AWARD NUMBER: W81XWH-13-1-0256

TITLE: Translational Advancement of Somatostatin Gene Delivery for Disease Modification and Cognitive Sparing in Intractable Epilepsy

PRINCIPAL INVESTIGATOR: Michael A. King, Ph.D.

CONTRACTING ORGANIZATION: University of Florida
Gainesville, FL 32610-0267

REPORT DATE: September 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2016	Annual	1Sep2015 - 31Aug2016

4. TITLE AND SUBTITLE
Translational Advancement of Somatostatin Gene Delivery for Disease Modification and Cognitive Sparing in Intractable Epilepsy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0256

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Michael A. King, Ph.D. (making@ufl.edu),
Brandi K. Ormerod, Ph.D. (bormerod@bme.ufl.edu),
Paul R. Carney, M.D. (paulcarney@unc.edu)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UNIVERSITY OF FLORIDA
207 GRINTER HALL
GAINESVILLE FL 32611-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
To test the safety and efficacy of somatostatin gene delivery as a potential therapeutic approach to epilepsy, an established rodent model is used in which electrical brain stimulation at current levels initially without effect gradually produce a persistent state where severe seizures occur reliably. Animals tested during the reporting period establish that somatostatin gene delivery after development of maximal seizure susceptibility can produce complete amelioration of a seizure-prone state. The therapeutic effect is essentially all or nothing. The responder rate is 30-40%, below the 70% observed when gene delivery preceded kindling, but comparable to extant antiepileptic medication. Responder and non-responder cohorts cannot be explained by variation in injection placement, transduction efficiency, electrographic seizure variables, effects on seizure-stimulated brain stem cell division or differentiation, or obvious brain pathology. Kindling increased new cell generation in hippocampus, and somatostatin gene expression reversed the effect of kindling.

15. SUBJECT TERMS
Epilepsy; seizure; kindling; somatatostatin; traumatic brain injury; gene delivery; adeno-associated viral vector; neurogenesis; inflammation; neurodegeneration; hippocampus, memory

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	39	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

STATEMENT OF WORK – 02/17/2107 (revised)
PROPOSED START DATE Sep 01, 2014

Site 1: PI, Michael King, PhD, University of Florida, 207 Grinter Hall, Gainesville FL 32611-0001

Specific Aim 1: Does SST gene transfer alter hippocampal neurogenesis in relation to behavioral or brain pathology during development of seizures in a rat kindling model of epileptogenesis?		
Major Task 1: Test effects of kindling alone on water maze and cytogenesis		
Subtask 1: Secure IACUC and ACURO approvals	1	Carney, King
Subtask 2: Kindling and testing kindling effects on cytogenesis [10 rats/group x 2 groups (sham-kindled, kindled; 4 hr survival) = 20 rats total]	2-6	Carney, Ormerod
Subtask 3: Kindling and testing kindling effects on water maze performance [10 rats per group x 2 groups (sham-kindled, kindled; 4 wk survival) = 20 rats total]	2-6	Carney, Ormerod
Subtask 4: Test kindling effects on cytogenesis and histopathology (histological analyses) [4 groups, 40 rats total from subtasks 2 & 3]	7-12	Ormerod, King
Major Task 2: Test therapeutic efficacy of SST gene delivery on kindled seizures and cognitive deficits in rats		
Subtask 1: Procure and validate GFP and SST gene delivery vectors	1-6	King, Carney
Subtask 2: Test GFP and SST vectors on acute cytogenesis (4 hr survival) [10 rats/group x 2 groups = 20 rats total]	12-18	Carney, Ormerod
Subtask 3: Test GFP and SST vectors on induced seizures (4 wk survival [10 rats/group x 2 groups = 20 rats total]	18-36	Carney
Subtask 4: Test GFP and SST vectors on seizure-related behavioral deficits [same animals as subtask 3]	18-36	Carney, Ormerod,
Milestone(s) Achieved: Establish effects of kindling on behavior, determine effects of SST on seizures and behavioral deficits; publication of 1-2 peer reviewed papers	36	
Major Task 3: Determine GFP and SST vector effects on seizure-related changes in cytogenesis		
Subtask 1: Histology and cell counts to assess SST effects on cell division (4 hr survival) and seizure-related pathology [same animals as subtask 2]	30-36	Carney, Ormerod, King,
Subtask 1: Histology and cell counts to assess SST effects on survival and phenotypic maturation (4 wk survival), and seizure-related pathology [same animals as subtask 3]	36-42	Carney, Ormerod, King,
Milestone(s) Achieved: Established effects of kindling and SST on cytogenesis and pathology, publication of 1-2 peer reviewed papers	36	Carney, Ormerod, King
Specific Aim 2: Does SST gene transfer alter inflammation over the time scale in which epileptogenesis occurs?		
Major Task 1: Generate biochemical group cohorts of Aim 1		
Subtask 1: Generate kindled rat cohorts of Aim 1 groups [10 rats/group x 8 groups = 80 rats total]	25-36	Carney
Subtask 2: BioPlex analysis of rats used in Aim 1 [same rats as subtask 1]	40-44	Ormerod
Milestone(s) Achieved: publication of 1-2 peer reviewed papers	48	
Extension Aims 1: Advanced vectors and optimized delivery		
Major Task 1: Generate biochemical group cohorts of Aim 1		
Subtask 1: Design and procure advanced vectors for optimization of efficacy	36-41	King, Carney
Subtask 2: Test new vs. previous SST vector effects on seizure susceptibility, water maze performance, related cytogenesis and pathology (histological analyses) [groups to be determined as necessary]	37-48	Carney, Ormerod, King

1. INTRODUCTION: Roughly 30% of epileptics cannot be satisfactorily treated by medication or surgery. Brain injury significantly increases risk for epilepsy, and the high incidence of brain injuries translates into substantial numbers of new intractable epilepsy cases in military personnel. The neuropeptide somatostatin (SST) synthesized in neurons exhibits synaptic release and activates receptor-effector cascades in target cells. Known peripheral interactions with classical inflammation mechanisms (TNFalpha, cytokines), and the presence of receptors on non-neuronal cells in brain, implicate brain SST in inflammation and cellular proliferation controls. Synaptic function and brain cell proliferation are both are disrupted in epilepsy and may contribute to neuropathology and functional impairment beyond immediate effects of seizures *per se*. Brain neurons expressing somatostatin are especially vulnerable to seizure-related loss, although this is limited in the kindling model where spontaneous seizures are rare and the total number of seizures is relatively low. Our initial tests demonstrated that intracranial somatostatin gene delivery prevented the evolution to high-level seizures in 70% of rats in the kindling model, when they were treated before initiation of the electrical brain stimulation-induced kindled seizure regimen. This impressive reduction in experimental epileptogenesis may provide strategies for new effective therapy for intractable epilepsy, but the efficacy mechanisms, safety, and application parameters must be established prior to informed clinical development. We predict that somatostatin gene transfer will counteract the effects of epileptic development even when initiated after recurrent seizures have established a distinct seizure-prone state. One potential mechanism based on known somatostatin properties could be a suppression of seizure-enhanced proliferation of hippocampal progenitor cells, their long-term phenotype differentiation, or their contribution to pro-convulsive networks. A second potential mechanism of hippocampal somatostatin gene delivery might be to reduce inflammation signaling that is stimulated by seizures and probably contributes to synaptic dysfunction, cell viability, and a progression to increasingly severe seizures. A thoroughly characterized rodent epilepsy model will be used as a platform to test the hypotheses. In this model temporal lobe electrical stimulation initially does not cause seizures, but gradually the same level of stimulation causes progressively more severe seizures over days to weeks. The seizure-prone state resulting from this kindling is measured by behavioral and electrographic severity appraisals, and the amount of damage displayed by cells in the brain *post-mortem*. The progressive hyperexcitability that develops in conjunction with lowered seizure thresholds is a therapeutic disease modification target distinct from the seizures they allow. Localized gene delivery using adeno-associated viral (AAV) vectors for SST or inactive control protein will be tested for ability to suppress seizure susceptibility and severity in fully kindled rats. Cellular proliferation and maturation mileposts, and brain inflammation pathway markers, will be evaluated to ascertain whether either are altered in relation to SST effects on seizure severity. If they are not altered then the efficacy of SST gene delivery against seizure susceptibility most likely depends on other mechanisms. The evolution of epilepsy between an initial insult and recurrent spontaneous seizures is the most opportune time for therapeutic intervention, because loss of important neuronal populations is likely to have already occurred when these emerge, and because seizures tend to become more severe and/or frequent once recurrent seizures begin. Our SST gene delivery uses vectors currently performing well in human clinical trials, and could provide a new, safe, and effective way to interfere with epileptogenesis and/or a developed epileptic state. Convergent direct and indirect actions of SST are likely to limit losses of brain circuitry from pathology or resection, and the associated functional impairments.

2. KEYWORDS: Epilepsy; seizure; kindling; somatatostatin; traumatic brain injury; gene delivery; adeno-associated viral vector; neurogenesis; inflammation; neurodegeneration; hippocampus, memory

3. ACCOMPLISHMENTS:

What were the major goals of the project?

In service of the need to establish preclinical efficacy and safety, 2 specific aims were pursued. The first was to determine whether therapeutic efficacy of SST gene delivery against seizure susceptibility was associated with effects on brain progenitor cell division, differentiation, and integration. The second was to determine whether therapeutic efficacy against seizures was associated with effects on brain inflammation.

Addressing these aims occurs in the context of establishing whether SST gene transfer could be effective and safe when initiated after a stable seizure-susceptible state was present, in contrast to our earlier testing where SST gene transfer preceded epileptogenesis. Thus the first objectives were to test whether the treatment is effective against seizures and associated pathologies, and whether it adversely affects brain structure or behavioral function (safety).

The project involves 2 parallel sets of animals, 1 destined for histological assessment and 1 for biochemical analyses (Table 1). Both sets use 8 identical groups of at least 10 rats per group, and all animals undergo surgical implantation of indwelling wire stimulation and recording electrodes fixed in a permanent connector glued to the skull. Kindled groups undergo repeated electrical stimulation with scoring of video and EEG records until reaching a 'fully kindled' criterion of 3 consecutive Racine grade 5 seizures. Sham-kindled rats are treated identically except that no current is delivered. DNA synthesis as a marker for dividing cells was quantified by counts of brain cells pulse-labeled with deoxyuridine analogues bromodeoxyuridine (BrdU) and ethynyldeoxyuridine (EdU) at different time points. Sham and kindled rats were injected with BrdU 2 days after reaching fully kindled status. Kindled rats in vector test groups were injected with BrdU 2 days after the first test stimulation (3 wks after surgical infusion of SST or GFP vector). Subgroups of the kindled/sham and SST/GFP vector animals were euthanized 4 hrs or 4 wks later. Some rats in the 4 wk survival cohorts were subjected to a regimen of behavioral testing using water maze escape spatial learning and memory tasks. During this interval seizure susceptibility was tested repeatedly to examine stability of the kindled state and therapeutic responses. These rats received (EdU) 4 hr prior to euthanasia to assay effects of enduring seizure susceptibility, behavioral testing, and therapeutic gene transfer on cell division, in relation to BrdU labeling. Phenotype immunolabeling will extend analysis of cell division, survival, differentiation, and integration. Parallel groups used in biochemical analyses will be used to measure treatment effects and interactions on numerous polypeptides involved in inflammation signaling. The revised design remains a 3 factor ANOVA (kindling, vector, survival time).

What was accomplished under these goals?

1) Major activities

UF IACUC 3rd year renewal was approved 3/2016. New SST and GFP vector preparations were generated 4/2016. SST vector nucleotide sequence was reconfirmed, and both vectors were validated by observation of expression in rat brains. All animals for the original aims have completed live testing and tissues have all been collected for histological and biochemical analyses.

Dr. Carney moved to the University of North Carolina in August 2016. All live animal work for the original efficacy and safety study was completed at UF prior to the Dr. Natarajan joining the UNC lab.

2) Specific objectives

- determine whether SST reduces seizure severity or threshold after kindling (efficacy)

Treatment group (N≥10):	Purpose:	Assessment
1.a. no vector, sham-kindled, BrdU, 4 hr survival	untreated baseline	Video/EEG, histology
1.b. no vector, fully kindled, BrdU, 4 hr survival	kindling effects on cell division	Video/EEG, histology
2.a. GFP vector, fully kindled, BrdU, 4 hr survival	does control vector alter kindling effects?	Video/EEG, behavior, histology
2.b. SST vector, fully kindled, BrdU, 4 hr survival	does sst vector alter kindling effects?	Video/EEG, behavior, histology
3.a. no vector, sham-kindled, BrdU, 4 wk survival	untreated baseline, extended survival	Video/EEG, behavior, histology
3.b. no vector, fully kindled, BrdU, 4 wk survival	kindling stability and effects, extended survival	Video/EEG, behavior, histology
4.a. GFP vector, fully kindled, BrdU, 4 wk survival	does control vector alter kindling effects?	Video/EEG, behavior, histology
4.b. SST vector, fully kindled, BrdU, 4 wk survival	does sst alter kindling effects?	Video/EEG, behavior, histology
5.a. no vector, sham-kindled, BrdU, 4 hr survival	untreated baseline	Video/EEG, biochem
5.b. no vector, fully kindled, BrdU, 4 hr survival	kindling effects on cell division	Video/EEG, biochem
6.a. GFP vector, fully kindled, BrdU, 4 hr survival	does control vector alter kindling effects?	Video/EEG, behavior, biochem
6.b. SST vector, fully kindled, BrdU, 4 hr survival	does sst vector alter kindling effects?	Video/EEG, behavior, biochem
7.a. no vector, sham-kindled, BrdU, 4 wk survival	untreated baseline, extended survival	Video/EEG, behavior, biochem
7.b. no vector, fully kindled, BrdU, 4 wk survival	kindling stability and effects, extended survival	Video/EEG, behavior, biochem
8.a. GFP vector, fully kindled, BrdU, 4 wk survival	does control vector alter kindling effects?	Video/EEG, behavior, biochem
8.b. SST vector, fully kindled, BrdU, 4 wk survival	does sst alter kindling effects?	Video/EEG, behavior, biochem

Table 1: Experimental groups. Groups 1 and 5 of kindled or sham-kindled control rats that did not receive vectors served to determine essential reference baseline effects of kindling alone on spatial learning and memory, hippocampal histological integrity, cytogenesis, and inflammation. Groups 5-8 are identical to 1-4, but because the biochemical analyses are incompatible with tissue fixation the brains must be harvested from rats euthanized by carbon dioxide inhalation. The 80 rats in this second study will be used in biochemical assays for brain inflammation in relation to vector and seizure severity.

- determine whether kindling alters mnemonic behavior
- determine whether kindling alters cytogenesis
- determine whether SST alters kindling-associated changes in cytogenesis (efficacy)
- determine whether SST alters kindling-related effects on mnemonic behavior (efficacy, safety)
- determine whether kindling stimulates histological or biochemical inflammation
- determine whether SST mitigates histological or biochemical kindling-associated inflammation (efficacy, safety)

The methodology involves use of a kindled seizure model in the experimental design presented summarized in Table 1. Brief applications of electrical current through permanently implanted brain electrode wires, at levels initially too weak to induce seizures, gradually become sufficiently potent to reliably induce severe seizures. Once rats for vector evaluation reach a criterion seizure susceptibility, they undergo surgical infusion of gene transfer vectors for somatostatin (SST) or an inert green fluorescent protein (GFP). After time for transgene expression to develop, rats are re-challenged over several weeks with the same electrical stimulation to test efficacy in terms of seizure susceptibility, severity, latency, duration, and variability. They also undergo acquisition, retention, reversal, and cued learning trials in a water escape spatial memory task sensitive to brain pathology often observed in seizure patients. Differences in outcome measures between vector groups are interpreted as therapeutic efficacy and referenced to effects of kindling in the absence of gene transfer. Histological methods to identify and quantify brain pathology, inflammation, and the generation and integration of new cells complement biochemical multiplex analyses of cytokine and inflammation reporter molecules in harvested tissue samples.

3) Significant results or key outcomes

A) *Kindling results in a stable state of increased seizure susceptibility.*

Rat#	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 19	Day 20	Day 22
C1	5	5			5			5						4.5				5
C2	5	5				5							5					
C3	5	5				5							5					
C4	2						2								3			
C5	2.5			4.5			3		5				3		2.5	2.5	5	
C6	1		5					2			5			5	5			
C7	4.5		5						5						5			
C8	5	5			5				5			5						
C9	5		0		0			5		5			5					
C10	4	4.5			5			5		5			5					

Figure 1: Kindled rats were challenged with delivery of original stimulation current on multiple occasions beginning (Day 1) 3 weeks after GFP control vector delivery. Initial retest scores were generally accurate for subsequent challenges and confirmed a sustained, stable kindled state. They conservatively underestimated later scores in 2 rats and in no cases overestimated averages of multiple tests. At this retest frequency, seizure scores reflect little probability of responses being refractory upon subsequent challenge.

Kindling to criterion resulted in a stable, enduring increase in seizure susceptibility in GFP control vector rats (Figure 1). This result extends a limited body of information on the stability and variability of the kindled state. It supports confidence in interpretations of therapeutic efficacy as unlikely to be artifacts of inadequate sampling frequency.

B) *Kindling results in an impairment of mnemonic behavior.*

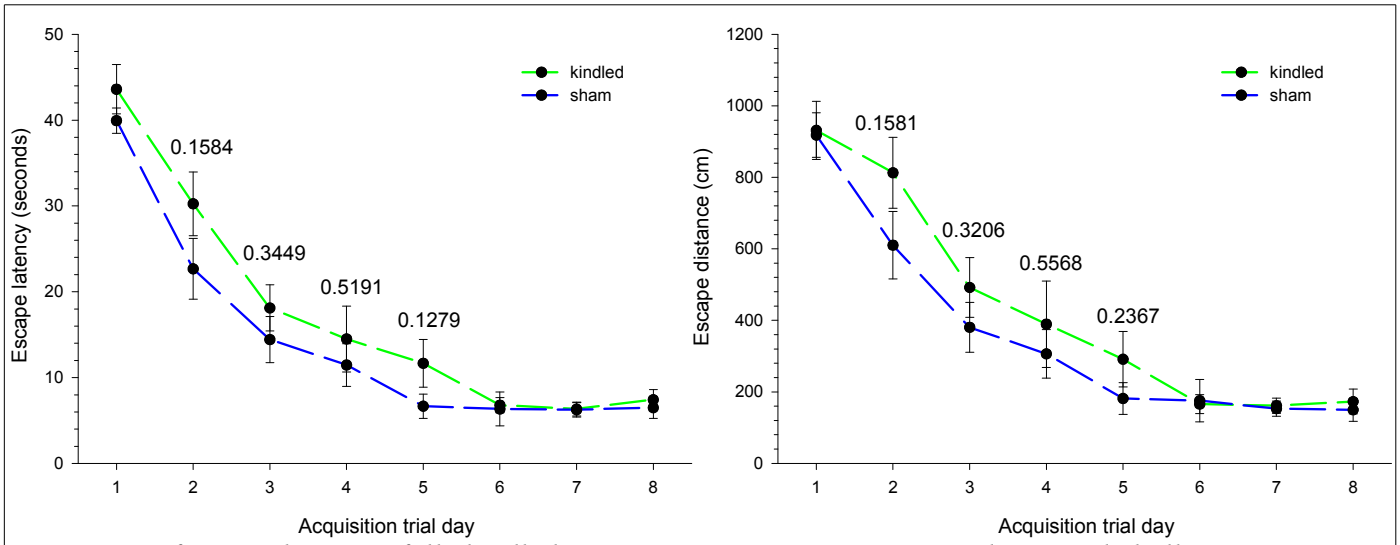


Figure 2: Left: Rats that were fully kindled prior to water maze training, and received challenge stimulation every other day during training, tended to require more time to escape than rats with implanted electrodes but no delivery of electrical current (sham kindled). SAS Proc GLM repeated measures ANOVA for between-subjects factor 'treatment' F(1,16)=3.21, Pr>F 0.0923. Both groups showed robust learning to equal proficiency (F(7,112)=64.90, Pr>F<.0001), without significant trial day x treatment (kindling) interaction F(7,112)=0.67, Pr>F 0.6976. Right: Effects were similar for swim distance prior to escape. SAS Proc GLM repeated measures ANOVA for between-subjects factor 'treatment' F(1,16)=1.73, Pr>F 0.2066. Both groups showed robust learning to equal proficiency (F(7,112)=42.02, Pr>F<.0001), without significant trial day x treatment (kindling) interaction F(7,112)=0.67, Pr>F 0.6961.

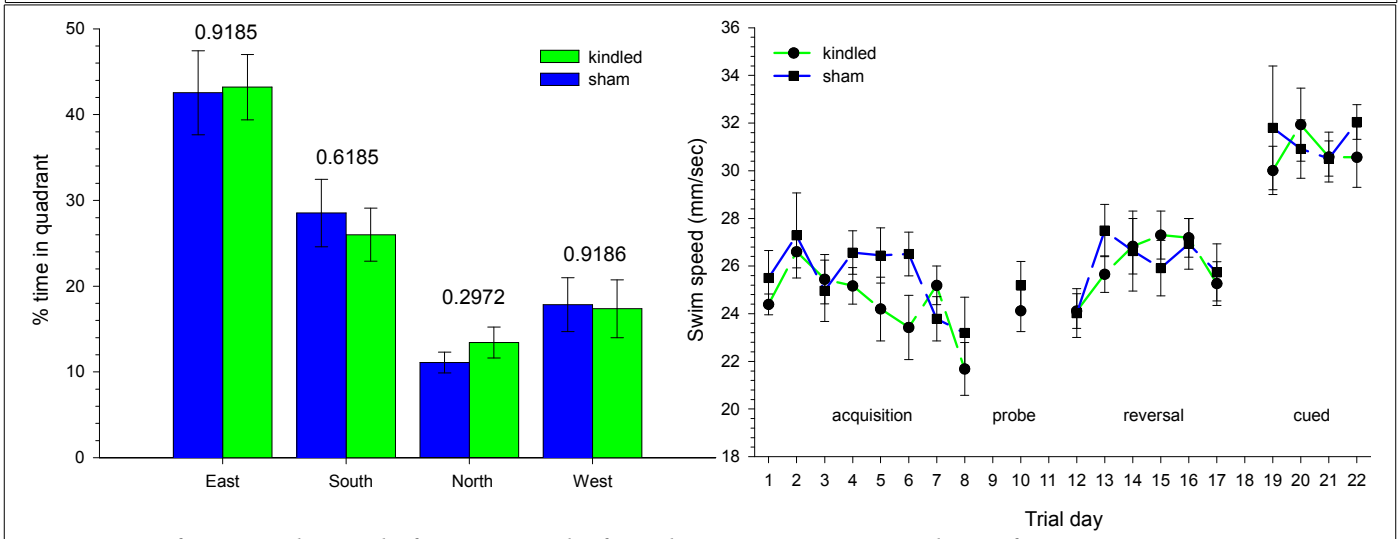


Figure 3: Left: In probe trials for escape platform location retention 2 days after acquisition training, kindled rats spent equivalent fractions of search time in target and non-target maze quadrants as sham-kindled rats. SAS Proc GLM ANOVA for between-subjects factor 'treatment' F(1,17), Pr>F above bars. Right: Swim speed did not differ between kindled and sham-kindled rats in any component of water maze testing. SAS Proc GLM repeated measures ANOVA F(1,16) 0.84, Pr>F 0.3719.

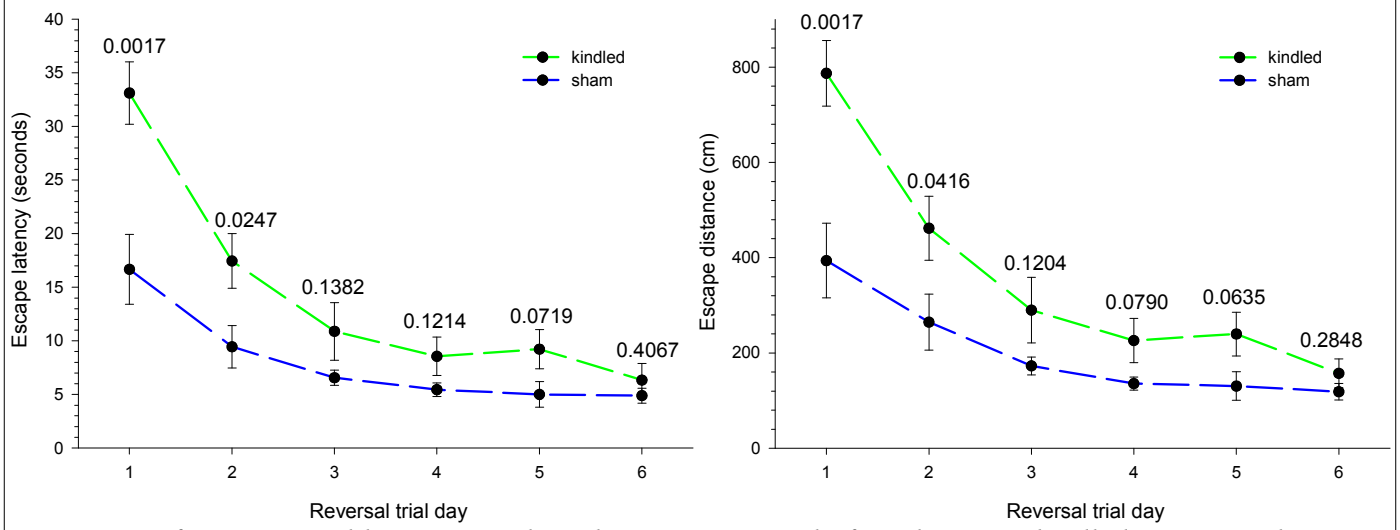


Figure 4: Left: In reversal learning trials with a new escape platform location, kindled rats swam longer before escape than sham-kindled rats, and required more training to attain equal proficiency. SAS Proc GLM repeated measures ANOVA for between-subjects factor 'treatment' F(1,16)=13.90, Pr>F 0.0018. Both groups showed learning to equal proficiency (F(5,80)=33.36, Pr>F<.0001), and there was a significant trial day x treatment (kindling) interaction F(5,80)=4.66, Pr>F 0.0009. Right: Results were similar for swim distance to escape. SAS Proc GLM repeated measures ANOVA for between-subjects factor 'treatment' F(1,16)=13.62, Pr>F 0.0020. Both groups showed learning to equal proficiency (F(5,80)=29.64, Pr>F<.0001), and there was a significant trial day x treatment (kindling) interaction F(5,80)=4.08, Pr>F 0.0024.

