

## EPILEPSY

## Ectopic HCN4 expression drives mTOR-dependent epilepsy in mice

Lawrence S. Hsieh<sup>1</sup>, John H. Wen<sup>1</sup>, Lena H. Nguyen<sup>1</sup>, Longbo Zhang<sup>1,2</sup>, Stephanie A. Getz<sup>1</sup>, Juan Torres-Reveron<sup>1\*</sup>, Ying Wang<sup>3</sup>, Dennis D. Spencer<sup>1</sup>, Angélique Bordey<sup>1,4†</sup>Copyright © 2020  
The Authors, some  
rights reserved;  
exclusive licensee  
American Association  
for the Advancement  
of Science. No claim  
to original U.S.  
Government Works

The causative link between focal cortical malformations (FCMs) and epilepsy is well accepted, especially among patients with focal cortical dysplasia type II (FCDII) and tuberous sclerosis complex (TSC). However, the mechanisms underlying seizures remain unclear. Using a mouse model of TSC- and FCDII-associated FCM, we showed that FCM neurons were responsible for seizure activity via their unexpected abnormal expression of the hyperpolarization-activated cyclic nucleotide-gated potassium channel isoform 4 (HCN4), which is normally not present in cortical pyramidal neurons after birth. Increasing intracellular cAMP concentrations, which preferentially affects HCN4 gating relative to the other isoforms, drove repetitive firing of FCM neurons but not control pyramidal neurons. Ectopic HCN4 expression was dependent on the mechanistic target of rapamycin (mTOR), preceded the onset of seizures, and was also found in diseased neurons in tissue resected from patients with TSC and FCDII. Last, blocking HCN4 channel activity in FCM neurons prevented epilepsy in the mouse model. These findings suggest that HCN4 play a main role in seizure and identify a cAMP-dependent seizure mechanism in TSC and FCDII. Furthermore, the unique expression of HCN4 exclusively in FCM neurons suggests that gene therapy targeting HCN4 might be effective in reducing seizures in FCDII or TSC.

## INTRODUCTION

Disorders caused by mutations in the mechanistic target of rapamycin (mTOR) pathway genes lead to mTOR hyperactivity, brain malformations, and life-long epilepsy in most patients (1, 2). Among this group of disorders, focal cortical dysplasia (FCD) and tuberous sclerosis complex (TSC) account for the largest population of children and young adults who undergo brain surgery to treat intractable epilepsy (3–5). Nearly two-thirds of these patients are refractory to treatment with antiepileptic drugs and experience life-long seizures, leading to a spectrum of neurocognitive and psychological disabilities (1). Upon failure of conventional antiepileptic drugs, surgical resection of the seizure foci and pharmacological inhibition of mTOR signaling in the case of TSC are the only available treatments for mTOR-dependent focal epilepsy, but neither option is fully effective (6, 7). Despite the identification of the molecular pathways dysregulated in TSC and FCD type II (FCDII) and the pathological characteristics associated with seizures (3, 8, 9), the mechanism of epileptogenesis remains to be fully elucidated, although several hypotheses of epileptogenicity have been proposed. These include abnormal reorganization of the circuitry within and surrounding focal cortical malformations (FCMs) and abnormal electrophysiological properties of FCM neurons (4, 10–12). In addition, the exact site and the cell types responsible for epileptogenic activity in humans remain controversial as some studies have recorded epileptic discharges both inside and outside of FCMs, whereas other studies have found FCMs to be electrically silent (12, 13). The

lack of consensus on the site of seizure generation further reflects our poor understanding of the epileptogenic mechanisms, which has hampered the identification of therapeutic targets and more effective treatment options.

Using a mouse model of TSC and FCDII (14), we show here that silencing FCM neurons prevented the occurrence of seizures, suggesting that FCM neurons might be responsible for seizure activity. To identify how FCM neurons drove seizures, we obtained patch clamp recordings in FCM neurons and identified an abnormal expression of hyperpolarization-activated cyclic nucleotide-gated potassium channel isoform 4 (HCN4), which is not present in normal pyramidal neurons. Although FCM neurons were depolarized and hence closer to the threshold for action potential generation, they required more current injection than controls to reach that threshold because of the cells' increased size and intrinsic conductance. Consistent with the fact that HCN4 channels are the most cyclic adenosine monophosphate (cAMP) sensitive of all HCN channels (15, 16), increasing intracellular cAMP induced repetitive firing of FCM neurons and suggested a possible mechanism of firing and seizure induction that differs from the traditional excitatory input-induced firing mechanism. HCN4 was also found in dysmorphic cortical neurons from patients with TSC and FCDII who underwent surgery for epilepsy treatment. Last, the use of a genetic approach to block HCN4 activity in FCM neurons prevented seizures in the mouse model. Collectively, our findings identified a potential mechanism responsible for seizure generation in mTOR-dependent FCM and suggest that a targeted gene therapy treatment option to prevent seizure initiation might be effective in reducing seizures in TSC and FCDII.

## RESULTS

## Silencing FCM neurons decreases seizure activity in mice

To investigate the mechanisms of seizure generation in FCM, we used a mouse model that recapitulates the focal nature of the cortical malformation and the cellular mosaicism of human FCDII and TSC brains (14, 17). To generate this model, we used in utero electroporation

<sup>1</sup>Department of Neurosurgery, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA. <sup>2</sup>Department of Neurosurgery, Xiangya Hospital, Xiangya Hospital, Central South University, 87 Xiangya Street, Changsha, Hunan 410008, China. <sup>3</sup>Emergency Department, Xiangya Hospital, Central South University, 87 Xiangya Street, Changsha, Hunan 410008, China. <sup>4</sup>Department of Cellular and Molecular Physiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA.

\*Present address: Department of Neurosurgery, University of Cincinnati Academic Health Center, 222 Piedmont Avenue, Suite 6200, Cincinnati, OH 45219, USA.

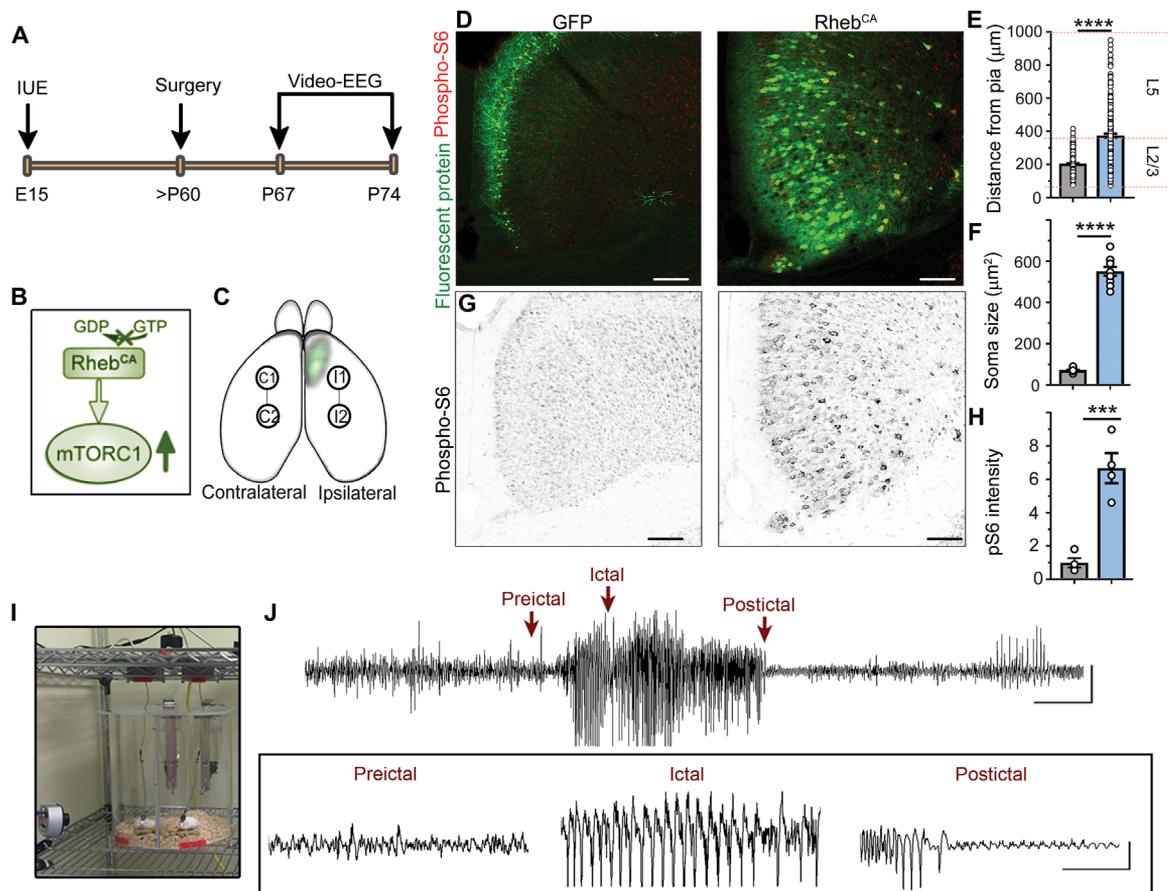
†Corresponding author. Email: angelique.bordey@yale.edu

(IUE) to induce mTOR hyperactivity in pyramidal neurons in a limited cortical area (Fig. 1A) (14, 17–21). IUE at embryonic day 15 (E15) specifically targets radial glia that generate layer 2/3 pyramidal neurons (Fig. 1B). To increase mTOR activity, we expressed a constitutively active mutant Ras homolog enriched in brain (Rheb) (Rheb<sup>CA</sup>), the canonical mTOR activator (Fig. 1B) (14, 18, 20, 22, 23). A green or red fluorescent protein (GFP or tdTomato) was co-expressed to label the transfected neurons. These mice developed a singular FCM in the medial prefrontal cortex (mPFC) (Fig. 1C) that displayed a pathology resembling that of human FCM, including the presence of misplaced and cytomegalic Rheb<sup>CA</sup>-expressing neurons that are interchangeably called FCM neurons (Fig. 1D). Whereas control GFP-expressing neurons are located in layer 2/3, FCM neurons are scattered across cortical layers 2 to 6 (Fig. 1E). In addition, FCM neurons showed an increase in soma size and immunoreactivity for phosphorylated protein S6 (phospho-S6), a readout of mTOR hyperactivation, compared to control neurons in mice electroporated with GFP only (Fig. 1, F to H). Mice containing FCM were visually observed to develop convulsive seizures by postnatal day 21 (P21). To better characterize the seizures, we obtained epidural electroencephalography (EEG) recordings combined with video monitoring for five to seven consecutive days in 3- to 4-month-old mice. Mice

containing FCM exhibited recurrent, Racine grade 4 to 5 seizures with classical interictal, tonic, clonic, and postictal periods (Fig. 1, I and J, and movie S1; seizure frequency and duration are shown in subsequent figures).

Using our mouse model of FCM, we previously reported that FCM neurons display depolarized resting membrane potentials (RMPs) compared to their control counterparts and are therefore closer to the threshold for generating action potentials (18). We thus examined whether silencing FCM neurons without normalizing their morphological abnormalities, which could contribute to seizure generation, would limit the incidence of seizures. To do this, we over-expressed inwardly rectifying potassium channels 2.1 (Kir2.1) that are expected to hyperpolarize neurons and decrease their membrane resistances (24). Using E15 IUE, we coexpressed Kir2.1 with Rheb<sup>CA</sup> (Fig. 2A). In the control condition, GFP was coelectroporated with Rheb<sup>CA</sup>. Kir2.1 expression in Rheb<sup>CA</sup> neurons did not prevent the formation of FCM (Fig. 2, A and B) and did not interfere with mTOR hyperactivity, as shown by quantification of soma size and phospho-S6 immunofluorescence, respectively (Fig. 2C). Furthermore, patch clamp recordings of Rheb<sup>CA</sup> neurons in acute slices from P26 to P42 validated that Kir2.1 expression did not alter membrane capacitance (a readout of soma size) but significantly hyperpolarized

