Received: 18 January 2019 | Revised: 24 February 2019 | Accepted: 15 March 2019

DOI: 10.1111/epi.14723

BRIEF COMMUNICATION

Epilepsia®

Targeted hippocampal GABA neuron ablation by Stable Substance P–saporin causes hippocampal sclerosis and chronic epilepsy in rats

Eugene Chun[1] | **Argyle V. Bumanglag**[2] | **Sara N. Burke**[3] | **Robert S. Sloviter**[2,4,5]

[1]Graduate Education in Biomedical Sciences Program, Morehouse School of Medicine, Atlanta, Georgia

[2]Neuroscience Institute, Morehouse School of Medicine, Atlanta, Georgia

[3]Department of Neuroscience, University of Florida, Gainesville, Florida

[4]Department of Neurobiology, Morehouse School of Medicine, Atlanta, Georgia

[5]Department of Pharmacology and Toxicology, Morehouse School of Medicine, Atlanta, Georgia

Correspondence
Robert S. Sloviter, Department of Neurobiology, Morehouse School of Medicine, Atlanta, GA.
Email: rssloviter@yahoo.com

Funding information
National Institute of Neurological Disorders and Stroke, Grant/Award Number: U54 NS083932; National Institute on Minority Health and Health Disparities, Grant/Award Number: 5R25GM058268, S21MD000101 and 5G12MD007602

Summary

Cryptogenic temporal lobe epilepsy develops in the absence of identified brain injuries, infections, or structural malformations, and in these cases, an unidentified preexisting abnormality may initiate febrile seizures, hippocampal sclerosis, and epilepsy. Although a role for GABAergic dysfunction in epilepsy is intuitively obvious, no causal relationship has been established. In this study, hippocampal GABA neurons were targeted for selective elimination to determine whether a focal hippocampal GABAergic defect in an otherwise normal brain can initiate cryptogenic temporal lobe epilepsy with hippocampal sclerosis. We used Stable Substance P–saporin conjugate (SSP-saporin) to target rat hippocampal GABA neurons, which selectively and constitutively express the neurokinin-1 receptors that internalize this neurotoxin. Bilateral and longitudinally extensive intrahippocampal microinjections of SSP-saporin caused no obvious behavioral effects for several days. However, starting ~4 days postinjection, rats exhibited episodes of immobilization, abnormal flurries of "wet-dog" shakes, and brief focal motor seizures characterized by facial automatisms and forepaw clonus. These clinically subtle behaviors stopped after ~4 days. Convulsive status epilepticus did not develop, and no deaths occurred. Months later, chronically implanted rats exhibited spontaneous focal motor seizures and extreme hippocampal sclerosis. These data suggest that hippocampal GABAergic dysfunction is epileptogenic and can produce the defining features of cryptogenic temporal lobe epilepsy.

KEYWORDS

cryptogenic temporal lobe epilepsy, dentate gyrus, hippocampal sclerosis, hippocampus, Stable Substance P–saporin

1 | INTRODUCTION

Brain injury–associated temporal lobe epilepsy with hippocampal sclerosis (TLE-HS+) is a common neurological condition in which the injured hippocampal formation becomes a suspected source of spontaneous epileptic seizures.[1–4] When TLE-HS+ develops without an identified cause, an unidentified preexisting abnormality presumably causes febrile seizures, hippocampal sclerosis, and temporal lobe epilepsy.[5–7]

Eugene Chun and Argyle V. Bumanglag contributed equally to this work.

The recent observation that the onset of focal epilepsy after experimental hippocampal injury is coincident with the initial neuron loss, rather than delayed,[8] is consistent with the "disinhibition" hypothesis of epileptogenesis, which states that injury-induced GABAergic dysfunction could be the immediate and sufficient cause of TLE-HS+.[8–11] The idea that injury reduces GABAergic inhibition, thereby initiating hippocampal epileptogenesis,[11] implies that when epilepsy develops in the absence of any identified brain injury or structural cause, perhaps a pre-existing genetic or developmental GABAergic defect is a primary epileptogenic trigger.[5,6] In this study, we tested the hypothesis that in patients with no history of brain injury or infection, and in whom no tumors or malformations can be detected, a subtle pre-existing hippocampal GABAergic defect could be a cause of cryptogenic TLE-HS+.

