In vivo imaging reveals that pregabalin inhibits cortical spreading depression and propagation to subcortical brain structures

Stuart M. Cain,1 Barry Bohnet, Jeffrey LeDue, Andrew C. Yung, Esperanza Garcia, John R. Tyson, Sascha R. A. Alles, Huili Han, Arn M. J. M. van den Maagdenberg, Piotr Kozlowski, Brian A. MacVicar, and Terrance P. Snutch

Migraine is characterized by severe headaches that can be preceded by an aura likely caused by cortical spreading depression (SD). The antiepileptic pregabalin (Lyrica) shows clinical promise for migraine therapy, although its efficacy and mechanism of action are unclear. As detected by diffusion-weighted MRI (DW-MRI) in wild-type (WT) mice, the acute systemic administration of pregabalin increased the threshold for SD initiation in vivo. In familial hemiplegic migraine type 1 mutant mice expressing human mutations (R192Q and S218L) in the Cav2.1 (P/Q-type) calcium channel subunit, pregabalin slowed the speed of SD propagation in vivo. Acute systemic administration of pregabalin in vivo also selectively prevented the migration of SD into subcortical striatal and hippocampal regions in the R192Q strain that exhibits a milder phenotype and gain of CaV2.1 channel function. At the cellular level, pregabalin inhibited glutamatergic synaptic transmission differentially in WT, R192Q, and S218L mice. The study describes a DW-MRI analysis method for tracking the progression of SD and provides support and a mechanism of action for pregabalin as a possible effective therapy in the treatment of migraine.

familial hemiplegic migraine type 1 | migraine | diffusion-weighted MRI | voltage-gated calcium channel | gabapentinoids

Migraine is a common debilitating episodic brain disorder that presents as severe headaches accompanied by other symptoms including nausea and vomiting. In approximately one-third of patients the headache phase is preceded by an aura, thought to be caused by cortical spreading depression (SD) (1). SD is characterized by electrocorticographic silencing and a directly coupled potential shift that is generated by spreading neuronal and glial depolarization. In addition to the aura in migraine, SD is associated with various pathological conditions, such as epilepsy, ischemic stroke, and subarachnoid hemorrhage (2–4). The SD wave front of brief neuronal excitation is followed by a long-lasting depolarization of invaded tissue inactive (5). SD induces cell swelling and the release of various neuroactive factors, including glutamate, potassium, protons, and prostaglandins that together contribute to the pathophysiological process (4). A clue to the importance of SD in migraine comes from the observation that in experimental models cortical SD triggers downstream headache mechanisms through the activation of trigeminal nerves and brainstem nuclei (6).

Familial hemiplegic migraine (FHM) is a monogenic form of migraine with aura accompanied by hemiparesis (1). FHM type 1 (FHM-1) is caused by missense mutations in the CACNA1A gene that encodes the α1A subunit of voltage-gated Cav2.1 (P/Q-type) calcium channels (7). FHM-1 mutations introduced into the orthologous Cacna1a gene produce transgenic mice with phenotypes that closely mimic both the milder (R192Q) and more severe (S218L) symptoms described in FHM-1 patients with these mutations (8, 9). FHM-1 mutations have been shown to produce an overall gain-of-function increase in calcium conductance at physiological membrane potentials (10, 11); given the well-established role of the channels in the calcium-mediated release of vesicular neurotransmitters, this increase can explain the increased synaptic activity observed in the mutant animals (12–16).

Although a number of preventative and abortive treatments are available, not all migraines are effectively treated, and hospitalization can be necessary for prolonged migraine attacks (2, 17). Gabapentinoids (gabapentin and pregabalin) are small-molecule drugs used clinically in the treatment of neuroarthropathic pain and partial seizures. Gabapentin was initially designed as a GABA analog, and pregabalin was developed to modulate GABA metabolism with the aim of generating new treatments for epilepsy. Instead, these drugs were shown to bind to the α2δ1/2 subunits of high-voltage-activated calcium channels with little direct effect on either GABA receptors or metabolism (18). Although, initial studies suggested that gabapentin may be effective in the treatment of migraine, it has since been shown to have only nominal potency in migraineurs (19). Pregabalin (Lyrica) displays more linear kinetics and a longer half-life than gabapentin (20) and has shown initially encouraging results as a potential treatment for migraine.
control vs. WT pregabalin: 

Pregabalin treatment significantly increased the SD

treatment (Fig. S1D) in either the WT (n = 5) or FHM-1 (n = 12) strains. SD could not be initiated in cerebellum, even when stimulation electrodes were placed directly in the vermis of the cerebellar cortex, suggesting that this region is refractory to SD (n = 16).

Pregabalin Slows SD Speed in R192Q and S218L FHM-1 Mutant Mice in Vivo. SD speed was calculated from the cortex as the wave front traveled from slice 5 to slice 8. Both R192Q and S218L mice displayed a faster SD than WT mice, and the speed was faster in S218L mice than in R192Q mice (WT vs. R192Q: P = 0.04; WT vs. S218L: P = 0.01; R192Q vs. S218L: P = 0.02, ANOVA) (Fig. 1C and Movies S1–S3). Notably, pregabalin treatment slowed SD speed significantly in both R192Q and S218L strains but had no effect in WT mice (WT control vs. WT pregabalin: P = 0.23; R192Q control vs. R192Q pregabalin: P = 0.003; S218L control vs. S218L pregabalin: P < 0.0001, t test) (Fig. 1C and Movies S4–S6).

A previous report found that SD is constrained to the cortex in WT mice but can invade the striatum in R192Q mice and can invade the striatum, hippocampus, and, occasionally, thalamus in S218L mice (28). Our data generally are in line with these findings, except that we did not observe SD invasion of the thalamus in S218L animals (Figs. 2 and 3 and Movies S1–S3). Of note, in R192Q mice we observed that SD invaded both the hippocampus and striatum, albeit with a significant delay of up to 1 min after the SD wave front had passed through the cortex. In contrast, in S218L mice the SD wave front invaded only subcortical structures almost simultaneously, consistent with the larger gain-of-function effect of this mutation. Notably, although pregabalin did not prevent the invasion of subcortical structures in S218L mice, it completely abolished spread into both striatum and hippocampus in four of five R192Q mice tested (Figs. 2 and 3 and Movies S2, S3, S5, and S6).

