Characterization and Therapeutic Screening of a Gain of Function Mutation in *Kcnt1* Utilizing Multielectrode Arrays

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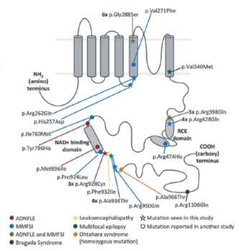
ID: 560.14

Rationale

- Whole exome sequencing has revealed that mutations in *KCNT1* are associated with a broad spectrum of epileptic encephalopathies

Identified *KCNT1* Mutations

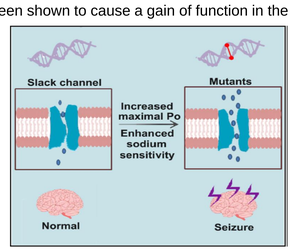


Møller and Heron *et al* 2015

- Molecular diagnosis via exome sequencing identified a missense mutation (c. c.2386T>C; p.Tyr796His) in the *KCNT1* gene (Y777H in mouse), as the variant causing pathology in a family of four with Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) as well as one *de novo* case.
- Clinical Features of ADNFLE:
  - Childhood onset (5 years) focal epilepsy syndrome with clusters of motor seizures arising from sleep.
  - Intellectual disability and various behavioral/psychiatric disorders
  - Refractory epilepsy

KCNT1 Function

- *KCNT1* (Slack) encodes a sodium dependent voltage-gated, outwardly rectifying potassium channel subunit.
- It is ubiquitously expressed in the brain throughout development. In the cortex, it is most highly expressed in the frontal lobe.
- KCNT1 contributes to the slow hyperpolarization following repetitive firing of action potentials.
- Epileptic *KCNT1* mutations have been shown to cause a gain of function in the channel via:
  - Increased Na^+ sensitivity
  - Increased K^+ current amplitude
  - Increased channel opening and open time



Tang *et al* 2016

- Gain of function in a potassium channel that is responsible for hyperpolarization paradoxically increases excitability, possibly explained via:
  - Enhanced Na^+ channels re-priming by faster action potentials repolarization
  - Secondary depolarizations caused by over-activation of the hyperpolarization-activated I_h currents
  - Preferential suppression of inhibitory interneurons, leading to an increased excitability of principal neurons

Funding Acknowledgements

This study was funded, in part, by Grant # NIH R37 NS031348 (to WNF)

The mouse model was generated by The Jackson Laboratory Center for Precision Genetics, funded by NIH grant U54 OD020351