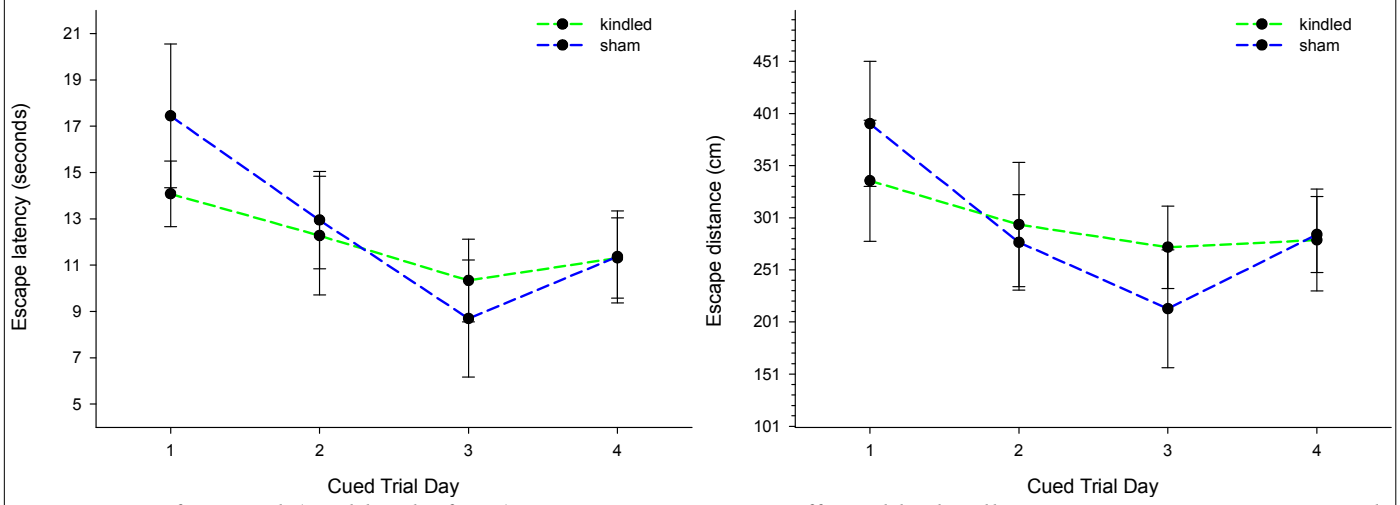


Figure 5: Left: Cued (visible platform) escape time was not affected by kindling. SAS Prog GLM repeated measures ANOVA F(1,16)=0.12, Pr>F 0.7300. There was significant learning over trial days(F(3,48)=3.08, Pr>F 0.0362) and no interaction with kindling (F(3,48)=0.48, Pr>F 0.6985). Right: Escape distance results were similar (kindling F(1,16)=0.01, Pr>F 0.9254) although improvement over days was not significant (F(3,48)=2.24, Pr>F 0.0959), nor were interactions (F(3,48)=0.49, Pr>F 0.6905).

Kindling alone had no significant effect on water maze acquisition (Figure 2), retention (Figure 3), or cued learning (Figure 4), but did result impair rats' abilities to learn a new location for the hidden escape platform (Figure 5). Response perseveration occurs with hippocampal pathology, and the result may indicate functionally relevant seizure-related damage not resolvable by histological assays. This result provides a new and more sensitive assay for effects on cognitive function earlier in epileptogenesis than has been available previously.

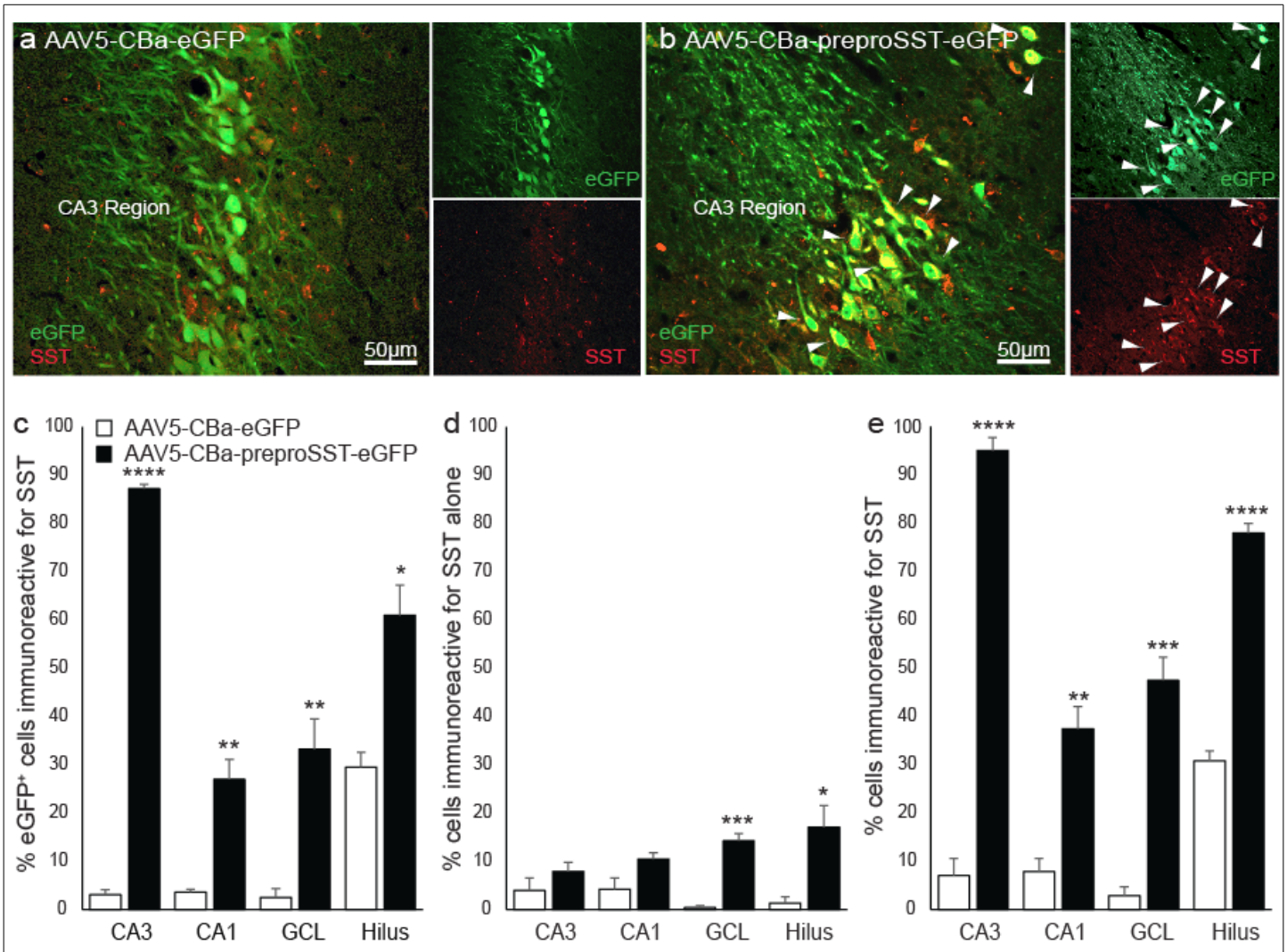


*Figure 6: Co-GFP and SST labeling confirm that cells infected with the AAV5-Cba-SST-GFP vector express SST. Confocal images show the CA3 region of a rat treated with the control vector (A) and a rat treated with the AAV5-CBa-preproSST-eGFP vector (B). Insets show eGFP expression (in green) and SST expression (in red) separately. (C) The percentage of at least 100 randomly selected eGFP+ cells examined in each hippocampal region that co-expressed SST neurons increased in the CA3 region (****p<0.0001) and CA1 region (**p<0.01) pyramidal cell layers, granule cell layer (GCL, **p<0.01) and hilus (*p<0.05) of preproSST vector-treated versus control vector-treated rats. (D) The percentage of eGFP-/SST+ neurons (presumably endogenous SST+ neurons) increased in the granule cell layer (GCL, ***p<0.001) and hilus (*p<0.05) but not the CA3 region and CA1 region pyramidal cell layers of preproSST vector-treated versus control vector-treated rats. (E) The percentage of neurons immunoreactive for transduced and/or endogenous SST increased in the CA3 region pyramidal cell layer (****p<0.0001) and CA1 region pyramidal cell layer (**p<0.01), the granule cell layer (GCL, ***p<0.001) and hilus (****p<0.0001) of the preproSST vector-treated versus control vector-treated rats (n=5 per group).*

C) *AAV-SST gene delivery mediates SST expression in hippocampal neurons.*

Immunoreactivity in principal hippocampal neurons indicates that preproSST RNA is translated to generate preproSST, with subsequent processing into SST14 or 28 peptide, in neuronal populations and subtypes that do not show detectable somatostatin content in control brains (Figure 6). This is

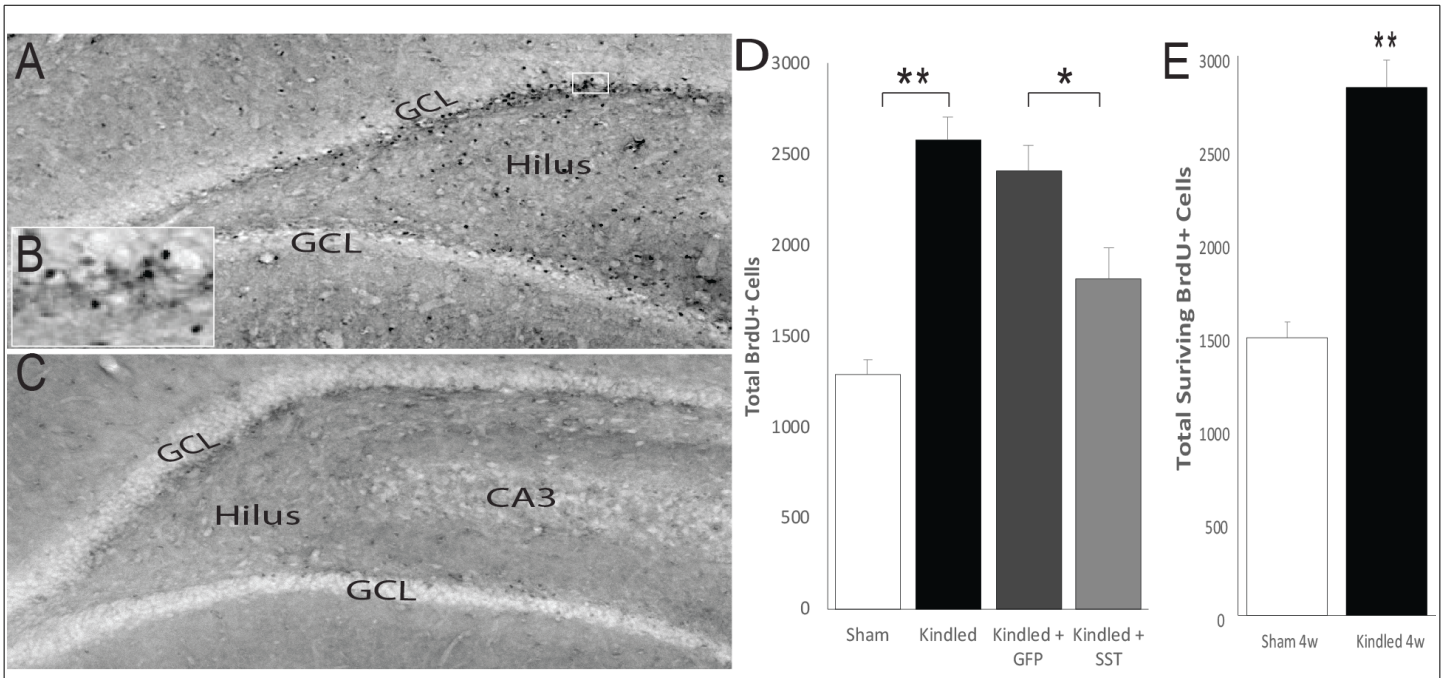


*Figure 7: SST normalizes the kindling-induced increase in total new cell (BrdU+) cell numbers. Representative light micrographs show new BrdU+ cells labeled using DAB in the dentate gyri of a kindled (A) and control rat (C). Inset B shows BrdU+ cells under 400x magnification. (D) The number of dividing cells labeled with BrdU 48h after the final test stimulation and 4h before perfusion significantly increased in kindled rats relative to controls ($p < 0.001$**) and was significantly lower in kindled rats treated with the pAAV5-Cba-SST-GFP versus control vector ($p < 0.05$*). (E) The number of surviving cells labeled with BrdU 48h after the final test stimulation and 4 weeks before perfusion was significantly increased in kindled rats ($p < 0.05$). The brains of kindled rats treated with the pAAV5-Cba-SST-GFP and control vector were collected in December 2016 and have been sectioned and immunostained by Ormerod and Leibowitz (completed in January), who are currently stereologically quantifying total surviving cell numbers.*

consistent with observed expression of prohormone convertases, and indicates that SST over-expression may prove therapeutically effective even after native SST neurons succumb due to preferential vulnerability to seizure effects. Immunoreactivity intensity suggests that SST abundance in transduced excitatory neurons may be lower on average than in transduced neurons with inhibitory phenotypes, but the presence of expression in both types suggests that diffusely distributed SST release (mediating sustained increased availability to cell surface receptors) could participate in therapeutic efficacy. Such release could be constitutive or synaptic, and mediate cell non-autonomous effects.

D) *Kindling increases the number of dividing progenitor cells, and AAV-SST counteracts the effects of kindling.*

Significantly more dividing progenitor cells were found in the dentate gyri of rats 48h after they reached the fully kindled state (BrdU was injected 4h before euthanasia; Figure 7). An increased number of new cells were detected in rats injected with BrdU 48h after reaching the fully kindled state that were euthanized 4 wks later, demonstrating the kindling-induced increase in new cell number is persistent. We are currently quantifying whether the new cells produced acquire neuronal or glial phenotypes in rats that survive 4 wks.

E) *Kindling increases proliferation of Type-1 neural stem cells, and SST gene transfer may counteract kindling effects.*

Normally, Type-1 neural stem cells are relatively quiescent and the proliferation of Type-2a and -2b neural progenitor cells generates new neurons. Kindling significantly increases the number of

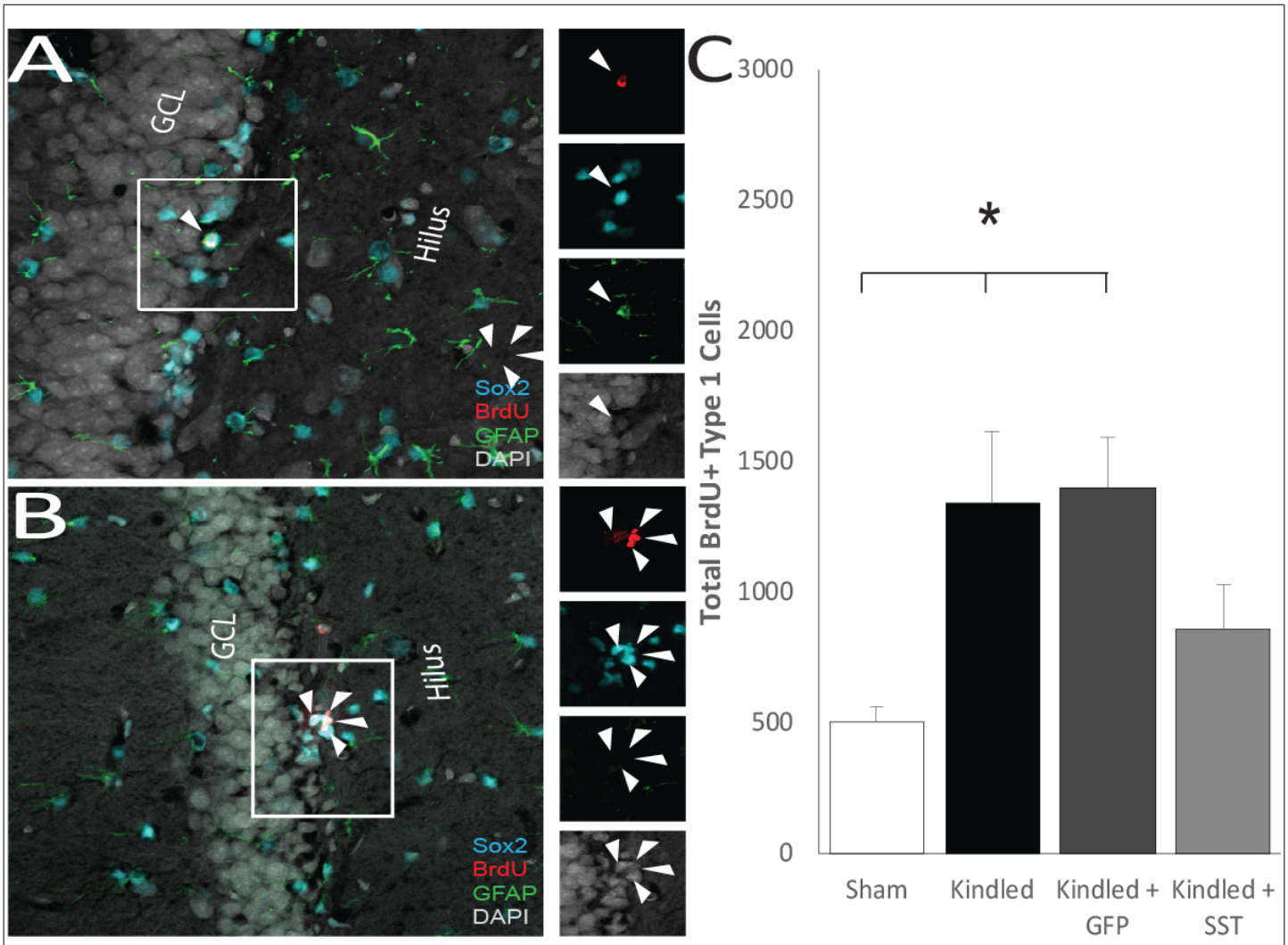


Figure 8: SST normalized the kindling-induced increase in dividing Type-1 progenitor cells. The Type-1, Type 2-a, Type-2b and Type-3 phenotype of dividing cells was quantified under confocal microscopy. At the 4h time point after BrdU, most BrdU+ cells (in red) are GFAP+ (in green)/SOX2+ Type-1 stem cells (A) or GFAP-/SOX2+ Type-2a progenitor (B). (C) The number of dividing Type-1 stem cells, which are normally relatively quiescent, significantly increases in the dentate gyri of kindled rats and kindled rats treated with the control vector ($p < 0.05$). However, treatment with the pAAV5-Cba-SST-GFP vector normalized the number of dividing Type-1 cells, suggesting that SST normalizes aberrant kindling-induced increases in neurogenesis.*

dividing Type-1 neural stem cells (Figure 8) but not Type-2a or -2b neural progenitor cells (data not shown). Fewer dividing dividing Type-1 cells labeled 4 hours before euthanasia were detected in SST vector-treated rats versus GFP-vector treated rats and the number observed in SST vector-treated rats was similar to the number detected in sham rats.

F) *Kindling stimulates hippocampal microglial activation, but SST gene transfer does not alter this inflammatory response.*

The fraction of activated microglia increased in dentate gyrus of kindled rats, but overall microglial number was not altered (Figure 9). Kindling thus appears to induce only a mild inflammatory response, consistent with clinical observations of early epilepsy. SST vector did not modify this response.

G) *SST can reduce seizure susceptibility <u>after</u> kindling (efficacy).*

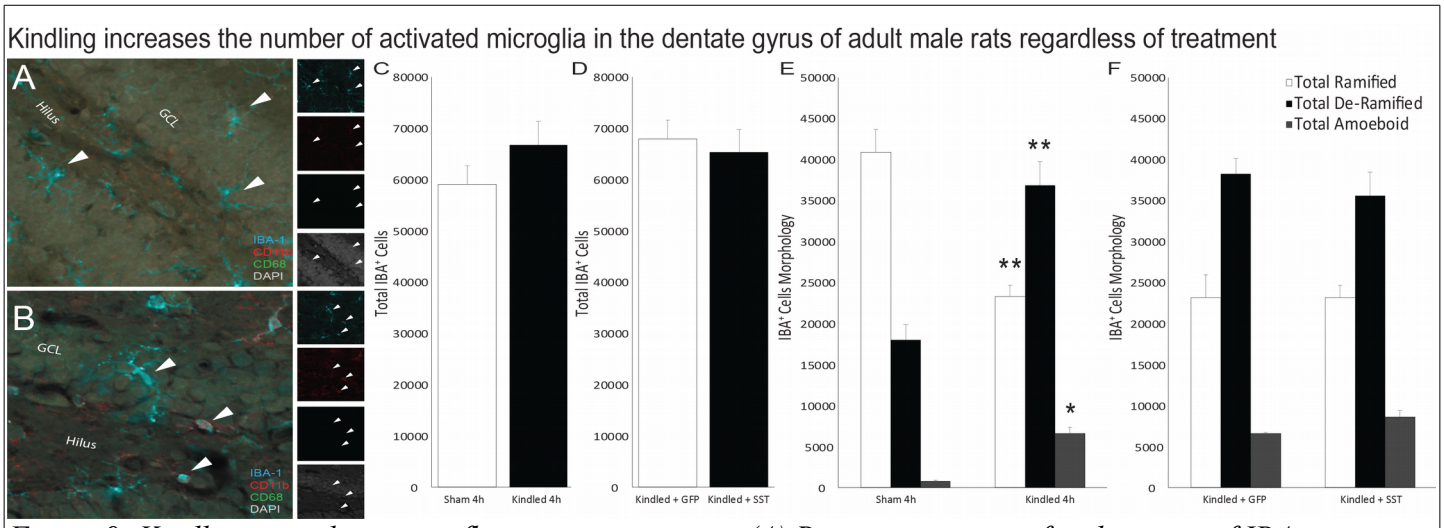


Figure 9: Kindling stimulates an inflammatory response. (A) Representative confocal images of IBA-1+/CD11b+ activated and amoeboid (B) and IBA-1+/CD11B- resting and ramified microglia. (C-D) Kindling did not alter the absolute number of IBA-1+ microglia in the dentate gyrus of adult rats even when accompanied with vector mediated SST or GFP gene delivery. (E) A morphological analysis revealed significantly more activated (black bars, $p < 0.001$) and highly activated (phagocytic, gray bars; $p < 0.05$) microglia and signficantly fewer resting microglia (open bars, $p < .001$) in the dentate gyri of kindled rats versus sham-kindled rats. (F) No difference were observed in microglia numbers of any type between kindled rats receiving preproSST or control vector. Protein has been harvested from the hippocampi of separate groups of sham, kindled and kindled rats and kindled rats treated with the control vector and the SST vector (n=5 per group).

Administration of AAV-SST after the evolution of a seizure-prone state can profoundly and persistently reduce the susceptibility to seizures that is otherwise stable in kindled rats. Reversal from a stable state of susceptibility to maximal intensity seizures to a stable completely refractory state is observed a substantial fraction of test animals (~40% to date)(Figure 10). Therapeutic efficacy shows a strong tendency to be all-or-nothing, be fully elaborated within 3 weeks of vector delivery, and to persist through several weeks of retesting seizure susceptibility (Figure 11).

H) *No single hippocampal subregion appears necessary or sufficient for maximum therapeutic efficacy.*

Complete suppression of seizure susceptibility has been observed in cases with expression restricted to dentate, CA3, or CA1, even unilaterally (Figure 12). Conversely, as we have increased the numbers of animals tested, we have observed animals that did not show a therapeutic response despite robust bilateral expression in one or more subregion. These results indicate that therapeutic efficacy may not require transduction of large brain volumes (e.g. entire human hippocampus or temporal lobe), but that understanding optimal application parameters will require a better understanding of functional effects on distributed synaptic networks.

