

Enhanced $K_{Na}1.1$ Channel Underlies Cortical Hyperexcitability and Seizure Susceptibility after Traumatic Brain Injury

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Abstract

Pathogenic variants of the sodium-activated potassium channel $K_{Na}1.1$, have been reported in multiple epileptic disorders. However, whether and how $K_{Na}1.1$ channel is involved in epileptogenesis after traumatic brain injury (TBI) remains unknown. Firstly, we used behavioral monitoring and EEG recording to examine physiological property, spontaneous seizure activity, and seizure susceptibility after TBI. We explored the changes of $K_{Na}1.1$ channel following TBI, including changes of subcellular distribution and expression pattern. Meanwhile, we performed patch-clamp recording to detect the neuronal excitability. Furthermore, we built TBI model using *kcnt1*^{-/-} mice and compared seizure activity with those on wild-type mice. We found severity-dependent seizure susceptibility in different degree of injured mice. Meanwhile, increased neuronal expression of $K_{Na}1.1$ channel, especially in inhibitory neurons, around the lesion was also observed following TBI with increased neuronal excitability including reduced firing rate of interneurons and imbalanced excitation and inhibition (E/I). Although the maximum frequency of action potential of *kcnt1*^{-/-} neurons was increased, *kcnt1*^{-/-} mice displayed decreased seizure susceptibility to the pentylenetetrazole (PTZ) after TBI. Taken together, this study suggests that pathologically enhanced expression and abnormally distributed $K_{Na}1.1$ channel after TBI contribute to disputed E/I and seizure susceptibility, which might provide a potential therapeutic target on the epileptogenesis after TBI.

eLife assessment

This potentially **valuable** study provides some evidence that upregulation of sodium-activated potassium channels contributes to neuronal hyperexcitability and seizures following traumatic brain injury. However, the evidence supporting a direct link is **incomplete**. This work will be of interest to epilepsy and ion channel researchers.

Introduction

Traumatic brain injury (TBI) afflicts approximately ten million people throughout the world annually, contributing to increased disability and mortality (Raymont, et al.,2010; Stein, et al.,2017). Post-traumatic epilepsy(PTE), a major complication, is a serious concern for neurotrauma patients and can result in the development of permanent epilepsy. The incidence of PTE varies from 4% to 53% (Frey,2003; Zhao, et al.,2012), mainly depending on the injury severity (Reid, et al.,2016). However, nearly one-third of patients are resistant to classical anti-seizure medications (ASMs) targeting rebalancing excitatory and inhibitory neurotransmission in the brain (Löscher, et al.,2013), suggesting that TBI may induce some hitherto unknown mechanisms leading to hypersynchronous activity and seizures.

The sodium-activated potassium channel $K_{Na}1.1$, encoded by *kcnt1* (*Slo2.2*) gene, is a voltage-gated K^+ channel that can be activated by a high cytoplasmic concentration of Na^+ . The $K_{Na}1.1$ channel is widely expressed throughout the central nervous system (CNS) (Rizzi, et al.,2016), and its functions include maintaining cellular resting membrane potential, modulating hyperpolarization following action potentials (APs), etc (Bhattacharjee and Kaczmarek,2005). Previous studies have reported patients carrying gain-of- function mutants of $K_{Na}1.1$ channel are mostly with clinically ‘drug-resistant epilepsy’, such as autosomal dominant nocturnal frontal lobe epilepsy (Heron, et al.,2012), malignant focal epilepsy with metastases (Barcia, et al.,2012; Ishii, et al.,2013), early onset epilepsy and encephalopathy (Ohba, et al.,2015), and Ohtahara syndrome (Martin, et al.,2014). In addition, Shore et al. (Shore, et al.,2020) found enhanced synaptic connectivity accompanied by network hyperexcitability and hyper-synchronicity after introducing a gain of function(GOF) variant of $K_{Na}1.1$ channel in mice, which attributed primarily to reduced excitability of ‘non-fast spiking’ GABAergic neurons’ and enhanced, homotypic connectivity in both excitatory and inhibitory neurons. Although increasing studies focused on the *kcnt1*-related genetic epilepsies, there is little study relating the $K_{Na}1.1$ channel to the acquired epilepsy. Therefore, whether $K_{Na}1.1$ channel is involved in epileptogenesis after TBI and how $K_{Na}1.1$ channel promotes the cortical hyperexcitability remains unknown.

In this study, we hypothesize that modulation of $K_{Na}1.1$ channel preferentially in inhibitory interneurons, contributes to hyperactivity of neurons in the peripheral cortex of lesions and enhanced seizure susceptibility, thereby promoting the occurrence of PTE. To further confirm the role of pathogenic upregulation of $K_{Na}1.1$ channel, we established TBI model using *kcnt1* knock-out (*kcnt1*^{-/-}) mice and found that reduced seizure susceptibility by eliminating the changes of $K_{Na}1.1$ after TBI. The results presented here are in accord with this hypothesis.

Results

Behavioral Test and Tissue Loss Evaluation in Different Degrees of Injured Mice

After TBI, we analyzed the corresponding parameters of mice from different degree of injured groups to sort out an appropriate TBI model (Fig. 1). Moderate and severe injury groups showed significant decreased hanging time at day 1 (Sham group: 553.2 ± 46.8 s, vs. Moderate group: 310.8 ± 72.8 s, $p < 0.01$; vs. Severe group: 49.9 ± 9.5 s, $p < 0.001$) and day 3 post-injury (Sham group: 588.3 ± 11.7 s, vs. Moderate injury group: 325.8 ± 64.3 s, $p < 0.01$; vs. Severe injury group: 156.2 ± 52.1 s, $p < 0.001$). Furthermore, severe injury group also showed significant decreased hanging time at day 7 post TBI (Sham group: 600 ± 0 s, vs. Severe injury group: 358.5 ± 72.2 s, $p < 0.01$). No significant difference was detected between sham and mild injury groups (Fig. 1B). We also took the

mortality within 2 weeks into account to attain a stable model. Compared with other groups, severe injury group had the highest mortality rate (4/16, 25%), whereas, mild and moderate injury groups had similar mortality rates (1/15 for Sham group, 2/15 for Mild group, 3/23 for Moderate group) (**Fig. 1C**). In the subsequent tissue evaluation, brain slices stained by cresyl violet at 7 days after TBI were compared with the sham group, moderate and severe injury groups had significant brain tissue loss, whereas the change in the mild group was not significant (Sham group: $1.1 \pm 0.5\%$, vs. Moderate group: $7.3 \pm 1.1\%$, $p = 0.03$; vs. Severe group: $19.0 \pm 1.3\%$, $p < 0.001$; Mild group: $2.1 \pm 1.52\%$, vs. Moderate group, $p = 0.04$; vs. severe group, $p < 0.001$) (**Fig. 1D, E**).

Severity-dependent Seizure Activity and Seizure Susceptibility after TBI in Mice

We analyzed the presence of spontaneous seizures of mice in groups with different degree of injury (**Fig. 2B**). While epileptiform activity was not observed in sham group, 14.3% (1/7) of mild group, 22.3% (2/9) of moderate group, and 33.3% (2/6) of severe group had at least one spontaneous seizure during consecutive 7 days of EEG recording at 3 months post-injury (**Fig. 2C**, left). Limited by the number of spontaneous seizures, there was no significant different among three groups in terms of seizure frequency and duration (**Fig. 2C**, middle and right). To compare seizure susceptibility among groups, mice were challenged with a sub-convulsant dose of PTZ (35 mg/kg) at 3 months post-TBI (**Fig. 2D**). Compared to sham, moderate and severe groups displayed shorter latency except with the mild injury group (Sham group: 600 ± 0 s, vs. Moderate group: 165.8 ± 16.9 s, $p < 0.001$; vs. Severe group: 128.0 ± 20.4 s, $p < 0.001$; vs. Mild group: 419.5 ± 84.5 s, $p = 0.61$, **Fig. 2E**). Moreover, moderate (80%) and severe (75%) groups also exhibited generalized convulsive seizures induced by PTZ injection during the 10-min observation period, while there in no generalized convulsive seizures in sham and mild groups. Specifically, the latency to generalized convulsions was significantly shorter in severe group compared to sham (Sham group: 600 ± 0 s, vs. Severe group: 322.8 ± 101.0 s, $p < 0.05$; **Fig. 2F**). In addition, the highest seizure severity scores in moderate and severe groups were significantly higher compared to sham and mild groups (Sham group: 1.0 ± 0 , vs. Moderate group: 5.0 ± 0.6 , $p < 0.001$; vs. Severe group: 5.5 ± 1.0 , $p < 0.001$; **Fig. 2G**). Furthermore, more mice in severe group (2/4, 50%) died of the prolonged tonic seizure activity after PTZ injection. In sum, these results led us to utilize the moderate injury TBI model with significant motor deficit (**Fig. 1B**), tissue loss (**Fig. 1E**), lower mortality rate (**Fig. 1C**), and higher seizure susceptibility (**Fig. 2**) for the rest of our studies.

Increased Expression of $K_{Na}1.1$ Channel after TBI

$K_{Na}1.1$ channel has been reported to be a potential therapeutic target in *KCNT1*-related epilepsies. However, whether $K_{Na}1.1$ channel participates in the epileptogenesis of TBI remains unknown. In this study, we collected perilesional neocortex at 1h, 6h, 12h, 1d, 3d, 7d, and 14d post-TBI and investigated spatial-temporal expression of $K_{Na}1.1$ channel (**Fig. 3A**). $K_{Na}1.1$ channel was robustly elevated in the injured ipsilateral cortex, peaking at 24 h post-TBI and maintained the high level 14 days later (Sham: 1, vs. Dpi-1h: 2.71 ± 0.31 , $p < 0.01$; vs. Dpi-6h: 3.88 ± 0.87 , $p < 0.01$; vs. Dpi-12h: 4.65 ± 1.54 , $p < 0.01$; vs. Dpi-24h: 9.39 ± 2.69 , $p < 0.01$; vs. Dpi-3d: 8.25 ± 2.47 , $p < 0.01$; vs. Dpi-7d: 7.83 ± 1.78 , $p < 0.001$; vs. Dpi-14d: 4.24 ± 1.36 , $p < 0.05$, **Fig. 3A**). By co-labeling $K_{Na}1.1$ channel and NeuN, it was clear that compared with the sham group, expression of $K_{Na}1.1$ channel around the lesion on mice of dpi-14d was significantly increased and more neurons co-labelled with $K_{Na}1.1$ in TBI group, as shown by increased fluorescence intensity (**Fig. 3B**).

