Isolated P/Q Calcium Channel Deletion in Layer VI Corticothalamic Neurons Generates Absence Epilepsy

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Introduction

Since the first description of cortical spike-wave seizures in a child with staring spells (Berger, 1933), identifying the essential neuronal circuitry underlying this pattern generation has been a central challenge in epilepsy research. Studies in inbred rat models (van Luijitaer and Zobeiri, 2014; Depaulis et al., 2015) and >20 defined monogenic mouse models (Maheshwari and Noebels, 2014) uniformly point to defects in the reciprocal interplay of long-range corticothalamic and thalamocortical circuitry during the stereotyped synchronous discharge, the electroencephalo-
graphic signature of absence epilepsy and related clinical syndromes. The canonical circuit defined in these models involves the near simultaneous activation of deep layer neurons in a low-threshold region of somatosensory cortex (Meeren et al., 2002; Polack et al., 2007) maintained by intrinsic rebound bursting of thalamic cells, whereas intracortical and intrathalamic GABAergic synaptic inhibition modulates both nodes of the loop (Huguenard and McCormick, 2007). However, in models where the dysfunctional gene is known, the defective protein is widely distributed throughout cells within and outside this network and the minimal elements necessary for the pathological discharge development are unknown.

The voltage-gated P/Q-type calcium channel was the first to be linked to the spike-wave phenotype and remains an index gene for the disorder. Loss of function mutations within the P/Q channel complex in the α pore-forming subunit encoded by Cacna1α, along with accessory β4 and α2δ-2 subunits, result in a phenotypic triad of spike-wave epilepsy, episodic dystonia, and ataxia (Maheshwari and Noebels, 2014). Discovered in the tuttering mouse, which contains a point mutation in Cacna1α that reduces current in single P/Q-type channels (Noebels and Sidman, 1979; Fischer et al., 1996; Wakamori et al., 1998); the absence phenotype appears in many other allelic, partial knockdown, and homozygous knock-out models of Cacna1α in mouse (Jun et al., 1999; Saito et al., 2009; Noebels, 2012), rat (Takuda et al., 2007), and human (Jouveucause et al., 2001; Imbrici et al., 2004). The P/Q channel is widely expressed at presynaptic terminals where, along with less effective N- and R-type channels, it regulates neurotransmitter release (Neher and Sakaba, 2008; Catterall et al., 2013). P/Q channel loss does not eliminate synaptic transmission, which can be rescued in part by N- and R-type-mediated exocytosis at central synapses (Qian and Noebels, 2000, 2001; Inchauspe et al., 2007), however, deficient P/Q function disrupts normal patterns of spontaneous and evoked release. Loss of P/Q increases jitter, depressing synaptic transmission fidelity (Depeurs et al., 2008), and targeted mutation of the IQ motif impairs Ca2+ calmodulin kinase modulation of short-term synaptic facilitation (Yan et al., 2014). An age- and synapse-specific switch from pure N-type to mixed N/P/Q-type dominant release occurs during early postnatal development, and most terminals in adult brain display roughly equally shared control of quantal release by these subtypes (Iwasaki et al., 2000).

In addition to altered transmission, mice bearing genomic P/Q subunit loss of function mutations display upregulation of low voltage-activated (LVA) T-type calcium currents in thalamic relay neurons, an important element of downstream circuit plasticity favoring spike-wave epilepsy generation (Zhang et al., 2002; Ernst et al., 2009; Cain and Snutch, 2013). This mechanism remains undefined, because there is no evidence for upregulation or alternative splicing of Cacna1α, the gene responsible for LVA calcium current in these cells (Ernst and Noebels, 2009). It is also unclear whether thalamic remodeling depends upon intrinsic loss of P/Q calcium current or abnormal synaptic input. Recently, a knock-in Cacna1α mouse with the YFP tagged channel flanked by loxP sites was created, allowing selective removal of Cacna1α from cre-expressing cells to investigate its role in microcircuits (Mark et al., 2011). To further elucidate the role of the P/Q channel within the thalamocortical spike-wave circuit and its ability to regulate lasting thalamic excitability changes, we generated mice with the channel removed in layer VI corticothalamic pyramidal neurons and investigated the impact of this single mutated projection on the generation of spike-wave epilepsy.