To target hippocampal GABA neurons selectively, we used a protease-resistant conjugate of substance P and the protein synthesis inhibitor saporin,[12] which is internalized via the neurokinin-1 (NK1) receptors that hippocampal GABA neurons selectively and constitutively express.[13] Because Stable Substance P–saporin conjugate (SSP-saporin) in a single locus within the dorsal hippocampus/dentate gyrus caused granule cell hyperexcitability, but not hippocampal sclerosis or epilepsy,[12] this study was designed to produce GABAergic dysfunction along a more longitudinally extensive expanse of the hippocampus/dentate gyrus.[11,14]

2 | MATERIALS AND METHODS

2.1 | Animal treatment

Male Sprague-Dawley rats (350-450 g, Envigo RMS) were treated in accordance with the National Institutes of Health (NIH) guidelines for the humane treatment of animals. All methods were approved by the Morehouse School of Medicine institutional animal care and use committee.

2.2 | Intrahippocampal microinjection

Rats were anesthetized (2%-3% isoflurane) and placed in a Kopf stereotaxic apparatus. Temperature was maintained at 37.0°C ± 0.2°C. The surgical area was shaved and cleaned with 70% alcohol and povidone-iodine. A midline incision exposed the skull, and holes were drilled at stereotaxic coordinates listed below.

Intrahippocampal injections of [Sar^9, $Met(O_2)^{11}$] SSP-saporin (0.4 ng/10 nL; IT-11-25, lot 113-79b; Advanced Targeting Systems) were performed using a 0.5-µL Neuros Syringe (Hamilton Company), which was lowered into four hippocampal sites along both the transverse and longitudinal hippocampal axes bilaterally. Coordinates from the bregma landmark were as follows: (1) ~3.5 mm anteroposterior (AP), ~2.5 mm mediolateral (ML), and ~3.6 mm dorsoventral (DV); (2) ~4.0 mm AP, ~2.0 mm ML, and ~3.6 mm DV; (3) ~4.5 mm AP, ~3.0 mm ML, and ~3.6 mm DV; and (4) ~5.0 mm AP, ~2.5 mm ML, and ~3.6 mm DV. Five minutes after lowering the microsyringe to the appropriate depth, 50 nL of SSP-saporin was injected at each site. Sterile autoclips were used to close the wound. Single doses of the analgesic carprofen (5 mg/kg subcutaneous [sc]) and the antibiotic enrofloxacin (10 mg/kg sc) were administered as described previously.[8] Control animals received intrahippocampal injections of phosphate-buffered saline vehicle (PBS; 0.1 mol·L^{-1} phosphate buffer, pH 7.4, in 0.9% NaCl wt/vol) or Blank-saporin in PBS (0.4 ng/10 nL; IT-21-25, lot 73-114; Advanced Targeting Systems), which is a nonspecific peptide-saporin conjugate control.

2.3 | Monitoring of behavior and dentate granule cell population activity

Behavior was initially observed intermittently after intrahippocampal SSP-saporin injections in five rats. When we noted during casual observation that longitudinally extensive SSP-saporin injections caused subtle behavioral seizures, a separate group of four SSP-saporin–injected rats was video-monitored continuously for 2 weeks postinjection. Months later, to determine whether SSP-saporin-injected animals were chronically epileptic, the initial group of five rats was implanted bilaterally with granule cell layer recording electrodes and perforant path stimulating electrodes, as previously described.[8] Continuous (24/7) monitoring for 1 week began immediately after recovery from anesthesia following electrode implantation, followed by perfusion-fixation. Digital depth recording data and video files were analyzed to detect spontaneous electrographic and behavioral seizures as previously described.[8]

2.4 | Perfusion-fixation and histology

After perfusion-fixation,[12] 50-µm-thick sections were cut and processed histologically as previously described.[12] Brightfield images were acquired on a Nikon E800M microscope with a Nikon DS-Fi2 digital camera and Nikon NIS-Elements imaging software. Adobe Photoshop CC2018 was used to construct figures, but not to change image content.