In addition to TBI mice, we also collected the lesion areas from patients with PTE and temporal neocortices from patients with mesial temporal lobe epilepsy (MTLE) as control group. The protein level of $K_{Na}1.1$ channel was elevated in the lesion of the patient with PTE compared with that in

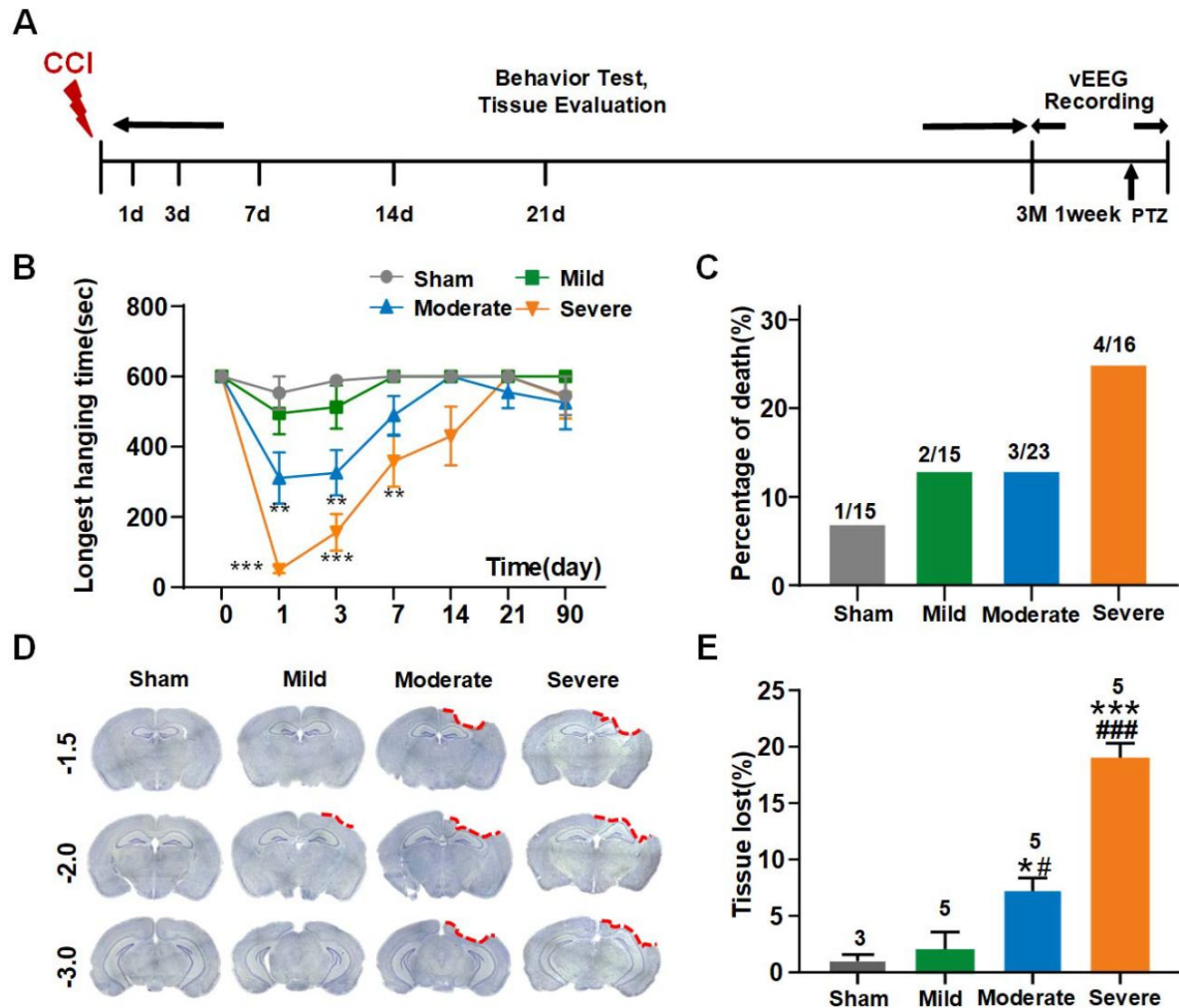


Fig. 1.

Evaluation of the degree of injury in CCI models. **A** Experimental procedure. Mice were subjected to different degrees injury with CCI models. Behavior test and tissue evaluation were performed at 1, 3, 7, 14, 21 days and 3 months post- traumatic brain injury (TBI). Skull electrodes were implanted at 11 weeks after brain damage. Long-term video-electroencephalogram (v-EEG) was monitored for one week, then followed by pentylenetetrazol (PTZ) injection. **B** Summary of behavior test in CCI models compared to sham groups at different time points. **C** Bar graph showing the percent mortality in CCI models compared to sham groups within 2 weeks. **D** Representative cresyl violet stained brain slices from three cortical regions in CCI models. **E** Summary of tissue loss in CCI models. (* $p < 0.05$, *** $p < 0.001$ vs. sham group; # $p < 0.05$, ### $p < 0.001$ vs. mild group, Error bars indicate standard error of the mean).

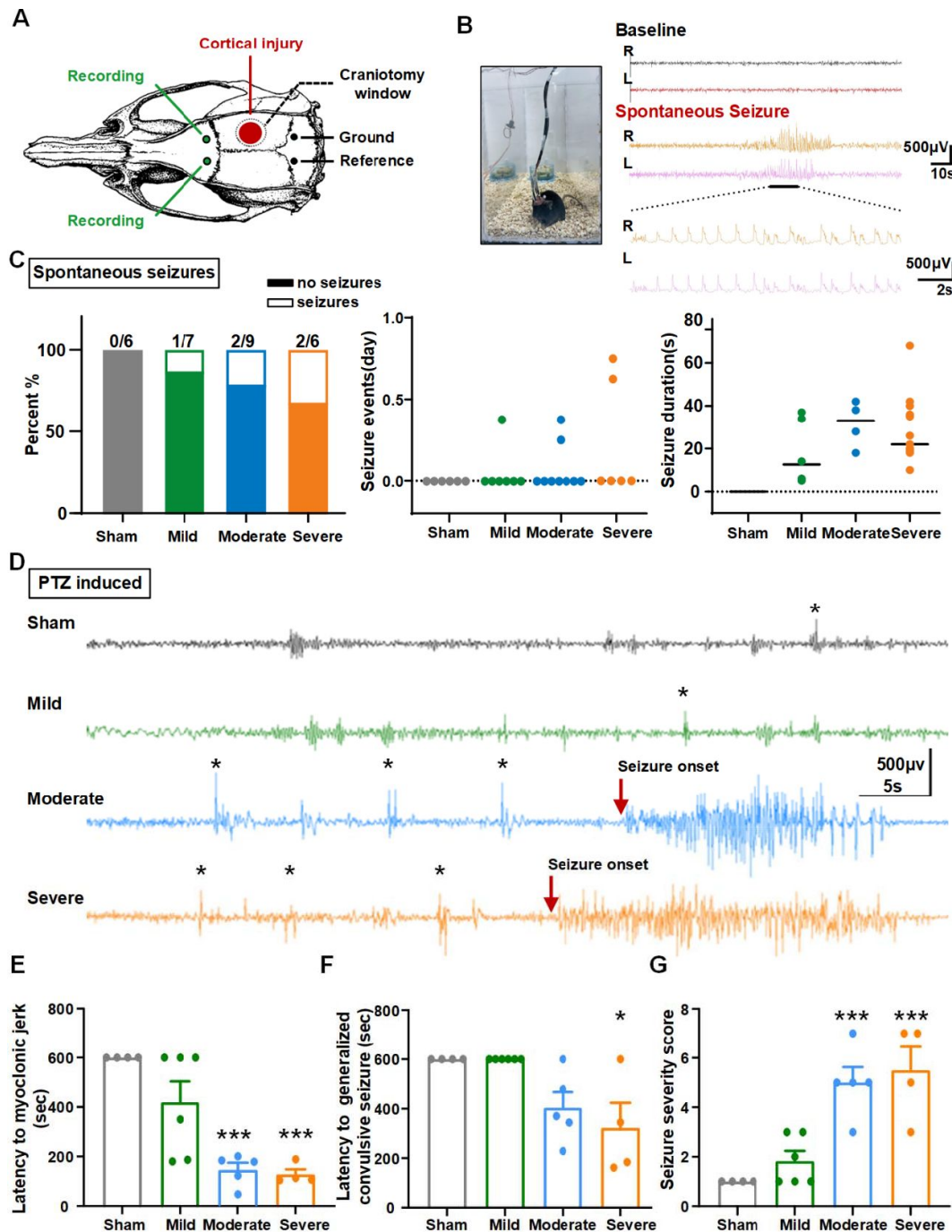


Fig. 2.

Characteristics of spontaneous seizures and pentylenetetrazol-induced seizure susceptibility in CCI models. **A** Locations of craniotomy window (black dotted circle), cortical injury (red circle), and placement of skull electrodes. **B** Long-term v-EEG monitor for 7 days on the TBI models. The epileptiform discharges of spontaneous seizure were showed in the right. **C** Evaluation of spontaneous seizures in the TBI models with different degree injury. It showed the percentage of spontaneous seizures (left), number of seizures per day (middle), and duration of seizures in TBI models. **D** Representative traces of ictal epileptiform discharges after pentylenetetrazol (PTZ) injection on the TBI models. *indicated interictal discharges. The red arrow indicated the onset of epileptiform discharges of generalized convulsive seizures. $N = 4-6$ for each group. **E** Quantification of latencies to myoclonic jerk. $N = 4-6$ for each group. **F** Quantification of latencies to generalized convulsive seizures. $N = 4-6$ for each group. **G** Quantification of seizure severity scores. $N = 4-6$ for each group. (* $p < 0.05$, *** $p < 0.001$. Error bars indicate standard error of the mean).