### Materials and Methods

**Mouse strains**

Mice of both sexes were maintained in the Baylor Developmental Neurogenetics breeding colony. The floxed Cacna1α<sup>Citrine</sup> (C57BL/6 129/SvJae) strain was obtained from Drs Melanie Mark and Stefan Herlitze at Ruhr University of Bochum. These mice have been characterized and show no change in channel function due to the construct (Mark et al., 2011). Tg[Ntsr1-cre]N220Gsat/Mmucd mice generated by the GENSAT project were obtained from the Mutant Mouse Regional Resource Center (www.mmrrc.org/catalog/sds.php?mmrrc_id = 17266). These mice show highly specific cre-driven reporter expression confined to large cortical layer VI pyramidal projection neurons. Cacna1α<sup>Citrine</sup> mice were crossed with Ntsr-1Cre cre mice to ablate Cacna1α in these neurons (Cacna1α<sup>Nsr1<sup>−/−</sup></sup>), To verify Cre expression in layer VI neurons, Ntsr1 cre mice were crossed with the reporter mouse B6.Cg-Gt (ROSA)26Sor tm1(WtHCGAGAGTAC/cre)Jabsf from The Jackson Laboratory. For physiology experiments, to identify layer VI cells with tdTomato expression, Ntsr1 cre mice, Cacna1α<sup>Citrine</sup> mice were crossed with the tdTomato reporter mice to generate Cacna1α<sup>Nsr1<sup>+/−</sup></sup>/tdTomato and Cacna1α<sup>Nsr1<sup>−/−</sup></sup>/tdTomato. Mouse breeding and all experiments were performed under IACUC approved protocols at Baylor College of Medicine. Genotyping primers were as follows: Cacna1α citrine forward: TATAT CAGTGGGCAGAACAAGC; Cacna1α citrine reverse: TTCCGCTTCA CATGGCCGACAAGCA; Cacna1α wild-type forward: GGGTTCTGACTTC CAAGGAACCC; Cacna1α wild-type reverse: ATGGCCGGACCCCTTAT; Ntsr-cre forward: GAGGCCACGGCCCTTCTA; Ntsr-cre reverse: CG GCAGAACGCAAGAACAT; tdTomato mutant forward: GGGATTA AAGGAGCAGGTATCC; tdTomato mutant reverse: CTGTTCCCTGTT AGGGCATGGG; tdTomato wild-type forward: AAGGGAGCTGCG AGTTGAGTA; tdTomato wild-type reverse: CTGTTCCTGTACGGC AGTG.

**Immunohistochemistry**

In situ hybridization

Hybridizing probes for Cacna1α mRNA transcripts were designed as described by Ernst et al. (2009). Sagittal sections of a representative Cacna1α<sup>Nsr1<sup>−/−</sup></sup> and Cacna1α<sup>Nsr1<sup>−/−</sup></sup> adult female brain were probed and analyzed by the Baylor College of Medicine RNA In Situ Hybridiza-
tation core, following standard procedures (Carson et al., 2005; Yayaoglu et al., 2005; Lein et al., 2007). Representative sections from each genotype were analyzed by scanning densitometry and the images pseudocolored for intensity of cellular Cacna1a mRNA expression.

Quantitative real-time PCR
Quantitative real-time PCR was used to assess regional mRNA transcript levels from Cacna1a

+/- , Cacna1a

crem and Cacna1a

-/- mice (N = 3 each) by dissection of the cortex, thalamus, and cerebellum. In brief, total RNA was extracted with TRIzol reagent (Invitrogen), quantified, and treated with DNase (Ambion). The Phusion kit from Ambion was used to reverse transcribe mRNA into cDNA. Using SyberGreen Advantage qPCR mix (Clontech Laboratories), probes for Cacna1a transcripts included forward: GCCTTCGACGACTACTCTT and reverse: GTGTTCCTCCAGTTAACATTCT. For GAPDH, primers included forward: AACAGCAACTCCCACTCTTC and reverse: CCTGT-TGCGTGGACCGTATT. Real-time qPCR was performed on an Applied Biosystems 7500 Real-Time PCR System. The average threshold cycle (Ct), which indicates the fractional cycle number of amplified PCR product that reaches a fixed threshold value, was determined for each sample by running three technical replicates. To report quantitative ratios between mice, we used the comparative threshold method (2-ΔΔCT) which uses an internal control (GAPDH) and compared both Cacna1a

crem mice and Cacna1a

-/- to Cacna1a

+/- mice (Schmittgen and Livak, 2008; Ernst et al., 2009).

EEG recordings
Silver wire electrodes (0.005 inch diameter) soldered to a micromanipulator connector were implanted bilaterally into the subdural space over frontal and parietal cortex of mice under Avertin anesthesia (250 mg/kg) 2 weeks before recording. Simultaneous EEG and behavioral monitoring was performed using a digital video-electroencephalograph (Harmonie 6.1, Stellate Systems) upon adult mice of either sex aged 8 weeks while moving freely in the test cage. EEG activity from adult wild-type (Cacna1a

+/- N = 3 and Cacna1a

crem N = 4) and mutant (Cacna1a

-/- N = 4) mouse genotypes was assayed during three separate recording sessions of at least 4 h each. To assess spike-wave discharge activity, a 12 h sample of EEG recording was analyzed in each mouse where spike-wave discharges (>1 s) were counted and reported as spike-wave discharge/b. To study the drug sensitivity of spike-wave seizures in Cacna1a

-/- mice, the T-type calcium channel antagonist, ethosuximide, was injected intraperitoneally at a concentration of 200 mg/kg following a 10 min baseline recording. After a 5 min postinjection recovery period, spike-wave discharges were counted for 10 min. In one Cacna1a

+/- mouse, bilateral electrodes were also positioned stereotaxically in ventrobasal thalamus (coordinates from bregma, 1.4 mm posterior, 1.5 mm lateral, and 3 mm depth) and electrode coordinates were verified visually following the recording session.

Slice electrophysiology
Evoked EPSCs in thalamic neurons. Coronal slices of a hemisphere from mice of either sex were cut at a 300 μm thickness and at an angle of 55° with the longitudinal axis to include intact corticothalamic circuitry (Agmon and Connors, 1991). The brain slices were first incubated at a temperature of 35°C for 45 min then incubated in ACSF at room temperature for 1 h before recording. The ACSF contained the following (in mM): 110 cesium gluconate, 10 HEPES, 8 NaCl, 1 MgCl2, 10 NaHCO3, 1.25 CaCl2, 280 mOsm, pH 7.2). All neurons were voltage-clamped at −70 mV. Access resistances were <25 MΩ and were compensated by 70%. Data were acquired using a MultiClamp 700B amplifier, digitized by Digidata 1332 and analyzed with pClamp 9.2 software (Molecular Devices). After establishment of stable whole-cell configuration, LVA currents were evoked by an initial step to −50 mV from prior hyperpolarization. Steady-state inactivation (SSI) of the current was analyzed using 2 s hyperpolarizing prepulses at varying holding membrane potentials from −110 to −40 mV. Data were fitted with Boltzmann’s equation using Prism software. The time dependency of de-inactivation was tested by changing the duration (0–2 s) of the −110 mV prepulses.