I) *Therapeutic efficacy may be vector dose-dependent.*

Rats received equivalent volumes of one of 2 different SST vector preparations required to complete the efficacy study. Preliminary data indicate a higher proportion of responders (6/13 vs. 1/9) with delivery of more vector genomes (Figure 13), despite a lack of correlation between therapeutic probability and histological extent or location of reporter gene expression. Data need to be replicated for within-prep dilution, and elimination of potential order effects.

J) *Failure to respond is not due to delayed expression.*

Rats that showed no therapeutic efficacy on the initial test did not show any significant difference on later tests (Figure 14). No responders or non-responders showed any indication for an increase or decrease in efficacy over time, suggesting that continued epileptogenesis is not likely to overtake a therapeutic effect, that tolerance does not develop, and that failure to respond is not likely due to slow maturation of transgene expression.

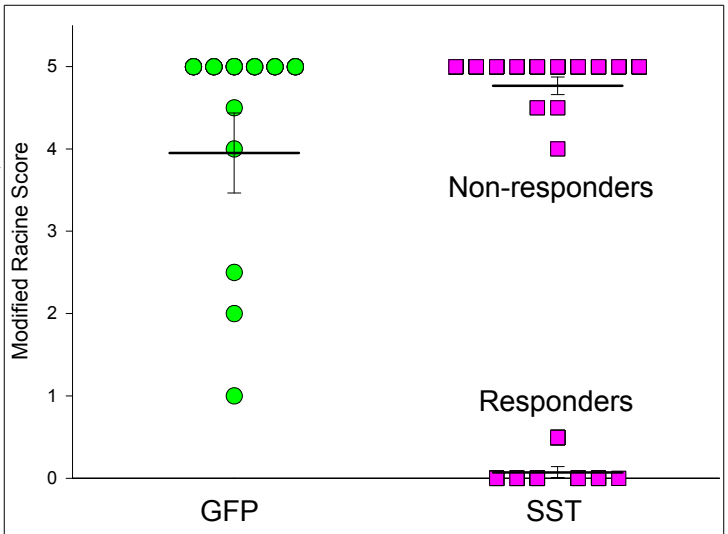


Figure 10: On the first electrical stimulation challenge 3 weeks after receiving vector, kindled GFP vector rats continued to show severe seizures. SST vector rats divided into distinct subgroups of responders that showed essentially complete seizure resistance, and non-responders that showed neither benefit nor harm. Individual rat scores and means +/- s.e.m.

K) *Seizure history may distinguish responders from non-responders*

Retrospective analysis of seizure parameters during kindling identified features that may be important for predicting efficacy and for pursuing therapeutic mechanisms. Stimulation current intensities (individual pre-kindling afterdischarge thresholds) were not predictive (Figure 15), though there was a trend for non-responders to have been kindled at currents higher than GFP control rats. Latency to severe seizure, number of stimulation episodes to become fully kindled, average severe seizure duration, and average afterdischarge duration also did not differ between responder and non-responder groups (Figure 16). The total amount of time spent in high-grade seizures was significantly greater in non-responders (Figure 17),

L) *SST gene transfer may relieve cognitive deficits associated with kindled seizures.*

Kindled rats treated with control GFP vector did not exhibit water maze spatial escape acquisition or retention deficits (Figures 18, 19), but demonstrated reversal learning deficits like those detected in rats that did not receive vectors (Figure 20). Despite a clear trend SST vector rats did not show significantly different reversal learning (daily change) compared with GFP vector rats, but did become more

	PTS-1	PTS-2	PTS-3	PTS-4	PTS-5	PTS-6 (+50 µA)	PTS-7 (+50 µA)	PTS-8 (+100 µA)
Rat1	0	0	0	0	0	0	0	0
Rat2	0	0	0	0	0	0	4	4
Rat3	0	0	5	0	0	5	ψ0	2
Rat4	0	0	0	5	5	0	2	5
Rat5	0	0	0	0	0	0	0	0
Rat6	5	5	5	5	5	0	0	0

Figure 11: SST vector effects are stable and persistent in rats that showed a complete therapeutic response (Score of 0) on 1st test (PTS-0). Post-test sessions (PTS) 6-8, during which the stimulus current was increased above AD threshold current, indicate that shift in seizure threshold could not explain the therapeutic effect. The data also indicate that neither tolerance nor a progressive increase in seizure susceptibility develop to diminish efficacy over the 4-6 week retesting interval. The timing of the repeated electrical stimulation challenges was comparable to that shown for non-responders.

Rat #	CA1		CA3		GCL		Hilus		Subiculum		Cortex	Thalamus
	Side1	Side2	Side1	Side2	Side1	Side2	Side1	Side2	Side1	Side2		
SST Non-Responders												
Rat 1	++	++	++	-	-	-	-	-	++	-	N	N
Rat 2	++	++	++	+++	-	+	-	+++	+	+	Y	N
Rat 3	++	++	++	++	-	+	-	++	-	-	Y	Y
Rat 4	++	+	+++	+++	++	++	+++	+++	+	-	Y	Y
Rat 5	++	++	+++	++	+++	+++	+++	+++	+	+	Y	N
Rat 6	++	++	+++	+	+	+	+++	+++	+	-	Y	N
SST Responders												
Rat 1	++	++	+++	++	+	-	+++	-	-	+	Y	N
Rat 2	++	+++	++	++	-	++	++	+++	+	-	Y	Y
Rat 3	+++	+++	-	-	-	-	-	-	++	++	Y	N
Rat 4	++	++	+++	++	++	+	+++	+++	++	-	Y	N
Rat 5	++	++	++	+	+	+	+++	++	+	+	N	N
Rat 6	+++	+++	++	++	-	+++	-	-	++	+	Y	N

Figure 12: Blind reconstruction and scoring of the spatial extent and location GFP reporter expression indicates that neither correlated significantly with whether rats were responders or not. Rats could be full responders without dentate gyrus expression, or fail to respond with robust bilateral expression across all hippocampal subregions. Inadvertent vector delivery to underlying thalamus or overlying cortex also could not explain efficacy.

proficient (terminal latency) in escaping to a new water maze hidden platform location. Learning or proficiency did not vary on cued (visible platform) learning, and swim speed did not differ between vector groups. The absence of sensorimotor or motivational effects supports the safety of SST gene delivery, and the effect on proficiency is consistent with a potential therapeutic benefit for seizure-related cognitive dysfunction.

4) Other achievements

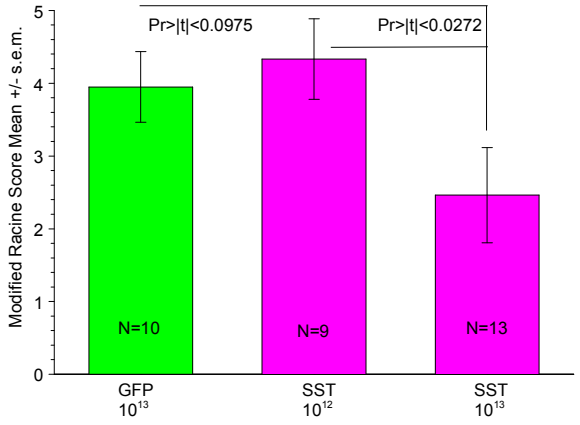
Gowri Natarajan successfully defended her doctoral dissertation in December 2016. Jeffrey Leibowitz successfully proceeded to PhD Candidacy after defending his qualifying examination in August 2016.

CoPIs King and Carney submitted a proposal for R21 funding to the NIH. Proposal R21NS098086 "Natural responders and nonresponders to antiepileptic gene therapy: physiological correlates, novel predictive biomarkers, validity across disease severity, and generalization to pharmacoresistance", was not scored and is unlikely to be pursued in the foreseeable future.

CoPIs Ormerod and Carney submitted a proposal for R01 fundiing to the NIH, to pursue questions about the mechanisms underlying therapeutic efficacy. The proposal, "Sustained SST treatment and seizure behavior: mechanism, efficacy and safety", was not scored.

CoPIs Carney, Ormerod, and King submitted a Congressionally Directed Medical Research Programs proposal "Somatostatin Gene Delivery to Enhance Long-Term Functional Recovery from Traumatic Brain Injury" for an Epilepsy Research Program Idea Development Award to DoD (Funding Opportunity W81XWH-16-ERP-IDA)11/2016 to explore the use of SST gene transfer in traumatic brain injury.

CoPIs Carney and King, and Ms. Natarajan, have begun a collaboration with Drs. Sanford and Shannon Boye at UF to develop more sophisticated SST vectors in anticipation of further translational studies. Self-complimentary transgene constructs will facilitate more rapid induction of expression with less catabolic loss of vector genomes prior to second strand (DNA) synthesis. Genetically engineered capsids can significantly increase transfection potency, and allow selective or broad cellular tropism in service of mechanistic requirements to maximize or restrict peptide availability. Advanced compact expression promoter constructs can broaden or narrow cellular targeting, as well as facilitate incorporation of reporter genes with minimal 'leaky' expression.



Treatment	Seizure free (%)	Low grade (%)	High grade (%)
GFP	0	30	70
SST low titer (10^12)	11.1	0	88.9
SST high titer (10^13)	46.2	7.7	46.2

Figure 13: Rats received equivalent volumes of one of 2 different SST vector preparations required to complete the efficacy study. Preliminary data indicate a higher proportion of responders (6/13 vs. 1/9) with delivery of more vector genomes, despite a lack of correlation between therapeutic probability and histological extent or location of expression. Data need to be replicated for within-prep dilution, and elimination of potential order effects. Independent factor 'vector' effect Pr>F 0.4652, vector x titer interaction Pr>F 0.0346. Least-squares means post-hoc comparisons between bars; SAS Proc GLM.

PI King and Ms. Natarajan have initiated collaboration with the UF Department of Chemistry to assay rat hippocampal SST levels in relation to SST and control vector administration. Dr. Kari Basso, Direcor of Mass Spectroscopy Services, is coordinating efforts to implement methods and generate translationally relevant data that will inform inquiry into therapeutic efficacy, mechanisms, and safety. Preliminary samples from untreated rats have returned novel information on multiple peptides (SST14, 28, and neuronostatin) coded by the SST gene. Samples from SST vector rats have been submitted for analysis.

PIs Carney and King have supervised a project conducted by Mr. Andrew Moss to characterize SST peptide and receptor abundance and localization in temporal lobe specimens resected from epileptic patients. This expands an initial case study conducted earlier by premedical undergraduate student Shahrukh Bengali. The data will provide novel information related to how limbic SST function changes earlier in epilepsy than has previously been examined.

What opportunities for training and professional development has the project provided?

Gowri Natarajan, a doctoral program student supported by CoPI Carney's subcontract, has continued to be integral to the project and highly productive throughout the reporting year. The project has provided her opportunities to develop unique expertise in experimental epilepsy, CNS gene therapy, qualitative and quantitative histological methodology, behavioral testing, and statistical analysis. She

Rat #	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 16	Day 20	Day 22	Day 24
NR1	4.5				4.5				5			4.5					
NR2	4.5				5			5		5							
NR3	5				5			5		5							
NR4	5	5			5		5										
NR5	4						4.5			5		5		5		5	
NR6	4						4.5							4.5			4.5
NR7	5				5			5		5			5		5		
NR8	5	5		1		5			5		5						
NR9	5		5		5			5		5			5				
NR10	5		5		4.5			5		5			1				
NR12	5		5		5			5		5			5				

Figure 14: SST non-responders also had consistent responses over multiple retests at original test currents. This indicates that failure to show therapeutic efficacy on test 1 was not due to delayed transgene expression, or within-subject variation in seizure severity, and that the first test accurately identifies non-responders.

has successfully defended her dissertation and joined Dr. Carney's laboratory for postdoctoral study at the University of North Carolina. She has generated all of the kindled animals. In addition she has performed all vector injections, and generated or participated in generating all of the histological material and behavioral data.

Dr. Ormerod supervises Mr. Jeffrey Leibowitz, who joined her lab early in 2014 as a Biomedical Engineering graduate student, in evaluating water maze behavior as well as neurogenesis and neuroinflammation in brains of rats kindled by Ms. Natarajan. Mr. Leibowitz's highly productive work has been and continues to be integral to successful completion of the project. He has performed the BrdU injections, perfusions, histological and immunohistochemical processing and microscopy necessary to generate the data on cytogenesis, new cell differentiation, and microglial indices of inflammation on data collected and for the work to be completed. He will also complete the BioPlex analyses of tissue samples.

How were the results disseminated to communities of interest?

A manuscript describing the seizure efficacy is now in print in Epilepsy Research. Two poster presentations were made at the 2016 Society for Neuroscience Annual Meeting. Copies of these are appended.

What do you plan to do during the next reporting period to accomplish the goals?

BKO and JL will complete the cytogenesis work and are currently confirming protein concentrations so that the Bioplex quantification and data analyses on inflammation proteins can be conducted March-May. These data will test how kindling stimulates inflammation, whether and how the SST vector impacts kindling-induced inflammation, and whether kindling or gene delivery affect survival and phenotypic differentiation of recently generated cells..

MK will complete histological analyses of neuronal and astroglial hippocampal pathology.

PRC, GN, and JZ will participate in kindling animals for MS, new vector development, and new seizure models (translational advancement safety & efficacy).

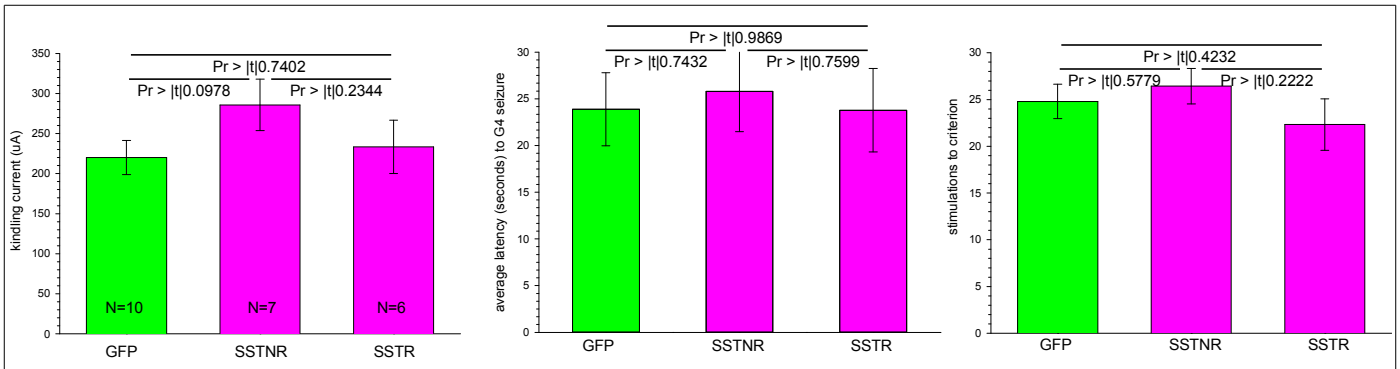


Figure 15: A retrospective analysis of kindling parameters was conducted to search for any that might be predictive for SST therapy responders and non-responders. Left: Stimulation current intensities (individual pre-kindling afterdischarge thresholds) were not predictive, though there was a trend for non-responders to have been kindled at currents higher than GFP control rats. SAS Proc GLM ANOVA Pr>F(2,22) 0.2308 for the independent factor 'pre-vector group'. Neither latency to sever seizure (center) nor number of stimulation episodes to become fully kindled (right) differed between responder and non-responder groups.

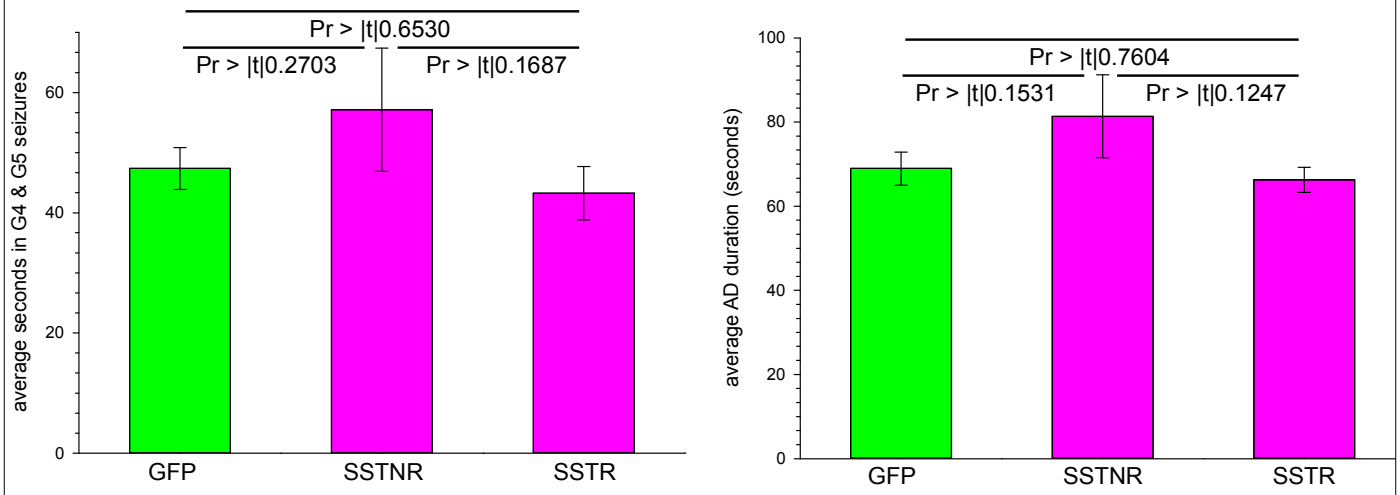


Figure 16: During kindling to criterion, average duration in high-grade seizures (left), or in afterdischarges (right), did not differ significantly between vector groups.

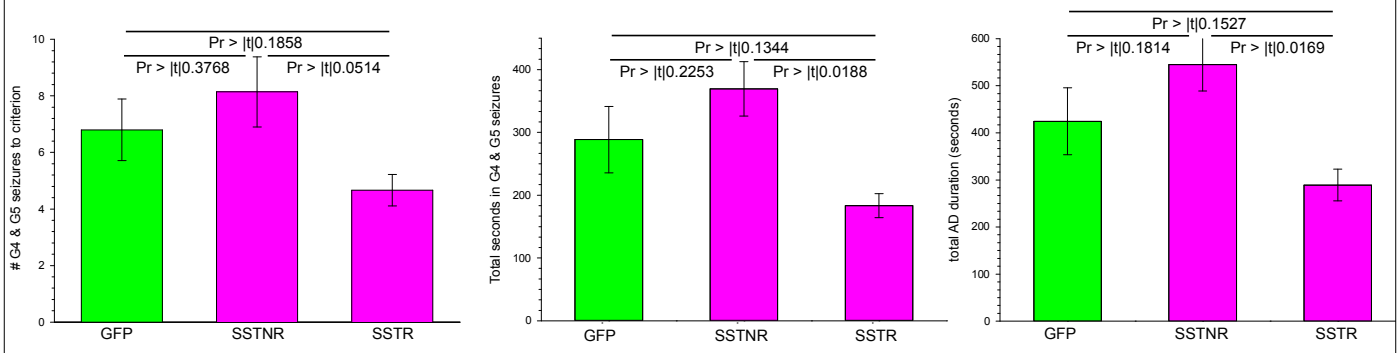


Figure 17: A trend was present for non-responders to have experienced more high-grade seizures during kindling to criterion (left). Total time in high-grade seizures (center), and total time spent in afterdischarges before exhibiting 3 consecutive grade 5 kindled seizures (right) were significantly higher in non-responders than in responders. Neither SST vector cohort differed significantly from GFP vector controls. SAS Proc GLM ANOVA Pr>F(2,21) 0.0537 for 'pre-vector group' effect on total AD duration.

All personnel will contribute to publication of results on behavior, cytogenesis, and inflammation. which is expected to produce 2-3 manuscripts (depending upon the outcome of the Bioplex-based

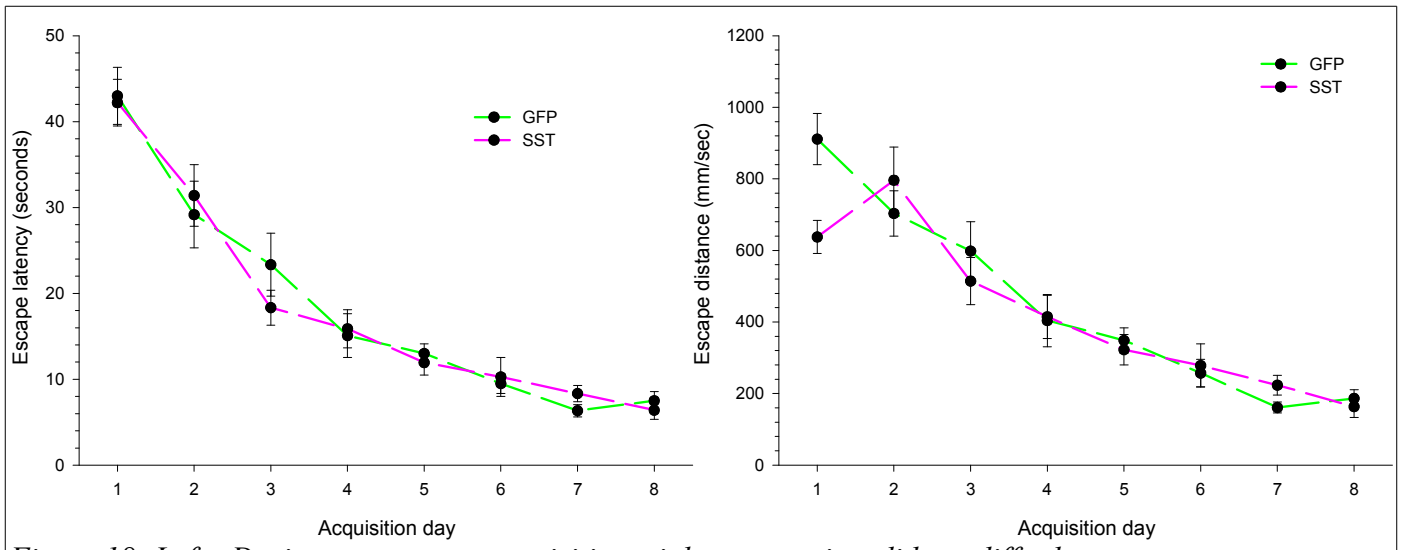


Figure 18: Left: During water maze acquisition trials, escape time did not differ between vector groups. SAS Proc GLM ANOVA for independent factor 'vector' F(1,215)=0.84 Pr>F 0.3719. Both groups showed robust acquisition of the escape task to equal proficiency. Right: Results were similar for distance to effect escape.