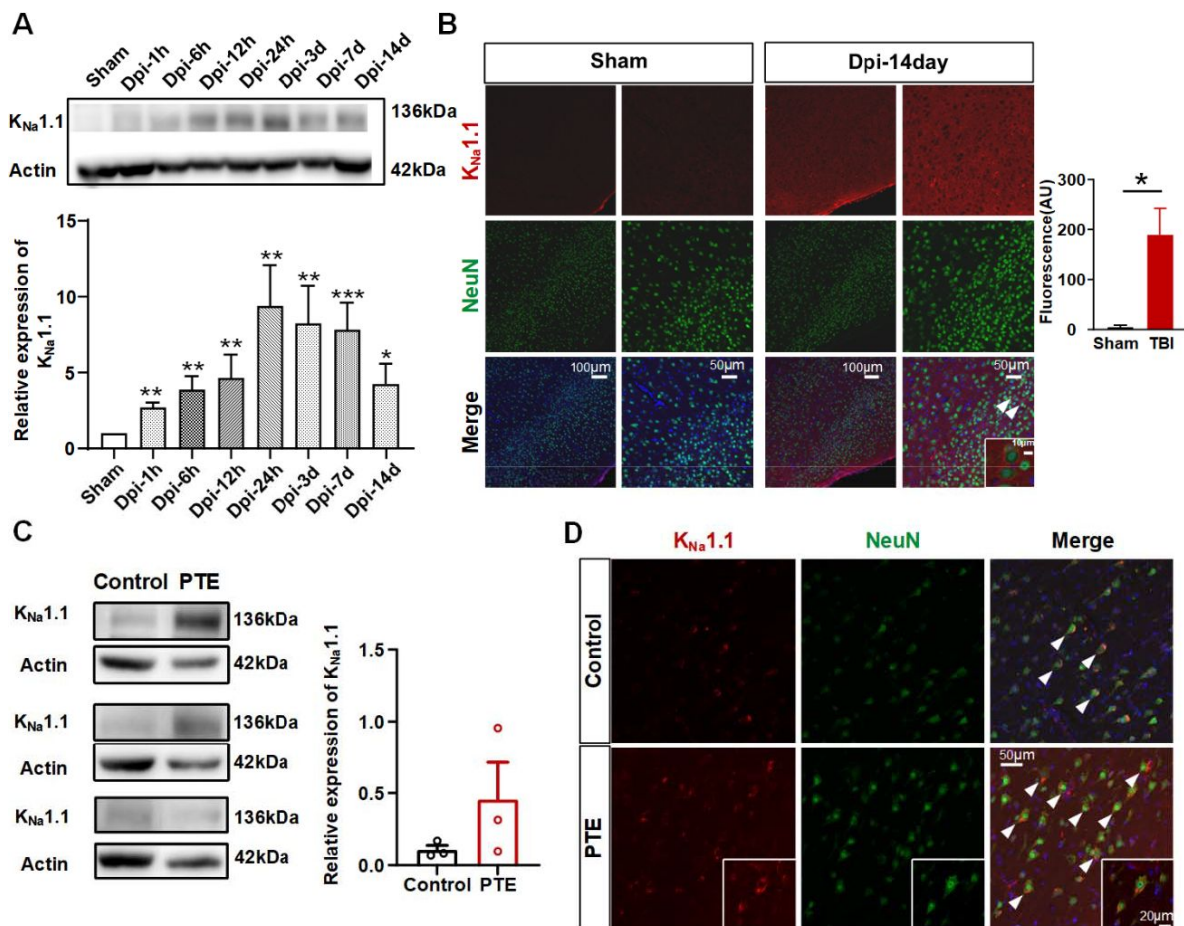


Fig. 3.

Increased $K_{Na}1.1$ channel expression after TBI. **A** The expression of $K_{Na}1.1$ protein at the different time points after TBI. $N = 5-7$ for each group. **B** On the day 14 after TBI, immunofluorescence staining showed enhanced fluorescence intensity and increased number of co-localized $K_{Na}1.1$ channel on neurons around lesions (white arrowhead). $N=3$ for each group. **C** The expression of $K_{Na}1.1$ channel on the lesion area on the neocortices from patients with PTE and patients with mesial temporal lobe epilepsy. $N=3$ for each group. **D** Immunofluorescence showed increased and more co-localized $K_{Na}1.1$ channel on neurons in the patient with PTE compared with the patient with mesial temporal lobe epilepsy. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars indicate standard error of the mean).

the patient with MTLE but without no significant difference (**Fig. 3C**). By co-labeling $K_{Na}1.1$ channel and NeuN, it was clear that compared with the patient with MTLE, the expression of $K_{Na}1.1$ channel was increased in the neurons of the patient with PTE (**Fig. 3D**).

Decreased Expression of GAD67 and Increased $K_{Na}1.1$

Preferentially in Inhibitory Interneurons on Dpi-14d Mice

Given that the protein expression of the GAD67 isoform is closely related to overall GABA levels (*Rimvall, et al., 1993*) and high expression activity of glutamate decarboxylase (GAD) is specific for mature GABAergic interneurons (*Le Magueresse and Monyer, 2013*), here we selected the GAD67 as the marker of GABAergic inhibition after TBI. Moreover, glutamatergic neurons have been identified by examining the expression of vesicular glutamate transporters (VGLUTs), especially VGLUT1 which is a subtype of VGLUTs and highly expressed in cortex (*Hussan, et al., 2022*). Consistent with imbalance of E/I in the patch recording, western blot showed that lower GAD67 level around the lesion on the Dpi-14d mice when compared with sham group (Sham: 1 vs. Dpi-14d: 0.77 ± 0.08 , $p < 0.05$, **Fig. 4A**). However, there was no significant difference in VGLUT1 level between Dpi-14d and sham groups (**Fig. 4A**). By co-labeling $K_{Na}1.1$ channel and VGLUT1, it was no significant difference in the number of co-labelled neurons between sham and Dpi-14d mice (**Fig. 4B**). In contrast, it is clear that compared with the sham group, increased number of co-localized GABAergic neurons with $K_{Na}1.1$ channel around the lesion on mice of dpi-14d (**Fig. 4C**).

Imbalance of Glutamatergic and GABAergic Neurons in Perilesional Cortical in Moderate TBI Mice

Nichols *et al.* validated that 14 days post-TBI is the earliest point in time to show epileptiform activity in vivo and in vitro, which may help define a critical time window or novel target for PTE prevention interventions (*Nichols, et al., 2015*). For the Dpi-14d mice, we assessed neuronal subtype-specific AP characteristics and synaptic activity by performing whole-cell patch-clamp recordings of neurons in the perilesional neocortex of post-injury moderate TBI mice (**Fig. 5A**). Compared with the sham group, maximum AP frequency (Sham: 45.3 ± 3.8 Hz vs. TBI: 32.2 ± 3.4 Hz, $p < 0.05$, **Fig. 5C**, **3D**) and afterhyperpolarization (Sham: 6.4 ± 1.2 mV vs. TBI: 10.8 ± 1.4 mV, $p < 0.05$, **Fig. 5C**, **3D**) of interneurons (INs) showed significant differences. However, there was no difference on these electrophysiological parameters of pyramidal neurons (PNs) (**Fig. 5B**, **3D**). We calculated the resting membrane between Sham and TBI groups and found there was no significant difference in both PNs and INs (data not shown). Next, we evaluated spontaneous excitatory and inhibitory post-synaptic currents (sEPSC and sIPSC) in TBI and sham groups. The sEPSC frequency of PNs in the TBI group was significantly higher than that of the sham group (Sham: 2.14 ± 0.31 Hz vs. TBI: 4.57 ± 0.86 Hz, $p < 0.05$, **Fig. 5E**, **3F**, left), while the sIPSC was markedly reduced (Sham: 1.74 ± 0.29 Hz vs TBI: 0.54 ± 0.12 Hz, $p < 0.001$, **Fig. 5E**, **3F**, middle), and excitation and inhibition (E/I) ratio was significantly increased (Sham: 0.38 ± 0.07 , vs TBI: 0.74 ± 0.07 , $p < 0.001$, **Fig. 5F**, right). This data indicates that the excitatory driving force of PNs in the cortex increased, while the inhibitory driving force decreased, hence the overall excitability was enhanced in the perilesional neocortex after TBI. The synaptic activity of INs showed no difference between sham and TBI groups (data not shown).

Improved Behavior and Decreased Seizure

Susceptibility on $kcnt1^{-/-}$ Mice after TBI

To figure out the functional role of $K_{Na}1.1$ channel in TBI, we created a loss of $kcnt1$ mouse line whose mutation site lies adjacent to the pore-formation domain of $K_{Na}1.1$ channel (**Fig. 6A**). We confirmed cohorts of wild-type and homozygous ($kcnt1^{-/-}$) littermates using tail PCR (**Fig. 6B**).