Characterization of intrinsic excitability in layer VI cortical neurons. For identification of layer VI cortical neurons, Cacna1a

+/- and Cacna1a

-/- mice of either sex were generated by additional mouse crosses to the tdTomato reporter mice, B6.Cg-Tg(Rosa26RtTA/cre/tdTomato)Jf10/J. Coronal corticothalamic slices (300 μm) were prepared from Cacna1a

+/- /tdTomato and age-matched Cacna1a

-/- /tdTomato mice of either sex. Slices were superfused with ACSF (in mM: 126 NaCl, 10 glucose, 1 MgSO4, 3 KCl, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, and 0.4 acid sorbic acid (equilibrated with 95% O2/5% CO2) at 2 ml/min.

The Cre-expressing layer VI cortical neurons were identified by their td-Tomato epi-fluorescence (excitation 560 ± 20 nm bandpass, emission 585 nm long-pass) and thalamic relay and reticular neurons were identified as described above. Patch pipettes were filled with an internal solution (in mM: 130 potassium gluconate, 10 HEPES, 1 MgCl2, 8 NaCl, 0.05 EGTA, 2 Na-ATP, 0.3 Na-GTP, pH adjusted to 7.2 with KOH) and had a 2–3 MΩ tip resistance. After establishment of stable whole-cell configuration, membrane excitability was tested in current-clamp mode. Membrane potential was held at −60 mV and a series of command currents (0.5 s, −200 to +200 pA) were injected. Signals were acquired at 20 kHz, amplified with a Multiclip 200B amplifier, digitized by Digidata1322A and analyzed with pClamp9.2 software (Molecular Devices). Series resistances >20 MΩ were included form analysis. Bridge balance was adjusted manually.

Behavioral analysis
Adult mice of either sex were trained over 3 d of trials on an automated rotating rod (Ugo Basile). Mice were placed carefully on the rotating rod and the latency to fall (seconds) was measured for each animal while the speed increased from 4 to 40 rpm over a 3 min interval. On the third day, with experimenters blinded to genotype, mice were serially tested three times and the average latency to fall is reported.

For gait analysis, with experimenters blinded to the genotype, adult mice of either sex were placed on a flat surface to observe spontaneous gait. Animals were scored on a graded ataxia scale of 0–3 (Guyenet et al., 2010), where normal movement included body weight supported on all limbs with abdomen off the ground scored a 0 for ataxia. Such mice could not be discerned from wild-type littermates. Slight limping or tremor scored a 1. A severe tremor, severe, flattened
pelvis, or hind limbs angled away from the body scored a 2. If
the mouse moved with more severe difficulty, it would score a 3.

For the ledge test, mice of either sex were lifted and placed onto the
lip of an empty plastic mouse cage. The mouse was observed to walk
along the ledge and lower itself into the cage. Easy movement along
the ledge and proper, graceful lowering into the cage scored a 0. Loss
of footing, but mostly coordinated mice scored a 1. Ineffective hind
leg movement or improper lowering into the cage scored a 2. Mice
that fell or refused to move even with gentle nudging scored a 3
(Guyenet et al., 2010).

Statistical analysis
Unless otherwise noted, the data presented represents the mean ±
SEM. Statistical tests were conducted as mentioned in the text using
either Student’s t tests, Kruskal–Wallis, or one-way ANOVAs as ap-
propriate using GraphPad Prism software. In some cases, where vari-
bances between the data populations were not equal, the Welch’s
correction was used on a t test and this is noted in the text. For
quantitative real-time PCR analysis, p values were generated by a
one-way ANOVA within each brain region comparing the three
different genotypes (∼Cacna1a^Ntsr^+/+, ∼Cacna1a^Citrine, ∼Cacna1a^Ntsr^-/-).

Results
Selective knockdown of P/Q-type calcium channels in layer VI
corticothalamic projection neurons
To selectively delete P/Q-type calcium channels from layer VI
pyramidal neurons, we crossed Cacna1a^Citrine (Mark et al., 2011)
mice with Tg^Ntsr1-Cre. The Cacna1a^Citrine mice consist of a condi-
tional knock-in of the Citrine gene in the first exon of Cacna1a sur-
rounded by loxP sites to permit Cre recombinase-mediated
removal of the P/Q-type calcium channel exon 1 (Mark et al.,
2011). Tg^Ntsr1-Cre mice, express Cre recombinase under the con-
trol of the neurotensin-1 (Ntsr1) promoter that selectively labels
a consistent subpopulation of layer VI neurons innervating thal-
ic relay neurons (Olsen et al., 2012; Bortone et al., 2014), as
well as collateral innervation of parvalbumin-positive interneu-
rions residing in the thalamic reticular nucleus (Bourassa and
Deschenes, 1995). To verify selective expression of Cre recombi-
nase in layer VI projection neurons in these mice, we crossed
male Tg^Ntsr1-Cre mice with female tdTomato reporter mice
(B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J). In coronal sec-

