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Characterization of kindled VGAT-Cre mice as a new animal model of temporal lobe epilepsy

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Abstract

Objective: Development of novel therapies for temporal lobe epilepsy is hindered by a lack of models suitable for drug screening. While testing the hypothesis that “inhibiting inhibitory neurons” was sufficient to induce seizures, it was discovered that a mild electrical kindling protocol of VGAT-Cre mice led to spontaneous motor and electrographic seizures. This study characterizes these seizures and investigates the mechanism.

Methods: Mice were implanted with electroencephalographic (EEG) headsets that included a stimulating electrode in the hippocampus before being electrically kindled. Seizures were evaluated by review of EEG recordings and behavior. γ -Aminobutyric acidergic (GABAergic) neurotransmission was evaluated by quantitative polymerase chain reaction, immunocytochemistry, Western blot, and electrophysiology.

Results: Electrical kindling of VGAT-Cre mice induces spontaneous recurring seizures after a short latency (6 days). Seizures occur 1–2 times per day in both male and female mice, with only minimal neuronal death. These mice express Cre recombinase under the control of the vesicular GABA transporter (*VGAT*), a gene that is specifically expressed in GABAergic inhibitory neurons. The insertion of Cre disrupts the expression of *VGAT* mRNA and protein, and impairs GABAergic synaptic transmission in the hippocampus.

Significance: Kindled VGAT-Cre mice can be used to study the mechanisms involved in epileptogenesis and may be useful for screening novel therapeutics.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

ETHICAL APPROVAL

We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Keywords

electrical hippocampal stimulation; GABA; spontaneous recurring seizures; temporal lobe epilepsy

1 | INTRODUCTION

There is a significant need to develop novel therapies for mesial temporal lobe epilepsy (TLE), a conclusion shared by the American Epilepsy Society Basic Science Committee, the International League Against Epilepsy Working Group for Preclinical Epilepsy Drug Discovery, and the National Advisory Neurological Disorders and Stroke Council.^{1,2} Current animal models of TLE have serious drawbacks that hinder drug development efforts. These models typically begin by inducing a long-lasting status epilepticus, by using either chemoconvulsants (eg, kainate, pilocarpine) or prolonged electrical stimulation.^{3–5} Many animals die during the procedure; mortality is 10%–30% in rats and ranges from 0% (intrahippocampal kainate⁵) to 90% (pilocarpine⁶) in mice. Animals that survive and develop spontaneous recurrent seizures (SRS) show extensive neuronal death throughout the brain.^{7,8} In contrast, postmortem analysis of human TLE patients shows either no detectable neuronal loss (ie, no hippocampal sclerosis in 20%–50% of cases) or restricted loss in hippocampal subfields.^{9,10} Seizure frequency in animal models varies greatly even within the same cohort of animals, and seizures often cluster.^{11,12} Evoked seizures in kindled animals overcome this lack of seizure predictability, but come with other problems: (1) seizure phenotype depends on where the stimulating electrode is placed; (2) only in extreme cases of “overkindling” (>200 stimulations) do animals become epileptic with spontaneous seizures¹³; and (3) chronic epilepsy induces changes in drug target expression, but compounds are tested on nonepileptic animals.¹⁴ Taken together, new antiseizure and antiepileptic drugs are needed, but current animal models with spontaneous limbic seizures are inadequate.

While testing the “GABA hypothesis of epilepsy,”¹⁵ we serendipitously discovered that electrical kindling of VGAT-Cre mice led to the development of spontaneous seizures. We recognized the importance of a novel animal model of TLE and focused our studies on characterizing seizure properties, neuronal death, and the mechanism for why kindling VGAT-Cre mice leads to epilepsy. We report that electrical stimulation is required; this is not a spontaneous seizure model. Spontaneous seizures develop approximately 7 days after kindling and average 1.4 seizures per day. Neuronal death assayed by either Fluoro-Jade C staining or counting anti-NeuN antibody–stained nuclei was virtually nonexistent. Insertion of Cre into the *VGAT* gene disrupted expression at both mRNA and protein level, and importantly, diminished γ -aminobutyric acidergic (GABAergic) synaptic transmission. These studies establish a novel animal model of TLE that avoids many of the pitfalls of post–status epilepticus models, possibly making it useful for screening novel antiseizure and antiepileptic drugs.

2 | MATERIALS AND METHODS

2.1 | Animals

VGAT-Cre mice, *Slc32a1^{tm2(cre)Low1}/J* (IMSR, Cat# JAX:016962, RRID:IMSR_JAX:016962 RRID:IMSR; described by Vong et al¹⁶), were obtained from the Jackson Laboratory. These mice were on a mixed background of FVB and 12956/SvEvTac mice. They were bred with C57BL/6J (IMSR, Cat# JAX:000664, RRID:IMSR_JAX:000664) for three generations at the University of Virginia, then maintained as homozygotes thereafter (data are from the N3F4 to N3F6 generation). Authentication was performed using single nucleotide polymorphism panels from the Jackson Laboratory. As noted, studies were also performed on C57BL/6J and the VGAT-Cre strain that is congenic with C57BL/6J (IMSR, Cat# JAX:028862, RRID:IMSR_JAX:028862). All mice were housed in the Pinn Hall vivarium under the following conditions: ad libitum access to food and water, 12-hour light and 12-hour dark cycles, and an enriched environment that was cleaned twice per week. Before surgery, mice were housed in polycarbonate cages in groups of fewer than five mice. For electroencephalographic (EEG) recording, mice were singly housed. Both male and female mice were used. All mice were age-matched.