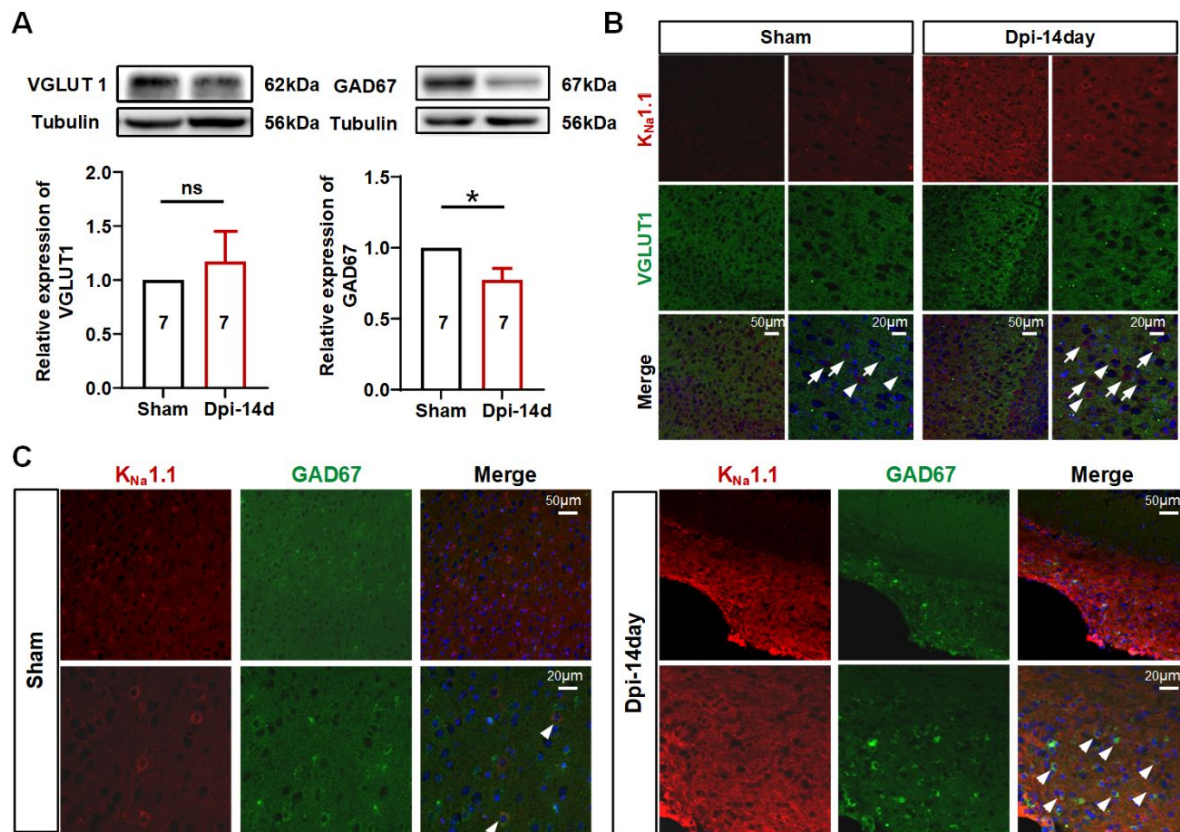


Fig. 4.

Increased $K_{Na}1.1$ preferentially in GABAergic inhibitory interneurons on Dpi-14d mice. **A** The expression of VGLUT1 and GAD67 protein after TBI. **B** At Dpi-14d, immunofluorescence staining showed the distribution of $K_{Na}1.1$ channel and VGLUT1 on neurons. The white arrowheads indicated the neurons with co-localized $K_{Na}1.1$ channel and VGLUT1. The white arrows indicated the neurons labelled with VGLUT1 but without $K_{Na}1.1$ channel. **C** At Dpi-14d, immunofluorescence staining showed the distribution of $K_{Na}1.1$ channel and GAD67 on neurons. The white arrowheads indicated the neurons with co-localized $K_{Na}1.1$ channel and GAD67. (*p < 0.05. Error bars indicate standard error of the mean).

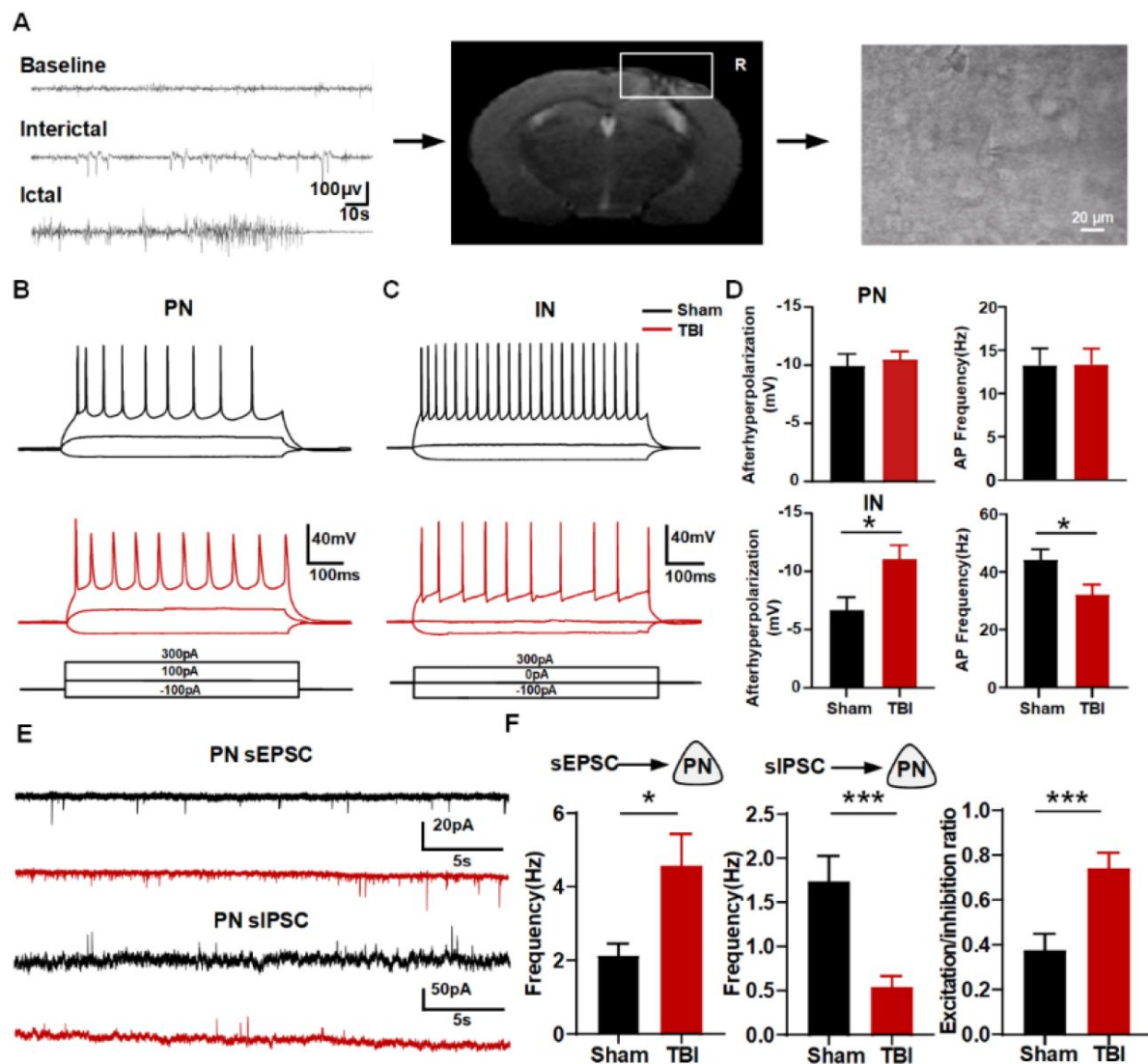


Fig. 5.

Altered electrophysiological characteristics of perilesional TBI neurons. **A** Macroscopic EEG recording showed interictal and ictal discharges in TBI mice. Neurons in layer II/III of perilesional cortex were selected for patch clamp recording. **B** Representative traces and action potential (AP) firing of PNs from sham and TBI groups with -100, 100, +300 pA current injection. **C** Representative traces and AP firing of INs from sham and TBI groups with -100, 0, +300 pA current injection. **D** Summary of afterhyperpolarization and AP frequency in PNs and INs. PNs: N = 18, 21 for sham and TBI groups. INs: N = 6 for sham and TBI groups. **E** Representative traces of spontaneous excitatory postsynaptic currents (sEPSC) and spontaneous inhibitory postsynaptic currents (sIPSC) of PNs (Black, sham group; red, TBI group). N = 18, 21 for sham and TBI groups. **F** Quantitative summary of sEPSC and sIPSC frequency and excitation/inhibition ratio of PNs. N = 18, 21 for sham and TBI groups. (* $p < 0.05$, *** $p < 0.001$. Error bars indicate standard error of the mean).

Genetic transmission of both alleles was normal, and no selective lethality was seen in either mutant genotype at any age. Western blot analysis of protein fractions isolated from the cortices of WT and *kcnt1*^{-/-} mice showed that K_{Na}1.1 channel was detected only in the WT mice, while K_{Na}1.1 expression was significantly higher than WT group at 7 days after TBI (**Fig. 6C**). Extracted brains from 10 to 12-week-old male *kcnt1*^{-/-} mice appeared similar in morphology and weight to their WT littermates (**Fig. 6D**), suggesting that loss of the *kcnt1* does not grossly alter brain development.

In addition, motor skill was tested by measuring the falling time and speed which animals were able to maintain balance on a rotarod with repeated trials. There was no significant difference in the running time or speed between WT and *kcnt1*^{-/-} mice (**Fig. 6E**). Furthermore, we compared the intrinsic membrane properties of cultured primary cortical neurons between *kcnt1*^{-/-} and WT mice (**Fig. 6F-H**). Although there was no significant difference in terms of AP threshold, rheobase, and resting membrane potential between groups, the maximum frequency of AP on *kcnt1*^{-/-} neurons was increased when compared with WT neurons (WT: 6.0 ± 1.5, vs. *kcnt1*^{-/-}: 12.1 ± 2.9, *p* < 0.05).

Decreased seizure susceptibility on *kcnt1*^{-/-} mice after TBI

We named these groups as Sham (WT mice), TBI (WT mice undergoing TBI), and *kcnt1*^{-/-}-TBI (*kcnt1*^{-/-} mice undergoing TBI). We evaluated the motor skill learning among different groups using rotarod test. We observed that *kcnt1*^{-/-}-TBI group improved their performance over the trials in the first day, and this improvement was maintained during the following days. However, the motor skill learning of sham group did not show significant difference during the time window. As for TBI group, it was markedly impaired in the motor skill at dpi-1 and dpi-3. Interestingly, *kcnt1*^{-/-}-TBI group was improved in procedural learning than TBI group, even sham group at dpi-14 (**Fig. 7B**), indicating the significance of eliminating the pathological changes of K_{Na}1.1 in epileptogenesis after TBI.

We next tested the effects of deletion of the rodent *kcnt1* gene on seizure susceptibility by administration of 35 mg/kg PTZ at 14 days after TBI. In all animals, seizures were observed within 15 minutes of PTZ administration. There was no significant difference in seizure susceptibility between *kcnt1*^{-/-}-sham and WT-sham groups. However, latency to convulsive seizures (including myoclonic jerk and generalized clonus) following PTZ showed longer latencies in *kcnt1*^{-/-}-TBI group compared with TBI group (*p* = 0.04, log rank test, **Fig. 7C**, left). Moreover, there was a significant difference in latency to generalized convulsive seizure, which were 174.7 ± 32.6 s and 269.8 ± 20.4 s for TBI and *kcnt1*^{-/-}-TBI group respectively (**Fig. 7C**, middle). However, compared with *kcnt1*^{-/-}-TBI group, we observed longer seizure duration and more severe seizures in TBI group as evidenced by significant differences in the distribution of Racine scores (Sham vs TBI, Kruskal-Wallis test, *p* < 0.0001; *kcnt1*^{-/-}-TBI vs TBI, Kruskal-Wallis test, *p* < 0.0001, **Fig. 7C**, right).