Figure 1. The Ntsr-1 cre drives expression of tdTomato in layer VI projection neurons to the thalamic reticular nucleus and ventrobasal thalamus. A, Stitched 10× images of the projections from layer VI through the internal capsule to the nRT and thalamus. Cell nuclei are marked with DAPI in blue, TdTomato staining in red indicates Ntsr Cre^+ cells, and FITC staining in green marks parvalbumin interneurons throughout the brain. Scale bar, 500 μm. B, Image (10×) of layer VI cortical projection neurons. Scale bar, 200 μm. C, Image (10×) of the nRT and thalamus shows no cre-driven cellular staining, but diffuse label due to axonal projections from layer VI neurons. Scale bar, 200 μm. D, Image (10×) of hippocampal dentate gyrus shows scattered cells expressing tdTomato. Scale bar, 200 μm.
tions of adult brain, tdTomato expression was clearly detected and confined to layer VI pyramidal neurons (Fig. 1A, B), axonal projections within the subcortical white matter (Fig 1A, 1C), and terminals in the ventrobasal thalamus and thalamic reticular nucleus (Fig. 1A, C). Within the deep layers of neocortex, the tdTomato-labeled cellular expression did not overlap with parvalbumin-positive cells, indicating that the interneurons within layer VI were spared (Fig. 1B). No cell-specific tdTomato staining was observed in other brain regions except for rare scattered cells detected in the dentate gyrus of the hippocampal formation (Fig. 1A, D) and no tdTomato-positive neurons were detected elsewhere in the forebrain or cerebellum.

To determine whether the expression of Cre recombinase in Cacna1aCitrine mice decreased the P/Q channel population in the terminal fields of layer VI projection neurons, immunohistochemistry was performed using a specific antibody to the P/Q channel (Fig. 2A). We used the ratio of the thalamic terminal field staining intensity over the intensity of the axon terminal-rich
hilar mossy fiber region of the dentate gyrus as an internal standard to control for staining variability between sections. We found significantly decreased P/Q staining intensity in the VAL, VPM, and VPL nuclei of the thalamus in Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} (0.83 ± 0.02, ANOVA, \( p = 0.0001, n = 5 \)) compared with wild-type mice (Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}} 1.00 ± 0.03, \( n = 6 \); Cacna1a\textsuperscript{Citrine} 1.03 ± 0.01, \( n = 5 \); Fig. 2B). Because these thalamic nuclei also receive thalamopetal axon terminals originating from nRT and other brain regions, there is substantial residual P/Q staining due to the overlapping presence of nonablated terminals. However, despite their innervation by layer VI axon collaterals, no measurable decreases of P/Q antibody labeling were detected in the nRT (ANOVA, \( p = 0.11 \); Fig. 2C) or deep cortical layer (ANOVA, \( p = 0.50 \); Fig. 2D) compared with the mossy fiber region of the dentate gyrus, presumably because these subregions comingle with an even larger population of nonablated axon terminals. These data confirm the specificity of Ntsr1 Cre ablation of P/Q calcium channels at layer VI terminals in the ventrobasal thalamus.

**Functional loss of presynaptic P/Q-type channels at the corticothalamic relay synapse in Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} mice**

To ascertain whether the channel ablation was reflected in functional knockdown of P/Q channel-mediated release at the layer VI-thalamic neuron terminals, we investigated synaptic transmission at the corticothalamic terminal synapses in the ventrobasal thalamus using an N-type channel-specific toxin to dissect the contribution of these two major presynaptic calcium channel subtypes. Mutation of presynaptic P/Q-type channels in tottering mice result in a shift of channel reliance of neurotransmitter release from P/Q-type to N-type at both corticohippocampal and mossy fiber hippocampal synapses (Qian and Noebels, 2000, 2001). As shown in the sample traces in Figure 3A, EPSCs evoked by paired-pulse stimulation of corticothalamic axons exhibit large amplitude paired pulse facilitation (PPF) of corticothalamic synaptic transmission in both Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}} and Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} animals (Fig. 3B; \( t \) test, \( p = 0.51 \)). In Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}} control brain slices, the mean PPF ratio was 3.4 ± 0.6 (\( n = 12 \)), which does not significantly differ from the Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} group (PPF ratio 3.2 ± 0.5, \( n = 12 \)). Application of 1 \( \mu M \) \( \omega \)-CTX GVIA, a N-type channel blocker, revealed that neurotransmitter release at the synapse in Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} slices was more sensitive to blockade of N-type channels than Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}} slices (Fig. 3C; \( t \) test, \( p = 0.0001 \)). On average, the N-type calcium channel toxin reduced the evoked EPSC amplitude by 71 ± 11\% (\( n = 12 \)) in Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} as compared with 50 ± 12\% (\( n = 12 \)) in the Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}} group. This evidence demonstrates a severe functional loss of presynaptic P/Q-type channels in layer VI presynaptic thalamic terminals in Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} mice.

**Layer VI cell intrinsic properties are unchanged in Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} mice**

To further investigate the extent of Cacna1a ablation on the excitability of the parent layer VI pyramidal neurons, which might impact levels of excitatory drive in descending corticothalamic axons to the thalamus, whole-cell recordings of these cells in Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}} and Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} mice labeled with the td-Tomato reporter were performed (Fig. 4A,B, representative trace; \( n = 9 \) cells each). There was no significant difference, at any injected voltage, in the number of action potentials generated in cells from Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}}/tdTomato and Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}}/tdTomato mice (Fig 4G; ANOVA interaction, \( p = 0.9118 \)). Additionally, no difference was seen in the resting membrane potential (Fig. 4D; \( t \) test, \( p = 0.30 \)), membrane resistance (Fig. 4E; \( t \) test, \( p = 0.10 \)), cell capacitance (Fig. 4F; \( t \) test, \( p = 0.85 \)), or frequency of spontaneous EPSCs (Fig. 4G; \( t \) test with Welch’s correction, \( p = 0.33 \)) of Cacna1a\textsuperscript{Nsr\textsuperscript{(+/-)}}/tdTomato or Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}}/tdTomato cells. This indicates that selective knock-out of Cacna1a in layer VI neurons does not significantly alter their intrinsic membrane firing properties, and results primarily in a specific impairment of neurotransmitter release.