2.2 | Animal surgeries

Surgeries were performed on 8-week-old mice using isoflurane anesthesia. A Kopf stereotaxic apparatus was used to guide implantation of an EEG recording headset that included a bipolar Teflon-coated stainless-steel stimulating electrode (A-M Systems, diameter = 0.008", #791400), bilateral supradural cortical electrodes, and a cerebellar reference electrode. Electrodes were connected to a six-pin pedestal (Plastics One), which was secured to the skull with dental cement. The depth electrode was placed in the left hippocampus, targeting the perforant path at the following coordinates (from bregma): 3 mm posterior, 3 mm lateral, and 3 mm depth. Animal discomfort was minimized with bupivacaine and ketoprofen (4 mg/kg delivered subcutaneously after surgery and every 24 hours as needed). All studies were performed in accordance with protocols approved by the Animal Care and Use Committee of the University of Virginia and ARRIVE guidelines.¹⁷

2.3 | Kindling protocol

After at least 1 week for recovery from surgery, mice were connected to a video-EEG monitoring system (AURA LTM64 using TWin software, Grass) via a flexible cable and commutator (Plastics One). One day later, we measured the afterdischarge threshold (ADT); the hippocampal electrode was connected to a constant current stimulator (A-M Systems, Model 2100), and then a 1-millisecond biphasic square wave pulse at 50 Hz was applied for 2 seconds. The current was initially set at 20 μ A and increased in 20- μ A increments until an electrographic discharge was observed (2 minutes between stimulations). The mean ADT was 108 μ A (range = 40–400). For kindling, the current intensity was set to 1.5 $\times$ the magnitude of ADT for that mouse and was delivered twice per day, every other day, with an interval between stimulations of at least 4 hours. Animals were considered fully kindled when stimulations evoked five consecutive seizures with a behavioral score of at least 5 (bilateral clonus with loss of posture control) on a modified Racine scale.¹⁸ Animals were

monitored by continuous video-EEG throughout the experiment (24 h/d, 7 d/wk). EEG data were digitized at 400 Hz, then stored on a file server along with video for subsequent analysis. Spontaneous seizures were defined as evolving spike-wave discharges with >2-Hz frequency and $3 \times$ the baseline amplitude lasting 15 seconds or longer. During analysis, electrographic seizures were confirmed by the corresponding video and a behavioral score was assigned.

2.4 | Histology

Mice were anesthetized and transcardially perfused with freshly prepared 4% paraformaldehyde solution (Electron Microscopy Sciences). Isolated brains were postfixed an additional 24–48 hours and then sliced using a Leica vibratome (horizontal slices of 40 μ m). Imaging was performed with an Olympus BX61WI microscope equipped with a spinning disk confocal unit and a Hamamatsu Orca 4 camera. Image acquisition and analysis used Olympus Cellsens software. Staining was performed on slices from a consistent depth above the anterior commissure (3–4 mm below bregma). Fluoro-Jade C stain (Histo-Chem) was used to measure neuronal death.^{8,19} Anti-NeuN rabbit monoclonal antibody (1:500, Abcam, Cat# ab177487, RRID:AB_2532109) was used to count neurons.²⁰ To visualize anti-NeuN labeling, we used Alexa Fluor 488–conjugated AffiniPure donkey antirabbit antibody (1:500, Jackson ImmunoResearch Laboratories; RRID:AB_2313584). Anti-VGAT mouse monoclonal antibody (1:200, Synaptic Systems, Cat# 131 011, RRID:AB_887872, clone 117G4) was used to measure VGAT expression.²¹ To visualize anti-VGAT labeling, we used Alexa Fluor 488–conjugated goat antimouse IgG3 antibody (1:500, Thermo Fisher Scientific, Cat# A-21151, RRID:AB_2535784).

2.5 | Quantitative reverse transcription polymerase chain reaction

Total RNA was purified from forebrain tissue using the Trizol plus PureLink RNA mini kit (Thermo Fisher Scientific, Cat# 12183555). Residual DNA was removed using the Turbo DNA-free kit (Thermo Fisher Scientific, Cat# M1907). Complementary DNA was synthesized from 500 μ g RNA using the iScript cDNA synthesis kit (BioRad, Cat#170–8890). Quantitative polymerase chain reaction (PCR) was performed using Bio-Rad Laboratories iQ SYBR green supermix and a MyIQ iCycler. Primers were designed using Primer-Blast.²² Primers for mouse VGAT were as follows: forward CCAAGATCACGGCGTGGGAA and reverse AAGCGATGACCAGGATGTTG. Primers for 18S rRNA subunit were used to normalize the data.²³ Validation included the following: established primer efficiency of >95%, sequenced PCR product to verify target amplification, and “no reverse transcriptase” controls to exclude contamination by DNA. Results are from three experiments each performed in triplicate using three C57BL/6 mice and 10 VGAT-Cre mice (five male, five female; similar results so pooled).