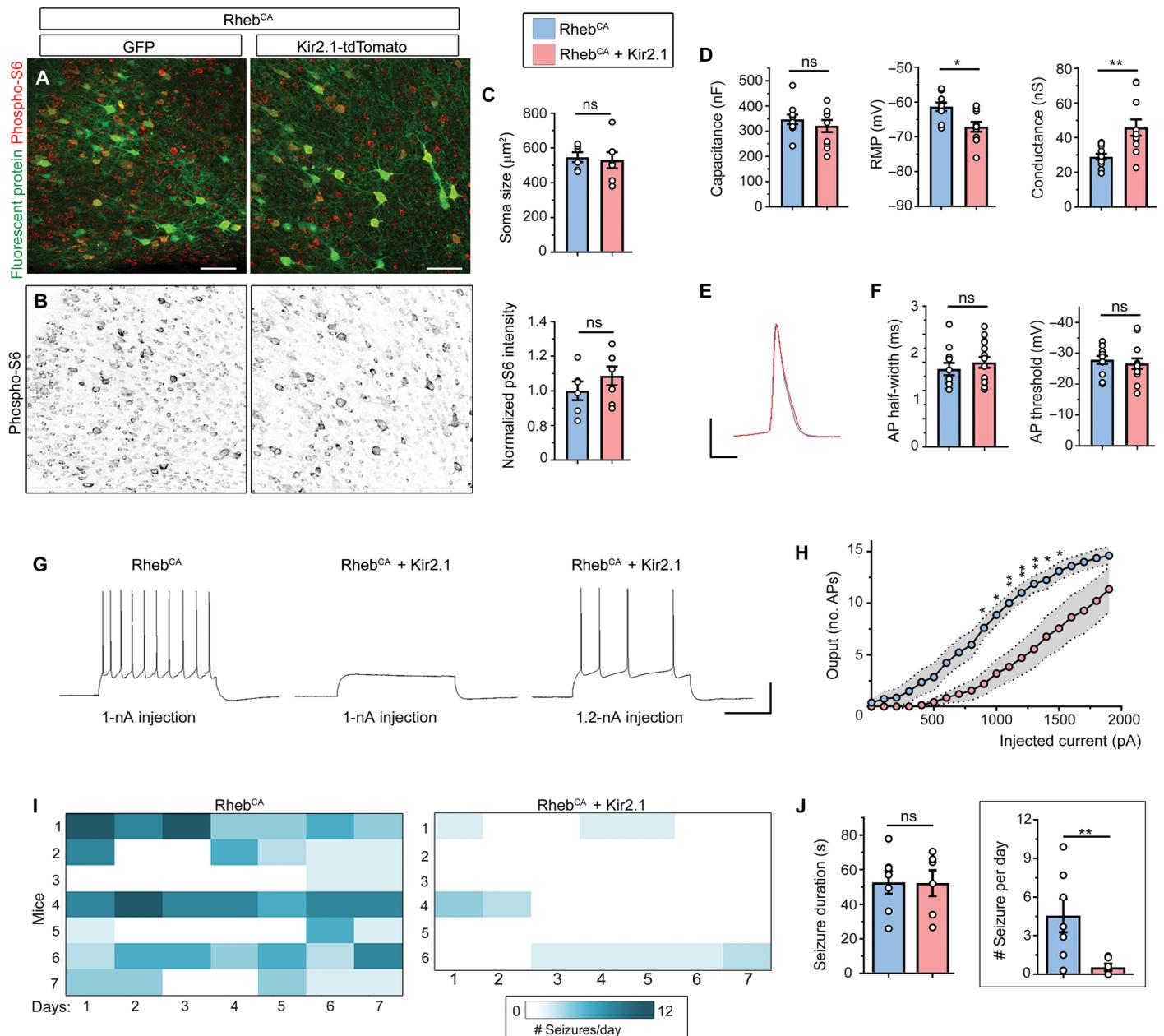
**Fig. 1. Model of mTOR-dependent FCM-associated seizures.**



and Rheb<sup>CA</sup>-expressing neurons. (I) Image of the video-EEG setup. (J) Representative examples of an EEG trace and higher temporal resolution traces in the inset. Scale bars, 10 s/200 µV and 2 s/200 µV (inset). Data are means ± SEM. \*\*\**P* < 0.001 and \*\*\*\**P* < 0.0001. Statistical analysis: Mann-Whitney *U* test (E), Student's *t* test (F and G). Exact *P* values can be found in table S4. GDP, guanosine diphosphate; GTP, guanosine triphosphate.

Rheb<sup>CA</sup> neurons and increased their membrane conductance ( $P < 0.05$  and  $P < 0.01$ , Student's  $t$  test, respectively; Fig. 2D). The properties of current-induced action potentials, including half-width and threshold, were unaltered by Kir2.1 expression (Fig. 2, E and F), but con-

sistent with the changes in RMP and membrane conductance, larger current injections were required for the generation of action potential in Kir2.1-expressing FCM neurons that resulted in a shift in the input (injected current amplitude)–output (number of action



**Fig. 2. Silencing cytomegalic neurons in mTOR-driven FCM prevents seizure activity.** (A) Confocal images of GFP and tdTomato fluorescence (pseudo-colored green) and phospho-S6 immunostaining (red) in coronal sections from 4-month-old littermate mice [used in (J)] expressing Rheb<sup>CA</sup> + GFP or + Kir2.1 (fused to tdTomato). Scale bars, 100 μm. (B) B&W phospho-S6 immunostaining from images shown in (A). (C) Bar graphs of normalized phospho-S6 immunofluorescence intensity and soma size of Rheb<sup>CA</sup> neurons coexpressing GFP (green) or Kir2.1 (pink). (D) Bar graphs of cell capacitance, resting membrane potential (RMP), and membrane conductance of Rheb<sup>CA</sup> neurons coexpressing GFP or Kir2.1. Patch clamp recordings were obtained in acute slices from P26 to P42 mice. (E) Superimposed individual action potentials (APs) from Rheb<sup>CA</sup> neurons in both conditions. Scale bar, 2 ms/40 mV. (F) Bar graphs of the action potential half-width and threshold. (G) Representative depolarization and action potentials upon current injection in Rheb<sup>CA</sup> coexpressing GFP or Kir2.1. Scale bars, 200 ms/40 mV. (H) Injected current amplitude against the mean number of action potentials for generating an input–output curve in Rheb<sup>CA</sup> neurons. The gray area outlines the SEM for each curve. (I) Heatmap of the number of seizures over a 7-day-long recording period in mice containing Rheb<sup>CA</sup> neurons with GFP or Kir2.1. (J) Bar graphs of the duration and number of seizures per day in the two conditions. Statistical analyses: Student's  $t$  test [(C, D, F, and J), seizure duration], two-way repeated measure ANOVA followed by Sidak posttest (H), and Mann Whitney  $U$  test [(J), seizure frequency]. Data are means ± SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . ns, not significant. Exact  $P$  values can be found in table S4.

potentials) curve (Fig. 2, G and H). Thus, expressing Kir2.1 rendered FCM neurons less excitable, in part, via hyperpolarizing their RMP further away from the action potential threshold. Mice overexpressing Kir2.1 in FCM neurons displayed a significant decrease in seizure frequency (from a mean of 4.5 to 0.5 seizures per day;  $P < 0.01$ , Student's  $t$  test) without affecting seizure duration (Fig. 2, I and J). Collectively, these data suggest that alterations in the electrical properties of FCM neurons are directly involved in seizure initiation and that silencing these neurons is sufficient to reduce the number of seizures in mice.

### FCM neurons abnormally express HCN channels, leading to depolarized RMP and cAMP sensitivity

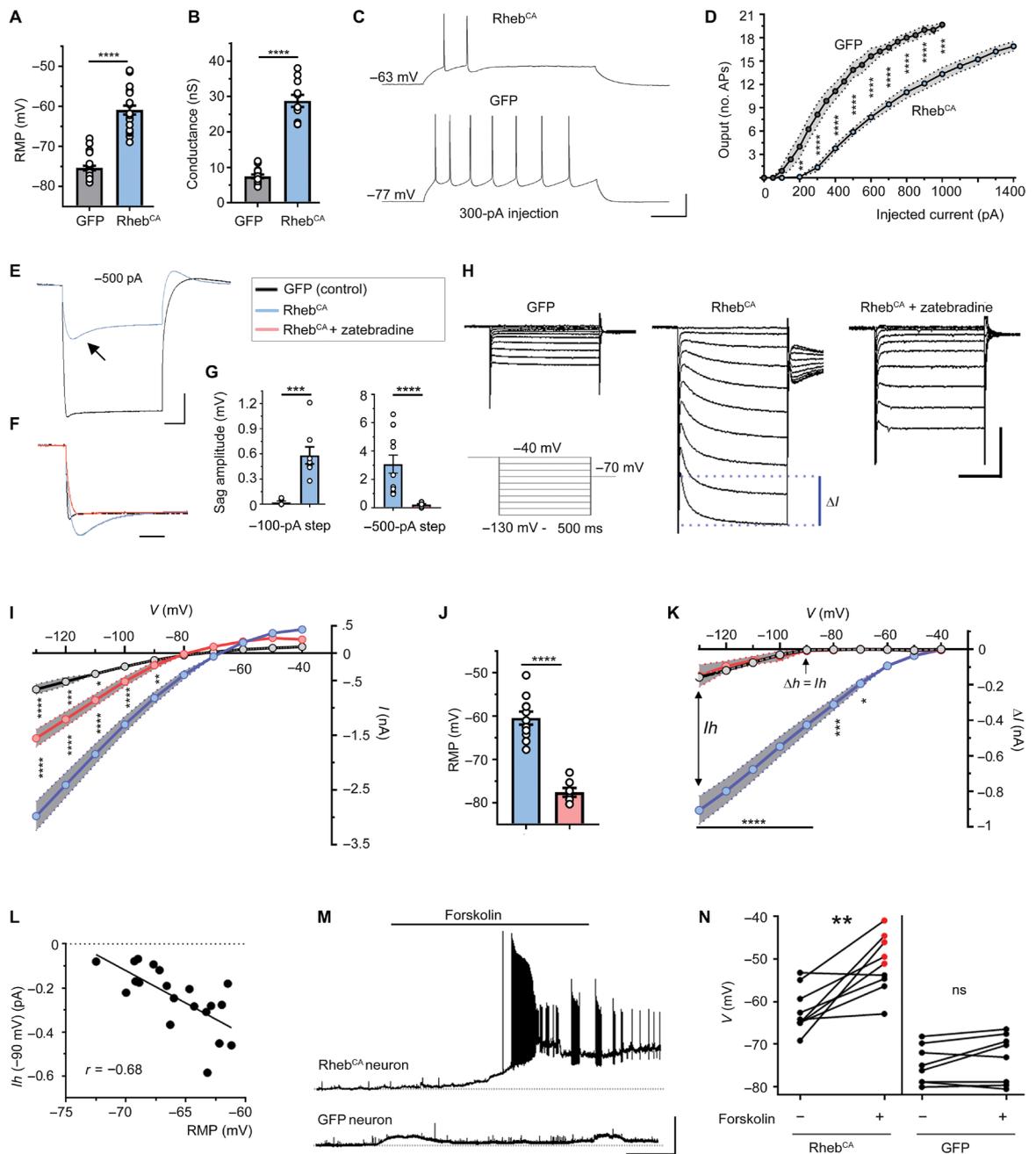
We next examined the electrical properties of FCM neurons using patch clamp recordings in acute brain slices from P26 to P42 mice. As we previously reported, we confirmed that FCM neurons displayed significantly depolarized RMP and increased conductance compared to control neurons in littermate mice expressing only GFP ( $P < 0.0001$ , Student's  $t$  test; Fig. 3, A and B) (21). Consistent with an increased conductance, there was a shift in the input (injected current from their RMP)–output (number of action potentials) curve, suggesting that Rheb<sup>CA</sup> neurons are less likely to generate action potentials from a given excitatory input (Fig. 3, C and D). Using hyperpolarizing current step, we noticed a robust “sag” response in Rheb<sup>CA</sup> neurons that was either minimal or mostly not present in control neurons (Fig. 3, E and G) (25). Such a sag response suggests the presence of HCN channels. Under control conditions, HCN channels are primarily expressed in deep but not in superficial 2/3 layer neurons (25), which are the neuronal population targeted by IUE at E15. In addition, HCN-mediated currents are known to control neuron RMP and may contribute to the depolarized RMP in Rheb<sup>CA</sup> neurons (15, 16, 26). Therefore, we preferentially focused on these channels. To validate that these sags were due to the presence of HCN channels, we recorded Rheb<sup>CA</sup> neurons in the presence of the HCN channel blocker, zatebradine. Zatebradine reduced the sag response in Rheb<sup>CA</sup> neurons (Fig. 3, F and G). Using voltage clamp, we applied 500-ms-long hyperpolarizing voltage steps (from  $-40$  to  $-130$  mV) to activate HCN channels (Fig. 3H). Rheb<sup>CA</sup> neurons displayed large hyperpolarization-activated inward currents that were much larger than in the control neurons (Fig. 3, H and I). These inward currents displayed a slow activation kinetics resembling that of HCN-mediated currents (called  $h$  currents), which required a 3-s-long voltage pulse to reach full amplitude (fig. S1), and were reduced in the presence of zatebradine (Fig. 3, H and I). However, zatebradine did not fully block the increased hyperpolarization-induced inward currents. This is likely due to an increase in the amplitude of inwardly rectifying K currents (Kir) in Rheb<sup>CA</sup> neurons compared to control, which accompanies the increase in cell size. As observed on the current–membrane potential curves (Fig. 3H), zatebradine shifted RMP of cells to hyperpolarized values that were similar to the RMP of control neurons (Fig. 3J and see Fig. 3A). This effect is consistent with a functional block of HCN channels. To quantify the  $h$  current amplitudes, we measured the amplitude difference ( $\Delta I$ ) between the onset and the end of the current trace using a 500-ms-long voltage step (see dotted line and  $I_h$  in Fig. 3H). Zatebradine significantly reduced  $\Delta I$  [ $P < 0.001$  at  $-80$  mV and  $P < 0.0001$  at  $-90$  and up, two-way repeated measures analysis of variance (ANOVA) followed by Tukey posttest; Fig. 3K]. In addition, there was no residual inward current at  $-90$  mV, consistent