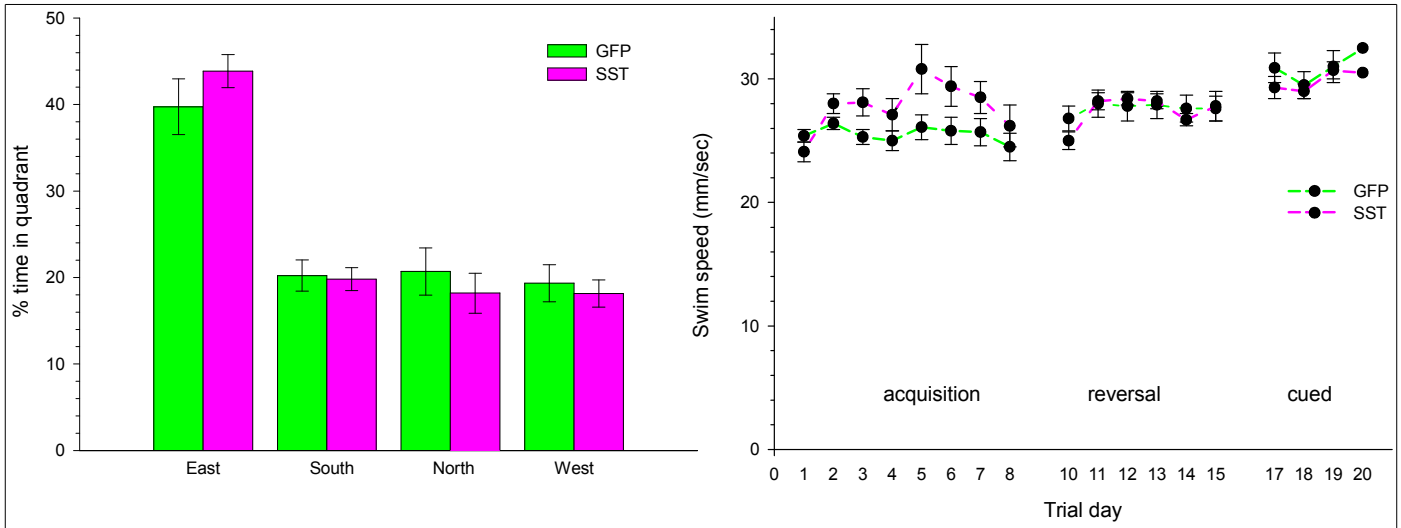


Figure 19: Left: During retention trials 2 days after acquisition training, both vector groups showed equivalent fractions of time in the target and non-target maze quadrants. SAS Proc GLM F(1,26)=1.32, Pr>F 0.2612. Right: Swim speed did not differ between vector groups.

inflammatory data). They will also participate in efforts to secure new funding necessary to move the technology toward clinical trials.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Gene delivery may provide a unique approach for localized treatment of epilepsies that are otherwise untreatable. Restoration of a seizure-free or mitigated severity condition by intracranial gene transfer could forestall or prevent the need for drastic surgical resection, or the need for continued use of powerful systemic medications that can limit daily activities. Furthermore, gene therapy may provide unique therapeutic alternatives in cases where epilepsy is more generalized or involves eloquent cortical regions that fully preclude surgical options. Anti-epileptic drugs are not effective anti-epileptogenics, so SST gene transfer offers a novel approach to limiting or even reversing the clinical evolution of seizures in brain-injured military personnel and veterans. Initial findings that significant therapeutic effects

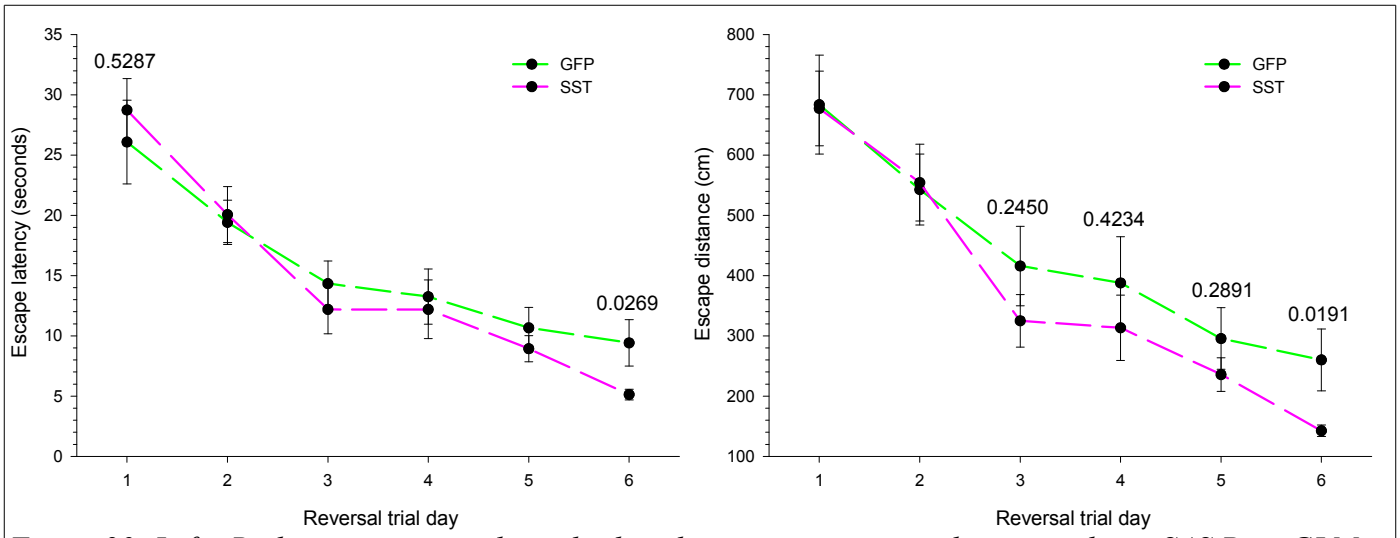


Figure 20: Left: Both vector groups showed robust learning over reversal training days. SAS Proc GLM repeated measures ANOVA F(5,125)=27.58, Pr>F<.0001. There was no significant effect of vector (F(1,25)=0.33 , Pr>F 0.5715), or interaction between vector and trial day (F(5,125)=0.74 , Pr>F 0.5974). SST vector rats showed a small but consistent trend to find the escape platform more quickly than GFP vector rats during reversal learning sessions 3-6, and were significantly more proficient on the 6th reversal trial day (F(1,26)=5.53, Pr>F 0.0269). Right: Vector effects on swim distance to escape were similar. SAS Proc GLM repeated measures ANOVA for independent factor 'vector' F(1,25)=1.48, Pr>F 0.2356. Both groups showed robust reversal learning over trial days (F(5,125)=26.21 Pr>F<.0001) and vector x trial day interactions were not significant (F(5,125)=0.50, Pr>F 0.7730). A trend for SST vector rats (N=15) to consistently locate the goal platform in shorter swim distances than GFP vector rats (N=12) emerged on day 3, and terminal proficiency was higher F(1,26)=6.28 Pr>F 0.0191.

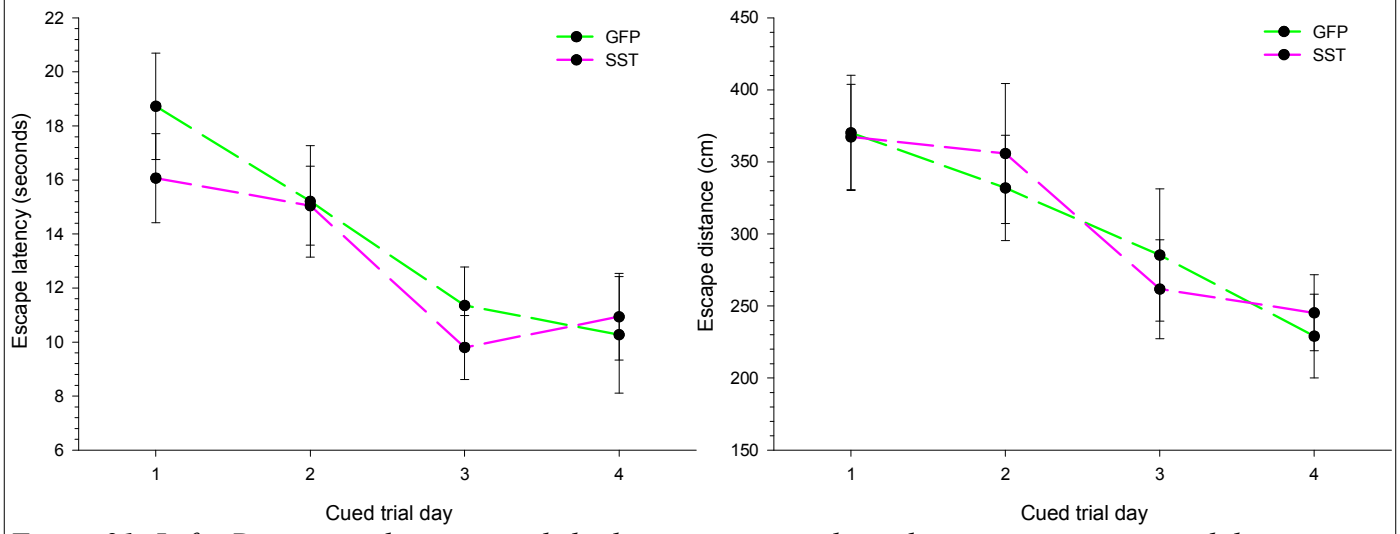


Figure 21: Left: During cued escape trials both vector groups showed improvement over trial days (F(3,75=7.35, Pr>F 0.0002) and reached equivalent proficiency. SAS Proc GLM repeated measures ANOVA found no effect of independent factor 'vector' (F(1,25)=0.87, Pr>F 0.3586), nor were interactions between vector and trial day significant (F(3,75)=0.34, Pr>F 0.7930). Right: Results were similar for escape distance during cued trials.

occur in a substantial fraction of test subjects support further investigation of mechanisms and application parameters necessary to refine and optimize efficacy and safety. The technology may also improve efficacy of other therapeutic approaches (pharmacological, dietary, etc.) in combination.

One impact of our results is that it does establish efficacy against some seizures involving temporal lobe circuitry. A long history of drug development for epilepsy confirms that efficacy in a kindling model can be highly predictive for efficacy in some but not necessarily all human seizure disorders. Epileptologists will be interested in exploring whether our experimental results extend to other seizure models, particularly those with recurrent spontaneous seizures missing from the kindling model.

A limitation of the kindling model has been a reliable functional impairment analogous to cognitive deficits in human temporal lobe epilepsy. The reversal learning deficits we report may provide a reliable approach to this in other studies of epilepsy mechanisms, consequences, and therapeutic development.

What was the impact on other disciplines?

Gene therapy is being actively developed or considered for many brain diseases refractory to pharmacological or surgical therapy. Our results provide further support of the safety and therapeutic power of AAV vector-mediated local gene delivery for *in situ* genetic correction, even when diseases are not overtly genetic. Polyfunctional signaling molecules like SST that can exert favorable actions on cytoprotection, cell generation, inflammation cascades, and neuronal excitability could constitute new ways to modulate pathophysiology common across epilepsy, stroke, aging, and trauma.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

The potential social benefits of improved epilepsy therapies are tremendous. Afflicted individuals can be severely constrained by seizures *per se*, and by their cognitive, psychic, and social consequences. Ameliorating these is likely to open new opportunities to work, travel, live independently, and contribute socially to many thousands of epileptics. Improved quality of individual's lives would be accompanied by considerable economic and medical resource allocation benefits to society at large.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Coincident to Dr. Carney's move to UNC a new IACUC protocol was submitted and approved. The procedures duplicate the UF IACUC and ACURO.

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals.

See above.

Significant changes in use of biohazards and/or select agents

None are used in this project.

6. PRODUCTS:

Publications, conference papers, and presentations

Authors: Natarajan G, Leibowitz JA, Zhou, J, Zhao Y, McElroy JA, King MA, Ormerod BK, Carney PR
Title: Adeno-associated viral vector-mediated preprosomatostatin expression suppresses induced seizures in kindled rat seizures in experimental temporal lobe epilepsy.
Journal: Epilepsy Research (2017), 130:81-92.
Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Authors: Natarajan G, Leibowitz J, Zhou J, King M, Ormerod B, Carney P
Title: Persistent somatostatin gene expression treats seizures in a subset of rats with experimental temporal lobe epilepsy.
Conference: Society for Neuroscience Annual Meeting 2016, abstract 592.11.*

Authors: Leibowitz JA, Natarajan G, Zhou J, King M, Carney P, Ormerod B
Title: SST treatment reverses kindling induced changes in adult hippocampal neurogenesis.
Conference: Society for Neuroscience Annual Meeting 2016, abstract 592.10.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Michael A. King, Ph.D.
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	making; orcid.org/0000-0001-5539-8552
Nearest person month worked:	4
Contribution to Project:	Vector design & procurement, seizure kindling, histological and statistical analysis, IACUC
Funding Support:	Dept. of Veterans Affairs, Army, NIH

Name:	Paul R. Carney, M.D.
Project Role:	coPI
Researcher Identifier (e.g. ORCID ID):	prcarney
Nearest person month worked:	3
Contribution to Project:	Epilepsy models
Funding Support:	

Name:	Brandi K. Ormerod, Ph.D.
Project Role:	coPI
Researcher Identifier (e.g. ORCID ID):	bormerod
Nearest person month worked:	3
Contribution to Project:	Histology, microscopy, behavior, biochemistry
Funding Support:	

Name:	Gowri Natarajan, Ph.D
Project Role:	Graduate student (to 12/2016), postdoctoral
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Animal surgery, kindling, behavioral testing
Funding Support:	UF College of Medicine graduate student stipend

Name:	Jeffrey Leibowitz
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12

Contribution to Project:	Cytogenesis, behavioral testing
Funding Support:	J. Crayton Pruitt Family Department of Biomedical Engineering Graduate Student Fellowship

Name:	Junli Zhou, Ph.D.
Project Role:	Post-doctoral associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Animal surgery, kindling
Funding Support:	

Name:	James McGuiness
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	bioplex training and technical assistance
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. King's NIH support from R01 MH080055 (PI: Lewis), "Development of Persistent Repetitive Behavior in Animals", ended 8/31/2016. His VA support from 1I21RX001396-01A1, "Somatostatin gene delivery to enhance long-term functional recovery from TBI", terminated 1/31/2017.

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS:

9. APPENDICES:

1. Natarajan et al. J. Epilepsy Research2016.pdf
2. Natarajan2016SFN.pdf
3. Leibowitz2016SFN.pdf

Epilepsy Research 130 (2017) 81–92

Contents lists available at www.sciencedirect.com

Epilepsy Research

journal homepage: www.elsevier.com/locate/epilepsyres

Original Research Paper

Adeno-associated viral vector-mediated preprosomatostatin expression suppresses induced seizures in kindled rats

Gowri Natarajan[a,b,c,d,e,f], Jeffrey A. Leibowitz[b], Junli Zhou[a,c,d], Yang Zhao[g], Jessica A. McElroy[a,c], Michael A. King[f,g,h], Brandi K. Ormerod[b,e,f], Paul R. Carney[a,b,c,d,e,f,*]

[a] Wilder Center of Excellence for Epilepsy Research, University of Florida, Gainesville, FL 32611, USA
[b] J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL 32611, USA
[c] Department of Pediatrics, University of Florida, Gainesville, FL 32611, USA
[d] Department of Neurology, University of Florida, Gainesville, FL 32611, USA
[e] Department of Neuroscience, University of Florida, Gainesville, FL 32611, USA
[f] McKnight Brain Institute, University of Florida, Gainesville, FL 32611, USA
[g] Department of Pharmacology and Therapeutics, University of Florida, Gainesville, FL 32611, USA
[h] NF/SG VA Medical Center, University of Florida, Gainesville, FL 32611, USA

ARTICLE INFO

Article history:
Received 10 August 2016
Received in revised form 4 December 2016
Accepted 4 January 2017
Available online 7 January 2017

Keywords:
Hippocampus
Gene therapy
Neuropeptides
Somatostatin
Kindling
Adeno-associated viral vectors

ABSTRACT

Somatostatin is expressed widely in the hippocampus and notably in hilar GABAergic neurons that are vulnerable to seizure neuropathology in chronic temporal lobe epilepsy. We previously demonstrated that sustained bilateral preprosomatostatin (preproSST) expression in the hippocampus prevents the development of generalized seizures in the amygdala kindling model of temporal lobe epilepsy. Here we tested whether sustained preproSST expression is anticonvulsant in rats already kindled to high-grade seizures. Rats were kindled until they exhibited 3 consecutive Racine Grade 5 seizures before adeno-associated virus serotype 5 (AAV5) vector driving either eGFP (AAV5-CBa-eGFP) or preproSST and eGFP (AAV5-CBa-preproSST-eGFP) expression was injected bilaterally into the hippocampal dentate gyrus and CA1 region. Retested 3 weeks later, rats that received control vector (AAV5-CBa-eGFP) continued to exhibit high-grade seizures whereas 6/13 rats that received preproSST vector (AAV5-CBa-preproSST-eGFP) were seizure-free. Of these rats, 5/6 remained seizure-free after repeated stimulation sessions and when the stimulation current was increased. These results suggest that vector-mediated expression of preproSST may be a viable therapeutic strategy for temporal lobe epilepsy.

1. Introduction

Over 20 million people continue to have seizures despite pharmacotherapy, leaving seizure-free rates unchanged for the last 15 years (Annegers et al., 1979; Brodie, 2005; Cascino, 2008; Kwan et al., 2010; Kwan and Brodie, 2000; Kwan et al., 2011). Developing novel strategies for these individuals is critical because drug resistant epilepsy is a potentially life-threatening condition accompanied by progressive cognitive impairment that compromises quality of life (Cramer, 1994; Kwan and Brodie, 2001, 2002). New surgical techniques, laser thermal ablation and responsive devices that detect seizures and automatically stimulate the brain to preempt them are exciting new options for these individuals, but they are in their infancy and have not yet substantially changed seizure-free rates (Cascino, 2008; Fisher et al., 2010; Jobst and Cascino, 2015; Morrell and RNS System in Epilepsy Study Group, 2011).

Viral vector-mediated neuropeptide gene delivery may open a promising treatment avenue for temporal lobe epilepsy (TLE; Riban et al., 2009; Vezzani, 2004) for individuals that are resistant to antiepileptic drug treatment or deemed not good candidates for resective epilepsy surgery. The temporal lobe structures involved in seizure genesis and propagation are permissive to neurotropic vector-mediated gene transfer, which has already demonstrated safety in clinical trials for a variety of human disorders (www.clinicaltrials.gov and Freese et al., 1997; McCown, 2004, 2010;

* Correspondence to: Department of Neurology, University of North Carolina at Chapel Hill, 170 Manning Drive, Campus Box 7025, NC 27599, USA.
E-mail address: paulcarney@unc.edu (P.R. Carney).

http://dx.doi.org/10.1016/j.eplepsyres.2017.01.002
0920-1211/

O'Connor et al., 1997; Riban et al., 2009; Vezzani, 2004, 2007; Weinberg and McCown, 2013; Weinberg et al., 2013). Some neuropeptides exhibit properties that could contribute to the effective treatment of seizures. For example, they may be neuroprotective and reduce excitability when released during high-frequency neuronal activity (Baraban and Tallent, 2004; Hökfelt, 1991). The endogenous expression and synaptic release of some neuropeptides (Csaba et al., 2004; Schwarzer et al., 1996; Simonato et al., 1998; Sperk et al., 1992) and the expression of their receptor subtypes (Csaba et al., 2005, 2004) is altered by seizure activity, suggesting a role for dysregulated neuropeptide signaling in seizure development and maintenance. Neuropeptides have been shown to suppress seizures in experimental epilepsy (Mazarati and Wasterlain, 2002; Zafar et al., 2012). Sustained adeno-associated viral (AAV) vector-mediated expression of galanin, neuropeptide Y or preprosomatostatin (preproSST) has been shown to delay epileptogenesis or suppress seizures in several animal models of TLE (Haberman et al., 2003; Kanter-Schlifke et al., 2007; Lin et al., 2003; McCown, 2006; Noè et al., 2008; Richichi et al., 2004; Sørensen et al., 2009; Woldbye et al., 2010; Zafar et al., 2012).

Somatostatin (SST) is a particularly attractive treatment candidate for TLE (Brazeau et al., 1973; Epelbaum, 1986; Tallent and Qiu, 2008; Vezzani and Hoyer, 1999). The 116 amino acid prepro-hormone preproSST is cleaved by proteases into biologically active SST-14, SST-28 and neuronostatin neuropeptides (Billova et al., 2007; Galanopoulou et al., 1995; Goodman et al., 1983; Samson et al., 2008; Tavianini et al., 1984; Winsky-Sommerer et al., 2000). In the naïve hippocampus, SST is predominantly expressed in CA1 region, CA3 region and hilar GABAergic interneurons (Freund and Buzsáki, 1996) although one report describes SST immunoreactivity in hippocampal granule neurons and pyramidal neurons (Billova et al., 2007). SST levels are responsive to neuronal activity and are altered across neurological diseases that include experimental and human TLE (Riekkinen and Pitkänen, 1990; Robbins et al., 1991). Specifically, SST expression and release is modulated by seizures (Csaba et al., 2004; Simonato et al., 1998; Tallent and Qiu, 2008; Vezzani and Hoyer, 1999). Moreover, a highly selective loss of SST-containing hilar GABAergic neurons occurs in both animal models and humans with TLE (Buckmaster and Dudek, 1997; Robbins et al., 1991; Sloviter, 1987; Sun et al., 2007). This neuronal loss along with changes in the morphology and connectivity of surviving SST-containing neurons has been postulated to mediate the chronic hyper-excitability associated with epileptogenesis (Peng et al., 2013; Zhang et al., 2009). SST knockout mice demonstrate increased severity of induced seizures (Buckmaster et al., 2002) and specific SST receptor (SSTR) agonists have been shown to effectively treat status epilepticus in experimental epilepsy (Aourz et al., 2011; Kozhemyakin et al., 2013). Together these data support the hypothesis that sustained hippocampal SST expression could ameliorate TLE. To this end, we previously demonstrated that sustained AAV5 vector-mediated hippocampal preproSST expression prevented the development of generalized or high-grade seizures in the majority of adult rats (Zafar et al., 2012).

The primary objective of the current study was to test whether sustained AAV5 vector-mediated hippocampal preproSST expression suppressed seizures when initiated *after* a stable seizure state was established in the rat amygdala kindling model (Goddard et al., 1969; McNamara et al., 1980; Sato et al., 1990). We hypothesized that sustained preproSST transgene expression in the hippocampi of *kindled* rats may be anticonvulsant and therefore a promising TLE treatment strategy. To test this hypothesis, an AAV serotype 5 vector was employed to bilaterally express the preproSST gene in the dentate gyrus and CA1 region of amygdala kindled adult rats. Our results showed that sustained hippocampal preproSST expression significantly reduced seizures in this experimental TLE model and that the preproSST anticonvulsant effect persisted over time.

2. Methods

2.1. Subjects

This study was conducted in accordance with Federal and University of Florida Institutional Animal Care and Use Committee policies regarding the ethical use of animals in research. Adult male Sprague Dawley rats (n = 23; 250–275 g upon arrival from Harlan) were housed in pairs in corncob-lined ventilated shoebox cages located in a standard colony room maintained at 24 ± 1 °C on a 12:12 h light:dark cycle (lights on at 0600 h). The rats were given Harlan Teklad Rodent Food Diet #7912 and reverse osmosis-filtered water *ad libitum* for the duration of the experiment.

Two weeks after arrival, rats were implanted with local electrical field potential recording and stimulating electrodes and allowed 10 days to recover from surgery. A baseline kindling session was employed to determine the after-discharge (AD) threshold current used for daily kindling sessions until rats reached the criterion of exhibiting 3 consecutive Racine Grade 5 seizures (Racine, 1972a,b). The following week, rats were randomly assigned to groups injected with either AAV5 vector driving preproSST and eGFP expression or control AAV5 vector driving eGFP expression. Three weeks later rats were retested to determine the stability and endurance of vector effects.

2.2. Bipolar electrode and connector strip preparation

Connector strips were 3D printed at the University of Florida Infinity Fabrication laboratory (http://fablab.arts.ufl.edu/). Bipolar twisted stimulating and recording electrodes were custom made in-house by cutting quadruple Teflon-coated 316 stainless steel wires (Sigmund Cohn Corp.; Mount Vernon, NY) into 6 cm lengths, removing the insulation at both ends and then soldering the male Amphenol gold pins (A-M systems; Sequim, WA) to both ends. Following this, the electrode wires with the Amphenol pins at either end were twisted. The loop generated at the end of the twisted wires was cut to produce an uninsulated tip that would make contact with the target tissue and deliver the administered current. For ground and reference screw electrodes, quadruple Teflon-coated 316 stainless steel wires (2.0 cm and 2.5 cm respectively), uninsulated at both ends were soldered to male Amphenol gold pins at one end. Stainless steel bone screws (FHC Inc.; Bowdoin, ME) were connected with a Unitek spot welder to the other end. Electrode wires were checked for continuity and impedance tested with an LCR/ESR meter (B&K Precision, Yorba Linda, CA). Only electrodes with impedance <1.8 Ω were implanted.

