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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

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13. SUPPLEMENTARY NOTES relationship. In all 3 of these experiments kindling stimulated microgliosis that showed at least partial recovery with longer survival, but somatostatin gene transfer had no effect on this. The conclusions supported by data from these 3 experiments are that dentate cytogenesis and microglial activation are stimulated in kindled rats, that a vector gene dose threshold may exist, and that somatostatin gene transfer may have a restorative effect on cytogenesis but this is not an absolute requirement for therapeutic efficacy. Separate groups of animals were used in a third study to evaluate whether kindling impaired hippocampal-dependent learning and memory, and whether the somatostatin gene transfer might show any behavioral toxicity in the form of impaired performance on hippocampal learning and memory tasks, or instead might ameliorate impairment associated with experiencing seizures. Consistent with a model of an early stage of epileptogenesis, kindled rats showed mild but detectable impairment. Somatostatin gene transfer had no adverse effects on the behavioral tasks and some improvement was observed relative to kindled rats that received a control vector. The results to date support further preclinical development of therapeutic strategies designed to supplant brain somatostatin (and/or neuronostatin) by AAV-based vectors or alternative methods.					
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14. ABSTRACT

Three studies have been completed. In the first, intracranial gene transfer of a somatostatin gene believed to produce localized constitutive expression of somatostatin and neuronostatin peptides in the rat hippocampus was found capable of full reversal of a state of pathological susceptibility to intense seizures. The result extends previous work to establish that full efficacy remains possible even when the gene transfer is conducted after susceptibility to intense seizures has developed. For reasons that remain unclear the effect appears to be all or none. A second study was undertaken to evaluate whether therapeutic efficacy of somatostatin gene delivery might derive from action on either endogenous cell proliferation in the dentate gyrus or seizure-associated inflammation reflected by activation of microglia. An initial experiment replicated previous demonstration that kindling stimulates dentate cytogenesis. A second experiment examined whether somatostatin gene delivery would alter kindling-associated cytogenesis and subsequent differentiation/survival in conjunction with seizure susceptibility. Unfortunately this experiment was confounded by only 1 of 4 somatostatin vector animals showing a convincing therapeutic response and 1 of the 4 control vector animals having spontaneous recovery from the kindled state. The limited therapeutic response may have been related to the potency of the somatostatin vector preparation generated for this experiment. A third experiment used higher and lower titer vectors and tested effects on dentate cytogenesis long after gene delivery and interposed recurrent challenge seizures. No (0/7) low-gene dose animals showed a therapeutic seizure reduction, but 4 of 6 high-gene dose rats did. The low numbers of responders, the occurrence of full seizure reduction in the apparent absence of normalized cytogenesis, and abnormal cytogenesis in the absence of increased seizure susceptibility make it premature to infer a causal relationship. In all 3 of these experiments kindling stimulated microgliosis that showed at least partial recovery with longer survival, but somatostatin gene transfer had no effect on this. The conclusions supported by data from these 3 experiments are that dentate cytogenesis and microglial activation are stimulated in kindled rats, that a vector gene dose threshold may exist, and that somatostatin gene transfer may have a restorative effect on cytogenesis but this is not an absolute requirement for therapeutic efficacy. Separate groups of animals were used in a third study to evaluate whether kindling impaired hippocampal-dependent learning and memory, and whether the somatostatin gene transfer might show any behavioral toxicity in the form of impaired performance on hippocampal learning and memory tasks, or instead might ameliorate impairment associated with experiencing seizures. Consistent with a model of an early stage of epileptogenesis, kindled rats showed mild but detectable impairment. Somatostatin gene transfer had no adverse effects on the behavioral tasks and some improvement was observed relative to kindled rats that received a control vector. The results to date support further preclinical development of therapeutic strategies designed to supplant brain somatostatin (and/or neuronostatin) by AAV-based vectors or alternative methods.

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 and regulation of cellular proliferation, along with the existence of receptors on neuronal and non-neuronal cells in brain, implicate brain SST in CNS (central nervous system) inflammation and proliferation. 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 SST are especially vulnerable to seizure-related loss in human patients, and loss of SST may contribute to development, maintenance, and pathology of epilepsy. Our initial tests demonstrated that intracranial SST gene delivery prevented the evolution to high-level seizures in 70% of rats in the kindling seizure 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 efficacy mechanisms, safety, and application parameters must be established prior to informed clinical development. Initial results led us to predict that SST gene transfer would not only abrogate development of seizure susceptibility, but could also counteract the effects of epileptic development even when initiated after recurrent seizures established a distinct seizure-prone (kindled) state. One potential mechanism based on known SST 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 SST 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 progressive hyperexcitability that develops in conjunction with lowered seizure thresholds is a therapeutic disease modification target distinct from the seizures they allow. The seizure-prone state resulting from this kindling is measured by behavioral and electrographic severity appraisals, the amount of damage displayed by cells in the brain post-mortem, assays for the proliferation and integration of new brain cells, and biochemical analytes reflecting the status of inflammatory processes. 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 would likely be due to 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 and synaptic networks is likely to have already occurred when these emerge, and because seizures tend to become more severe, frequent, and resistant to pharmacotherapy as recurrent seizures accumulate. 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 disease pathology or therapeutic resection, and the associated functional impairments.

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

3. ACCOMPLISHMENTS

What were the major goals of the project?

The central hypothesis of this project is that SST gene delivery will oppose adverse effects of kindling on hippocampal cell proliferation and inflammation. **In service of the need to establish preclinical efficacy and safety data, 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, or with pathological effects on brain structure or cognition.** SST gene transfer was predicted to alter hippocampal neurogenesis in relation to behavioral or brain pathology during development of seizures in a rat kindling model of epileptogenesis. Experiments addressing this aim comprised SOW Tasks 1-4 (revised version July 2016). **The second aim was to test the hypothesis that SST gene transfer would alter inflammation pathology and biochemical signaling cascades over the time scale in which epileptogenesis occurs.** Experiments addressing this aim comprised SOW Tasks 1, 2, 5, & 6.

The aims were addressed 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 primary objectives of the project were intended to explore whether the treatment could be effective in epileptic patients to mitigate against seizures and associated pathologies, without adversely affecting brain structure or behavioral function.

SOW milestones (original SOW):

Task 1. ORP approval process (month 1)(100% completed): UF IACUC approved 3/18/2013; ACURO submitted; UF IACUC 3rd year renewal approved 3/2016

Task 2. Preparation and validation of gene transfer vectors (months 1-2)(100% completed): Original vectors were prepared by University of Florida Vector Core Facility and validated by DNA sequencing and expression testing in vivo prior to the award. New vector preparations were obtained 4/2016.

Task 3. Experiment 1: SST vector effects on dentate gyrus cell proliferation and integration after initial seizure (months 3-20)(revised): The 2016 revised SOW substituted an expanded Task 4 to include sham-kindled and kindled groups receiving no vector, for the purpose of establishing kindling effects alone. It was agreed by the PIs that control groups for potential effects of kindling alone on hippocampal cytogenesis would be more important for testing therapeutic efficacy than groups administered vectors after initial seizure events.

Task 4. Experiment 2: SST vector effects on dentate gyrus cell proliferation and integration after repeated seizures (months 18-35)(90% completed): All the experimental groups for this revised task have been filled. Because water maze behavioral testing may alter hippocampal neurogenesis, behavioral testing was restricted to groups committed to biochemical analyses. To evaluate proliferation long after initial kindling or vector administration, some groups received EdU 4 hours prior to euthanasia in addition to BrdU near the initial attainment of a kindled state. Histological labeling of proliferating cells, surviving cells, and their phenotypes, and quantification of each, were completed. Qualitative assessment of gliosis, neuronal loss, and oxidative stress were conducted in a subset of histological material available after microgliosis and proliferation/survival samples were used. Statistical analyses and hypothesis testing of kindling and vector effects have been completed, and a manuscript has been submitted for publication of results.

Task 5: Experiment 3: SST vector effects on dentate gyrus inflammation after initial seizure (months 3-20)(revised): The 2016 revised SOW substituted an expanded Task 6 that included sham-kindled and kindled groups receiving no vector, to establish kindling effects alone. It was agreed by the PIs that control groups for potential effects of kindling alone on hippocampal cytogenesis would be more important for testing therapeutic efficacy than groups administered vectors after initial seizure events.

Task 6: Experiment 4: SST vector effects on dentate gyrus inflammation after repeated seizures (months 18-35)(75% completed): As with the Task 3 revision it was determined that establishing kindling effects alone would be more informative than testing the effects of the initial seizure, so treatment groups were substituted in the revised SOW to that effect. All the sham, kindled, and vector experimental groups for this task have been filled. Because water maze behavioral testing may alter hippocampal neurogenesis, behavioral testing originally planned for all groups was restricted to groups committed to biochemical analyses. Histological labeling of microglial cells, their activation phenotypes, and quantification of each, were completed. Statistical analyses and hypothesis testing of kindling and vector effects have been completed, and a manuscript is being finalized for publication of results. Brains for biochemical analyses have been prepared from a separate cohort used for behavioral testing but not histological evaluation, and are stored in anticipation of completing the supported BioPlex evaluations.

Task 7: Safety and efficacy with advanced vectors (50% completed): Drs. Carney, King, and Natarajan have collaborated with UF vector design experts to test several novel AAV vector constructs that might improve the dosing, distribution, or expression characteristics of SST gene transfer. We have not tested new vectors in kindled rats, and because the results to date do not indicate markedly superior performance of the new vectors we probably will not pursue this further in the absence of funding.

SOW 2/2017 revision: A modified SOW was approved with a no-funds extension, based on project status and new objectives for year 4. Although the major objectives were not altered there were some revisions of experimental design that differ from the prior version. Progress is described below in relation to the most recent approved SOW, although most of the work is identical to that described above.

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

UF IACUC approved 3/18/2013; ACURO submitted; UF IACUC 3rd year renewal approved 3/2016

Subtask 2: Kindling and testing kindling effects on cytogenesis

[10 rats per group x 2 groups (sham-kindled, kindled, 4 hours survival) = 20 rats total]

Evaluation of cellular proliferation was conducted upon identifying 2 groups (sham-kindled or kindled) of 5 rats meeting kindling and electrode placement inclusion criteria. These animals correspond to Experiment 1, short survival (Figure 1).

Evaluation of survival of cells generated after kindling was conducted upon identifying 2 additional groups of 5 (sham-kindled or kindled) that met inclusion criteria and survived 4 weeks after BrdU administration to mark dividing cells. These animals correspond to Experiment 1, long survival (Figure 1).

Subtask 3: Kindling and testing kindling effects on water maze performance

[10 rats per group x 2 groups (sham-kindled, kindled, 4 week survival) = 20 rats total]

Because behavioral testing can itself alter cytogenesis, animals used for water maze performance evaluation were not used for evaluating cell proliferation or survival. Separate (from vector experiment) groups of rats meeting kindling and/or electrode placement inclusion criteria were thus generated for behavioral testing (sham-kindled N=9, kindled N=11). These animals correspond to Experiment 1, long survival (Figure 1) but did not receive BrdU injections.

Subtask 4: Test kindling effects on cytogenesis and histopathology (histological analyses)

[4 groups, 40 rats total from subtasks 2 & 3]

Immunolabeling, quantification, and statistical analysis of data have been completed for the animals included in Major Task 1.

Major Task 2: Test therapeutic efficacy of SST gene delivery against kindled seizures and related cognitive deficits in rats

Subtask 1: Procure and validate GFP and SST gene delivery vectors

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. One SST vector preparation was outsourced to compare with reagent prepared in house with respect to efficacy, economy, and safety.

Subtask 2: Test GFP and SST vector effects on acute cytogenesis (4 hours survival)

[10 rats per group x 2 groups = 20 rats total]

Experiment 2 (Figure 1) rats were injected with BrdU after kindling, to mark cells proliferating shortly after kindling that might be present after an additional 4 week survival interval. They then received intracranial delivery of either SST or GFP control vector 3 weeks prior to recurrent challenge seizure stimulation. The same rats received EdU 4 hours prior to euthanasia to mark acute proliferation. Groups of 4 were identified meeting electrode placement and vector injection inclusion criteria.

Subtask 3: Test GFP and SST vector effects on induced seizures (4 week survival)

[10 rats per group x 2 groups = 20 rats total]

Vector effects on seizures were evaluated in the same animals used for Subtask 2. Recurrent challenge stimulation was introduced to evaluate possible buildup or decay of effects, examine temporal variability in seizure susceptibility or vector efficacy, and test internal validity of the initial test as a reliable index of seizure susceptibility for resolving therapeutic efficacy.

In addition, the animals generated for Experiment 3 (Figure 1) were also evaluated for effects on seizure susceptibility, and included 2 SST vector doses (low, N=7; high, N=6; GFP, N=4).

Subtask 4: Test GFP and SST vector effects on seizure-related behavioral deficits

[same animals as subtask 3]

Because behavioral testing can itself alter cytogenesis, animals used for testing cognitive effects were not used for cytogenesis evaluation. The animals described for Major Task 3 were used to satisfy this objective. After applying inclusion criteria 9/12 GFP and 8/15 SST vector animals were used in analyses.

Milestone(s) Achieved: Establish effects of kindling on behavior, determine effects of SST on seizures and associated behavioral deficits; publication of 1-2 peer reviewed papers

A manuscript describing vector effects on seizure susceptibility has been published (Natarajan et al. '2017). A manuscript describing gene transfer effects on cytogenesis and survival has been submitted (Liebowitz et al. '18, Appendix). A manuscript describing vector effects on water maze performance is in preparation.

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 hours survival) and seizure-related pathology

[same animals as Major Task 2, Subtask 2]

Experiment 2 (Figure 1) rats were injected with BrdU after kindling to mark cells proliferating shortly after kindling and surviving an additional 4 weeks. They then received intracranial delivery of either SST or GFP control vector 3 weeks prior to recurrent challenge seizure stimulation. Groups of 4 were identified meeting electrode placement and vector injection inclusion criteria. The same rats received EdU 4 hours prior to euthanasia to mark acute proliferation.

Microglial activation was evaluated quantitatively in all these brains. Qualitative histopathology was evaluated on a subset of tissue available after cytogenesis and microgliosis analyses.

Experiment 3 kindled rats were injected with BrdU 6 weeks after gene transfer and euthanized 4 hours later for evaluation of acute proliferation at similar to delay as experiment 2 but using the same thymidine analog as used to mark early proliferation in experiments 1 & 2, and omitting the early injection (Figure 1).

Subtask 2: Histology and cell counts to assess SST effects on survival and phenotypic maturation (4 week survival), and seizure-related pathology
[same animals as subtask 1, experiment 2]

Milestone(s) Achieved: Establish effects of kindling on cytogenesis and pathology, determine effects of SST on seizure-related changes in cytogenesis and pathology; publication of 1-2 peer reviewed papers

A manuscript describing vector effects on seizure susceptibility has been published (Natarajan et al. '2017). A manuscript describing gene transfer effects on cytogenesis and survival has been submitted (Liebowitz et al. '18, Appendix). A manuscript describing vector effects on water maze performance is in preparation.

Specific Aim 2: Does SST gene transfer alter inflammation cascades over the time scale in which epileptogenesis occurs?

Major Task 1: Generate biochemical group cohorts of Aim 1

Subtask 1: Generate kindled animal cohorts of Aim 1 groups

[10 rats per group x 8 groups = 80 rats total]

Kindled animal cohorts for this task have been generated and tested for seizure susceptibility.

Brain samples have been generated and are stored by Dr. Ormerod.

Subtask 2: BioPlex biochemical analysis of animals used in Aim 1

[same animals as subtask 1]

These samples are awaiting processing, but the absence of vector effects on inflammation as reflected by microgliosis indicates that further pursuit may not be productive.

Milestone(s) Achieved: publication of 1-2 peer reviewed papers

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

Drs. Carney, King, and Natarajan have collaborated with UF vector design experts to test several novel AAV vector constructs that might improve the dosing, distribution, or expression characteristics of SST gene transfer.

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]

We have not tested new vectors in kindled rats, and because the results to date do not indicate markedly superior performance of the new vectors we probably will not pursue this further in the absence of new funding.

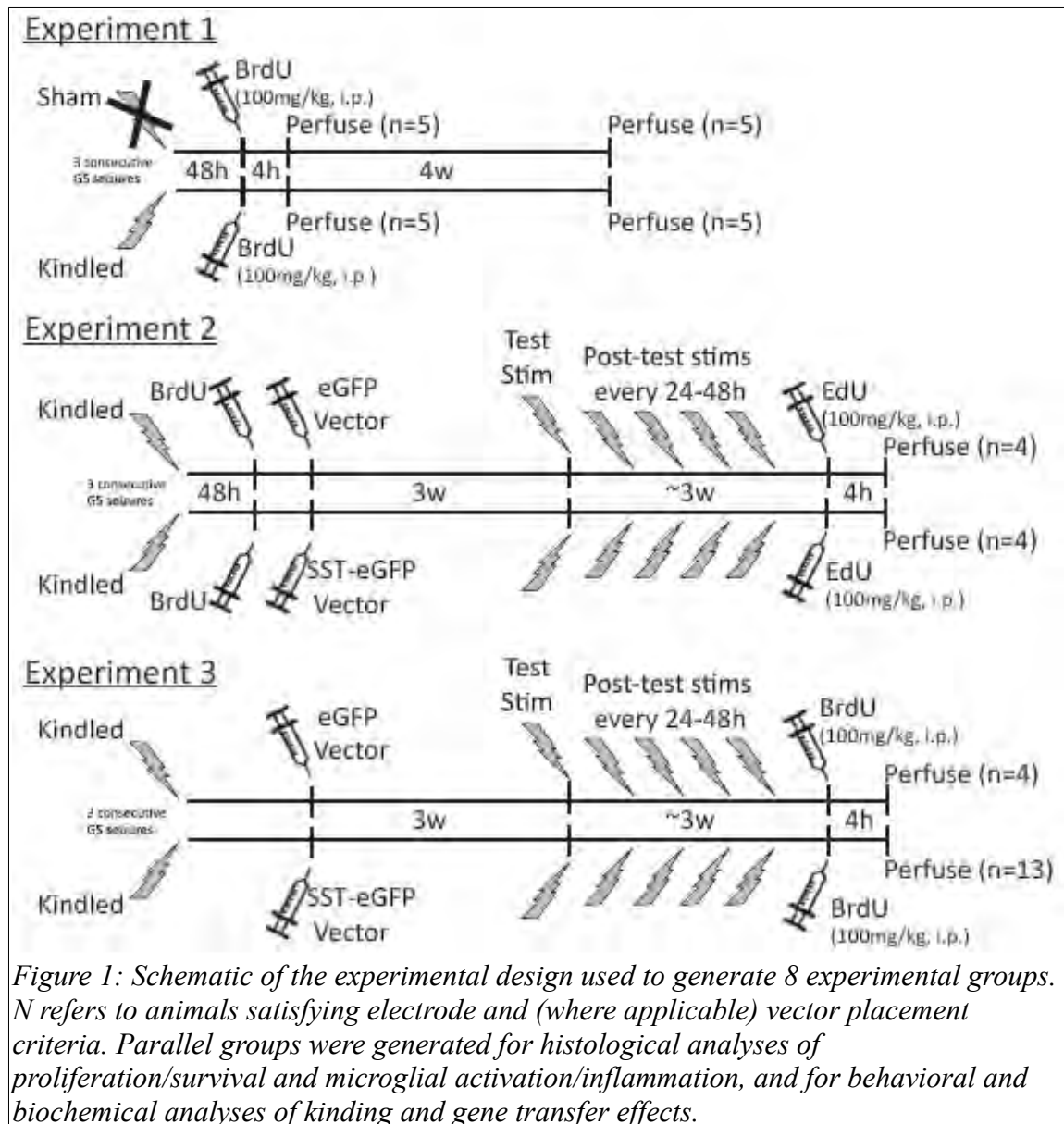
What was accomplished under these goals?

1) major activities: A review of the experimental design (Figure 1) and methods will serve to describe the major activities occupying the participants of the project. The overall experimental design for the project involved 2 parallel sets of rats, 1 for which brains would be collected for histological assessment of cell proliferation, microglial activation, and pathology, and 1 for which brains would be committed to biochemical analyses incompatible with chemical tissue fixation. All animals underwent surgical implantation of indwelling

wire stimulation and recording electrodes fixed in a permanent connector glued to the skull. Kindled groups underwent 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 were treated identically except that no current was delivered. The kindling process develops a persistent state of abnormally elevated susceptibility to seizures, thought to approximate conditions early in the evolution of epilepsy when relatively few seizures have occurred and seizure-associated brain pathology is mild or undetectable with current clinical methods. The probability that therapeutic intervention can succeed is significantly higher before such pathology develops.

2) specific objectives: The objective of Experiment 1 was to determine whether kindling altered cytogenesis at the time a robust state of seizure susceptibility was established, and to evaluate the long-term development and endurance of cells generated at this time. To minimize acute effects of seizures, administration of the thymidine analog bromodeoxyuridine (BrdU) to label DNA in dividing cells was delayed 48 hours from the last kindled seizure. Kindled and sham groups (with unstimulated implanted electrodes) were euthanized 4 hours or 4 weeks after systemic BrdU administration, without induction of any further seizures after reaching criterion. Rats were euthanized by anesthetic overdose, thoracotomy, and vascular perfusion fixation. Brains were processed to prepare tissue sections and histological quantification of BrdU-labeled cell numbers, along with numbers of cells labeled with immunomarkers identifying phenotypes of dividing and differentiating cells. Other samples of sections were immunolabeled with accepted markers for resting and activated microglia reflecting tissue inflammation. Proliferating cell numbers were estimated as the product of all labeled cell body profile counts through the full section thickness multiplied by the interpolated volume of the dentate gyrus granule cell and subgranular subregions traced on microscope images. Microglia numbers were estimated by optical fractionator stereology, in which exhaustive counts in multiple systematic random samples completely internal to section tissue volume were multiplied by the reciprocal of volume sampling fraction. SAS statistical software was used for data normalization, summary statistics, and simple correlations among response measures. General Linear Models analysis of variance was used to test hypotheses about kindling and survival duration effects, and Adaptive Regression was used to evaluate relative contributions of independent factors to outcome measures.

The objective of Experiment 1 was to establish reference points for kindling and survival effects. The objective of Experiments 2 and 3 was to examine whether SST gene transfer might alter seizure susceptibility in association with effects of kindling on cytogenesis and differentiation. For Experiment 2, kindled groups received BrdU 2 days after reaching criterion. A week later, stereotaxic intracranial infusions of control or test adeno-associated viral vectors were made at 2 loci in each hippocampus of animals under general anesthesia. The test vector contained the gene for full-length human preprosomatostatin, the parent peptide sequence from which somatostatin (SST) is enzymatically derived, plus a downstream green fluorescent protein (GFP) biologically inert reporter gene place so that GFP protein could only express if the SST gene did. Control vector contained only the GFP gene. Rat and human have 97% preproSST nucleotide homology, and the derived SST amino acid sequences are identical in both species. After 3 weeks for surgical recovery and the full emergence of transgene expression, rats were challenged for ~3 weeks at 0.5-1 electrical stimulation per day (Figure 2). For most post-tests the same current levels were used as those selected for initial kindling. Sufficient challenge post-tests were imposed to determine stability of kindling and therapeutic efficacy, and after multiple tests at original current levels most rats were challenged with 1-3 additional tests 50 and 100 uA above initial current to assess whether therapeutic efficacy reflected a change in threshold or an unconditional resistance to seizure. The repeated challenge stimulations returned novel information on the stability and potential mechanisms underlying the kindled state and vector effects over a duration ensuring sustained maximal expression of vector genes. Administration of a second thymidine analog, 5-ethynyl-2'-deoxyuridine, (EdU) 2 days after the final post-test was conducted to compare proliferation after recurrent (challenge) seizures, potential therapeutic vector effects, and a prolonged period of elevated seizure susceptibility. The same 4 hour survival was used as for Experiment 1. After selected survival intervals rats were euthanized by anesthetic overdose, thoracotomy, and vascular perfusion fixation. Brains were processed for frozen sectioning and histological detection of proliferation, phenotype, microglial activation, and general pathology.



Phenotyping, counting, and statistical analysis of proliferating, differentiating, and microglial cells used methods identical to Experiment 1.

Experiment 3 also tested vector effects. No proliferation marker was administered immediately after kindling. BrdU was administered at a time comparable to when EdU was given in Experiment 2, after prolonged post-testing for vector efficacy and stability.

Individual AAV vector preparations yield about 500 ul, and are not uniform in the number of transducing vector genomes per unit volume of the final reagent. In the course of generating sufficient numbers of animals meeting experimental inclusion criteria for electrode and infusion placement, it became necessary to use 2 different SST vector preparations in Experiment 3 than what had been used in Experiment 2, but this provided an opportunity to begin testing vector dose effects.

Because behavioral testing may alter hippocampal cyto genesis in rodents, examination of kindling and vector effects on cognitive performance was conducted in the parallel experimental treatment groups committed to biochemical analyses of inflammation. The objective of behavioral testing was to assess whether beneficial SST vector effects on seizure susceptibility were associated with amelioration of seizure-related cognitive impairment (therapeutic efficacy), or whether SST vectors exhibited any adverse effects on cognition

independent of effects on seizures (safety). As with Experiment 3, groups of sham-kindled and kindled rats received surgical infusion of SST, GFP, or no vector 3 weeks before retesting for seizure susceptibility. After allowing surgical recovery and development of transgene expression, they were subjected to a regimen of behavioral testing using water maze escape spatial learning and memory tasks. Rats were first trained to escape random placement in the water tank by locating a fixed-position submerged (hidden) platform (5 trials a day for 8 days)(acquisition), and then tested on a memory probe trial 48 hours after training (retention). Response perseveration was tested on reversal trials using a novel hidden platform position (5 trials a day for 6 days) and finally, sensorimotor ability was tested on cued trials (visible escape platform, 8 trials a day for 2 days). Escape latencies and pathlengths in acquisition trials were used as measures of learning, % duration and % number of crossings in each quadrant during probe trials as a measure of long-term memory, and swim speeds as a measure of sensorimotor ability.

During the water maze testing interval seizure susceptibility was challenged intermittently as in Experiments 2 & 3 to examine stability of the kindled state and therapeutic responses. The revised design was a 3 factor ANOVA (kindling, vector, survival time) analyzed using General Linear Model statistical procedures for hypothesis testing. After seizure and behavioral testing was complete rats were euthanized by carbon dioxide and brains rapidly harvested for storage at -80 degrees C for batch processing in biochemical analyses (Bioplex).

The objective of new vector development was to test potentially superior transgene constructs, expression reporters, and AAV capsid variants that might improve the incidence or magnitude of efficacy.

SOW objectives defined securing and maintaining animal care and use approvals, the acquisition and validation of gene delivery reagents, milestone stages for processing treatment groups in the experimental design including analyses and reporting of results, and the initial development of refined reagents and methodology in anticipation of subsequent translational efforts.

3) significant results:

I. Kindling results in an enduring and stable state of increased seizure susceptibility. In GFP control vector rats, kindling to criterion resulted in a persistent, non-decrementing increase in seizure susceptibility (Figure 2). After 3 weeks of transgene expression, multiple challenge stimulation episodes days apart did not result in further progression of a seizure prone state. These novel data extend a limited body of information on the stability and variability of the kindled state, and how kindled seizures faithfully reflect clinical variation in seizure severity during human epileptogenesis. They justify confidence that observed therapeutic efficacy is unlikely to be an artifact of transient susceptibility to seizures, or a random consequence of inadequate sampling frequency. As discussed below, the novel data from repeated tests also address the internal validity of using the first challenge trial as an exclusive single measure of seizure susceptibility.

II. Kindling results in an impairment of mnemonic behavior. In a set of animals comparable to Experiment 1 but without thymidine analog administration, water maze performance was evaluated beginning the day after reaching the kindling criterion of 3 consecutive grade 5 seizures. Kindling alone had no significant effect on water maze acquisition (Figure 3), retention (Figure 4), or cued learning (Figure 5), but did result in impaired learning of a new location for the hidden escape platform (Figure 6). Response perseveration occurs with hippocampal pathology, and the impaired reversal learning 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. Although seizures can have a transient effect on mnemonic performance that may account for deficits in kindling model rats, our reversal testing was begun 10 days after the last seizure and extended to 16 days, well beyond the endurance of transient kindled seizure effects reported in the literature. Our data suggest a persistent cognitive inflexibility that has been proposed to relate to defective neurogenesis (Anacker & Hen '17). Human epileptics frequently develop persistent memory problems that are likely to be prevented by interrupting seizure recurrence and cumulative subclinical pathology of individual seizures.

III. In kindled rats hippocampal BrdU incorporation is increased 48-52 hours after the first 3 consecutive grade 5 seizures. In Experiment 1 (Figure 1) immunoreactivity of the thymidine analog BrdU was used to identify brain cells that were synthesizing new DNA (i. e. replicating)(Figure 7) during a 4 hour availability window 2 days after the last kindling seizure (or an equivalent timepoint in sham-kindled controls). Profile counts of BrdU-positive cells from a sample of brain tissue sections in which hippocampus was present were multiplied by the inverse fraction of the number of counted to the total number of hippocampal sections, to estimate total numbers of dividing cells in each animal.

About twice as many BrdU-immunolabeled cells were found in the dentate gyrus of rats that survived 4 hours following BrdU injections they received 2 days after reaching fully-kindled criteria of 3 consecutive grade 5 seizures (Figure 8A). This most likely reflects an increase in the proliferation rate of hippocampal progenitor cells. Such an increase might be a lasting effect associated with maintenance of a seizure-susceptible state (kindled), or a transient effect related to seizures in the recent past.