with the lack of Kir currents at this voltage (Fig. 3K). Throughout the rest of the manuscript,  $I_h$  current amplitude will then be measured as  $\Delta I$  at  $-90$  mV. Consistent with the normalization of RMP with zatebradine, larger  $h$  currents were associated with more depolarized RMPs in Rheb<sup>CA</sup> neurons (Fig. 3L). The amplitude of the  $h$  currents was also dependent on the amount of electroporated Rheb<sup>CA</sup> plasmid and, thus, the degree of mTOR activation (fig. S1). HCN channels contribute to the generation of rhythmic firing in neurons and heart cells (27, 28) and display different sensitivity to intracellular cAMP concentrations (15, 16). We thus examined whether increasing cAMP in Rheb<sup>CA</sup> neurons would be sufficient to trigger spontaneous, repetitive firing independent of depolarizations. We bath-applied a well-characterized cell-permeable adenylate cyclase activator, forskolin, to increase intracellular cAMP in acute brain slices containing Rheb<sup>CA</sup> neurons. When recorded at RMP, all Rheb<sup>CA</sup> neurons were depolarized by forskolin and 4/9 neurons generated repetitive action potentials (Fig. 3, M and N). This effect did not occur in control superficial layer neurons (layer 2/3; Fig. 3, M and N), which express very few or no HCN channels. Collectively, these data indicate that Rheb<sup>CA</sup> neurons express ectopic HCN channels and the abnormal presence of these channels contribute to the depolarized RMP of Rheb<sup>CA</sup> neurons and their repetitive firing upon increase in intracellular cAMP.

### Ectopic HCN4 expression in FCM neurons

HCN channels are encoded by four genes, *HCN1* to *HCN4*, with different expression patterns throughout the brain (15). In the adult cortex, deep-layer pyramidal neurons predominantly express HCN1 and display low HCN2 expression (29). HCN3 and HCN4 display weak, diffuse expression in the cortex thought to arise from subcortical projections, and HCN4 expression has recently been found in neuronal cell bodies scattered in the cortex that may be GABAergic neurons (29–31). Consistent with the previous finding on HCN1 and HCN2 (29), we observed HCN1 staining in presumed apical dendrites of deep-layer neurons and weak, diffuse HCN2 staining in the cortex (Fig. 4A). However, we found no changes in HCN1 and HCN2 staining pattern in the ipsilateral cortex containing FCM compared to the contralateral cortex of 2-month-old mice (Fig. 4, A, B, and E, and fig. S2, A and B). We found no HCN3 staining in the cortex and no change between ipsilateral and contralateral cortex (Fig. 4E and fig. S2C). However, we identified strong HCN4 immunostaining in the cortex containing Rheb<sup>CA</sup> neurons that was higher than that in the contralateral cortex (Fig. 4, C to E). The increase in HCN4 channel expression in the ipsilateral versus contralateral cortex suggests that aberrant HCN channel expression does not result from recurrent seizures. About 83% of Rheb<sup>CA</sup> neurons displayed HCN4 immunoreactivity that decorated their soma, dendrites, and axons ( $n = 4$  mice; Fig. 4, F and G). One of the HCN4 antibodies (Alomone Labs) was previously validated in conditional knockout mice (30). The specificity of both HCN4 antibodies was further confirmed by small interfering RNA (siRNA)–mediated HCN4 knockdown in vitro (fig. S3). Furthermore, these antibodies showed immunoreactivity in pyramidal neurons electroporated with an HCN4 overexpression plasmid and not in the surrounding non-electroporated cells (fig. S4). Last, cells in mice electroporated with GFP instead of Rheb<sup>CA</sup> did not display HCN4 expression (Fig. 4, H and I). Thus, Rheb<sup>CA</sup>-expressing neurons display selective HCN4 expression that was absent in control pyramidal neurons.

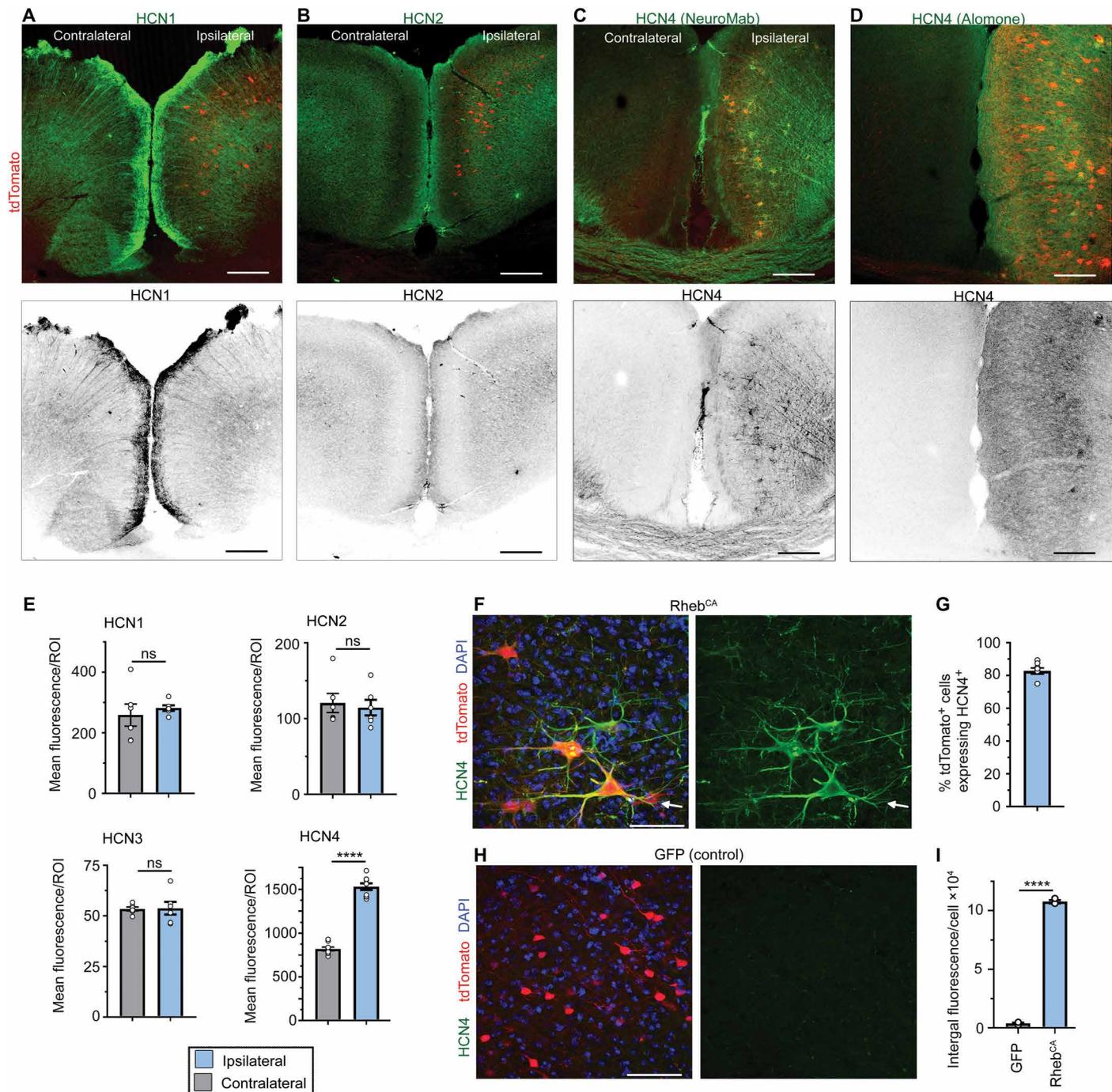
**Fig. 3. Abnormal HCN-mediated currents in FCM neurons are depolarizing and confer cAMP-dependent firing.** (A and B) Bar graphs of the RMP and conductance of control (GFP) and Rheb<sup>CA</sup> neurons recorded in littermate P26 to P42 mice. (C) Representative depolarization and action potentials upon current injection in Rheb<sup>CA</sup> or control (GFP) neurons. Scale bars, 100 ms/40 mV. (D) Injected current amplitude plotted as a function of the mean number of action potentials for generating an input-output curve in Rheb<sup>CA</sup> neurons. The gray area outlines the SEM for each curve. (E) Representative voltage traces in response to a -500-pA step in neurons expressing GFP (control) or Rheb<sup>CA</sup> neurons. Scale bars, 100 ms/40 mV. (F) Voltage traces in response to a -500-pA step from GFP-expressing neurons (black), Rheb<sup>CA</sup>-expressing neurons (green), and Rheb<sup>CA</sup>-expressing neurons in the presence of zatebradine (40 μM, red). Voltage responses were rescaled and superimposed after recording. Scale bar, 100 ms. (G) Plots of the sag amplitude following a -100-pA step from GFP or Rheb<sup>CA</sup> neurons (left) or a -500-pA step protocol from Rheb<sup>CA</sup> neurons under vehicle or zatebradine condition (right). (H) Representative current traces in cortical GFP or Rheb<sup>CA</sup> neurons with vehicle or zatebradine. Protocol: Conditioning step to -40 mV followed by 10-mV hyperpolarizing steps from -130 to -40 mV. Scale bars, 200 ms/1 nA. The blue dotted lines illustrate where the difference in current amplitude ( $\Delta I$ ) was measured within each voltage step to generate current-voltage ( $\Delta I$ -V) curves in (K). (I) Current amplitude ( $I$ , measured at the end of the trace) versus the voltage in each condition. The gray area indicates the SEM for each curve. (J) Bar graphs of the RMP for each condition. (K)  $\Delta I$ -V curves. At -90 mV,  $\Delta I$  corresponds to  $I_h$ . Statistics is for Rheb<sup>CA</sup> versus Rheb<sup>CA</sup> + zatebradine. (L) Scatter plot of  $I_h$  (measured at -90 mV) against RMP. Two-tailed Pearson  $r$  with correlation coefficients. (M and N) Voltage traces during bath application of forskolin on Rheb<sup>CA</sup> and control neurons (M) and resulting plots of the voltage changes (N). The red dots indicate the neurons (4/9) that generated repetitive firing upon forskolin application. Scale bars, 5 min/30 mV. Data are means  $\pm$  SEM. Statistical analyses: Unpaired Student's  $t$  test (A, B, and J), Mann-Whitney  $U$  test (G), paired Student's  $t$  test (N), two-way repeated measure ANOVA, followed by Sidak posttest (D) and by Tukey's posttest (I and K). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . Exact  $P$  values can be found in table S4.