For further visualization and quantitative comparisons of DW-MRI images during SD enhancement, a custom Matlab script was designed to detect the SD wavefront automatically and to represent it in each slice as a heatmap in which cold colors correspond to early and hot colors to late SD appearance. Representative examples shown in Fig. S2 emphasize the cortical confinement of SD in WT mice and the subcortical invasion in FHM-1 mice. Furthermore, they confirm the marked delay in the arrival of the SD wave front in striatum and hippocampus of R192Q mice. Finally, this image analysis tool confirmed that pregabalin administration slows SD speed in both strains of FHM-1 mice and prevents subcortical invasion of SD in R192Q mice.

Pregabalin Slows the Speed of SD in S218L Mutant Brain Slices in Vitro. To examine the effects of pregabalin on SD further, we used IOS imaging on acute brain slices from FHM-1 and WT mice. In this preparation SD is visualized as an increase in brightness resulting from the increased transparency of the brain tissue caused by cell swelling during the depolarization (29). Bath application of 40 mM KCl induced SD in brain tissue, occasionally from more than one focal point (Fig. 4). The SD wave front traveled across the cortex and invaded (and sometimes was initiated in) the caudate putamen in brain slices from both FHM-1 and WT mice (Fig. 4A). In agreement with previous findings (8, 9), the speed of the cortical SD wave front was significantly faster in brain slices from R192Q and S218L animals than in slices from WT mice (WT vs. R192Q: P = 0.01, ANOVA; WT vs. S218L: P = 0.001, ANOVA) (Fig. 4A and B). Pregabalin pretreatment (1 h) had no significant effect on SD speed in brain slices from WT mice, but it significantly slowed SD in brain slices from both R192Q and S218L mice. R192Q control vs. R192Q pregabalin: P = 0.26, t test; R192Q control vs. R192Q pregabalin: P = 0.03, t test; S218L control vs. S218L pregabalin: P = 0.0005, t test) (Fig. 4B). No significant difference in the degree of localized cell swelling correlated with ΔIOS (29) was observed either among strains or as a result of pregabalin pretreatment (Fig. 4C).

![Fig. 1. DW-MRI in vivo. (A) Diagram showing the setup for DW-MRI scanning. (B) Mean in vivo data for the SD stimulation threshold in vehicle- and pregabalin-pretreated mice: WT control = 35.0 ± 6.5 μC (n = 5); R192Q control = 35.8 ± 9.3 μC (n = 6); S218L control = 10.0 ± 2.2 μC (n = 6); WT pregabalin = 96.0 ± 18.6 μC (n = 5); R192Q pregabalin = 58.0 ± 10.2 μC (n = 5); S218L pregabalin = 30.5 ± 11.4 μC (n = 8). (C) Mean data for wave-front speed: WT control = 4.9 ± 0.2 mm/min (n = 5); R192Q control = 6.4 ± 0.1 mm/min (n = 6); S218L control = 5.7 ± 0.4 mm/min (n = 5); WT pregabalin = 4.8 ± 0.6 mm/min; R192Q control = 5.3 ± 0.3 mm/min (n = 5); S218L pregabalin: 6.3 ± 0.1 mm/min (n = 8). *P < 0.05, one-way ANOVA with Tukey’s post hoc test (between strains) and paired sample t test (control versus pregabalin treatment).](#)
Pregabalin Acutely Inhibits CaV2.1 Calcium Channel Complexes Containing the α2δ1 Subunit. To confirm pregabalin’s effects on CaV2.1-mediated calcium currents, electrophysiological recordings were performed in human-derived neuroblastoma SH-SYSY cells transiently expressing the recombinant human CaV2.1 subunit coexpressed with β2 and either α2δ1 or α2δ3 auxiliary subunits. The effect of pregabalin pretreatment was assessed at a concentration of 500 μM, previously reported to inhibit synaptic activity in the mouse brainstem auditory system (30). The peak calcium current amplitude elicited by repetitive square depolarizations from a holding potential of −110 mV to 0 mV was reduced by 27% (n = 8) when 500 μM pregabalin was applied to SH-SYSY cells expressing CaV2.1 cotransfected with β2 and α2δ1 subunits (Fig. 5A). Conversely, currents obtained upon coexpression with the β2 and α2δ3 subunits were unaffected by pregabalin (Fig. 5A). The selective inhibitory effect on CaV2.1 channels coexpressed with the α2δ1, but not with the α2δ3 auxiliary subunit was observed across a range of voltage steps elicited by a current–voltage relationship (Fig. 5B). These results are consistent with gabapentinoids’ higher binding affinity for the α2δ1 subunit than for the α2δ3 subunit (31).

Pregabalin Suppresses Spontaneous and Evoked Synaptic Activity. We next examined whether the effects of pregabalin on intrinsic neuronal excitability and/or synaptic activity levels could explain the differential alterations in the threshold and speed of SD in WT and FHM-1 mice. Whole-cell patch-clamp recordings in hippocampal acute slices were used because the DW-MRI analyses revealed that SD invasion occurred in this region in S218L and R192Q mice but not in WT mice. Potential differences in global hippocampal synaptic activity were investigated by recording spontaneous excitatory postsynaptic currents (sEPSCs) (see Fig. S4B) in the absence of any stimulation or current injection, using whole-cell voltage-clamp recordings with picrotoxin in the patch pipette to block GABAergic inhibitory PSCs (IPSCs). Pregabalin (500 μM) significantly reduced the amplitude and increased the sEPSC interval (i.e., decreased the sEPSC frequency) in CA1 neurons in slices from both WT and R192Q mice (Fig. 5C and D and Figs. S3 and S4A). Rather unexpectedly, pregabalin was found to increase amplitude and decrease the interevent interval in S218L CA1 neurons. At a lower concentration (100 μM) pregabalin had no effect on sEPSC amplitude or frequency in WT neurons, reduced the frequency of sEPSCs in both R192Q and S218L neurons, and increased the amplitude of sEPSCs in S218L CA1 neurons (Fig. 5E and Fig. S4A).