Discussion

In this study, we firstly evaluated the properties of TBI mice with different degrees of injury and found its severity-dependent behavior deficit, tissue damage and seizure activities. Next, we selected the moderate TBI mice as study subjects and found enhanced seizure susceptibility, increased neuronal excitability including reduced firing rate of interneurons and imbalanced excitation and inhibition (E/I) in the moderate TBI model combining EEG recordings with patch clamp recordings. Increased expression of K_{Na}1.1 channel in TBI mice, preferentially in inhibitory interneurons, may underly in the disputed E/I and increased seizure susceptibility. Meanwhile, a rise in K_{Na}1.1 channel was also found in patients with PTE compared with controls from patients with MTLE. To determine the functional role of K_{Na}1.1 channel after TBI, *kcnt1*^{-/-} mice undergoing TBI displayed improved motor learning skill and decreased seizure susceptibility to the PTZ.

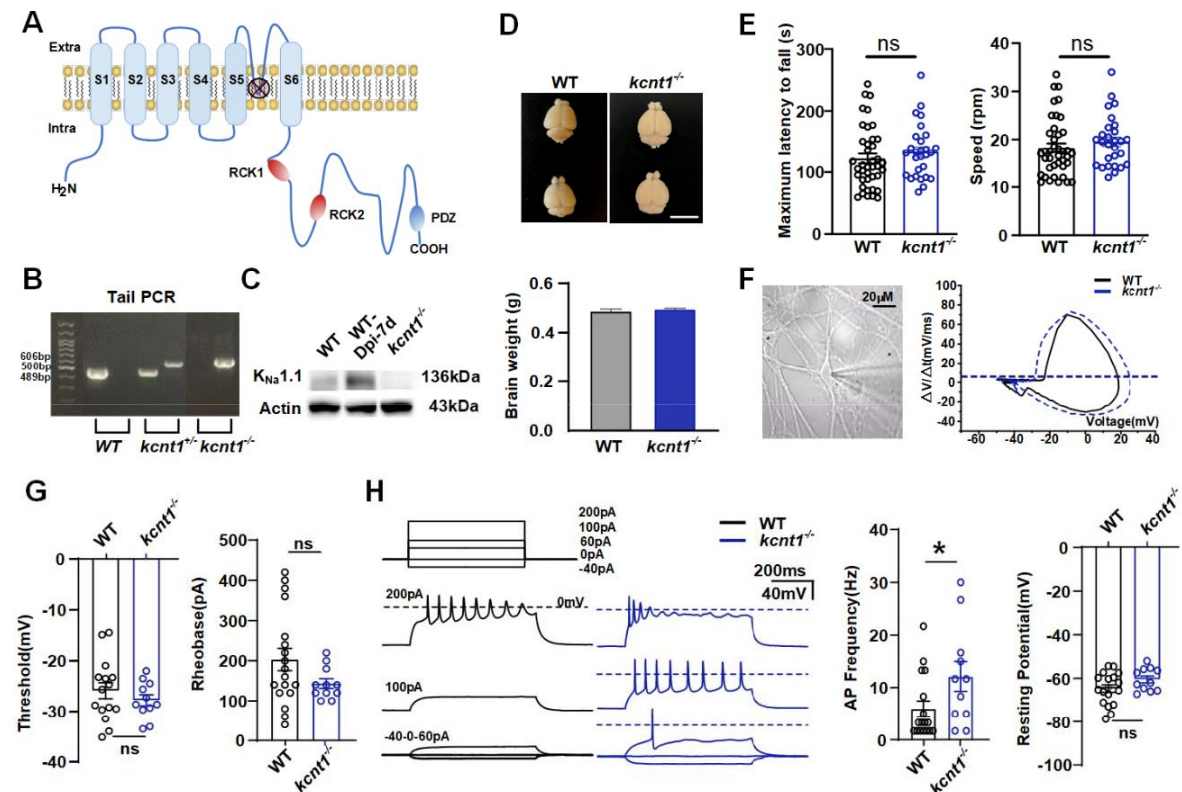


Fig. 6.

Evaluation of homozygous *kcmt1*^{-/-} mice and age-matched wild type controls. **A** Diagram of K_{Na}1.1 protein in the *kcmt1*^{-/-} mice showed corresponding mistranslated site in its pore-formation domain. **B** PCR verification of *kcmt1* exon 3-11 deletion. The predicted amplicons for WT and the exon 3-11 deleted mutant were 489 bp and 606 bp, respectively. The corresponding sample from the WT, *kcmt1*^{+/-}, and *kcmt1*^{-/-}. **C** Western blots showed K_{Na}1.1 expression from the WT, WT-TBI (for a positive control), and *kcmt1*^{-/-} mice. **D** Representative image showed similar gross morphology of brains dissected from 10 to 12-week-old male WT, *kcmt1*^{-/-} mice. N = 7 in WT and *kcmt1*^{-/-} groups, respectively. Scar bar represented the length of 1cm. **E** Evaluation of motor task in *kcmt1*^{-/-} and WT mice (WT, black; *kcmt1*^{-/-}, blue). N = 40, 28 in WT and *kcmt1*^{-/-} groups, respectively. **F** Representative diagram showed cultured primary cortical neuron from *kcmt1*^{-/-} mice. dV/dt is plotted as function of membrane voltage for the action potentials (APs) (WT, black; *kcmt1*^{-/-}, blue). Horizontal dotted lines correspond to the dV/dt value that is 10% of peak dV/dt for a given cell. **G** Comparisons of AP threshold and rheobase of cultured primary cortical neuron from WT and *kcmt1*^{-/-} mice. N = 17, 11 in WT and *kcmt1*^{-/-} groups, respectively. **H** -40, 0, 60, 100, and 200 pA current injections (0.6 s) were used to elicit firing in WT and *kcmt1*^{-/-} (blue) neurons from a holding potential of -70 mV. Comparisons of AP frequency and resting membrane potential of cultured primary cortical neuron from *kcmt1*^{-/-} and WT mice. N = 17, 11 in WT and *kcmt1*^{-/-} groups, respectively. (*p < 0.05. Error bars indicate standard error of the mean).

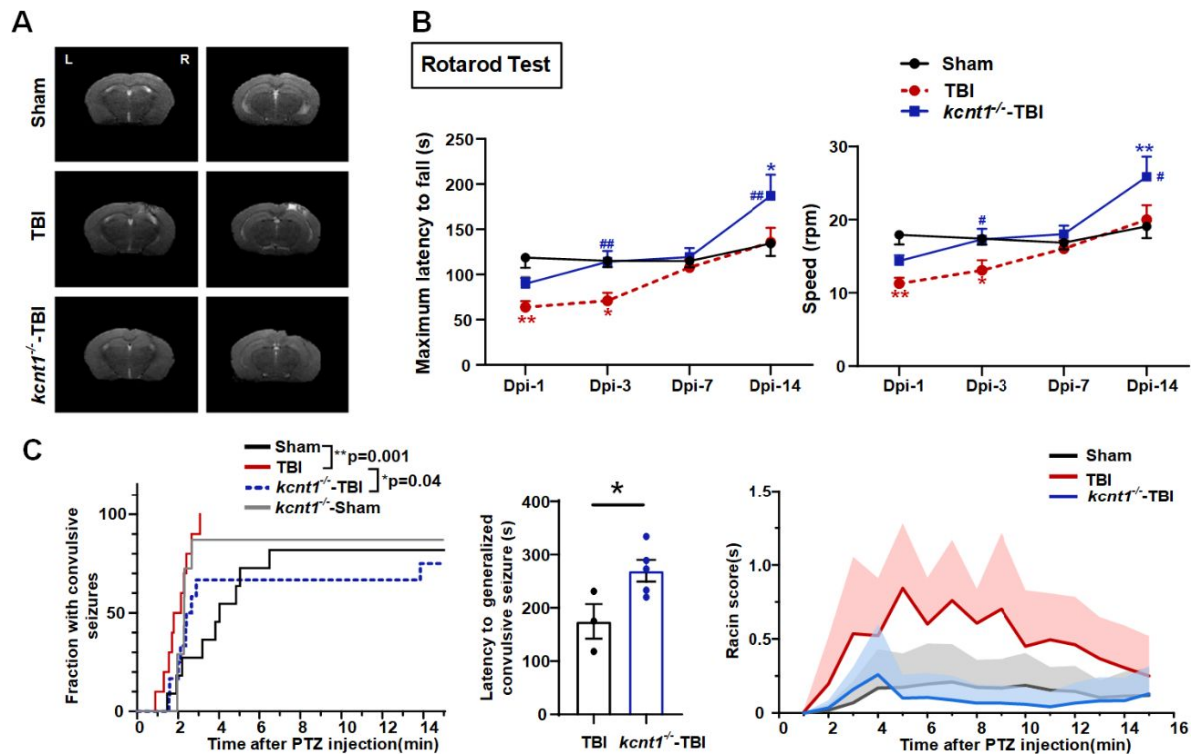


Fig. 7.

Decreased seizure susceptibility in homozygous *kcmt1*^{-/-} mice after TBI. **A** Representative MRI image showed both TBI and *kcmt1*^{-/-}-TBI mice appeared lesions in the cortex after TBI. **B** The motor skill learning of WT mice and *kcmt1*^{-/-} mice after TBI were improved (accelerating rotarod) over successive trials. N = 11, 17, 16 for Sham, TBI, and *kcmt1*^{-/-}-TBI groups, respectively. **C** The latency to seizure, including generalized and partial seizure, and distribution of Racine scores after injection of PTZ. N = 11, 9, 7, 12 for Sham, TBI, *kcmt1*^{-/-}-sham, and *kcmt1*^{-/-}-TBI groups, respectively. (*p < 0.05, **p < 0.01, vs. sham group; #p < 0.05, ##p < 0.01 vs. TBI group, Error bars indicate standard error of the mean).