NewYork-Presbyterian

Columbia University Medical Center

National Institutes of Health

Spontaneous Recurrent Seizures in a Mouse Model of *KCNT1*^{Y796H}

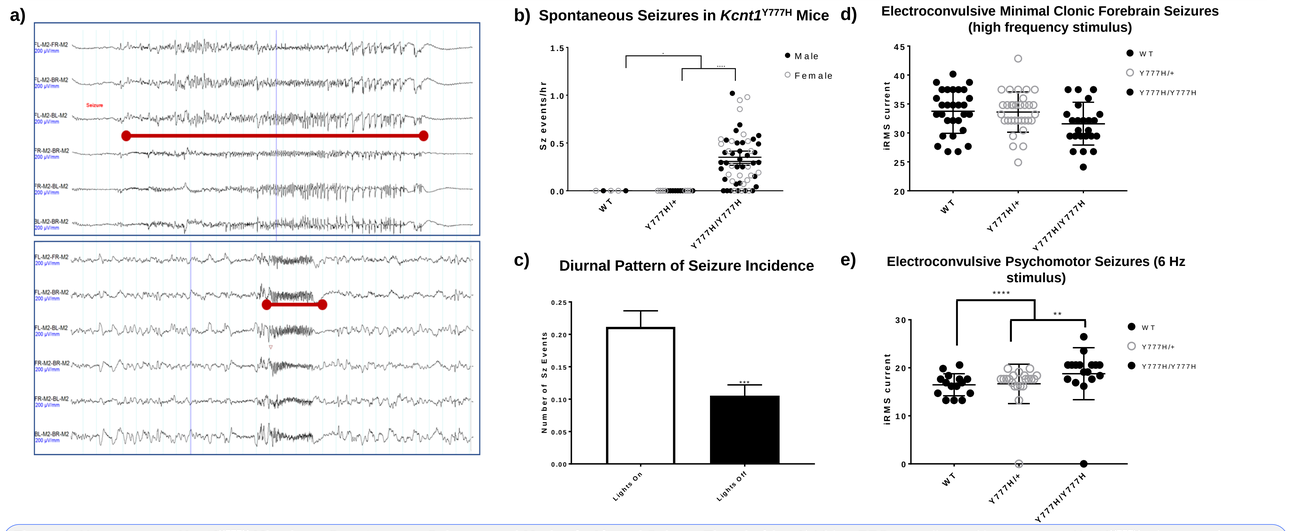


Figure 1. Homozygous *Kcnt1*^{Y777H} Mice have Spontaneous Seizures and Modestly Altered Seizure Thresholds. A) Video-EEG recordings of homozygous *Kcnt1*^{Y777H} mice reveal spontaneous generalized tonic-clonic seizures, short abortive events and interictal spiking (see video and methods). B) EEG studies show no seizure events in wildtype or heterozygous *Kcnt1*^{Y777H} mice, but an average rate of 0.32 events per hour in homozygous mice with 84% having at least one seizure. C) Seizures occur significantly more during sleep phase (lights on) rather than wake phase (lights off). D) Homozygous *Kcnt1*^{Y777H} show a trend towards lower electroconvulsive threshold to minimal clonic forebrain seizures, compared to control littermates. D) Homozygous *Kcnt1*^{Y777H} mice demonstrate significant resistance to the 6 Hz electroconvulsive stimulus (inducing a so-called "psychomotor" partial seizure), compared to control littermates.

Increased Spontaneous and Evoked Activity of *Kcnt1*^{Y777H} Neurons *In Vitro*

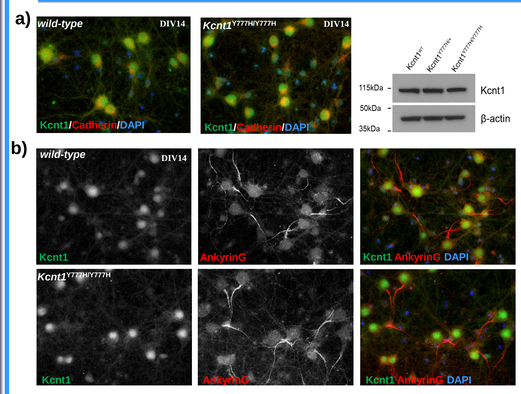


Figure 2. Normal Localization of *Kcnt1* in Homozygous Neurons Compared to Wildtype. A) Immunocytochemistry revealing co-localization of Kcnt1 at the membrane with cadherin and Western Blot of adult mouse brain membrane fraction showing equal amounts of Kcnt1 expression between genotypes. B) no co-localization of Kcnt1 at the axon initial segment with AnkyrinG.

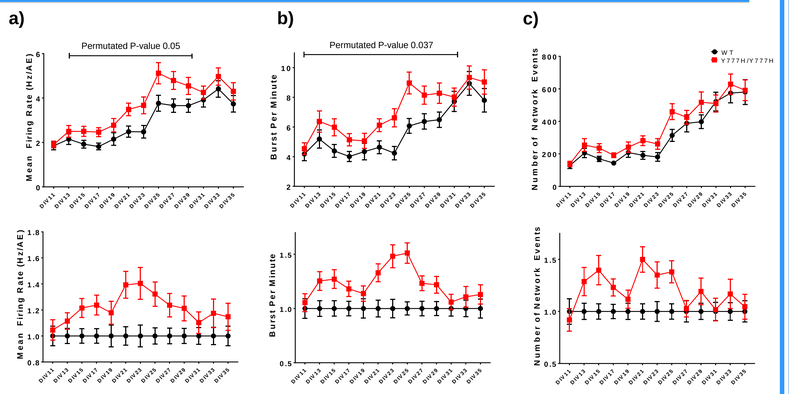


Figure 3. Multielectrode Array (MEA) Recordings Reveal Increased Firing and Bursting in *Kcnt1* Homozygous Neurons Compared to Wildtype. A) Recordings from wildtype and homozygous *Kcnt1*^{Y777H} neurons reveals a higher firing rate and B) higher bursting rate emerges during neuronal maturation. C) Homozygous *Kcnt1*^{Y777H} neurons have a greater number of synchronous network events (>25% of well active simultaneously) during neuronal maturation compared to wildtype. Top row is averaged from 5 different litters each run on a different MEA preparation, and the bottom row represents the same data normalized to wildtype.

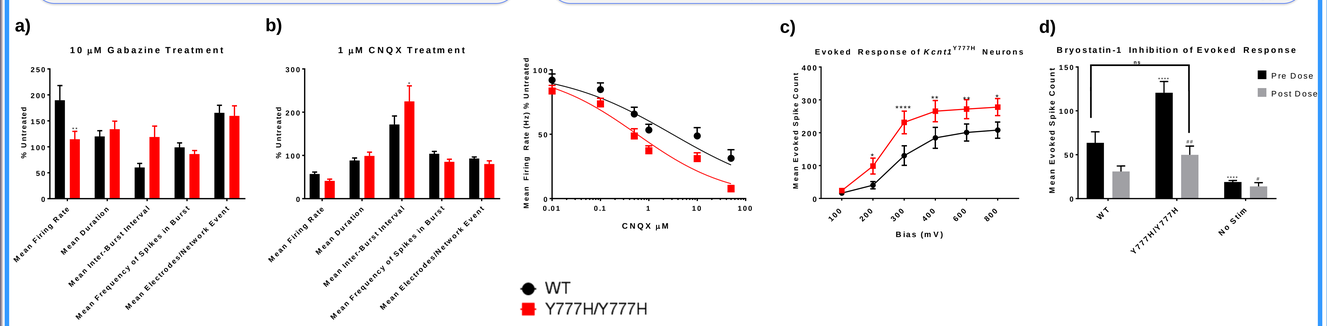
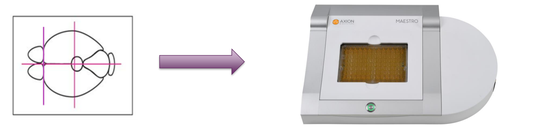
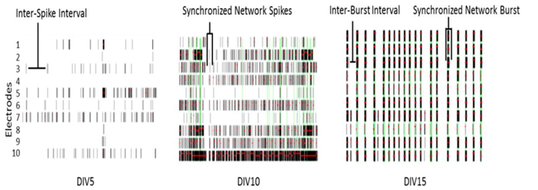