**Abnormal HCN4 expression is mTOR dependent and precedes seizure onset**

To assess whether increased mTOR activity was responsible for the abnormal expression of HCN4, we treated mice with the mTOR inhibitor

rapamycin using the treatment paradigm (1 mg/kg every 48 hours from postnatal day 1 to 2 months of age) that prevented FCM and the development of seizures (14). Rapamycin treatment prevented the expression of HCN4 in Rheb<sup>CA</sup> neurons compared to vehicle-treated

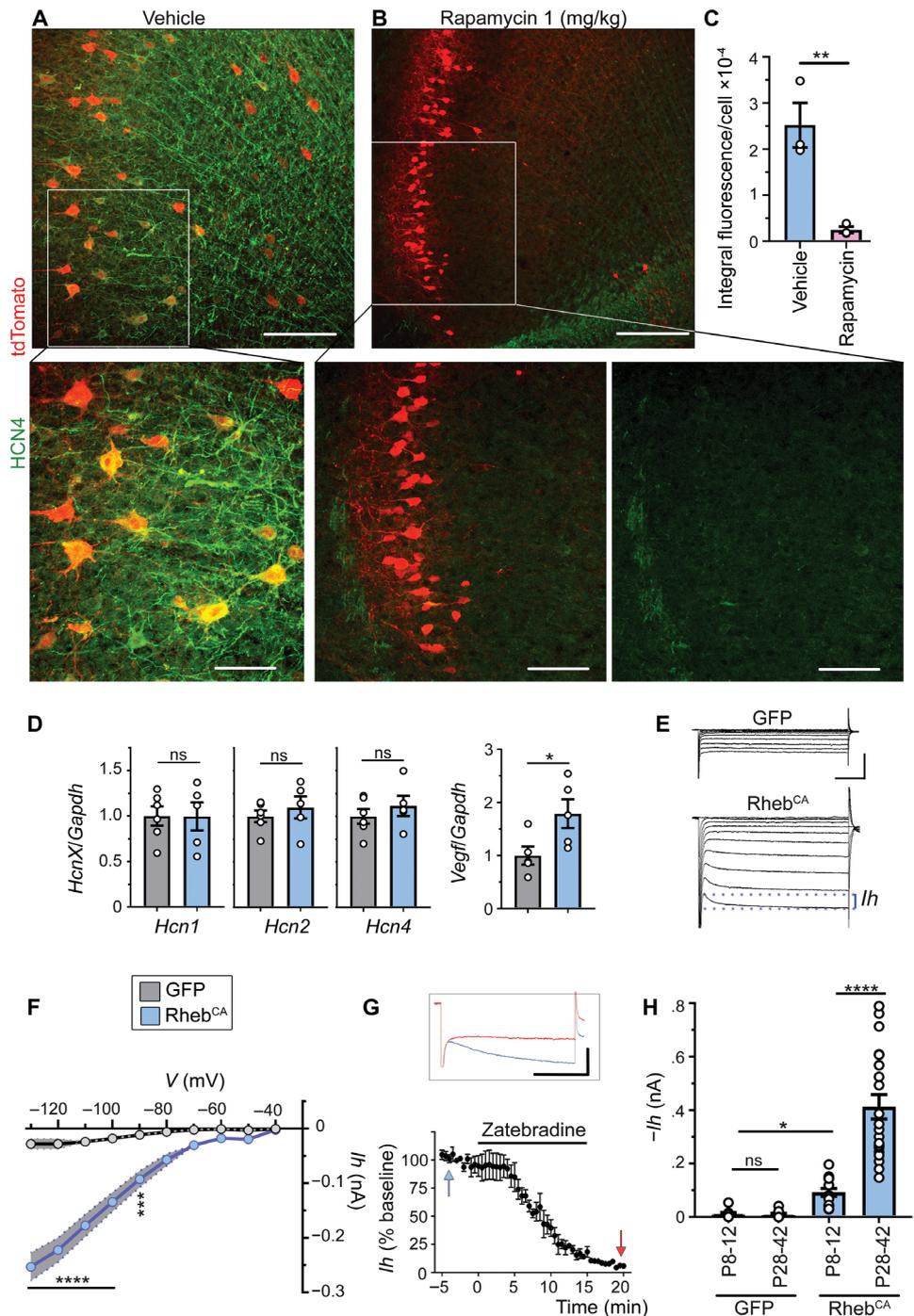


**Fig. 4. FCM neurons display abnormal mTOR-dependent HCN4 expression.** (A to D) Immunostaining for HCN1 (A), HCN2 (B), and HCN4 (C) in coronal sections containing Rheb<sup>CA</sup> neurons coexpressing tdTomato. HCN4 staining was performed using antibodies from NeuroMab and Alomone. The bottom images display HCN staining in B&W. Scale bars, 200 μm. The brain section in (D) was more caudal than the ones in (A) to (C), resulting in a larger cortex. In addition, Rheb<sup>CA</sup> concentration of 2.5 μg/μl instead of 2 μg/μl was used. (E) Quantification of HCN1-HCN4 immunostaining in the ipsilateral versus contralateral cortex. ROI, region of interest. (F) Higher magnification images of HCN4 (green, NeuroMab antibody), 4',6-diamidino-2-phenylindole (DAPI), and tdTomato. Scale bar, 60 μm. (G) Quantification of the percentage (%) of tdTomato<sup>+</sup> cells expressing HCN4<sup>+</sup>. (H) Images of HCN4 immunostaining, tdTomato fluorescence, and DAPI in GFP electroporated mice. Scale bar, 60 μm. (I) Bar graph of integral fluorescence per GFP or Rheb<sup>CA</sup> cell. Data are means ± SEM. Statistical analyses: Paired Student's *t* test [(E) for HCN1, HCN3, and HCN4], unpaired Student's *t* test (I), and Wilcoxon test [(E), for HCN2], \*\*\*\**P* < 0.0001.

mice (Fig. 5, A to C). mTOR not only is a master regulator of protein translation but also can lead to increases in mRNA. Therefore, we examined whether *Hcn4* mRNA was increased in *Rheb<sup>CA</sup>*- compared to GFP-electroporated cortices from 3- to 4-month-old mice. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed for *Hcn1*, *Hcn2*, and *Hcn4*, as well as *Vegf*, which is increased in hyperactive mTOR conditions (32, 33). The expression of *Hcn1*, *Hcn2*, and *Hcn4* mRNA were unchanged, whereas the expression of *Vegf* mRNA was significantly increased ( $P < 0.05$ , Student's *t* test; Fig. 5D). Considering that HCN channel expression has been shown to be up- or down-regulated by seizures (34), we examined whether *Rheb<sup>CA</sup>* neurons would express HCN channels during postnatal cortical development before the onset of convulsive seizures, which were visible in >P21 mice. Recordings in slices from P8 to P12 mice showed that *Rheb<sup>CA</sup>* neurons displayed zatebradine-sensitive *I<sub>h</sub>* currents that were greater than that in control GFP neurons recorded in littermate mice ( $P < 0.001$  at  $-90$  mV and  $P < 0.0001$  at  $-100$  to  $-130$  mV, two-way repeated measures ANOVA followed by Tukey's posttest; Fig. 5, E to G). In addition, there was a significant increase in *I<sub>h</sub>* currents in *Rheb<sup>CA</sup>* neurons during development from age group P6-P12 to P28-P42 ( $P < 0.0001$ , one-way ANOVA) but no change in control neurons (Fig. 5H). These data indicate that mTOR hyperactivity participates in the aberrant expression of HCN4 in FCM neurons before the onset of convulsive seizures.

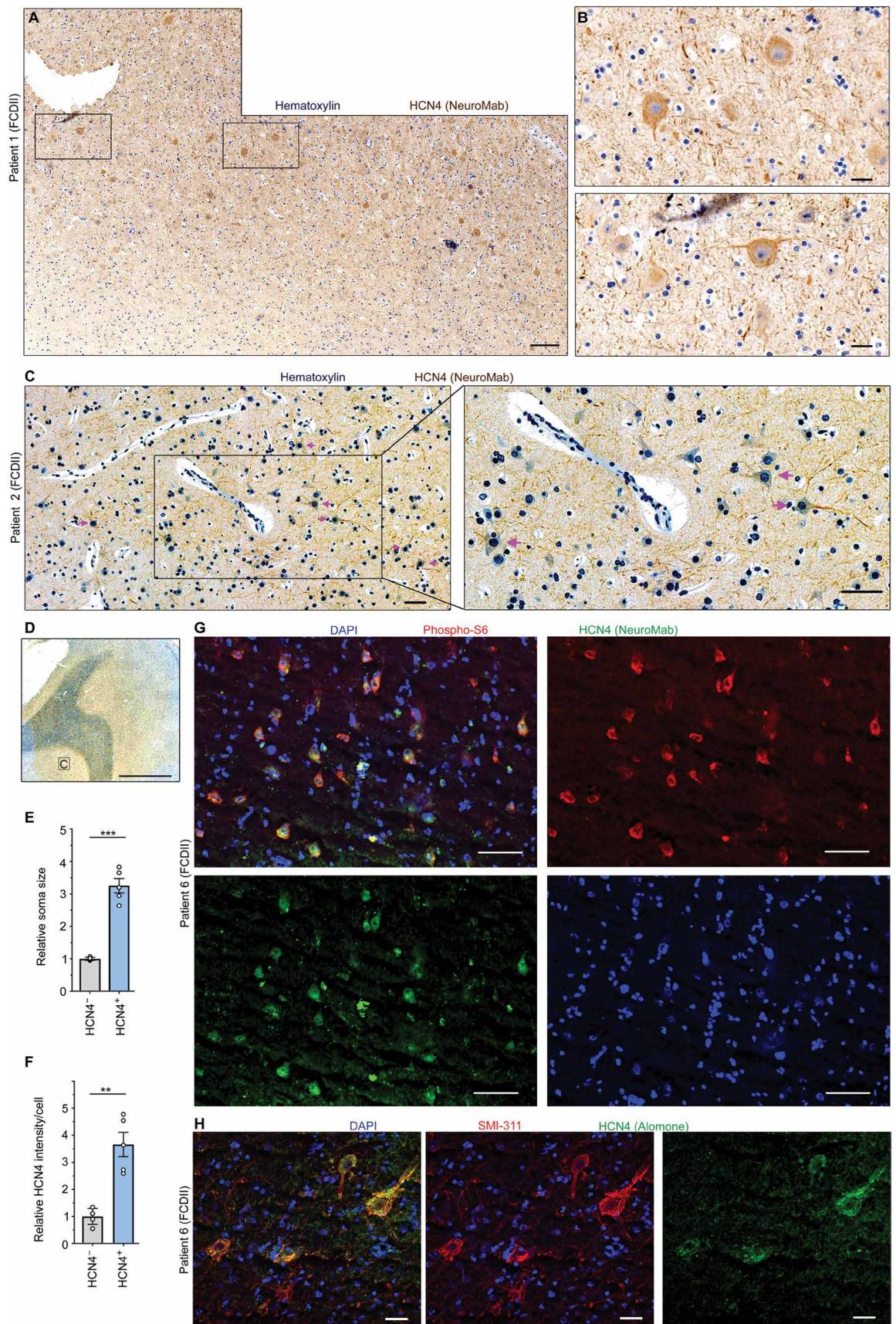
**HCN4 is abnormally expressed in human TSC and FCDII neurons**

Data presented above indicate that HCN4 channels confer a spiking advantage in *Rheb<sup>CA</sup>* neurons that otherwise would not be able to generate repetitive firing upon cAMP stimulation. To examine whether abnormal HCN4 expression occurs in patients with FCM, we obtained cortical tissue from nine patients who underwent surgery for epilepsy due to FCM. Details about the samples (patient age, sex, fixation type, and medical notes) and the staining performed are summarized in table S1. Seven patients had the histopathological diagnosis of FCDII, and two patients had TSC. Patients



**Fig. 5. HCN4 expression is mTOR-dependent and precedes seizure onset.** (A and B) HCN4 immunostaining (NeuroMab) and tdTomato fluorescence in *Rheb<sup>CA</sup>* mice treated with vehicle (A) or rapamycin (B) and higher-magnification images. Scale bars, 140 and 60 μm, respectively. (C) Integral fluorescence in cells expressing *Rheb<sup>CA</sup>* from mice treated with vehicle or rapamycin. (D) Normalized qRT-PCR values for *Hcn1*, *Hcn2*, and *Hcn4* as well as *Vegf* divided by *Gapdh* from microdissected cortices containing GFP or *Rheb<sup>CA</sup>*. (E and F) Representative current traces in P8 to P12 cortical neurons expressing GFP (control) or *Rheb<sup>CA</sup>*. Scale bars, 100 ms/500 pA. The blue dotted lines illustrate where the *I<sub>h</sub>* current amplitude (*I<sub>h</sub>*) was measured within each voltage step to generate *I<sub>h</sub>*-V curves (F). (G) Plot of the zatebradine block of *I<sub>h</sub>* (measured at  $-90$  mV) over time in a *Rheb<sup>CA</sup>* neuron. Left inset: Traces of zatebradine block at  $-90$  mV in *Rheb<sup>CA</sup>* neurons. Scale bars, 200 ms/100 pA. (H) Bar graphs of  $-I<sub>h</sub>$  at the different ages under control and *Rheb<sup>CA</sup>* conditions. Statistical analyses: Unpaired Student's *t* test. (C), two-way repeated measures ANOVA followed by Tukey's posttest (F), and one-way ANOVA (H). Data are means ± SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\*\* $P < 0.0001$ . Exact *P* values can be found in table S4.