2.6 | Western blot analysis

Hippocampal tissue from VGAT-Cre and C57BL/6 animals (n = 4 each) was acutely isolated and lysed in radioimmunoprecipitation assay lysis buffer as described previously.²⁴ Fifteen micrograms of protein was resolved on 10% sodium dodecyl sulfate–polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and blotted with anti-VGAT antibody (1:500, same as above). The blots were developed using a chemiluminescence reagent

(PerkinElmer) and imaged using a Chemidoc Touch imaging system (Bio-Rad Laboratories). The blots were reprobed with anti- β -actin antibody (1:10 000, Sigma-Aldrich, Cat# A2228, RRID:AB_476697). The intensity of VGAT signal was quantified using the Image Lab program (Bio-Rad Laboratories) and normalized to that of β -actin expression.

2.7 | Electrophysiology

GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded from dentate granule cells and CA1 pyramidal neurons of acutely isolated hippocampal slices of C57BL/6 and VGAT-Cre mice using standard whole-cell patch-clamp techniques.²⁵ Slices were perfused with oxygenated artificial cerebrospinal fluid containing DL-2-amino-5-phosphonopentanoic acid (50 $\mu\text{mol}\cdot\text{L}^{-1}$) and 6,7-dinitroquinoxaline-2,3-dione (20 $\mu\text{mol}\cdot\text{L}^{-1}$). The recording electrode was filled with an internal solution containing (in $\text{mmol}\cdot\text{L}^{-1}$) 153.3 CsCl, 1 MgCl_2 , 10 hydroxyethylpiperazine ethane sulfonic acid, and 5 ethyleneglycoltetraacetic acid, pH adjusted to 7.2 with CsOH (osmolarity = 285–290 mOsm). The electrode shank contained adenosine triphosphate Mg^{2+} salt (4 $\text{mmol}\cdot\text{L}^{-1}$). Whole-cell capacitance and series resistance were adjusted to 70% with a 10-microsecond lag. Recordings were performed at 30°C from neurons voltage-clamped at –60 mV. Currents were analyzed offline using MiniAnalysis software (Synaptosoft) as described previously.²⁵

2.8 | Statistics and scientific rigor

Statistical analysis was performed using Prism software (GraphPad, version 8.3, RRID:SCR_002798). Data were first analyzed for normality, and then with either parametric or nonparametric tests as appropriate. The number of animals is represented by “n.” In histology experiments, we analyzed multiple images for each animal, then used the average result for statistical analysis. Only the initial experiments comparing adeno-associated viruses (AAVs) were performed blind. The key finding that kindled VGAT-Cre mice develop spontaneous seizures was independently reproduced by John Williamson and Jaideep Kapur.

3 | RESULTS

3.1 | VGAT-Cre mice develop spontaneous limbic seizures after electrical kindling

The original objective was to test the “GABA hypothesis of epilepsy,” that loss of inhibitory GABA neuron function is sufficient to cause seizures. Our approach was to inhibit GABAergic neurons selectively by overexpressing an engineered leak potassium channel described previously (TrekM⁸). To achieve neuronal subtype selectivity, we used VGAT-Cre mice and injected them with AAVs that require Cre recombinase for expression. A proven method for conferring Cre-dependence to AAV-mediated gene delivery is to flank an inverted copy of the gene of interest with wild-type and mutant loxP sites, the FLEX or DIO switch.²⁶ A common application of this method has been to deliver optogenetic payloads such as channelrhodopsin-2 (Chr2) into various Cre-driver mice.^{27,28} Our original study design was to inject AAVs (AAV-DIO-TrekM or AAV-DIO-Chr2 as control), implant VGAT-Cre mice with an EEG recording headset that included a depth electrode in the hippocampus, wait 4 weeks for AAV expression, electrically kindle the mice, and then monitor them 24 h/7 d by video-EEG (Protocol 1, Figure 1A). In keeping with the GABA

hypothesis, VGAT-Cre mice injected with AAV-DIO-TrekM and kindled developed spontaneous electrographic seizures that were accompanied by full tonic-clonic motor seizures (Racine class 5²⁹). Unexpectedly, VGAT-Cre mice injected with the control, AAV-DIO-Chr2, also developed SRS after electrical kindling. Furthermore, there was no statistical difference between the percentage of animals that developed SRS between the AAV groups (AAV-DIO-Chr2, 4/8 = 50%; AAV-DIO-TrekM, 9/11 = 82%; $P = .3$, Fisher exact test).