Differentiating proliferating cells labeled with BrdU were further categorized as Type 1, 2, or 3 based upon immunoreactivity for glial fibrillary acidic protein (GFAP), SOX2, doublecortin (DCX), and neuronal nuclear protein (NeuN)(Type-1: GFAP+/SOX2+; Type-2: GFAP-/SOX2+; Type 3 GFAP-/SOX2-/DCX+/NeuN+). Types 2a and 2b were further distinguished by the colocalization of SOX2 and DCX (2a, GFAP-/SOX2+/DCX-; 2b, SOX2+/DCX+)(Figure 7). Total numbers of cells of each type were estimated as the product of the fraction for each type times the estimated total number of BrdU+ cells in each animal.

Type 1 cells demonstrated a robust kindling effect (Figure 8B, Table 1). Type 2 absolute cell numbers were not affected (Figure 8C) but the distribution of both types was significantly altered (Figure 8D, E). Type 2a and 2b fractions were reduced equivalently (Table 2), pooled for further analyses, and not phenotyped separately in vector effects testing (Experiments 2 & 3). Type 3 cells were rare and did not contribute substantially to observed effects.

IV. Survival and phenotypic differentiation of hippocampal cells generated 48-52 hours after the first 3 consecutive grade 5 seizures are not different from sham-kindled rats. The increase in new cells generated

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 2: Kindled rats were challenged with electrical brain stimulation at original kindling current levels on multiple occasions beginning (Day 1) 3 weeks after GFP control vector delivery (Figure 1, Experiment 3). Initial retest scores were generally predictive for subsequent challenges that confirmed a sustained, stable state of elevated seizure susceptibility. They conservatively underestimated later scores in 2 rats and in no cases overestimated averages of multiple tests. At this retest frequency and duration, seizure scores also demonstrated little probability of responses being refractory upon subsequent challenge. From Natarajan et al. '17.

48-52 hours after reaching fully kindled status that was observed with 4 hour survival was sustained in kindled rats that survived 4 weeks after BrdU injection (Figure 8A). In both sham control and kindled rats, the numbers of BrdU+ cells did not change over a 4 week survival, indicating that survival was not altered by a sustained seizure-prone state, in the presumed absence of new seizures (spontaneous seizures are rare in this kindling model).

Cells labeled with BrdU immediately after kindling to criterion in animals that survived 4 weeks were also phenotyped for differentiation into distinct cell types based on immunoreactivity for doublecortin (DCX), (NeuN), and GFAP (DCX+/NeuN-, 'immature'; DCX+/NeuN+ 'transitioning neuron'; DCX-/NeuN+ 'mature neuron'; GFAP+ 'astrocyte'; oligodendrocyte progenitor marker rabbit anti-NG2 'oligodendroglia')(Figure 9). As for proliferating cells, total numbers of surviving cells of each type were estimated as the product of the fraction for each type times the estimated total number of BrdU+ cells in each animal.

In both sham-kindled controls and kindled rats, BrdU-labeled cell numbers were not different after 4 week survival than from their respective 4 hours survival estimates. More immature (Figure 8F) and mature BrdU+ neurons (Figure 8H, J) were found in kindled rat hippocampus, with a significant decrease in both the number and proportion of new cells that differentiated into astrocytes (Figure 8I, N; Table 2).

This result indicates that new cells generated in relation to kindling or recent seizures survive and differentiate. Differentiation in the kindled brain does not appear different from that in the normal brain, distinguishing it from effects on cell formation. If there is no compensatory loss of pre-existing cells, stimulation of cell division by recurrent seizures in epilepsy could mediate cumulative and persistent increases in hippocampal cell numbers.

V. Kindling stimulates sustained microglial activation in the dentate gyrus. Microglia in tissue sections from sham-kindled and kindled rat brains harvested 2 days and 4 weeks after reaching criterion kindling (with individual sham-kindled animals paired with each kindled animal time) were immunolabeled to identify all (Iba1) and activated (CD11b) microglia (resting microglia were defined as Iba1+/CD11b-)(Figure 10). Optical fractionator methods were used to estimate the total numbers of each type within the dentate granule cell layer (GCL) and hilus.

In GCL kindling stimulated a small acute increase in total microglia that was sustained 4 weeks (Figure 11 A). The number (B) and fraction (D) of resting GCL microglia was decreased at 4 hours, and number but not fraction recovered by 4 weeks. The number (C) and fraction (E) of activated GCL microglia was elevated at 4 hours, and the fraction but not number showed significant recovery by 4 weeks.

In the dentate hilus kindling did not stimulate an acute increase in total microglia (Figure 11 F), but at 4 weeks the estimated total numbers were higher in kindled than in control rats. The numbers (G) and fraction (I) of resting hilar microglia were lower at 4 hours and both recovered significantly by 4 weeks. Numbers (H) and fraction (J) of activated hilar microglia were higher in 4 hours and 4 week kindled rats. The fraction of activated hilar microglia exhibited significant recovery although the absolute numbers did not.

Kindling thus appears to induce only a mild inflammatory response, consistent with clinical observations of early epilepsy. Differences in hilus and GCL effects may signal that alternative mechanisms and sequencing of microglial activation and recovery occur in proliferative zones than where differentiated granule cell perikarya define the primary anatomical feature. Thymidine analog incorporation was not obvious in conjunction with increased total microglial cell numbers. A kindling-stimulated increase of >10,000 microglia per side was identified with a 'resting' phenotype marker, yet BrdU-labeled cells were not observed outside the subgranular proliferation zone (Figure 7, 9) and only about 1500 new cells per side were reported by BrdU. Future studies will need to address whether proliferation of microglia occurred prior to the 48 hour post-seizure BrdU administration time, or the possibility that Iba1 immunolabeling as used may not have revealed in sham controls all microglia stimulated by kindling to become immunoreactive.

III. AAV-SST gene delivery mediates expression in hippocampal neurons. In gene transfer experiments an AAV serotype 5 vector omitting (control) or including the entire SST gene (Figure 12) was surgically infused to

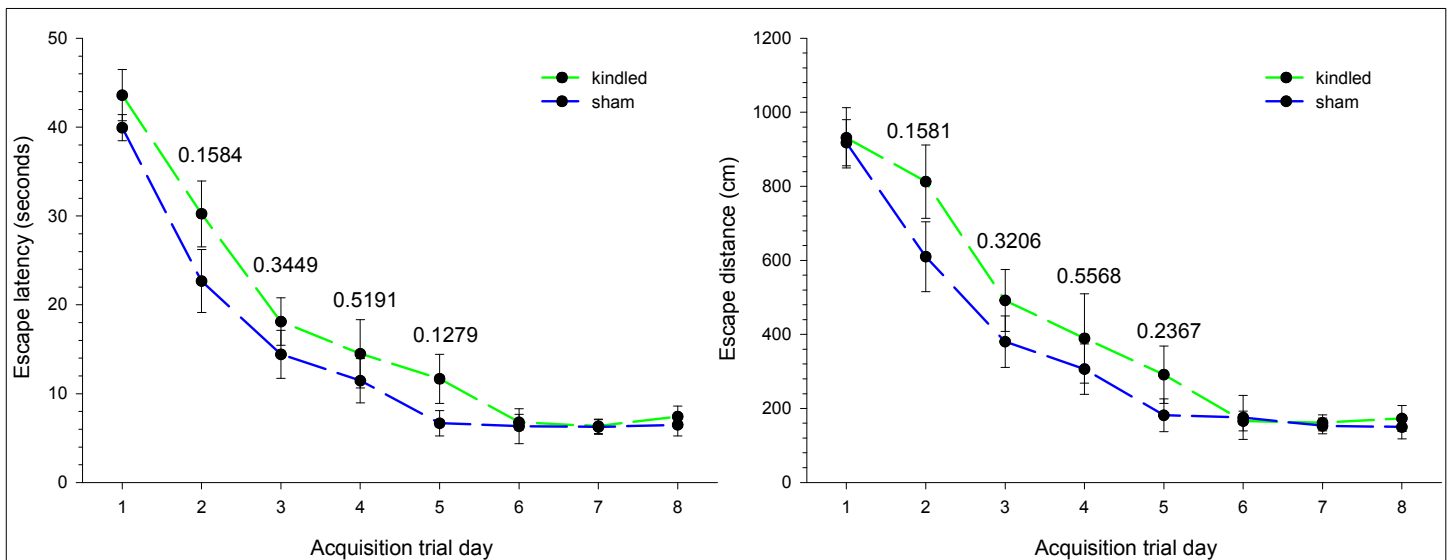


Figure 3: Left: Rats that were fully kindled prior to water maze acquisition training, and received challenge stimulation every other day during training, required no more time to escape than rats with implanted electrodes but no delivery of electrical current (sham kindled). SAS Proc GLM repeated measures ANOVA indicated only a trend 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 $\times$ treatment (kindling) interaction ($F(7,112)=0.67$, $Pr>F$ 0.6976). Right: Effects were similar for swim distance prior to escape (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 $\times$ treatment (kindling) interaction ($F(7,112)=0.67$, $Pr>F$ 0.6961).

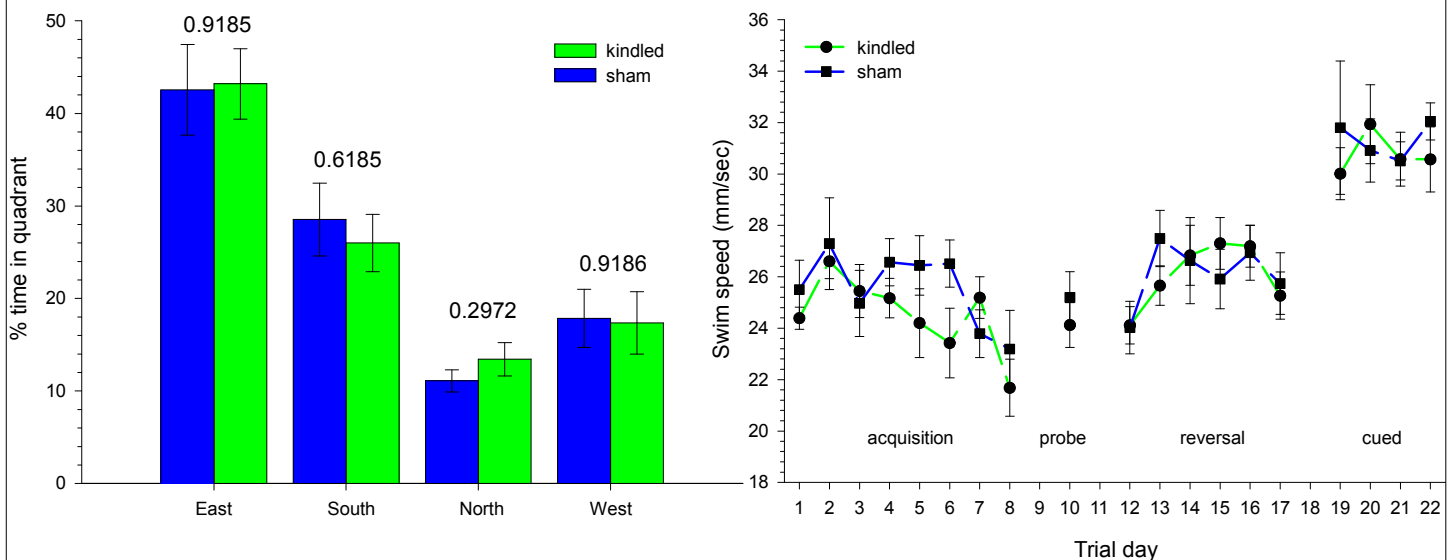


Figure 4: Left: In probe trials testing retention for water maze escape platform location 2 days after acquisition training, kindled rats spent equivalent fractions of search time in target and non-target maze quadrants as sham-kindled rats. SAS Proc GLM ANOVA for between-subjects factor 'treatment' ($F(1,17)$ not significant), Least-squares means post-hoc $Pr>F$ above bars. Right: Swim speed did not differ between kindled and sham-kindled rats in any component of water maze testing ($F(1,16)$ 0.84, $Pr>F$ 0.3719), indicating that kindling did not impose a gross motor performance deficit or altered motivation to escape immersion.

hippocampal CA1 and dentate gyrus bilaterally during the week following attaining kindling criterion (Figure 1). After allowing 3 weeks for surgical recovery and establishment of stable expression levels, rats began seizure testing over 3 more weeks.

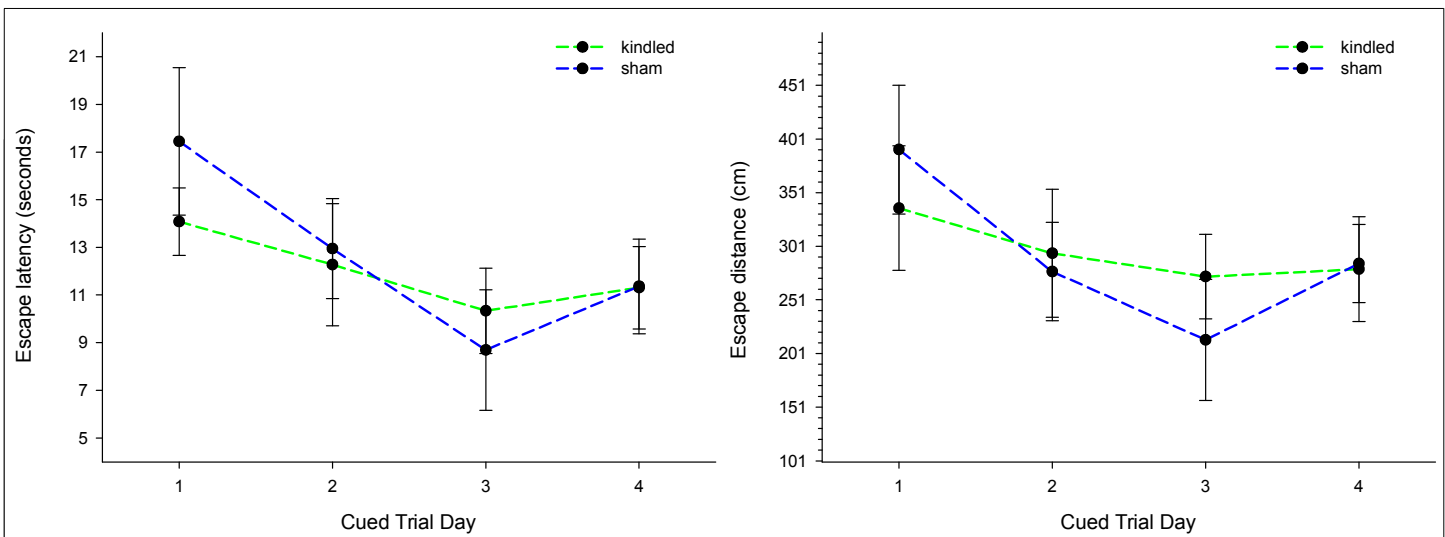


Figure 5: Left: Cued (visible platform) escape time in the water maze task was not affected by kindling. SAS Proc GLM repeated measures ANOVA $F(1,16)=0.12$, $Pr>F$ 0.7300. There was significant learning over trial days ($F(3,48)=3.08$, $Pr>F$ 0.0362) and no interaction with kindling ($F(3,48)=0.48$, $Pr>F$ 0.6985). Right: Escape distance results were similar (kindling $F(1,16)=0.01$, $Pr>F$ 0.9254); 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).

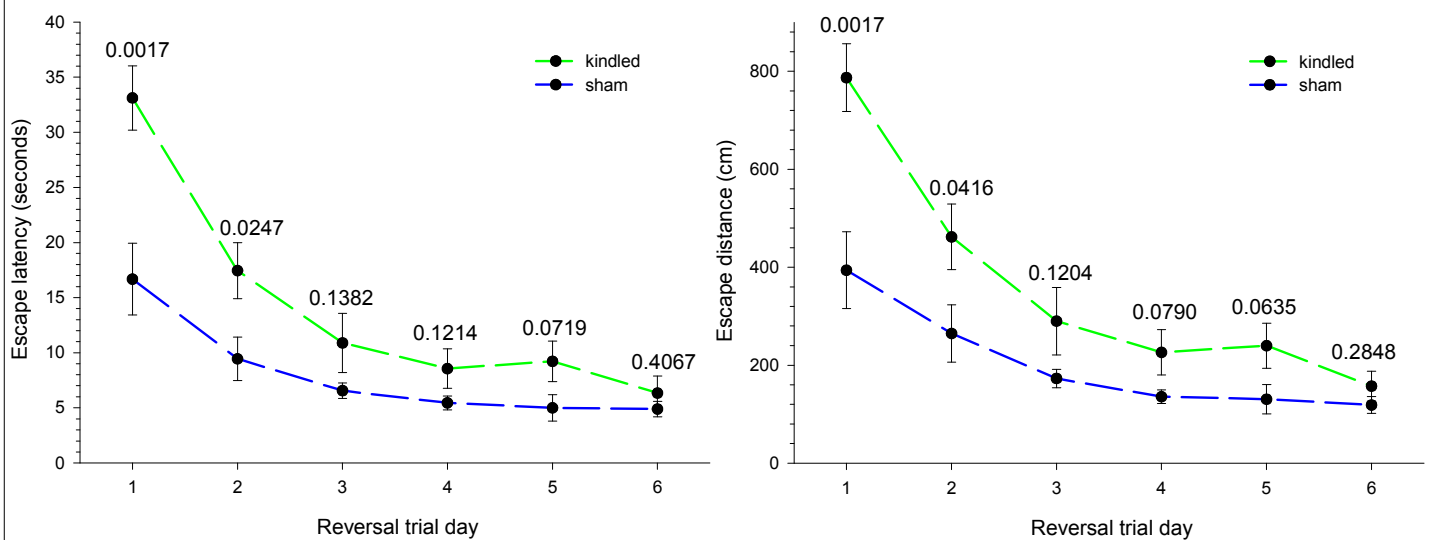


Figure 6: Left: In water maze reversal learning trials requiring trained rats to learn a new escape platform location starting the day after initial retention (probe) trials, kindled rats swam longer before escape than sham-kindled rats, and required more training to attain equal proficiency. SAS Proc 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 (final trial performance) ($F(5,80)=33.36$, $Pr>F$ <.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 (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).

The stereotaxic coordinate and infusion volume parameters for delivering AAV vectors facilitated transduction of a substantial fraction of the entire hippocampal formation (Figure 13). For SST vectors, 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 SST content in control brains (Figure 14). This is consistent with observed expression of the

prohormone convertases that derive SST from its precursor peptide, and indicates that SST over-expression may prove therapeutically effective even after native SST neurons succumb due to preferential vulnerability to seizure effects: other neurons assuming a vector-defined SST phenotype may mediate therapeutic actions. Immunoreactivity intensity suggests that SST abundance may be lower on average in transduced excitatory neurons than in transduced neurons with inhibitory phenotypes. However, in the absence of further information this could represent more release just as much as less expression. The presence of expression in both types does indicate 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 wherever in the brain hippocampal neurons maintain synaptic terminals.

IV. SST can reduce seizure susceptibility after kindling (therapeutic efficacy). 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 in ~40% of all SST vector test animals, and 4/6 Experiment 3 SST animals that received the highest vector dose (Figure 15). Therapeutic efficacy shows a strong tendency to be all-or-nothing, to be fully elaborated within 3 weeks of vector delivery, and to persist through several weeks of retesting seizure susceptibility

(Figure 16). This power and endurance bode well for therapeutic refinement, and support the hypotheses that seizure susceptibility neither progresses spontaneously to gradually erode therapeutic efficacy, nor develops pharmacological tolerance to SST as a result of sustained agonist availability following gene transfer.

Following the convention adopted in our earlier studies (Zafar et al. '10), our initial operational definition of 'responder' animals was a score of 0 in response to the initial challenge stimulation at the original test current (I) level. The collection of multiple challenge seizure data in Experiment 2 and 3 rats encouraged a critical re-evaluation of this arbitrary definition (Table 1). Classification accuracy and internal consistency were found to improve with a definition of responders based on average post-test scores ≤ 1 or < 2 . However, statistical comparisons of proliferation and inflammation variables were conducted using both schemes and will be presented for many of the analyses in this document.

Because the raw seizure scores are ordinal but not necessarily linear response measures, we analyzed both raw scores and rank-transformed normalized scores in statistical analyses. This provides an alternative approach to the non-parametric ANOVA methods used in the Leibowitz manuscript. The SAS procedure RANK was used on raw Test 1 and average for all challenges at original stimulation current (AvOrigI) values from Experiment 1-3

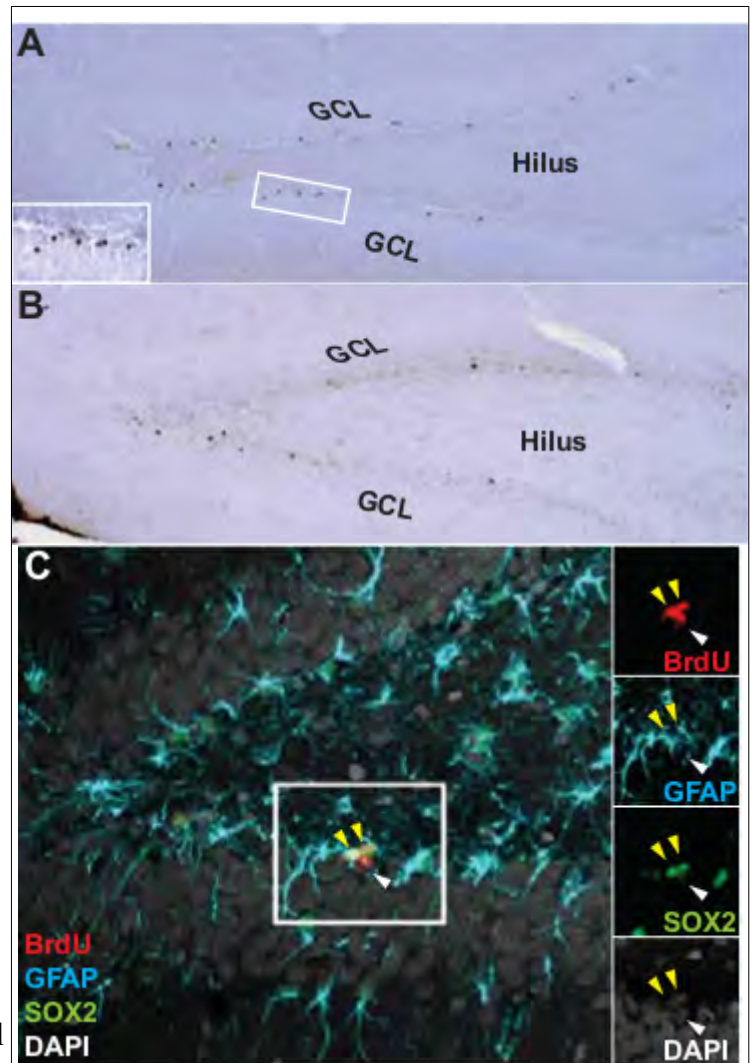


Figure 7: A, B: BrdU-immunoreactive cells in dentate granule cell layer (GCL) and hilar subgranular layer. C. Multiple markers were used to define subtypes of proliferating cells identified by BrdU immunoreactivity. From Liebowitz et al. submitted.

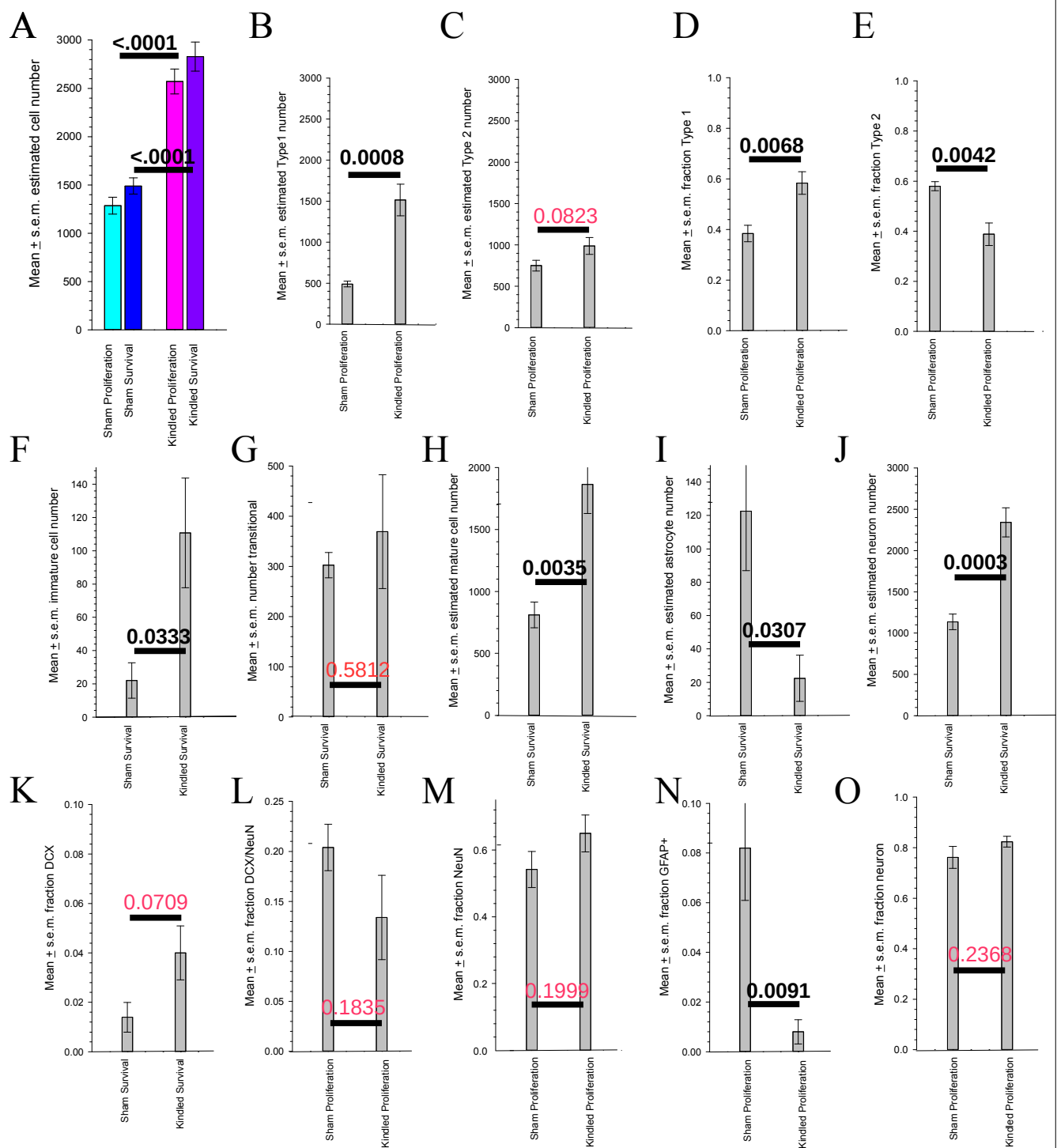


Figure 8: A. Estimated total BrdU+ cell number in kindled and control rat dentate gyri 4 hours and 4 week after reaching criterion. B-E: A sample of BrdU-immunolabeled cells was assessed for colocalization of glial fibrillary acidic protein (GFAP) and sex determining region Y-box 2 (SOX-2) to phenotype proliferating cells. Kindling increased the estimated number (sample fraction $\times$ estimated total BrdU+ number) of only Type 1 proliferating cells, but the distribution of both Types 1 and 2 were both altered (D,E). F-O: To phenotype surviving cells, one sample of BrdU-immunolabeled cells was assessed for colocalization of doublecortin (DCX) and neuronal nuclear protein (NeuN) (DCX+/NeuN-, immature (F, K); DCX+/NeuN+, transitioning (G, L); DCX-/NeuN+, mature neurons (H, M)). A second sample was assessed for colocalization of GFAP (astrocytes, (I, N)) and NG2 (oligodendrocytes, too few for analysis). Cells in this sample with BrdU+ only were considered 'neurons' (J, O). Numbers (F-J) and phenotypic distribution (sample fraction) (K-O) of surviving cells were also altered in kindled rats, with absolute and relative increases in neuronal differentiation (H) primarily at the expense of astrocytes (I, N). Bold values are p values from SAS GLM least-squares means post-hoc comparisons, red values reflect $p > 0.05$.