3 | RESULTS

3.1 | Histological effects of a single intrahippocampal injection of SSP-saporin or Blank-saporin

A single unilateral injection of SSP-saporin in the volume and concentration used for all injections was effective in reducing parvalbumin (PV) immunoreactivity, which is a marker of GABAergic basket and chandelier cells,[13] 2 weeks after injection (Figure 1). As previously described,[12] there were no

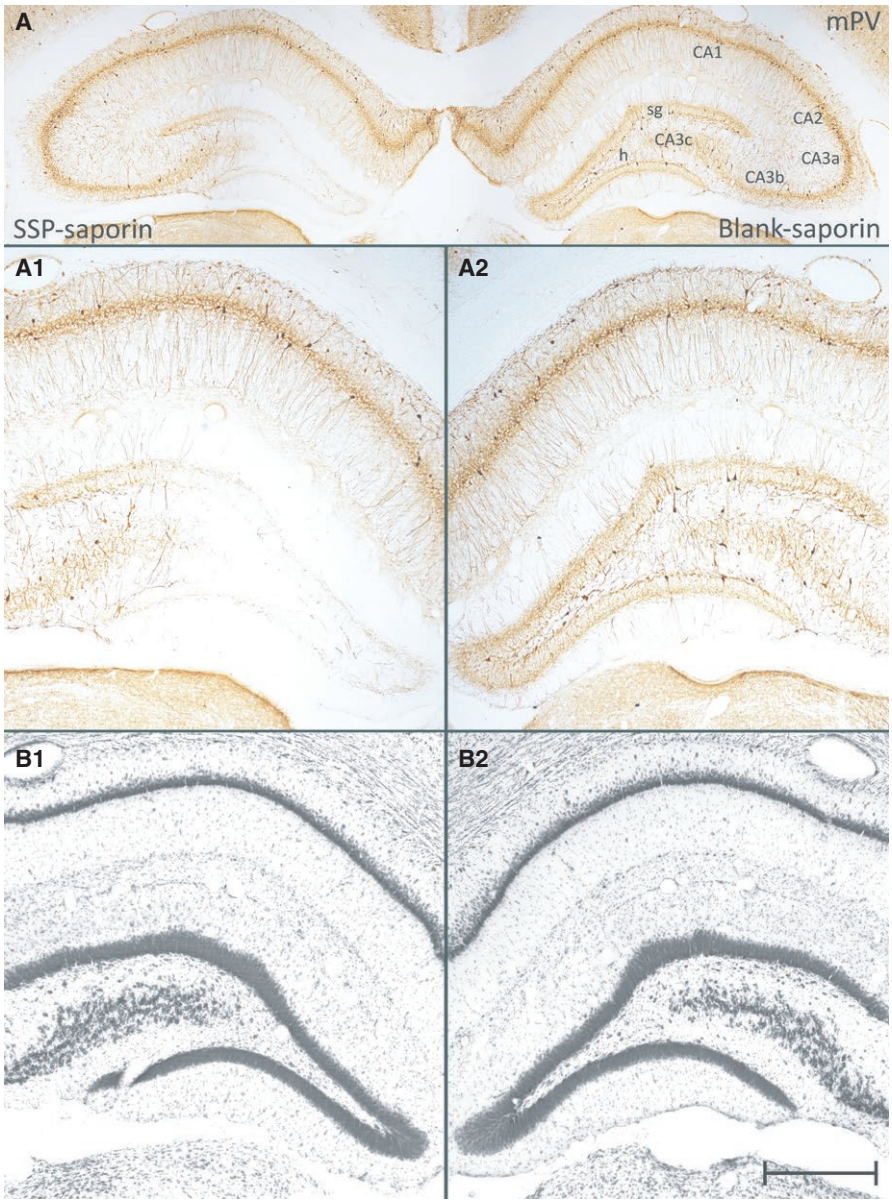

FIGURE 1 Loss of parvalbuminlike immunoreactivity (PV-LI) and survival of hippocampal principal neurons 15 days after intrahippocampal injection of Stable Substance P–saporin conjugate (SSP-saporin). A, A single unilateral injection of 50 nL of SSP-saporin into the dentate gyrus decreased PV-LI, whereas the contralateral control injection of Blank-saporin produced no detectable loss of PV-LI. h, hilus; sg, stratum granulosum. A1, A2, Expanded views of the dentate gyrus from the same section as A. B1, B2, Adjacent Nissl-stained section showing survival of hippocampal principal cells. Scale bar in B2 = 1053 μm in A, 525 μm in A1-B2. mPV, monoclonal parvalbumin antibody. [Colour figure can be viewed at wileyonlinelibrary.com]

apparent extrahippocampal changes in PV or NK1 immunoreactivity (data not shown). Blank-saporin conjugate injected into the contralateral hippocampus had no detectable effect on PV or NK1 immunoreactivity in the same immunostained sections (Figure 1). After establishing the effectiveness of SSP-saporin following a single unilateral injection of the volume and concentration used for all injections, we proceeded to inject SSP-saporin bilaterally in both the transverse and longitudinal planes.