To examine the effect of pregabalin on electrically evoked EPSPs (eEPSPs) in hippocampal slices, a paired-pulse stimulation protocol was applied to glutamatergic CA3 axons (Schaffer collaterals) while simultaneously recording voltage responses using current-clamp recordings in CA1 soma (Fig. 5F). To ameliorate cell-to-cell variability, eEPSP amplitude was normalized to the control eEPSP peak. Although pregabalin (500 μM) significantly reduced the amplitude of eEPSPs in both WT and R192Q neurons, it had no significant effect on S218L CA1 neurons. At a lower concentration (100 μM), pregabalin inhibited eEPSP amplitude only in R192Q CA1 neurons. No significant effect of pregabalin was observed on paired-pulse facilitation (Fig. 5F).

Discussion
One aim of this study was to develop advanced neuroimaging methodologies that permit the in vivo visualization of SD in a
only the S218L CaV2.1 channel gain-of-function mutation was in S218L mice than in WT and R192Q animals, suggesting that FHM-1 mutations; (7) threshold and speed are differentially affected by R192Q and S218L findings concerning SD and the actions of pregabalin are (8) may exhibit functional effects in FHM-1 and WT mice. Our key findings concerning SD and the actions of pregabalin are (i) SD threshold and speed are differentially affected by R192Q and S218L FHM-1 mutations; (ii) in both FHM-1 strains SD invades the striatum and hippocampus, albeit with a notable delay in R192Q animals; (iii) SD does not invade the cerebellum in any strain or under any stimulation protocol; (iv) the SD threshold is increased by pregabalin in WT but not in FHM-1 mice; (v) pregabalin slows SD velocity in both FHM-1 strains but not WT mice; and (vi) SD invasion of subcortical structures is suppressed by pregabalin in animals expressing the milder R192Q change but not in animals expressing the more severe S218L FHM-1 mutation.

SD Threshold and Speed Are Differentially Affected by the R192Q and S218L FHM-1 Mutations. We observed a lower SD threshold in vivo in S218L mice than in WT and R192Q animals, suggesting that only the S218L CaV2.1 channel gain-of-function mutation was sufficiently pathophysiological to promote the initiation of SD as a result of cortical electrical depolarization. This finding was somewhat unexpected because previous data indicated a lower SD threshold associated with both FHM-1 mutations (8, 9) and also because both R192Q and S218L mice display enhanced glutamatergic activity in cortical neurons (15, 16, 32). Notably, the use of isoflurane (instead of urethane) in the current study may have differentially affected the SD threshold. Also, the carbon fiber electrodes used for stimulation in the MRI scanner are larger in diameter than the metal electrodes used previously, and this difference may have affected stimulation sensitivity (9). If the stimulation threshold is modulated solely by the level of synaptic excitability, a lower SD threshold would be expected in both S218L and R192Q mice. Our findings may indicate that the SD threshold is not linked directly to synaptic activity but instead is linked to enhanced basal neuronal excitability in the cortex. This notion fits with previous data demonstrating that cortical neurons from S218L mice display calcium currents with a distinct leftward shift in activation properties that is less pronounced in R192Q mice. As a result, S218L neurons are predicted to have the ability to conduct calcium at rest, endowing them with the ability to modulate neuronal excitability at a range of membrane potentials normally considered subthreshold (13). Although the SD threshold was found to be decreased only in S218L animals, we observed an increase in cortical SD conduc tion velocity, similar to that described previously (8, 9), in both R192Q and S218L FHM-1 strains. The gain-of-function alterations of CaV2.1 channels containing R192Q or S218L mutations likely

Fig. 4. IOS imaging in acute coronal brain slices. (A) Representative images of brain slices from WT, R192Q, and S218L mice following KCl (40 mM) application to initiate SD. (B) Mean IOS data for SD wave-front speed: WT control: 2.9 ± 0.3 mm/min (n = 20); R192Q control: 5.4 ± 0.4 mm/min (n = 16); S218L control: 5.8 ± 0.7 mm/min (n = 14); WT pregabalin: 2.5 ± 0.1 mm/min (n = 14); R192Q pregabalin: 4.2 ± 0.3 mm/min (n = 14); S218L pregabalin: 2.9 ± 0.9 mm/min (n = 13). (C) Mean data for the IOS signal in control and pregabalin-pretreated brain slices. *P < 0.05, one-way ANOVA with Tukey’s post hoc test (between strains) and two-sample t test (control versus pregabalin treatment).

