenotype of the heterozygous mice investigated. In fact, in their previous paper the Authors state that "...Kcnt1-Y777H heterozygous mice did not exhibit any detectable epileptiform activity" (first sentence on page 4). However, in the present manuscript, they indicate twice in the discussion section that these mice exhibit "infrequent seizures". This relevant difference needs to be clarified to correctly attribute to the novel pathophysiological mechanism a role in seizure occurrence. Were such infrequent seizures clearly identified on the EEG, or were behavioral seizures? Could the authors quantify this "infrequent" value? This is crucial also to place in the proper perspective the Discussion statement regarding "... the increased INaP contribution to ... network hyperexcitability and seizures".

Also, some statistical analysis seems to be missing. For example, I could not find any for the data shown in Fig. 6. Thus, the following statement: "the model PV neurons responded to KCNT1 GOF with decreased AP firing and an increased rheobase" requires proper statistical evaluation.

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