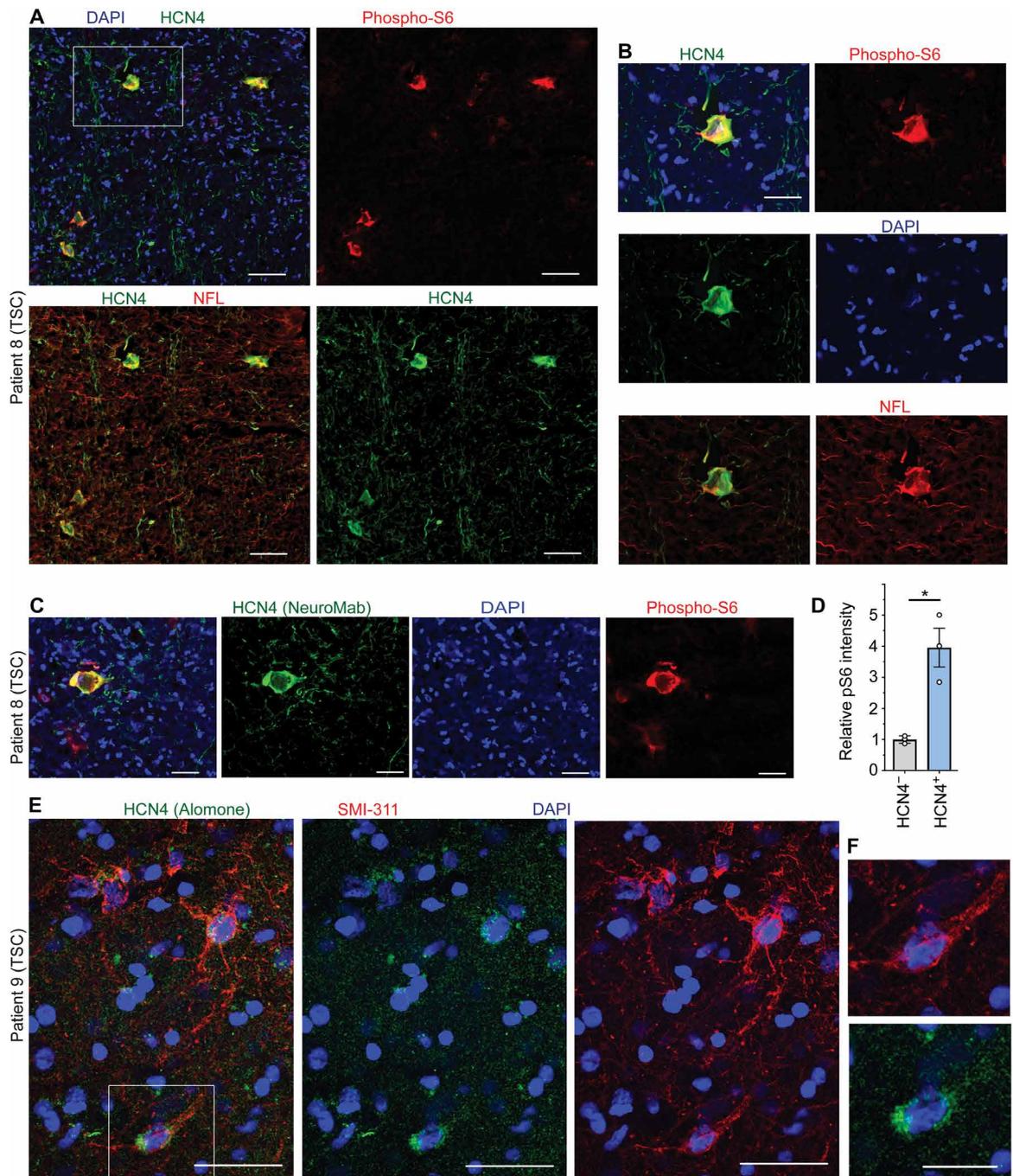
**Fig. 6. Ectopic HCN4 expression in diseased neurons in human FCDII cortices.** (A and B) Staining for HCN4 in patient 1 with FCDII at low (A) and high magnifications (B). Images in (B) were approximately from the squares in (A). Scale bars, 250  $\mu\text{m}$  (A) and 30  $\mu\text{m}$  (B). (C) Staining for HCN4 in patient 2 with FCDII at low and high magnifications. The pink arrows point to HCN4-positive cells. Scale bars, 250 and 30  $\mu\text{m}$ . (D) Image at a lower magnification for patient 2. Scale bar, 2600  $\mu\text{m}$ . (E and F) Relative soma size (E) and HCN4 intensity (F) in HCN4-positive and HCN4-negative cells averaged per FCDII sample. (G and H) Immunostaining for HCN4 and phospho-S6 (G) or SMI-311 (H) in FCDII tissue from patient 6 contained with DAPI. Scale bars, 70  $\mu\text{m}$  (G) and 30  $\mu\text{m}$  (H). Statistical analysis: Paired Student's *t* test.  $**P < 0.01$  and  $***P < 0.001$ .



had FCM detected on magnetic resonance imaging and underwent EEG with a combination of subdural grid and depth electrodes before FCM resection. Patients were identified as FCDII after surgery on the basis of pathological examination of the hematoxylin-stained resected tissue and identification of hallmarks of FCDII, including cortical dyslamination and the presence of cytomegalic neurons. In all the samples ( $n = 7$  FCDII and  $n = 2$  TSC), we performed HCN4 immunostaining and observed enlarged cells with strong HCN4 immunoreactivity (NeuroMab and Alomone antibodies in all nine samples and in three frozen samples, respectively; Figs. 6 and 7 and figs. S5 to S7). Upon quantification, HCN4-positive cells displayed

increased soma size compared to normal-appearing, surrounding cells that had no or weak HCN4 staining (mean of 15 to 53 analyzed cells per sample,  $n = 7$  hematoxylin-stained samples; Fig. 6, E and F). HCN4-positive fibers were visible in six of nine samples (Fig. 6) and may correspond to either processes of diseased neurons or thalamic inputs or both (35). In three frozen samples (two FCDII and one TSC), we coimmunostained for HCN4 and phospho-S6 (Figs. 6, G, and 7, A to C, and fig. S7, A to C). HCN4-positive cells displayed increased phospho-S6 compared to surrounding cells (13 to 14 cells analyzed per sample; Fig. 7D). In addition, in three frozen samples used for immunofluorescence (two FCDII and one TSC), we

**Fig. 7. Ectopic HCN4 expression in diseased neurons in human TSC cortices.** (A and C) Immunostaining for HCN4 and phospho-S6 TSC tissue from patient 8 costained with DAPI. Scale bars, 70  $\mu\text{m}$  (A) and 40  $\mu\text{m}$  (C). (B) Higher magnification of the cell shown in the white square in (A). Scale bar, 35  $\mu\text{m}$ . (D) Quantification of phospho-S6 (pS6) intensity in HCN4<sup>+</sup> and HCN4<sup>-</sup> cells. Statistical analysis: Paired Student's *t* test. \* $P < 0.01$ . (E) Immunostaining for HCN4 and SMI-311 in TSC tissue from patient 9 costained with DAPI. Scale bars, 40  $\mu\text{m}$ . (F) Higher magnification of the cell shown in the white square in (E). Scale bar, 20  $\mu\text{m}$ . NFL, neurofilament light chain.



coimmunostained for SMI-311, which is a pan-neuronal neurofilament (NF) that is accumulated in the soma and dendrites of dysmorphic neurons in human and murine FCD and is considered a marker of these neurons [Fig. 6H and fig. S7 (D to F) for FCDII; Fig. 7 (E and F) for TSC] (10, 14, 36). Every HCN4-positive cell was immunoreactive for SMI-311, identifying them as dysmorphic neurons ( $n = 7$  to 15 cells per sample). Collectively, these data show that both mouse FCM neurons and dysmorphic human neurons from patients with TSC and FCDII display abnormal expression of HCN4 channels.

### Overexpressing HCN4 in control neurons does not lead to convulsive seizures

We next examined whether ectopic expression of HCN4 alone was sufficient to trigger convulsive seizures (27, 28, 37, 38). To do so, we expressed a plasmid encoding HCN4 together with tdTomato or GFP using IUE at E15 to target layer 2/3 neurons. As reported above, HCN4 antibodies detected HCN4 immunostaining selectively in pyramidal neurons electroporated with HCN4 plasmid (fig. S4). Neurons overexpressing HCN4 were located in layer 2/3 as in control condition and did not display increased soma size or increased phospho-S6 staining compared to contralateral layer 2/3 neurons (fig. S8, A to C). In acute slices of P21 to P43 mice, HCN4-overexpressing neurons displayed larger hyperpolarization-activated inward currents and mean depolarized RMP compared with control neurons (fig. S8, D to F). HCN4-overexpressing neurons displayed a mean input-output (number of action potentials) curve similar to that of control neurons, although firing was detected earlier (fig. S8, G and H). We next performed 7-day-long video-EEG recordings of mice expressing HCN4 channels. None of the HCN4-expressing mice displayed tonic-clonic seizures ( $n = 7/7$ ; fig. S8K). Nonetheless, five of seven mice displayed epileptiform discharges containing spikes, spike trains, or waves [defined in (39)] that were absent in GFP-expressing mice (fig. S8, I to K). These data show that HCN4 overexpression in control pyramidal neurons of the mPFC without morphological alterations typical of Rheb<sup>CA</sup> neurons was not sufficient to trigger convulsive seizures.

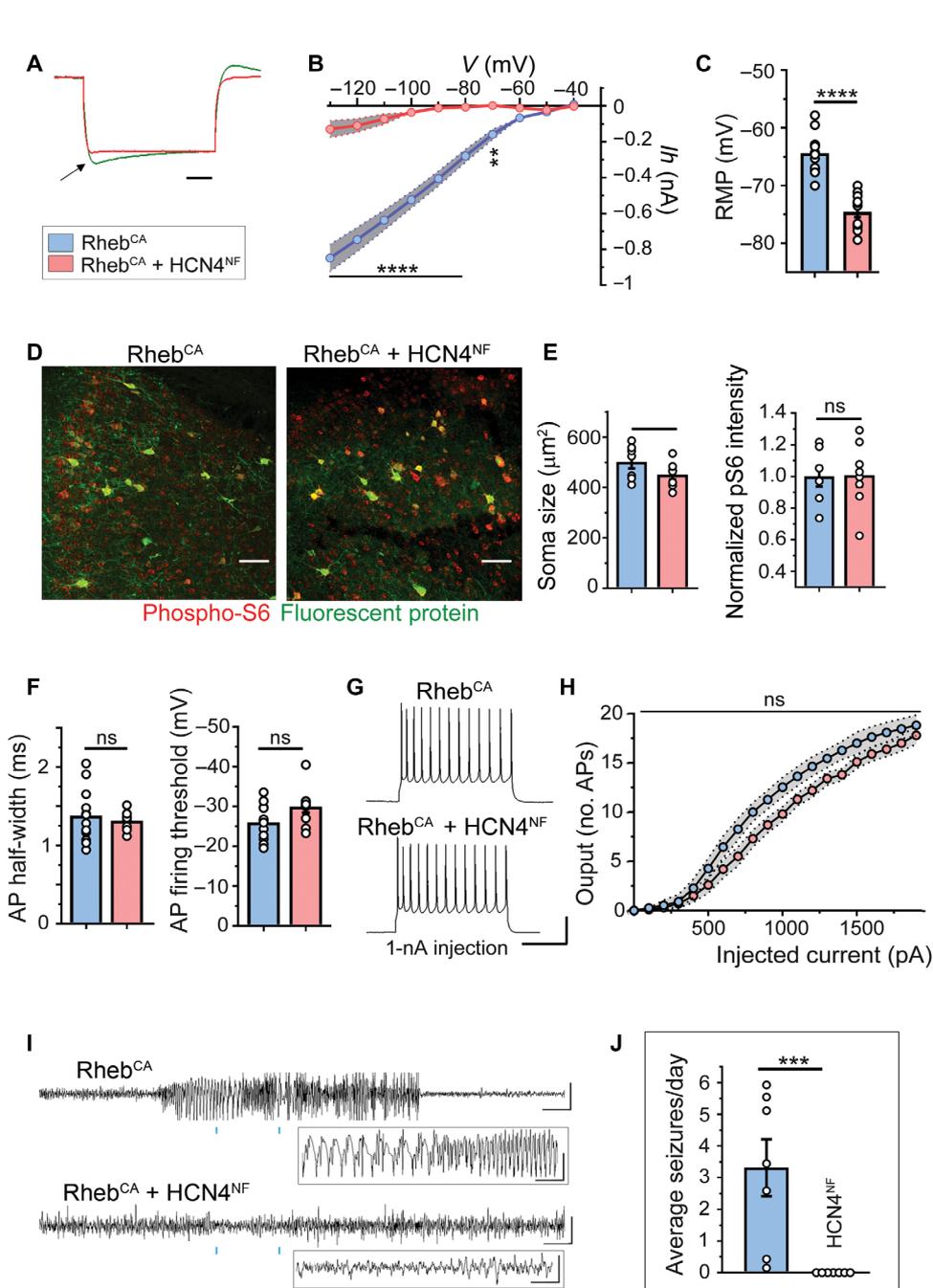
### Blocking HCN4 activity prevents the establishment of epilepsy

To then address whether the ectopic expression of HCN4 channels in Rheb<sup>CA</sup> neurons contributed to seizure generation in FCM-expressing mice, we blocked HCN channel activity *in vivo*. To do so, we expressed nonfunctional HCN4 (HCN4<sup>NF</sup>) subunits that were generated by adding two mutations into HCN4 (40). HCN4<sup>NF</sup> subunits are expected to form heteromers with endogenous HCN4 subunits and render the endogenous HCN4 channel unable to conduct ions, thus acting as dominant negative. Both the sag responses observed in current clamp and the *h* currents recorded in voltage clamp in Rheb<sup>CA</sup> neurons were eliminated in Rheb<sup>CA</sup> neurons that coexpressed HCN4<sup>NF</sup> (Fig. 8, A and B). Furthermore, HCN4<sup>NF</sup> expression hyperpolarized the RMP of Rheb<sup>CA</sup> neurons (Fig. 8C) similarly to what was shown with the HCN channel blocker zatebradine in Fig. 3. Similar to the experiments with Kir2.1, expressing HCN4<sup>NF</sup> in Rheb<sup>CA</sup> neurons did not interfere with mTOR hyperactivity as measured by phospho-S6 immunofluorescence and soma size (Fig. 8, D and E). HCN4<sup>NF</sup> expression in Rheb<sup>CA</sup> neurons did not alter the properties of action potentials (Fig. 8F) or the input-output curve (Fig. 8, G and H). Thus, expressing HCN4<sup>NF</sup> in Rheb<sup>CA</sup> neurons did not alter their ability to generate action potentials, but it blocked the activity of endogenous HCN4 channels and hyperpolarized RMP. Last, a

7-day-long continuous video-EEG monitoring of mice containing Rheb<sup>CA</sup> neurons expressing HCN4<sup>NF</sup> revealed that these mice had no seizures, whereas littermate mice containing Rheb<sup>CA</sup> neurons (without HCN4<sup>NF</sup>) displayed a mean of three daily convulsive seizures (Fig. 8, I and J). Together, these data indicate that the abnormal expression of HCN4 channels in FCM neurons is necessary for generating seizures and blocking HCN4 channels is sufficient to decrease seizure activity.