Rather than continue this line of experimentation, we focused on validating kindling of VGAT-Cre mice as a new model of TLE (no AAV injections). Figure 1 compares EEG traces from an electrically evoked seizure to a spontaneous seizure observed later in the same mouse. Both types of seizures show evolving high-amplitude spike-wave discharges and postictal suppression. To exclude the effects of AAV, we next injected saline only (Protocol 2, Figure 1D, $n = 8$, two females, six males, 8 weeks old). To exclude the effects of surgically implanting an EEG headset with a depth electrode, we also video-EEG monitored these mice for 4 weeks before kindling. No spontaneous seizures were observed at this time. We next electrically kindled these mice, and about 1 week later they developed spontaneous recurring seizures. Figure 1E shows the time courses of spontaneous seizure development observed in Protocols 1 and 2.

We next studied the strain dependence of kindling-induced spontaneous seizures using Protocol 3 (Figure 1D). The preliminary results were obtained with a VGAT-Cre line that was on a mixed background.¹⁶ The mice originally obtained from the Jackson Laboratories (*Slc32a1^{tm2(cre)Lowl/J}*, strains FVB and 129S6) were backcrossed three times to C57BL/6. The line was maintained by crossing homozygotes. These mice retain their agouti coat color (VGA). Genotyping confirmed VGA mice are on a mixed background (87.5% C57BL/6). Subsequently, Lowell and Warden deposited VGAT-Cre mice that were fully congenic with C57BL/6 (JAX Lab #028862) and have black coats (VGB). Electrical kindling of VGB mice led to the development of spontaneous seizures just as with the VGA strain (Figure 1F, $n = 7$). Taken together, 80% of kindled VGAT-Cre mice developed spontaneous seizures. In contrast, kindling of C57BL/6 mice did not lead to the development of spontaneous seizures ($n = 4$).

3.2 | Seizure characteristics in VGAT-Cre mice

Because electrical stimulation of VGAT-Cre mice leads to the development of spontaneous seizures, we pooled the results obtained with the different strains and protocols. We also pooled the sexes, as both male and female mice developed spontaneous seizures (male, 21/28 = 75%; female, 11/12 = 92%; $P = .4$, Fisher exact test). As detailed in Materials and Methods, electrical kindling of the hippocampus was performed twice per day at $1.5 \times$ the minimal amount of current required to trigger an afterdischarge (ADT). The criteria used to define the fully kindled state was five Racine class 5 seizures.²⁹ Figure 2 shows exemplar diaries of spontaneous seizure frequency in three mice (A) and results from a large cohort of kindled VGAT-Cre mice. We did not measure seizure frequency for 4 weeks in enough animals to make statistically significant conclusions on how seizure frequency changes with time. The diaries shown in Figure 2 were from a long-term experiment performed by

Williamson and Kapur (n = 6). Analysis of variance analysis of seizure frequency binned by 1-week intervals did not show any significant difference. Death evoked by electrical stimulation (sudden unexpected death in epilepsy) was only observed in three mice (6%). The average number of stimulations to reach the fully kindled state was 14 (10%–90% percentile range = 8–20, n = 40, Figure 2B). Most kindled VGAT-Cre mice developed spontaneous electrographic and behavioral seizures (32/40, 80%). These seizures developed with an average latency of 6 days after the last electrical stimulation (10%–90% percentile range = 1–9, n = 21, Figure 2C). Surprisingly, eight mice developed spontaneous seizures before reaching the fully kindled state (negative values in Figure 2C). Spontaneous seizure frequency was 1.4 seizures/d (10%–90% percentile range = 0.4–2.6, n = 24, Figure 2D). The behavioral component for each spontaneous seizure was scored using a modified Racine scale described previously,¹⁸ then averaged for that mouse. The average score was 4.2 (10%–90% percentile range = 3.9–4.5, n = 24, Figure 2E), indicating that most were bilateral, tonic-clonic seizures. There was one mouse that experienced electrographic seizures that were not accompanied by a motor component (Vgat-6). This statistical outlier was included in the analysis. Clustering of seizures was not apparent, with most animals seizing 1–2 times every day. Consecutive seizure-free days were observed, but the average was only 3 (10%–90% percentile range = 1–7, n = 24, Figure 2F).

3.3 | Lack of neuronal death

Post-status epilepticus models of TLE have been shown to have extensive neuronal death using either Fluoro-Jade dyes or counts of neurons or their nuclei (anti-NeuN staining). We used Fluoro-Jade C, which proved useful in our studies with pilocarpine-induced status epilepticus.⁸ Horizontal brain slices from the center-to-ventral hippocampus showed no signs of significant staining in either shams (headset but no stimulation) or kindled animals that developed seizures (Figure 3A). Note that the threshold for detection was set near the level of autofluorescence to allow statistical comparison. Positive controls included staining dorsal slices that included the stimulating electrode (see inset in Figure 3A) and an animal that developed status epilepticus (Figure S1). Studies of Fluoro-Jade staining after pilocarpine-induced status epilepticus have shown that its green fluorescent signal decays significantly in 1–2 weeks.⁷ Our studies were performed on animals perfused 1 day after the last spontaneous seizure from a cohort of animals that had on average 10 seizures. However, this time point corresponds to 11 days after the last electrical kindling stimulation. Therefore, these studies would capture seizure-induced death, but underestimate kindling-induced death. Counting neurons that survive overcomes these limitations.