**Functional knock-out of P/Q calcium channels in layer VI projection neurons results in spike-wave epilepsy**

To assess whether reduction of P/Q calcium channels in layer VI projection neurons is sufficient to produce spike-wave epilepsy, video-EEG experiments were performed on wild-type and Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} mice (Fig. 5A). During extensive monitoring, Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} mice (\( n = 4 \)) displayed frequent seizures (33.2 ± 5.8 spike-wave discharges/h, Kruskal–Wallis, \( p < 0.0001 \); range: 0–156 spike-wave discharges/h), consisting of bilaterally synchronous, 5–7 spikes/s discharges that always coincided with behavioral arrest of the mice. Depth electrodes placed in the ventrobasal thalamus, as well as over frontal cortex detected concomitant spike-wave activity in both regions of one Cacna1a\textsuperscript{Nsr\textsuperscript{(-/-)}} mouse, confirming the involvement of both structures in the aberrant discharges (Fig. 5C). In contrast, no
EEG abnormalities were found in Cacna1aNtsr(+/+) (n = 3) or Cacna1aCitrine (n = 4) mice (Fig. 5B).

To further characterize the spike-wave seizures, we investigated whether they were sensitive to ethosuximide, a drug with T-type calcium channel blocking activity, which effectively suppresses seizures in toterting and all other reported SWD mouse models. Figure 5D shows a sample of baseline EEG traces recorded before and after 200 mg/kg intraperitoneal ethosuximide injection, demonstrating the similar rapid suppression of spike-wave seizures in this model. In three Cacna1aNtsr(−/−) mice, we saw spike-wave discharges decrease from 2.4 ± 0.7 spike-wave seizures/min to 0 ± 0 spike-wave seizures/min (data not shown).

Selective knock-out of P/Q calcium channels in cortical layer VI output neurons increases T-type calcium currents in thalamic relay and reticular neurons

LVA (T-type) calcium currents in thalamic relay neurons are required for spike-wave seizures (Kim et al., 2001), and elevation of this current in thalamic relay neurons is a common, sufficient, but not essential, feature of mutant mice with spike-wave seizures (Zhang et al., 2002; Song et al., 2004; Ernst et al., 2009). Thus, we examined whether similar membrane current remodeling of ventrobasal thalamic relay neurons occurred in Cacna1aNtsr(−/−) mutant mice. Whole-cell LVA currents in response to membrane depolarization were recorded from thalamic relay neurons in brain slices maintained in vitro. LVA current was reliably evoked in both Cacna1aNtsr(+/+) and Cacna1aNtsr(−/−) slices with typical fast kinetics (Fig. 6A; 10–90% decay) of 44.1 ± 11.7 ms (Cacna1aNtsr(+/+), n = 11) and 39.5 ± 4.7 ms (Cacna1aNtsr(−/−), n = 10) and strong dependency on prior membrane hyperpolarization. Peak LVA current density was significantly elevated 1.7-fold in ventrobasal thalamic neurons of Cacna1aNtsr(−/−) (12.38 ± 1.15 pA/pF, n = 10) slices compared with wild-type (7.36 ± 1.05 pA/pF, n = 11) neurons (t test, p = 0.0044, data not shown). In addition, SSI, defined by half-maximum voltage of the prior hyperpolarization voltage, showed a significant depolarizing shift in the mutant neurons (Fig. 6B; –82.2 ± 2.1 mV Cacna1aNtsr(+/+), n = 11; –75.3 ± 1.2 mV Cacna1aNtsr(−/−), n = 10; t test, p = 0.0109) indicating that LVA currents can be evoked by a smaller hyperpolarizing input in the mutant neurons. In contrast, the dependency on duration of the hyperpolarizing prepulse was not significantly altered (data not shown). We also examined rebound burst firing in ventrobasal thalamus and found no difference between Cacna1aNtsr(+/+) (1.14 ± 0.14 bursts, n = 7) and Cacna1aNtsr(−/−) (1.29 ± 0.18 bursts, n = 7) thalamic relay neurons (representative trace Fig. 6C; t test, p = 0.55). Additionally, we observed no significant difference in resting membrane potential (Fig. 6D; t test, p = 0.80), membrane resistance (Fig. 6E; t test, p = 0.18), latency to burst (Fig. 6F,G, representative trace; t test, p = 0.41), or number of action potentials per burst (Fig. 6H; t test, p = 0.99).