### DISCUSSION

To examine whether Rheb<sup>CA</sup> neurons may be hyperexcitable and thus trigger seizures, we expressed Kir2.1 channels in Rheb<sup>CA</sup> neurons to hyperpolarize them (18). Kir2.1 expression hyperpolarized Rheb<sup>CA</sup> neurons and increased their membrane conductance, leading to a right shift in the input-output curve without preventing increased cell size, mTOR hyperactivity, and misplacement in mice. Rheb<sup>CA</sup> neurons expressing Kir2.1 were thus less excitable and less responsive to depolarization. As a result of this manipulation, mice had fewer seizures, suggesting that alterations in Rheb<sup>CA</sup> neurons' electrical properties contribute to seizures. Using patch clamp recordings to examine electrical properties, we confirmed that Rheb<sup>CA</sup> neurons have depolarized RMP and are thus closer to the threshold for generating action potentials. However, they also displayed increased conductance and required a larger depolarizing current injection to reach firing threshold. Therefore, Rheb<sup>CA</sup> neurons are less responsive to depolarization than control neurons. These findings seemed to contradict the conclusion from the Kir2.1 experiment, suggesting that Rheb<sup>CA</sup> neurons should not trigger seizures. However, we identified the aberrant expression of HCN-mediated currents, which provided an explanation for the increased likelihood of Rheb<sup>CA</sup> neurons to generate action potentials independent of depolarizing inputs but dependent on intracellular cAMP concentrations, as further detailed below. In young adult mice, Rheb<sup>CA</sup> neurons expressed larger inward currents than in controls, including a combination of HCN-mediated and presumed Kir-mediated currents. Outward currents could not be examined in Rheb<sup>CA</sup> mouse neurons because of unclamped firing upon depolarizing voltage pulses. Because of the large increase in Rheb<sup>CA</sup> neuron size, it was not surprising to find larger Kir-mediated currents, which are expressed in control layer 2/3 pyramidal neurons (41, 42). However, finding HCN-mediated currents was unexpected because control layer 2/3 pyramidal neurons do not express such currents, as confirmed here. HCN channels, particularly HCN2 and HCN4, are sensitive to intracellular cAMP concentrations and that high concentrations lead to increased HCN-mediated currents (15, 16). Considering that the concentrations of cAMP increases in developing hippocampal neurons (43) and could be altered in disease condition, it is conceivable that the increase in HCN-mediated currents could also be due to increased cAMP concentrations in Rheb<sup>CA</sup> neurons. We could not directly measure cAMP concentrations in Rheb<sup>CA</sup> neurons. However, using immunostaining, we identified the selective expression of HCN4 that was not present in control neurons, and expressing HCN4<sup>NF</sup> led to a reduction of HCN-mediated currents. These data suggest that HCN-mediated currents in Rheb<sup>CA</sup> neurons are primarily due to the ectopic expression of HCN4 channels independent of changes in cAMP concentrations. Increasing intracellular cAMP concentrations with forskolin was sufficient to trigger the firing of Rheb<sup>CA</sup> neurons. Therefore, although Rheb<sup>CA</sup>



**Fig. 8. Blocking HCN4 channel activity in FCM neurons prevents epilepsy.** (A and B) Representative voltage traces of Rheb<sup>CA</sup> neurons with and without nonfunctional HCN4 (HCN4<sup>NF</sup>) channel expression. Neurons were recorded in acute slices from P21 to P35 littermate mice electroporated with Rheb<sup>CA</sup> + GFP or Rheb<sup>CA</sup> + HCN4<sup>NF</sup> (+tdTomato). (B) *I*-*h*-*V* curve in each condition. (C) Bar graphs of RMP. Recordings were obtained in acute slices from P21 to P35 mice. (D) Confocal images of GFP and tdTomato fluorescence (pseudo-colored green) and phospho-S6 immunostaining (red) in coronal sections from 4-month-old mice expressing Rheb<sup>CA</sup> + GFP or + HCN4<sup>NF</sup> (+tdTomato). Scale bars, 80 μm. (E) Bar graphs of soma size and normalized (to GFP control cells) phospho-S6 immunofluorescence for neurons expressing Rheb<sup>CA</sup> + GFP or Rheb<sup>CA</sup> + HCN4<sup>NF</sup>. (F) Bar graphs of the action potential threshold and half-width. (G) Representative depolarization and action potentials upon current injection in Rheb<sup>CA</sup> neurons with and without HCN4<sup>NF</sup>. Scale bars, 200 ms/40 mV. (H) Input-output curves of Rheb<sup>CA</sup> neurons with and without HCN4<sup>NF</sup>. The gray area indicates the SEM for each curve. (I) Representative EEG traces in Rheb<sup>CA</sup> mice with and without HCN4<sup>NF</sup>. (J) Bar graphs of the number of seizures per day. Data are means ± SEM. Statistical analyses: Two-way repeated measure ANOVA followed by Sidak posttest (B and H), Student's *t* test (C, E, and F), and Mann Whitney *U* test (J). \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001. Exact *P* values can be found in table S4.

neurons have an increased membrane conductance and require more current injection to reach firing threshold, they are more depolarized and thus closer to the firing threshold, and an increase in intracellular cAMP concentrations, as opposed to excitatory input-induced depolarization, could act as the trigger to induce Rheb<sup>CA</sup> neuron firing. These data show an unanticipated mechanism of excitability that is consistent with a decrease in the excitatory drive [onto Rheb<sup>CA</sup> neurons (18)]. Cortical pyramidal neurons receive multiple inputs [including noradrenergic and dopaminergic innervation from the locus coeruleus (44) and the ventral tegmental area (42, 45)] that activate receptors, leading to cAMP increases. Noradrenaline or dopamine released from these inputs could thus increase cAMP and activate HCN4 channels that would lead to firing and seizures. It is conceivable that preventing aberrant increases in cAMP, through inhibition of these specific receptors, might contribute to reduce seizure activity.

Among the different HCN channels, we found the selective expression of HCN4 channels in Rheb<sup>CA</sup> neurons. HCN4 expression was absent in control pyramidal neurons in our sections, consistent with previous reports showing no expression in cortical pyramidal neurons in adult mice despite the presence of mRNA (29–31, 46, 47). Finding HCN4 channels in neurons that normally do not express these channels may seem unexpected. However, as shown in the developing hippocampus (43, 48), HCN4 channels are expressed perinatally in the cortex and are markedly decreased in young adults (P30) (31). Considering that HCN4 expression was mTOR-dependent and mTOR increases protein translation, it is conceivable that the ectopic HCN4 expression results from increased translation of mRNA already present in pyramidal neurons. Consistent with this idea is the lack of changes in *Hcn4* mRNA expression in gene arrays performed in cortical tuber samples from individuals with TSC (49–51).

Our findings add to a large body of literature on HCN expression and seizures. Several studies have reported alterations in the expression of HCN, particularly HCN1 and HCN2, in different

types of epilepsy in both mice and humans [for review (34, 52)] (53). Regarding HCN4 channels, a study reported no change in HCN4 expression in the hippocampus after febrile seizures despite changes in HCN1 (54). However, loss-of-function mutations in HCN4 have been associated with benign myoclonic epilepsy (55), and increased HCN4 expression in the hippocampal dentate gyrus has been reported in individuals with sudden unexpected death (56). Nevertheless, it was unclear whether the alterations in HCN4 expression preceded seizure occurrence or were responsible for seizures. Here, we identified the abnormal expression of HCN-mediated currents in Rheb<sup>CA</sup> neurons as early as P8, about 2 weeks before the onset of convulsive seizures (observed starting at P21). In addition, HCN4 expression was not detected in the contralateral hemispheres lacking FCM but experiencing epileptiform activity. Overexpression of HCN4 channels in control layer 2/3 pyramidal neurons was not sufficient to cause convulsive seizures despite depolarizing them. However, expressing nonfunctional HCN4 channels in Rheb<sup>CA</sup> neurons that act as dominant negative prevented seizures. These data show that HCN4 channels in FCM neurons are necessary for seizures. The lack of convulsive seizure generation with HCN4 channel expression is expected, considering that FCM neurons display an array of alterations, such as increased dendrite complexity and axon lengths (16, 21, 22). Thus, our data suggest that a combination of alterations is required for seizures, but blocking one single alteration, such as ectopic HCN4 expression or increased filamin A expression (57), is sufficient to prevent seizures.

Identifying ectopic HCN4 has several important clinical implications. At the present time, there are no HCN blockers that are selective for HCN4. However, our findings support HCN4 as a prime candidate for short hairpin RNA (shRNA)-based gene therapy for treating seizures associated with FCDII and TSC that exhibit FCMs. While ectopic HCN4 expression was eliminated with rapamycin treatment, directly targeting HCN4 for epilepsy treatment would prevent the severe adverse events that occur when using the higher rapamycin doses necessary to improve efficacy (6). Furthermore, considering that HCN4 is downstream of mTOR signaling, our findings are likely applicable to other mTORopathies resulting from mutations in the mTOR and GAP activity toward RAGs (GATOR) pathway genes (58).

We acknowledge several limitations to our study. First, we did not use a dual construct containing Rheb<sup>CA</sup> and Kir2.1-mCherry or HCN4. A few cells will thus express Kir2.1-mCherry or HCN4 without expressing Rheb<sup>CA</sup>. Could these cells contribute to the decrease in seizures? This is unlikely for the following reasons: The coelectroporated vectors are expressed in the same cell type, layer 2/3 pyramidal neurons, and ~92% of the electroporated neurons will colocalize both plasmids (59). Considering that IUE targets about 10% of layer 2/3 neurons, we do not anticipate that silencing 0.8% of layer 2/3 control neurons intermingled with Rheb<sup>CA</sup> neurons would be sufficient to prevent seizures. In the case of HCN4<sup>NF</sup>, the control layer 2/3 pyramidal neurons do not express HCN, and hence, the expression of nonfunctional channels is like expressing channelrhodopsin, which has no impact on the cell biophysics. Second, although we found that the increase in HCN4 (protein) is mTOR dependent and there is no increase in its mRNA, the mechanisms leading to this increase remain unknown. It is, however, possible that a small increase in *Hcn4* mRNA was not detectable in microdissected tissue. Third, although it has been shown that blocking another molecular player, filamin A, can reduce seizures in adults (57), it remains to be examined whether blocking HCN4

activity in adults once epilepsy is established can also reduce seizures. Fourth, our seizure data are only acquired using a mouse model of the TSC. It is unknown whether data obtained in mice would be applicable to humans. Nevertheless, considering that our mouse model recapitulates the pathological features observed in humans (14, 17, 18, 20, 23) and the increase in HCN4 is also observed in the cortices of patients with TSC and FCDII, we hope that our seizure data will be applicable to humans. Last, it is essential to assess whether viral delivery of an shRNA against *Hcn4* would reduce seizures without leading to brain inflammation. It would be preferential to express the shRNA selectively in pyramidal neurons using specific promoter to avoid targeting interneurons, some of which may express HCN4, and validate that the viral vector infects diseased neurons. In addition, it will be necessary to assess whether one or multiple viral injections are required to cover the cortical malformations and assess how many diseased neurons need to be infected to reduce seizures.

In conclusion, we have provided evidence that enlarged, dysmorphic mutant neurons in mouse and human FCMs express HCN4 channels that are normally absent in cortical neurons in adults. In mice, this ectopic HCN4 channel expression is mTOR dependent, precedes the development of epilepsy, and contributes to the generation of seizures by depolarizing FCM neurons that are then closer to the firing threshold and by enhancing their cAMP-dependent excitability. Our findings add to the body of literature on HCN channels in epilepsy and highlight a possible mechanism of seizures in mice that involves HCN4 channels that have high cAMP sensitivity. This mechanism might help explain how sensory stimulations leading to the activation of cAMP-generating dopaminergic or adrenergic inputs onto FCM neurons would trigger seizures. In addition, the unique expression of HCN4 channels in dysmorphic FCM neurons provides a potential target for reducing seizures in TSC and FCDII.