Fig. 5. Pregabalin acutely inhibits CaV2.1 Ca2+ currents and both spontaneous and evoked synaptic activity. (A) Time course of pregabalin (500 μM) on currents recorded from SH-SY5Y cells cotransfected with the CaV2.1 αδ1 and αδ2 (n = 8) (Left) or with αδ1 (n = 4) (Right) subunits. Insets show representative currents (black, control; grey, pregabalin) and mean fractional inhibition (Right). (B) Mean CaV2.1 current density-voltage relationships obtained before and after the application of pregabalin. Insets show representative currents. (C) Schematic of acute hippocampal brain slice preparation used in C-E for whole-cell voltage-clamp recordings in CA1 neurons. Shown are mean data for the effect of 500 μM pregabalin on sEPSC amplitude (Upper) and the interevent interval (Lower). WT: n = 7 cells, 4 animals; R192Q: n = 6 cells, 4 animals; S218L: n = 9 cells, 4 animals. (D) Representative current traces taken from 60-μs voltage-clamp recordings at CA1 soma showing the effect of pregabalin (500 μM) on sEPSCs in WT, R192Q, and S218L FHM-1 strains. (Scale bars: horizontal, 100 ms; vertical 10 pA) (E) Mean data for the effect of 100 μM pregabalin on sEPSC amplitude (Upper) and the interevent interval (Lower). WT: n = 5 cells, 3 animals; R192Q: n = 4 cells, 3 animals; S218L: n = 5 cells, 3 animals. Insets show representative eEPSPs from WT, R192Q, and S218L CA1 neurons (black, control; grey, pregabalin); WT: n = 6 cells, 4 animals; R192Q: n = 6 cells, 4 animals; S218L: n = 8 cells, 4 animals. (Inset scale bars: horizontal, 50 ms; vertical, 5 mV.) **P < 0.05.
underlie the observed increases in SD speed in these strains; this possibility is supported by studies demonstrating enhanced synaptic activity in cortical neurons from both R192Q (15, 32) and S218L mice (16).

**SD Spreads to Subcortical Structures in Both FHM-1 Strains but Not in WT Mice.** Several mechanisms, such as interneuron-mediated signaling or passive diffusion of neuroactive substances through extracellular fluid (5), have been proposed to contribute to the spread of SD. In R192Q mice, a progressive spread of SD to subcortical structures was observed particularly in slice 5 (bregma -2.50 mm). Typically, the SD propagated ventrolaterally from the dorsomedial cortex until reaching the entorhinal cortex, followed by the delayed appearance of SD first in the ventral hippocampus and then in the dorsal hippocampus. Similarly, this pattern of SD propagation was observed in slice 6 in R192Q mice, with SD spreading into the striatum (Figs. 2 and 3 and Movie S3). These results suggest that failure-points where SD propagation is limited normally exist between certain interconnected areas, for example the entorhinal cortex–subiculum and piriform cortex–striatum, and that a diffusional and/or synaptic barrier must be bridged for SD to spread further. We speculate that neurons expressing CaV2.1 channels containing either R192Q or S218L mutations enable the spread of SD across subcortical failure-points.

Hippocampal SD has been reported previously after direct stimulation of the CA1 region in Sprague–Dawley rats (33), indicating that SD can indeed be initiated in this brain region but likely does not receive sufficient input from cortical SD and/or that a barrier in this region prevents the propagation of cortically initiated SD. Invasion of SD into the striatum has been observed in some rat models (34), and in our IOS slice preparation we regularly observed SD invasion and initiation in the striatum of WT mice. Because WT animals did not display subcortical SD in vivo, it is likely that, like the hippocampus, the striatum is capable of SD but cannot normally propagate the wave in response to adjacent cortical SD. It has been suggested that in FHM-1 mice, SD propagation into the striatum and hippocampus via the amygdala and subiculum, respectively, and that lower neuronal densities in these areas may be responsible for limiting SD spread in WT animals (28). Our data from R192Q animals support this view with respect to the anatomical entry point of SD into these structures for this strain. In S218L mice, in which SD spreads immediately from the adjacent cortex to subcortical structures, the stronger gain-of-function effect of the mutation on CaV2.1 channels apparently generates an SD wave that is sufficiently powerful to traverse the corpus callosum. Of further interest is that SD is not observed to propagate into the thalamus for either of the FHM-1 mutations, although this propagation has been reported to occur in a percentage of S218L mice (28). It should be considered that in the study by Eikermann-Haerter et al. (28) the electrophysiological measurements of SD were made under pentobarbital anesthesia, whereas our experiments were performed under isoflurane anesthesia; the two anesthetics may have distinct effects on SD invasion into the thalamus (35). Despite this difference, both studies indicate that the white matter of the internal capsule appears to be a particularly difficult region for SD to traverse.

It is noteworthy that we did not observe SD in the cerebellum, even when stimulation electrodes were placed directly into the cerebellar cortex. In agreement with our findings, cerebellar SD has not been reported previously in the FHM-1 mouse strains, although it has been observed in rats (36). Strong cerebellar symptoms in FHM-1 patients, including those with the S218L mutation, are well described, and altered synaptic activity has been reported in cerebellar synapses in S218L mice (12, 37). Our results suggest that the FHM-1–mediated cerebellar symptoms are not associated with functional changes that might occur as a result of any recurrent SD within the cerebellum.

**SD Threshold Is Increased by Pregabalin in WT but Not in FHM-1 Mice.** Gabapentin has been shown to increase the threshold for SD in vivo (38), and we hypothesized that pregabalin also might alter SD. Furthermore, gabapentin and pregabalin are known to bind to the α2δ1/2 auxiliary subunit of CaV2.1 calcium channels, and gabapentinoid-mediated inhibition of calcium currents has been reported both acutely (24, 30, 39) and via chronic effects on channel trafficking (25, 40). In the current study pregabalin was effective in increasing the threshold for stimulation to induce SD in WT but not R192Q or S218L mice. This finding suggests that pregabalin may be an effective treatment for preventing migraine with aura in migraineurs without FHM-1 mutations. In contrast, in FHM-1 patients the gain-of-function phenotypes conferred to CaV2.1 channels may be too severe for pregabalin to be therapeutically efficacious. We did, however, observe a trend toward increased threshold following acute pregabalin treatment in FHM-1 mice, and therefore pregabalin should not necessarily be ruled out as a chronically administered preventative treatment for FHM-1 patients.