Visual inspection of slices stained with anti-NeuN did not appear to have significant neuronal loss. Neuronal counts were performed on confocal images taken at ×20 magnification from brain regions known to show neuronal loss in TLE: dentate hilus, CA1 pyramidal cell layer, and layers II and III of the entorhinal cortex (Figure 3B–D). Neuronal counts were not significantly different between naive controls (no headset) and kindled mice that developed SRS.

3.4 | Mechanistic studies

The goal of our next studies was determining whether VGAT-Cre mice show deficits in GABAergic neurotransmission. To drive the expression of Cre recombinase from the VGAT promoter, Vong et al¹⁶ used homologous recombination to insert an IRES-Cre cassette after the stop codon of the VGAT coding region (Figure 4A). We tested the hypothesis that this insertion disrupted the expression of VGAT mRNA using quantitative reverse transcription PCR (RT-qPCR). Total RNA was purified from forebrains of control C57BL/6 and naive VGAT-Cre mice (VGA), converted to cDNA, and then compared using qPCR with validated primers. Abundance of mRNA was calculated using the C_t method.³⁰ Figure 4Ba shows representative traces of PCR product formation for the control gene, ribosomal 18S (*Rn19s*), and the *VGAT* gene (*Slc32a1*). On average, VGAT product formation from VGAT-Cre mice required one more cycle than from C57BL/6 mice, reflecting a 50% reduction in VGAT-Cre mRNA (Figure 4Bb, $P < .01$; C57BL/6, $n = 3$; VGAT-Cre, $n = 10$). Further supporting our hypothesis were in vitro studies using reporter assays that also found a 50% decrease in expression by inserting the IRES-Cre cassette (Figure S2).

We next tested whether decreased expression of VGAT mRNA resulted in decreased expression of VGAT protein. Immunocytochemistry studies were performed on brain slices of control C57BL/6 and naive VGAT-Cre mice using a validated mouse monoclonal anti-VGAT antibody³¹ and an Alexa Fluor 488–conjugated goat antimouse antibody. Representative images of the dentate clearly show decreased staining in slices from VGAT-Cre mice (Figure 4Ca). The sum fluorescent signal was calculated from $\times 20$ confocal images of the dentate, CA3, CA1, and subiculum. On average, VGAT-Cre mice had 50% lower anti-VGAT signal than C57BL/6 controls (Figure 4Cb, $P < .001$; C57BL/6, $n = 6$; VGAT-Cre, $n = 9$). Western blot analysis used the same anti-VGAT antibody as above. The proteins detected from hippocampal samples were similar to the predicted molecular weight of VGAT protein (57 kDa, Figure 4Da, top). VGAT signal was normalized to β -actin (Figure 4Da, bottom). A significant reduction in VGAT protein expression was also observed by Western blot analysis (41%, Figure 4D, $P < .05$; C57BL/6, $n = 4$; VGAT-Cre, $n = 4$).

Because VGAT is the main transporter for GABA into synaptic vesicles, we hypothesized that its reduced expression would result in decreased GABA-mediated synaptic transmission. Whole-cell voltage-clamp recordings were performed on CA1 neurons using acutely isolated hippocampal slices and solutions to isolate GABA-mediated currents.²⁵ Averaged traces of spontaneous IPSCs recorded from VGAT-Cre mice showed a clear decrease in amplitude as compared to those recorded from C57BL/6 mice (Figure 4Ea). On average, the sIPSC amplitude was decreased 23% (Figure 4Eb, $P = .02$; C57BL/6, $n = 7$; VGAT-Cre, $n = 11$ mice). A similar result was also obtained from recordings of dentate granule cells (Figure S3A,B). No statistically significant difference was observed for sIPSC rise time ($P = .54$), decay rate ($P = .06$), or sIPSC frequency ($P = .53$; Figure 3C–E).

4 | DISCUSSION

This is the first study to discover a mouse strain that develops limbic seizures after mild electrical kindling. Electrical kindling of rodents has been studied extensively, and spontaneous seizures have only been observed in a few cases of overkindling.^{13,32,33} Most

animal models of TLE begin with inducing a prolonged bout of status epilepticus with either chemoconvulsants (eg, kainate, pilocarpine) or electrical stimulation.³⁴ Drawbacks to these post–status epilepticus models include high mortality, extensive neuronal death, seizure clustering, and electrographic abnormalities that are counted as seizures but are not accompanied by motor seizures (intrahippocampal kainate³⁵). These problems have hampered drug development efforts for spontaneous seizures in mesial TLE.