## MATERIALS AND METHODS

Details of the methods are provided in the Supplementary Materials.

### Study design

Our research objective was to investigate whether, and if so how, FCM neurons contributed to seizure generation in our mouse model of TSC/FCD. We have extensive experience with the mouse model that we developed that is based on using IUE of Rheb<sup>CA</sup>. To assess whether FCM neurons could contribute to seizures, we expressed selective ion channels, Kir2.1, to hyperpolarize FCM neurons. After finding that Kir2.1 hyperpolarized FCM neurons and decreased seizure frequency, we obtained whole-cell patch clamp recordings to assess the biophysics of FCM neurons. Using patch recordings followed by immunostaining, we identified the expression of HCN4 in FCM neurons. Antibodies were validated with siRNA in cell lines. The Alomone lab was validated by other labs. To then block the activity of HCN4 channels, we expressed nonfunctional HCN4 channels that acted as dominant negative, which was validated with patch clamp recordings. Experimental and control animals were littermates, although several litters had to be used for most experiments, and none were excluded from analysis at the time of harvest. Experimental animals were lost before or during seizure recordings either because of sudden death by seizure or because of technical issues with the recording system. No attempt was made to segregate results on the basis of gender or sex. Analysis was performed blindly,

and seizure analysis was performed by two investigators. Blinding was performed at multiple points during the experiments. For example, after video-EEG recording, the recording files were renamed for analysis by investigators blinded to the treatment condition.

Normality was checked with D'Agostino and Pearson normality test and reported in data file S1. Sample sizes were estimated on the basis of our previous experience both for assessing neuronal biophysical properties and seizure activity. The sample size calculation was performed using power analysis with G\*Power 3 (60). For each set of experiments, the sample size was estimated for an effect size of 50 to 70% using SD calculated from the control population and a power at 0.8 ( $\beta = 0.2$ ) and an  $\alpha$  of 0.05. Each experiment was reproduced at least three times with  $n \geq 3$ , as detailed in table S4. We removed one outlier in Fig. 2F using Grubbs' test, as indicated in data file S1. There was no impact on statistical results (no significant differences). The brain of every recorded mouse was examined after EEG recording to make sure that the mouse with no seizure had proper electro-eporation. Raw data are reports as separate Excel document in data file S1.

### Statistical analyses

All analyses were conducted blindly knowing only the arbitrarily assigned animal ID (independent of electroporation condition). Statistical tests and plots were performed using Prism 7 (GraphPad Software Inc.), with  $P < 0.05$  for significance for all experiments. Specific statistical tests used for each experiment are described in table S4. Data are presented as means  $\pm$  SEM.

### SUPPLEMENTARY MATERIALS

stm.sciencemag.org/cgi/content/full/12/570/eabc1492/DC1

Materials and Methods

Fig. S1. The concentration of Rheb<sup>CA</sup> influences *h* current amplitudes.

Fig. S2. HCN1 to HCN3 immunostaining.

Fig. S3. siRNA validation of HCN4 antibodies.

Fig. S4. HCN4 antibodies detect HCN4 overexpression.

Fig. S5. HCN4 immunostaining in patient 2 with FCDII.

Fig. S6. HCN4 immunostaining in patients 3 to 5 with FCDII.

Fig. S7. HCN4 and phospho-S6 or SMI-311 immunostaining in patient 7 with FCDII.

Fig. S8. HCN4 overexpression does not lead to convulsive seizures.

Table S1. Patient information.

Table S2. Constructs used.

Table S3. Primary and secondary antibodies.

Table S4. Summary of statistical tests.

Movie S1. Example of a convulsive seizure in a mouse containing an FCM.

Data file S1. Raw data.

[View/request a protocol for this paper from Bio-protocol.](#)

### REFERENCES AND NOTES

- R. J. Leventer, R. Guerrini, W. B. Dobyns, Malformations of cortical development and epilepsy. *Dialogues Clin. Neurosci.* **10**, 47–62 (2008).
- P. B. Crino, Focal cortical dysplasia. *Semin. Neurol.* **35**, 201–208 (2015).
- I. M. Najm, H. B. Sarnat, I. Blümcke, Review: The international consensus classification of focal cortical dysplasia—A critical update 2018. *Neuropathol. Appl. Neurobiol.* **44**, 18–31 (2018).
- L. C. Wong-Kissel, T. Blauwblomme, M.-L. Ho, N. Boddaert, J. Parisi, E. Wirrell, R. Nabbout, Challenges in managing epilepsy associated with focal cortical dysplasia in children. *Epilepsy Res.* **145**, 1–17 (2018).
- K. Zhang, W.-H. Hu, C. Zhang, F.-G. Meng, N. Chen, J.-G. Zhang, Predictors of seizure freedom after surgical management of tuberous sclerosis complex: A systematic review and meta-analysis. *Epilepsy Res.* **105**, 377–383 (2013).
- J. A. French, J. A. Lawson, Z. Yapici, H. Ikeda, T. Polster, R. Nabbout, P. Curatolo, P. J. de Vries, D. J. Dlugos, N. Berkowitz, M. Voi, S. Peyrard, D. Pelov, D. N. Franz, Adjunctive everolimus therapy for treatment-resistant focal-onset seizures associated with tuberous sclerosis (EXIST-3): A phase 3, randomised, double-blind, placebo-controlled study. *Lancet* **388**, 2153–2163 (2016).
- R. Stevelink, M. W. Sanders, M. P. Tuinman, E. H. Brilstra, B. P. Koeleman, F. E. Jansen, K. P. Braun, Epilepsy surgery for patients with genetic refractory epilepsy: A systematic review. *Epileptic Disord.* **20**, 99–115 (2018).
- P. H. Iffland II, P. B. Crino, Focal cortical dysplasia: Gene mutations, cell signaling, and therapeutic implications. *Annu. Rev. Pathol.* **12**, 547–571 (2017).
- M. Wong, Mammalian target of rapamycin (mTOR) activation in focal cortical dysplasia and related focal cortical malformations. *Exp. Neurol.* **244**, 22–26 (2013).
- Y. Wang, J. S. Greenwood, M. E. Calcagnotto, H. E. Kirsch, N. M. Barbaro, S. C. Baraban, Neocortical hyperexcitability in a human case of tuberous sclerosis complex and mice lacking neuronal expression of TSC1. *Ann. Neurol.* **61**, 139–152 (2007).
- S. Abdijadid, G. W. Mathern, M. S. Levine, C. Cepeda, Basic mechanisms of epileptogenesis in pediatric cortical dysplasia. *CNS Neurosci. Ther.* **21**, 92–103 (2015).
- L. Kannan, S. Vogrin, C. Bailey, W. Maixner, A. S. Harvey, Centre of epileptogenic tubers generate and propagate seizures in tuberous sclerosis. *Brain* **139**, 2653–2667 (2016).
- P. Major, S. Rakowski, M. V. Simon, M. L. Cheng, E. Eskandar, J. Baron, B. A. Leeman, M. P. Frosch, E. A. Thiele, Are cortical tubers epileptogenic? Evidence from electrocorticography. *Epilepsia* **50**, 147–154 (2009).
- L. S. Hsieh, J. H. Wen, K. Claycomb, Y. Huang, F. A. Harrsch, J. R. Naegele, F. Hyder, G. F. Buchanan, A. Bordey, Convulsive seizures from experimental focal cortical dysplasia occur independently of cell misplacement. *Nat. Commun.* **7**, 11753 (2016).
- R. B. Robinson, S. A. Siegelbaum, Hyperpolarization-activated cation currents: From molecules to physiological function. *Annu. Rev. Physiol.* **65**, 453–480 (2003).
- E. E. Benarroch, HCN channels: Function and clinical implications. *Neurology* **80**, 304–310 (2013).
- L. H. Nguyen, T. Mahadeo, A. Bordey, mTOR hyperactivity levels influence the severity of epilepsy and associated neuropathology in an experimental model of tuberous sclerosis complex and focal cortical dysplasia. *J. Neurosci.* **39**, 2762–2773 (2019).
- T. V. Lin, L. Hsieh, T. Kimura, T. J. Malone, A. Bordey, Normalizing translation through 4E-BP prevents mTOR-driven cortical mislamination and ameliorates aberrant neuron integration. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 11330–11335 (2016).
- D. M. Feliciano, T. Su, J. Lopez, J.-C. Platel, A. Bordey, Single-cell Tsc1 knockout during corticogenesis generates tuber-like lesions and reduces seizure threshold in mice. *J. Clin. Invest.* **121**, 1596–1607 (2011).
- X. Gong, L. Zhang, T. Huang, T. V. Lin, L. Miyares, J. Wen, L. Hsieh, A. Bordey, Activating the translational repressor 4E-BP or reducing S6K-GSK3 $\beta$  activity prevents accelerated axon growth induced by hyperactive mTOR in vivo. *Hum. Mol. Genet.* **24**, 5746–5758 (2015).
- L. Zhang, T. Huang, S. Teaw, A. Bordey, Hypervascularization in mTOR-dependent focal and global cortical malformations displays differential rapamycin sensitivity. *Epilepsia* **60**, 1255–1265 (2019).
- C. A. Lafourcade, T. V. Lin, D. M. Feliciano, L. Zhang, L. S. Hsieh, A. Bordey, Rheb activation in subventricular zone progenitors leads to heterotopia, ectopic neuronal differentiation, and rapamycin-sensitive olfactory micronodules and dendrite hypertrophy of newborn neurons. *J. Neurosci.* **33**, 2419–2431 (2013).
- L. Zhang, C. M. Bartley, X. Gong, L. S. Hsieh, T. V. Lin, D. M. Feliciano, A. Bordey, MEK-ERK1/2-dependent FLNA overexpression promotes abnormal dendritic patterning in tuberous sclerosis independent of mTOR. *Neuron* **84**, 78–91 (2014).
- M. Xue, B. V. Atallah, M. Scanziani, Equalizing excitation–inhibition ratios across visual cortical neurons. *Nature* **511**, 596–600 (2014).
- A. J. Albertson, S. B. Williams, J. J. Hablitz, Regulation of epileptiform discharges in rat neocortex by HCN channels. *J. Neurophysiol.* **110**, 1733–1743 (2013).
- S. Gasparini, D. DiFrancesco, Action of the hyperpolarization-activated current (*I<sub>h</sub>*) blocker ZD 7288 in hippocampal CA1 neurons. *Pflügers Arch.* **435**, 99–106 (1997).
- H. C. Pape, D. A. McCormick, Noradrenaline and serotonin selectively modulate thalamic burst firing by enhancing a hyperpolarization-activated cation current. *Nature* **340**, 715–718 (1989).
- S. Herrmann, F. Hofmann, J. Stieber, A. Ludwig, HCN channels in the heart: Lessons from mouse mutants. *Br. J. Pharmacol.* **166**, 501–509 (2012).
- T. Notomi, R. Shigemoto, Immunohistochemical localization of *I<sub>h</sub>* channel subunits, HCN1–4, in the rat brain. *J. Comp. Neurol.* **471**, 241–276 (2004).
- M. Zobeiri, R. Chaudhary, A. Blaich, M. Rottmann, S. Herrmann, P. Meuth, P. Bista, T. Kanyshkova, A. Lüttjohann, V. Narayanan, P. Hundehage, S. G. Meuth, M. N. Romanelli, F. J. Urbano, H.-C. Pape, T. Budde, A. Ludwig, The hyperpolarization-activated HCN4 channel is important for proper maintenance of oscillatory activity in the thalamocortical system. *Cereb. Cortex* **29**, 2291–2304 (2019).
- A. Battefeld, N. Rocha, K. Stadler, A. U. Bräuer, U. Strauss, Distinct perinatal features of the hyperpolarization-activated non-selective cation current *I<sub>h</sub>* in the rat cortical plate. *Neural Dev.* **7**, 21 (2012).
- D. M. Feliciano, S. Zhang, J. L. Quon, A. Bordey, Hypoxia-inducible factor 1 $\alpha$  is a Tsc1-regulated survival factor in newborn neurons in tuberous sclerosis complex. *Hum. Mol. Genet.* **22**, 1725–1734 (2013).