**Pregabalin Slows SD in FHM-1 Strains but Not in WT Mice.** We demonstrated that pregabalin slowed SD in S218L and R192Q but not WT mice. As discussed, cortical synapses that express CaV2.1 channels containing the R192Q and S218L mutations display enhanced excitatory, but not inhibitory, neurotransmission (16, 32), and a leftward shift in the activation curve of CaV2.1 channels containing the S218L mutation allows calcium conduction at resting membrane potentials (16). Together these properties would permit subthreshold modulation of excitability by CaV2.1 in S218L mice, providing a potential mechanism underlying the more severe phenotype in this strain. Pregabalin has a direct inhibitory effect on calcium-mediated glutamate release in brain slices from neocortex (41) and entorhinal cortex (42). In addition, pregabalin displays two- to threefold enhanced efficacy in the depression of noradrenaline release upon sustained depolarization as compared with brief stimulation (26, 43). The long-lasting depolarization that occurs during SD may further increase the efficacy of pregabalin on neurotransmitter release when attenuating the synaptic gain of function resulting from R192Q and S218L mutations. Overall, pregabalin’s greater effectiveness in slowing SD speed in FHM-1 mice than in WT mice directly supports our hypothesis that pregabalin has a functional inhibitory effect on native CaV2.1 channels.

**SD Invasion of Subcortical Structures Is Abolished by Pregabalin in R192Q Mice.** A notable finding from our DW-MRI experiments is that pregabalin prevented the subcortical invasion of SD in four of five R192Q animals. Given that pregabalin did not prevent subcortical invasion in S218L mice, we would argue that pregabalin suppresses excitability sufficiently to prevent SD propagation through failure points that block the spread of SD into subcortical structures in the milder R192Q phenotype mice. Previous studies have demonstrated that pregabalin inhibits vesicle trafficking in hippocampal neurons, reducing the readily releasable pool (44) and attenuating vesicle release (45). In hippocampal brain slices, we observed that pregabalin (500 μM) effectively suppressed both spontaneous and evoked synaptic activity in WT and R192Q CA1 neurons but not in S218L CA1 neurons. This strain-specific inhibition of evoked synaptic function provides a molecular mechanism for pregabalin to suppress the invasion of the hippocampus in R192Q mice but not in S218L mice. Although SD did not invade the hippocampus spontaneously in WT mice, pregabalin’s efficacy on synaptic activity in WT CA1 neurons suggests that it may provide an effective preventative treatment to limit the subcortical progression of migraine in non-FHM-1 migraineurs and in milder forms of FHM-1.

In summary, we find that SD is limited to defined brain regions by as yet unknown synaptic and/or diffusional barriers involving CaV2.1 (P/Q-type) calcium channel–mediated excitability and that these barriers can be bridged by the consequences of FHM-1 mutations. Furthermore, pregabalin is effective in increasing the threshold for SD in WT animals, in slowing SD velocity in FHM-1 mice, and in preventing SD subcortical propagation associated with the milder R192Q FHM-1 mutation. These findings support the notion that pregabalin may be effective acutely but have
not addressed its effects with chronic drug administration. Although gabapentin has recently fallen from favor as a preventative treatment in migraine (21–23). The current study provides insights into the mechanism of action of pregabalin and supports its therapeutic potential in both non-REM and patients with milder FHM-1 mutations.

Materials and Methods

Full details on methods used for DW-MRI, brain slice IOS, cell culture, and electrophysiology can be found in the Materials and Methods. All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and the University of British Columbia Animal Care Committee (see SI Materials and Methods).

ACKNOWLEDGMENTS. This work was supported by a Brain Canada Multi-Investigator Research Initiative Grant with matching support from Genome BC, the Michael Smith Foundation for Health Research, and the Koerner Foundation. T.P.S. and S.M.C. were supported by a Pfizer Neuropathic Pain Award. T.P.S. also was supported by Canadian Institutes of Health Research (CIHR) Operating Grant 10677 and by the Canadian Research Chair (CRC) in Biotechnology and Genomics-Neurobiology. S.M.C. was supported by a research grant from the BC Epilepsy Society. B.A.M. was supported by a CRC in Neuroscience, CIHR Operating Grants 148397, 8545, and 115121, T.C.E–117869 in the framework of European Research Area Network NEURON, and the Fondation Leducq.
Supporting Information

Cain et al. 10.1073/pnas.1614447114

SI Materials and Methods

Animals. WT, transgenic R192Q, and S218L littermates postnatal day (P)20–P40 male and female mice were used in all experiments and were genotyped as previously described (8, 9).

Acute Brain Slice Preparation. Animals were anesthetized using isoflurane [5% in oxygen (vol/vol)] and were killed by cervical dislocation. The brains were removed rapidly and transferred to ice-cold sucrose-artificial cerebral spinal fluid (sucrose-asCSF) containing 214 mM sucrose, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 11 mM glucose, 2.5 mM KCl, 0.5 mM CaCl₂, 6 mM MgCl₂, bubbled with 95% O₂:5% CO₂. Brain tissue was glued to a cutting chamber in a vibrating microtome (VT 1200; Leica), which then was filled with ice-cold sucrose-asCSF.

IOS. Coronal brain slices (350 μm thick) were cut at the level of the striatum and incubated at 33–35 °C in aCSF containing 126 mM NaCl, 2.5 mM KCl, 1.5mM NaH₂PO₄, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, bubbled with 95% O₂:5% CO₂. Slices were transferred to a recording chamber superfused with aCSF and were maintained at 33–35 °C. Slices were pretreated with pregabalin or aCSF (control) for 60 min, and treatment continued via the perfusate for the duration of the experiment. Brain slices were visualized using a differential interference contrast (DIC) microscope (Axioskop 2-FS Plus; Carl Zeiss) and an infrared camera (IR-1000; DAGE MTI). SD was initiated using KCl (40 mM), and IOS transients indicative of SD were recorded using custom software as described by Zhou et al. (29). ImageJ (NIH) software was used to analyze SD spread. Pixels displaying SD were selected for each cell that was marginally subthreshold for action potential initiation by KCl application to the rat cortex with simultaneous measurement of diffusion coefficient (ADC). More recent, MRI was used to examine the cells with 180-ms square pulses of increasing amplitude from −60 mV to +45 mV (Vh = −120 mV).