Although there has been a long-held view that GOF of $K_{Na}1.1$ channel in the brain plays a pivotal role in the *kcnt1*-related epilepsy, we demonstrate for the first time that enhanced $K_{Na}1.1$ channel after TBI with changed distribution on different subtypes of neurons, which contributes to loss of cortical inhibition and might promote the epileptogenesis on acquired epilepsy model following TBI. In doing so, this research was able to reveal a critical target for prevention and treatment during the critical subacute post-traumatic period.

Severity-dependent deficits prompt moderate CCI model a suitable study subject

Well-designed preclinical PTE models can help to evaluate mechanisms involved in PTE which cannot be determined in human patients. The animal model of TBI mentioned here mimics focal brain trauma, which is prone to the development of PTE compared with other models ([Hunt, et al., 2009](#) [↗](#)). Similar with human studies, one critical risk factor of PTE on the CCI model is the injury severity ([Curia, et al., 2011](#) [↗](#)). In this study, we utilized sham and three severity levels of CCI mice and found that neurological function deficits, cortical tissue loss and increased susceptibility to PTZ were severity-dependent after TBI. Indeed, the more severe the injury, the more pronounced features observed. However, to mimic the clinical characteristics of TBI patients as closely as possible and acquire a lower mortality, the moderate TBI model was recommended as a more appropriate model for additional research.

One study found that the incidence of spontaneous seizures was only 9% in mice followed up for 9 months after TBI, with fairly infrequent seizures (0.2-0.3 seizures/day) ([Bolkvadze and Pitkänen, 2012](#) [↗](#)). Inconsistent with the previous study, spontaneous seizures in our study are only about 0.3 seizures/day. Capturing these sporadic seizures is challenging in terms of animal numbers and long-term recording, which hinders high-throughput studies. Although the clinical relevance of enhanced susceptibility to PTZ remains controversial, evidence suggests that PTZ-evoked hyperexcitability is associated with the potential to develop to spontaneous seizures ([Blanco, et al., 2009](#) [↗](#); [Rattka, et al., 2011](#) [↗](#)). Here, we employed PTZ as a measure to evaluate the seizure susceptibility by injecting a sub-convulsive dose into mice after injury. Subsequent EEG monitoring revealed that the response of mice with moderate or severe injuries to proconvulsant PTZ was significantly faster and stronger than that observed in sham and mild injury groups, indicating that the increase in seizure susceptibility was proportional to the degree of damage, consistent with clinical patients ([Annegers, et al., 1998](#) [↗](#)).

Imbalance of E/I in perilesional neocortex might contribute to epileptogenesis

Nichols et al. ([Nichols, et al., 2015](#) [↗](#)) proposed that these changes begin early after trauma and result in seizure susceptibility and subclinical electrographic abnormality before PTE occurs. They validated that day 14 post-TBI is the earliest time point to show epileptiform activity *in vivo* and *in vitro*. Although partial seizure is the dominant seizure type after brain trauma, few studies have definitively determined which cortical regions are selectively predisposed to epileptogenic vulnerability. Human postsurgical pathology revealed iron deposition, nonspecific gliosis and fibrotic meningo-cortical scarring occurred adjacent to the injured cortex, which has been identified as a potential epileptic zone ([Clifton, et al., 1981](#) [↗](#); [Marks, et al., 1995](#) [↗](#)). However, there are few corresponding functional studies that evaluated the consistency between epileptogenic area and perilesional neocortex. In fact, the excitability of neural networks and hyperactivity have changed significantly before post-traumatic epileptogenic foci can be identified.

However, most studies mainly focus on the macroscopic epilepsy expression with neglect on microscopic detection in the epileptogenic mechanisms. Thus, Farrell et al. ([Farrell, et al., 2019](#) [↗](#)) highlighted that micro-macro disconnection should be noticed in uncovering the detailed epileptogenic mechanisms. Here, inspired by enhanced neuronal activities after TBI using EEG

recording, we performed patch clamp recording on the perilesional neocortex as well to resolve this disconnection. Our results indicated that the intrinsic membrane properties of PNs remain unchanged after CCI. Contrary to PNs, AP characteristics were altered in inhibitory INs, possibly reflecting unique TBI-induced changes to inhibitory INs. At the synaptic level, the frequency of sEPSC and sIPSC in PNs were markedly altered following CCI, and E/I ratio was significantly increased, indicating that the overall excitability was enhanced in the perilesional neocortex after TBI. Overall, our results suggested that specific cortical neuronal microcircuits, such as increased glutamatergic signaling and reduced GABAergic inhibition, may initiate and facilitate the spread of epileptiform activity in the perilesional neocortex after TBI. Consistent with Cantu et al. (Cantu, et al., 2015 [DOI](#)), we speculate that hyperexcited network at macroscale is due to imbalance between E/I and the loss of GABAergic control at microscale.

The role of enhanced $K_{Na}1.1$ channel in epileptogenesis of TBI

Since *kcnt1* was confirmed as an epilepsy-associated gene in 2012, GOF mutations have been reported to be closely related to “refractory epilepsy” in infants. Increasing studies expand current understanding of the epileptogenesis mechanisms of *kcnt1*-associated epilepsy from remodeling heterologous channel with pathogenic variants to initial characterization of mouse models. However, our study firstly reported that enhanced $K_{Na}1.1$ channel also participated in epileptogenesis in acquired epilepsy models. Herein, we provided the evidence that expression of $K_{Na}1.1$ channels was elevated in neurons from the perilesional cortex. Our results showed that increased number of co-localized GABAergic neurons with $K_{Na}1.1$ channel around the lesion on the dpi-14d mice, which indicated enhanced $K_{Na}1.1$ channels preferentially in inhibitory interneurons, contributed to imbalanced E/I. However, the role of enhanced $K_{Na}1.1$ channel in epileptogenesis of TBI still remains unclear. Consistent with previous studies about the detailed mechanisms of *kcnt1*-related epilepsy, one plausible explanation of enhanced $K_{Na}1.1$ channels after TBI is that the variants in *kcnt1* may result in an imbalance of its expression in excitatory and inhibitory neurons (Tang, et al., 2016 [DOI](#)). In other words, when $K_{Na}1.1$ channel is over-expressed in inhibitory neurons, it will increase overall neuronal excitability by reducing the excitability of inhibitory neurons (Bhattacharjee, et al., 2002 [DOI](#); Joshi, et al., 2012 [DOI](#)). In addition, changing synaptic connectivity caused by GOF in $K_{Na}1.1$ channels possibly participates in the epileptogenic process. Shore and colleagues introduced a GOF mutation of K_{Na} channel (KCNT1-Y777H) into mice and, interestingly, they observed a cell-type-specific difference on K_{Na} currents. They further provided evidence of synaptic rewiring, including homotypic synaptic connectivity (Shore, et al., 2020 [DOI](#)). Another reasonable explanation for these conflicting findings is that there is an abnormal distribution of $K_{Na}1.1$ channel at the subcellular level. Our previous study found that activation of $K_{Na}1.1$ channel has a braking effect on the initiation of Aps (Martinez-Espinosa, et al., 2015 [DOI](#)). When $K_{Na}1.1$ channel was overexpressed in non-initiating regions of actions potentials, such as the peripheral axon, then the braking effect was reduced and neuronal excitability was increased. However, these hypotheses all need further investigation.

To confirm the role of $K_{Na}1.1$ channels in TBI, we produced *kcnt1*^{-/-} mice by ruining channel pore domain and compared behavior tests and neuronal properties between WT and *kcnt1*^{-/-} mice. Although WT and *kcnt1*^{-/-} mice displayed similar motor activity in rotarod test, neurons from *kcnt1*^{-/-} mice were found with increased AP firing rate. As for seizure susceptibility by administration of PTZ, there was no significant difference in latency to seizures between sham and *kcnt1*^{-/-}-sham mice. However, *kcnt1*^{-/-}-TBI group was improved in procedural learning and reduced in the seizure susceptibility than TBI group at dpi- 14. In our study, consistent with previous studies, we found that the maximum frequency of AP of *kcnt1*^{-/-} neurons was increased when compared with WT neurons, indicating that $K_{Na}1.1$ might be a protective modulator in a physiological manner. However, pathological redistribution of K_{Na} channels may contribute to imbalanced E/I and promote the epileptogenesis. In contrast to genetic *KCNT1*-related epilepsy, our study emphasizes that the re-distribution of $K_{Na}1.1$ channel following TBI breaks the balance of E/I and underlies the epileptogenesis, which widen the current understanding of acquired epilepsy.

Outlooks and limitations

This study had several inevitable limitations. Firstly, in terms of the exact role of upregulation of $K_{Na}1.1$ channel after TBI, we admitted that we could not solve it undisputedly in this study. When seizure occurs, hypersynchronous electrical activity calls for a sudden influx in Na^+ and activates $K_{Na}1.1$ channel which is sensitive to Na^+ . Byers et al. reported that $K_{Na}1.1$ channel, activated by neuronal overexcitation, contribute to a protective threshold to suppress the induction of seizure-like activity not only in established genetic but also pharmacologically primed seizure models (Byers, et al., 2021 [↗](#)). Although we found the distribution difference in $K_{Na}1.1$ channel between PNs and INs after TBI, we could not exclude other factors which might regulate the function of $K_{Na}1.1$ channel, such as factors linked with K_{Na} channels by modulate intracellular Na^+ or interact with K_{Na} channels via effecting its Na^+ sensitivity (Aoki, et al., 2018 [↗](#); Tamsett, et al., 2009 [↗](#)). Secondly, there is the absence of reversing verification with established *kcnt1* over-expression animals undergoing TBI. Given that neurons are very sensitive to expressed levels of $K_{Na}1.1$ channel, it is hard to titrate the right amount of $K_{Na}1.1$ channel expression. Thirdly, the size of our patient cohort was relatively small. In the future, a larger cohort of patients should be recruited to confirm the change and role of $K_{Na}1.1$ in patients with PTE, which might provide a novel direction for treatment.

Materials and Methods Animals

A null mutant-*kcnt1*^{-/-} mice were obtained that were initially generated on a C57BL/6J background by deleting the exon 3-11 of *kcnt1* gene sequence (NCBI: 227632) and homologous recombining in murine embryonic stem cells. *Kcnt1*^{-/-} and white-type C57BL/6 background strain (Vital River Laboratory Animal Technology Co., Ltd, Beijing, China) were subsequently backcrossed at least 2 generations prior to testing. Adult male WT and *kcnt1*^{-/-} male mice (8-10 weeks old) were housed in an animal facility with suitable temperature and humidity, a 12h light-dark cycle, and free access to food and water. All procedures were approved by the Animal Care and Use Committee of Capital Medical University in accordance with the Guide for the Care and Use of Laboratory Animals.