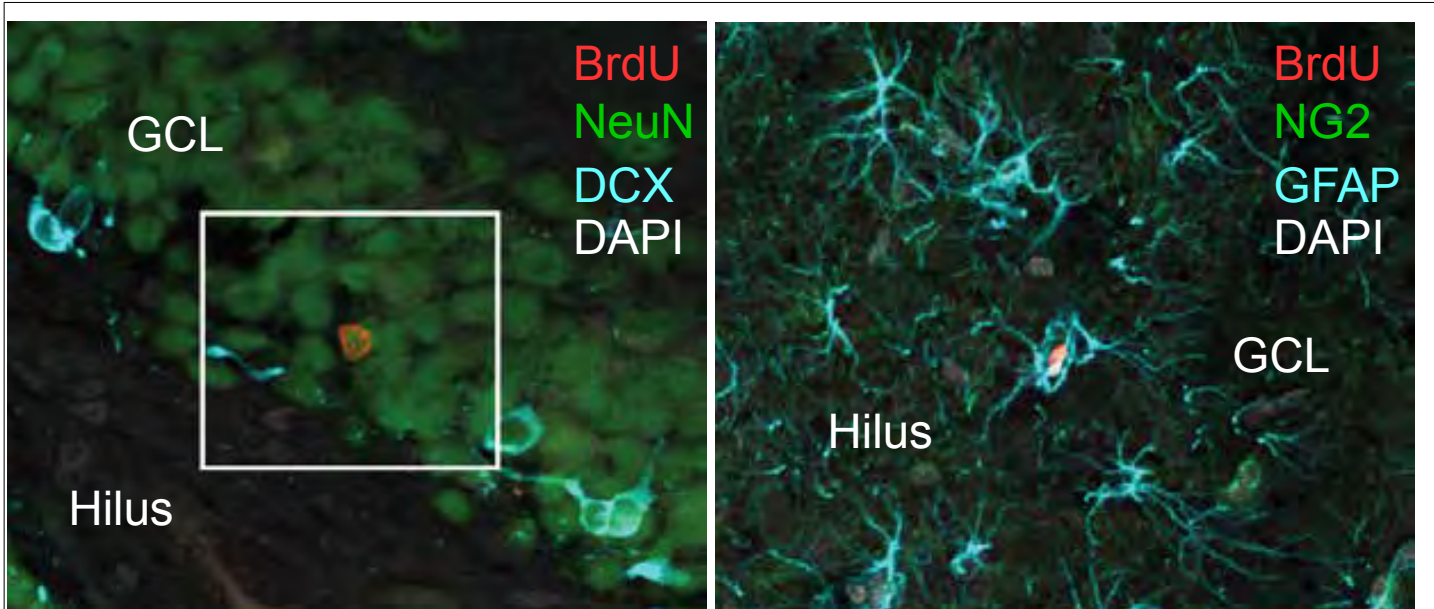


Figure 9: Phenotyping cells labeled with BrdU administered 48 hours after final kindling seizure and surviving 4 weeks. One sample of sections was colabeled with neuronal maturation markers (left), and another sample labeled with astrocyte (GFAP) and oligodendrocyte (NG2) markers. Modified from Liebowitz et al. submitted.

	Type-1 NSC (GFAP ⁺ / Sox2 ⁺)	Type-2a NPC (GFAP ⁺ / Sox2 ⁺)	Type-2b NPC (Sox2 ⁺ /DCX ⁺)	Immature Neuron (DCX ⁺)	Transition Neuron (DCX ⁺ / NeuN ⁺)	Mature Neuron (NeuN ⁺)	Astrocyte (GFAP ⁺)
Experiment 1							
Sham-kindled	38.5±3.2	58.0±1.8	11.0±3.4	1.4±0.6	20.7±2.3	54.0±5.4	8.1±2.1
Kindled	58.2±4.4	38.9±4.4	7.1±3.2	4.0±1.2	13.5±4.2	65.0±5.5	0.8±0.4
Experiment 2							
eGFP vector-treated	63.3±4.5	32.9±4.2		2.4±1.9	2.5±0.4	82.1±4.7	2.5±0.5
SST-eGFP vector-treated	59.7±5.6	34.0±5.4		1.6±1.1	3.1±1.4	81.2±2.2	7.4±2.5
Experiment 3							
eGFP vector-treated	54.0±0.6	40.9±1.1		-	-	-	-
SST-eGFP NR	64.5±1.8	32.3±1.8		-	-	-	-
SST-eGFP R	39.6±4.4	55.6±4.0		-	-	-	-

Table 1: Phenotype % distribution among proliferating and surviving cell populations.

data to scale each to a common range, and permit the use of parametric analysis of variance for hypothesis testing.

Vector effects on seizure susceptibility were revealed for rank-normalized seizure scores (Figure 17 E-H) that were not detected if raw scores (Figure 17 A-D) were used in parametric analyses. These data also demonstrate differences between using ranked Test 1 and ranked AvOrigI as seizure susceptibility indices. A vector effect between Experiment 3 GFP and low gene dose SST non-responders is observed regardless of whether Test 1 (Figure 17 E) or AvOrigI (F) is used to classify. However, this vector effect is not seen using ranked AvOrigI scores (G, H).

Detection of vector effects on seizure susceptibility was also found to depend on consideration of gene dose and responder subgroups (Figure 18). No significant differences were resolved with data collapsed across responder category and SST vector dose (Figure 18 right). When gene dose was separated but not responder and non-responder animals, vector showed a significant main effect on rank-normalized Test 1 seizure scores (df 6,26 Type III SS 1059.642857, Mean Square 176.607143, F=2.70 Pr > F 0.0360), based on differences between Experiment 3 subgroups (Figure 18 bottom center). However, there was no vector effect on the average of all

seizure scores obtained at original stimulation current levels (df 6,26 Type III SS 525.1130952, Mean Square 87.5188492, $F=1.07$, $Pr > F$ 0.4071) (Figure 18 upper row center); neither SST dose subgroup differed from GFP controls. Segregating clear responders and non-responders revealed vector effects that were undetectable in pooled data. For both rank-normalized Test 1 and AvOrigI, vector effects emerged when responders and nonresponders were classified and vector dose was included in the model, in particular differences between GFP and SST high dose subgroups (Figure 18 left). The numerical result is consistent with the number of responders at low (0/7), middle (1/4), and high (4/6) vector doses.

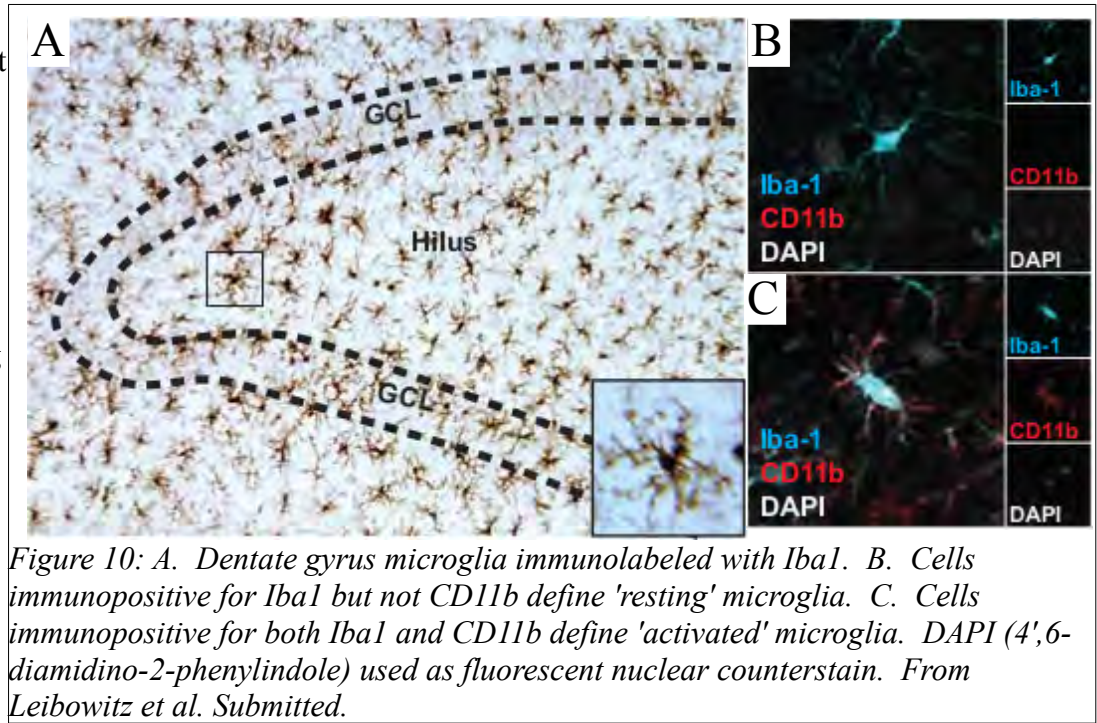


Figure 10: A. Dentate gyrus microglia immunolabeled with Iba1. B. Cells immunopositive for Iba1 but not CD11b define 'resting' microglia. C. Cells immunopositive for both Iba1 and CD11b define 'activated' microglia. DAPI (4',6-diamidino-2-phenylindole) used as fluorescent nuclear counterstain. From Leibowitz et al. Submitted.

VII. AAV-SST delivered after kindling counteracts kindling/recent seizure effects on proliferation, but may be neither necessary nor sufficient for therapeutic reduction in seizure susceptibility.

SST vector significantly antagonized the increase in the number of dividing dentate hilus progenitor cells labeled with BrdU 48-52 hours after the last seizure (Figure 20). ANOVA showed that transgene had a significant effect on the number of cells labeled with thymidine analogs (i.e. Experiment 2-3 data) (df 3,32 $F=6.75$ $p=0.0146$). However, *post hoc* tests could not resolve differences between any experiment groups if vector dose or responder classification were not included in the model (Figure 19 bottom). Experiment was not a significant factor (df 1,32 $p=0.3958$), nor was there an interaction between transgene and experiment (df 1,32 $p=0.2859$). Inclusion of vector dose (Figure 19 center) and responder classification (Figure 19 top) allowed detection of subgroup differences, but even the subgroup of 7 animals that received the low SST vector dose without significant seizure efficacy (Exp. 3 SST lo NR) showed a significant reduction in kindling effects on proliferation compared with GFP vector. This calls into question the possibility of a causal relationship between seizure susceptibility and proliferation. Control GFP vector had no effect compared to kindled rats, and numbers of cells revealed by EdU in control subgroups were equivalent to those obtained with BrdU. Examination of between-subjects variability of thymidine analog-immunolabeled cell number estimates showed lower variability in no-vector and GFP-vector subgroups (Figure 20) relative to most of the SST vector subgroups. The distributions for SST vector animals were uniformly shifted to lower number estimates than corresponding GFP vector subgroups, as would be consistent with partially effective doses. Variability for EdU-based estimates (Experiment 2) was greater than for BrdU-based estimates in the same animals, but the significance of this is not clear in light of the small group size.

Despite a significant reduction in mean cell numbers in SST vector animals that showed a therapeutic effect on seizure severity, several individual animals demonstrate that reduction in proliferation is neither necessary nor sufficient for therapeutic reduction of seizure severity (Figure 21).

Analysis of the phenotypes of dividing and surviving cells showed selective effects of SST gene transfer. Estimates of Type 1 cell numbers showed a robust potentiation by kindling that was comparable in both GFP

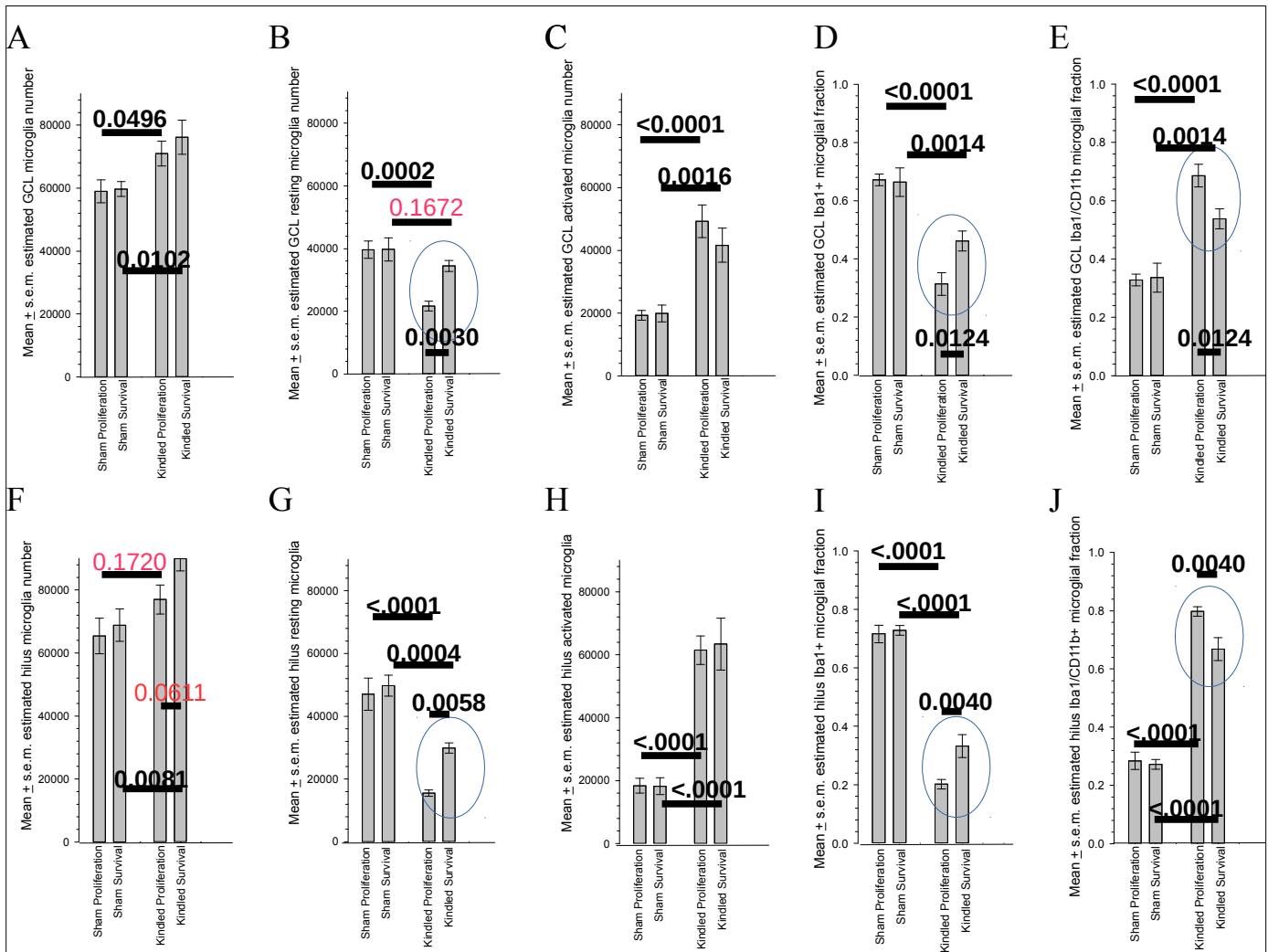


Figure 11: Kindling stimulated acute and sustained microglial activation in dentate gyrus granule cell layer (GCL, A-E) and hilus (F-J). Initial decreases in numbers (B, G) and proportion of Iba1+/CD11b- resting (D, I) microglia show significant recovery by 4 weeks (ovals), along with some reciprocal changes in CD11b+ activated microglia, more so in the GCL (C, E) than in hilus (H, J). Bold values are *p* values from SAS GLM least-squares means post-hoc comparisons between bar pairs; red values reflect *p* > 0.05.

groups (Figure 22 A). Experiment 2 SST vector rats (3 NR, 1 R) did not differ from GFP controls, but Experiment 3 responders (N=4/6, high vector dose) had fewer Type 1 cells than every other subgroup except Experiment 2 responder and sham-kindled animals.

Although the total estimated number of Type 2 cells tended to respond to experimental conditions similarly to Type 1 cells, the interaction (kindling, experiment, transgene, dose) was not significant and no subgroup differences in total estimated Type 2 cell number were detected (Figure 22 B). Nor were there any kindling, vector, or experiment subgroup differences in the population of BrdU+ cells that did not label for Type 1 or 2 markers (Figure 22 C). No GFAP-only (Sox-) cells were detected in the BrdU+ sample populations.

In addition to affecting the *number* of Type 1 cells, a significant treatment interaction was observed for the *fraction* of this subpopulation (kindling, experiment, transgene, vector dose interaction df 8,26 Type III SS 0.40101190, Mean Square 0.05012649, *F*=9.18, *Pr* > *F*<.0001). Post-hoc comparisons revealed that kindling significantly increased the proportion of Type 1 proliferating cells (Figure 22 D), and the kindling effect was consistently observed across GFP vector controls and non-responders in Experiments 2 and 3. Responders

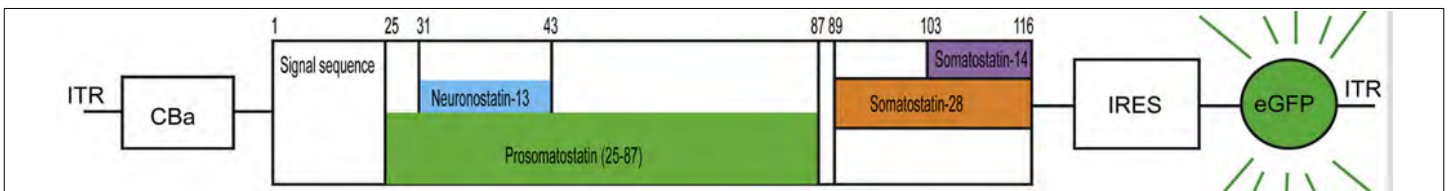


Figure 12: The AAV5-CBa-preproSST-eGFP vector construct contains the 116 amino acid preproSST. An N-terminal signaling sequence directs the translocation of the translated preproSST product into endoplasmic reticulum where it is cleaved at positions 24–25. The remaining 92 amino acid prohormonepreproSST undergoes alternative cleavage by specific proteases to yield the different mature peptide products SST-14, SST-28 (the N-terminal extension of SST-14) and the more recently discovered neuronostatin. The hybrid CMV early enhancer/CBa promoter was used to drive expression of the preproSST transgene plus an internal ribosomal entry site (IRES) and a downstream eGFP reporter. Control vector omitted the SST gene. Plasmids were packaged into serotype 5 AAV capsids. From Natarajan et al. '2017.

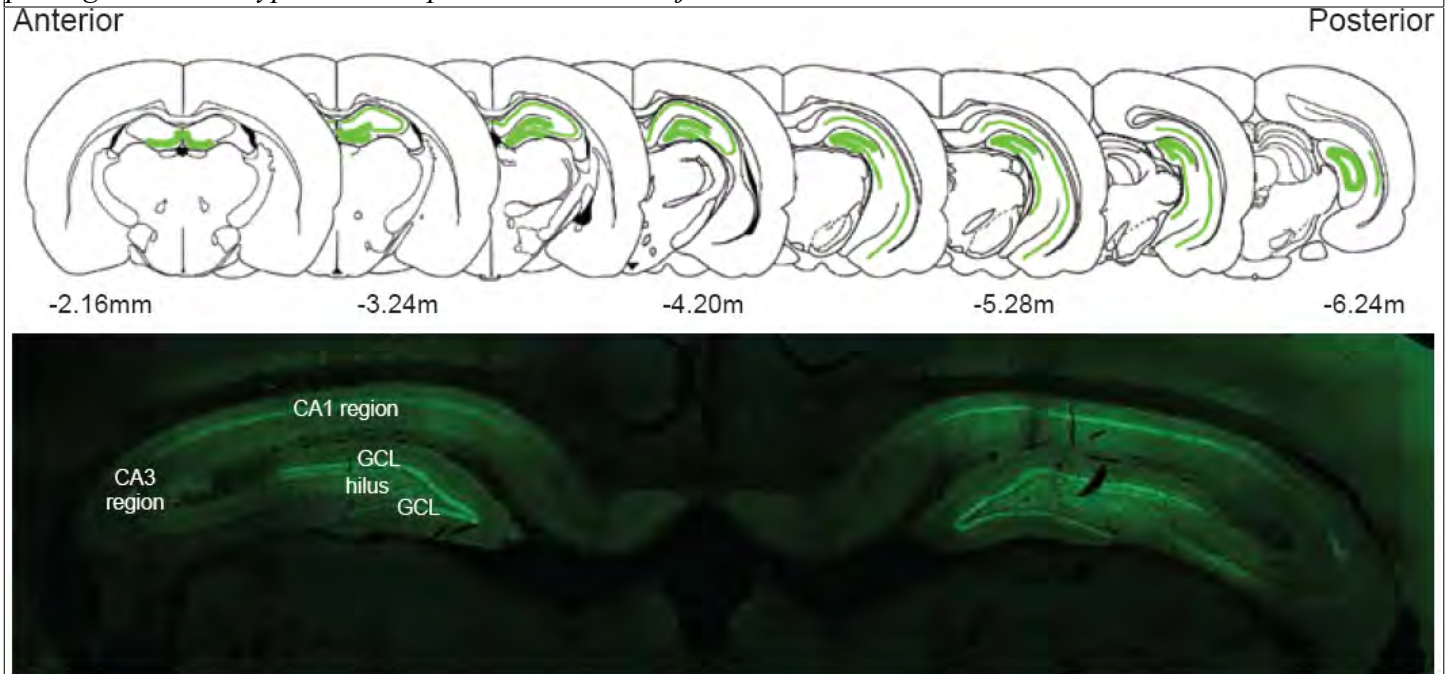


Figure 13: Upper: Vector reporter expression mapped to standard rat brain atlas plates. Lower: Epifluorescent GFP reporter shows vector transgene expression distributed throughout much of the hippocampus bilaterally in this coronal section. From Leibowitz et al., in preparation 2017; see also Natarajan et al. '17 Figure 3.

comprising the high-dose SST subgroup in Experiment 3 showed total cell number estimates significantly lower than other kindled groups and equivalent to sham-kindled control animals.

Type 2 total cell number estimates were, like Type 1, higher in kindled than sham kindled animals, but not significantly (Figure 22 B). Average Type 2 cell number was equivalent in kindled and GFP vector controls in both Experiment 2 and 3, as well as non-responders. Experiment 2 and 3 GFP groups both had significantly fewer Type 2 cells than sham-kindled, consistent with kindling suppressing Type 2 generation as seen in Experiment 1. For animals that showed a seizure response ($AvOrigI \leq 2$), more Type 2 cells were estimated in Experiment 2, comparable to sham-kindled rats (Figure 22 B), but with $N=1$ significance is questionable. Experiment 3 responders had Type 2 cell number estimates equivalent to sham-kindled controls, and significantly higher than non-responders and GFP controls.

The fraction as well as the number of Type 2 cells showed a significant treatment interaction (kindling, experiment, transgene, dose; df 8,26 Type III SS 0.39121929, Mean Square 0.04890241, $F = 10.42$, $Pr > F < .0001$). Opposite to the effect on Type 1 cells, the Type 2 fraction was significantly reduced by kindling, and

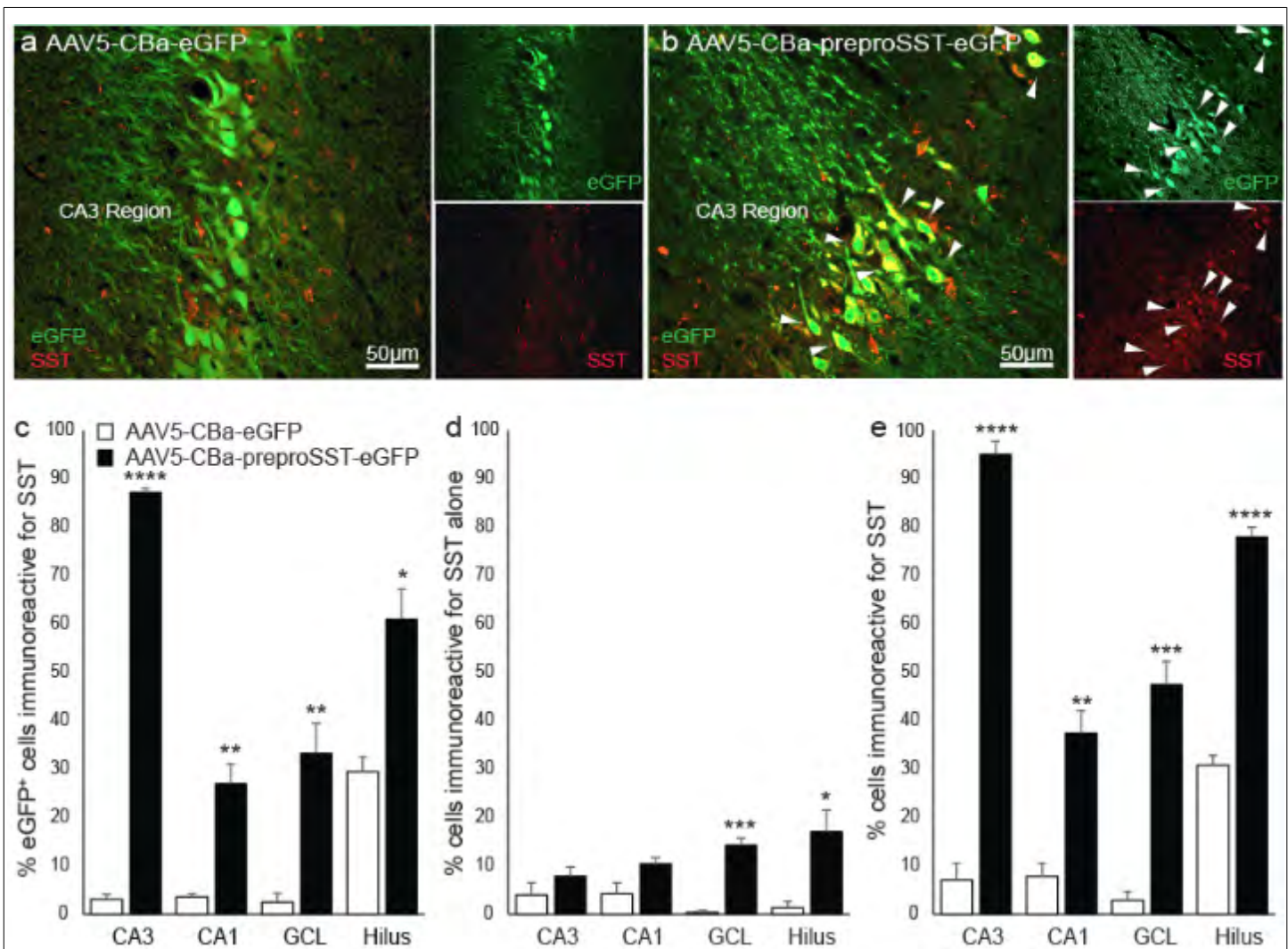


Figure 14: In animals that received the AAV5-SST-GFP vector (A), more and different cells contain SST immunofluorescence than in animals that received control vector (B). Left, composite; right, eGFP emission (green) and SST localization (red) confocal imaging channels. (C) The percentage of at least 100 randomly selected GFP+ cells examined in each hippocampal region that co-localized SST was higher in CA3 (**** $p < 0.0001$) and CA1 region (** $p < 0.01$) pyramidal cell layers, as well as in the dentate gyrus granule cell layer (GCL, ** $p < 0.01$) and hilus (* $p < 0.05$) of SST vector versus control vector rats. Values <100% suggest unequal expression, accumulation, and/or detection sensitivity for GFP and SST. (D) The percentage of GFP-/SST+ neurons (presumably endogenous SST+ neurons) increased in the GCL (*** $p < 0.001$) and hilus (* $p < 0.05$) but not the CA3 region and CA1 region pyramidal cell layers of SST vector versus control vector rats. (E) In subregions where transgene expression was observed the percentage of neurons immunoreactive for vector-derived or endogenous SST was higher in the CA3 region pyramidal cell layer (**** $p < 0.0001$), CA1 region pyramidal cell layer (** $p < 0.01$), the granule cell layer (GCL, *** $p < 0.001$) and hilus (**** $p < 0.0001$) of the SST vector-treated versus control vector-treated rats ($n = 5$ per group).

this was maintained in GFP vector groups (Figure 20 D). SST vector did not have any effect on this in Experiment 2, but in Experiment 3 responders differed significantly from non-responders and GFP controls (both experiments) and were not different from sham-kindled animals or the lone Experiment 2 responder. The effects on numbers and relative proportions of dividing cells indicate that SST may reinstate the same regulatory mechanisms governing dentate proliferation that are disrupted in a seizure-susceptible state.