Electrodes were made from borosilicate capillaries and had a resistance of 3–4 MΩ when filled with an internal solution containing 105 mM CsMeSO₄, 11 mM EGTA, 10 mM Hepes, 25 mM TEA-Cl, and 5 mM ATP-Mg (pH 7.2; 290 mOsml/kg). The extracellular solution contained 5 mM CaCl₂, 1 mM MgCl₂, 88 mM CsCl, 40 mM TEA-Cl, 10 mM Hepes, and 10 mM glucose (pH 7.4; 305 mOsml/kg). Osmolality was adjusted with D-mannitol.

Acute Brain Slice Electrophysiology. Horizontal brain slices (350-μm thick) were cut from the level of the ventral hippocampus and were incubated at 33–35 °C in aCSF bubbled with 95% O₂:5% CO₂. Slices were transferred to a recording chamber superfused with aCSF and were maintained at 33–35 °C. CA1 neurons were visualized using a DIC microscope and infrared camera (Slicer-Scope 6000; Scientifica) and were visually identified by their location, morphology, and orientation. All recordings were undertaken using a Multichannel 700B amplifier and pClamp software version 10 (Molecular Devices). The recording chamber was grounded with an Ag/AgCl pellet.

Whole-cell current-clamp recordings were undertaken using fire-polished borosilicate glass pipettes (4–6 MΩ) filled with the intracellular solution containing 120 mM K-glucuronate, 10 mM Hepes, 1 mM MgCl₂, 1 mM CaCl₂, 11 mM KCl, 11 mM EGTA, 4 mM MgATP, and 0.5 mM NaGTP, with pH adjusted to 7.2 using KOH and osmolarity adjusted to 290 mOsml/kg using D-mannitol. The liquid junction potential for current-clamp solutions was calculated as +13.5 mV, but data shown were not corrected. To evaluate the basic input-output action potential frequency response to hyperpolarization and depolarization, DC current was injected from −110 pA to +200 pA in 10-pA increments for a duration of 1,000 ms at the cell’s intrinsic resting membrane potential. Paired-pulse stimulation was delivered via a concentric electrode placed in the CA3 Schaffer collaterals using a S48 square-pulse stimulator (Grass Technologies). A stimulation voltage was selected for each cell that was marginally subthreshold for action potential firing. Although the stimulation current varied from cell to cell the same stimulation current was used to elicit eEPSPs recorded before and after pregabalin in the same cell. Stimulation was applied with a 67-ms interpulse interval, with each pair of pulses applied four times (30-s intersweep interval), and the mean for each eEPSP response was taken to minimize variability between cells. Membrane potential responses under current-clamp conditions were sampled at 50 kHz and filtered at 10 kHz. Bridge balance was monitored during recordings, and any neurons displaying bridge balance values greater than 20 MΩ were discarded. Capacitance neutralization was performed between 3.8 and 4.2 pF.

Whole-cell voltage-clamp recordings were performed using the same intracellular solution and in aCSF. To evaluate eEPSCs, cells were held at a membrane potential of −70 mV during a 60-s gap-free recording. Data acquisition was sampled at 20 kHz and filtered at 2.4 kHz. Recordings with a series resistance greater than 20 MΩ were discarded, and series resistance was compensated to 70%.

MRI. The first described use of MRI to measure SD involved its initiation by KCl application to the rat cortex with simultaneous acquisition of DW echo-planar and T2-weighted images (46). This method requires acquisitions with diffusion weighting in several directions and postprocessing to measure the apparent diffusion coefficient (ADC). More recently, MRI was used to examine the...
subcortical spread of SD in S218L mice with a 20-s time resolution (28). We adapted the technique by acquiring images with diffusion weighting in only one direction to view SD as a clear increase in voxel intensity, increasing the time resolution to 8 s and circumventing the need to calculate the ADC. During SD neurons swell because of water influx, which results in attenuation of the average diffusion constant in the pixel. This effect produces less signal attenuation when a DW gradient is applied, thereby increasing pixel intensity in DW-MR images.

Animals were anesthetized using isoflurane for the duration of surgical preparation and MRI scanning. Mice were placed in a stereotaxic frame, and an incision was made in the scalp. Two holes were drilled in the skull over the right hemisphere of the visual cortex (hole 1: bregma -4.5 mm, lateral 1.5 mm; hole 2: bregma -4.5 mm, lateral 2.5 mm), and sterile carbon fiber electrodes (World Precision Instruments) were inserted into each hole to a depth of 0.5 mm into the cortex. Glue was used to cement the electrodes into position, and the wound was sutured and covered with surgical tape.

The mouse was laid supine in the MRI cradle, and the stimulation electrodes were fed through the center of a custom RF coil and then were connected to a constant current unit exterior to the magnet bore and controlled via a stimulator (Grass Technologies). Respiratory and heart rates were monitored during scanning to control anesthesia depth.

MRI experiments were carried out on a 7-T animal scanner (Bruker). An RF coil with a 70-mm i.d. volume was used for pulse transmission, and the MRI signal was received with an actively decoupled surface coil with a 14-mm diameter. T2-weighted MRI was used to acquire high-resolution structural images before DW-MRI scanning. DW-MRI was acquired using diffusion-weighted spin-echo echo planar imaging with a b-value of 1,800 s/mm² (echo time/repetition time = 29/2,000 ms, four shots, field of view = 2 × 2 cm, matrix size = 64 × 64, slice thickness = 1.25 mm, eight interleaved slices, 100 repetitions in 13 min 20 s) in 13-min intervals, during which four stimulations were applied to the visual cortex at 1, 4, 7, and 10 min. Stimulation intensity was started at 0.25 mA × 100 ms (25 μC), and the amperage or duration was increased to generate an incrementally increasing charge stimulation (scan set 1: 25, 50, 100, and 200 μC; scan set 2: 300, 400, 500, and 600 μC; scan set 3: 700, 800, 900, and 1,000 μC). If no SD occurred, the 13-min DW-MRI scan was repeated with the next level of stimulation intensities.