Human Tissue

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Tiantan Hospital, Capital Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

TBI Models

For TBI models, mice were anesthetized with 3% isoflurane in 67% N₂/30% O₂ mixture, placed in a stereotaxic frame, and maintained with 1.5% isoflurane. Brain injury was delivered using controlled cortical impact (CCI) equipment (Leica 9969S; Wetzlar, Germany). We performed a craniotomy (4 mm diameter) from the bregma over the right cortex region (center: AP -2.00 mm, RL +2.00 mm). CCI equipment was mounted on the right stereotaxic arm at a 15° angle from vertical. The diameter of the impactor was 3 mm (flat tip). Impact depths were 0.5 mm (mild), 1.0 mm (moderate), or 1.5 mm (severe), delivered with a velocity of 3.0 m/s and 100 ms duration. Sham-injured mice received only craniotomy and were not subjected to CCI impact. The timeline diagram depicts timing of the experiments through the TBI protocol and corresponding experiments (Fig. 1A [↗](#)).

Primary Cortical Neuron Culture

For primary neuron cultures, cortices from WT and *kcnt1*^{-/-} mice aged postnatal day 0-1 and of either sex were dissected in cold phosphate-buffered saline (PBS). The tissue was then cut into pieces and digested with 0.05% trypsin (T1300; Solarbio, Beijing, China) and DNAase (01086251; Thermo Fisher Scientific, Waltham, MA, USA) for 10-20 min. Digestion was terminated by adding Dulbecco's Modified Eagle Medium (DMEM; 11995-065; Gibco, Amarillo, TX, USA) supplemented with 10% fetal bovine serum (10099-141C, Gibco), 10% horse serum (04-004-1A; Biological Industries, Beit Haemek, Israel), and 50× penicillin/streptomycin (15140-122, Gibco). After filtering the cell suspension with 70 µm mesh, it was centrifuged at 1000 rpm, dispersed, and resuspended with DMEM. Next, cells were mechanically counted and added at densities of 1×10^5 and 1×10^6 cells/well to poly-D-lysine pre-coated 24-well or 6-well plates, respectively. After 4h incubation to allow for cell attachment, DMEM was replaced with NBA Plus [Neurobasal-A medium (10888-022, Gibco) supplemented with 1× GlutaMAX (35050-061, Gibco), 1× B27 supplement (17504-044, Gibco), and 100× penicillin/streptomycin]. Cells were treated with cytarabine (5 µM) and cultured for 3-5 days to inhibit the proliferation of gliocytes. Every 2 days, 50% of the media was replaced with fresh NBA Plus.

Behavioral Tests

In the behavioral 75% alcohol was prepared to eliminate substances and smell that left in machine between two mice to be tested. For the rotarod test, mice were pre-trained on 3 consecutive days on the rotarod rotating for 3 times with the interval of 5 min. Mice were then tested at the initial speed of 4 rpm, accelerating speed ranging from 4 to 30 rpm within 5 min. Time and speed spent on the rotating rod until falling off were recorded for each performance. Each test including three repetitions with an inter-trial interval of 5 min and the means from these three runs were analyzed for each mouse.

For the hanging test to evaluate the movement, coordination and muscle condition of mice, a vertical 2 mm diameter smooth metal rod was fixed in the platform. Mouse grasped the wire with the two forepaws only, started the timer when the mouse was released. When the mouse fell off, the timer was stopped immediately and the hanging time was recorded. If the mouse could hang for more than 10 min, it was placed back in the cage. Falling mice were allowed to try up to two more times, at 2-minute intervals, and the maximum hanging time was recorded for further analysis ([Aartsma-Rus and van Putten, 2014](#)).

Electrode implantation and video-electroencephalogram (v-EEG) recordings

Continuous v-EEG monitoring was performed for 1 week (7 days/week, 24 h/day). Intracranial screw electrodes (1*2 mm; Plexon, Dallas, TX, USA) were implanted 1 or 12 weeks post-TBI. One recording electrode was positioned anterior to the craniectomy window, and another was located contralaterally to the region. Reference and ground electrodes were placed in the ipsilateral and contralateral occipital bone, respectively (**Fig. 2A**). Mice were placed in Plexiglass cages and connected via tether to the v-EEG recording system (NT 9200 with a sample rate of 1000 Hz, Thermo Nicolet Corporation, USA, **Fig. 2B**). A modified "Seizure Severity Score" was used as a criterion for response to pentylenetetrazol (PTZ) ([Semple, et al., 2017](#); [Van Erum, et al., 2019](#)): 0 (no response), 1 (behavioral arrest), 2 (mouth and face twitches), 3 (head nodding and forelimb clonus), 4 (rearing), 5 (generalized convulsive seizure), 6 (continual generalized seizures), and 7 (status epilepticus resulting in death). The frequency of EEG abnormalities and severity of epileptic seizures were manually reviewed and scored based on both EEG and video recordings.

Cresyl Violet Staining

Sections were put onto gelatin-covered adhesive glass slides, stained with cresyl violet for 5 min, and dehydrated in a series of 70%, 80%, 90%, and 100% alcohol for a few seconds each. Next, sections were cleaned twice with xylene for 2 min each. Images were acquired using an EVOS FL Auto 2 Imaging System (Thermo Fisher Scientific). Cresyl violet-stained coronal sections were imaged and histological lesion volumes were quantified with ImageJ software (An, *et al.*, 2016) (<http://imagej.nih.gov>). The percentage of injured volume was calculated using the formula previously described by An *et al.* (An, *et al.*, 2016): $[(VC-VL)/VC] \times 100\%$, where VC is the volume of the contralateral hemisphere of the lesion, and VL is the volume of the ipsilateral hemisphere of the lesion.

Immunofluorescence Staining

Brain slices and primary neurons were post-fixed with 4% PFA, washed three times with PBS, incubated for 30 min with 0.3% Triton, and then blocked with 1% goat serum for 1h to avoid binding of non-specific antibodies. Sections were then incubated at 4°C overnight with the following primary antibodies: anti- $K_{Na}1.1$ (1:300; TA326566, Origene, Rockville, MD, USA), anti-NeuN, which is specific to nuclei and perinuclear cytoplasm of most of the neurons in the central nervous system of mammals and no glial cells (1:300; 26975-1-AP, Proteintech, Rosemont, IL, USA), anti-GABA-synthesizing enzyme glutamic acid decarboxylase 67 (GAD67) (1:300; ab213508, Abcam), anti-vesicular glutamate transporters1 (VGLUT1) (1:300; ab227805, Abcam). Secondary antibodies conjugated to Alexa Fluor 488 or 594 (1:1000; Invitrogen) were applied for 1.5h at room temperature. Slides were mounted with Fluoroshield containing DAPI (Ab104139; Abcam) and observed with a confocal microscope (LSM710; Zeiss, Oberkochen, Germany).

Western Blotting

Fresh brain tissues were removed around the lesion and were lysed with radioimmunoprecipitation assay buffer (R0010, Solarbio) containing protease inhibitors and phosphatase inhibitors, and then centrifuged at 12,000 rpm at 4 °C for 10 min. Protein concentrations were determined using a Pierce™ BCA Protein Assay Kit (23227, Thermo Fisher Scientific). Immunoblotting was performed with the following antibodies: anti-GAD67 (1:500), anti-VGLUT1 (1:500), Anti- $K_{Na}1.1$ (1:500), and tubulin (1:1000), β -actin (1:1000). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (1:10000; Applygen) was used as the secondary antibody, and the signal was visualized using Super ECL Plus substrate (P1050, Applygen).

Slice Electrophysiology

For fourteen days post injury (Dpi-14) mice, as well as sham mice, were deeply anesthetized with isoflurane and decapitated. Brain tissues were immediately submerged in ice-cold (0-4 °C) and oxygenated (95% O₂ and 5% CO₂) cutting solution containing (in mM): 126 NaCl, 25 NaHCO₃, 10 D-glucose, 3.5 KCl, 1.5 NaH₂PO₄, 0.5 CaCl₂, 10 MgCl₂ (pH 7.35 and 340 mOsm). The brain was cut into 300 μ m coronal slices with a Leica VT1000S. After incubating slices in fresh artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 3.5 KCl, 1.0 MgCl₂, 2.0 CaCl₂, 1.5 NaH₂PO₄, 25 NaHCO₃, and 10 D-glucose; pH 7.3-7.4 and 310 mOsm) at 37 °C for 30 min, they were allowed to recover at room temperature for another 1 hour and then transferred to a submerged recording chamber located on an upright microscope (BX51WI; Olympus), which was perfused with oxygenated ACSF at a rate of 2-2.5 ml/min at the room temperature (22-23 °C). Whole-cell recordings were performed on cortical neurons visualized using infrared differential interference contrast microscopy using a Multiclamp 700B (Molecular Devices, San Jose, CA, USA) and Clampex 10.5 software (Molecular Devices). Data were acquired at 10 kHz and low-pass filtered at 1 kHz. Recording electrodes (resistance of 3-6 M Ω) were prepared from borosilicate glass capillaries (BF150-86-75; Sutter Instruments, Novato, CA, USA) using a vertical pipette puller (PC-100;

Narishige, Tokyo, Japan). The pipette solution contained (in mM): 136 K-gluconate, 17.8 HEPES, 1 EGTA, 0.6 MgCl₂, 4 ATP, 0.3 GTP, 12 creatine phosphate (pH 7.3 adjusted with KOH and osmolarity maintained at 290-300 mOsm).