2.3. Surgical implantation of electrodes

Surgical procedures were conducted as described previously (Zafar et al., 2012). Rats were sedated with xylazine (10 mg/kg, subcutaneous) before anesthesia was induced with 4% isoflurane in 1 L/min oxygen and maintained at 1.5% isoflurane in 0.5 L/min oxygen. Anesthetized rats were placed in a Kopf stereotaxic frame and their shaven heads sterilized with alternating scrubs of 1% povidone-iodine solution and 70% ethanol. A midline incision exposed bregma and lambda. Two bipolar electrodes (330 μm d) were implanted bilaterally in the amygdala (−2.2 mm AP, ±4.8 mm ML, −8.3 mm DV; Paxinos and Watson, 2007) to stimulate and record activity in the left and right hemispheres counterbalanced randomly across groups. Two small diameter plastic hex nuts containing removable screws were affixed to the skull over dentate gyrus (−3.8 mm AP, ±1.8 mm ML) and CA1 region (−3.8 mm AP, ±1.8 mm ML) target coordinates (Paxinos and Watson, 2007) to keep the skull free of dental cement for later vector delivery. Ground and reference metal screw electrodes were

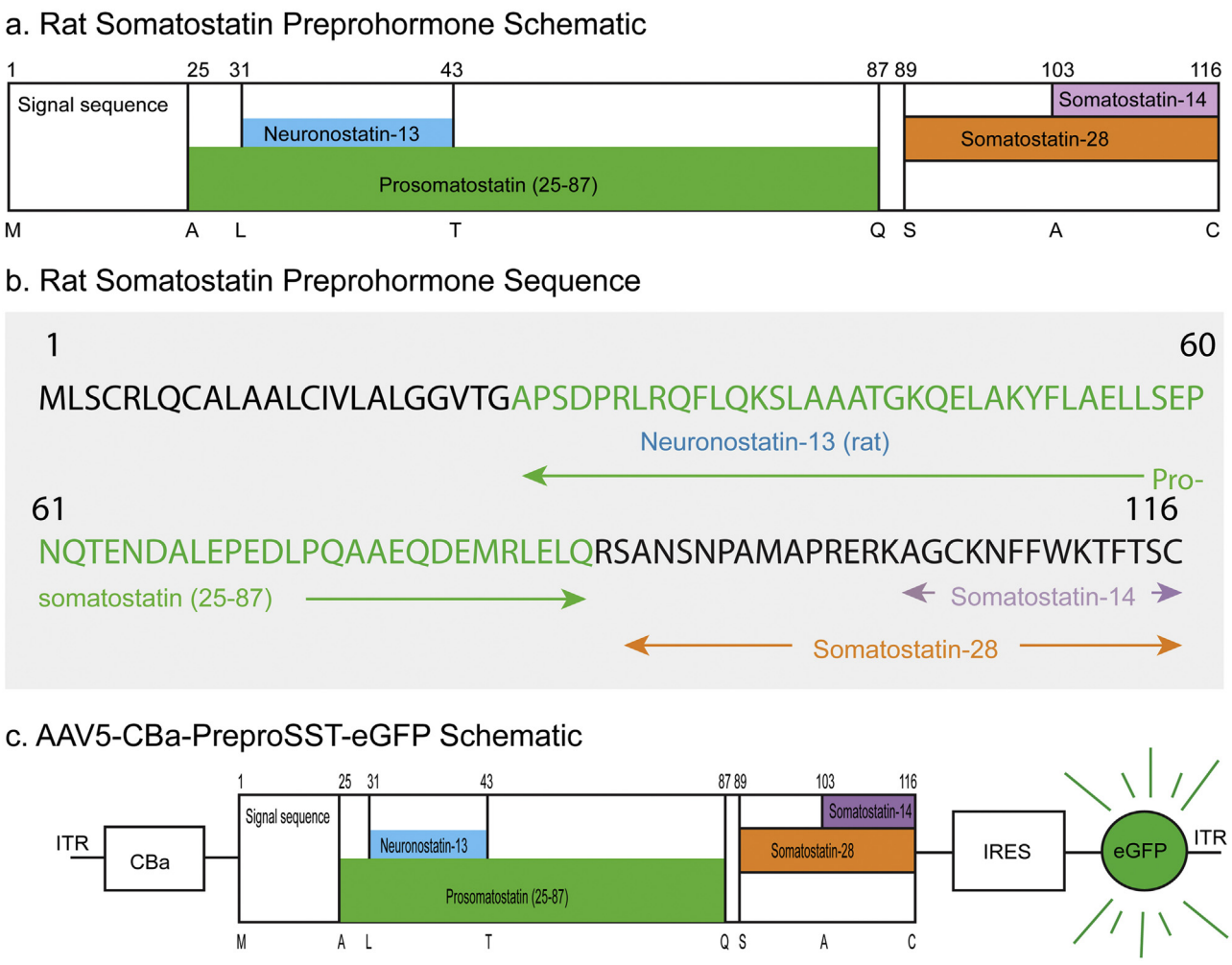


Fig. 1. Representation of the a) preproSST schematic b) preproSST amino acid sequence and c) AAV5-CBa-preproSST-eGFP vector construct. a, b) The 116 amino acid preproSST contains an N-terminal region of 24 amino acids. This signaling sequence directs the translocation of the translated preproSST product into the endoplasmic reticulum following which it is cleaved off at positions 24–25 (glycine-alanine) of the preproSST molecule (Goodman et al., 1983). The remaining 92 amino acid prohormone proSST undergoes alternative cleavage by specific proteases (Billova et al., 2007; Brakch et al., 1995; Galanopoulou et al., 1995; Goodman et al., 1983; Winsky-Sommerer et al., 2000) to yield the different mature peptide products SST-14, SST-28 (the N-terminal extension of SST-14) and the more recently discovered neuronostatin (Samson et al., 2008). c) The hybrid CMV early enhancer/CBa promoter was used to drive expression of the preproSST transgene tagged with a downstream eGFP reporter. The CBa-preproSST-eGFP plasmid contained an IRES sequence between the preproSST and the eGFP coding sequences that made eGFP expression an inert reporter of preproSST expression in this vector (Wong et al., 2002). This plasmid construct was packaged into serotype 5 AAV capsids. The final dot blot titer value was 4.19×10^{13} vg/mL. Adapted with permission from (Phoenix Pharmaceuticals, 2016).

implanted rostral to bregma and caudal to lambda respectively. Electrodes were attached to male Amphenol pins for insertion into connector strips before the entire electrode assembly was secured with dental cement anchored by skull bone screws.

2.4. Electrical kindling

Kindling sessions were initiated 10 days after surgery. Synchronized behavioral and electroencephalographic (EEG) data for all rats were recorded across kindling sessions and after vector treatment. ADs were defined as spikes >1 Hz with amplitudes at least twice the pre-stimulation baseline amplitude detected in the EEG (Zafar et al., 2012). Baseline EEG AD threshold currents were determined for each rat by identifying the minimum current intensity (starting at 50 μA and then increased in 50 μA steps delivered 1 min apart) at which a standard 2 s, 1 ms pulse duration, 50 Hz biphasic square wave pulse generated an AD (Racine, 1972a; Zafar et al., 2012). Kindling sessions were initiated 24 h later and conducted twice daily (>6 h apart) using each rat's AD threshold current.

Seizures were graded according to the Racine scale, a method by which seizure severity is quantified in animal models of epilepsy (Racine, 1972b). Briefly, Grade 0 included no evidence of behavioral seizure; Grade 1 included staring, immobility, and facial movements; Grade 2 included head nodding and chewing; Grade 3 included unilateral forelimb clonus; Grade 4 included bilateral forelimb clonus with rearing and Grade 5 included bilateral forelimb clonus with rearing, loss of balance, and falling. Kindling sessions were terminated once the rats exhibited 3 consecutive Grade 5 seizures. Upon reaching this criterion, rats were assigned randomly to the AAV5-CBa-preproSST-eGFP group (n = 13) or the AAV5-CBa-eGFP group (n = 10). The synchronized behavioral and EEG data for all rats across kindling sessions and after vector treatment on test and post-test sessions were analyzed blinded to score: (1) latency (s) to Grade 4 seizure after the onset of electrical stimulation (2) AD duration (s), (3) time (s) spent in high-grade seizure and (4) seizure grade (Grade 0–5).

2.5. Plasmids and vector construct

CBa-preproSST-eGFP and CBa-eGFP plasmids were packaged into serotype 5 AAV capsids by the University of Florida vector core facility (gtc.ufl.edu/core/vector-core-lab.htm). Final dot blot

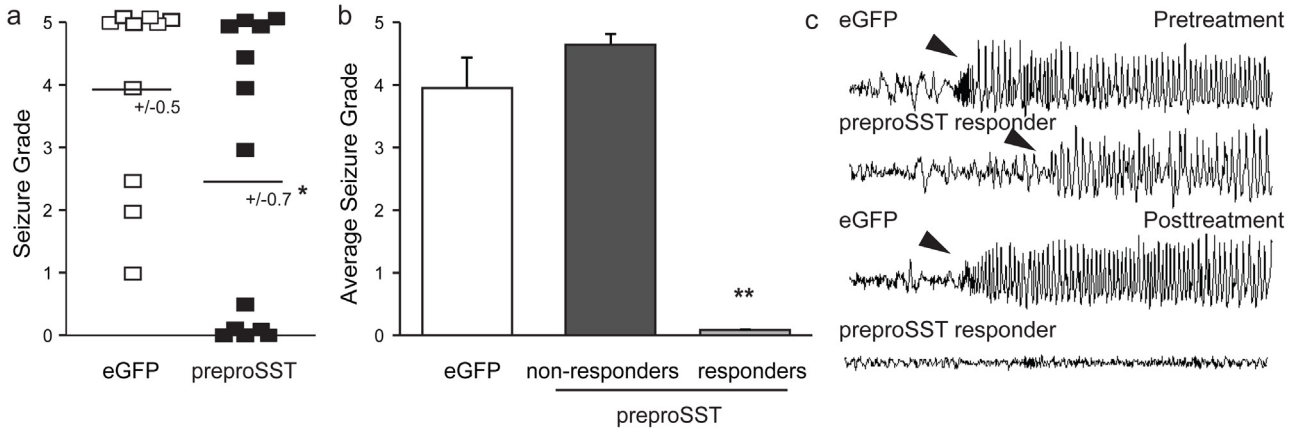


Fig. 2. Sustained preproSST expression prevented induced seizures in a subset of kindled rats. AAV5-CBa-preproSST-eGFP-treated rats (n = 13) and AAV5-CBa-eGFP-treated rats (n = 10) were given a test session 3 weeks after vector injection at the AD threshold current to test whether sustained preproSST expression could prevent seizure behavior. a) The average seizure grade produced by the preproSST-treated rats was significantly lower than the average seizure grade produced by eGFP-treated rats (*$p < 0.05$, one-tailed). The scatterplot analysis of individual seizure grades revealed that a subset of preproSST-treated rats (preproSST-treated responders, n = 6/13, 100% Grade 0 in the 6 rats) was completely seizure-free relative to eGFP controls (n = 10, 0% Grade 0, 10% Grade 1, 20% Grade 2–3, 10% Grade 4, 60% Grade 5). The seizure grade remained largely unchanged in the remaining preproSST-treated rats (preproSST-treated non-responders, n = 7/13, 14.3% Grade 3, 14.3% Grade 4, 71.4% Grade 5 in the 7 rats). b) The seizure grade varied between the eGFP-treated, preproSST-treated responder and preproSST-treated non-responder groups such that the average seizure grade was significantly lower in the preproSST-treated responder (**$p < 0.01$) but not in the preproSST-treated non-responder ($p = 0.73$) group relative to the eGFP-treated control group. c) Severe ADs characteristic of Grade 5 seizures were recorded from the amygdala, of a representative rat in the AAV5-CBa-eGFP-treated group and also an eventual preproSST-treated responder rat from the AAV5-CBa-preproSST-eGFP-treated group. Three weeks after vector expression, during the test session, ADs were absent in the preproSST-treated responder rat relative to the eGFP-treated rat that produced severe ADs. Arrows indicate the onset of ADs. All data are represented as mean ± SEM.

Table 1
Seizure grade exhibited by preproSST-treated responder rats on post-test sessions (PTS).

	PTS-1	PTS-2	PTS-3	PTS-4	PTS-5	PTS-6 (+50μA)	PTS-7 (+50μA)	PTS-8 (+100μA)
Rat 1	0	0	0	0	0	0	0	0
Rat 2	0	0	0	0	0	0	4	4
Rat 3	0	0	5	0	0	5	0*	2
Rat 4	0	0	0	5	5	0	2	5
Rat 5	0	0	0	0	0	0	0	0
Rat 6	5	5	5	5	5	0	0	0

*The current employed for this rat during this session was the AD threshold current. Post-test sessions during which the stimulus current was increased above the AD threshold current are shown in grey.

titer values were 4.19×10^{13} vector genomes (vg)/mL for AAV5-CBa-preproSST-eGFP and 8.9×10^{12} vg/mL for AAV5-CBa-eGFP. The plasmid sequence consisted of the hybrid cytomegalovirus enhancer/chicken β-actin (CBa) promoter (Klein et al., 2002; Niwa et al., 1991) driving either preproSST transgene tagged with a downstream eGFP reporter (pAAV5-CBa-preproSST-eGFP) expression or eGFP (pAAV5-CBa-eGFP) expression. The CBa-preproSST-eGFP plasmid contained an internal ribosomal entry site (IRES) sequence between the preproSST and the eGFP coding sequences that made eGFP expression an inert reporter of preproSST expression in this vector (Wong et al., 2002). Fig. 1 shows the preproSST sequence and the AAV5-CBa-preproSST-eGFP vector construct.

2.6. AAV vector injections

The week after reaching fully kindled criterion, rats were infused bilaterally with AAV5-CBa-preproSST-eGFP vector (n = 13) or AAV5-CBa-eGFP control vector (n = 10) in the dentate gyrus (−3.8 mm AP, ±1.8 mm ML, −3.4 mm DV) and CA1 region (−3.8 mm AP, ±1.8 mm ML, −2.4 mm DV; Paxinos and Watson, 2007). Anesthetized rats were mounted in the stereotaxic frame and the placeholder screws were removed from the hex nuts placed over dentate gyrus and CA1 region targets. Holes were drilled through the skull using the affixed hex nuts as guides to expose the surface of the brain. Vectors were injected using a 10 μL Hamilton syringe fitted with a 27G steel needle (Hamilton, Reno, NV) attached to an infusion system (Cole-Parmer, Vernon Hills, IL). A 2 μL volume of the vector was delivered in each of 4 injection sites (i.e. 2 μL of vector was injected into the left and right CA1 region and dentate gyrus) through polyaryletherketone tubing (ID 0.381 mm, OD 0.794 mm, length 0.5 m; Restek; Bellafonte, PA) by convection-enhanced delivery (Bankiewicz et al., 2000; Bobo et al., 1994) at a flow rate of 0.3 μL/min. The injection needle was held in place for 3–5 min to allow for uniform vector distribution and to minimize backflow.

2.7. Post vector injection testing

After a 3-week interval to ensure that vector expression plateaued (Klein et al., 2002; Zafar et al., 2012), each rat was given a single test kindling session at the original AD threshold current. Rats were subsequently kindled 2–3 times per week for up to 3 additional weeks during post-test sessions to determine the stability and persistence of vector effects. Rats that exhibited Grade 0 seizures on the test session were also tested at higher currents (+50 μA, +50 μA, +100 μA above the AD threshold current) on the final 3 post-test sessions. As with kindling sessions, the synchronized behavioral and EEG data for all rats in test and post-test sessions administered after vector treatment were scored blind to

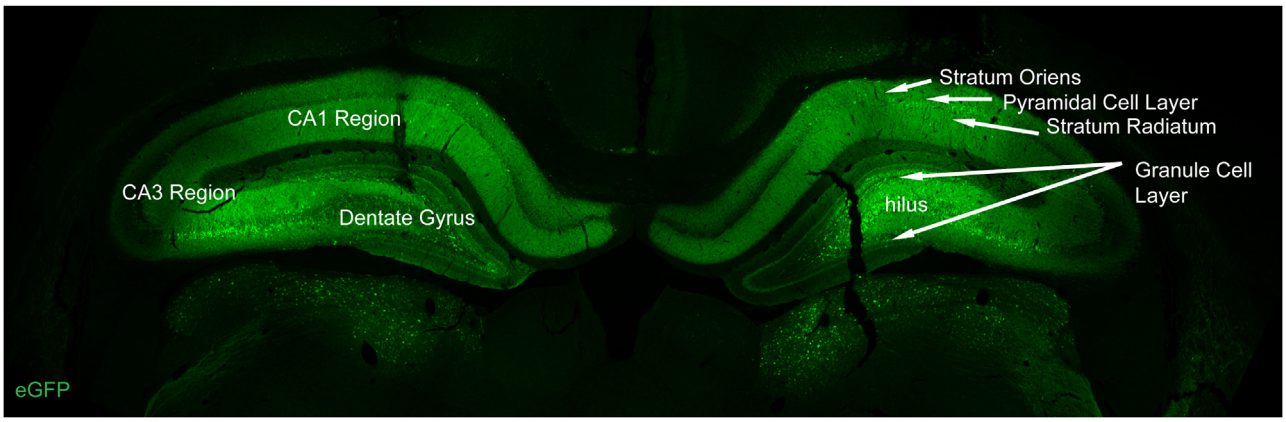


Fig. 3. AAV5-CBa-preproSST-eGFP transduction within the hippocampus of a rat in the preproSST vector-treated group. Robust eGFP expression was observed bilaterally within the dentate hilus, granule cell layer, CA3 region and CA1 region of the hippocampus. In this rat a relatively small number of thalamic neurons ventral to the hippocampus also showed eGFP expression.

Table 2
eGFP expression in hippocampal and extrahippocampal regions.

Rat #	CA1		CA3		GCL		Hilus		Subiculum		Cortex	Thalamus
	Side1	Side2	Side1	Side2	Side1	Side2	Side1	Side2	Side1	Side2		
SST Non-Responders												
Rat 1	++	++	++	–	–	–	–	–	++	–	N	N
Rat 2	++	++	++	+++	–	+	–	+++	+	+	Y	N
Rat 3	++	++	++	++	–	+	–	++	–	–	Y	Y
Rat 4	++	+	+++	+++	++	++	+++	+++	+	–	Y	Y
Rat 5	++	++	+++	++	+++	+++	+++	+++	+	+	Y	N
Rat 6	++	++	+++	+	+	+	+++	+++	+	–	Y	N
SST Responders												
Rat 1	++	++	+++	++	+	–	+++	–	–	+	Y	N
Rat 2	++	+++	++	++	–	++	++	+++	+	–	Y	Y
Rat 3	+++	+++	–	–	–	–	–	–	++	++	Y	N
Rat 4	++	++	+++	++	++	+	+++	+++	++	–	Y	N
Rat 5	++	++	++	+	+	+	+++	++	+	+	N	N
Rat 6	+++	+++	++	++	–	+++	–	–	++	+	Y	N

The presence or absence of eGFP expression is denoted by '+' and '–' symbols respectively. The number of '+' symbols represents the qualitative robustness of eGFP expression in each region, where '+' represents a few scattered eGFP+ cells, '++' denotes a moderate number of eGFP+ cells and "+++" denotes that most cells were eGFP+. Scattered cells were detected in the cortices and thalamic region of some rats. 'N' denotes no eGFP+ cells detected and 'Y' denotes eGFP+ cells detected. Note: One preproSST-treated non-responder rat was excluded from the analysis because its tissue was damaged during histology.

compare latencies to Grade 4 seizure, AD durations, time spent in high-grade seizures, and seizure grade.

2.8. Perfusion and histology

Forty-eight hours after the final post-test stimulation, rats were deeply anesthetized with an intraperitoneal xylazine (10 mg/kg)/ketamine (80 mg/kg) cocktail injection and then perfused with ice cold isotonic saline followed by ice cold freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were extracted and post-fixed overnight in perfusate and then cryoprotected in 30% sucrose PBS solution for ~3 days. The brains were sectioned coronally at 40 μm through the rostral-caudal extent of the hippocampus on a freezing stage microtome (American Optical Corporation, Buffalo, N.Y., USA) and brain sections were stored in cryoprotectant solution (25% glycerol, 30% ethylene glycol and 45% 0.1 M PBS) at −20° C.

2.9. Electrode tract and gene expression analysis

Electrode and vector injection placements were confirmed by brightfield and epifluorescence microscopy by an experimenter who was blind to the treatment conditions and treatment outcomes. Placements were localized through the anterior-posterior extent of the dorsal hippocampus (−2.04 mm to −5.4 mm AP to bregma, DV <5 mm; Paxinos and Watson, 2007) and in underlying subcortical regions and overlying cortical regions using the GFP filter on an Olympus IX71 epifluorescence microscope.

2.10. SST immunohistochemistry

Sections from 3 representative rats in the AAV5-CBa-preproSST-eGFP group and the AAV5-CBa-eGFP group were immunolabeled with an SST specific antibody to confirm SST expression. The sections were rinsed repeatedly in tris-buffered saline (TBS) prior to immunolabeling and between each step. The sections were blocked in a solution of 3% normal donkey serum and 0.1% Triton-X in TBS and then incubated overnight at 4 °C in rabbit polyclonal SST primary antibody (1:5000, Peninsula laboratories; Peng et al., 2013) dissolved in antibody diluent (1% normal donkey serum and 0.1% Triton-X in TBS). The following day, the sections were incubated in anti-rabbit secondary antibody conjugated to cy3 (1:500, Jackson laboratories) in antibody diluent for 4 h at room temperature, incubated with DAPI (1:10,000; Chemicon) for 10 min and then mounted under PVA-DABCO (2.5% diazobicyclooctane in TBS with 10% polyvinyl alcohol and 20% glycerol). At least 50 immunolabeled neurons per hippocampal region were examined through their z-plane to confirm eGFP expression and/or SST immunoreactivity

using a Zeiss meta LSM 710 fully spectral laser scanning confocal microscope with 405, 488, 543 and 633 nm laser lines (Thornwood, N.Y., USA) under a 20× objective and 2.3× digital zoom. The percentages of neurons producing transgenic SST ($eGFP^+/SST^+$ neurons), endogenous SST ($eGFP^-/SST^+$ neurons) and transgenic and/or endogenous SST ($eGFP^+/SST^+$ neurons and $eGFP^-/SST^+$ neurons) were compared between AAV5-CBa-preproSST-eGFP-treated and AAV5-CBa-eGFP-treated rats (n = 3 per group) across hippocampal regions to confirm that the AAV5-CBa-preproSST-eGFP vector drove SST expression.

2.11. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 24 software (www.onthehub.com/spss). *T*-tests (preproSST versus eGFP treatment) and one-way ANOVAs (eGFP controls versus preproSST-treated responders versus preproSST-treated non-responders) were used to compare group differences for dependent variables (kindling sessions to criterion, latency to Grade 4 seizure, AD duration, number and time in high-grade (Grade 4/5) seizures and percentages of $eGFP^+$ and/or SST^+ neurons). Group differences in dependent variables yielding non-parametric data (seizure grade after vector treatment, AD threshold currents) were compared using Mann–Whitney *U* tests (preproSST versus eGFP treatment) or Kruskal Wallace ANOVA tests (eGFP controls versus preproSST-treated responders versus preproSST-treated non-responders) followed by Mann–Whitney *U* tests. Significant effects revealed by the Kruskal Wallace ANOVA were explored with a Mann–Whitney *U* test. Effect sizes were calculated using Cliff's *d* (Cliff, 1993). Alpha levels were set at $p < 0.05$ and reported as two-tailed unless stated otherwise.

3. Results

3.1. Kindling acquisition proceeded similarly in rats assigned to the preproSST or eGFP control group

Behavioral changes in all 23 rats progressed through the classic Racine scale (Racine, 1972b) from Grades 0–5 across kindling sessions. The average AD threshold current across rats was 243.5 ± 16.4 μA. Rats exhibited their first Grade 5 seizures after 20.1 ± 1.3 sessions (~10 days) and reached the fully kindled criteria of 3 consecutive Grade 5 seizures after 24.7 ± 1.1 sessions (~12 days).

Rats assigned to the preproSST and eGFP vector groups required similar numbers of kindling sessions to reach criterion (eGFP = 24.8 ± 1.8 and preproSST = 24.5 ± 1.7; $t_{(21)} = 0.11$; $p = 0.92$) and exhibited similar AD threshold currents (eGFP = 220 ± 21.3 μA and preproSST = 261.5 ± 23.4 μA; $U = 46.5$, $n_1 = 10$, $n_2 = 13$, $p = 0.20$). They also exhibited similar numbers of high-grade (Grade 4/5) seizures during kindling acquisition (eGFP = 6.8 ± 1.1 and preproSST = 6.5 ± 0.9; $t_{(21)} = 0.19$; $p = 0.85$), latencies to Grade 4 seizure (eGFP = 23.9 ± 3.9 s and preproSST = 24.9 ± 3.0 s; $t_{(21)} = -0.20$; $p = 0.22$), time spent in high-grade seizures (eGFP = 47.4 ± 3.5 s and preproSST = 50.7 ± 6.0 s; $t_{(21)} = 0.45$; $p = 0.66$) and AD durations (eGFP = 69.0 ± 3.9 s and preproSST = 74.4 ± 5.7 s; $t_{(21)} = -0.73$; $p = 0.66$).

3.2. Sustained preproSST expression produced seizure resistance in a subset of rats

Fig. 2a shows the individual and vector group seizure grade exhibited during the test session. The average seizure grade exhibited by preproSST-treated rats (2.5 ± 0.7) was significantly lower than the average seizure grade exhibited by eGFP-treated rats (4.0 ± 0.5; $U = 39$, $n_1 = 13$, $n_2 = 10$, $p = 0.048$, one-tailed). The effect size for this analysis ($d = -0.40$) approached Cliff's (Cliff, 1993) convention for an intermediate effect. Fig. 2a also shows that of the eGFP-treated rats 10% exhibited Grade 1 seizures, 20% exhibited Grade 2 seizures and 70% exhibited Grade 4/5 seizures. For preproSST-treated rats, 46% remained seizure-free while 54% exhibited seizures higher than Grade 3 on the test session. On the basis of this response separation, preproSST-treated rats were divided into preproSST-treated responder and preproSST-treated non-responder groups (Fig. 2b). Seizure grade varied between the eGFP-treated, preproSST-treated responder and preproSST-treated non-responder groups ($H_{(2,20)} = 14.2$; $p < 0.001$), such that seizure grade was significantly lower in the preproSST-treated responder ($p < 0.01$), but not in the preproSST-treated non-responder ($p = 0.73$) group relative to the eGFP-treated control group (Fig. 2b). Importantly, the effect size of the difference between eGFP-treated and preproSST-treated responder groups ($d \geq -0.99$) far exceeded Cliff's (Cliff, 1993) convention for a large effect.