DW-MRI data were postprocessed in Matlab by averaging across all the images in a series per slice, and the brain exterior borders were defined to create masks that were applied to each time point in the series. A prestimulation baseline set of time points was defined, averaged, and subtracted from each image in the series. These were imported to ImageJ and then a lookup table was applied and the color image showing SD was superimposed on the original DW-MRI greyscale image for each timepoint. Time-series slices were rearranged and packaged into .bov files for import to VisIt and were reconstructed into a 3D time series.

To generate SD heatmaps, scans were imported to Matlab, and the first three repetitions were discarded because of high signal intensity. A Matlab script was created to define the SD wave front by thresholding the increased pixel intensity and was vectored to define the direction of SD spread. In each slice, a 3 × 3 voxel ROI was defined in an area of the brain unaffected by the SD event, and the mean value was calculated for each repetition. Subsequent repetitions were normalized to the mean value of each repetition to normalize any global fluctuations in signal intensity. For each slice an ROI was defined outside the head, and a mean and standard deviation were calculated. A mask was applied automatically to each image by including only voxels in which the voxel intensity over all repetitions was greater than the mean intensity of the noise plus three times the standard deviation of the noise. The mean voxel intensity then was calculated for the masked region over all repetitions. The appearance of the SD wave front in a voxel then was defined as the first repetition in which, for two repetitions in a row, the intensity of the voxel was greater than the mean intensity of the voxel plus three times the standard deviation of the noise. Hot/cold colors were applied automatically by calculating the time when each pixel crossed threshold relative to the initiation of SD.

Although SD was visible throughout slices 3–8, slices 3 and 4 contained imaging artifacts (minor image distortion). Although the electrodes are made of carbon, they still produce magnetic susceptibility artifacts that prevent accurate analysis of exactly when the SD first appears in these slices (Fig. S1C). Therefore the SD speed was calculated as the distance between slices 5 and 8 (3.75 mm apart) divided by the time required for the SD wave front first to appear in slice 8 minus the time required for the SD wave front first to appear in slice 5.

**Drugs.** Pregabalin (generously provided by Pfizer) was dissolved directly in extracellular solution for heterologous expression system experiments, in aCSF for in vitro acute brain slices, and in 0.9% sterile saline (10 mL/kg; weight/vol) for in vivo MRI experiments. In humans, the maximum recommended daily dose of pregabalin is 600 mg, roughly equating to 8.5 mg/kg (47). However, studies using pregabalin in mice to assess its use as an antiepileptic drug have demonstrated that a dose of 20 mg/kg (i.p.) is required to achieve a 50% reduction in multiple electric shock (MES)-induced seizures, but a dose of 110 mg/kg (i.p.) is necessary to achieve a 95% reduction (47). Conversely, in rats only approximately one-tenth of this dose is required to achieve a similar effect on MES-induced seizures. Therefore, the pharmacokinetic profile of pregabalin likely results in a greatly increased clearance in mice as compared with rats. In the absence of reported CSF levels following systemic pregabalin administration in mice, we used a single 160-mg/kg, i.p. dose aimed at maximizing the likelihood of achieving an effective concentration in vivo.

**Data Analysis.** Electrophysiological data analysis was performed using Clampfit (v9 and v10; Molecular Devices). IOS was performed using Zen (Blue edition; Zeiss) and ImageJ (v 1.50d; NIH). DW-MRI analyses were performed using MATLAB (v 2014a; MathWorks) and ImageJ (v 1.50d; NIH). 3D reconstruction was performed using VisIt (v2.9.1; Lawrence Livermore National Laboratory). Graphing and statistical analyses were performed using Origin (v8.6; OriginLab). Data followed a normal distribution, and statistical significance was calculated using Student’s two-sample t test (paired where relevant). One-way ANOVA with Tukey’s post hoc test was used for multiple comparisons. Cumulative distributions were compared using the Kolmogorov–Smirnov test. Data are plotted as mean ± SE.
Fig. S1. DW-MRI in vivo. (A) Diagram showing the positioning of carbon fiber electrodes for electrical SD initiation during DW-MRI scanning. (B, Upper) 3D representation of electrode position within the visual cortex (48). (Lower) T2-weighted MRI coronal image displaying electrode positioning in the visual cortex. (C) DW-MRI images showing the eight coronal slice acquisitions covering the whole brain from cerebellum to frontal cortex in 1.25-mm thick slices. The red circle defines the ROI used for the time-course plot of SD spread in D. (D) Time-course plot showing mean pixel intensity in the cerebellum during SD; a.u., arbitrary units.
Fig. S2. Spatiotemporal dynamics of SD spread. (A) DW-MRI images corresponding to the slices in B–D are shown for anatomical reference. (B–D) A custom Matlab script was designed to define the SD wave front in representative examples of SD spread in WT (B), R192Q (C), and S218L (D) mice. Upper rows in each panel show vehicle control-treated animals, and lower rows in each panel show pregabalin-treated animals. Pixels becoming activated in early and late time periods after the initial appearance of SD in slice 5 are depicted by cold and hot colors, respectively. A heat-second scale is shown on the right of each panel for reference.