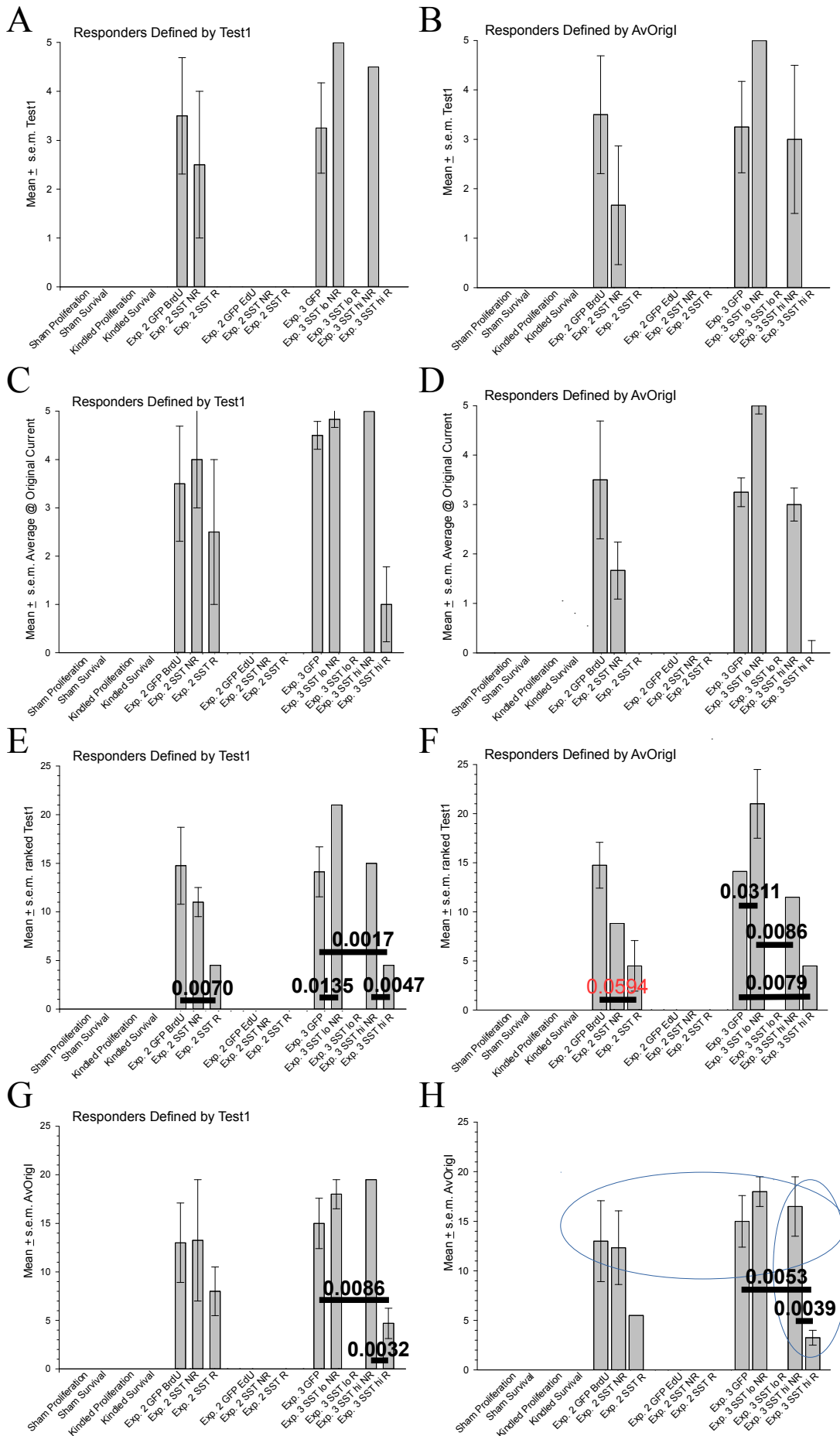


Figure 17: Seizure measure normalization and effects by responder definition

Figure 17 (previous page): Vector effects on severity of electrically stimulated seizures depend on responder definition and data normalization. No significant vector-related differences were resolved for raw Test 1 seizure scores using Test 1 = 0 (A) or raw average seizure score at original stimulation current level (AvOrigI < 2) to classify responders (B) (Exp 3 SST hi R bar mean = 0, s.e.m = 0 by definition in A, B); nor were significant effects resolved for raw AvOrigI using Test 1 = 0 (C), or for AvOrigI < 2 (D) to classify responders. After rank-normalizing seizure scores (E-H) parametric ANOVA showed clear vector effects on both outcome measures. Compared with raw scores, rank-normalized Test 1 and AvOrigI showed closer correspondence between GFP group and SST vector nonresponders, especially for AvOrigI and responders defined by AvOrigI < 2 (cf. H, horizontal oval, vs. D). This approach also returned a more distinct vector effect in responders (H, vertical oval). Bold values are *p* values from SAS GLM least-squares means post-hoc comparisons between bar pairs; red values reflect *p* > 0.05.

There were no significant interactions of kindling, experiment, or vector on the estimated number of cells immunoreactive for thymidine analogue (EdU, experiment 2, BrdU, experiment 3) but negative for markers for type 1 or 2 cells (A), or the estimated number of type 2 cells (C). Kindled rats had higher estimates of type 1 cells than sham controls (B), and seizure responders in experiment 3 had estimates lower than non-responders or GFP controls, and equivalent to sham-kindled controls. For each type, values for GFP control groups in experiments 2 & 3 were statistically equivalent to no-vector experiment 1 kindled rat data, consistent with equivalent validity for labeling proliferating cells between EdU and BrdU, and an absence of vector surgery or AAV transduction effects. The fractions of types 1 (E) and 2 (F) also showed interactions and significant within-experiment differences demonstrating kindling and vector effects. Values were equivalent to sham-kindled controls. Black bars represent *p* < 0.05 differences by Duncan's multiple range.

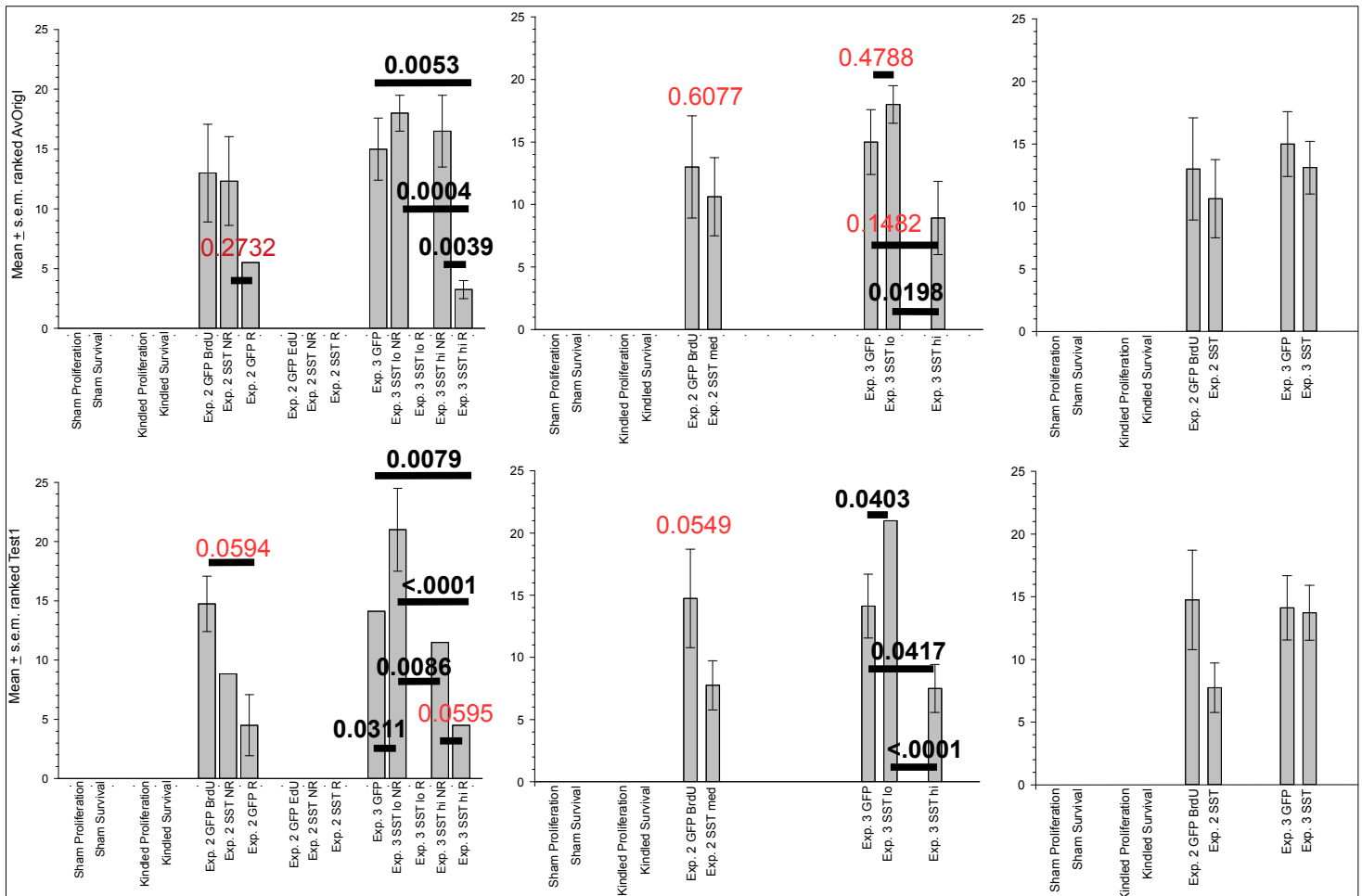


Figure 18: Vector effects on seizure susceptibility require consideration of dose and responder classification.

Survival and differentiation were examined in Experiment 1 and Experiment 2 cells labeled with BrdU (Figure 23). One sample of brain tissue sections was scored for presence of markers for astrocytes and oligodendrocytes in a random sample of cells positive for thymidine analogs. Another sample of sections was scored for colocalization of neuron differentiation markers in cells positive for the thymidine analog.

Most cells in the samples of surviving cells labeled with BrdU and glial markers were immunopositive only for BrdU. A strong interaction among kindling, experiment, transgene, and dose was observed for estimated total number of these cells (df 4,13 Type III SS 6039303.564, Mean Square 1509825.891 $F=15.45$, $Pr > F < .0001$). The proportion of these cells was elevated in the kindled vs. sham group ($p=0.0285$), and the kindling effect in Experiment 1 was observed in the GFP vector subgroup in Experiment 2 (Figure 23 A, Kindled survival vs. Exp. 2 GFP EdU $p=0.0529$). Estimated total numbers of thymidine analog-only labeled cells did not differ between SST vector non-responder and GFP controls, but were significantly lower in SST vector non-responders than in kindled rats ($p=0.0195$).

For the new cell survival sample labeled with BrdU and glial markers, about 10% of BrdU+ cells in the sham-kindled survival group were immunopositive for the astrocyte marker GFAP (Figure 21 A,B). A significant interaction among kindling status, experiment, transgene, and dose was observed for total estimated astrocyte number (sample fraction *dU+ x estimated total number of *dU+ cells)(df 4,13 Type III SS 55824.76416, Mean Square 13956.19104, $F=3.68$, $Pr > F 0.0326$). Despite an approximate doubling of the number of BrdU+ cells in the kindled survival group, far fewer astrocytes contributed to this population and the proportion dropped about an order of magnitude relative to the sham-kindled group ($p=0.0279$). The net result was that the number of new astrocytes did not differ significantly between sham and kindled rats. The results in Experiment 2 were difficult to interpret. GFP subgroup astrocyte numbers did not differ from either sham or kindled Experiment 1 animals, and no difference were detected between any Experiment 2 subgroups.

Oligodendrocytes were only observed in 1 subgroup and thus no subgroup differences in either number or fraction could be detected.

There were no significant treatment subgroup differences in either the fraction or estimated total of surviving cells immunoreactive for BrdU and none of the neuronal differentiation markers (Figure 23 C). Nor were there fraction or number differences for “immature” neurons colocalizing BrdU and doublecortin (DCX) (Figure 23 D). For “transitioning” neurons positive for BrdU, DCX, and NeuN (Figure 23 E), there

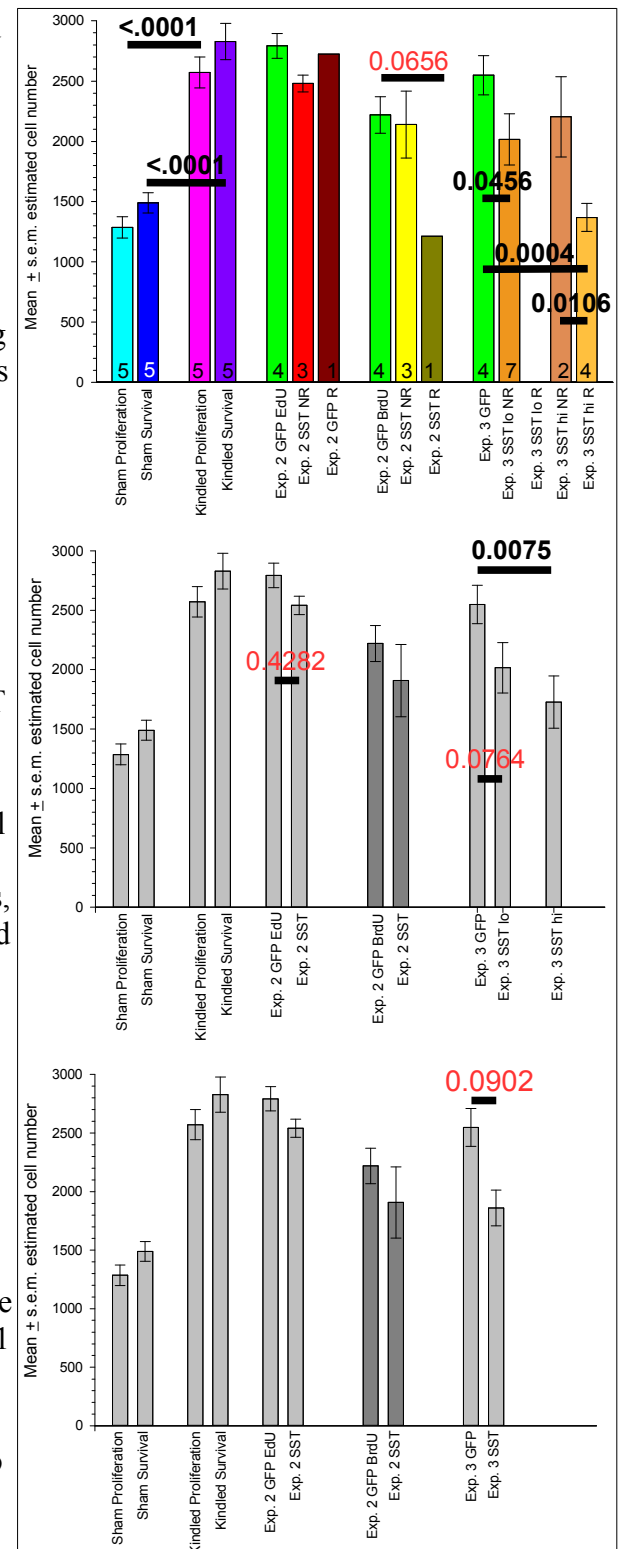


Figure 19: Top, thymidine labeled cell numbers across experiment, vector dose, and responder classification. N at bar base. Middle, the same data pooling responders and nonresponders. Bottom, same data collapsed over vector dose and responder classification. Experiment 1 (no vector) data included for comparison.

was a significant interaction (df 4,13 Mean Square 346532.4707, Type III SS 86633.1177, $F=3.99$, $Pr > F 0.0251$) but no discernable kindling or transgene effects. The fraction of mature “neurons” with BrdU and NeuN but not DCX showed a significant experiment x transgene x dose x responder interaction (df 4,13 Type III SS 0.24627278, Mean Square 0.06156819, $F=5.28$, $Pr > F 0.0095$), such that Experiment 2 subgroups had higher proportions of than Experiment 1 groups, but no kindling or transgene comparisons were significantly different. There was a robust experiment x transgene x dose x responder interaction for the estimated total number of surviving mature neurons (df 4,13 Type III SS 3823225.914, Mean Square 955806.479, $F=5.87$, $Pr > F 0.0063$), with a strong kindling effect consistent in the GFP and SST NR subgroups (Figure 23 F). The value for the lone responder was close to the estimate for sham controls.

IX. SST gene transfer does not alter the inflammatory response observed in the dentate gyrus of kindled rats. Estimated numbers of Iba1+ microglia in the dentate gyrus granule cell layer (GCL) were stable across the survival intervals in Experiment 1 sham and kindled groups (Figure 24A). Numbers were significantly elevated in kindled rats at both time points. The kindling effect was not observed in the GFP vector group, and no transgene effects were detected in SST responders or non-responders defined by average seizure score ≤ 2 over all original current level post-tests. The kindling effect was mediated by a transient decrease in the number (Figure 24B) and proportion (Figure 24D) of resting microglia (Iba1+, CD11b-) that showed significant recovery over the survival interval (ovals). Numbers of resting microglia at the long survival were not different from sham-kindled animals. Increased numbers of activated microglia (CD11b+) were observed in kindled animals (Figure 24C) and did not change significantly over survival time. The fraction of activated microglia did show significant recovery but remained elevated compared to sham controls (Figure 24E). Experiment 3 vector animals showed less of a kindling effect and no transgene effect even in the high titer responder subgroup.

In the dentate hilus kindling did not stimulate a significant immediate increase in the total estimated number of microglia (Figure 24F), but after long survival higher numbers were present compared to sham-kindled long survival animals (but not short survival kindled rats). Vector animals in Experiment 3 were not significantly different from either sham or

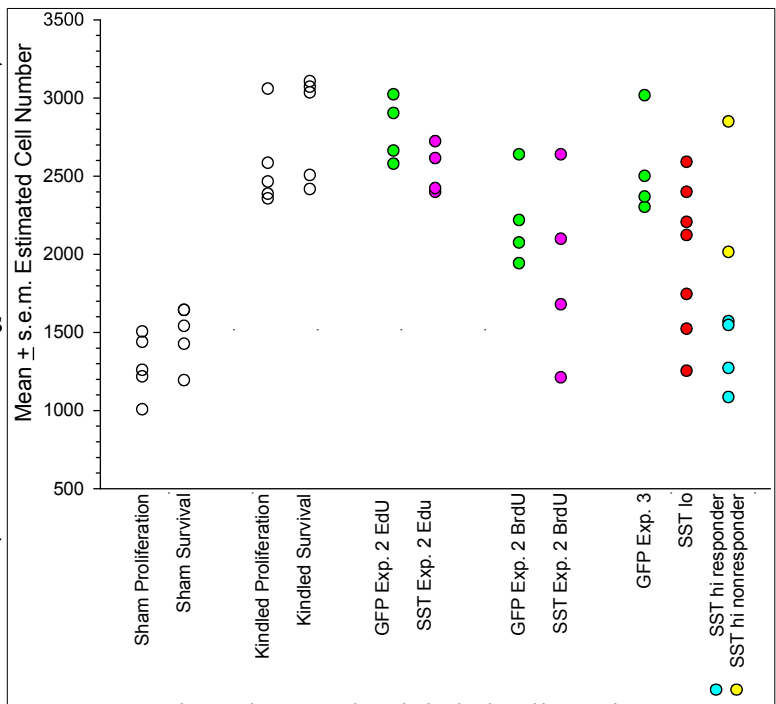


Figure 20: Thymidine analog labeled cell number estimates showed considerable variability that tended to be greater in SST vector subgroups, and in line with variable therapeutic efficacy.

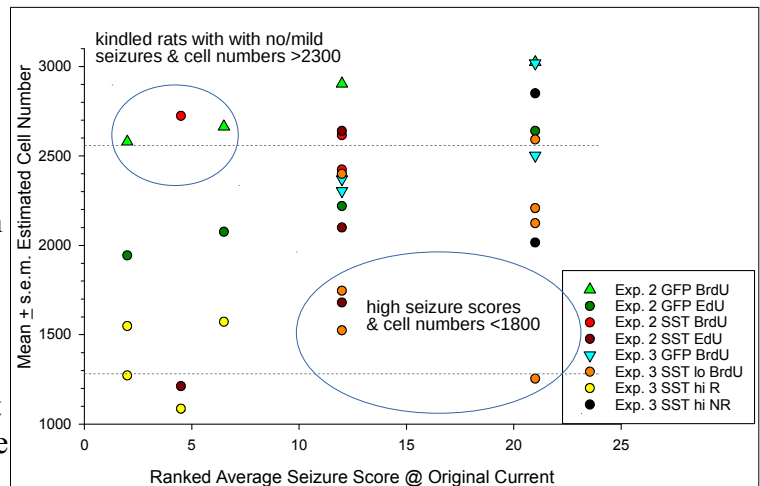
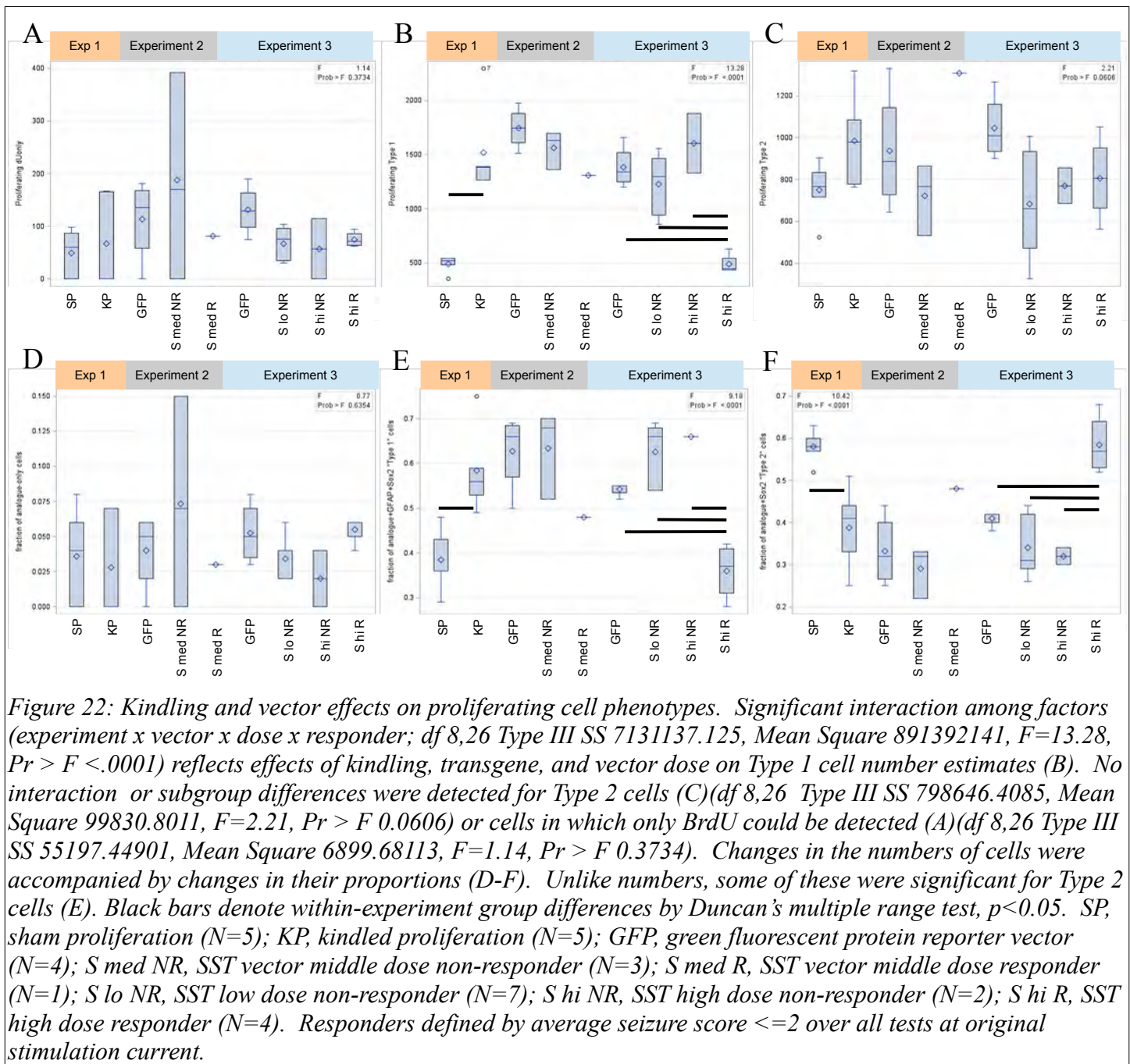


Figure 21: Although 5 SST vector rats showed a robust therapeutic reduction in seizure severity ($AvOrigI < 2$), 1 sustained proliferation above the average (upper dashed line) for kindled rats (left oval). Two GFP vector rats also showed proliferation above the average kindling effect but little or no susceptibility to seizures. Other rats showed little or no SST vector effect on seizures but exhibited low proliferation, in 1 case below the average for unkindled rats (lower dashed line) with no seizure reduction at all.



kindled groups, and showed no transgene effects. As in the GCL, resting hilus microglial numbers and fractions were reduced by kindling (Figure 24G, I) and showed significant recovery (oval). Vector animals (kindled) tended to show these differences, although the lowest titer subgroup (7/7 non-responders) showed reduced numbers even from GFP controls, and responders (4/6 highest-titer rats) did not demonstrate a significant restoration. Activated microglial numbers were also similar in hilus as in GCL, with a clear immediate increase in numbers (Figure 24H) and fraction (Figure 24J), and significant recovery in the fraction but not number. Vector rats showed less robust hilar microglial activation than kindled Experiment 1 rats. No transgene effects were observed for number, but higher fractions of activated microglia were found in SST non-responder subgroups (both low and high titer) compared with GFP controls. SST responders were not different from GFP or non-responder rats.

X. 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,

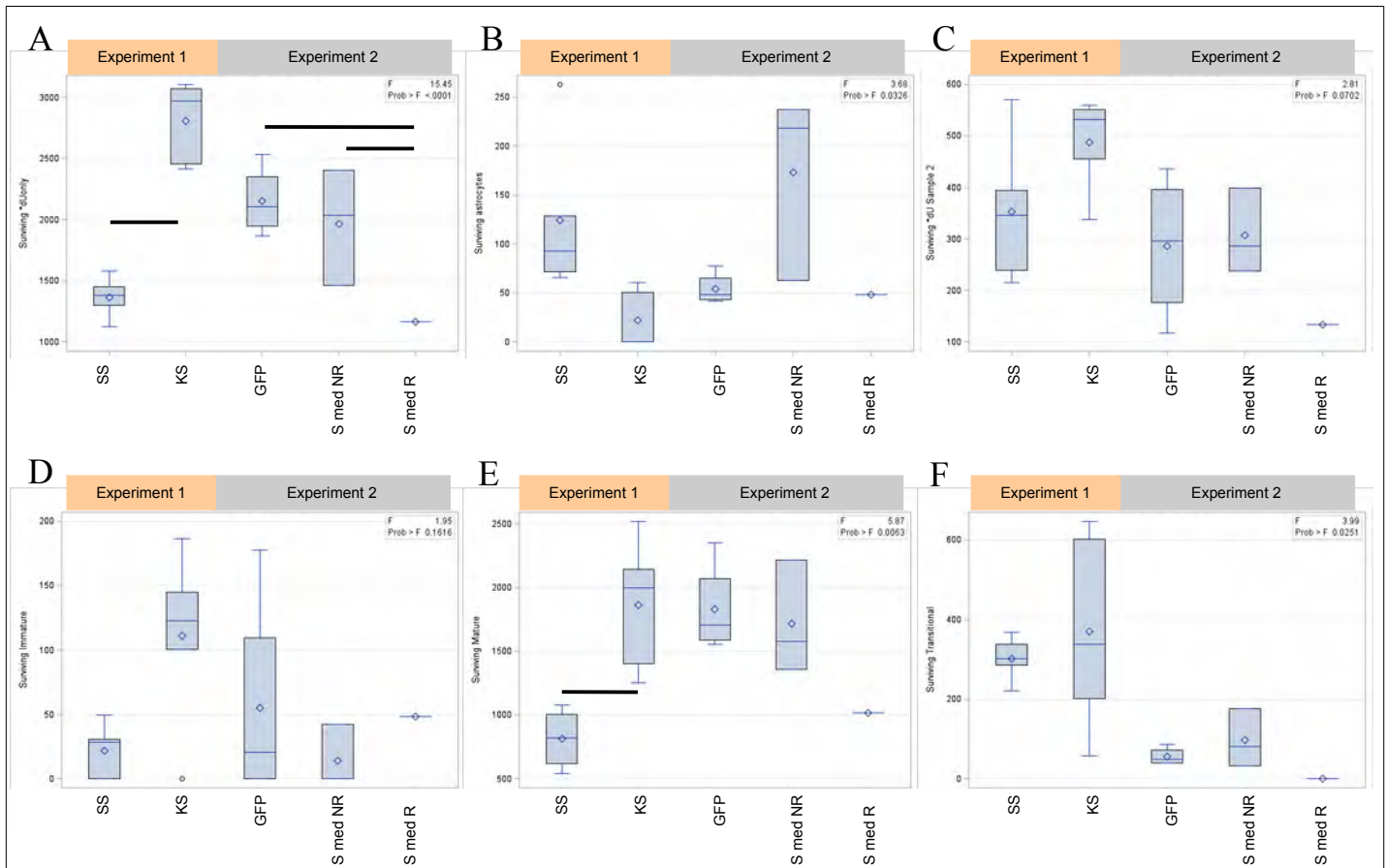


Figure 23: Kindling and vector effects on phenotypes of cells surviving 4 weeks after BrdU injection. Black bars denote within-experiment group differences by Duncan's multiple range test, $p < 0.05$. Survival 4 weeks after BrdU injection revealed that kindling increased the estimated number of cells that were immunoreactive for BrdU but not glial markers (A). Fewer were found in the GFP than the kindled group, and the single responder in Experiment 2 did not differ from sham-kindled controls but had fewer cells than GFP or non-responder experiment 2 rats. The number of astrocytes differed only between the KS and SST non-responders (B), suggesting a possible vector effect independent of effects on seizure susceptibility. Within or across experiments 1 and 2 no kindling effect was observed on the estimated number of BrdU-labeled cells that were not immunoreactive for neuronal differentiation markers (C), but the number was reduced in the direction of normal in the single responder animal. Estimates of immature neurons did not differ by kindling or vector, or responder classification (D). Kindling elevated the estimated number of mature neurons (E), with equivalent levels in experiment 2 GFP controls, and a value equivalent to normal (SS) in the responder animal. Kindling had no effect on transitioning neuron number (F). Both GFP and the SST responder had lower estimates than the experiment 1 kindled survival group, but did not differ from sham-kindled or SST non-responder groups. The validity of the Duncan result for S med R ($N=1$) is limited but vector effects tend to indicate a return toward SS control levels. Survival of 4 weeks did not alter numbers compared to proliferation assessed at 4 hours (Figure 20). SS, sham survival ($N=5$); KS, kindled survival ($N=5$); GFP, green fluorescent protein reporter vector ($N=4$); S med NR, SST vector middle dose non-responder ($N=3$); S med R, SST vector middle dose responder ($N=1$). Responders defined by average seizure score ≤ 2 over all tests at original stimulation current. Comparisons not shown are not significant.

CA3, or CA1, even unilaterally (Figure 25). Conversely, as we increased the numbers of animals tested, we observed animals that did not show a therapeutic response despite robust bilateral expression in one or more subregion. These results encourage hope that therapeutic efficacy may not require transduction of large brain volumes (e.g. entire human hippocampus or temporal lobe), but reinforce that understanding optimal application parameters will require a better understanding of functional effects on distributed synaptic networks.