Figure 4. Pharmacological Evaluation on MEA Demonstrates Altered Response of Homozygous *Kcnt1*^{Y777H} Neurons to Synaptic Inhibitors. A) Addition of $GABA_A$ antagonist gabazine reveals decreased excitatory response of homozygous *Kcnt1*^{Y777H} neurons, N = 17 wells. B) Homozygous *Kcnt1*^{Y777H} neurons show increased sensitivity of CNQX mediated inhibition of firing, IC50 of 3.061 ±0.83 and 0.57 ±0.11 for Wildtype and homozygous respectively, N =30 wells. IC50 determined by fit to non-linear regression shown in black for wildtype and red for homozygous neurons. Results represent averages from 3 different litters(gabazine) and 4 different litters (CNQX) each run on a different MEA preparation. C) Increased evoked response to electrical stimuli in homozygous *Kcnt1*^{Y777H} neurons (N = 12 wells) and D) inhibition of electrically evoked response in both genotypes after treatment with PKC inhibitor Bryostatin-1.

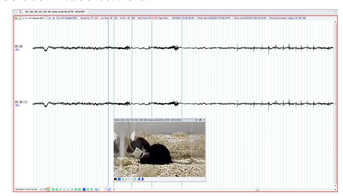
Methods

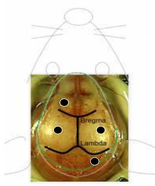
- CRISPR/Cas9 knock-in mouse model was generated at the mouse reference sequence coordinate (Y777H) orthologous to the human Y796H variant. The mutation was generated and is maintained and studied on the C57BL/6NJ strain background for results in this presentation.
- Activity analysis of cultured neurons from *Kcnt1*^{Y777H} mice utilizing Maestro MEA system (Axion BioSystems). Briefly primary cortical neurons from P0 pups were dissociated and plated on 48-well MEA plates, with each well containing 16 electrodes. Neurons were maintained in NBA/B27 media and recorded for 15 minutes every other day. Parameters including firing rate, bursting properties, and network properties were obtained using an in house program. For pharmacological treatment, plates were recorded to obtain baseline reference prior to addition of drug and values are reported as a percentage of the untreated reference.





- Seizure susceptibility was determined using electroconvulsive threshold (ECT) tests, using the Ugo Basile Electroconvulsive Device and transcorneal electrodes, as described in Frankel, 2001 Genomics (PMID: 11414758). High frequency ECT settings: 299 Hz, 1.6 ms pulse width, 0.2 s duration, variable current. Low frequency ECT settings: 6 Hz, 0.2 ms pulse width, 3 s shock duration, variable current. Tests were performed approximately daily in individual mice until threshold was reached. Integrated root mean square (iRMS) was calculated from stimulus parameters to describe threshold, and group means were calculated for genetic analysis.
- Spontaneous seizure events detected by video-EEG . Adult mice (6 wks or older) were implanted with electrodes for continuous EEG monitoring. Subdural cortical electrodes were implanted under general anesthesia as indicated in the image below. Signal was detected with a Grael 48 EEG amplifier and acquired on a computer using Profusion 5 software (Compumedics, USA), synchronized to a high resolution video camera





Summary

- Homozygous *Kcnt1*^{Y777H} (equivalent to human Y796H) mice have frequent, recurrent (non-lethal) spontaneous generalized tonic-clonic seizures (GTCS), readily observed in video-EEG.
  - Variety of EEG features including short (< 10 second) events with nominal motor involvement, and longer (> 30 second) full GTCS events.
  - The seizure incidence (both major and minor events) was about 2-fold higher with lights on compared with lights off, suggesting that the mouse may phenocopy sleep-associated seizures seen in human KCNT1 ADFLNE
- *Kcnt1* localization and expression is not altered by the mutation.
  - *Kcnt1* also shows expression in both glutamatergic and GABAergic neurons.
- Homozygous neurons fire and burst significantly more during neuronal development compared to wildtype and have increased evoked response to electrical stimuli.
- Inhibitors of synaptic transmission have differential effects based on genotype hinting towards altered transmission in homozygous neurons.
- PKC inhibitor Bryostatin-1 shown previously in heterologous expression to decrease KCNT1 current reverses evoked phenotype.

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