Fig. S3. Cumulative probability analysis of sEPSC amplitude and interevent intervals in hippocampal CA1 neurons. Cumulative probability plots for sEPSC amplitude (Upper Row) and interevent interval (Lower Row) are shown for (Left) WT (control: n = 2,985; 500 μM pregabalin: n = 2,064), (Center) R192Q (control: n = 564; 500 μM pregabalin: n = 363), and (Right) S218L (control: n = 6,325; 500 μM pregabalin: n = 7,363) mice. *P < 0.01, Kolmogorov–Smirnov test.
Concentration-dependent effects of pregabalin on glutamatergic sEPSC amplitude and frequency. (A) The table shows mean data for peak amplitude, interevent interval, time to peak, and time to 50% decay for sEPSCs recorded in acute hippocampal brain slices before and after administration of pregabalin at a concentration of 100 µM (WT: n = 5 cells, 3 animals; R192Q: n = 4 cells, 3 animals; S218L: n = 5 cells, 3 animals) and 500 µM (WT: n = 7 cells, 4 animals; R192Q: n = 6 cells, 4 animals; S218L: n = 9 cells, 4 animals). (B, Left) Representative voltage-clamp traces before (Upper) and after (Lower) the application of AMPA (CNQX; 20 µM) and NMDA (D-APV; 100 µM) receptor blockers. (Right) The number of events (Upper) and the amplitude distribution histogram (Lower) for sEPSCs before and after the application of AMPA and NMDA receptor blockers. *p < 0.05.

Movie S1. DW-MRI imaging of cortical SD spread in WT vehicle-treated mice. SD is constrained to cortex. (Upper) Eight coronal postprocess images (1.25-mm slice thickness) of a representative WT mouse brain from rostral (slice 8, Upper Left) to caudal (slice 1, Lower Right). Each slice is animated (8 s per frame) to show the propagation of SD through the brain. A color map is applied to show the brain (cold colors) and SD wave (hot colors). (Lower) A 3D reconstruction of the postprocessed brain across the same range of time points encompassing SD propagation to show the brain (blue) and SD wave (yellow). Time is synchronized in the two datasets. “STIM” denotes time of electrical stimulation to initiate SD in the visual cortex. In WT animals SD is constrained to the cortex.

Movie S1
Movie S2. DW-MRI imaging of cortical and subcortical SD spread in vehicle-treated R192Q mice. (Upper) Eight coronal postprocess images (1.25-mm slice thickness) of a representative R192Q mouse brain from rostral (slice 8, Upper Left) to caudal (slice 1, Lower Right). Each slice is animated (8 s per frame) to show the propagation of SD through the brain. A color map is applied to show the brain (cold colors) and SD wave (hot colors). (Lower) A 3D reconstruction of the postprocessed brain across the same range of time points encompassing SD propagation to show the brain (blue) and SD wave (yellow). Time is synchronized in the two datasets. “STIM” denotes time of electrical stimulation to initiate SD in the visual cortex. In five of six R192Q animals, SD spreads across the cortex but also from the ventral side of the brain, and with a delay, to the striatum and hippocampus.

Movie S2

Movie S3. DW-MRI imaging of cortical and subcortical SD spread in vehicle-treated S218L mice. SD propagates to the hippocampus and striatum without delay. (Upper) Eight coronal postprocess images (1.25-mm slice thickness) of a representative S218L mouse brain from rostral (slice 8, Upper Left) to caudal (slice 1, Lower Right). Each slice is animated (8 s per frame) to show the propagation of SD through the brain. A color map is applied to show the brain (cold colors) and SD wave (hot colors). (Lower) A 3D reconstruction of the postprocessed brain across the same range of time points encompassing SD propagation to show the brain (blue) and SD wave (yellow). Time is synchronized in the two datasets. “STIM” denotes time of electrical stimulation to initiate SD in the visual cortex. In S218L animals SD spreads across the cortex and also to the striatum and hippocampus in all mice, without delay and apparently from the neighboring cortex.

Movie S3
Movie S4. DW-MRI imaging of cortical SD spread in pregabalin-treated WT mice. Pregabalin does not affect SD spread or velocity. (Upper) Eight coronal postprocess images (1.25-mm slice thickness) of a representative WT mouse brain from rostral (slice 8, Upper Left) to caudal (slice 1, Lower Right). Each slice is animated (8 s per frame) to show the propagation of SD through the brain. A color map is applied to show the brain (cold colors) and SD wave (hot colors). (Lower) A 3D reconstruction of the postprocessed brain across the same range of time points encompassing SD propagation to show the brain (blue) and SD wave (yellow). Time is synchronized in the two datasets. “STIM” denotes time of electrical stimulation to initiate SD in the visual cortex. Time is synchronized in all three datasets. Pregabalin does not affect SD propagation in WT animals.

Movie S4

Movie S5. DW-MRI imaging of cortical SD spread in pregabalin-treated R192Q mice. Pregabalin slows the SD velocity and prevents spread to subcortical structures. (Upper) Eight coronal postprocess images (1.25-mm slice thickness) of a representative R192Q mouse brain from rostral (slice 8, Upper Left) to caudal (slice 1, Lower Right). Each slice is animated (8 s per frame) to show the propagation of SD through the brain. A color map is applied to show the brain (cold colors) and SD wave (hot colors). (Lower) A 3D reconstruction of the postprocessed brain across the same range of time points encompassing SD propagation to show the brain (blue) and SD wave (yellow). Time is synchronized in the two datasets. “STIM” denotes time of electrical stimulation to initiate SD in the visual cortex. In four of five R192Q animals, pregabalin slows the speed of SD propagation across the cortex and suppresses spread to the striatum and hippocampus.

Movie S5
Movie S6. Diffusion-weighted MRI imaging of cortical and subcortical SD spread in pregabalin-treated S218L mice. Pregabalin slows SD velocity only. (Upper) Eight coronal postprocess images (1.25-mm slice thickness) of a representative S218L mouse brain from rostral (slice 8, Upper Left) to caudal (slice 1, Lower Right). Each slice is animated (8 s per frame) to show the propagation of SD through the brain. A color map is applied to show the brain (cold colors) and SD wave (hot colors). (Lower) A 3D reconstruction of the postprocessed brain across the same range of time points encompassing SD propagation to show the brain (blue) and SD wave (yellow). Time is synchronized in the two datasets. “STIM” denotes time of electrical stimulation to initiate SD in the visual cortex. In S218L animals pregabalin slowed the speed of SD propagation across the cortex but does not suppress subcortical invasion.

Movie S6