We also investigated intrinsic excitability and LVA currents in the thalamic reticular nucleus (nRT). Similar to thalamic relay neurons, peak LVA current was significantly elevated by 1.86-fold (t test, p = 0.001) in Cacna1aNtsr(−/−) nRT neurons (17.67 ± 2.00 pA/pF, n = 10) compared with Cacna1aNtsr(+/+) neurons (9.48 ± 0.57 pA/pF, n = 10; Fig. 7A). In contrast to ventrobasal thalamus, the SSI voltage was not altered in nRT neurons (Fig. 7B;
Layer VI P/Q Defect Activates Spike-Wave Network

Neurological phenotype of Cacna1a\textsuperscript{Ntsr\text{--/-}} mice

Genomic mutations reducing Cacna1a function produce mice with various degrees of ataxia; however, the functional contribution of descending corticothalamic projections to abnormal motor control has never been assessed. To ascertain whether the layer VI P/Q channel ablation in Cacna1a\textsuperscript{Ntsr\text{--/-}} mice lead to a motor coordination phenotype, we performed three assays for balance, agility, and gait. Using a rotating rod, we found no significant differences between the

\( n = 3 \) per genotype) using the comparative threshold method (see Materials and Methods), Cacna1a\textsuperscript{Ntsr\text{+/+}} mice were used as a control for Cacna1a\textsuperscript{Ntsr\text{--/-}} and Cacna1a\textsuperscript{Citrine} mice. The cerebellum, cortex, and thalamus were dissected from each mouse and revealed no significant difference of Cacna1g mRNA transcript levels (Fig. 8E). Within the thalamus, mRNA levels of Cacna1a\textsuperscript{Ntsr\text{--/-}} were 1.15 \( \pm 0.14 \)-fold of Cacna1a\textsuperscript{Ntsr\text{+/+}} levels and Cacna1a\textsuperscript{Citrine} transcript levels were 1.26 \( \pm 0.05 \)-fold (one-way ANOVA, \( p = 0.16 \)). Similarly, there was not a significant difference found in cortex mRNA levels (Cacna1a\textsuperscript{Ntsr\text{--/-}} 1.01 \( \pm 0.11 \)-fold; Cacna1a\textsuperscript{Citrine} 1.09 \( \pm 0.12 \)-fold, one-way ANOVA, \( p = 0.76 \)) or in cerebellum (Cacna1a\textsuperscript{Ntsr\text{--/-}} 0.92 \( \pm 0.01 \)-fold; Cacna1a\textsuperscript{Citrine} 0.92 \( \pm 0.09 \)-fold, one-way ANOVA, \( p = 0.73 \)). The finding of comparable mRNA levels between the wild-type and mutant is consistent with the lack of significant detectable transcriptional changes in other related spike-wave mouse models, including sg, tg, and lh (Zhang et al., 2002; Song et al., 2004), and suggest that the measured current alterations are more likely explained by alternative post-translational modifications that modulate T-type currents (Chemin et al., 2014; Todorovic and Jevtovic-Todorovic, 2014).

Figure 5. Cacna1a\textsuperscript{Ntsr\text{--/-}} mice have spike-wave epilepsy. A, Representative 12 s traces of wild-type (Cacna1a\textsuperscript{Ntsr\text{+/+}}, Cacna1a\textsuperscript{Citrine}) and Cacna1a\textsuperscript{Ntsr\text{--/-}} mice electroencephalographic recordings from bilateral electrodes over frontal cortex. Cacna1a\textsuperscript{Ntsr\text{--/-}} mice display stereotyped 5–7 spike/s spike-wave discharges. Scale bar, 500 \( \mu \)V and 500 ms. B, Spike-wave discharge rates from Cacna1a\textsuperscript{Ntsr\text{+/+}} \( n = 3 \), 12 h/mouse; Cacna1a\textsuperscript{Citrine} \( n = 4 \), 12 h/mouse; and Cacna1a\textsuperscript{Ntsr\text{--/-}} mice, \( n = 4 \), 12 h/mouse. Spike-wave discharges were only seen in Cacna1a\textsuperscript{Ntsr\text{--/-}} mice. C, Representative bilateral EEG recording from the frontal cortex with depth electrodes placed in the ventrobasal thalamus show spike-wave discharges in both regions of the brain. Scale bar, 500 \( \mu \)V and 500 ms. D, Representative EEG recording from a Cacna1a\textsuperscript{Ntsr\text{--/-}} frontal cortex before and after intraperitoneal injection of 200 mg/kg ethosuximide shows suppression of spike-wave discharges after drug exposure. Scale bar, 500 \( \mu \)V and 500 ms.
wild-type groups (Cacna1aNtr(+/+), n = 9; Cacna1aNtr(Citrine), n = 6) and Cacna1aNtr(−/−) (n = 9) adult mice (Fig. 9A; ANOVA, p = 0.17). Similarly, no significant difference in gait was detected between wild-type (Cacna1aNtr(+/+), n = 9; Cacna1aNtr(Citrine), n = 6) and Cacna1aNtr(−/−) (n = 9) mice (Fig. 9B; ANOVA, p = 0.17). A small but significant difference (0.85 ± 0.19 compared with 0.27 ± 0.14 and 0.18 ± 0.12, ANOVA, p = 0.017), in performance score was found between Cacna1aNtr(−/−) mice (n = 13) and their wild-type (Cacna1aNtr(+/+), Cacna1aNtr(Citrine)) littermates (n = 11) using a ledge test (Guyenet et al., 2010) to assay motor coordination (Fig. 9C).

Unlike genomic ataxic mutant models of Cacna1a, it was not possible to visually detect adult Cacna1aNtr(−/−) mice with certainty by their gait or spontaneous motor behavior, indicating that the severity of these motor phenotypes is accounted for by P/Q deficits outside of the corticothalamic pathway. We also did not detect any episodes of repetitive limb dyskinesia like those observed in the tg mutant.