EEG traces from representative rats (Fig. 2c) illustrate kindling and vector effects. The top 2 traces were obtained from an eGFP-treated and preproSST-treated responder rat respectively, on the final kindling session prior to vector injection and show EEG ADs (the arrow shows AD onset) coincident with a Grade 5 behavioral seizure. The third and fourth traces were obtained from the same rats after vector injection. The AAV5-CBa-eGFP vector-treated control rat exhibited EEG ADs coincident with a Grade 5 behavioral seizure, whereas the AAV5-CBa-preproSST-eGFP vector-treated rat exhibited neither EEG ADs nor a behavioral seizure.

3.3. The effects of vector on kindled seizure susceptibility were stable and persistent

Vector-treated rats were given 2–3 post-test sessions per week for up to 3 weeks. Responders that exhibited Grade 0 seizures on the initial test session (n = 6/13 AAV5-CBa-preproSST-eGFP-treated rats) underwent 5 subsequent post-test sessions using their AD threshold currents and then 3 more sessions during which the stimulus current was delivered at 50 μA, 50 μA and then 100 μA above their AD threshold current. On the first 5 post-test sessions, 3/6 rats remained completely seizure-free, 2 rats exhibited 1 or 2 high-grade seizures among multiple Grade 0 seizures and 1 rat repeatedly exhibited high-grade seizures. On the last 3 sessions when higher current intensities were employed, 2/3 rats that were seizure-free across post-test sessions remained seizure-free while 1 rat exhibited high-grade seizures at the highest stimulus currents. The 2 rats that exhibited high-grade seizures during 1 or 2 post-test sessions produced variable grade seizures at higher stimulus currents. The rat that produced high-grade seizures on all the post-test sessions was seizure-free (Table 1).

eGFP-treated and preproSST-treated non-responders underwent 3–5 post-test sessions over a 2–3 week period using the AD threshold current for each rat. Since all preproSST-treated non-responder rats exhibited Grade 4–5 seizures during each of these sessions, current intensities were not increased during final sessions. Of the eGFP-treated rats, 9/10 exhibited seizures equal to or greater in severity across post-test sessions to those exhibited during the test session. The remaining eGFP-treated rat exhibited Grade 5 seizures on all but 2 post-test sessions during which it exhibited Grade 0 seizures.

3.4. eGFP expression was similar in preproSST-treated responders and non-responders

eGFP expression was found within the dentate gyrus (hilus, subgranular zone and granule cell layer), the CA1 region (pyramidal cell layer, stratum oriens and stratum radiatum) and the CA3 region (predominantly in the pyramidal cell layer) and in several cases, the

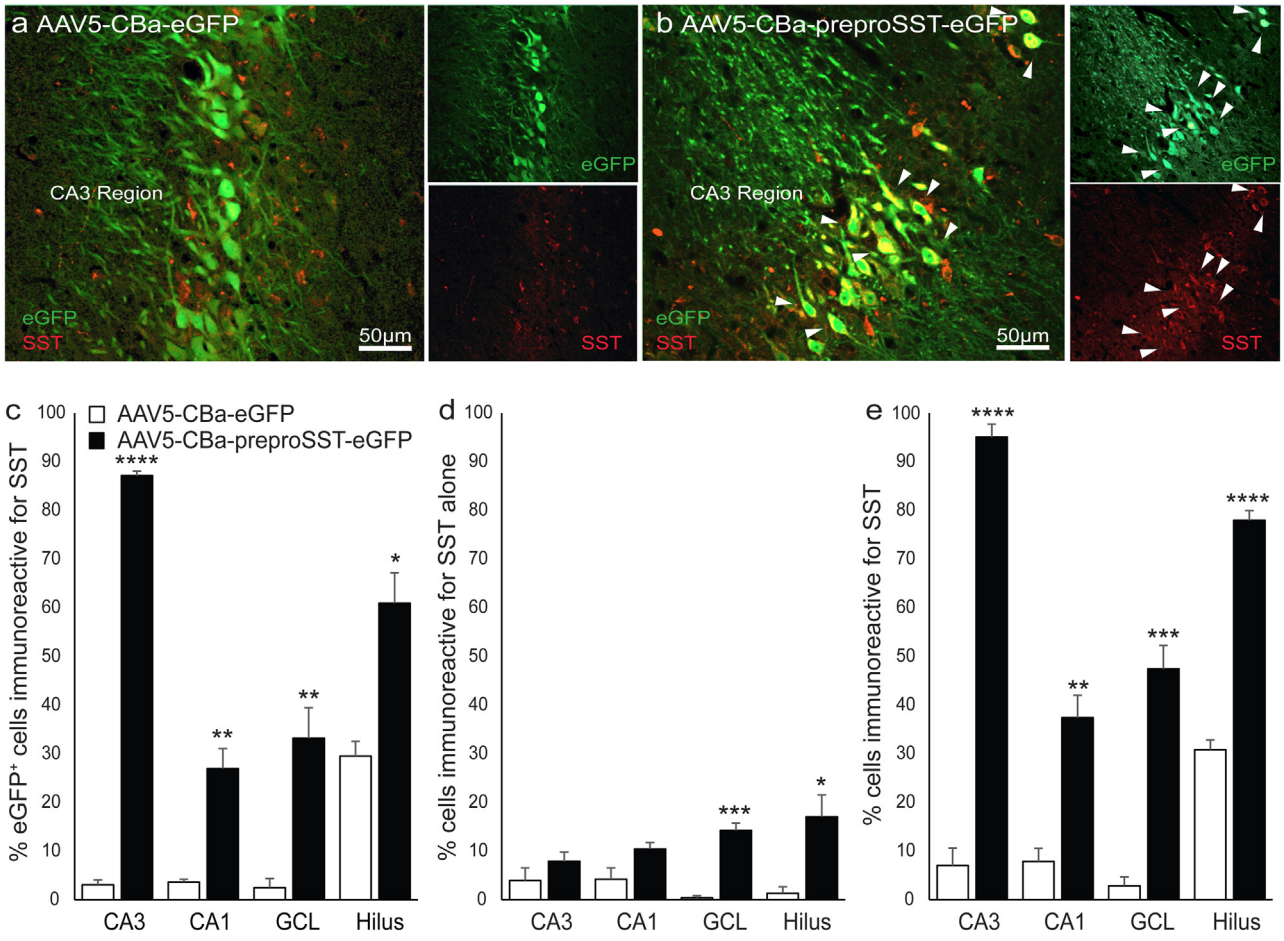


Fig. 4. Coincident eGFP and SST localization was consistent with vector transduction of hippocampal principal neurons. a, b) Examples of native eGFP$^+$ neurons (in green), eGFP$^+$/SST$^+$ (both green and red) and SST$^+$ (in red) neurons found in the CA3 hippocampal region of rats treated with the a) control or b) preproSST vector. Few eGFP$^+$ neurons in the pyramidal cell layer were immunoreactive for SST in eGFP vector-treated rats whereas most of eGFP$^+$ neurons were immunoreactive for SST in the preproSST-treated rats. Scale bar represents 50 μm. Arrows indicate co-labeled neurons. c) The percentage of eGFP$^+$/SST$^+$ neurons increased in the CA3 region (****$p < 0.0001$) and CA1 region (**$p < 0.01$) pyramidal cell layers, granule cell layer (GCL, **$p < 0.01$) and hilus (*$p < 0.05$) of preproSST vector-treated versus control vector-treated rats. d) The percentage of eGFP$^-$/SST$^+$ neurons increased in the granule cell layer (GCL, ***$p < 0.001$) and hilus (*$p < 0.05$) but not the CA3 region and CA1 region pyramidal cell layers of preproSST vector-treated versus control vector-treated rats. e) The percentage of neurons immunoreactive for transduced and/or endogenous SST increased in the CA3 region pyramidal cell layer (****$p < 0.0001$) and CA1 region pyramidal cell layer (**$p < 0.01$), the granule cell layer (GCL, ***$p < 0.001$) and hilus (****$p < 0.0001$) of the preproSST vector-treated versus control vector-treated rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

subiculum of the dorsal hippocampus of vector-treated rats (Fig. 3). Table 2 shows eGFP expression in each region of the hippocampus of preproSST-treated responder and non-responder rats. Note that 1 preproSST-treated non-responder rat was excluded from this analysis because its brain was frozen prior to cryopreservation making the resolution of fine detail difficult to resolve. However, the rat was retained in other analyses because eGFP$^+$ cells could be resolved grossly throughout hippocampal tissue.

In all rats, bilateral eGFP expression was detected in at least 1 major hippocampal region. In most rats, eGFP expression was detected through the anterior-posterior extent of 1 region on at least 1 side of the dorsal hippocampus. Moderate to strong eGFP expression was detected through the CA1 region of all rats and through the CA3 region of all rats except for 1 of the preproSST-treated responder rats. In these regions, eGFP expression could be localized or diffuse. eGFP expression was undetectable in the dentate gyrus of 1 preproSST-treated responder and 1 preproSST-treated non-responder rat but was detected in the dentate gyrus of all other rats. Intense localized eGFP expression was detected in the hilus and sub-granular zone of all rats except 1 preproSST-treated non-responder and 2 preproSST-treated responder rats and the granule cell layer was intensely transduced in a few rats. Cells in the subiculum were similarly transduced in responder and non-responder rats. Overall, eGFP expression patterns did not differ overtly between preproSST-treated responder and non-responder rats with respect to the laterality of expression or the hippocampal regions transduced. There were also no overt differences between groups with respect to the localization or morphologies of the cells transduced in each region.

Extrahippocampal eGFP expression was also compared across responder and non-responder rats. Only 3 preproSST-treated rats (1 responder and 2 non-responders) had eGFP expression in the thalamic region ventral to the hippocampus. Cortical eGFP expression dorsal to the hippocampus was found in all but 2 rats and was consistent between preproSST-treated responders and non-responders.

3.5. Co-labeling for eGFP and SST was consistent with vector transduction in hippocampal principal neurons

Our IRES vector employs downstream eGFP expression to report preproSST transcription and presumably the generation of SST peptide in transduced neurons (Wong et al., 2002). Fig. 4a and b show examples of eGFP$^+$, eGFP$^+$/SST$^+$ and SST$^+$ neurons found in the

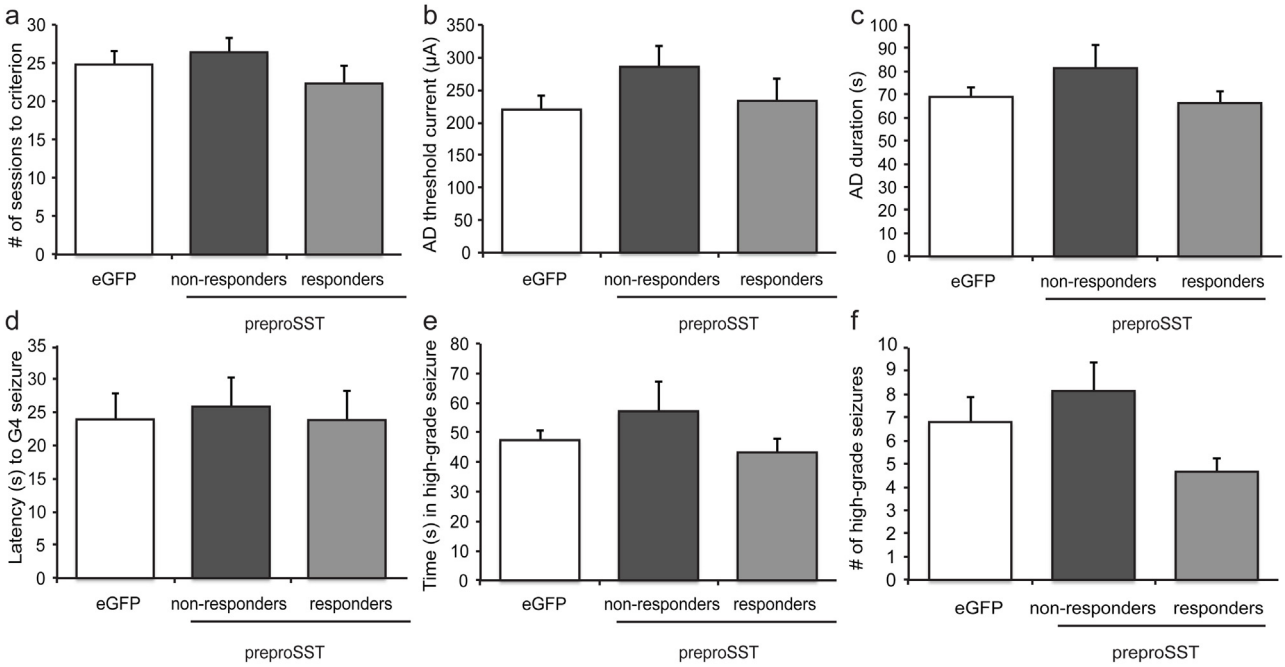


Fig. 5. PreproSST-treated responders and non-responders exhibited similar behavioral responses and EEG characteristics during kindling acquisition. An omnibus ANOVA did not reveal significant differences between preproSST-treated responders, preproSST-treated non-responders and eGFP-treated rats in a) the number of sessions to criterion b) the AD threshold current c) AD duration d) latency to Grade 4 seizure and e) time spent in high-grade seizure. Although preproSST-treated responders specifically appeared to produce fewer high-grade (Grade 4/5) seizures during kindling acquisition relative to preproSST-treated non-responders, this was not overall statistically significant between preproSST-treated responders, preproSST-treated non-responders and eGFP-treated rats (f).

CA3 region of rats treated with the control (Fig. 4a) or preproSST (Fig. 4b) vector. Fig. 4 also shows the percentages of $eGFP^+/SST^+$ neurons (Fig. 4c), $eGFP^-/SST^+$ neurons (Fig. 4d) and $eGFP^+/SST^+$ plus $eGFP^-/SST^+$ neurons (Fig. 4e). Our initial assumption was that the population of $eGFP^+/SST^+$ neurons expressed transgenic SST and that the population of $eGFP^-/SST^+$ neurons naturally expressed SST. Few $eGFP^+$ neurons were immunoreactive for SST in the granule or pyramidal cell layers of control vector-treated rats whereas most $eGFP^+$ neurons were immunoreactive for SST in the CA3 region pyramidal cell layer and ~30% of $eGFP^+$ neurons were immunoreactive for SST in the CA1 region pyramidal cell layer and dentate gyrus granule cell layers of preproSST vector-treated rats (Fig. 4c). In both groups, a few $eGFP^-/SST^+$ neurons were found typically outside of the CA1 and CA3 region pyramidal cell layers and in the hilus of the dentate gyrus. The majority of hilar neurons were $eGFP^+$ making the distinction between neurons expressing transgenic and natural SST more difficult and reinforcing that at least among hilar $eGFP^+/SST^+$ neurons, $eGFP^+$ neurons were immunoreactive for transgenic and/or endogenous SST. In control rats, ~30% of hilar neurons were $eGFP^+/SST^+$ and this percentage surprisingly doubled in the preproSST vector-treated rats (Fig. 4c).

SST was co-labeled in a greater percentage of $eGFP^+$ CA3 region ($t_{(4)} = 59.78$; $p < 0.0001$) and CA1 region ($t_{(4)} = 5.60$; $p < 0.01$) pyramidal cell layer neurons, granule cell layer neurons ($t_{(4)} = 4.75$; $p < 0.01$) and hilar neurons ($t_{(4)} = 4.50$; $p < 0.05$) in preproSST vector-treated rats versus control vector-treated rats (Fig. 4c). The percentage of $eGFP^-/SST^+$ neurons increased in the granule cell layer ($t_{(4)} = 8.92$; $p < 0.001$) and hilus ($t_{(4)} = 3.33$; $p < 0.05$) but not the CA3 region ($t_{(4)} = 1.23$; $p =$ n.s.) or CA1 region ($t_{(4)} = 2.37$; $p =$ n.s.) pyramidal cell layers of preproSST vector-treated rats versus control vector-treated rats (Fig. 4d). The percentage of neurons immunoreactive for transduced and/or endogenous SST increased in the CA3 region pyramidal cell layer ($t_{(4)} = 19.63$; $p < 0.0001$), CA1 region pyramidal cell layer ($t_{(4)} = 5.55$; $p < 0.01$), granule cell layer ($t_{(4)} = 8.63$; $p < 0.001$) and hilus ($t_{(4)} = 16.84$; $p < 0.0001$) of preproSST vector-treated versus control vector-treated rats (Fig. 4e).

3.6. PreproSST-treated responders and non-responders exhibited similar behavioral responses and EEG characteristics during kindling acquisition

Retrospective analysis of kindling properties revealed that the eGFP, preproSST-treated responder and preproSST-treated non-responder groups required equivalent numbers of kindling sessions to reach criterion ($F_{(2,20)} = 0.80$; $p = 0.46$; Fig. 5a). All groups also exhibited similar AD threshold currents ($H_{(2,20)} = 2.771$; $p = 0.25$; Fig. 5b), AD durations ($F_{(2,20)} = 1.57$; $p = 0.232$; Fig. 5c), latencies to Grade 4 seizure ($F_{(2,20)} = 0.07$; $p = 0.93$; Fig. 5d) and time spent in high-grade seizures ($F_{(2,20)} = 1.124$; $p = 0.35$; Fig. 5e). While preproSST-treated responders appeared to produce fewer high-grade seizures than controls or preproSST-treated non-responders, the omnibus ANOVA demonstrated that the effect was not statistically significant ($F_{(2,20)} = 2.17$; $p = 0.14$; Fig. 5f).

4. Discussion

The results of this study showed that vector-mediated ectopic preproSST expression prevented seizures in a significant fraction of kindled rats. The large therapeutic effect size encourages development of SST gene therapy for TLE. Control rats continued to exhibit high-grade evoked seizures despite displaying equivalent kindling acquisition characteristics to preproSST-treated rats. The presence or absence of an effect of the preproSST vector was consistent across repeated kindling sessions. SST was co-labeled in $eGFP^+$ neurons in the pyramidal cell layer of the CA1 and CA3 regions and in $eGFP^+$ neurons in the granule cell layer. Co-labeling was significantly elevated in these regions following treatment with the preproSST vector relative to treatment with control vector. eGFP expression patterns, behavioral responses and EEG characteristics

during kindling acquisition could not explain differences between preproSST-treated responder and non-responder groups.

The relevance of the kindling model to human TLE has been debated but there is agreement that the model approximates early clinical seizure susceptibility with limited neuropathology and has contributed significantly to our understanding of how limbic networks change in TLE and to the development of anti-epileptic drugs (Bertram, 2007; Löscher, 2002b; McNamara et al., 1980; Sato et al., 1990). Repeated kindling stimulations produce robust evoked seizures that are reliable across time and multiple tests (Brandt et al., 2004; Kalynchuk, 2000; Pinel and Rovner, 1978). We add to this paradigm by showing that rats exhibited robust evoked seizures when challenged even once after a month-long break from kindling sessions, supporting that changes underlying seizure susceptibility are relatively permanent (Goddard et al., 1969). In fact, spontaneous seizure refractoriness was observed in 1 kindled rat, consistent with previous reports (Freeman and Jarvis, 1981; Löscher and Köhling, 2010; Mucha and Pinel, 1977; Stripling and Russell, 1989). Our observation that neither the AAV5 vector nor eGFP expression affected seizure behavior supports the viability of developing gene therapy approaches for epilepsy treatment in TLE models.

The most exciting outcome of our study was that a significant fraction of preproSST-treated rats were seizure-free when challenged during the test session and even across post-test sessions. The clinical relevance of complete seizure freedom in this subset of rats was showcased by the very large effect size produced by the difference in seizure grade between responder (Grade 0) and eGFP-treated controls (~Grade 5) on the test session and even by the encouraging intermediate effect size produced by the difference in seizure grade between preproSST-treated responder and non-responder groups pooled and eGFP-treated controls. These findings expand our previous work showing that sustained preproSST expression prevented the acquisition of a fully kindled state (Zafar et al., 2012) and encourage the pre-clinical development of sustained preproSST expression as an effective non-destructive therapeutic alternative to surgery in antiepileptic drug-resistant TLE. Of course, testing the efficacy of this approach in chronic TLE models (Bertram and Cornett, 1994; Löscher, 2002b; Morimoto et al., 2004) will be important next steps for advancing the strategy to clinic.

Identifying the side effects of sustained ectopic AAV vector-mediated SST expression is also an important step in translating this strategy to clinic. Indeed, safer alternatives to antiepileptic drugs that can aggravate TLE-associated cognitive decline are desirable (Carreño et al., 2008; Kwan and Brodie, 2001). We are currently testing the effects of sustained preproSST expression on spatial cognition and neuroinflammation in kindled and control rats. Expanding this work across behavioral tasks sensitive to other cognitive domains will be an important pre-clinical step. Notably, overt effects of sustained preproSST expression on measures of general health (i.e. body mass, porphyrin production and grooming) were not observed in the current study. Although sustained SST expression could lead to the disinhibition of inhibitory interneurons (Pfeffer, 2014), changes in seizure frequency or ADs indicative of hippocampal network disinhibition were not detected. We previously confirmed that sustained AAV vector-mediated preproSST expression did not produce gross neurodegeneration using Fluoro-Jade C (unpublished data), but experiments quantifying cell stress and death markers in this context would validate whether cell viability is compromised. Our data add to the growing picture that AAV vector-mediated gene delivery and sustained neuropeptide expression is relatively safe, although future work should document how variability in injection sites, transduction efficiency, vector titers and survival times impact safety measures.

Our analysis of eGFP expression patterns indicated that preproSST expression in any single hippocampal region could mediate a strong therapeutic response. For example, eGFP expression in 1 responder rat was largely restricted to the hippocampal CA1 region (Table 2). The effect of ectopically expressed SST was likely mediated through 1 or more of the SSTR subtypes that are expressed throughout the hippocampus (Viollet et al., 2008). Although endogenous SST is largely expressed by a subpopulation of inhibitory neurons (Freund and Buzsáki, 1996), SST modulates excitability across hippocampal regions. For example, the application of SST to CA1 and CA3 regions suppresses epileptiform activity presynaptically by inhibiting glutamate release and postsynaptically by modulating voltage sensitive potassium channels (Boehm and Betz, 1997; Kozhemyakin et al., 2013; Moore et al., 1988; Qiu et al., 2008; Tallent and Siggins, 1997, 1999). In addition, SST can modulate lateral perforant path long-term potentiation (Baratta et al., 2002). Testing how the specific transduction of cell subtypes and subregions impacts hippocampal excitability and the therapeutic efficacy of sustained SST expression, as well as identifying the SSTR subtypes that mediate these effects, could provide insight about how to improve responder rates.

While the all-or-none response of preproSST vector-treated rats is puzzling, non-responder subpopulations are not atypical for antiepileptic therapies (Löscher, 2002a; Löscher et al., 1993). We did find that responders exhibited fewer high-grade seizures than eGFP-treated controls and non-responders during kindling sessions prior to treatment (Fig. 5), but this difference was insufficiently powered to achieve statistical significance. Interestingly, individuals with epilepsy who exhibit fewer seizures prior to the onset of antiepileptic drug treatment are more likely to respond to the treatment (Brodie, 2005; Kwan and Brodie, 2000; Sillanpää, 1993). Consistent with this idea, response rates were higher in our previous study that treated rats with AAV-preproSST *before* kindling sessions (Zafar et al., 2012) than in the current study that treated rats *after* they exhibited 3 consecutive Grade 5 seizures.

A number of factors could have varied between the responder and non-responder groups. It is possible that responders and non-responders exhibited overall differences in levels of mature SST or neuronostatin. Mass spectrometric analyses in progress will compare inter-rat and regional transgene peptide content differences, although qualititative comparison could not discriminate explanatory variations. Apart from potential differences in transcription, translation and derivation of transgene peptides, the expression of catabolic proteases cleaving SST or even neuronostatin in cells transduced to express preproSST (Billova et al., 2007; Brakch et al., 1995) could have varied across subregions within the hippocampus. Natural variation in SSTR expression changes related to kindling or gene transfer could also be linked to responder rates. Kindled seizures transiently upregulate SST peptide immunoreactivity, which is followed by the transient downregulation of $SSTR_2$ immunoreactivity in the rat hippocampus (Csaba et al., 2004; Tallent and Qiu, 2008; Vezzani and Hoyer, 1999). However, rats in the current study were perfused weeks after transient kindling-induced changes in SST and $SSTR_2$ would be observable. Importantly, the onset of our treatment may have been initiated after the critical period for modulating this or other transient mechanisms in some rats. Consistent with this idea, response rates were higher in our previous study that treated rats with AAV-preproSST *before* kindling sessions.

There may also be time points after which reversing more permanent changes hypothesized to underlie seizure development and maintenance becomes more difficult. For example, early SST treatment could prevent the seizure-induced neuronal loss and sprouting of SST-containing neurons in TLE models (Buckmaster and Dudek, 1997; Peng et al., 2013; Sloviter, 1987; Sun et al., 2007; Zhang et al., 2009). Kindling drastically increases neural progenitor

cell division that leads to the accumulation of young hyperplastic neurons with aberrant morphologies and connectivity that are hypothesized to contribute to seizure maintenance (Botterill et al., 2015; Parent et al., 2007). SST could prevent the accumulation of aberrant neurons through anti-proliferative effects on neural progenitor cells (Lamberts et al., 1991). The ability of SST to potentially reverse the chronic inflammatory response that accompanies TLE (Basivireddy et al., 2013; Vezzani et al., 2011) is likely less time sensitive. The anticonvulsant properties of preproSST could be byproducts of its modulatory effects on seizure-associated cell death, aberrant neurogenesis or neuroinflammation but the efficacy of SST treatment on these factors could be time sensitive.