3.2 | Acute behavioral effects of longitudinally extensive intrahippocampal SSP-saporin injections

Bilateral SSP-saporin injection into each dorsal dentate gyrus caused no obvious behavioral effects for several days after injection. However, episodes of immobilization followed by abnormally prolonged flurries of "wet-dog" shakes were typically observed. Brief seizures characterized by forepaw clonus and rearing also occurred intermittently in these SSP-saporin–injected rats. An additional group of four identically injected rats was continuously video-monitored to determine the time course of these acute behaviors, and to determine whether convulsive status epilepticus ever developed. This initial interval of acute and subtle abnormal behavior, and occasional focal seizures, began ~4 days postinjection and lasted ~4 days, after which the overall behavioral state of SSP-saporin–injected animals appeared normal when rats were handled or passively observed. Convulsive status epilepticus did not develop, and no deaths occurred.

3.3 | Chronic epilepsy and hippocampal pathology 3-4 months after SSP-saporin injection

Five consecutively injected animals were bilaterally implanted with stimulating and recording electrodes 3-4 months postinjection, and monitored continuously for 1 week prior to perfusion-fixation, or until they had two spontaneous behavioral seizures. This was done to determine whether animals were chronically epileptic, and whether spontaneous behavioral seizures were temporally associated with spontaneous granule cell layer epileptiform discharges, as recently described in perforant path-stimulated epileptic rats.[8] All five rats exhibited clinically obvious behavioral seizures. Four of the five rats exhibited at least two clinically obvious seizures. The remaining animal exhibited one clinically obvious seizure during the monitoring period. All spontaneous seizures consisted of brief immobilization, facial automatisms, and unilateral or bilateral forepaw clonus that progressed to rearing, and all were preceded by granule cell layer epileptiform discharges (Figure 2). Chronic epileptic seizures were not the product of the implanted electrodes, because spontaneous epileptic seizures were also observed >10 months postinjection in five nonimplanted SSP-saporin–injected rats.

In a rat in which one recording electrode was located within the granule cell layer of one shrunken hippocampus (Figure 2B), and the contralateral electrode tip was located within a neuron-depleted region of the contralateral sclerotic hippocampus (Figure 3F; electrode track not shown), the contralateral electrode did not detect any of the large-amplitude population spikes shown in Figure 2. This suggests that the recording shown in Figure 2 was a locally generated event. If this electrode had been detecting discharges originating from outside the hippocampus, the contralateral electrode would have recorded the same events similarly. Furthermore, the population spikes in the epileptiform discharge (Figure 2) were of identical morphology and polarity as the granule cell population spikes evoked by perforant path stimulation, as previously demonstrated in epileptic rats subjected to prolonged perforant path stimulation.[8] These observations indicate that the population discharges were locally generated granule cell population discharges.

Histological analysis revealed that all five SSP-saporin–injected rats exhibited relatively extreme hippocampal sclerosis (Figures 2B and 3). The patterns of cell loss and hippocampal sclerosis included classic hippocampal sclerosis, in which many dentate granule cells survived (Figures 2B and 3B-E), and maximal sclerosis, in which nearly all hippocampal principal cells degenerated (Figure 3F). Different extents of neuron loss were observed in different rostrocaudal locations of the same hippocampus.