4.1 | Electrically kindled VGAT-Cre mice as a new model of TLE

The goal of our preliminary experiments was to test the hypothesis that loss or dysfunction of GABAergic inhibitory neurons was sufficient to cause spontaneous seizures. To accomplish selective inhibition of these neurons, we relied on a mouse line where Cre recombinase was knocked into the vesicular GABA transporter gene.¹⁶ These VGAT-Cre mice were injected with AAVs with Cre-dependent payloads and electrically kindled using stimulation protocols developed by Lothman and Williamson.^{18,36} Key details of the protocol include that a short 1-millisecond pulse was delivered at 50 Hz for 2 seconds, mice were stimulated twice per day, and kindling was terminated after five consecutive tonic-clonic seizures. Kindled VGAT-Cre mice developed spontaneous seizures regardless of the type of AAV injected; therefore, we stopped injecting AAV and focused our studies on validating their use as a novel model of TLE. Control mice that were either injected with saline instead of AAV, or had no injection, also developed seizures, but only after electrical stimulation of the hippocampus. This result demonstrates that VGAT-Cre mice are not spontaneously epileptic, that is, this is not a model of epileptic encephalopathy. These key findings were independently replicated by investigators with extensive experience with rodent models (Drs Kapur and Williamson).^{18,36}

Initial studies were performed on a VGAT-Cre strain developed in our vivarium. This strain is on a mixed background: 87.5% C57Bl/6J and 12.5% 129S1/FVB. Numerous studies have documented the effect of strain on seizure inducibility. In particular, FVB mice have been reported to have seizures³⁷ and to be susceptible to chemoconvulsant-induced neuronal death.³⁸ Therefore, we tested a congenic C57Bl/6J strain of VGAT-Cre mice that are commercially available, finding that kindling also induces the subsequent appearance of spontaneous seizures. In contrast, kindling of wild-type C57Bl/6J did not lead to spontaneous seizures. This was not due to an inherent difference in evoked seizure threshold, as our ADT values are similar between VGAT-Cre (103 μ A) and C57Bl/6J mice (74 μ A¹⁸). Taken together, it is the presence of Cre recombinase in the *VGAT* gene that renders these mice epilepsy-prone, with no discernible effect of background strain.

Studies next focused on determining the seizure characteristics that differentiate the VGAT-Cre model from post–status epilepticus models. Mortality was very low (6%) and was evoked by electrical stimulation that produced a prolonged tonic contraction. In contrast, mortality in post–status epilepticus mouse models can be as high as 90%,⁶ commonly occurring during status epilepticus, but also during the recovery period (dehydration, loss of appetite).

In most cases, spontaneous seizures developed about 1 week after mice reached the fully kindled state. However, in some cases these seizures began even before the animals were

fully kindled. This result suggests that electrical stimulation can trigger epilepsy by a separate mechanism that is independent of the brain circuits modified by the kindling process. The involvement of distinct circuits provides another important reason for drug testing on spontaneous seizures instead of evoked seizures in kindled animals. These results also show that there is no true latency period. The onset of seizures was well fit by a Boltzmann equation as predicted by the continuous-function hypothesis elaborated by Ben-Ari and Dudek³⁹ (results not shown).

4.2 | Seizure characteristics of epileptic VGAT-Cre mice

Spontaneous seizures detected by EEG were almost always associated with tonic-clonic motor seizures. The exception was one mouse (Vgat6) that had primarily focal electrographic seizures that did not generalize to the motor cortex. The average behavioral seizure score (BSS) for all epileptic mice was between 4 and 5, using a modified Racine class scoring system: BSS4 for bilateral forelimb clonus, BSS5 for rearing and falling, and BSS6 for running fits.¹⁸ Therefore, both electrographic and behavioral seizures were similar to those observed in post-status epilepticus TLE models.

Spontaneous seizure frequencies ranged between 0.4 and 2.6 per day (10%–90% range). Although most mice seized regularly, the distribution of longest interseizure intervals was not Gaussian, with six of 24 mice having seizure-free gaps of >5 days. Nonetheless, most mice had seizure frequencies that are amenable for drug screening using methods developed by the Epilepsy Therapy Screening Program.⁴⁰

4.3 | Little or no hippocampal sclerosis

Two techniques were used to detect the presence of neuronal death in brain slices from epileptic VGAT-Cre mice: (1) staining with the dye Fluoro-Jade C¹⁹ and (2) counting neuronal nuclei that were stained with anti-NeuN antibody.²⁰ In both controls and epileptic animals, there was Fluoro-Jade C staining around the stimulating electrode. This electrode is a twisted pair of Teflon-coated stainless-steel wires, which are lowered into the hippocampus. Therefore, it is not surprising that there is damage as the electrode progresses into the brain.⁴¹ Nevertheless, we did not find evidence for death of seizure-susceptible neurons such as those in the dentate hilus, CA1 cell layer, or entorhinal cortex. Previous studies of neuronal death after electrical kindling of rodents also have found few or no overt signs of hippocampal sclerosis.^{42–44} In contrast, death is widespread throughout the brain in post-status epilepticus animals, with significant loss of GABAergic interneurons, CA1 and CA3 neurons, and mossy cells.^{13,45} Human hippocampal sclerosis follows a continuum from no sclerosis in up to 40% of cases,⁴⁶ to restricted neuronal loss in dentate hilus, to classical sclerosis in ~60% of cases, and to total sclerosis with extensive loss of neurons in all hippocampal subfields.⁴⁷ Therefore, the significant proportion of human patients who have no hippocampal sclerosis provides external validity to the kindled VGAT-Cre model.