Discussion

Together with its rich monogenic landscape, absence epilepsy is an especially complex synchronization disorder to physiologically dissect, since the identity of critical disease microcircuitry underlying the phenotype in each model is obscured by widespread and largely functionally redundant expression of...
the disease-causing mutant protein. In the case of P/Q-type calcium channelopathy, none of the models expressing genomic P/Q channel defects reveal which limb, if any, of the spike-wave circuit is inherently sufficient to generate the distinctive discharge, because P/Q channels contribute to synaptic release at each of its nodes as well as the remainder of the nervous system.

Here we used conditional cre-driven mutagenesis of Cacna1a within a highly targeted subpopulation of layer VI neocortical excitatory pyramidal cells to pinpoint synapses originating from a solitary cell type within the canonical thalamocortical network, and determined that impaired synaptic release at their terminals is sufficient for the genesis of generalized, spike-wave absence epilepsy. Seizures generated by ablating P/Q channels from layer VI pyramidal neuron terminals were phenomenally identical to those present in genomic Cacna1a mutations, including spike burst frequency (5–7 spikes/s), concomitant behavioral arrest, and ethosuximide sensitivity. We also determined that impairment of layer VI projections to the thalamus was not sufficient to account for the ataxia or episodic dystonia characteristic of genomic Cacna1a deficiency. This conditional mutant model reduces the network complexity required to solve a mechanism for the aberrant spike-wave phenotype to a single, defined, cortical synaptic output and may simplify the analysis and modeling of critical dynamic firing patterns and downstream molecular plasticity that contribute to a stable spike-wave synchronization phenotype.
Layer VI neurons: isolated dynamic release defect at the distant corticothalamic projection synapse remodels thalamic neuron excitability

The “top down” suppressant feedback of layer VI output following thalamic afferent input to a cortical column has been confirmed in visual (Olsen et al., 2012) and somatosensory (Crandall et al., 2015) cortex, where it acts as an inhibitory gain control of ascending sensory-evoked responses. Electrical stimulation of corticothalamic fibers also alters intrinsic oscillation frequency in this circuit (Bal et al., 2000; Blumenfeld and McCormick, 2000). Thus, although a complete silencing of this synapse might be expected to impede rhythmicity, we found that P/Q channel-deficient layer VI synaptic output modified thalamic T-currents, rebound burst firing, and released aberrant network oscillatory activity. At the layer VI–thalamic relay synapse, we found evidence for shared P/Q- and N-type calcium channel control of evoked neurotransmitter release, with partial compensation by non-P/Q presynaptic calcium channel subtypes as described at other descending cortical and intrahippocampal central excitatory synapses (Qian and Noebels, 2000, 2001). Despite preservation of unitary evoked release events, the dynamic properties of P/Q deficient synapses are altered because of unequal coupling of N-type channel subunits to vesicular release nanodomains; tightly coupled P/Q channels trigger fast, synchronous exocytosis, whereas N-type channels mediate less synchronous release (Neher and Sakaba, 2008; Catterall et al., 2013). Thus P/Q-deficient synapses, when analyzed at motor nerve terminals or the presynaptic Calyx of Held, show an increase in synaptic release delay (jitter; Depeetis et al., 2008) and impaired short-term facilitation (Inchauspe et al., 2007) that, depending on the degree of rescue.
by N- and R-type calcium channels, may significantly alter network synaptic synchrony. This strongly implies a role for P/Q-mediated short-term plasticity at corticothalamic synapses in preserving normal patterns of thalamic oscillations.

Along with functional release defects that impact dynamic circuit behavior, we also detected a persistent postsynaptic excitability change in relay cells, similar in magnitude to that found previously in genomic spike-wave mutant models, which was due solely to the isolated corticofugal synaptic defect (Tsakiridou et al., 1995; Zhang et al., 2002, 2004; Broicher et al., 2008; Ernst et al., 2009). Importantly, in our conditional model, the transsynaptic enhancement of thalamic LVA current occurred in the presence of wild-type P/Q-type channel expression in the relay cells, demonstrating that T-type current remodeling is presynaptically driven, and independent of the native P/Q-type channel status in the postsynaptic cells. Transsynaptic plasticity in thalamocortical pathways due to abnormal deprivation of afferent sensory input during early brain development and later in adulthood is well documented in somatosensory circuits (Yu et al., 2012; Li et al., 2013). Our findings clearly indicate that altered release from descending cortical inputs is also sufficient to persistently modify intrinsic thalamic membrane properties and have clear implications for neurodevelopment. Because we found that a similar magnitude of LVA calcium current enhancement (~50%) due to overexpression of a BAC transgene for Cacna1g T-type channels is sufficient to produce the spike-wave phenotype (Ernst et al., 2009), and the majority of native Cacna1g expression is within thalamic relay cells, our results add to the evidence that thalamic T-type remodeling is a sufficient, although not necessarily exclusive, molecular basis for the spike-wave phenotype.

We previously identified spike-wave discharges following conditional ablation of Cacna1a in cerebellar circuits in purky (Mark et al., 2011) and quirky mutants (Maejima et al., 2013). Although thalamic T-type currents were not measured, both models disturb developmental patterns of thalamic signaling, suggesting a similar mechanism. However, these cre-promoter lines also showed sparse forebrain cellular expression, whereas in the case of the NTSR-cre driver studied here, reporter expression was confined to the cortical layer VI neurons, and we detected limited expression only among a small number of hippocampal cells unrelated to thalamocortical circuitry.