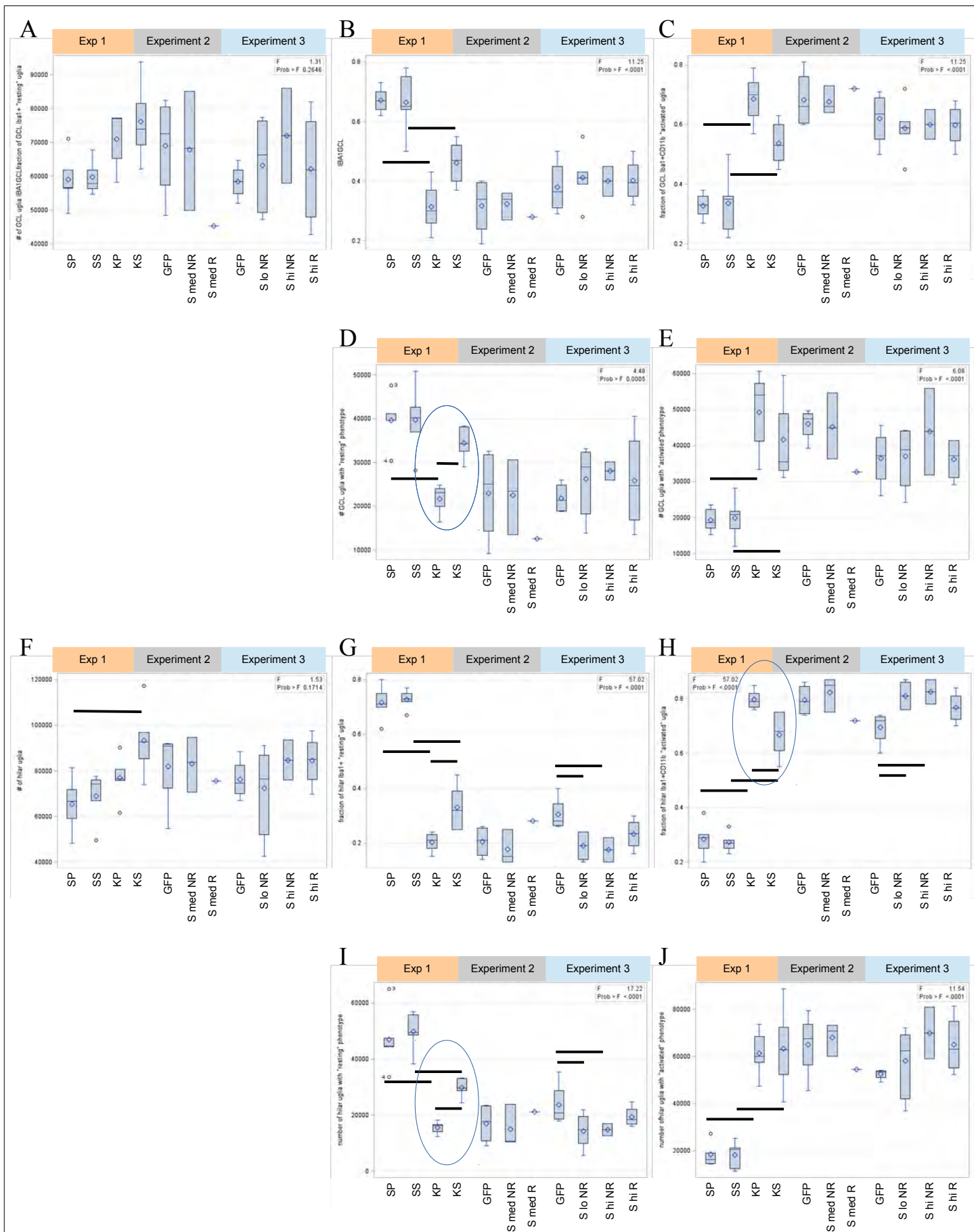


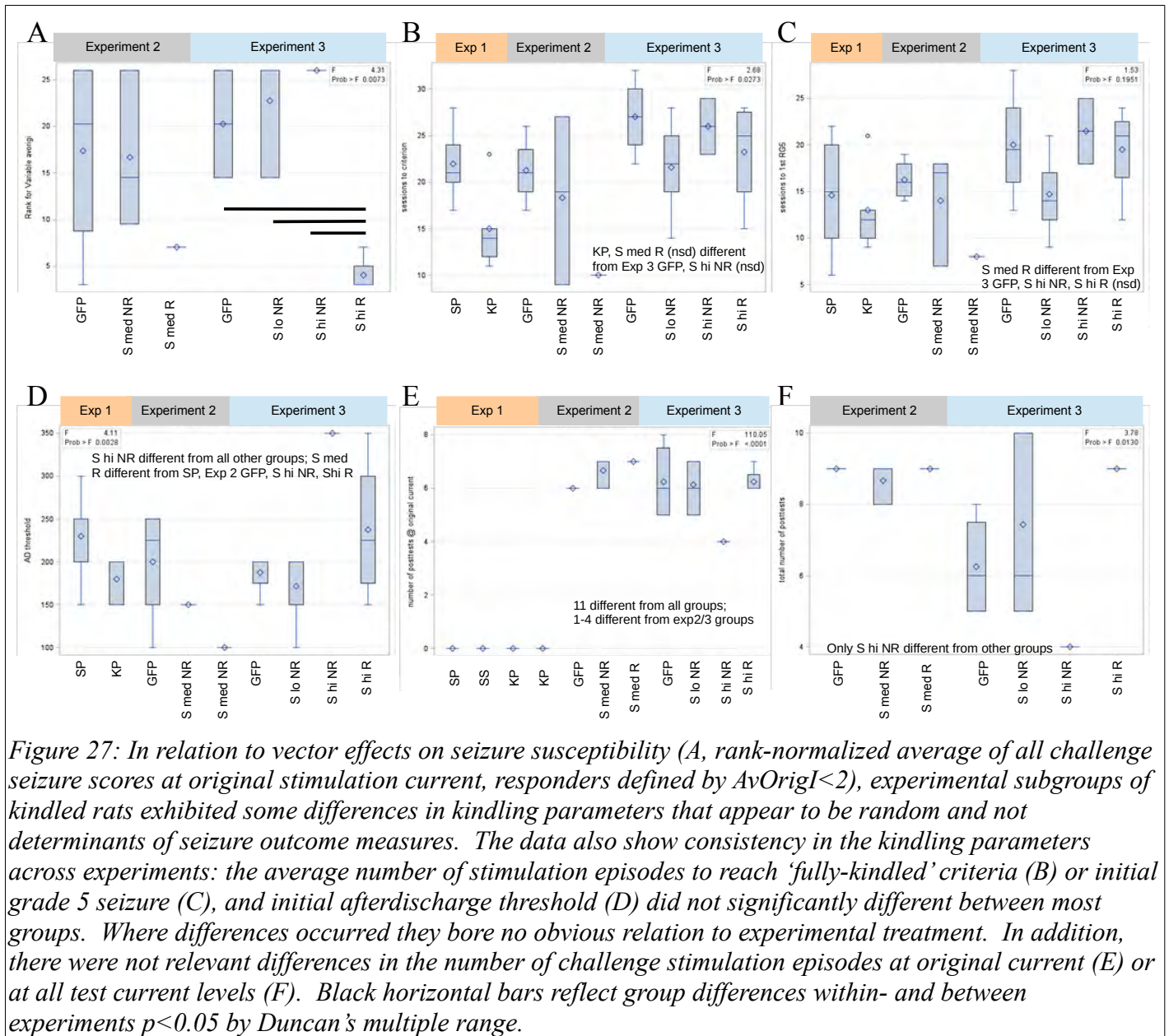
Figure 24: Kindling and gene transfer effects on dentate gyrus microglia.

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 25: Qualitative transgene expression and localization in relation to reduction of first challenge seizure score to 0 ('responder'). No single subregion is necessary or sufficient for a therapeutic response.

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 26: 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. From Natarajan et al. '17.



XI. Therapeutic efficacy may be vector dose-dependent. Due to the limited yield (ca. 500 ul) of AAV vector preparations with the methods used, 3 preparations were needed to generate the animals used to date. Rats received equivalent volumes of one of 3 different SST vector preparations required to complete the efficacy study. Inherent variation in the titer of purified vector preps thus led to 3 subpopulations that received different numbers of AAV genomes infused. Assuming that each preparation is equally potent with respect to the transfection efficiency (i.e. multiplicity of infection), the 3 subgroups can be examined for possible transgene dose effects. For the lowest titer, 7/7 rats failed to show consistent evidence of a therapeutic response (a reduction from 3 consecutive seizure scores of 5 to an average seizure score ≤ 2 over all challenges at original stimulation current). For the middle titer 1/4 rats was a clear responder, and 4/6 were responders in the highest titer subgroup. As shown above (Figures 17-19), therapeutic efficacy could be missed if data were not segregated by responder status and SST vector dose. An adaptive regression procedure was performed to examine the relative contribution of each independent variable on whether or not an animal that received AAV vector became a responder (defined as $AvOrigI \leq 1$ for this analysis). For all Experiment 2 and 3 animals (N=17), the 2 most important variables were number of high-grade seizures experienced during kindling (relative value 100) and titer (relative value 79.88). Thus despite insufficient formal dose-response data the

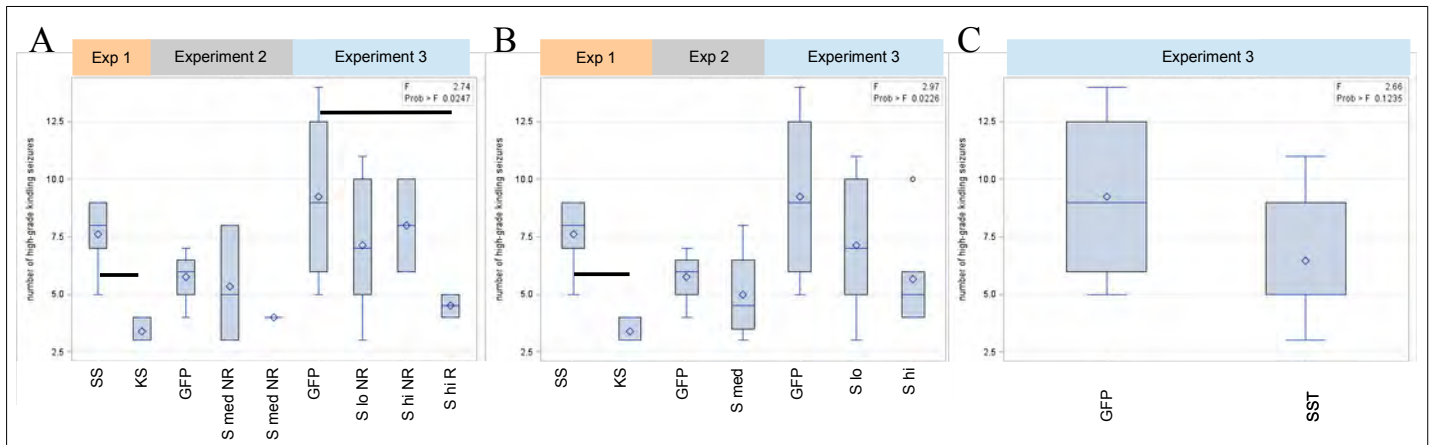


Figure 28: The number of high-grade seizures experienced during kindling sessions was lower in responders that received the high titer SST vector, when defined by $AvOrigI < 2$. Significant differences among Exp. 3 responder subgroups are lost when responder (B) or vector dose subgroups (C) are pooled. The difference may be important in light of the small number of responders ($N=4$), and because the number of high-grade kindling seizures showed a significant main effect on proliferation/survival but NOT seizure outcome measures. Gene transfer may have worked in responders because those animals had a less developed state of seizure susceptibility related to cumulative seizure history.

results support a positive relationship between vector dose and therapeutic efficacy. They also predict a threshold effect in which efficacy is not detectable below some minimal amount of transfection/transduction.

XII. Failure to show a therapeutic response is not due to delayed expression. Rats that showed no therapeutic efficacy on the initial test often did not show any significant difference on later tests (Figure 2, Table 2, Figure 27). No responders or non-responders showed any indication for a sustained 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.

XIII. Seizure history may distinguish responders from non-responders. Most of the independent variables related to kindling were unrelated to the probability that an animal would exhibit a therapeutic response (Figure 26). However, the number of seizures an animal experienced in the course of becoming fully kindled varied (Figure 28). In the small subset of rats that showed a therapeutic response ($N=4$ in Experiment 3, Figure 28A), the number of seizures experiences during kindling was significantly less than GFP controls, and this difference does not become apparent unless responders are considered (Figure 28 B) along with vector dose (Figure 28 C). In light of the small number of responder animals in this study it must be considered possible that the reversal of seizure susceptibility could be due to inherent impermanence of a kindled state related to a milder cumulative seizure history, rather than to any therapeutic effect of the SST vector.

XIV. 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 (Figure 29) or retention (Figure 30) deficits, but demonstrated reversal learning deficits like those detected in rats that did not receive vectors (Figure 31). Despite a clear trend SST vector rats did not show significantly different reversal learning (daily change) compared with GFP vector rats, but they did reach a higher level of proficiency (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 (Figure 32). 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.

XV. Neither kindling nor AAV vector transduction induce significant neuronal loss. A sample of sections was examined for the presence of neurons injured or recently dead using the fluorescent dye fluoro-Jade C (FJC). Few examples of labeled cells were observed following application of methods we have used in multiple

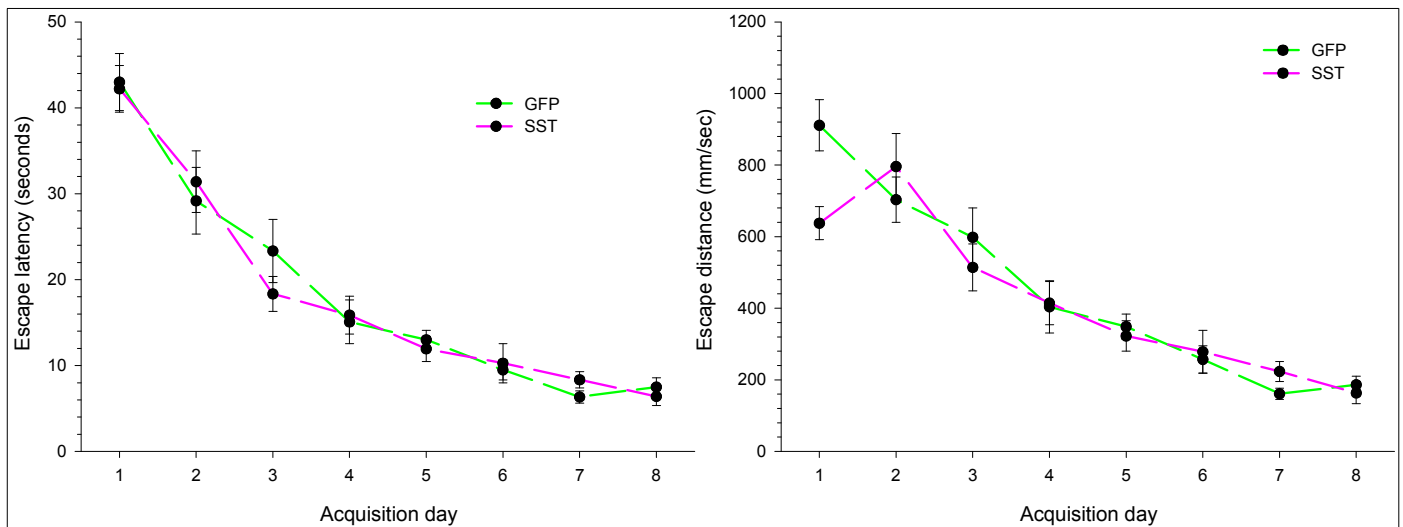


Figure 29: Left: During water maze acquisition trials, escape time did not differ between vector groups. SAS Proc GLM ANOVA for independent factor 'vector' $F(1,215)=0.84$ $Pr>F$ 0.3719. Both groups showed robust acquisition of the escape task to equal proficiency. Right: Results were similar for distance to effect escape.

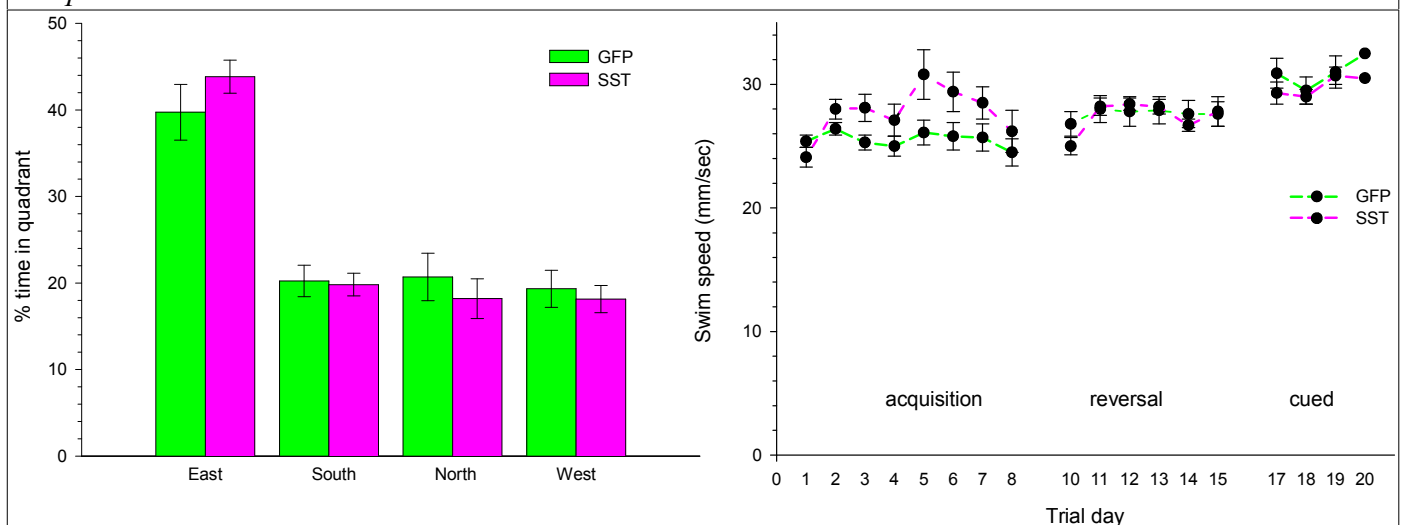


Figure 30: Left: During retention trials 2 days after acquisition training, both vector groups showed equivalent fractions of time in the target and non-target maze quadrants. SAS Proc GLM $F(1,26)=1.32$, $Pr>F$ 0.2612. Right: Swim speed did not differ between vector groups.

studies of traumatic brain tissue injury and neurodegenerative disease. This may be a technical issue related to long storage of tissue sections before they could be processed, but kindling is known to involve little detectable neuronal loss. The objective was to primarily to determine whether vector contributed to any additional neuronal loss as a safety issue. Qualitative examination indicates that FJC staining in SST vector brains was no different from controls, with levels approaching zero for each. With respect to evaluating vector and transgene safety it is notable that SST vector transduction does induce neuronal loss different from GFP vector. Images are not shown as the view is essentially black when no FJC labeled objects are present.

A second approach to qualitatively assess potential neuronal cell loss related to kindling, vector, or transgene made use of Richardson staining, a relatively neuron-selective Nissl method compatible with immunohistochemistry (Figure 33). Comparisons of Richardson staining across a sample of animals from different experimental groups corroborate the FJC observations. Few pyknotic neurons (injured/dead) were evident in any animal. No qualitative evidence was observed for kindling or vector-related effects on cell body layer width, area, or cell density. There was no obvious shrinkage of neuropil layers or hippocampal transverse

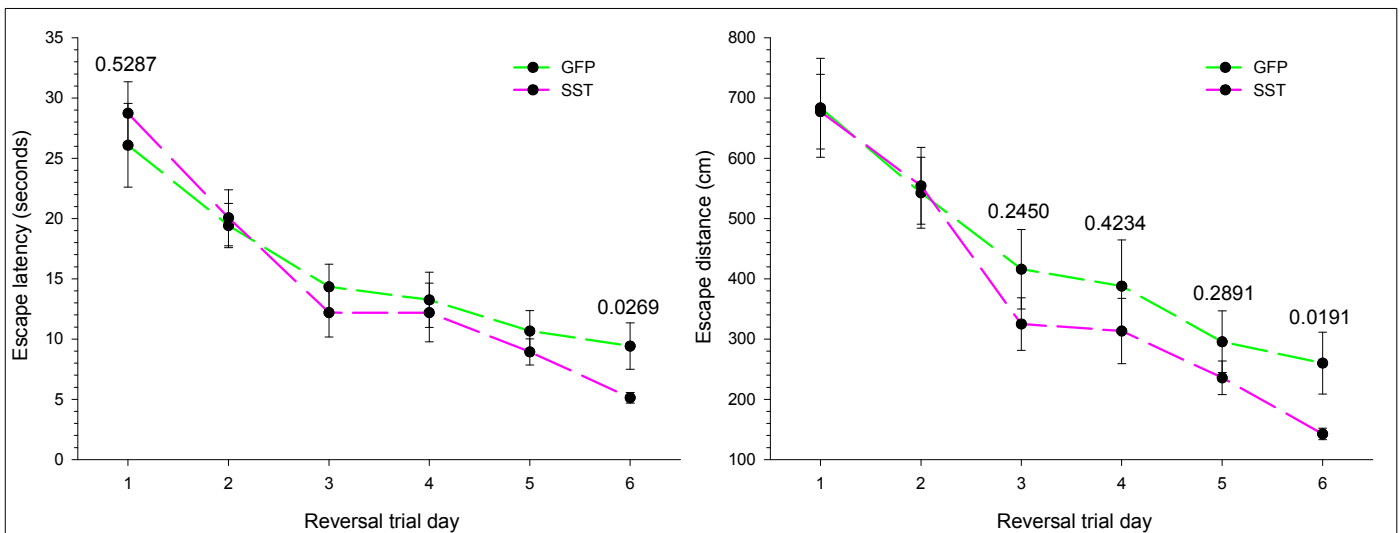


Figure 31: Left: Both vector groups showed robust learning over reversal training days. SAS Proc GLM repeated measures ANOVA $F(5,125)=27.58$, $Pr>F<.0001$. There was no significant effect of vector ($F(1,25)=0.33$, $Pr>F 0.5715$), or interaction between vector and trial day ($F(5,125)=0.74$, $Pr>F 0.5974$). 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 $\times$ 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 $F(1,26)=6.28$, $Pr>F 0.0191$.

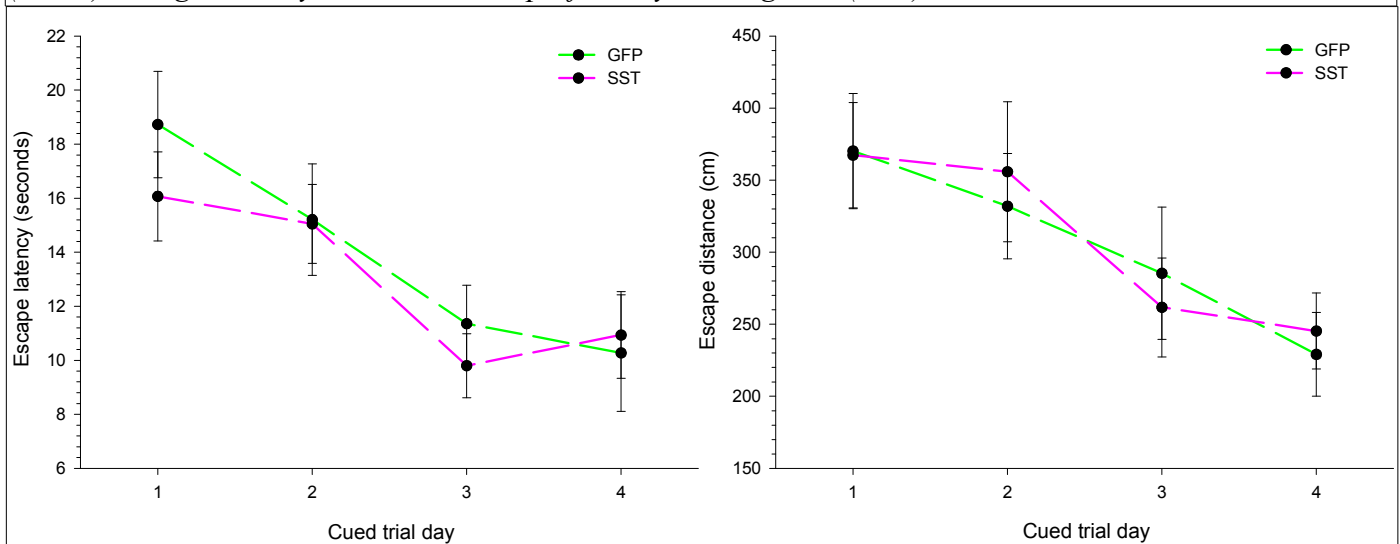


Figure 32: Left: During cued escape trials both vector groups showed improvement over trial days ($F(3,75)=7.35$, $Pr>F 0.0002$) and reached equivalent proficiency. SAS Proc GLM repeated measures ANOVA found no effect of independent factor 'vector' ($F(1,25)=0.87$, $Pr>F 0.3586$), nor were interactions 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.

width. With respect to evaluating vector and transgene safety it is notable that SST vector transduction does not produce any detectable pathology in relation to GFP vector.

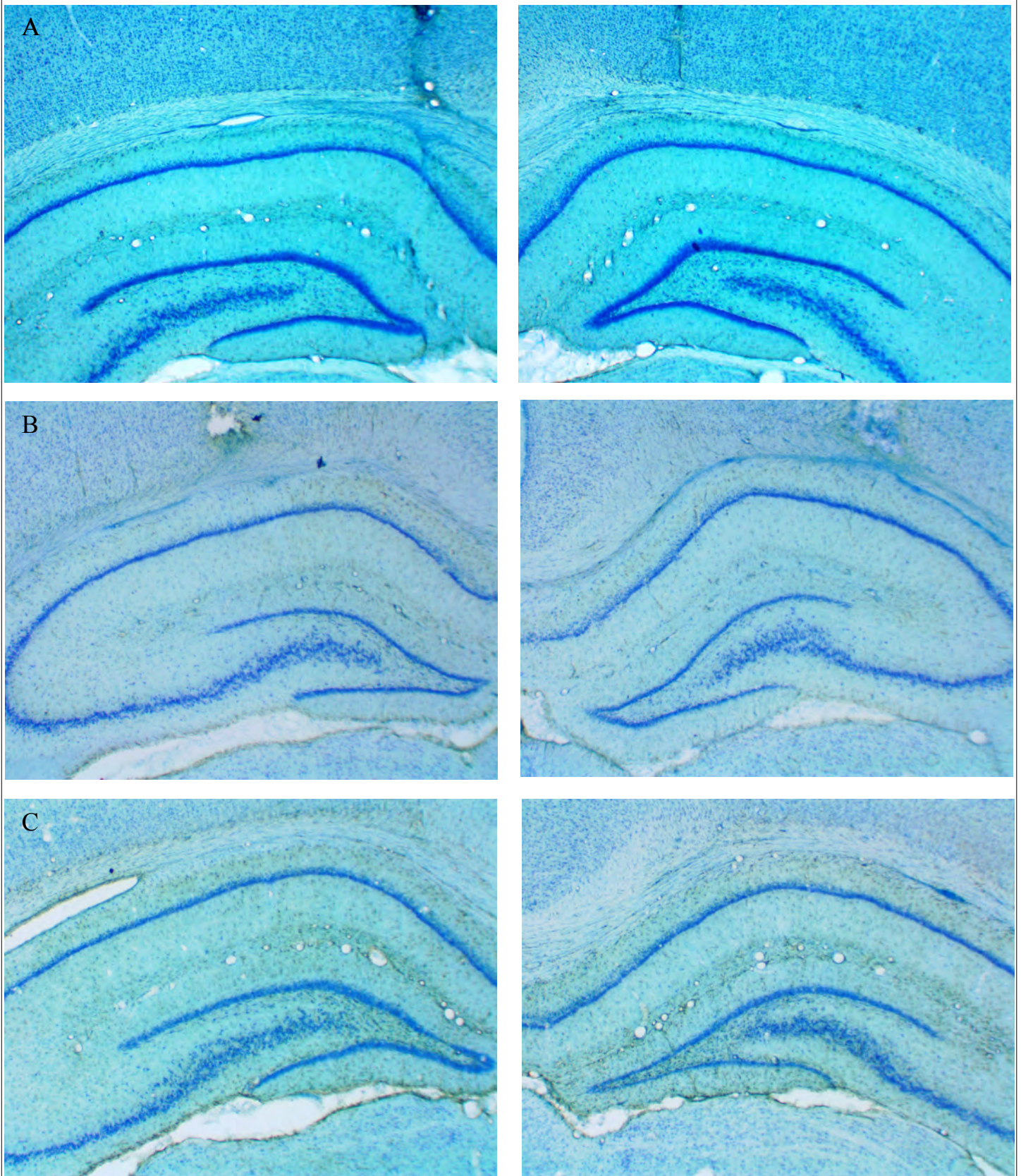


Figure 33: Qualitative evaluation of hippocampal neuronal populations with Richardson's stain and astrocytosis with GFAP. A, GFP vector; B, SST responder; C, SST non-responder. No remarkable differences were observed in cell layer thickness or cell density in any hippocampal subregion; 2X magnification.