33. W. E. Parker, K. A. Orlova, G. G. Heuer, M. Baybis, E. Aronica, M. Frost, M. Wong, P. B. Crino, Enhanced epidermal growth factor, hepatocyte growth factor, and vascular endothelial growth factor expression in tuberous sclerosis complex. *Am. J. Pathol.* **178**, 296–305 (2011).
34. Y. Noam, C. Bernard, T. Z. Baram, Towards an integrated view of HCN channel role in epilepsy. *Curr. Opin. Neurobiol.* **21**, 873–879 (2011).
35. S. J. Cruikshank, O. J. Ahmed, T. R. Stevens, S. L. Patrick, A. N. Gonzalez, M. Elmaleh, B. W. Connors, Thalamic control of layer 1 circuits in prefrontal cortex. *J. Neurosci.* **32**, 17813–17823 (2012).
36. I. Blümcke, E. Aronica, H. Miyata, H. B. Sarnat, M. Thom, K. Roessler, B. Rydenhag, L. Jehi, P. Krsek, S. Wiebe, R. Spreafico, International recommendation for a comprehensive neuropathologic workup of epilepsy surgery brain tissue: A consensus Task Force report from the ILAE Commission on Diagnostic Methods. *Epilepsia* **57**, 348–358 (2016).
37. J. Stieber, S. Herrmann, S. Feil, J. Löster, R. Feil, M. Biel, F. Hofmann, A. Ludwig, The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 15235–15240 (2003).
38. S. Y. Abbas, S.-W. Ying, P. A. Goldstein, Compartmental distribution of hyperpolarization-activated cyclic-nucleotide-gated channel 2 and hyperpolarization-activated cyclic-nucleotide-gated channel 4 in thalamic reticular and thalamocortical relay neurons. *Neuroscience* **141**, 1811–1825 (2006).
39. N. Kane, J. Acharya, S. Beniczky, L. Caboclo, S. Finnigan, P. W. Kaplan, H. Shibasaki, R. Pressler, M. van Putten, A revised glossary of terms most commonly used by clinical electroencephalographers and updated proposal for the report format of the EEG findings. Revision 2017. *Clin. Neurophysiol. Pract.* **2**, 170–185 (2017).
40. P. Mesirca, J. Alig, A. G. Torrente, J. C. Müller, L. Marger, A. Rollin, C. Marquilly, A. Vincent, S. Dubel, I. Bidaud, A. Fernandez, A. Seniuk, B. Engeland, J. Singh, L. Miquerol, H. Ehmke, T. Eschenhagen, J. Nargeot, K. Wickman, D. Isbrandt, M. E. Mangoni, Cardiac arrhythmia induced by genetic silencing of ‘funny’ (f) channels is rescued by *GIRK4* inactivation. *Nat. Commun.* **5**, 4664 (2014).
41. L. Saenz del Burgo, R. Cortes, G. Mengod, J. Zarate, E. Echevarria, J. Salles, Distribution and neurochemical characterization of neurons expressing GIRK channels in the rat brain. *J. Comp. Neurol.* **510**, 581–606 (2008).
42. G. Radnikow, D. Feldmeyer, Layer- and cell type-specific modulation of excitatory neuronal activity in the neocortex. *Front. Neuroanat.* **12**, 1 (2018).
43. R. Surges, A. L. Brewster, R. A. Bender, H. Beck, T. J. Feuerstein, T. Z. Baram, Regulated expression of HCN channels and cAMP levels shape the properties of the h current in developing rat hippocampus. *Eur. J. Neurosci.* **24**, 94–104 (2006).
44. D. J. Chandler, W.-J. Gao, B. D. Waterhouse, Heterogeneous organization of the locus coeruleus projections to prefrontal and motor cortices. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 6816–6821 (2014).
45. K. Grzelka, P. Kurowski, M. Gawlak, P. Szulczyk, Noradrenaline modulates the membrane potential and holding current of medial prefrontal cortex pyramidal neurons via  $\beta_1$ -adrenergic receptors and HCN channels. *Front. Cell. Neurosci.* **11**, 341 (2017).
46. L. M. Monteggia, A. J. Eisch, M. D. Tang, L. K. Kaczmarek, E. J. Nestler, Cloning and localization of the hyperpolarization-activated cyclic nucleotide-gated channel family in rat brain. *Brain Res. Mol. Brain Res.* **81**, 129–139 (2000).
47. S. Moosmang, M. Biel, F. Hofmann, A. Ludwig, Differential distribution of four hyperpolarization-activated cation channels in mouse brain. *Biol. Chem.* **380**, 975–980 (1999).
48. A. L. Brewster, Y. Chen, R. A. Bender, A. Yeh, R. Shigemoto, T. Z. Baram, Quantitative analysis and subcellular distribution of mRNA and protein expression of the hyperpolarization-activated cyclic nucleotide-gated channels throughout development in rat hippocampus. *Cereb. Cortex* **17**, 702–712 (2007).
49. K. R. Martin, W. Zhou, M. J. Bowman, J. Shih, K. S. Au, K. E. Dittenhafer-Reed, K. A. Sisson, J. Koeman, D. J. Weisenberger, S. L. Cottingham, S. T. DeRoos, O. Devinsky, M. E. Winn, A. D. Cherniack, H. Shen, H. Northrup, D. A. Krueger, J. P. MacKeigan, The genomic landscape of tuberous sclerosis complex. *Nat. Commun.* **8**, 15816 (2017).
50. J. D. Mills, E. A. van Vliet, B. J. Chen, M. Janitz, J. J. Anink, J. C. Baayen, S. Idema, S. Devore, D. Friedman, B. Diehl, M. Thom, C. Scott, R. Thijs, E. Aronica, O. Devinsky, Coding and non-coding transcriptome of mesial temporal lobe epilepsy: Critical role of small non-coding RNAs. *Neurobiol. Dis.* **134**, 104612 (2020).
51. K. Boer, P. B. Crino, J. A. Gorter, M. Nellist, F. E. Jansen, W. G. Spliet, P. C. van Rijen, F. R. Wittink, T. M. Breit, D. Troost, W. J. Wadman, E. Aronica, Gene expression analysis of tuberous sclerosis complex cortical tubers reveals increased expression of adhesion and inflammatory factors. *Brain Pathol.* **20**, 704–719 (2010).
52. C. A. Reid, A. M. Phillips, S. Petrou, HCN channelopathies: Pathophysiology in genetic epilepsy and therapeutic implications. *Br. J. Pharmacol.* **165**, 49–56 (2012).
53. F. David, N. Çar çak, S. Furdan, F. Onat, T. Gould, Á. Mészáros, G. Di Giovanni, V. M. Hernández, C. S. Chan, M. L. Lörincz, V. Crunelli, Suppression of hyperpolarization-activated cyclic nucleotide-gated channel function in thalamocortical neurons prevents genetically determined and pharmacologically induced absence seizures. *J. Neurosci.* **38**, 6615–6627 (2018).
54. A. Brewster, R. A. Bender, Y. Chen, C. Dube, M. Eghbal-Ahmadi, T. Z. Baram, Developmental febrile seizures modulate hippocampal gene expression of hyperpolarization-activated channels in an isoform- and cell-specific manner. *J. Neurosci.* **22**, 4591–4599 (2002).
55. G. Camprostrini, J. C. DiFrancesco, B. Castellotti, R. Milanese, T. Gnechi-Ruscione, M. Bonzanni, A. Bucci, M. Baruscotti, C. Ferrarese, S. Franceschetti, L. Canafoglia, F. Ragona, E. Freri, A. Labate, A. Gambardella, C. Costa, C. Gellera, T. Granata, A. Barbuti, D. DiFrancesco, A loss-of-function *HCN4* mutation associated with familial benign myoclonic epilepsy in infancy causes increased neuronal excitability. *Front. Mol. Neurosci.* **11**, 269 (2018).
56. M. B. Papanicolou, A. Butt, C. Moore, P15-T increased HCN4 expression in hippocampus of SUDEP patients. *Clin. Neurophysiol.* **130**, e42 (2019).
57. L. Zhang, T. Huang, S. Teaw, L. H. Nguyen, L. S. Hsieh, X. Gong, L. H. Burns, A. Bordey, Filamin A inhibition reduces seizure activity in a mouse model of focal cortical malformations. *Sci. Transl. Med.* **12**, eaay0289 (2020).
58. P. H. Iffland II, V. Carson, A. Bordey, P. B. Crino, GATORopathies: The role of amino acid regulatory gene mutations in epilepsy and cortical malformations. *Epilepsia* **60**, 2163–2173 (2019).
59. N. W. Hartman, T. V. Lin, L. Zhang, G. E. Paquetel, D. M. Feliciano, A. Bordey, mTORC1 targets the translational repressor 4E-BP2, but not S6 kinase 1/2, to regulate neural stem cell self-renewal in vivo. *Cell Rep.* **5**, 433–444 (2013).
60. F. Faul, E. Erdfelder, A.-G. Lang, A. Buchner, G\*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39**, 175–191 (2007).

**Acknowledgments:** We thank Drs. J. Stieber and A. Ludwig (Institute of Experimental and Clinical Pharmacology and Toxicology, Erlangen, Germany) for the pcDNA3-hHCN4 plasmid and Drs. K. Hanada and T. Maehama (National Institute of Infectious Diseases) for the Rheb<sup>CA</sup> plasmid. **Funding:** This work was supported by NIH/NINDS R21 NS093510 (A.B.), DoD TS150058 (A.B.), R01 NS111980 (A.B.), the Swebilius Foundation (A.B.), a Brown Coxe Postdoctoral Fellowship (L.H.N.), an American Epilepsy Society Postdoctoral Fellowship (L.H.N.), NIH/NICHD F32 HD095567 (L.H.N.), and the National Natural Science Foundation of China 81671123 (L.Z.). We thank the Tuberous Sclerosis Alliance for providing one TSC sample. **Author contributions:** L.S.H. and A.B. designed the experiments. L.S.H. performed the patch clamp and EEG experiments and analysis. J.H.W. did most of the immunostaining. L.H.N. did some HCN1-HCN3 immunostaining, the antibody validation using siRNA, and EEG analysis for the HCN4<sup>NE</sup> experiment. S.A.G. did some HCN4 immunostaining. L.Z. performed some IUE. D.D.S., J.T.-R., Y.W., and L.Z. provided human tissue samples. A.B., L.S.H., and L.H.N. wrote the manuscript. **Competing interests:** L.S.H. and A.B. are coinventors on a patent application PCT/US2020/020994 entitled “Methods of treating and diagnosing epilepsy.” A.B. is an unpaid consultant for the Tuberous Sclerosis Alliance and has been a paid consultant for AskBio Inc. **Data and materials availability:** All the data associated with this study are present in the paper or the Supplementary Materials.

Submitted 8 April 2020

Accepted 31 July 2020

Published 18 November 2020

10.1126/scitranslmed.abc1492

**Citation:** L. S. Hsieh, J. H. Wen, L. H. Nguyen, L. Zhang, S. A. Getz, J. Torres-Reveron, Y. Wang, D. D. Spencer, A. Bordey, Ectopic HCN4 expression drives mTOR-dependent epilepsy in mice. *Sci. Transl. Med.* **12**, eabc1492 (2020).

## Ectopic HCN4 expression drives mTOR-dependent epilepsy in mice

Lawrence S. Hsieh, John H. Wen, Lena H. Nguyen, Longbo Zhang, Stephanie A. Getz, Juan Torres-Reveron, Ying Wang, Dennis D. Spencer and Angélique Bordey

*Sci Transl Med* **12**, eabc1492.  
DOI: 10.1126/scitranslmed.abc1492

### Out of place channels drive seizures

Focal cortical malformations (FCMs) develop in a group of diseases caused by mutations in the mTOR pathway. Patients often develop pharmacoresistant epilepsy; however, the molecular mechanisms underlying seizure occurrence are not completely understood. Now, Hsieh *et al.* performed in vitro and in vivo studies in a mouse model of FCM to understand the mechanism of seizure generation. The authors showed that ectopic HCN4 channels expressed in FCM neurons were responsible for seizure generation by depolarizing the resting membrane potential. Expression analysis in cortical tissue from patients with FCM showed increased expression of HCN4, suggesting that HCN4 could be targeted for treating seizures in patients with FCM.

#### ARTICLE TOOLS

<http://stm.sciencemag.org/content/12/570/eabc1492>

#### SUPPLEMENTARY MATERIALS

<http://stm.sciencemag.org/content/suppl/2020/11/16/12.570.eabc1492.DC1>

#### RELATED CONTENT

<http://stm.sciencemag.org/content/scitransmed/11/521/eaaw8954.full>  
<http://stm.sciencemag.org/content/scitransmed/4/161/161ra152.full>  
<http://stm.sciencemag.org/content/scitransmed/10/443/eaar3796.full>  
<http://stm.sciencemag.org/content/scitransmed/9/409/eaam6072.full>

#### REFERENCES

This article cites 60 articles, 9 of which you can access for free  
<http://stm.sciencemag.org/content/12/570/eabc1492#BIBL>

#### PERMISSIONS

<http://www.sciencemag.org/help/reprints-and-permissions>

Use of this article is subject to the [Terms of Service](#)

---

*Science Translational Medicine* (ISSN 1946-6242) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science Translational Medicine* is a registered trademark of AAAS.

Copyright © 2020 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works