**Layer VI neurons: local excitatory and inhibitory control of the spike-wave circuit**

Intracortical circuitry plays a major role in spike-wave seizure generation, beginning with the terminals of regular spiking deep pyramidal neurons of layer VI whose output we targeted in this study. In vivo microelectrode analysis in somatosensory cortex of an inbred rat model of spike-wave epilepsy revealed that layer VI cells show the strongest coupling to the cortical EEG seizure compared with neurons in layer IV and their activation systematically preceded firing in more superficial layers (Polack et al., 2007). In wild-type mice, dual whole-cell recording in disinhibited cortical slices demonstrate that layer VI intracortical terminals are essential for horizontal synchrony during epileptiform burst (Golshani and Jones, 1999).

Recent details on the functional connectivity of the layer VI pyramidal cell projectome reveal an elaborate intracortical syn-
aptic microcircuitry activated by these axon collaterals, and how they differ between motor (Yamawaki and Shepherd, 2015) and sensory cortex (Thomson, 2010). Layer VI neurons activate layer V cells (Kim et al., 2014), and this projection is stronger in sensory than motor cortex. Thalamic afferents contact layer V1 as well as layer IV neurons (Constantinople and Bruno, 2013), but have diverse regional and laminar patterns (Clasca et al., 2012). Layer IV projects back to layer VI (Tanaka et al., 2011), and this local excitatory loop is faster and stronger in the neocortical microcircuit than that driven from the thalamic projection.

Along with these excitatory connections, there is a strong feedforward input onto a diverse and translaminar group of inhibitory neurons (Bortone et al., 2014). In rat neocortex, fast spiking interneurons in layer V ramify around pyramidal cell somata and show P/Q-mediated synaptic depression, whereas burst firing interneurons synapsing on pyramidal cell dendrites show N-type-mediated facilitation (Ali and Nelson, 2006). Adding to this anatomical complexity, it is not yet known whether P/Q-type release coupling predominates at these intracortical synapses, and how mutation affects their development. In cerebellar cortex, the P/Q channel is the predominant adult form at excitatory synapses (Baur et al., 2015), however in neocortex, thalamocortical-evoked EPSPs remain N-type dominant (Iwasaki et al., 2000). There are distinct and still unexplained differences in P/Q channel-mediated release at excitatory versus inhibitory synapses (Ermolyuk et al., 2013). Analysis of a gain-of-function mutation in Cacna1a showed enhanced transmission at intracortical excitatory synapses, while sparing release at fast-spiking interneurons, suggesting that presynaptic calcium channels are differentially sensitive to modulation in different cell types (Vecchia et al., 2014).

If a synaptic release defect is present in layer VI intracortical axon collaterals similar to that found at the distant thalamic synapse, the isolated P/Q deficiency in this model creates an imbalance of network excitation and inhibition at both the intracortical and thalamic nodes. We found no intrinsic excitability difference in the P/Q channel-obliterated layer VI neurons themselves, and therefore little evidence that altered firing properties per se lead to seizure generation. Additional evidence favoring the complex disruption of finely tuned signaling between intracortical excitatory and inhibitory neurons during generalized seizures was found following conditional P/Q ablation in mice using cre-drivers for parvalbumin+ (Nr2c2,1bac′), somatostatin (SSTb′), and pyramidal (Enk+′) neurons. P/Q-deficient synaptic impairment of these interneuron subtypes only produced spike-wave seizure patterns when simultaneously removed from both inhibitory and excitatory cortical neurons (Rossignol et al., 2013). Because the degree of excitation and inhibition at each synapse in this loop varies depending on the pattern of activity (Crandall et al., 2015), detailed modeling of this circuitry will be essential to visualize the dynamic balance of synaptic strength underlying the aberrant oscillating activity.

References

Agmon A, Connors BW (1991) Thalamocortical responses of mouse somatosen-


Bortone DS, Olsen SR, Scanziani M (2014) Translaminar inhibitory cells recruited by layer 6 corticthalamic suppressors visual cortex. Neur-

on 82:474–485. CrossRef Medline

Bourassa J, Deschênes M (1995) Corticothalamic projections from the primary visual cortex in rats: a single fiber study using biocytin as an antero-


ous 384–399. CrossRef Medline


Cain SM, Snutch TP (2013) T-type calcium channels in burst-firing, net-


Cain SM, Tyson JR, Jones KL, Snutch TP (2015) Thalamocortical neu-

rons display suppressed burst-firing due to an enhanced Ih current in a genetic model of absence epilepsy. Pflugers Arch 467:1367–1382. CrossRef Medline


Clasca F, Rubio-Garrido P, Jabaudon D (2012) Unveiling the diversity of thalamo-


Depaulis A, David O, Charpier S (2015) The genetic absence epilepsy strain from Strasbourg as a model to decipher the neuronal and network mech-


Depetris RS, Nuñer SI, Uchitel OD, Urbano FJ (2008) Altered synaptic syn-

chrony in motor nerve terminals lacking P/Q-calcium channels. Synapse 62:466–471. CrossRef Medline

Dreyfus FM, Tschetter A, Errington AG, Renger JJ, Shin HS, Uebele VN, Crumelli V, Lambert RC, Leresche N (2010) Selective T-type calcium channel block in thalamic neurons reveals channel redundancy and physi-


Ernst WL, Zhang Y, Yoo JW, Ernst SJ, Noebels JL (2009) Genetic enhance-


Golshani P, Jones EG (1999) Synchronized paroxysmal activity in the devel-


Medline


Bomben et al. • Layer V P/Q-Dependent Slices Depolarize Spike-Network