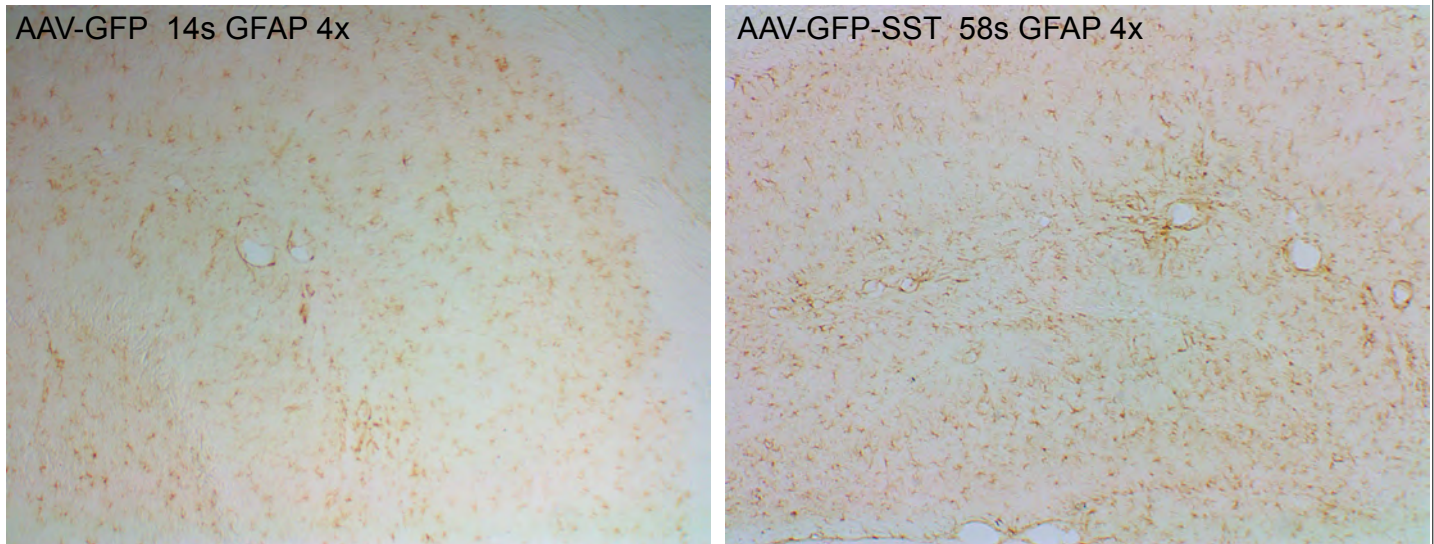


Figure 34: Immunolabeling for glial acidic fibrillary protein (GFAP) in brain sections near injection sites revealed little astrogliosis reflected by reactive cellular morphology or any overall increase in tissue immunoreactivity. Left, control GFP vector; right, SST vector responder.

XVI. Neither kindling nor AAV vector transduction induce significant astrogliosis. Astrocyte labeling with antibodies to the cytoskeletal marker glial fibrillary acidic protein (GFAP) in young adult rats varies considerably both regionally with animals and between animals (Figure 34). Increased GFAP labeling intensity, especially accompanied by characteristic changes in astrocyte morphology, can denote brain injury. No evidence was observed of differences between animals related to kindling, gene transfer, transgene, or seizure susceptibility responder status. With respect to evaluating vector and transgene safety it is notable that SST vector transduction does not induce astrogliosis different from GFP vector.

XVII. Neither kindling nor AAV vector transduction induce significant microgliosis. Microglial labeling with Iba1 (Figure 35) revealed a large hippocampal population that tended to be uniform in size, morphology, and labeling intensity within and across animals. This indicates at most a mild microglial activation. Like the quantitative data, there did not appear to be any significant relationship between rats that showed a therapeutic response on seizures and any microglial properties. With respect to evaluating vector and transgene safety it is notable that SST vector transduction does not induce microglial activation different from GFP vector. SST vector transduction does induce microgliosis different from GFP vector.

XVIII. Gene transfer did not alter kindling-related phagocytic microglial activation. Immunolabeling with phagocyte (activated microglia and macrophage) marker CD68 was elevated in kindled and vector-infused animals (Figure 36). Phagocytic cells tended to be most abundant in the subgranular layer of the dentate gyrus where neurogenesis occurs, and along needle tracks from vector infusion injury. No clear relationship was evident between therapeutic response and either numbers or location of phagocytic microglia. However, SST vector transduction did not induce phagocytosis different from GFP vector, indicating that SST overexpression did not have any pathological effect or interaction.

XIX. Oxidative stress was observed in kindled rats but less evident in SST responder animals. Immunolabeling with a polyclonal antibody to the lipid peroxidation marker 4-hydroxynonenal (4HNE) was observed in kindled animals, standing out from background and non-specific labeling by several distinctive features (Figure 37). In hippocampus the labeling often filled neuronal perikarya and dendritic arborizations, with dendrites often more intensely immunoreactive than cell bodies. Oxidative stress marker immunolabeling was observed in GFP and SST non-responder animals but not observed in SST vector responders. Verification

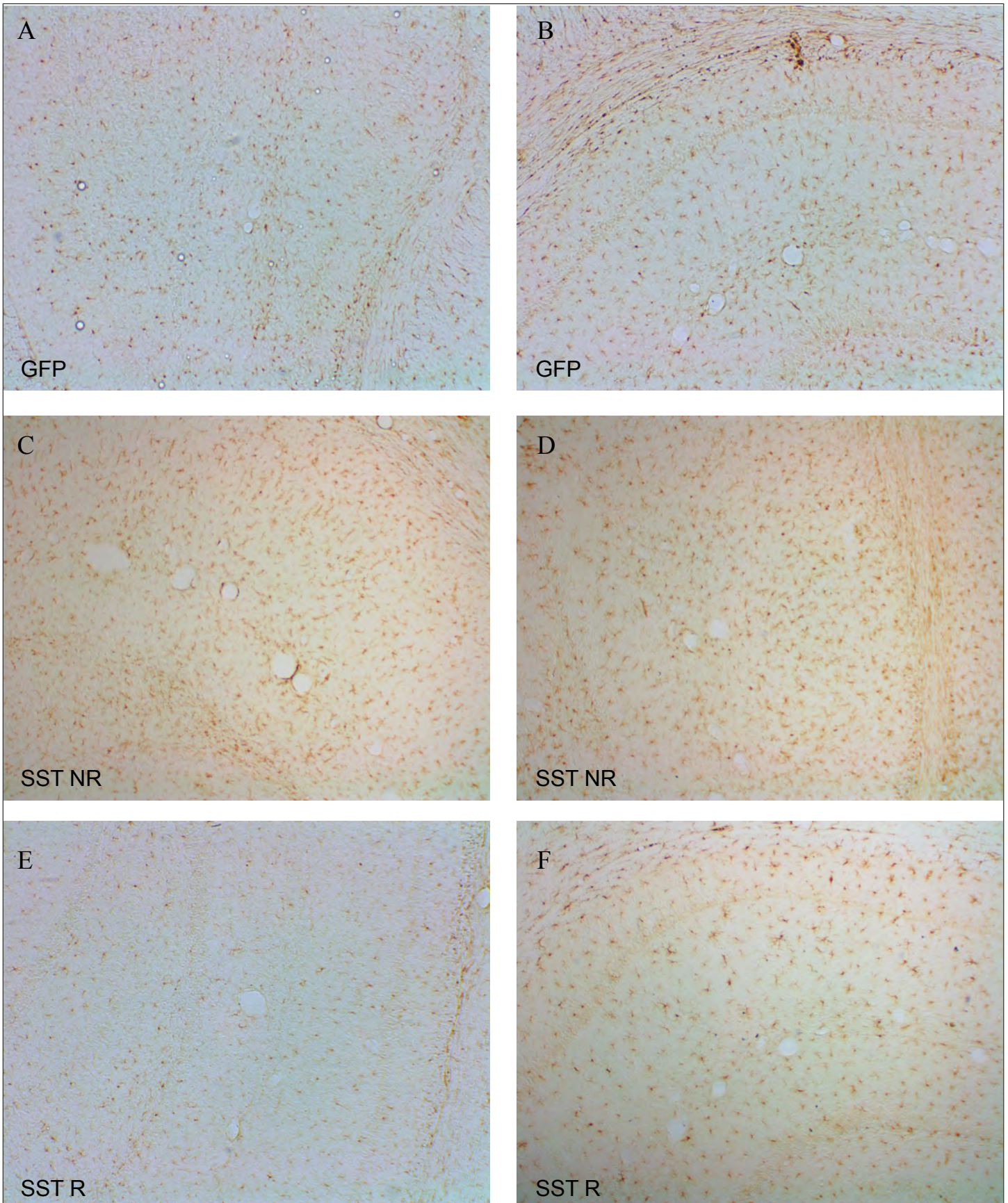


Figure 37: Iba1 immunolabeling in hippocampus (4x) does not appear to vary systematically by vector or responder condition in kindled rats. Neither the number nor the morphological distribution show distinct differences related to gene transfer or seizure susceptibility response.

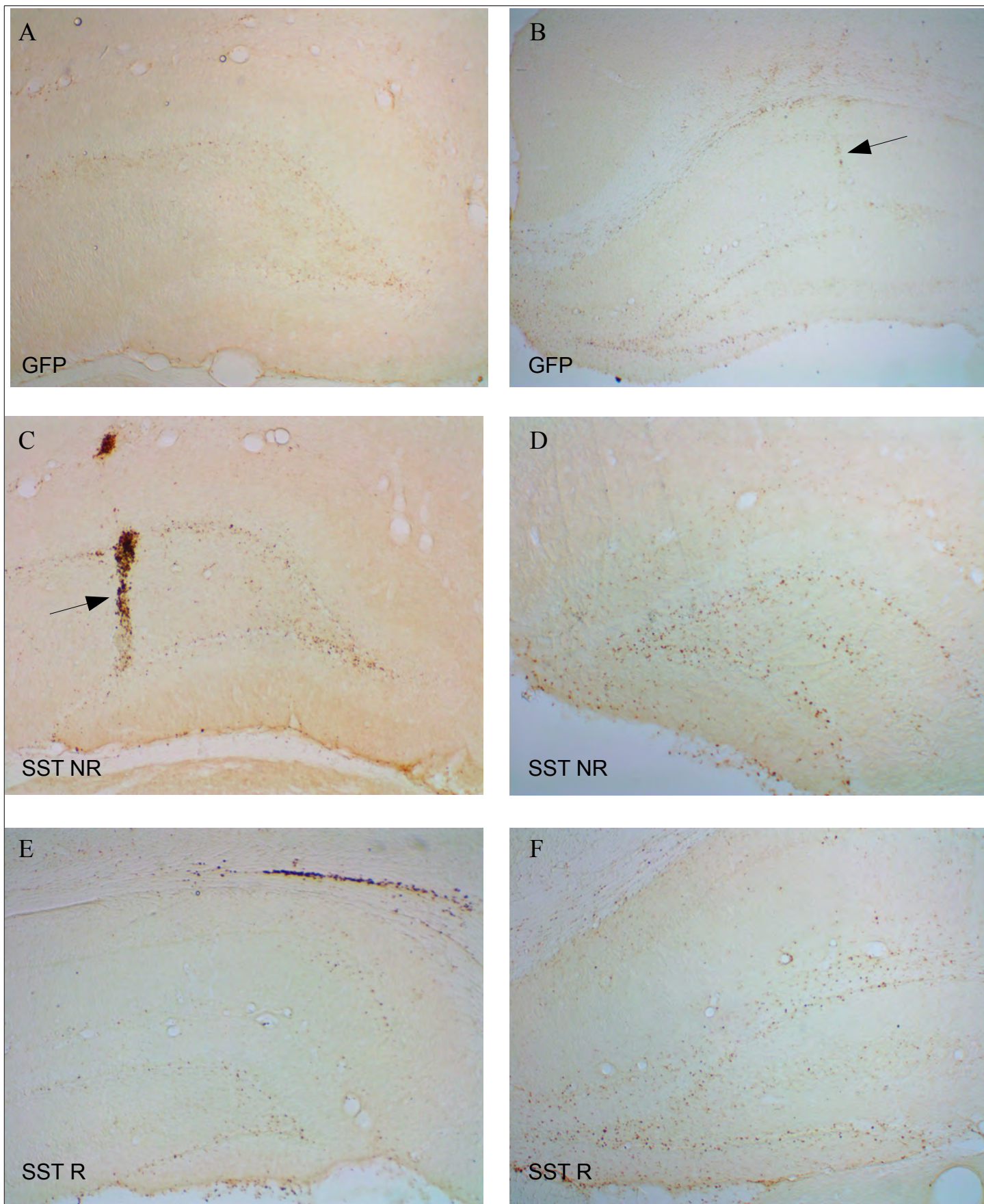


Figure 35: CD68 immunolabeling shows hippocampal phagocytic microglia. Interanimal variability and small numbers of animals prevent discrimination of transgene or responder subgroup differences. Arrows indicate vector infusion needle track injury.

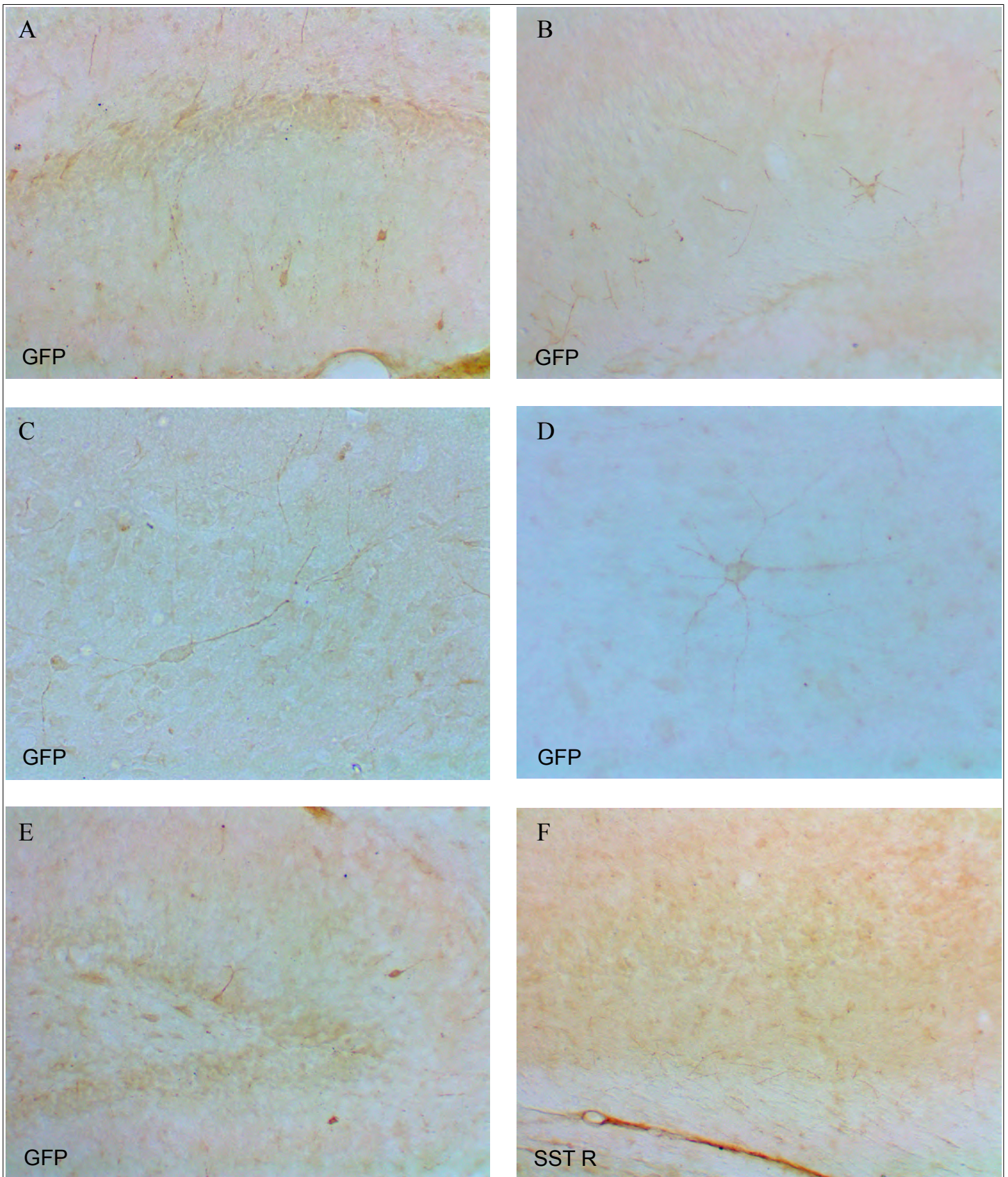


Figure 36: 4HNE immunolabeling in dentate gyrus of kindled rats shows neurons observed in the subgranular and molecular layers in GFP (A-E) and SST non-responders (not shown) but not in responders (F) 10x

of this possible correlate and its relevance to therapeutic mechanisms of SST will require additional study, but lipid peroxidation has been reported in the rat amygdala kindling model (Frantseva MV1, Perez Velazquez JL, Tsoraklidis G, Mendonca AJ, Adamchik Y, Mills LR, Carlen PL, Burnham MW. Oxidative stress is involved in seizure-induced neurodegeneration in the kindling model of epilepsy. *Neuroscience*. 2000;97(3):431-5).

4) other achievements

Gowri Natarajan successfully defended her doctoral dissertation (Appendix) December 2016. Jeffrey Leibowitz successfully defended his doctoral dissertation in November 2017.

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 funding 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 submitted a proposal in August 2017 for R21 funding to the NIH. Proposal “Somatostatin gene transfer for seizures in animal models of Alzheimer's disease” did not receive a score encouraging for resubmission.

CoPIs Carney and King, and Dr. Natarajan, collaborated with Drs. Sanford and Shannon Boye at UF to develop more sophisticated SST vectors in anticipation of further translational studies:

- We tested an alternative fluorescent reporter, mCherry, that may have less potential for biological activity than GFP. GFP toxicity has been reported in some model systems, although none of the standard measures for cell loss or tissue injury appear to occur with vectors we have studied.
- We examined capsid variants (Y436+719F) that might transduce more vigorously or in more cells for a given administered dose, relative to wild-type AAV5 or AAV2 serotypes. 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.
- We examined self-complimentary (sc) transgene constructs that are intended to bypass the need for single-stranded AAV DNA in conventional AAV vectors to form double-stranded DNA essential for gene transcription. Self-complimentary transgene constructs will facilitate more rapid induction of expression with less catabolic loss of vector genomes prior to second strand (DNA) synthesis. This also required substitution of a new constitutive promoter for the relatively large hybrid chicken beta actin/cytomegalovirus promoter in the vectors used for the reported studies. Of 2 tested, the synapsin-1 (syn-1) promoter was superior to a small

chicken beta actin (smCBa) construct. Advanced compact expression promoter constructs can broaden or narrow cellular targeting, as well as facilitate incorporation of reporter genes with minimal 'leaky' expression.

- We examined an alternative strategy to the IRES (internal ribosomal entry site) approach used in our vectors to date. To express both a therapeutic transgene and a reporter gene that can reveal cells making the therapeutic gene, we implemented the PEPS construct (Davies, J., Holter, J.L., Knight, D., Beaucourt, S.M., Murphy, D., Carter, D.A., Wells, T., 2004. Manipulating sorting signals to generate co-expression of somatostatin and eGFP in the regulated secretory pathway from a monocistronic construct. *J Mol Endocrinol* 33, 523-532). This is smaller and may be more reliable than IRES with respect to not producing reporter unless the therapeutic gene is transcribed.
- Pilot studies of these variant vectors were conducted in normal adult rats. The most promising was a self-complimentary expression construct using mCherry reporter driven by synapsin-1 promoter and packaged in capsid variant Y436+719F AAV5. Further testing was suspended when Dr. Natarajan left the project and translational funding proposals based on the improved vectors were unsupported.

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, Director 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 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 neurogenesis and neuroinflammation in brains of rats kindled by Ms. Natarajan. He has performed the histological processing and microscopy necessary to generate the data on cytotogenesis, differentiation, and microglial indices of inflammation. He will also develop expertise in 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*. A manuscript describing kindling and vector effects on dentate gyrus cytogenesis and microglial activation was submitted in June 2018 to *Experimental Neurology*. Two poster presentations were made at the 2016 Society for Neuroscience Annual Meeting, and one at the 2016 Biomedical Engineering Society conference.. Copies of these are appended.

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 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. The potential beneficial effects of the SST vector on cognitive impairment related to a history of seizures could have a substantial impact on epilepsy therapeutics. AAV vector-based delivery of SST gene constructs may prove to be the best route to efficacy, but the result may indicate that alternative methods for engaging therapeutic mechanisms activated by enhanced SST receptor agonist availability could also be effective, and perhaps simpler, safer, and more economical.

What was the impact on other disciplines?

Gene transfer has the potential to treat a variety of neurological conditions. Our studies add to a growing body of evidence that AAV vectors offer safe and enduring supplementation of gene expression in the nervous system. This could easily extend to other neurodegenerative diseases.

The functional role for neurogenesis in the adult brain is not yet clear. The studies from this project contribute to efforts to understand whether and how adult neurogenesis might participate in the development and/or recovery from brain disease or injury.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Epilepsy is a debilitating disease that can compromise the mental abilities of afflicted individuals, and prevent them from conducting normal daily activities. New technologies capable of therapeutic management of epilepsy have the potential to restore independence and self-sufficiency that have not been amenable to existing clinical approaches.

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

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications.

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 rats.

Journal: Epilepsy Res. 2017 Feb;130:81-92. doi: 10.1016/j.epilepsyres.2017.01.002.

Authors: Leibowitz JA, Natarajan G, Zhou, J, King, MA, Carney PR, Ormerod BK.

Title: Sustained somatostatin gene expression reverses kindling-induced increases in the number of dividing Type-1 neural stem cells in the hippocampi of behaviorally responsive rats.

Journal: Experimental Neurology, submitted 6/2018

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

Nothing to Report.

Other publications, conference papers, and presentations. (*Appendix)

Authors: Leibowitz J, Natarajan G, Asokan A, King M, Carney P, Ormerod B.

Title: Kindling Increases Type-1 Progenitor Cell Division in the Dentate Gyrus of Adult Rats

Conference: BMES 2015, Program Number: P-Fr-712

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:	9
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 (to 11/2017)
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 McGuinness
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: This report describes collaboration among the 3 coPIs at laboratories in the University of Florida and the University of North Carolina. All animal studies were conducted jointly at the University of Florida with the exception of year 4 advanced vector development conducted at the University of North Carolina. Contributions to project tasks by each PI adhered to the designations outlined in the approved Statement of Work.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

9. APPENDICES:

1. Natarajan et al. J. Epilepsy Research2016.pdf
2. Leibowitz et al., ExperimentalNeurology2018submitted.pdf
3. Natarajan2016SFN.pdf
4. Leibowitz2016SFN.pdf



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

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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.

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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).

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; Noë 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 preprohormone preproSST is cleaved by proteases into biologically active SST-14, SST-28 and neuronostatin neuropeptides (Bilova et al., 2007; Galanopoulou et al., 1995; Goodman et al., 1983; Samson et al., 2008; Taviani 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 (Bilova 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 corn-cob-lined ventilated shoebox cages located in a standard colony room maintained at $24 \pm 1^\circ\text{C}$ 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.

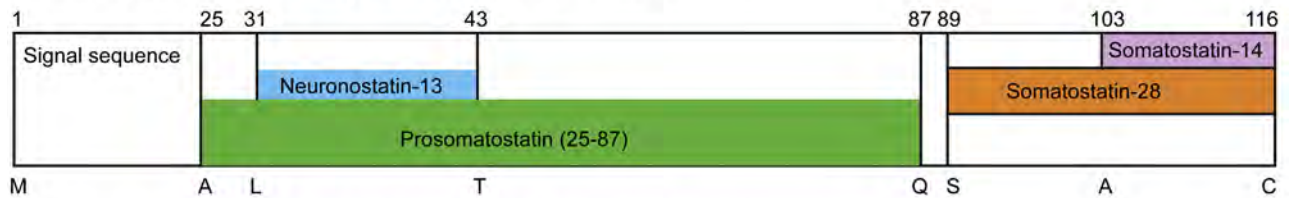
2.2. Bipolar electrode and connector strip preparation

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 $<1.8 \Omega$ were implanted.

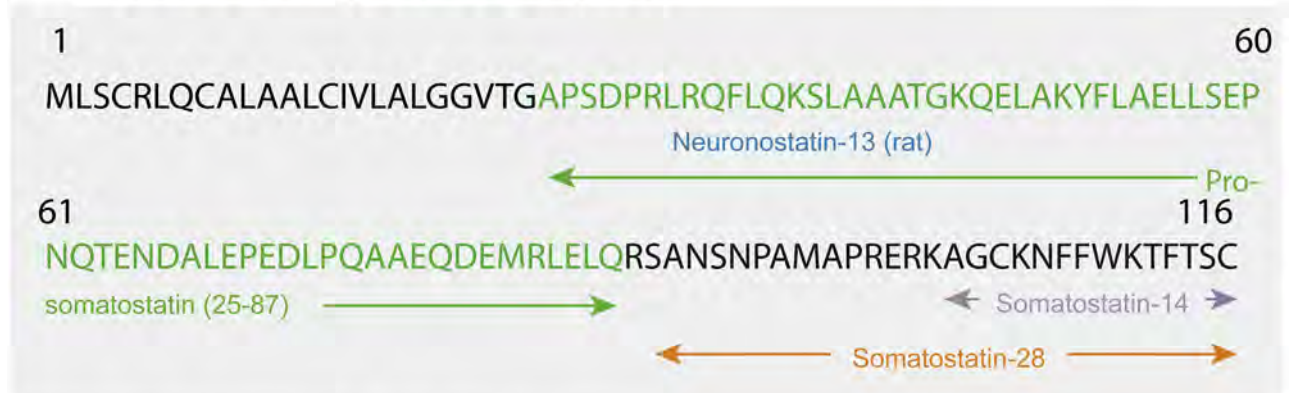
2.3. Surgical implantation of electrodes

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

a. Rat Somatostatin Preprohormone Schematic



b. Rat Somatostatin Preprohormone Sequence



c. AAV5-CBa-PreproSST-eGFP Schematic

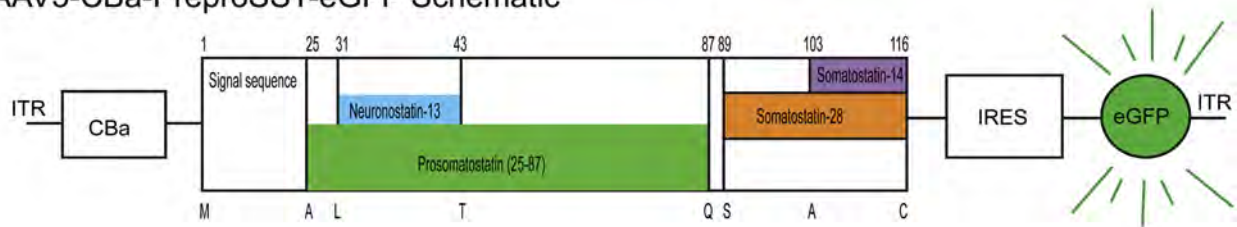


Fig. 1. Representation of the a) preproSST schematic b) preproSST amino acid sequence and c) AAV5-CBa-preproSST-eGFP vector construct. a, b) The 116 amino acid preproSST contains an N-terminal region of 24 amino acids. This signaling sequence directs the translocation of the translated preproSST product 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 μ A and then increased in 50 μ A 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

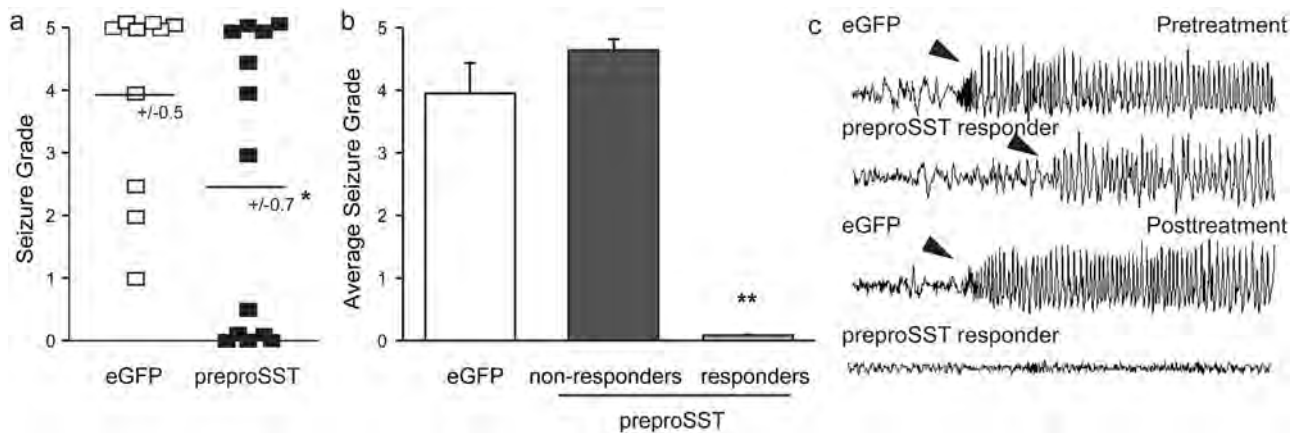


Fig. 2. Sustained preproSST expression prevented induced seizures in a subset of kindled rats. AAV5-CBa-preproSST-eGFP-treated rats ($n = 13$) and AAV5-CBa-eGFP-treated rats ($n = 10$) were given a test session 3 weeks after vector injection at the AD threshold current to test whether sustained preproSST 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 $\pm$ 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 (+50 μ A)	PTS-7 (+50 μ A)	PTS-8 (+100 μ A)
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 μ L Hamilton syringe fitted with a 27G steel needle (Hamilton, Reno, NV) attached to an infusion system (Cole-Parmer, Vernon Hills, IL). A 2 μ L volume of the vector was delivered in each of 4 injection sites (i.e. 2 μ L 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 μ L/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 μ A, +50 μ A, +100 μ A above the AD threshold current) on the final 3 post-test sessions. As with kindling sessions, the synchro-