Here we show the first preclinical evidence that sustained preproSST expression initiated *after* stable seizures are evoked in experimental TLE is anticonvulsant in a subset of rats. The opportunity to achieve a seizure-free state with superior responder rates in epilepsy gene therapy intervention also supports development and optimization of better gene delivery strategies like AAV capsid shuffling (Gray et al., 2010), the use of capsid mutated (Petrs-Silva et al., 2009; Zhong et al., 2008; Zhong et al., 2007) and self-complementary AAV vectors (McCarty et al., 2003; McCarty et al., 2001) that can possibly enhance transgene expression and provide superior transduction (Srivastava, 2016). Future studies might also test whether the use of promoters to drive expression selectively in specific cell types might improve responder rates (Nathanson et al., 2009). Nevertheless, our findings demonstrated that sustained preproSST expression treated induced seizures in kindled rats and advance a novel gene therapy intervention using this neuropeptide. Testing therapeutic efficacy in chronic TLE models that exhibit spontaneous recurrent seizures will be an important step forward for translational advancement.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We thank the Vector Core Lab, Powell Gene Therapy Center at the University of Florida for packaging the AAV5-CBa-preproSST-eGFP and AAV5-CBa-eGFP vectors. We thank Dr. David Stanley and Immanuel B.H. Samuel for help with exporting the EEG data and Dr. Francisco Delgado for technical support and insightful discussions. This research was supported by the Department of Defense Congressionally Directed Medical Research Programs (CDMRP) Grant Number PR121769, the Wilder Center of Excellence for Epilepsy Research, the Children's Miracle Network and NIH Grant #R03 AGO49411. This work was also supported by resources provided by the North Florida/South Georgia Veterans Health System, Gainesville, FL. The contents do not represent the views of the U.S Department of veterans Affairs or the United States Government.

References

Annegers, J.F., Hauser, W.A., Elveback, L.R., 1979. Remission of seizures and relapse in patients with epilepsy. Epilepsia 20 (6), 729–737.
Aourz, N., De Bundel, D., Stragier, B., Clinckers, R., Portelli, J., Michotte, Y., et al., 2011. Rat hippocampal somatostatin sst3 and sst4 receptors mediate anticonvulsive effects in vivo: indications of functional interactions with sst2 receptors. Neuropharmacology 61 (8), 1327–1333.
Bankiewicz, K.S., Eberling, J.L., Kohutnicka, M., Jagust, W., Pivirotto, P., Bringas, J., et al., 2000. Convection-enhanced delivery of AAV vector in parkinsonian monkeys; in vivo detection of gene expression and restoration of dopaminergic function using pro-drug approach. Exp. Neurol. 164 (1), 2–14.
Baraban, S.C., Tallent, M.K., 2004. Interneuron diversity series: interneuronal neuropeptides-endogenous regulators of neuronal excitability. Trends Neurosci. 27 (3), 135–142.
Baratta, M.V., Lamp, T., Tallent, M.K., 2002. Somatostatin depresses long-term potentiation and Ca2+ signaling in mouse dentate gyrus. J. Neurophysiol. 88 (6), 3078–3086.
Basivireddy, J., Somvanshi, R.K., Romero, I.A., Weksler, B.B., Couraud, P.O., Oger, J., et al., 2013. Somatostatin preserved blood brain barrier against cytokine induced alterations: possible role in multiple sclerosis. Biochem. Pharmacol. 86 (4), 497–507.
Bertram, E.H., Cornett, J.F., 1994. The evolution of a rat model of chronic spontaneous limbic seizures. Brain Res. 661 (1–2), 157–162.
Bertram, E.H., 2007. The relevance of kindling for human epilepsy. Epilepsia 48 (Suppl. 2), 65–74.
Billova, S., Galanopoulou, A.S., Seidah, N.G., Qiu, X., Kumar, U., 2007. Immunohistochemical expression and colocalization of somatostatin, carboxypeptidase-E and prohormone convertases 1 and 2 in rat brain. Neuroscience 147 (2), 403–418.
Bobo, R.H., Laske, D.W., Akbasak, A., Morrison, P.F., Dedrick, R.L., Oldfield, E.H., 1994. Convection-enhanced delivery of macromolecules in the brain. Proc. Natl. Acad. Sci. U. S. A. 91 (6), 2076–2080.
Boehm, S., Betz, H., 1997. Somatostatin inhibits excitatory transmission at rat hippocampal synapses via presynaptic receptors. J. Neurosci. 17 (11), 4066–4075.
Botterill, J.J., Brymer, K.J., Caruncho, H.J., Kalynchuk, L.E., 2015. Aberrant hippocampal neurogenesis after limbic kindling: relationship to BDNF and hippocampal-dependent memory. Epilepsy Behav. 47, 83–92.
Brakch, N., Galanopoulou, A.S., Patel, Y.C., Boileau, G., Seidah, N.G., 1995. Comparative proteolytic processing of rat prosomatostatin by the convertases PC1, PC2, furin, PACE4 and PC5 in constitutive and regulated secretory pathways. FEBS Lett. 362 (2), 143–146.
Brandt, C., Ebert, U., Löscher, W., 2004. Epilepsy induced by extended amygdala-kindling in rats: lack of clear association between development of spontaneous seizures and neuronal damage. Epilepsy Res. 62 (2–3), 135–156.
Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., et al., 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179 (4068), 77–79.
Brodie, M.J., 2005. Diagnosing and predicting refractory epilepsy. Acta Neurol. Scand. Suppl. 181, 36–39.
Buckmaster, P.S., Dudek, F.E., 1997. Neuron loss, granule cell axon reorganization, and functional changes in the dentate gyrus of epileptic kainate-treated rats. J. Comp. Neurol. 385 (3), 385–404.
Buckmaster, P.S., Otero-Corchón, V., Rubinstein, M., Low, M.J., 2002. Heightened seizure severity in somatostatin knockout mice. Epilepsy Res. 48 (1–2), 43–56.
Carreño, M., Donaire, A., Sánchez-Carpintero, R., 2008. Cognitive disorders associated with epilepsy: diagnosis and treatment. Neurologist 14 (6 Suppl. 1), S26–34.
Cascino, G.D., 2008. When drugs and surgery don't work. Epilepsia 49 (Suppl. 9), 79–84.
Cliff, N., 1993. Dominance Statistics: Ordinal Analyses to Answer Ordinal Questions., pp. 494–509.
Cramer, J.A., 1994. Quality of life for people with epilepsy. Neurol. Clin. 12 (1), 1–13.
Csaba, Z., Richichi, C., Bernard, V., Epelbaum, J., Vezzani, A., Dournaud, P., 2004. Plasticity of somatostatin and somatostatin sst2A receptors in the rat dentate gyrus during kindling epileptogenesis. Eur. J. Neurosci. 19 (9), 2531–2538.
Csaba, Z., Pirker, S., Lelouvier, B., Simon, A., Videau, C., Epelbaum, J., et al., 2005. Somatostatin receptor type 2 undergoes plastic changes in the human epileptic dentate gyrus. J. Neuropathol. Exp. Neurol. 64 (11), 956–969.
Epelbaum, J., 1986. Somatostatin in the central nervous system: physiology and pathological modifications. Prog. Neurobiol. 27 (1), 63–100.
Fisher, R., Salanova, V., Witt, T., Worth, R., Henry, T., Gross, R., et al., 2010. Electrical stimulation of the anterior nucleus of thalamus for treatment of refractory epilepsy. Epilepsia 51 (5), 899–908.
Freeman, F.G., Jarvis, M.F., 1981. The effect of interstimulation interval on the assessment and stability of kindled seizure thresholds. Brain Res. Bull. 7 (6), 629–633.
Freese, A., Kaplitt, M.G., O'Connor, W.M., Abbey, M., Langer, D., Leone, P., et al., 1997. Direct gene transfer into human epileptogenic hippocampal tissue with an adeno-associated virus vector: implications for a gene therapy approach to epilepsy. Epilepsia 38 (7), 759–766.
Freund, T.F., Buzsáki, G., 1996. Interneurons of the hippocampus. Hippocampus 6 (4), 347–470.
Galanopoulou, A.S., Seidah, N.G., Patel, Y.C., 1995. Heterologous processing of rat prosomatostatin to somatostatin-14 by PC2: requirement for secretory cell but not the secretion granule. Biochem. J 311 (Pt. 1), 111–118.
Goddard, G.V., McIntyre, D.C., Leech, C.K., 1969. A permanent change in brain function resulting from daily electrical stimulation. Exp. Neurol. 25 (3), 295–330.
Goodman, R.H., Aron, D.C., Roos, B.A., 1983. Rat pre-prosomatostatin. Structure and processing by microsomal membranes. J. Biol. Chem. 258 (9), 5570–5573.
Gray, S.J., Blake, B.L., Criswell, H.E., Nicolson, S.C., Samulski, R.J., McCown, T.J., et al., 2010. Directed evolution of a novel adeno-associated virus (AAV) vector that crosses the seizure-compromised blood-brain barrier (BBB). Mol. Ther. 18 (3), 570–578.
Hökfelt, T., 1991. Neuropeptides in perspective: the last ten years. Neuron 7 (6), 867–879.
Haberman, R.P., Samulski, R.J., McCown, T.J., 2003. Attenuation of seizures and neuronal death by adeno-associated virus vector galanin expression and secretion. Nat. Med. 9 (8), 1076–1080.

Jobst, B.C., Cascino, G.D., 2015. Resective epilepsy surgery for drug-resistant focal epilepsy: a review. JAMA 313 (3), 285–293.
Kalynchuk, L.E., 2000. Long-term amygdala kindling in rats as a model for the study of interictal emotionality in temporal lobe epilepsy. Neurosci. Biobehav. Rev. 24 (7), 691–704.
Kanter-Schlifke, I., Toft Sørensen, A., Ledri, M., Kuteeva, E., Hökfelt, T., Kokaia, M., 2007. Galanin gene transfer curtails generalized seizures in kindled rats without altering hippocampal synaptic plasticity. Neuroscience 150 (4), 984–992.
Klein, R.L., Hamby, M.E., Gong, Y., Hirko, A.C., Wang, S., Hughes, J.A., et al., 2002. Dose and promoter effects of adeno-associated viral vector for green fluorescent protein expression in the rat brain. Exp. Neurol. 176 (1), 66–74.
Kozhemyakin, M., Rajasekaran, K., Todorovic, M.S., Kowalski, S.L., Balint, C., Kapur, J., 2013. Somatostatin type-2 receptor activation inhibits glutamate release and prevents status epilepticus. Neurobiol. Dis. 54, 94–104.
Kwan, P., Brodie, M.J., 2000. Early identification of refractory epilepsy. N. Engl. J. Med. 342 (5), 314–319.
Kwan, P., Brodie, M.J., 2001. Neuropsychological effects of epilepsy and antiepileptic drugs. Lancet 357 (9251), 216–222.
Kwan, P., Brodie, M.J., 2002. Refractory epilepsy: a progressive, intractable but preventable condition? Seizure 11 (2), 77–84.
Kwan, P., Arzimanoglou, A., Berg, A.T., Brodie, M.J., Allen Hauser, W., Mathern, G., et al., 2010. Definition of drug resistant epilepsy: consensus proposal by the ad hoc task force of the ILAE commission on therapeutic strategies. Epilepsia 51 (6), 1069–1077.
Kwan, P., Schachter, S.C., Brodie, M.J., 2011. Drug-resistant epilepsy. N. Engl. J. Med. 365 (10), 919–926.
Löscher, W., Köhling, R., 2010. Functional, metabolic, and synaptic changes after seizures as potential targets for antiepileptic therapy. Epilepsy Behav. 19 (2), 105–113.
Löscher, W., Rundfeldt, C., Hönack, D., 1993. Pharmacological characterization of phenytoin-resistant amygdala-kindled rats, a new model of drug-resistant partial epilepsy. Epilepsy Res. 15 (3), 207–219.
Löscher, W., 2002a. Animal models of drug-resistant epilepsy. Novartis Found. Symp. 243, 149–159 (discussion 159-166 180-145).
Löscher, W., 2002b. Animal models of epilepsy for the development of antiepileptogenic and disease-modifying drugs. A comparison of the pharmacology of kindling and post-status epilepticus models of temporal lobe epilepsy. Epilepsy Res. 50 (1–2), 105–123.
Lamberts, S.W., Krenning, E.P., Reubi, J.C., 1991. The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocr. Rev. 12 (4), 450–482.
Lin, E.J., Richichi, C., Young, D., Baer, K., Vezzani, A., During, M.J., 2003. Recombinant AAV-mediated expression of galanin in rat hippocampus suppresses seizure development. Eur. J. Neurosci. 18 (7), 2087–2092.
Mazarati, A., Wasterlain, C.G., 2002. Anticonvulsant effects of four neuropeptides in the rat hippocampus during self-sustaining status epilepticus. Neurosci. Lett. 331 (2), 123–127.
McCarty, D.M., Monahan, P.E., Samulski, R.J., 2001. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. Gene Ther. 8 (16), 1248–1254.
McCarty, D.M., Fu, H., Monahan, P.E., Toulson, C.E., Naik, P., Samulski, R.J., 2003. Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. Gene Ther. 10 (26), 2112–2118.
McCown, T.J., 2004. The clinical potential of antiepileptic gene therapy. Expert Opin. Biol. Ther. 4 (11), 1771–1776.
McCown, T.J., 2006. Adeno-associated virus-mediated expression and constitutive secretion of galanin suppresses limbic seizure activity in vivo. Mol. Ther. 14 (1), 63–68.
McCown, T.J., 2010. The future of epilepsy treatment: focus on adeno-associated virus vector gene therapy. Drug News Perspect. 23 (5), 281–286.
McNamara, J.O., Byrne, M.C., Dasheiff, R.M., Fitz, J.G., 1980. The kindling model of epilepsy: a review. Prog. Neurobiol. 15 (2), 139–159.
Moore, S.D., Madamba, S.G., Joëls, M., Siggins, G.R., 1988. Somatostatin augments the M-current in hippocampal neurons. Science 239 (4837), 278–280.
Morimoto, K., Fahnestock, M., Racine, R.J., 2004. Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog. Neurobiol. 73 (1), 1–60.
Morrell, M.J., RNS System in Epilepsy Study Group, 2011. Responsive cortical stimulation for the treatment of medically intractable partial epilepsy. Neurology 77 (13), 1295–1304.
Mucha, R.F., Pinel, P.J., 1977. Postseizure inhibition of kindled seizures. Exp. Neurol. 54 (2), 266–282.
Nathanson, J.L., Jappelli, R., Scheeff, E.D., Manning, G., Obata, K., Brenner, S., et al., 2009. Short promoters in viral vectors drive selective expression in mammalian inhibitory neurons, but do not restrict activity to specific inhibitory cell-types. Front. Neural Circuits 3, 19.
Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108 (2), 193–199.
Noè, F., Pool, A.H., Nissinen, J., Gobbi, M., Bland, R., Rizzi, M., et al., 2008. Neuropeptide Y gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy. Brain 131 (Pt. 6), 1506–1515.
O'Connor, W.M., Davidson, B.L., Kaplitt, M.G., Abbey, M.V., During, M.J., Leone, P., et al., 1997. Adenovirus vector-mediated gene transfer into human epileptogenic brain slices: prospects for gene therapy in epilepsy. Exp. Neurol. 148 (1), 167–178.
Parent, J.M., Jessberger, S., Gage, F.H., Gong, C., 2007. Is neurogenesis reparative after status epilepticus? Epilepsia 48 (Suppl. 8), 69–71.
Paxinos, G., Watson, C., 2007. The Rat Brain In Stereotaxic Coordinates. Elsevier.
Peng, Z., Zhang, N., Wei, W., Huang, C.S., Cetina, Y., Otis, T.S., et al., 2013. A reorganized GABAergic circuit in a model of epilepsy: evidence from optogenetic labeling and stimulation of somatostatin interneurons. J. Neurosci. 33 (36), 14392–14405.
Petrs-Silva, H., Dinculescu, A., Li, Q., Min, S.H., Chiodo, V., Pang, J.J., et al., 2009. High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. Mol. Ther. 17 (3), 463–471.
Pfeffer, C.K., 2014. Inhibitory neurons: vip cells hit the brake on inhibition. Curr. Biol. 24 (1), R18–20.
Phoenix Pharmaceuticals, I., 2016. Rat Somatostatin Preprohormone Schematic, from http://www.phoenixpeptide.com.
Pinel, J.P., Rovner, L.I., 1978. Experimental epileptogenesis: kindling-induced epilepsy in rats. Exp. Neurol. 58 (2), 190–202.
Qiu, C., Zeyda, T., Johnson, B., Hochgeschwender, U., de Lecea, L., Tallent, M.K., 2008. Somatostatin receptor subtype 4 couples to the M-current to regulate seizures. J. Neurosci. 28 (14), 3567–3576.
Racine, R.J., 1972a. Modification of seizure activity by electrical stimulation. I. After-discharge threshold. Electroencephalogr. Clin. Neurophysiol. 32 (3), 269–279.
Racine, R.J., 1972b. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr. Clin. Neurophysiol. 32 (3), 281–294.
Riban, V., Fitzsimons, H.L., During, M.J., 2009. Gene therapy in epilepsy. Epilepsia 50 (1), 24–32.
Richichi, C., Lin, E.J., Stefanin, D., Colella, D., Ravizza, T., Grignaschi, G., et al., 2004. Anticonvulsant and antiepileptogenic effects mediated by adeno-associated virus vector neuropeptide Y expression in the rat hippocampus. J. Neurosci. 24 (12), 3051–3059.
Riekkinen, P.J., Pitkänen, A., 1990. Somatostatin and epilepsy. Metabolism 39 (9 Suppl. 2), 112–115.
Robbins, R.J., Brines, M.L., Kim, J.H., Adrian, T., de Lanerolle, N., Welsh, S., et al., 1991. A selective loss of somatostatin in the hippocampus of patients with temporal lobe epilepsy. Ann. Neurol. 29 (3), 325–332.
Sørensen, A.T., Nikitidou, L., Ledri, M., Lin, E.J., During, M.J., Kanter-Schlifke, I., et al., 2009. Hippocampal NPY gene transfer attenuates seizures without affecting epilepsy-induced impairment of LTP. Exp. Neurol. 215 (2), 328–333.
Samson, W.K., Zhang, J.V., Avsian-Kretchmer, O., Cui, K., Yosten, G.L., Klein, C., et al., 2008. Neuronostatin encoded by the somatostatin gene regulates neuronal, cardiovascular, and metabolic functions. J. Biol. Chem. 283 (46), 31949–31959.
Sato, M., Racine, R.J., McIntyre, D.C., 1990. Kindling: basic mechanisms and clinical validity. Electroencephalogr. Clin. Neurophysiol. 76 (5), 459–472.
Schwarzer, C., Sperk, G., Samanin, R., Rizzi, M., Gariboldi, M., Vezzani, A., 1996. Neuropeptides-immunoreactivity and their mRNA expression in kindling: functional implications for limbic epileptogenesis. Brain Res. Brain Res. Rev. 22 (1), 27–50.
Sillanpää, M., 1993. Remission of seizures and predictors of intractability in long-term follow-up. Epilepsia 34 (5), 930–936.
Simonato, M., Bregola, G., Beani, L., Vezzani, A., Sala, R., Raiteri, M., et al., 1998. Time- and region-specific variations in somatostatin release following amygdala kindling in the rat. J. Neurochem. 70 (1), 252–259.
Sloviter, R.S., 1987. Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. Science 235 (4784), 73–76.
Sperk, G., Marksteiner, J., Gruber, B., Bellmann, R., Mahata, M., Ortler, M., 1992. Functional changes in neuropeptide Y- and somatostatin-containing neurons induced by limbic seizures in the rat. Neuroscience 50 (4), 831–846.
Srivastava, A., 2016. Adeno-associated virus: the naturally occurring virus versus the recombinant vector. Hum. Gene Ther. 27 (1), 1–6.
Stripling, J.S., Russell, R.D., 1989. Twenty-four-hour post-seizure inhibition during limbic kindling requires seizure generalization. Neurosci. Lett. 99 (1–2), 208–213.
Sun, C., Mtchedlishvili, Z., Bertram, E.H., Erisir, A., Kapur, J., 2007. Selective loss of dentate hilar interneurons contributes to reduced synaptic inhibition of granule cells in an electrical stimulation-based animal model of temporal lobe epilepsy. J. Comp. Neurol. 500 (5), 876–893.
Tallent, M.K., Qiu, C., 2008. Somatostatin: an endogenous antiepileptic. Mol. Cell. Endocrinol. 286 (1–2), 96–103.
Tallent, M.K., Siggins, G.R., 1997. Somatostatin depresses excitatory but not inhibitory neurotransmission in rat CA1 hippocampus. J. Neurophysiol. 78 (6), 3008–3018.
Tallent, M.K., Siggins, G.R., 1999. Somatostatin acts in CA1 and CA3 to reduce hippocampal epileptiform activity. J. Neurophysiol. 81 (4), 1626–1635.
Tavianini, M.A., Hayes, T.E., Magazin, M.D., Minth, C.D., Dixon, J.E., 1984. Isolation, characterization, and DNA sequence of the rat somatostatin gene. J. Biol. Chem. 259 (19), 11798–11803.
Vezzani, A., Hoyer, D., 1999. Brain somatostatin: a candidate inhibitory role in seizures and epileptogenesis. Eur. J. Neurosci. 11 (11), 3767–3776.
Vezzani, A., French, J., Bartfai, T., Baram, T.Z., 2011. The role of inflammation in epilepsy. Nat. Rev. Neurol. 7 (1), 31–40.
Vezzani, A., 2004. Gene therapy in epilepsy. Epilepsy Curr. 4 (3), 87–90.
Vezzani, A., 2007. The promise of gene therapy for the treatment of epilepsy. Expert Rev. Neurother. 7 (12), 1685–1692.
Viollet, C., Lepousez, G., Loudes, C., Videau, C., Simon, A., Epelbaum, J., 2008. Somatostatinergic systems in brain: networks and functions. Mol. Cell. Endocrinol. 286 (1–2), 75–87.

Weinberg, M.S., McCown, T.J., 2013. Current prospects and challenges for epilepsy gene therapy. Exp. Neurol. 244, 27–35.
Weinberg, M.S., Samulski, R.J., McCown, T.J., 2013. Adeno-associated virus (AAV) gene therapy for neurological disease. Neuropharmacology 69, 82–88.
Winsky-Sommerer, R., Benjannet, S., Rovère, C., Barbero, P., Seidah, N.G., Epelbaum, J., et al., 2000. Regional and cellular localization of the neuroendocrine prohormone convertases PC1 and PC2 in the rat central nervous system. J. Comp. Neurol. 424 (3), 439–460.
Woldbye, D.P., Angehagen, M., Gøtzsche, C.R., Elbrønd-Bek, H., Sørensen, A.T., Christiansen, S.H., et al., 2010. Adeno-associated viral vector-induced overexpression of neuropeptide Y Y2 receptors in the hippocampus suppresses seizures. Brain 133 (9), 2778–2788.
Wong, E.T., Ngoi, S.M., Lee, C.G., 2002. Improved co-expression of multiple genes in vectors containing internal ribosome entry sites (IRESes) from human genes. Gene Ther. 9 (5), 337–344.
Zafar, R., King, M.A., Carney, P.R., 2012. Adeno associated viral vector-mediated expression of somatostatin in rat hippocampus suppresses seizure development. Neurosci. Lett. 509 (2), 87–91.
Zhang, W., Yamawaki, R., Wen, X., Uhl, J., Diaz, J., Prince, D.A., et al., 2009. Surviving hilar somatostatin interneurons enlarge, sprout axons, and form new synapses with granule cells in a mouse model of temporal lobe epilepsy. J. Neurosci. 29 (45), 14247–14256.
Zhong, L., Zhao, W., Wu, J., Li, B., Zolotukhin, S., Govindasamy, L., et al., 2007. A dual role of EGFR protein tyrosine kinase signaling in ubiquitination of AAV2 capsids and viral second-strand DNA synthesis. Mol. Ther. 15 (7), 1323–1330.
Zhong, L., Li, B., Mah, C.S., Govindasamy, L., Agbandje-McKenna, M., Cooper, M., et al., 2008. Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. Proc. Natl. Acad. Sci. U. S. A. 105 (22), 7827–7832.