4.4 | Support for the GABA hypothesis of epilepsy

The VGAT-Cre mouse model was engineered by homologous recombination resulting in the insertion of an IRES-Cre cassette after the stop codon in the *VGAT* gene.¹⁶ We hypothesized that this insertion disrupted the stability of VGAT mRNA, leading to decreased VGAT

protein expression. This hypothesis was first tested at the mRNA level using RT-qPCR. Comparison of VGAT mRNA levels in VGAT-Cre mice to C57Bl/6J (both naive) revealed a 50% decrease in forebrain samples. The hypothesis was then tested at the protein level using immunocytochemistry and Western blotting. Both methods gave similar results, a 40%–50% decrease in VGAT protein. Because VGAT is the key transporter of GABA into synaptic vesicles, we next tested for reduced GABA-mediated currents using electrophysiology on acute brain slices. Again, a comparison to C57Bl/6J mice revealed a ~20% reduction in spontaneous inhibitory synaptic currents onto either dentate granule cells or CA1 neurons. Two possible explanations for why this decrease was not as large as the decrease in VGAT protein are that (1) we recorded sIPSCs rather than miniature IPSCs, which would have been a more accurate test of vesicular filling; and (2) synaptic responses are determined more by the number of postsynaptic GABA_A channels than the quantal size of GABA release.⁴⁸

Considerable evidence supports the “GABA hypothesis of epilepsy,” which states that reduced function of GABAergic neurons is a major cause of epilepsy.¹⁵ A variety of mechanisms have been described, such as death of inhibitory GABAergic neurons, changes in GABA receptor expression, and inherited mutations in both GABA receptors⁴⁹ and GABA transporters (GAT-1³⁵). We conclude that reduced VGAT expression leads to overexcitation of circuits modulated by GABAergic neurons, and that electrical kindling acts as a second hit that leads to epilepsy in VGAT-Cre mice.

Supplementary Material

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Key Points

- Electrical kindling of VGAT-Cre mice is sufficient to trigger epileptogenesis that culminates in spontaneous electrographic seizures accompanied by tonic-clonic motor seizures
- The uniformity in seizure frequency should allow for screening of novel antiseizure drugs on spontaneous seizures in a TLE model
- Insertion of Cre recombinase into the *VGAT* gene disrupted its function, leading to decreased GABAergic transmission, which is a common cause of epilepsy in humans

FIGURE 1.

Electrical kindling of VGAT-Cre leads to the development of spontaneous seizures. A, Schematic showing the original experimental design (Protocol 1). Mice were 8 weeks old at the time of the surgery to inject adeno-associated virus (AAV), insert the stimulating electrode into the hippocampus, and implant the electroencephalographic (EEG) recording headset. B, EEG recorded during a kindling stimulation to the hippocampus of a VGAT-Cre mouse. The stimulation protocol was a 2-second train of 1-millisecond biphasic square pulses at 40- μ A constant current delivered at 50 Hz (labeled stim.). C, EEG trace of a spontaneous (Spont.) seizure recorded from the same mouse. This seizure was accompanied by bilateral tonic-clonic motor convulsions (BSS5). Scale bar refers to B and C. D, Schematic showing the experimental designs of Protocols 2 and 3. Again, mice were 8

weeks old at the time of the surgery to implant the EEG recording headset and stimulating electrode. E, F, Kaplan-Meier curves of the mice that developed spontaneous seizures as a function of days of video-EEG recording. E, Development of spontaneous recurring seizures (SRS) in the local colony of VGAT-Cre mice. The original experiment tested the effect Cre-dependent AAV (dark red line, n = 12). Mice in Protocol 2 were injected with saline (sham), then video-EEG monitored for 4 weeks prior to kindling (green line, n = 6). F, Development of SRS using Protocol 3 in the following three mouse lines: (1) the local VGAT-Cre line (VGA, red line, n = 8), the VGAT-Cre line that is congenic with C57Bl/6 mice (VGB, blue line, n = 17), and C57Bl/6 (dotted line, n = 4)

FIGURE 2.