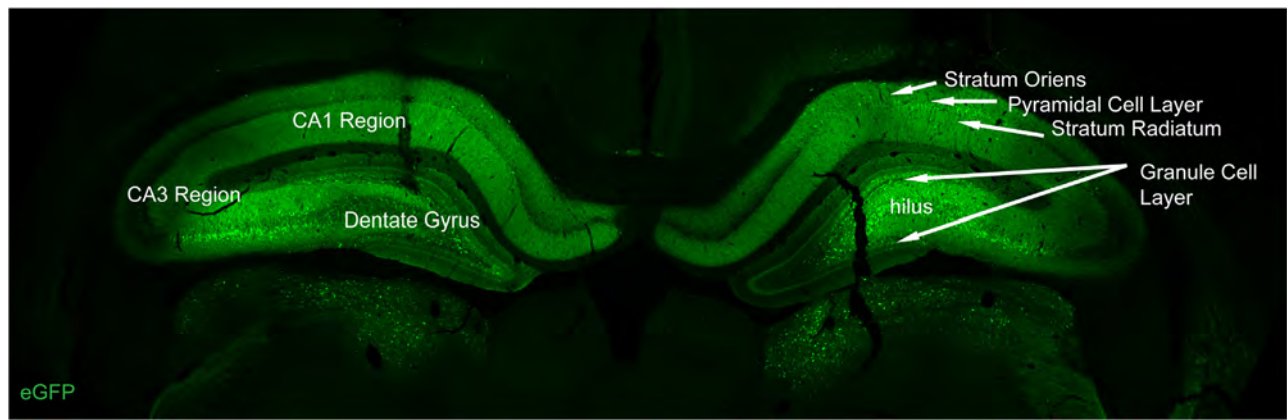


Fig. 3. AAV5-CBa-preproSST-eGFP transduction within the hippocampus of a rat in the preproSST vector-treated group. Robust eGFP expression was observed bilaterally within the dentate hilus, granule cell layer, CA3 region and CA1 region of the hippocampus. In this rat a relatively small number of thalamic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

nized behavioral and EEG data for all rats in test and post-test sessions administered after vector treatment were scored blind to 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 μ m 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 $\times$ objective and 2.3 $\times$ 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 Wallance 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 Wallance 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 $243.5 \pm 16.4 \mu\text{A}$. 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 = $220 \pm 21.3 \mu\text{A}$ and preproSST = $261.5 \pm 23.4 \mu\text{A}$; $U = 46.5$, $n_1 = 10$, $n_2 = 13$, $p = 0.20$). 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 $50 \mu\text{A}$, $50 \mu\text{A}$ and then $100 \mu\text{A}$ 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

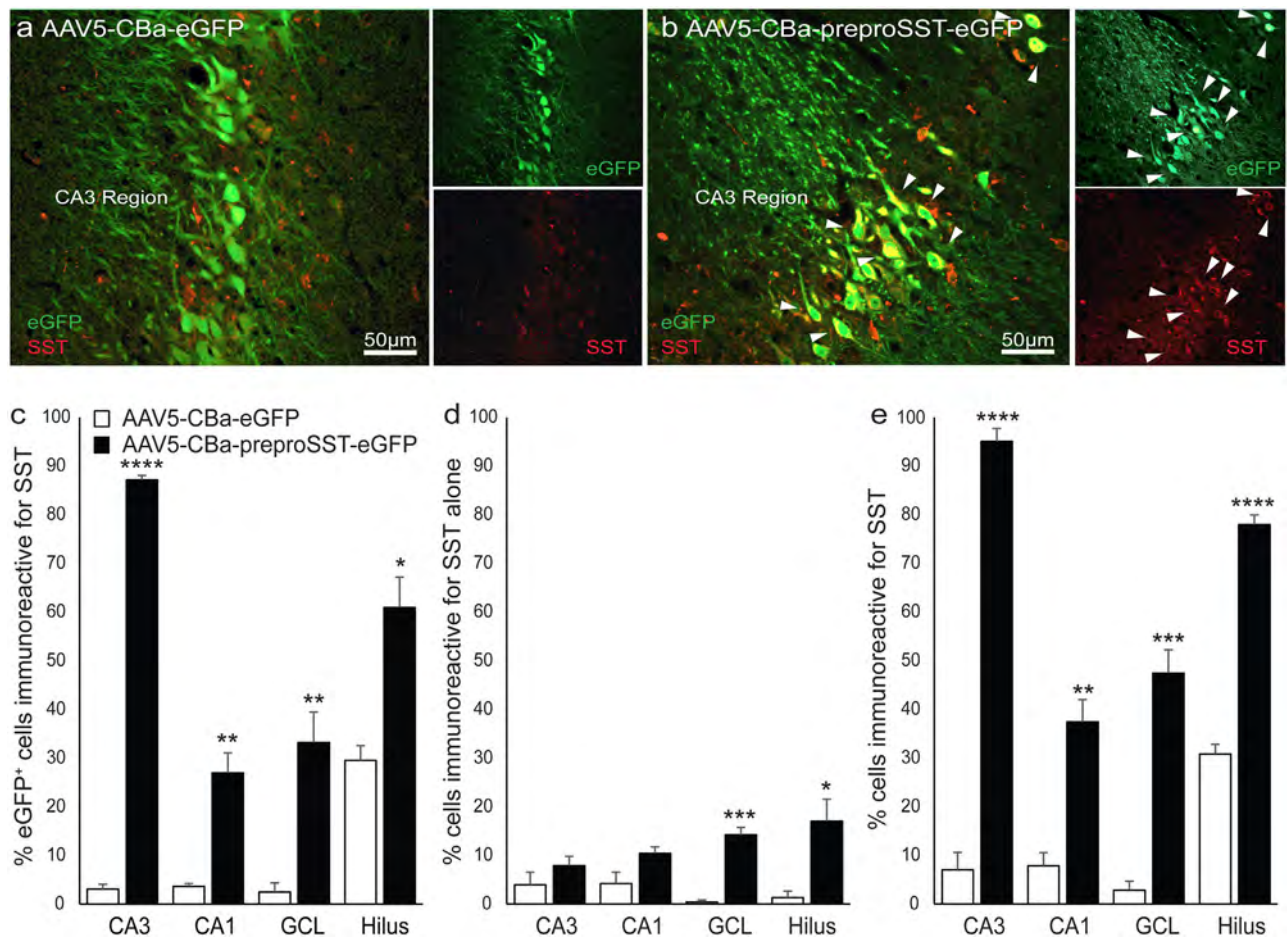


Fig. 4. Coincident eGFP and SST localization was consistent with vector transduction of hippocampal principal neurons. a, b) Examples of native eGFP⁺ neurons (in green), eGFP⁺/SST⁺ (both green and red) and SST⁺ (in red) neurons found in the CA3 hippocampal region of rats treated with the a) control or 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 μ m. 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).

cell layer, stratum oriens and stratum radiatum) and the CA3 region (predominantly in the pyramidal cell layer) and in several cases, the 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 pep-

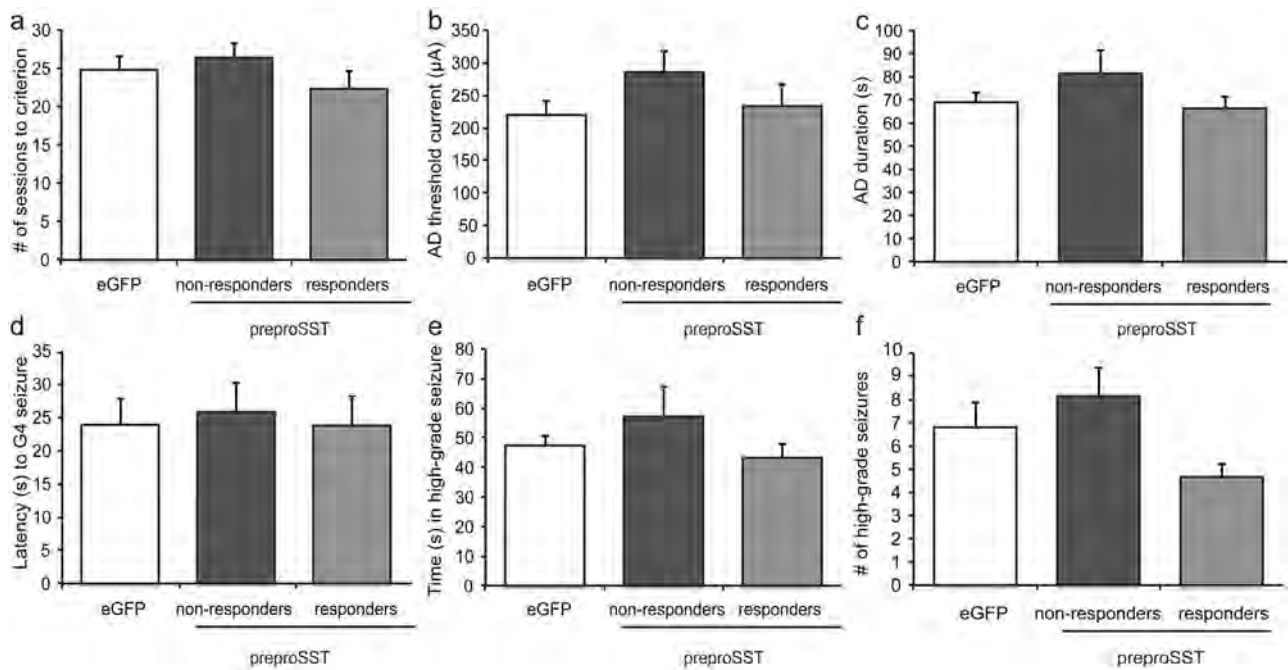


Fig. 5. PreproSST-treated responders and non-responders exhibited similar behavioral responses and EEG characteristics during kindling acquisition. An omnibus ANOVA did not reveal significant differences between preproSST-treated responders, preproSST-treated non-responders and eGFP-treated rats in a) 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).

tide in transduced neurons (Wong et al., 2002). Fig. 4a and b show examples of eGFP⁺, eGFP⁺/SST⁺ and SST⁺ neurons found in the 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 ($t_{(4)} = 1.23$; $p = \text{n.s.}$) or CA1 region ($t_{(4)} = 2.37$; $p = \text{n.s.}$) 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 qualitative 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; Brackch 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 SSTR₂ 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 SSTR₂ 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 (Petr-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.

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Corresponding Author: Dr. Brandi K Ormerod, PhD

Corresponding Author's Institution: J. Crayton Pruitt Family Department of Biomedical Engineering and McKnight Brain Institute, University of Florida

First Author: Jeffrey A Leibowitz, PhD

Order of Authors: Jeffrey A Leibowitz, PhD; Gowri Natarajan, PhD; Junli Zhou, PhD; Michael A King, PhD; Paul R Carney, M.D.; Brandi K Ormerod, PhD

Abstract: Neurogenesis persists throughout life in the hippocampus of all mammals, including humans. In the healthy hippocampus, relatively quiescent Type-1 neural stem cells (NSCs) can give rise to more proliferative Type-2a neural progenitor cells (NPCs), which generate neuronal-committed Type-2b NPCs that mature into Type-3 neuroblasts. Many Type-3 neuroblasts survive and mature into functionally integrated granule neurons over several weeks. In kindling models of epilepsy, neurogenesis is drastically upregulated and many new neurons form aberrant connections that could support epileptogenesis and/or seizures. We have shown that sustained vector-mediated hippocampal somatostatin (SST) expression can both block epileptogenesis and reverse seizure susceptibility in fully kindled rats. Here we test whether adeno-associated virus (AAV) vector-mediated sustained SST expression modulates hippocampal neurogenesis and microglial activation in fully kindled rats. In Experiment 1, we found significantly more dividing Type-1 NSCs 4 h after BrdU injection and a corresponding increased number of surviving new neurons 4 weeks after BrdU injection in the hippocampi of kindled versus sham-kindled rats. Increased numbers of activated microglia were found in the granule cell layer and hilus of kindled rats at both time points. In Experiment 2, we found similar numbers of dividing EdU+ Type-1 NSCs and -2 NPCs and surviving BrdU+ neurons and glia in the hippocampi of kindled rats injected intrahippocampally with either eGFP or SST-eGFP vector. Variability in responses to SST-eGFP (2/4 rats exhibited Grade 0 seizures in the test session) led us to conduct Experiment 3. In Experiment 3, we found fewer dividing BrdU+ Type-1 NSCs in the hippocampi

of SST-eGFP vector-treated responder rats (5/13 rats) that did not exhibit induced seizures on the test session relative to SST-eGFP vector-treated non-responders and eGFP vector-treated controls that exhibited high-grade seizures on the test session. The number of activated microglia was upregulated in the GCL and hilus of kindled rats, regardless of vector treatment. These data support the hypothesis that sustained SST expression may exert antiepileptic effects by normalizing neurogenesis and suggests that the abnormally high proliferating Type-1 NSC numbers may be a cellular mechanism of epilepsy.



College of Engineering
J. Crayton Pruitt Family
Department of Biomedical Engineering
www.bme.ufl.edu

1250 Center Drive.
PO Box 116131
Gainesville FL 32611
352.273.8125 Ph
352.273.9221 Fax

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Experimental Neurology
Editor in Chief
Dr. A. Hoke

Dear Dr. Hoke,

We are submitting a manuscript entitled "Sustained somatostatin gene expression reverses kindling-induced increases in the number of dividing Type-1 neural stem cells in the hippocampi of behaviorally responsive rats" for exclusive consideration of publication as a research article in *Experimental Neurology*.

The paper demonstrates that AAV vector-mediated expression of preprosomatostatin is anticonvulsant and normalizes neurogenesis in a subset of amygdala-kindled rats. We believe that our manuscript would be of interest to a broad readership including those interested in epilepsy therapeutics, AAV gene therapy, the mechanism/function of adult neurogenesis and neuropeptides including somatostatin.

Thank you for your consideration of our work. Please address all correspondence concerning this manuscript to my PhD student Jeffrey Leibowitz (jleibowitz@bwh.harvard.edu) or me at the University of Florida (bormerod@bme.ufl.edu).

Sincerely,

A handwritten signature in black ink, appearing to read 'B. Ormerod', with a stylized, cursive script.

Brandi K. Ormerod, PhD
Associate Professor

Reviewer Suggestions:

1. Dr. Daniela Kaufer

Associate Professor, Berkeley

danielak@berkeley.edu

Dr. Kaufer is an expert in epilepsy and the effects of stress on CNS variables including neurogenesis.

2. Dr. Jack Parent

Professor and Co-director, Epilepsy Program, Department of Neurology and Hospital, U Michigan

parent@umich.edu

Dr. Parent has published some of the pioneering work describing the relationship between neurogenesis and epilepsy.

3. Dr. Ashok Shetty

Professor, Department of Molecular and Cellular Medicine, Texas A&M University

shetty@medicine.tamhsc.edu

Dr. Shetty is an expert in the functional consequence of changes in rates of adult neurogenesis across disease models that include neurogenes.

4. Dr. Gunter Sperk

Department of Pharmacology, Medical University Innsbruck, 6020 Innsbruck, Austria

guenther.sperk@i-med.ac

Dr. Sperk is an expert in the effects of vector-mediated peptide delivery on seizures in animal models of epilepsy.

Highlights

- SST gene transfer prevents kindling-induced seizures in some fully-kindled rats
- Kindling increases Type-1 NSC division and microglial activation in rat hippocampi
- SST gene transfer normalizes Type-1 NSC division in treatment responders
- SST treatment does not affect microglial activation in the hippocampi of kindled rats
- SST gene transfer may mediate effects on kindled seizures through neurogenesis

Sustained somatostatin gene expression reverses kindling-induced increases in the number of dividing Type-1 neural stem cells in the hippocampi of behaviorally responsive rats

Jeffrey A. Leibowitz, Ph.D.¹, Gowri Natarajan, Ph.D.^{2,6,7}, Junli Zhou, M.D.^{6,7}, Michael A. King, Ph.D.²⁻⁵, Paul R. Carney, M.D.^{6,7}, Brandi K. Ormerod, Ph.D.^{1-3*}

¹J. Crayton Pruitt Family Department of Biomedical Engineering, ²Department of Neuroscience, ³McKnight Brain Institute, ⁴Department of Pharmacology and Therapeutics and ⁵NF/SG VA Medical Center, University of Florida, Gainesville, FL 32611, U.S.A. ⁶Department of Neurology and Pediatrics, ⁷Neuroscience program, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, U.S.A.

*Corresponding Author:

Brandi K. Ormerod

J. Crayton Pruitt Family Department of Biomedical Engineering

Herbert Wertheim College of Engineering

University of Florida

1275 Center Drive, JG296

Gainesville, FL 32611-0296, U.S.A

Tel: +1-352-273-8125

Fax: +1-352-273-9221-

E-mail: bormerod@bme.ufl.edu

Abstract

Neurogenesis persists throughout life in the hippocampus of all mammals, including humans. In the healthy hippocampus, relatively quiescent Type-1 neural stem cells (NSCs) can give rise to more proliferative Type-2a neural progenitor cells (NPCs), which generate neuronal-committed Type-2b NPCs that mature into Type-3 neuroblasts. Many Type-3 neuroblasts survive and mature into functionally integrated granule neurons over several weeks. In kindling models of epilepsy, neurogenesis is drastically upregulated and many new neurons form aberrant connections that could support epileptogenesis and/or seizures. We have shown that sustained vector-mediated hippocampal somatostatin (SST) expression can both block epileptogenesis and reverse seizure susceptibility in fully kindled rats. Here we test whether adeno-associated virus (AAV) vector-mediated sustained SST expression modulates hippocampal neurogenesis and microglial activation in fully kindled rats. In *Experiment 1*, we found significantly more dividing Type-1 NSCs 4 h after BrdU injection and a corresponding increased number of surviving new neurons 4 weeks after BrdU injection in the hippocampi of kindled versus sham-kindled rats. Increased numbers of activated microglia were found in the granule cell layer and hilus of kindled rats at both time points. In *Experiment 2*, we found similar numbers of dividing EdU⁺ Type-1 NSCs and -2 NPCs and surviving BrdU⁺ neurons and glia in the hippocampi of kindled rats injected intrahippocampally with either eGFP or SST-eGFP vector. Variability in responses to SST-eGFP (2/4 rats exhibited Grade 0 seizures in the test session) led us to conduct Experiment 3. In *Experiment 3*, we found fewer dividing BrdU⁺ Type-1 NSCs in the hippocampi of SST-eGFP vector-treated responder rats (5/13 rats) that did not exhibit induced seizures on the test session relative to SST-eGFP vector-treated non-responders and eGFP vector-treated controls that exhibited high-grade seizures on the test session. The number of activated microglia was upregulated in the GCL and hilus of kindled rats, regardless of vector treatment. These data support the hypothesis that sustained SST expression may exert antiepileptic effects by normalizing neurogenesis and suggests that the abnormally high proliferating Type-1 NSC numbers may be a cellular mechanism of epilepsy.

Key Words: Temporal lobe epilepsy, adult neurogenesis, neural stem cells (NSCs), neural progenitor cells (NPCs), Type-1 NSCs, Type-2 NPCs, hippocampus, somatostatin (SST), kindling, adeno-associated viral (AAV) vectors, gene therapy

Highlights

- SST gene transfer prevents kindling-induced seizures in some fully-kindled rats
- Kindling increases Type-1 NSC division and microglial activation in rat hippocampi
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- SST gene transfer may mediate effects on kindled seizures through neurogenesis

1. Introduction

Thousands of granule neurons are added daily throughout life to the hippocampal dentate gyrus (Altman, 1962; Cameron et al., 1993; Eriksson et al., 1998). Neurogenesis in the healthy adult hippocampus consists of several steps that can begin with the proliferation of relatively quiescent Type-1 neural stem cells (NSCs) but more regularly begins with the proliferation of uncommitted Type-2a neural progenitor cells (NPCs). Type-2a NPCs can give rise to neuron-committed Type-2b NPCs that differentiate into Type-3 neuroblasts. About one half of these neuroblasts survive and mature into functional neurons over several weeks (Cameron and McKay, 2001; Filippov et al., 2003; Kempermann et al., 2004; Seri et al., 2001; van Praag et al., 2002). Exposure to stimuli like physical activity or social and environmental enrichment increases neurogenesis primarily by stimulating Type-2 NPC proliferation or by promoting new neuron survival, respectively (Kronenberg et al., 2003; Olson et al., 2006; van Praag et al., 1999b). Generally, the increased number of new neurons stimulated by these factors in aging models or in young animals housed in standard colony conditions is associated with better scores in hippocampus-dependent tasks (Gonçalves et al., 2016; Speisman et al., 2013a; Speisman et al., 2013b; van Praag et al., 2005). Whether altering this natural process produces aberrant neurogenesis or impairs function is currently unclear.

A wide range of epilepsy models has demonstrated that chemical and electrical kindling leads to a supraphysiological increase in hippocampal neurogenesis (Nakagawa et al., 2000; Parent et al., 2006; Parent et al., 1998). Newborn neurons produced in the kindled brain often exhibit ectopic migration, aberrant mossy fiber sprouting and abnormal hilar basal dendrites (Fournier et al., 2010; Fournier et al., 2013; Jessberger et al., 2007b; Kron et al., 2010; Parent et al., 2006; Parent et al., 1998; Pekcec and Potschka, 2007). The exaggerated production of new neurons with aberrant connectivity has been hypothesized to contribute to epileptogenesis, seizure maintenance and the

cognitive impairments exhibited by individuals with epilepsy (Botterill et al., 2015; Cho et al., 2015; Fournier et al., 2013; Jessberger and Parent, 2015; Scharfman and Hen, 2007). Indeed, ablating neurogenesis can reduce the frequency of spontaneous seizures and the occurrence of aberrant seizure-induced plasticity after chemical kindling (Cho et al., 2015; Jung et al., 2004; Jung et al., 2006; Kron et al., 2010) and driving ectopic or exaggerated neurogenesis can produce seizures (Pun et al., 2012; Scharfman et al., 2005; Scharfman et al., 2002). Additionally, anti-epileptic drugs can block the neurogenic effects of kainic acid and protect rats from seizure-induced cognitive impairment (Jessberger et al., 2007a). Interestingly, targeted gamma irradiation, anti-mitotic agents and genetic ablation strategies that drastically reduce neurogenesis also reduce the number and duration of seizures (Cho et al., 2015; Hüttmann et al., 2003; Jung et al., 2004; Monje et al., 2002; Parent et al., 1999; Pekcec et al., 2008; Steiner et al., 2008). These data suggest that aberrant neurogenesis could support the development and maintenance of seizures in temporal lobe epilepsy and in animal models of temporal lobe epilepsy.

We have shown that sustained adeno-associated viral (AAV) vector-mediated somatostatin (SST) expression can block epileptogenesis in adult rats and prevent seizures in a subgroup of rats already amygdala-kindled to a seizure-susceptible state (Natarajan et al., 2017; Zafar et al., 2012). These effects are consistent with the observations that inhibitory SST-expressing hilar neurons are selectively vulnerable to death in individuals with epilepsy and in animal temporal lobe epilepsy models, that reduced SST levels increase kindling rates and seizure severity, and that SST receptor agonists have anticonvulsant effects (Aourz et al., 2011; Bezchlibnyk et al., 2007; Buckmaster and Dudek, 1997; Buckmaster et al., 2002; Monno et al., 1993; Robbins et al., 1991; Sloviter, 1987; Vezzani et al., 1991). We hypothesized that since SST is anti-proliferative in other tissues (Leu et al., 2008; Nolan et al., 2007), sustained SST expression may normalize kindling-induced changes in adult hippocampal neurogenesis. We expected that if kindling-induced seizures were related to upregulated and perhaps aberrant neurogenesis, then normalizing neurogenesis with sustained

vector-mediated SST gene expression may reduce seizure severity in rats kindled to a seizure-susceptible state.

In *Experiment 1*, we tested the effects of amygdala kindling on the number and phenotype of dividing NSCs/NPCs and their progeny. In *Experiment 2*, we tested whether sustained vector-mediated SST expression impacted the number and phenotype of NSCs/NPCs and their progeny in the hippocampi of kindled rats. In *Experiment 3*, we tested whether sustained SST expression differentially impacted the effects of kindling on neurogenesis in responder and non-responder rats. Since adult neurogenesis is sensitive to neuroinflammation (Ek Dahl et al., 2003; Monje et al., 2003; Ormerod et al., 2013) and kindling stimulates a neuroinflammatory response (Khurgel and Ivy, 1996; Plata-Salamán et al., 2000), we quantified the effects of kindling on resting and activated microglia numbers and the strength of their relationship with dividing NSC and NPC numbers or surviving new neuron numbers in each experiment.

2. Materials and methods

2.1 Subjects

All rats used in this study were treated in accordance with Federal and University of Florida Institutional Animal Care and Use Committee regulations regarding the ethical use of animals for experimentation. Adult male Sprague Dawley rats (n = 45 250-275 g; Envigo) were housed in pairs upon arrival in corn-cob lined ventilated shoebox cages located in a standard colony room maintained on a 12:12 h light:dark cycle (lights on at 6:00AM) at $24 \pm 1^{\circ}\text{C}$. Following surgical implantation of electrodes, rats were housed individually to minimize the likelihood of head-stage losses. In either case, they were given free access to Harlan Teklad Rodent Food Diet #7912 and reverse osmosis-filtered water for the duration of each experiment.

Figure 1 illustrates the methods used in *Experiments 1-3*. In all experiments, rats underwent amygdala kindling in twice daily sessions (≥ 6 h apart) until they exhibited a Grade 5 seizure in 3 consecutive sessions. *Experiment 1* tested the effects of kindling on the number of dividing BrdU⁺

Type-1 NSCs and Type-2 NPCs and on the number and phenotype of surviving BrdU⁺ new cells. Sham-kindled rats (no electrical stimulation was administered) and kindled rats were injected with BrdU 48 h after a kindled rat achieved criterion. Rats were perfused 4 h later to quantify and phenotype dividing BrdU⁺ cells or 4 weeks later to quantify and phenotype surviving BrdU⁺ cells (n = 5 rats per group). *Experiment 2* tested the effects of sustained SST expression on the number of dividing EdU⁺ Type-1 NSCs and Type-2 NPCs and on the number and phenotype of BrdU⁺ surviving new cells in kindled rats. Rats were injected with BrdU 48 h after achieving criterion and then eGFP or SST-eGFP (n = 4 rats per group) vector was infused bilaterally into their hippocampi the following week. After a 3-week recovery, the rats were given a test session, post-test sessions every 48 h for ~2.5 weeks, injected with EdU 48 h after the final post-test session and then perfused 4 h later to quantify the number and phenotype of dividing EdU⁺ cells and of surviving BrdU⁺ cells. *Experiment 3* tested whether kindling differentially affected the number of dividing BrdU⁺ Type-1 NSCs or Type-2 NPCs in eGFP vector-treated (n = 4), SST-responder rats that exhibited Grade 0 seizures on the test stimulation (n = 5) and SST-non-responders that exhibited seizures $\geq$ Grade 1 on the test stimulation (n = 8) rats. eGFP vector or SST-eGFP vector was infused into the hippocampi of rats the week after they achieved criterion. After a 3-week recovery, the rats were given a test session, post-test sessions every 48-72 h over ~3 weeks, injected with BrdU 48 h after the final post-test session and then perfused 4 h later to quantify and phenotype dividing BrdU⁺ cells. Relationships between numbers of microglia and numbers of dividing NSCs, dividing NPCs and new neurons were tested in each experiment.

2.2 Electrode Implantation

Two weeks after arrival, rats were implanted with bipolar local electrical field potential recording and stimulating electrodes (330 μ m d; impedance confirmed as $< 1.8 \Omega$) that were made in-house, as detailed previously (Natarajan et al., 2017; Zafar et al., 2012). Briefly, anesthetized rats were placed in a Kopf stereotaxic frame so that a bipolar electrode could be implanted into each

amygdala (-2.2 mm AP, $\pm$ 4.8 mm ML, -8.3 mm DV) according to Paxinos and Watson (Paxinos and Watson, 2007) to record and stimulate activity in the left and right hemispheres of the rats, counterbalanced within and between groups. In *Experiments 2 and 3*, small plastic hex nuts with removable screws were placed over dentate gyrus and CA1 region target coordinates to keep the skull free of dental cement so that vector could be delivered at a later time point. Ground and reference electrodes were implanted rostral to bregma and caudal to lambda, respectively. All electrodes were then attached to male Amphenol pins that were inserted into plastic connector strips that were 3D-printed at the University of Florida Infinity Fabrication laboratory (<http://fablab.arts.ufl.edu/>). The entire assembly was secured with dental cement to bone screws anchored to the skull.