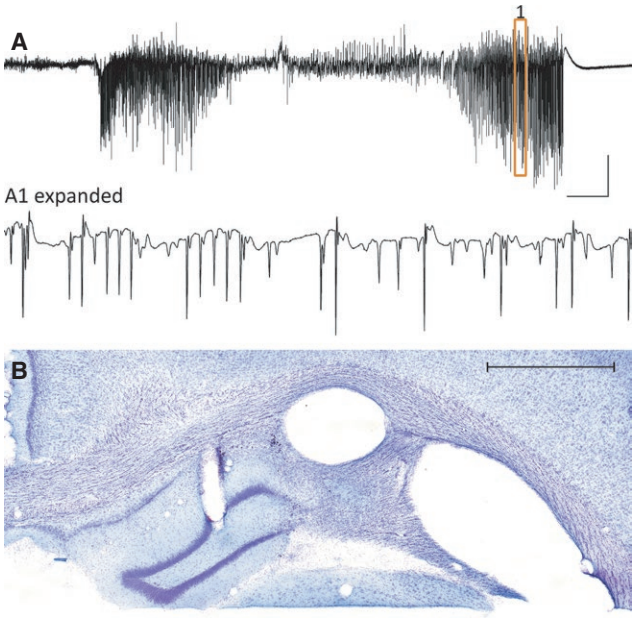

FIGURE 2 Spontaneous granule cell layer activity in the awake state 96 days after Stable Substance P–saporin conjugate (SSP-saporin) injection. A, Spontaneous epileptiform discharge temporally associated with a clinically obvious behavioral seizure. The high-amplitude granule cell layer activity preceded the onset of forepaw clonus by ~5 seconds. A portion of the compressed trace in A is expanded to show the high-amplitude granule cell population spikes within the epileptiform discharge. B, Nissl-stained coronal section from the rat in A confirming the location of the recording electrode tract within the dentate gyrus, and showing the extent of hippocampal shrinkage (compare to the control hippocampal section shown in Figure 3A). Perfusion-fixation was performed 102 days after SSP-saporin injection. Note that this animal exhibited an obvious loss of dentate hilar neurons and CA1-CA3 pyramidal cells. The contralateral hippocampus from this animal is shown below in Figure 3F (Rat #05). Calibration bars in A = 6 seconds and 4 mV in the compressed trace, 65 milliseconds and 4.7 mV in the expanded trace. Calibration bar in B = 1 mm [Colour figure can be viewed at wileyonlinelibrary.com]

Immunocytochemical localization of NK1 receptors and other hippocampal GABA neuron markers was not performed, because the combined GABA neuron loss caused by SSP-saporin[12] and seizure activity[9,10] cannot be differentiated. The selective effects of SSP-saporin treatment on GABA neuron markers[13] have been described in detail previously.[12]

3.4 | Control injections

Identical injections of Blank-saporin (n = 3) or PBS (n = 3) produced no obvious abnormal behaviors during the first week postinjection. All control animals implanted with recording and stimulating electrodes 4 months postinjection did not exhibit chronic spontaneous seizures during the observation period, or hippocampal sclerosis (Figure 3A).