As previously described ([Cheng, et al., 2022](#); [Shore, et al., 2020](#)), electrophysiological parameters recorded in our experiments included intrinsic membrane properties, and synaptic activities. We first divided neurons into excitatory pyramidal neurons (PNs) and non-fast spiking interneurons (INs) according to different morphological characteristics. PNs usually have clear apical and basal dendrites while INs are featured with small body size and obscure apical dendrite. Next, we utilized the electrophysiological parameters described in previous studies ([Avermann, et al., 2012](#); [Shore, et al., 2020](#)) to distinguish PNs and INs. PNs showed obvious AP adaption with a wide AP half-width during AP trains. INs were confirmed if their maximum mean firing rates were in the range of 30-60 Hz, and with a larger input resistance and smaller AP half-width than PNs. Protocols were reported in a previous study ([Shore, et al., 2020](#)). The threshold amplitude of sEPSCs was above 5 pA, while sIPSCs were two or three times that of baseline standard deviation (SD). The excitation/inhibition ratio = (sEPSC frequency * charge) / (sEPSC frequency * charge + sIPSC frequency * charge).

Cultured Cell Electrophysiology

Whole-cell recordings were performed on primary cortical neurons in parallel on the same day (day 13-16 in vitro), and viewed with an infrared differential interference contrast microscope with an Olympus 40× water immersion lens (BX51WI; Olympus, Tokyo, Japan) at room temperature (22-23°C). Standard extracellular solution contained the following (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 MgCl₂, and 2 CaCl₂ (pH 7.35 and 320 mOsm). The internal solution, protocols, and determination of electrophysiological parameters were conducted as described above for slice recordings.

Statistical Analysis

All results were presented as mean ± SEM. Statistical analyses were done using GraphPad Prism 8 or SPSS 22 (IBM, Armonk, NY, USA). All data distributions were assessed with the Shapiro-Wilk test. Unpaired two-tailed Student's t test, Kruskal-Wallis test, one-way ANOVA with Tukey's post hoc tests, and two-way ANOVA with Sidak's post hoc tests were used to determine statistical significance (p < 0.05).

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Competing interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Author contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Ru Liu and Lei Sun. The original draft was written by Ru Liu. Formal analysis, visualization, review and editing were performed by Du Le and Xi Guo. Data curation, review and editing, project administration, resources, and supervision of the research were performed by Qun Wang and Jianping Wu. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All procedures were approved by the Animal Care and Use Committee of Capital Medical University in accordance with the Guide for the Care and Use of Laboratory Animals. The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Tiantan Hospital, Capital Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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Reviewer #1 (Public Review):

Summary

General Comments

The authors present an interesting study that aims to resolve the contribution of the sodium-activated potassium channel (KNa1.1) to acquired, trauma-induced epilepsy. To this end, the authors first aim to develop a mouse model that consistently generates seizures. Using controlled cortical impact (CCI) methods to induce traumatic brain injuries (TBIs) that range from mild to severe, the authors demonstrate that behavioral deficits correlate with the extent of brain damage. Interestingly, despite the differences in behavioral scores, the spontaneous seizure phenotype was similar across mice with a range of TBI-associated tissue loss. However, when challenged with the chemoconvulsant pentylenetetrazol (PTZ), mice with more severe TBI exhibited more severe seizures.

After establishing a model of moderate TBI, the authors then show that moderate brain injury transiently upregulates the perilesional expression of KNa1.1. Moreover, the authors provide some evidence that the expression of inhibitory neuron markers is downregulated in the perilesional region following moderate TBI, whereas the expression of excitatory neuron markers is unchanged. Consistent with this finding is the functional observation that neurons receive less inhibitory signaling following TBI, whereas excitatory signaling is unchanged. Inhibitory neurons also fire less robustly following moderate TBI.

The authors then show that deletion of KNa1.1 in mice provides a moderate level of protection against pharmacologically induced seizures following TBI. In aggregate, the authors propose a model wherein inhibitory neuron-specific upregulation of KNa1.1 following TBI selectively reduces the excitability of inhibitory neurons. In turn, this reduced

excitability of inhibitory neurons promotes perilesional tissue hyperexcitability and, ultimately, seizures. Although this model is compelling, readers should be aware that the authors only utilized PTZ-induced seizures following TBI to resolve differences between WT and KO animals. It remains unclear whether WT mice have a robust spontaneous seizure phenotype 14 days after moderate TBI, and whether deleting KNa1.1 reduces this spontaneous seizure phenotype. In general, the combined use of TBI and a chemoconvulsant to evaluate epileptic phenotypes diminishes this reviewer's enthusiasm for the clinical impact of the author's conclusions; although, I appreciate that "capturing [spontaneous] seizures is challenging in terms of animal numbers and long-term recording, which hinders high-throughput studies" (line 302).

Finally, the authors seemed to have missed an opportunity to determine if the electrophysiological changes observed in WT mice following TBI (i.e., Figure 5) are eliminated in the KNa1.1 KO mouse. That is, the authors show that KNa1.1 contributes to the intrinsic firing properties of uninjured tissue (Figure 6). But does deleting KNa1.1 also restore inhibitory neuron excitability associated with TBI? The inclusion of such data would strengthen the conclusion that the reduced inhibitory neuron excitability following TBI is indeed the result of changes in KNa1.1 expression.

Strengths:

- (1) The development of a TBI model with a range of behavioral phenotypes.
- (2) The inclusion of knockout mouse.
- (3) The inclusion of functional data regarding the intrinsic and synaptic properties of neurons following TBI.

Weaknesses:

- (1) A missed opportunity to better utilize the knockout animal to test the hypothesis that KNa1.1 drives changes in intrinsic excitability following TBI.
- (2) The combined use of TBI and chemoconvulsants to suss out differences in seizure phenotypes.

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Reviewer #2 (Public Review):

Summary:

Authors hypothesized that modulation of KNa1.1 channel specifically in inhibitory interneurons contributes to the hyperactivity of neurons in the peripheral cortex at the lesion site, enhances seizure susceptibility to PTZ-induced seizures, and promotes the occurrence of PTE. They test this hypothesis in a mouse model of TBI induced by controlled cortical impact in wild-type and *kcnt1* knock-out (KO) mice. The authors performed a series of experiments including behavioral assessment, electrographic recordings in vivo and in vitro, western blotting, and immunofluorescence imaging with the goal of investigating the contributory role of KNa1.1 channel to post-traumatic epileptogenesis.

Strengths:

The hypothesis is innovative, focusing on the specific role of the KNa1.1 channel in the development of PTE post-TBI. The use of a comprehensive set of techniques, including EEG, whole cell patch clamp, western blot, immunofluorescence imaging, and behavioral assessments, provides a diverse data set. The study makes initial steps in correlating specific molecular changes with functional outcomes in TBI models, offering potential pathways for therapeutic intervention.

Weaknesses:

1. The study presents interesting findings on early changes in protein expression and electrophysiological properties following TBI. However, I would like to draw attention to the timeline of EEG and cellular assessments that require further clarification or consideration. The patch clamp recordings and other assays were conducted within 14 days post-TBI, while EEG recordings with or without PTZ testing were performed at 3 months post-injury. This temporal gap leaves a period where changes in electrophysiological properties and PTE status are not accounted for. Since epileptogenesis post-TBI involves a dynamic process spanning from hyperacute and acute phases to chronic development, capturing these changes continuously or at least at more frequent intervals (on and off bi-weekly) could provide a more comprehensive understanding of this progression. In the current study design, the one-week duration of EEG recordings at the 3-month timepoint raises the possibility that some seizures might have occurred undetected between the early post-injury phase and the EEG recording period. This gap could potentially affect the interpretation of results, especially when correlating early post-injury cellular changes with later seizure activity and thresholds and hence is a significant limitation to data interpretation. Experiments using western blots, immunofluorescence, and patch clamp were done at an early timepoint hence the relevance of these datasets to PTE status outcome is not established.
2. While referencing Nichols et al., 2015, to justify the 14-day timeline for characterizing seizures is understandable, it is important to consider differences in animal models (juvenile rats in Nichols vs. adult mice in the present study) which might influence the generalizability of the findings.
3. Behavior: Authors performed behavioral assays using the rotarod technique evaluating the hanging time of mice with different severity of TBI (mild, moderate, severe). The purpose of the testing is explained as 'to sort out an appropriate TBI model'. The authors also measured mortality rates 'to attain a stable model'. It is not clear what is assumed by the terms 'appropriate' or 'stable' model. Furthermore, the relevance of this to post-traumatic epileptogenesis is unclear. Additionally, the mortality in the Sham group within 2 weeks of craniectomy is not explained.
4. Seizure assessment: authors report seizure severity in PTZ-induced seizures but no mention about the severity of spontaneous seizures between different TBI severity modalities. When characterizing the PTZ-induced seizures, the mild TBI group does not have generalized seizures. Does this mean that all 6 tested animals in the mild TBI group had exclusively focal seizures? What about the spontaneous seizure occurrence: were all seizures generalized or were any focal too? Did that differ between mild, moderate, and severe TBI?
5. Experiments with KCNT1 KO mice: in all experiments with a mutant mouse line, authors only used them in TBI group. Without the Sham group, it is difficult to discern whether any observed changes in seizure susceptibility in the KCNT1 KO TBI group are due to gene deletion, the TBI, or a combination of both. This group would provide a crucial comparison point to isolate the effects of the KCNT1 knockout from those of TBI. This limits the ability to make comprehensive conclusions about the role of the KCNT1 gene in seizure susceptibility following TBI.

6. While the current study showed interesting data about the KNa1.1 changes early after TBI, the study design and disconnect between early and late electrophysiology experiments timeline, does not establish a correlative or causative link between KNa1.1 and post-traumatic epileptogenesis since it remained unresolved whether KCNT1 KO mice developed no PTE (or less severe/ less frequent seizures at 3 months) compared with WT mice and what are the seizure properties of KCNT1 KO Sham mice compared to WT TBI and Sham groups. The hypothesis was that modulation of KNa1.1 channel specifically in inhibitory interneurons contributes to the hyperactivity of neurons in the peripheral cortex at the lesion site, enhances seizure susceptibility to PTZ-induced seizures, and promotes the occurrence of PTE. The part about 'promotes the occurrence of PTE' was not established.
 7. NeuN is not the best marker of neurons in the context of TBI since TBI affects its expression patterns which will influence the interpretation of co-localization results. Unlike NeuN, Nissl staining is less likely to be affected by factors that alter protein expression, such as TBI. Therefore, it can be a more stable marker for identifying neurons in injured brain tissue.
 8. Statistics: Authors report only SEM, which shows the precision of the mean and it will decrease as the sample size increases and does not reflect the data variability.
- <https://doi.org/10.7554/eLife.93304.1.sa0>