Characteristics of seizures in VGAT-Cre mice. Aa-Ac, Representative diaries of seizure frequency from three kindled VGAT-Cre mice. Video and electroencephalogram (EEG) were recorded continuously. B, Number of electrical stimulations required to fully kindle VGAT-Cre mice. The criteria used to define fully kindled mice were five consecutive evoked seizures that showed bilateral tonic-clonic motor seizures with or without running fits. The average number of stimulations was 14 ± 1 (mean $\pm$ SEM, $n = 40$). Three mice died during the kindling process, immediately after a stimulation. Most, but not all, kindled mice developed spontaneous recurrent seizures (SRS; $32/40 = 80\%$). C, Latency from the last electrical stimulation to the first spontaneous seizure. Data shown include one statistical outlier (ROUT method, $Q = 1\%$). Eight mice developed SRS before being fully kindled (negative values in yellow shaded area). Excluding these mice, the average latency was 5.7 ± 1.1 days (mean $\pm$ SEM, $n = 22$). D, Average seizure frequency, 1.4 ± 0.2 seizures/d (mean $\pm$ SEM, $n = 24$). Six kindled mice were excluded: three that were not EEG monitored, two that had a single seizure, and the one that entered status epilepticus (G036, Figure S1). E, Average behavioral score during a spontaneous seizure using a modified Racine scale ($n = 24$). F, Longest interseizure interval (ISI) in epileptic VGAT-Cre mice, 2.9 ± 0.5 days (mean $\pm$ SEM, $n = 24$).

FIGURE 3.

Neuronal death assayed by Fluoro-Jade C (FJC) staining and counts of anti-NeuN-positive neurons. Aa, Representative images of FJC staining in the hippocampal dentate of sham controls (mice implanted with electroencephalographic headsets but not kindled), and kindled animals that developed spontaneous recurrent seizures (SRS). Also shown is an image of the area around the stimulating electrode in a mouse that was kindled and developed seizures (inset labeled Electrode). The dentate granule cell layer is outlined. Images were acquired with a $\times 10$ objective. Image analysis used Cellsens software and a fixed threshold for detection that was just above background in sham controls. The sum Fluoro-Jade signal was calculated by summing the area of each object times its intensity in each image, then averaged over all slices for that animal. Ab, Quantification of the FJC

signal in either the dentate hilus (Sham, SRS) or around the stimulating electrode. Hilar signal was determined in slices that were further from bregma (−3 to −4 mm) than the electrode (−2.5 mm). Each symbol represents the average results from 3–4 brain slices for each animal: sham, $n = 4$; SRS, $n = 8$; and electrode, $n = 5$. Statistical analysis confirmed normal distributions with the Shapiro-Wilk test, followed by ordinary one-way analysis of variance and Holm-Sidak multiple comparisons test (ns, not significant; *** $P < .001$). B-D, Neuronal counts determined by anti-NeuN staining in the hippocampal dentate hilus (B), CA1 pyramidal cell layer (C), and layers II and III of the entorhinal cortex (EC; D). Images from the center of the respective brain region were acquired with a $\times 20$ objective in confocal mode. Each symbol represents the average results from 3–4 brain slices for each animal: naive (no surgery) group, $n = 7$; and SRS group, $n = 12$). Statistical analysis confirmed normal distributions with the Shapiro-Wilk test, followed by an unpaired t test (ns, not significant). Scale bar in lower right corner of all images represents 100 μm

FIGURE 4.

VGAT mRNA, protein, and function are reduced in homozygous VGAT-Cre mice. A, Diagram illustrating the design of the IRES-Cre insertion into the *VGAT* gene (16). Homozygous VGAT-Cre mice were used for all studies, therefore, both alleles of the *VGAT* gene carried the insertion (cds, coding sequence; UTR, untranslated region). B, Quantitative reverse transcription polymerase chain reaction (PCR) was performed using forebrain samples. Ba, Typical data collected during the quantitative PCR reaction for C57BL/6 (green traces) and VGAT-Cre samples (red traces; both are average of three replicates). SYBR green fluorescence was normalized to the maximum observed for each primer pair. The control “housekeeping gene” was ribosomal 18S RNA, which was used to normalize and determine the C_t relative to the results with the VGAT primers. Bb, C_t values were

normalized to the average observed for all C57BL/6 mice, followed by an unpaired *t* test (***P* < .01; C57BL/6, *n* = 3; VGAT-Cre, *n* = 10). Ca, Representative images of anti-VGAT staining of the dentate of C57BL/6 and VGAT-Cre mice (identical exposure and contrast). Images were acquired with a ×20 objective in confocal mode (scale bar 100 μm). Cb, Quantitative analysis of the sum anti-VGAT signal (area × intensity), normalized to average results obtained from C57BL/6 brain slices (unpaired *t* test, *****P* < .001; C57BL/6, *n* = 6; VGAT-Cre, *n* = 9). Data from each mouse includes dentate, CA3, CA1, and subiculum. Da, Representative image of a Western blot containing samples from one C57BL/6 mouse (WT) and two VGAT-Cre mice. Molecular weight standards are shown on the left. The blot was first stained with anti-VGAT antibody (top), and then stripped and reblotted with β-actin (bottom). Db, Western blot analysis of VGAT protein expression relative to β-actin (unpaired *t* test; **P* < .05; *n* = 4 in each group). Ea, Averaged GABA spontaneous spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from CA1 neurons in acute hippocampal slices from either C57BL/6 (black trace) or VGAT-Cre mice (red trace). Eb, The mean of median sIPSCs was 23% smaller in slices from C57BL/6 than VGAT-Cre mice (*n* = 7 cells from five animals, *n* = 11 cells from seven animals, respectively, unpaired two-tailed *t* test, **P* < .02)