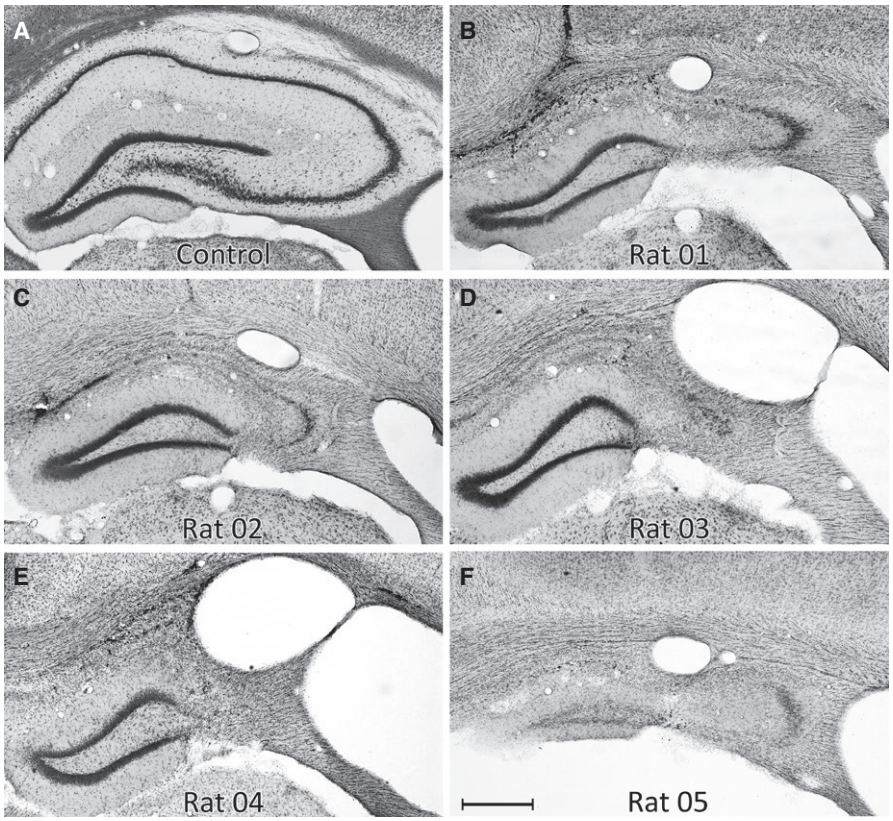

FIGURE 3 Hippocampal morphology in six different animals after intrahippocampal Stable Substance P–saporin conjugate (SSP-saporin) or vehicle injection. A, Control animal 209 days after phosphate-buffered saline vehicle injection. B-F, Hippocampal sclerosis in all five experimental animals injected with SSP-saporin. Note minimal variability in four of the five consecutively injected rats, but virtually complete hippocampal sclerosis in F. Survival periods: A, 209 days; B, C, 125 days; D, 138 days; E, 123 days; F, 102 days. Scale bar: 630 μm in A and 500 μm in B-F

4 | DISCUSSION

In this study, hippocampal GABA neurons were targeted for selective elimination to test the hypothesis that a focal GABAergic defect could be a primary epileptogenic mechanism.[12,14] The finding that SSP-saporin injection reliably produced extreme hippocampal sclerosis without giving any clinical indication early in the process that a significant and selective hippocampal injury was occurring is remarkable and unprecedented. This finding suggests that a preexisting focal GABAergic defect may be sufficient to initiate a clinically subtle focal excitotoxic insult that replicates the defining features of cryptogenic TLE-HS+[1–4] and may be the rat analog of prolonged febrile seizures in children.[5–7] Earlier attempts to demonstrate the epileptogenic potential of selective hippocampal GABAergic dysfunction[12,15–17] may have been unsuccessful because the extent of GABA neuron loss was too spatially limited,[12] or because the experimental designs did not sufficiently involve the dentate gyrus,[15–17] which plays a central role in producing hippocampal sclerosis and chronic granule cell–onset epilepsy.[8–11]

Although it is not currently possible to identify the ultimate neuronal source of any spontaneous seizure, we use the term *granule cell–onset seizures* to describe behavioral seizures in which the first behavioral sign was reliably preceded by high-amplitude granule cell layer activity, as previously described.[8] Our use of the term *granule cell–onset seizures* is similar to the term *hippocampal-onset seizures*, used clinically to describe behavioral seizures preceded by hippocampal discharges, even though the actual initiating neuronal source of the seizures cannot be identified.

Future experiments that explore and exploit the results of this initial finding will determine the site specificity of the effect and will use chronic depth recording in awake animals to determine: (1) whether the initial behaviors that occur during the first week after SSP-saporin injection reflect a subtle initial state of nonconvulsive or minimally convulsive dentate granule cell status epilepticus[8] in rats that may correspond to prolonged febrile seizures in children[5–7]; (2) precisely when, after SSP-saporin injection, clinical epilepsy begins, and how it progresses; and (3) whether suppression of the initial, presumably excitotoxic, injury prevents hippocampal sclerosis and chronic epilepsy.

SSP-saporin may be the first nonconvulsive injectable molecule that replicates the defining features of cryptogenic TLE-HS+ and does so without involving convulsive status epilepticus, widespread extrahippocampal brain damage, or any lethality.[18] Future studies will optimize and characterize this method, which may yield the most etiologically realistic TLE-HS+ model yet developed.

ACKNOWLEDGMENTS

Eugene Chun was supported by Minority Biomedical Research Support of the Research Initiative for Scientific Enrichment program (5R25GM058 268) and the Research

Centers in Minority Institutions program (5G12MD007602). Research reported in this publication was also supported by the NIH National Institute on Minority Health and Health Disparities under award number S21MD000101 and by NIH grant NS083932. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. We thank Dr Daniel H. Lowenstein for useful discussions and constructive criticism of the manuscript.

CONFLICT OF INTEREST

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

ORCID


Robert S. Sloviter https://orcid.org/0000-0002-6231-3171

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How to cite this article: Chun E, Bumanglag AV, Burke SN, Sloviter RS. Targeted hippocampal GABA neuron ablation by Stable Substance P–saporin causes hippocampal sclerosis and chronic epilepsy in rats. *Epilepsia*. 2019;60:e52–e57. https://doi.org/10.1111/epi.14723