Racine Grade 5 seizures. Subsequently, AAV serotype 5 vector driving eGFP (AAV5-CBa-eGFP) or rat preproSST and eGFP (AAV5-CBa-preproSST-eGFP) gene expression was injected bilaterally into the hippocampus dentate gyrus and CA1 region. Three weeks later, rats were re-tested at periodic intervals using previously effective seizure-evoking current intensities. Kindled rats treated with AAV5-CBa-eGFP vector continued to consistently exhibit Grade 5 seizures upon repeated retesting whereas a significant fraction of AAV5-CBa-preproSST-eGFP-treated rats were persistently seizure free even with prolonged stimulus repetition. Repetitive seizures and excessive drug burden lead to severe cognitive impairment in individuals with chronic, intractable TLE. We first tested the effects of kindling epileptogenesis on spatial learning, memory and cognitive flexibility using the Morris water maze swim task. We demonstrated that kindling epileptogenesis lead to a significant initial learning impairment on reversal trials relative to sham-stimulated rats. Sustained vector-mediated expression of preproSST in the hippocampus of kindled rats did not worsen this cognitive deficit relative to kindled rats treated with eGFP vector. Together, these preclinical results suggest that SST maybe an effective and safe therapeutic candidate for TLE. Our findings encourage the translational advancement of this non-destructive therapeutic intervention that could be administered prior to last-resort neurosurgical resection in individuals where pharmacotherapy has failed to achieve seizure control.

SPECIFIC AIMS

Aim 1: Determine whether intrahippocampal preproSST gene transfer has putative antiepileptic properties *in vivo* in a rat model of TLE – ***therapeutic efficacy***.
Aim 2: Determine the effects of intrahippocampal preproSST gene transfer *in vivo* on hippocampus dependent learning and memory – ***safety and cognitive sparing properties.***

EXPERIMENT DESIGN

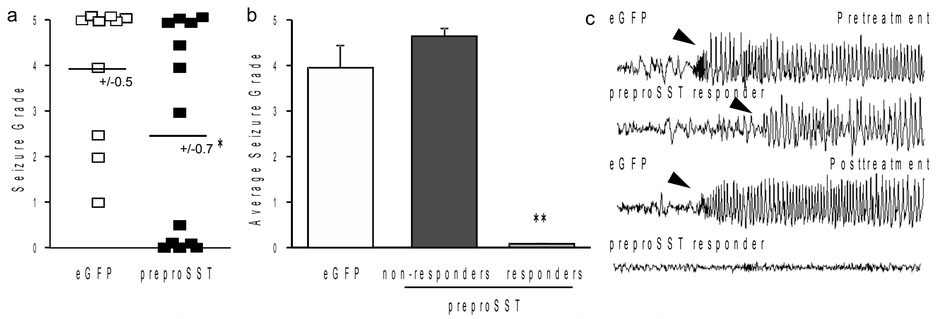
Aim 1

Aim 2

1. ***2.***

RESULTS

Sustained preproSST expression produced seizure resistance in a subset of rats



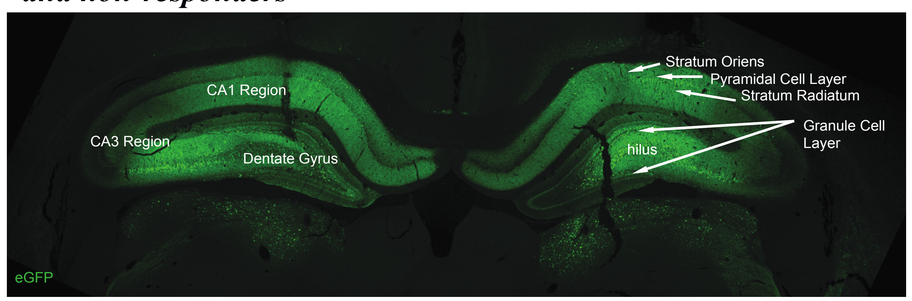
Sustained preproSST expression prevented induced seizures in a subset of kindled rats a) The average seizure grade produced by the preproSST-treated rats (n=13) was significantly lower than the average seizure grade produced by eGFP-treated rats [(n=10), *p<0.05, one-tailed]. **b)** The average seizure grade was significantly lower in the preproSST-treated responder (**p<0.01) but not in the preproSST-treated non-responder (p=0.73) group relative to the eGFP-treated control group. **c)** Severe after-discharges (ADs) characteristic of Grade 5 seizures were recorded from the amygdala, of a representative rat in the AAV5-CBa-eGFP-treated group and also an eventual preproSST-treated responder rat from the AAV5-CBa-preproSST-eGFP-treated group. Three weeks after vector expression, during the test session, ADs were absent in the preproSST-treated responder rat relative to the eGFP-treated rat that produced severe ADs. Arrows indicate the onset of ADs..

Vector effects were stable and persistent

	PTS-1	PTS-2	PTS-3	PTS-4	PTS-5	PTS-6 (+50 µA)	PTS-7 (+50 µA)	PTS-8 (+100 µA)
Rat1	0	0	0	0	0	0	0	0
Rat2	0	0	0	0	0	0	4	4
Rat3	0	0	5	0	0	5	ψ0	2
Rat4	0	0	0	5	5	0	2	5
Rat5	0	0	0	0	0	0	0	0
Rat6	5	5	5	5	5	0	0	0

Seizure grade exhibited by preproSST-treated responder rats on post-test sessions (PTS). Each PTS was spaced 48 hours apart. The last 3 PTS were administered at higher AD threshold currents.

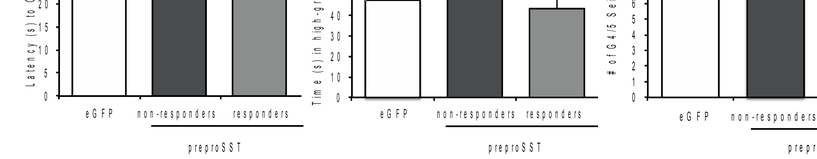
eGFP expression was similar in preproSST-treated responders and non-responders



AAV5-CBa-preproSST-eGFP transduction within the hippocampus of a representative rat in the preproSST vector-treated group Robust eGFP expression was observed bilaterally within the dentate hilus, granule cell layer, CA3 region and CA1 region of the hippocampus. In this rat a relatively small number of thalamic neurons ventral to the hippocampus also showed eGFP expression.

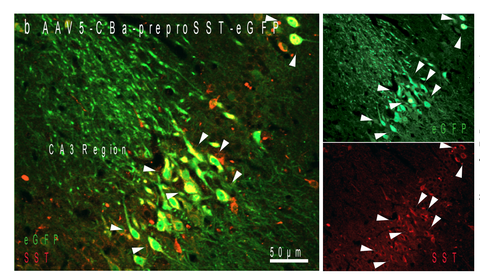
Rat #	CA1		CA3		GCL		Hilus		Subiculum		Cortex	Thalamus
	Side1	Side2	Side1	Side2	Side1	Side2	Side1	Side2	Side1	Side2		
SST Non-Responders												
Rat 1	++	++	++	-	-	-	-	-	++	-	N	N
Rat 2	++	++	++	+++	-	+	-	+++	+	+	Y	N
Rat 3	++	++	++	++	-	+	-	++	-	-	Y	Y
Rat 4	++	+	+++	+++	++	++	+++	+++	+	-	Y	Y
Rat 5	++	++	+++	++	+++	+++	+++	+++	+	+	Y	N
Rat 6	++	++	+++	+	+	+	+++	+++	+	-	Y	N
SST Responders												
Rat 1	++	++	+++	++	+	-	+++	-	-	+	Y	N
Rat 2	++	+++	++	++	-	++	++	+++	+	-	Y	Y
Rat 3	+++	+++	-	-	-	-	-	-	++	++	Y	N
Rat 4	++	++	+++	++	++	+	+++	+++	++	-	Y	N
Rat 5	++	++	++	+	+	+	+++	++	+	+	N	N
Rat 6	+++	+++	++	++	-	+++	-	-	++	+	Y	N

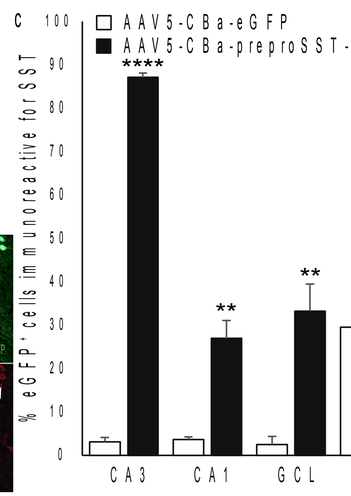
eGFP expression in hippocampal and extrahippocampal regions The presence or absence of eGFP expression is denoted by '+' and '-' symbols respectively. The number of '+' symbols represents the qualitative robustness of eGFP expression in each region, where '+' represents a few scattered eGFP$^+$ cells, '++' denotes a moderate number of eGFP$^+$ cells and "+++" denotes that most cells were eGFP$^+$. Scattered cells were detected in the cortices and thalamic region of some rats. 'N' denotes no eGFP$^+$ cells detected and 'Y' denotes eGFP$^+$ cells detected.



PreproSST-treated responders and non-responders exhibited similar behavioral responses and EEG characterist epileptogenesis a) the number of sessions to criterion **b)** the AD threshold current **c)** AD duration **d)** latency to Grade 4 **e)** time spent in high-grade seizure were similar between preproSST-treated responders, non-responders and eGFP-treate Although preproSST-treated responders specifically appeared to produce fewer high-grade (Grade 4/5) seizu epileptogenesis relative to preproSST-treated non-responders, this was not overall statistically significant **(f)**.

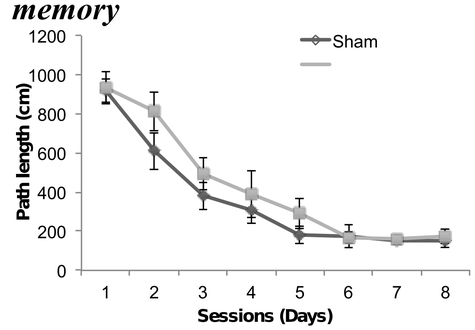
Co-labeling for eGFP and SST was consistent with vector transduction in hippocampal principal neurons





Coincident eGFP and SST localization was consistent with vector transduction of hippocampal principal neurc Examples of native eGFP$^+$ neurons (in green), eGFP$^+$/SST$^+$ (both green and red) and SST$^+$ (in red) neurons found in hippocampal region of rats treated with the control **(a)** or preproSST **(b)** vector. **c)** The percentage of eGFP$^+$/SST$^+$ neurons in the CA3 region (****p<0.0001) and CA1 region (**p<0.01) pyramidal cell layers, granule cell layer (GCL, **p<0.01) (*p<0.05) of preproSST vector-treated versus control vector-treated rats.

Kindling epileptogenesis did not impair spatial learning and memory



Performance of kindled rats (n=9) relative to sham-stimulated rats (n=9) in the Morris water maze swim task for a learning and b) spatial memory/retention a) kindled rats and sham-stimulated rats travelled equal distances (kindle sham; $F_{(1,16)}$=1.73; p=0.207) across training sessions (days 1-8; $F_{(7,112)}$=42.02; p=0.000 and interaction effect of seizures× sessions: $F_{(7,112)}$=0.67; p=0.696) to locate the hidden platform **b)** Percent duration in each quadrant did not differ sign between sham and kindled rats during the 48-hour memory probe trial.

Kindling epileptogenesis lead to a significant initial learning impairment on reversal trials

Performance of kindled rats (n=9) relative to sham stimulated rats (n=9) in the Morris water maze swim task learning a) kindled rats travelled longer distances to locate the hidden platform relative to sham-stimulated rats (kindled $F_{(1,16)}$=13.63; p=0.002) across training sessions (days 1-6; $F_{(5,80)}$=29.64; p=0.000 and interaction effect: seizures×traini $F_{(5,80)}$=4.08; p=0.002). Specifically, kindled rats travelled significantly longer distances relative to sham-stimulated rats o day 2. * Represents p<0.05 and ***represents p<0.0005 **b)** There were no significant effects of kindling epileptogenesis (ki sham; $F_{(1,16)}$=0.002; p=0.964) across training sessions (days 1-6; $F_{(5,80)}$=5.607; p=0.000 and interaction effect: seizures×train $F_{(5,80)}$=1.244; p=0.297) on swim speeds during hidden water maze reversal trials.

0.05) but not BrdU⁺/GFAP⁺/Sox2⁺ Type 2 NPCs. In these rats, IBA⁺ microglia number was similar between groups but a greater proportion of IBA⁺/CD11b⁺ microglia ($p < 0.001$) and number of activated ($p < 0.001$) and highly activated ($p < 0.05$) microglia was detected in kindled rats. To test whether SST expression blocked seizure behavior and normalized kindling-induced aberrant neurogenesis, male Sprague Dawley rats were kindled to 3 consecutive Grade 5 seizures and were then injected bilaterally into the CA1 region and dentate gyrus (2µl/site) with pAAV-CBa-GFP control vector (n=5) or pAAV-GFP-SST vector (n=5) to drive SST expression. Three weeks later, rats were given 2-3 test stimulations/week for three weeks, injected with BrdU (100 mg/kg, i.p.) 48h after the final stimulation and perfused 4h later. The average seizure grade was significantly reduced in pAAV-GFP-SST versus pAAV-CBa-GFP rats ($p < 0.05$). Our preliminary data show that pAAV-GFP-SST-treated rats had significantly fewer dividing BrdU⁺ NPCs than GFP-treated rats ($p < 0.01$) and tended to have fewer Type 1 NPCs ($p = 0.06$) and we are currently quantifying markers of neuroinflammation in these rats. These data support the hypotheses that 1) kindling upregulates Type-1 NPC division, 2) stimulates a neuroinflammatory response and 3) that SST expression may alleviate seizure behavior in epileptic rats by normalizing neurogenesis.

Background

Epilepsy is a neurological disorder which affects millions worldwide. It can be characterized by recurrent seizures that can produce brain damage and death [1]. Temporal lobe epilepsy (TLE) is the most common type of focal epilepsy [1]. A model of TLE, amygdala kindling, can be used to identify the mechanisms underlying the development and maintenance of seizure behavior. Briefly, repeated sub-threshold electrical stimulation delivered to the amygdala results in progressively worsening seizure behavior, on the Racine scale [2,3] from 1 (mouth and facial movement) to 5 (rearing and falling with bilateral forelimb clonus).

We have previously shown that somatostatin (SST) gene delivery to the hippocampus by AAV vector prevents seizure development in 70% of rats subjected to amygdala kindling [5] and may prevent the development of kindling-induced seizure behavior and water maze memory probe trial deficits [unpublished data]. These results suggest that overexpression of certain peptides through gene delivery by safe vectors may hold promise as an alternative treatment strategy for seizure disorders that are refractory to drugs and not amenable for surgical resection.

Interestingly, the hippocampal dentate gyrus produces thousands of new neurons each day [6] and limbic kindling increases progenitor cell proliferation and new neuron production 2- to 8-fold, often in ectopic locations [7, 8, 9]. New neuron numbers correlate with scores in behavioral tasks that depend upon hippocampal integrity [11, 12, 13] but whether the aberrantly high production of new neurons contributes to epileptogenesis and impaired cognition in the kindled brain is unknown. Additionally, it is unclear which sub-population of dividing progenitor cells contributes to the drastic increase in proliferation seen after a stimulation.

Hippocampal neural progenitor cells behavior is affected by neuroinflammatory environment [14,15,16,17,18] and kindling can produce both acute and chronic neuroinflammatory responses. SST has been shown to be immunosuppressive in a variety of contexts [4].

Here, we test whether SST gene delivery reverses seizure behavior through normalization of neurogenesis and neuroinflammatory signaling in a rat amygdala kindling model of epilepsy.

ENGINEERS *for* LIFE.

injected with BrdU (100mg/kg, i.p.) 48h after their last stimulation and perfused with 4% paraformaldehyde 4h later.

Enzyme Substrate immunostaining. Sections were washed in tris-buffered saline (TBS; pH 7.4) between steps. Endogenous peroxidase was quenched in 0.3% H_2O_2 for 10m, DNA was denatured with 2N HCl for 20m at 37°C followed by blocking in 3% normal donkey serum (in TBS with 0.1% triton-x) before an overnight incubation in rat anti-BrdU (1:500; AbD Serotec) at 4°C. The following day, sections were incubated in biotinylated anti-rat IgG (Jackson ImmunoResearch, 712-065-150, 1:500) for 4h at RT and then complexed within avidin-biotin horseradish peroxidase (PK6100; Vector Laboratories) before being reacted with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB; D9015; Sigma) and 0.5% H_2O_2. Sections were mounted on glass microscope slides and dried overnight before being dehydrated using an alcohol series and cover-slipped under permount.

Fluorescent immunostaining. Sections were washed in TBS between steps. Sections were blocked in 3% normal donkey serum (in TBS with 0.1% triton-x) before being incubated overnight in either neuronal progenitor cell (goat anti-SOX2, 1:500, Santa Cruz Biotechnology; chicken anti-GFAP, 1:1000, Encor Biotechnology), glial/inflammatory (rabbit anti-Iba1, 1:1000, Wako Chemicals; mouse anti-CD11b, 1:500, Millipore; goat anti-CD68, 1:500, Santa Cruz Biotechnology), or somatostatin (rabbit anti-somatostatin, 1:5000, Peninsula Laboratories) primary antibodies at 4°C. The next day, sections incubated in the appropriate cross-adsorbed secondary antibodies (from Jackson; 1:500) for 4h at RT. The NPC-stain sections were then paraformaldehyde-fixed, rinsed twice in 0.9% NaCl, and then incubated in 2N HCl at 37°C to denature DNA before an overnight incubation in rat anti-BrdU (1:500; AbD Serotec) at 4°C. The next day, sections were incubated in Cy3 anti-rat secondary (1:500; Jackson ImmunoResearch) for 4h at RT, stained with DAPI (Calbiochem; 1:10,000) and then mounted under diazobicyclooctane (DABCO).

New cell number and phenotypes. Microglial and neuronal progenitor cell were obtained on every 12th section using a Zeiss Axio Observer Z1 inverted microscope with 40X objective and StereioInvestigator software. The neuronal progenitor/stem cell phenotypes of > 50 BrdU⁺, the microglial phenotype of >100 Iba1⁺, and the expression of SST of >100 GFP⁺ cells per rat were confirmed by examining the entire diameter of DAPI/BrdU⁺, DAPI/Iba1⁺, and DAPI/GFP⁺ cells using a Zeiss LSM 710 confocal microscope (405, 488, 543, and 633 laser lines).

Statistical analyses. Data were analyzed using STATISTICA with α-levels set at $p = 0.05$. Group differences were compared with Student's T-tests or Kruskal Wallace ANOVAs.

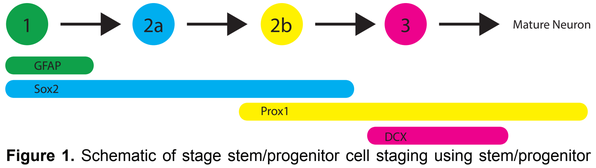


Figure 1. Schematic of stage stem/progenitor cell staging using stem/progenitor cell neuronal protein markers.

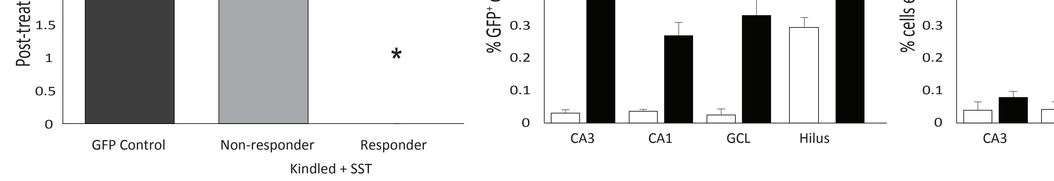


Figure 2 (A) Cresyl violet stained atlas figure [19] showing the bilateral placement of electrodes in the amygdala as well as AAV-Cba-GFP and AAV-Cba-SST-GFP vecto revealed that SST-responders exhibited significantly lower seizure grades than GFP control rats and SST non-responders (p values < 0.05). (C,D) Representative conf treated with control GFP (C) or preproSST (D) vector. (E) The percentage of GFP⁺ cells colabeled with SST was increased significantly in the CA3 ($p < 0.0001$), CA1 (p control vector treated rats. (F) The percentage of SST⁺ only cells was increased in the GCL ($p < 0.001$) and hilus ($p < 0.05$) but not CA3 or CA1 subregions of the hippc in the CA3 ($p < 0.0001$), CA1 ($p < 0.01$), GCL ($p < 0.001$), and hilus ($p < 0.0001$) subregions of preproSST versus control vector treated rats.

Somatostatin gene delivery reverses kindling-increased division of Type-1 progen

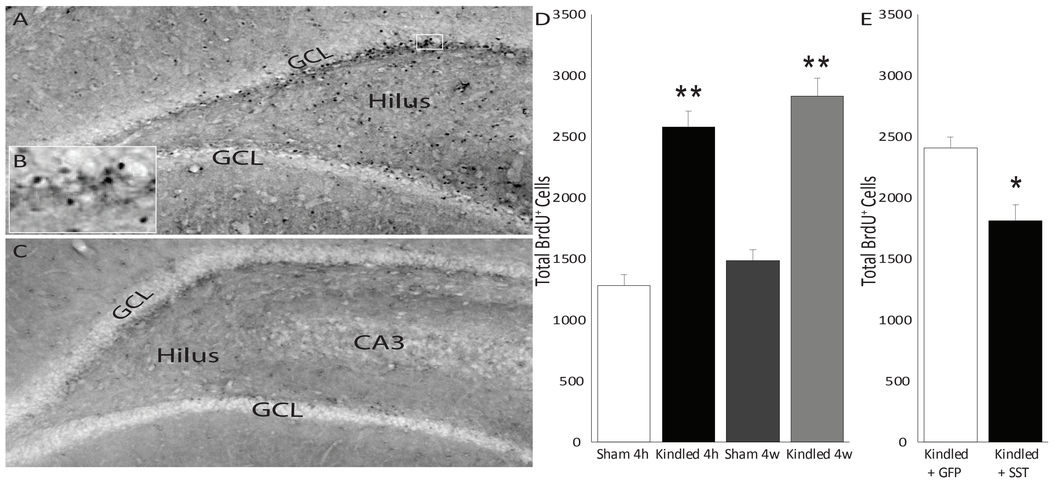


Figure 3. Kindled rats had higher levels of Type-1 progenitor cell division and SST gene delivery reversed this affect. (A-C) DAB stained BrdU⁺ cells are shown for kindl liferation is increased in kindled rats ($p < 0.001$) versus control and an increase in suriviving BrdU⁺ cells at 4w post-injection ($p < 0.001$). (E) There is a decrease in pro was used to reveal the neuronal progenitor cell phenotypes of fluorescent immune-labeled BrdU⁺ cells (in red) in the dentate grus of the hippocampus . Type-1 Neur beled cells in kindled rats had a type-1 NPC expression of GFAP and SOX2 ($p < 0.05$). The total number of new neurons in the granule cell layer was calculated as (#E rats receiving preproSST versus control vector.

Kindling increases the number of activated microglia in the dentate gyrus of adult

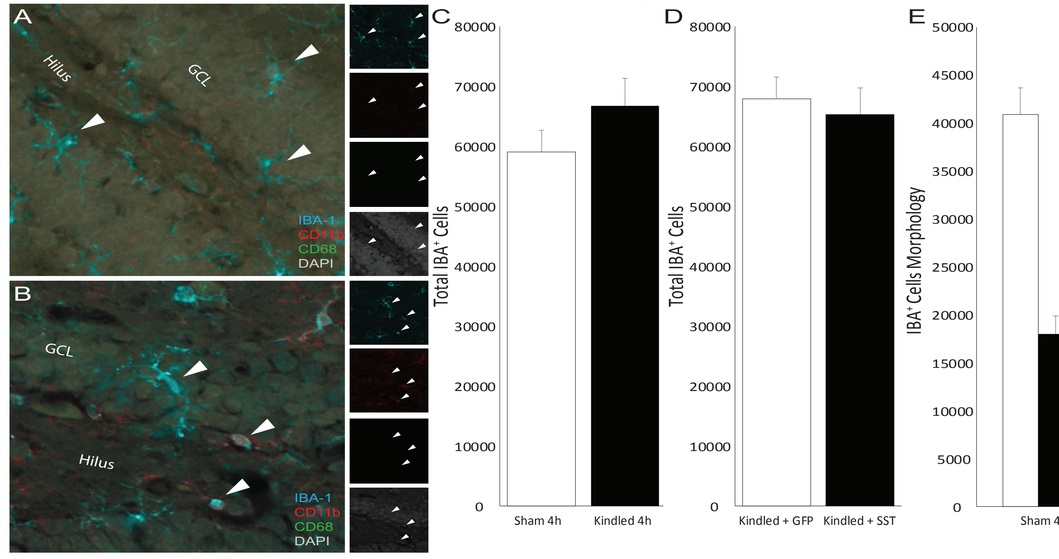


Figure 4. There is an inflammatory response to kindling. (A) Representative confocal images of IBA-1⁺/CD11b⁺ activated and amoeboid (B) and IBA-1⁺/CD11B⁻ restir the dentate gyrus of adult rats even when accompanied with vector mediated SST or GFP gene delivery. (E) A morphological analysis revealed significantly more acti microglia ($p < .001$) in the dentate gyri of kindled rats versus sham-kindled rats. (F) No difference in microglia numbers of any type between preproSST and control