2.3 Electrical Kindling

Synchronized behavioral and electroencephalographic (EEG) data were recorded in all kindling, test and post-test sessions to grade behavioral seizures and to confirm the presence or absence of afterdischarges (ADs) or epileptiform bursts (Racine et al., 1972). After a 10 d recovery from surgery, rats were tested in a baseline session to identify the AD threshold current that would be employed in all subsequent kindling, test and post-test sessions. A standard 2 s, 1 ms pulse duration, 50 Hz biphasic square wave pulse was delivered at 1 min inter-stimulus intervals (initially at 50 μ A but stepped up 50 μ A in intensity every delivery) until an AD was observed in the EEG (Natarajan et al., 2017; Racine, 1972; Zafar et al., 2012). An AD was defined as spikes >1 Hz with amplitudes greater than twice the spike amplitudes detected in the baseline EEG (Natarajan et al., 2017; Zafar et al., 2012). Sham-kindled rats were treated similarly to kindled rats but did not receive electrical current.

Beginning 24 h later, the AD threshold current identified for each rat was delivered in kindling sessions administered twice daily ($\geq$ 6 h apart) until that rat exhibited a Grade 5 seizure in 3 consecutive sessions. Seizures were graded according to the Racine scale of seizure severity in animal epilepsy models (Racine, 1972), in which Grade 0 reflects no behavioral change; Grade 1

reflects staring, immobility, and facial movements; Grade 2 reflects head nodding and chewing; Grade 3 reflects unilateral forelimb clonus; Grade 4 reflects bilateral forelimb clonus with rearing; Grade 5 reflects bilateral forelimb clonus with rearing, loss of balance, and falling. In *Experiments 2* and *3*, a single test session was administered 3 weeks after vector injection to quantify the effect of vector treatment on seizure grade, followed by post-test sessions to quantify the resilience of the effects (see **Figure 1** and the subjects section for details).

The synchronized behavioral and EEG data collected during kindling sessions were scored to quantify: 1) AD threshold currents (μA); 2) number of sessions to exhibit the first Grade 5 seizure; 3) number of high-grade (Grade 4-5) seizures; and 4) sessions to criterion (the number of sessions required to exhibit a Grade 5 seizure over 3 consecutive sessions). Seizure grades were scored and the presence or absence of ADs were confirmed in EEGs on test and post-test sessions.

2.4 BrdU and EdU preparation and injections

Bromodeoxyuridine (BrdU; Sigma Aldrich, St. Louis, MO) and ethynyldeoxyuridine (EdU; Life Technologies, Eugene, OR) were dissolved in freshly prepared sterile isotonic saline at a concentration of 20 mg/ml and 10 mg/ml, respectively. BrdU (100 mg/kg) was injected intraperitoneally (i.p.) 48 h after rats reached criterion in *Experiments 1* and *2* or 48 h after the final post-test session in *Experiment 3*. EdU (100 mg/kg) was injected i.p. 48 h after the final post-test session in *Experiment 2*.

2.5 AAV vector construct and injections

We detailed construction of the AAV5-CBa-preproSST-eGFP (the ‘SST-eGFP vector’) and the AAV5-CBa-eGFP (the ‘eGFP vector’) vector previously (Natarajan et al., 2017). The plasmid sequence included the hybrid cytomegalovirus enhancer/chicken β -actin (CBa) promoter driving the expression of either a preproSST transgene plus a downstream eGFP reporter, or an eGFP reporter alone. An internal ribosomal entry site sequence was inserted between preproSST and eGFP coding sequences of the SST-eGFP vector so that eGFP expression reported preproSST expression. Briefly,

the CBa-eGFP plasmid was packaged into serotype 5 AAV capsids in one batch for both Experiments 2 and 3 (gtc.ufl.edu/core/vector-core-lab.htm; titer = 8.9×10^{12} vg/ml) and the CBa-SST-eGFP plasmid was packaged into serotype 5 AAV capsids in one batch for *Experiment 2* (SAB tech Inc., Philadelphia, PA; titer = 1.0×10^{13} vg/ml) and in 2 batches for *Experiment 3* (gtc.ufl.edu/core/vector-core-lab.htm; titer = 6.1×10^{12} vg/ml or 4.9×10^{13} vg/ml). In *Experiment 3*, SST-eGFP vector-treated rats were grouped because we found no statistically significant effects of SST-eGFP vector titer on test session seizure grade, post-test session seizure grade or on histological variables associated with neuroinflammation or neurogenesis.

In the rat hippocampus, preproSST yields proSST that is cleaved into neuronostatin-13 by a yet to be identified peptidase and into either SST-14 by prohormone convertase 1 (PC1) and PC2 or the N-terminal expanded isoform SST-28 by furin-like peptidases, such as carboxypeptidase-E (CPE) and furin (Bilova et al., 2007; Samson et al., 2008). Pan SST antibodies reveal strong SST expression in hippocampal principal neurons and interneurons, high-level CPE and PC2 expression and low to moderate PC1 expression (Bilova et al., 2007; Viollet et al., 2008). While SST-14 is the predominant natural CNS SST isoform both SST-14 and SST-28 are expressed in the brain and bind with similar affinities to the four G protein-coupled SST receptors (SSTRs) expressed in the hippocampus (Csaba and Dournaud, 2001; Dun et al., 2010; Olias et al., 2004; Reisine and Bell, 1995; Viollet et al., 2008). In the hippocampus, neuronostatin-13/SST-14 protein concentration ratios and expression of the putative neuronostatin receptor GPR107 are extremely low and numbers of neuronostatin producing neurons are sparse (Dun et al., 2010; Samson et al., 2016; Samson et al., 2008). While the effects of the SST-eGFP vector could be produced by SST, neuronostatin or a combination of both peptides, these data suggest that the effects of the SST-eGFP vector are likely mediated through sustained SST expression.

In *Experiments 2* and *3*, eGFP or SST-eGFP vector was injected bilaterally into the dentate gyri and CA1 regions of rats in the week after they achieved criterion, as we described previously

(Natarajan et al., 2017). Anesthetized rats were mounted in a Kopf stereotaxic frame and the placeholder screws were removed from the hex nuts that had been placed over injection target regions during the initial electrode placement surgery so that holes could be drilled. The eGFP or SST-eGFP vector was infused through a 10 μ l Hamilton syringe fitted with a 27G steel needle by an infusion system (Cole-Parmer, Vernon Hills, IL) into each dentate gyrus (-3.8 mm AP, $\pm$ 1.88 mm ML, -3.4 mm DV) and CA1 region (-3.8 mm AP, $\pm$ 1.88 mm ML, -2.4 mm DV) according to Paxinos and Watson (Paxinos and Watson, 2007) at a flow rate of 0.3 μ l/min. A 2 μ l volume was infused into each of the 4 injection sites.

2.6 Perfusion and histology

Four hours or 4 weeks after the final cell synthesis marker injection, rats were anesthetized with a xylazine (10 mg/kg) and ketamine (80 mg/kg) cocktail injected i.p. and then perfused with ice-cold isotonic saline followed by ice-cold, freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA) in phosphate-buffered saline (PBS). Brains were extracted, post-fixed overnight in perfusate at 4°C and then cryoprotected in 30% sucrose solution for 2-3 d. The brains were then sectioned coronally at 40 μ m through the rostral-caudal extent of the hippocampus on a freezing stage microtome (American Optical Corporation; Buffalo, NY). Brain sections were stored in cryoprotectant solution (25% glycerol, 30% ethylene glycol and 45% 0.1M PBS) at -20°C until processed immunohistochemically.

Electrode placements in the amygdalae of all rats were confirmed in histological sections. **Figure 2** shows a schematic of the hippocampus regions that the vector infusion targeted and a representative image of eGFP expression in the hippocampus of a kindled rat. An experimenter blind to the vector treatment group and seizure grades of the rats confirmed bilateral eGFP expression through at least 1 major hippocampal region (the CA1 region, CA3 region and/or dentate gyrus) in all rats. Moderate to strong bilateral eGFP expression was found through the CA1 and CA3 region of most rats and eGFP expression was also noted in the cortex dorsal to and thalamic regions ventral to

the hippocampus of some rats. No overt differences in eGFP expression were found between eGFP vector-treated, SST-eGFP vector-treated responder and non-responder groups.

2.7. Immunohistochemistry

Immunohistochemistry was performed on free-floating sections and the sections were rinsed with tris-buffered saline (TBS; pH 7.4) repeatedly between steps. Blocking solution and antibody diluent was 3% normal donkey serum and 0.1% Triton-X in TBS.

2.7.1. Enzyme substrate immunostaining and Click-IT chemistry. BrdU⁺, EdU⁺, and Iba-1⁺ cells were revealed enzymatically on separate sets of sections so that total cell numbers could be estimated using stereological principles. To reveal BrdU⁺ and Iba-1⁺ cells, sections were incubated in 0.3% H₂O₂ for 10 min at room temperature (RT) to quench endogenous peroxidase and then in blocking solution for 20 min at RT. Sections being processed to reveal BrdU were next rinsed in 0.9% NaCl and then incubated in 2N HCl for 20 min at 37°C to denature DNA. Sections were then incubated overnight at 4°C in rat anti-BrdU (1:500; AbD Serotec, Raleigh, NC) or rabbit anti-Iba-1 (1:1000; Wako Chemicals, Wako, TX) and then in biotinylated anti-rat IgG or anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA), respectively for 4 h at RT. The sections were incubated in avidin-biotin horseradish peroxidase (Vector Labs, Burlingame, CA) for 2 h at RT and then reacted in a solution of 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, St. Louis, Mo) and 0.5% H₂O₂ for ~3 min. EdU⁺ cells were revealed on sections processed using the Click-iT EdU Colorimetric IHC detection kit (Life Technologies, Eugene, OR) according to kit instructions using kit reagents. Briefly, sections were incubated in 0.3% H₂O₂ for 10 min at RT to quench endogenous peroxidase, Trypsin-EDTA buffer for 20 min at RT to permeabilize the tissue, freshly prepared Click-IT reaction cocktail for 1 h at RT, streptavidin-peroxidase reaction cocktail for 2 h at RT and then reacted in DAB reaction buffer for ~3 min. All sections were mounted on Fisher Superfrost microscope slides, dried overnight, dehydrated under an ethanol series, cleared in xylene and then coverslipped under Permount (Thermo Fisher Scientific). Sections stained to reveal EdU⁺ and Iba-1⁺

cells were counterstained with 0.25% Cresyl Violet acetate (Sigma Aldrich) during dehydration to assist with cell counting.

2.7.2. Fluorophore immunostaining. The phenotypes of dividing BrdU⁺ cells, surviving BrdU⁺ cells, dividing EdU⁺ cells, and Iba-1⁺ microglia were each revealed on separate sets of 3-4 sections from a randomly selected 1 in 12 series for each rat using maximally cross-adsorbed fluorophore-conjugated secondary antibodies.

EdU⁺ cells were first revealed using the Click-iT EdU Alexa Fluor 555 imaging kit (Life Technologies, Eugene, OR) according to kit instructions. Briefly, sections were reacted in freshly prepared Click-IT reaction cocktail for 1 h at RT and then fixed in 4% paraformaldehyde for 10 min at RT. On separate sections, BrdU⁺ cells were also first revealed by rinsing sections in 0.9% NaCl and then incubating them in 2N HCl for 20 min at 37°C to denature DNA. The sections were incubated in blocking solution for 20 min at RT and then overnight at 4°C in rat anti-BrdU (BrdU; 1:500; AbD Serotec, Raleigh, NC). The following day, the sections were incubated in Cy3-conjugated donkey anti-rat IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at RT and then fixed in 4% paraformaldehyde for 10 min at RT.

After revealing EdU⁺ or BrdU⁺ cells, sections were incubated overnight at 4°C in a cocktail containing 1) the Type-1 NSC and astrocyte marker chicken anti-glial fibrillary acidic protein (GFAP; 1:1000; EnCor Biotechnology, Alachua, FL) and the Type-2a and -2b marker rabbit anti-SOX2 (SOX2; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), 2) the immature neuronal marker goat anti-doublecortin (DCX; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and the mature neuronal marker mouse anti-Neuronal Nuclei (NeuN; 1:500; Chemicon, Temecula, CA), or 3) chicken anti-GFAP (GFAP; 1:1000; EnCor Biotechnology, Alachua, FL) and the oligodendrocyte progenitor marker rabbit anti-NG2 (1:1000; Millipore; Chemicon, Temecula, CA). To phenotype Iba-1⁺ cells, sections were incubated in rabbit anti-Iba-1 (1:1000; Wako Chemicals, Wako, TX) and the monocyte/macrophage activation marker CD11b (mouse anti-CD11b; 1:100; Chemicon, Temecula,

CA) overnight at 4°C. After overnight incubation in primary antibodies, all sections were incubated in fully cross-adsorbed cocktails of fluorophore-conjugated secondary antibodies of the appropriate species (1:500; Jackson ImmunoResearch, West Grove, PA) for 4 h at RT to reveal primary antibodies. All sections were incubated in 4',6-diamidino-2'-phenylindole (DAPI; 1:10,000; Calbiochem, San Diego, CA) for 10 min at RT and then mounted under diazobicyclooctane (DABCO; 2.5% DABCO, 10% polyvinyl alcohol and 20% glycerol in TBS).

2.8 Cell Counting and Stereology

Total BrdU⁺ and EdU⁺ cells were estimated using fractionator principles (Boyce et al., 2010; Kempermann et al., 1997; Noori and Fornal, 2011; Ormerod et al., 2013; Speisman et al., 2013a; Speisman et al., 2013b; West et al., 1991). Because BrdU⁺ and EdU⁺ cells are located irregularly and relatively infrequently throughout the subgranular zone and granule cell layer, they were counted exhaustively on every 12th systematically uniform section (240 µm apart) through the rostral-caudal extent of the dentate gyrus (10 sections per rat) under a 40x objective on a Zeiss Axio Observer Z1 inverted microscope. Only round or oval cells were included in these counts. Total EdU⁺ and BrdU⁺ cell numbers were estimated using the formula $N_{\text{Total}} = \sum Q^- \times 1/\text{ssf}$, where $\sum Q^-$ represents the number of counted cells and ssf is the section sampling fraction (1/12). The average number of dividing BrdU⁺ cells in sham-kindled (107 ± 7 cells, CV = 0.15, CE = 0.07), and kindled (214 ± 11 cells, CV = 0.11, CE = 0.05) rats and surviving BrdU⁺ cells in sham-kindled (124 ± 7 cells, CV = 0.13, CE = 0.07) and kindled (235 ± 12 cells, CV = 0.12, CE = 0.05) rats in *Experiment 1*, dividing EdU⁺ cells in eGFP (185 ± 13 cells, CV = 0.14; CE = 0.07) and SST-eGFP (159 ± 25 cells, CV = 0.32, CE = 0.16) vector-treated rats and surviving BrdU⁺ cells in eGFP (233 ± 9 cells, CV = 0.07, CE = 0.04) and SST-eGFP (212 ± 7 cells, CV = 0.06, CE = 0.03) vector-treated rats in *Experiment 2* and dividing BrdU⁺ cells in eGFP vector-treated (212 ± 13 cells, CV = 0.13, CE = 0.06), SST-eGFP non-responder (177 ± 16 cells, CV = 0.25, CE = 0.09) and SST-eGFP responder (120 ± 10 cells, CV = 0.18, CE = 0.08) rats in

Experiment 3 that were counted was sufficient to ensure reliable estimates between groups (Boyce et al., 2010; Larsen, 1998).

The total number of Iba-1⁺ cells was estimated using optical fractionator principles (Bañuelos et al., 2013; Boyce et al., 2010; Ormerod et al., 2013; West et al., 1991). Iba-1⁺ cells were counted through the GCL and hilus using Microbrightfield StereoInvestigator software (Williston, VT). Cells were counted on every 12th systematically uniform section (240 µm apart) through the rostral-caudal extent of the dentate gyrus (10 sections per rat) under a 40x objective on a Zeiss Axio Observer Z1 inverted microscope. Iba-1⁺ cells were estimated using the formula: $N_{\text{Total}} = \sum Q^- \times 1/\text{ssf} \times 1/\text{asf} \times t/h$, where $\sum Q^-$ represents the number of counted cells, ssf is the section sampling fraction (1/12), asf is the area sampling fraction or the ratio of the counting frame area to the total area of the fractionator sampling grid (~1/15 for Iba-1⁺ cells), t is the average section thickness and h is the height of the dissector (0.010 mm). A guard zone of 4 µm was used during Iba-1⁺ cell counting to avoid sectioning artifacts. and Iba-1⁺ cells in sham-kindled (174 ± 6 cells, CV = 0.17, CE = 0.04) and kindled (225 ± 9 , CV = 0.17, CE = 0.04) rats in *Experiment 1*, eGFP (193 ± 9 cells, CV = 0.14; CE = 0.05) and SST-eGFP vector-treated (175 ± 13 cells, CV = 0.21, CE = 0.07) rats in *Experiment 2* and in eGFP (206 ± 11 cells, CV = 0.14, CE = 0.05), SST-eGFP responders (217 ± 14 cells, CV = 0.20, CE = 0.06) and SST-eGFP non-responders (211 ± 12 cells, CV = 0.23, CE = 0.06) in *Experiment 3* that were counted was sufficient to ensure reliable stereological estimates (Boyce et al., 2010; Larsen, 1998).

Phenotypic markers were confirmed in each analysis for at least 45 BrdU⁺ and EdU⁺ and at least 100 Iba-1⁺ cells in quadruple fluorescent-stained sections under a 20x objective (with 2.5x digital zoom) using a Zeiss LSM 710 meta scanning confocal microscope with 405 nm (to excite DAPI), 488 nm (to excite FITC on non-vector-treated tissue), 510 nm, 543 nm (to excite Cy3), 594nm (to excite Alexa 594) and 633 nm (to excite Cy5) laser lines. DAPI⁺/BrdU⁺, DAPI⁺/EdU⁺ and DAPI⁺/Iba-1⁺ cells were scanned through their full 'z-dimension' to confirm co-expression of one or more phenotypic marker. Total numbers of cells estimated stereologically were multiplied by the % of cells expressing

each phenotype to calculate the total number of dividing Type-1 NSCs, dividing Type-2 NPCs, new neurons, new astrocytes, new oligodendrocyte precursors, resting microglia and activated microglia. Percentages of BrdU⁺ and EdU⁺ expressing phenotypic markers are reported in **Supplemental Table 1**.

2.9 Statistical Analysis

Statistical analyses were performed using Version 13 Statistica software (Tulsa, Oklahoma; rented from <http://onthehub.com/>). Behavioral variables (number of sessions to the 1st Grade 5 seizure, number of Grade 4/5 seizures, sessions to criterion, AD threshold current) collected prior to vector injection were compared between groups and the effects of the independent variables (kindling and vector treatment) on total EdU⁺, BrdU⁺ or IBA-1⁺ cell numbers were tested with analyses of variance (ANOVAs) and revealed by Newman-Keuls post-hoc tests when compared between 3 groups or tested with Student's t-tests when compared between 2 groups. The effects of the independent variables (kindling, vector injection and dentate gyrus subregion) on numbers of dividing cells (Type-1 NSCs, Type-2a NPCs, Type-2b NPCs), numbers of surviving cells (neurons and glia) and numbers of microglia (resting and activated) were tested with repeated measures ANOVAs and revealed by Newman-Keuls post-hoc tests. The effect of vector treatment and AD current increases on test and post-test session seizure grade was assessed using a Mann-Whitney U test for 2 groups or a Kruskal Wallance ANOVA followed by a Mann-Whitney U test for 3 groups. Effect sizes were calculated using Cliff's *d* (Cliff, 1993). Pearson product moment correlation coefficient analyses tested relationships between variables. All figures report means ($\pm$ S.E.M.). The α -level was set at $p < 0.05$ and reported as two-tailed.

3. Results

3.1 Neurogenesis and microglial activation are potentiated in the hippocampi of kindled rats.

In *Experiment 1*, we tested whether the number and phenotype of BrdU⁺ cells and the number and activation state of Iba-1⁺ microglia varied in the hippocampi of sham-kindled and kindled rats that

were injected with BrdU 48 h after kindled rats achieved criterion and then perfused either 4 h or 4 weeks later (see **Figure 1**). The average AD threshold current employed during kindling sessions was $205.0 \pm 15.7 \mu\text{A}$ and kindled rats exhibited their 1st Grade 5 seizure after 13.8 ± 1.8 sessions (~7 d), exhibited 5.5 ± 0.8 high-grade seizures during kindling sessions and achieved kindling criterion after 18.5 ± 1.8 sessions (~9 d).

3.1.1. More dividing Type-1 NSCs and activated microglial were detected in the hippocampi of kindled rats. To test the effects of kindling on the number of Type-1 NSCs, Type-2 NPCs and activated microglia, rats were injected with BrdU 48 h after kindled rats achieved criterion and then perfused 4 h after BrdU injection. **Figure 3** shows a representative microphotograph of DAB-stained dividing BrdU⁺ cells (in brown) in the dentate gyrus of **(A)** a kindled rat (the inset shows BrdU⁺ cells under 63x magnification) and **(B)** a sham-kindled rat. **Figure 3C** shows a representative confocal image of DAPI-stained (in gray) dividing BrdU⁺ (in red) cells expressing the Type-1 NSC marker GFAP (in cyan) and/or the Type-2 NPC marker SOX2 (in green). The **Figure 3C insets** show examples of Type-1 GFAP⁺/SOX2⁺ NSCs (yellow arrows) and a Type-2 GFAP⁻/SOX2⁺ NPC (white arrow) in single detection channels for each signal.

Figure 3D shows that the mean ($\pm$ S.E.M.) total number of dividing BrdU⁺ cells was significantly higher in the dentate gyri of kindled versus sham-kindled rats ($t_{(8)} = 8.26$; $p < 0.001^{***}$). **Figure 3E** shows the total number of dividing Type-1 NSCs, Type-2a NPCs and Type-2b NPCs in the dentate gyri of sham-kindled and kindled rats. Note that because similarly rare BrdU⁺ cells expressed Type-3 neuroblast phenotypes in either sham-kindled or kindled rats 4 h after BrdU injection ($< 1\%$ in either group), they were excluded from further analyses (see **Supplemental Table 1** for percentages of BrdU⁺ cells expressing each phenotype). The total number of dividing cells varied by phenotype ($F_{(2,16)} = 26.47$; $p < 0.001$), group ($F_{(1,8)} = 51.50$; $p < 0.001$) and the interaction between phenotype and group ($F_{(2,16)} = 10.00$; $p < 0.001$). Overall, more dividing cells were found in the dentate gyri of kindled versus sham-kindled rats ($p < 0.001$) and generally, most dividing cells were Type-1 NSCs (p

< 0.01 versus Type-2a and $p < 0.001$ versus -2b NPCs) followed by Type-2a NPCs ($p < 0.001$ versus Type-2b NPCs). The latter effect was primarily due to the increased number of dividing Type-1 NSCs in the dentate gyri of kindled versus sham-kindled rats ($p < 0.001^{***}$). In fact, while similar numbers of dividing Type-1 NSCs and -2a NPCs were found in the dentate gyri of sham-kindled rats, dividing Type-1 NSCs outnumbered dividing Type-2a NPCs in kindled rats ($p < 0.001^{***}$). These data show that the number of Type-1 NSCs is upregulated in the dentate gyri of kindled rats.

Figure 3F shows a representative photomicrograph of DAB-stained Iba-1⁺ microglia (in brown; the inset shows Iba-1⁺ cells under 63x magnification). Representative confocal images of a DAPI-labeled (in gray) resting CD11b⁻/Iba-1⁺ (in cyan) microglia and a DAPI-labeled activated CD11b⁺ (in green)/Iba-1⁺ (in cyan) microglia in the dentate gyrus of a kindled rat are shown in **Figures 3G** and **3H**, respectively. The insets show each signal in single detection channels. Iba-1⁺/CD11b⁻ microglia exhibited classic resting morphologies with small round cell bodies and extensive branching while Iba-1⁺/CD11b⁺ microglia exhibited classic activated morphologies with larger cell bodies and shorter, thicker cell processes. **Figure 3I** shows the mean ($\pm$ S.E.M.) total number of Iba-1⁺ microglia in the GCL and hilus of sham-kindled and kindled rats. In both groups combined, more Iba-1⁺ microglia were found in the hilus than GCL of all rats combined ($F_{(1,8)} = 6.81$; $p < 0.05^*$), but microglia numbers neither varied by group ($F_{(1,8)} = 3.95$; $p = 0.08$) nor by the overall interaction ($F_{(1,8)} = 0.01$; $p = 0.93$). **Figure 3J** shows the mean ($\pm$ S.E.M.) total number of Iba-1⁺ microglia expressing CD11b⁻ resting versus CD11b⁺ activated states in the GCL and hilus of sham-kindled and kindled rats. The total number of microglia varied region ($F_{(1,8)} = 6.81$; $p < 0.05$), the interaction between group and activation state ($F_{(1,8)} = 105.24$; $p < 0.0001$) and the overall interaction ($F_{(1,8)} = 11.29$; $p < 0.01$) but did not vary by group, activation state or other interactions between the independent variables (all p values ≥ 0.08). In both groups combined, more microglia were found in the hilus versus GCL ($p < 0.05$). However, the number of resting microglia was lower while the number of activated microglia was higher in the GCL ($p < 0.01^{**}$ and $p < 0.001^{***}$, respectively) and hilus (both p values $< 0.001^{***}$)

of kindled versus sham-kindled rats. In sham-kindled rats, more resting versus activated microglia were found in both the GCL ($p < 0.01^a$) and hilus ($p < 0.001^a$). In these rats, ratios of activated versus resting microglia were 0.48 in the GCL and 0.95 in the hilus and the ratio of activated microglia in the hilus versus GCL was 0.95. In kindled rats, more activated than resting microglia were found in the GCL ($p < 0.001^b$) and hilus ($p < 0.001^b$), and the number of activated microglia was elevated in the hilus versus GCL ($p < 0.05^c$). In these rats, ratios of activated versus resting microglia were 2.28 in the GCL and 3.97 in the hilus and the ratio of activated microglia in the hilus versus GCL was 1.25. These data demonstrate that numbers of activated microglia are increased in the GCL and more robustly in the hilus of kindled rats, just after their last Grade 5 seizure.

The total number of dividing Type-1 NSCs correlated positively with the total number of activated GCL ($r_{(10)} = 0.90$; $p < 0.0001$) and hilar ($r_{(10)} = 0.80$; $p < 0.01$) microglia and negatively with the total number of resting GCL ($r_{(10)} = -0.85$; $p < 0.01$) and hilar ($r_{(10)} = -0.75$; $p < 0.05$) microglia. Numbers of dividing Type-2 NPCs were unrelated to numbers of dividing Type-1 NSCs, resting microglia or activated microglia. These data suggest that microglial activation and Type-1 NSC proliferation may be related processes in the hippocampi of kindled rats.

3.1.2. Microglial activation persists for several weeks in the hippocampi of kindled rats.

To test whether NSCs and NPCs that divide in the hippocampi of kindled rats produce similar numbers of surviving neurons and glia and whether kindling produces a persistent microglial response, rats were injected with BrdU 48 h after achieving criterion and then perfused 4 weeks later (see **Figure 1**). **Figure 4A** shows a representative confocal image of a 4 week-old BrdU⁺ cell (in red) expressing the mature neuronal protein NeuN⁺ (in green). Immature DCX⁺ neurons (in cyan) and transitioning NeuN⁺/DCX⁺ neurons are also shown. **Figure 4B** shows a representative confocal image of a 4 week-old BrdU⁺ cell (in red) expressing the astrocyte protein GFAP (in cyan). NG2⁺ oligodendrocyte precursor cells are shown in green. Because less than 1% of BrdU⁺ cells expressed

NG2 in the dentate gyri of either sham-kindled or kindled rats, new oligodendrocyte precursors were excluded from further analyses.

Figure 4C shows that the total number of surviving 4 week-old BrdU⁺ cells was significantly higher in the hippocampi of kindled versus sham-kindled rats ($t_{(8)} = 7.77$; $p < 0.0001^{***}$). **Figure 4D** shows the mean ($\pm$ S.E.M.) total number of new BrdU⁺ astrocytes and neurons expressing immature, transitioning and mature phenotypes (see **Supplemental Table 1** for the percentages of BrdU⁺ cells expressing each phenotype). This number varied by group ($F_{(1,8)} = 28.76$; $p < 0.001$), phenotype ($F_{(3,24)} = 60.99$; $p < 0.001$), and the overall interaction ($F_{(3,24)} = 11.52$; $p < 0.001$). A greater number of differentiated BrdU⁺ cells was found in the hippocampi of kindled versus sham-kindled rats ($p < 0.001$), primarily because more mature new neurons were found in kindled versus sham-kindled rats ($p < 0.001^{***}$). Irrespective of group and consistent with a 4-week survival period, most new cells had differentiated into mature neurons ($p < 0.001^a$ versus all other phenotypes) followed by transitioning neurons ($p \leq 0.05^b$ versus immature neurons and astrocytes). In the dentate gyri of kindled versus sham-kindled rats, the 2.1-fold increase in total new neuron number detected 4 weeks after BrdU reflected the 2-fold increase in dividing cells detected at 4 h after BrdU, suggesting that increased new neuron numbers are a function of increased numbers of dividing cells rather than changes in the differentiation or survival of new neurons.

Figure 4E shows the mean ($\pm$ S.E.M.) total number of Iba-1⁺ microglia in the GCL and hilus of sham-kindled and kindled rats 4 w after their last kindling session (see **Figure 3F** for examples of Iba-1⁺ microglia). This number varied by group ($F_{(1,8)} = 10.24$; $p < 0.05$) and region ($F_{(1,8)} = 11.06$; $p < 0.05$) but not by the overall interaction ($F_{(1,8)} = 1.02$; $p = 0.34$). More microglia were detected in kindled versus sham-kindled rats ($p < 0.05^*$) and more microglia were found in the hilus versus GCL ($p < 0.05^*$) of both groups combined. **Figure 4F** shows the mean ($\pm$ S.E.M.) total number of resting and activated microglia in the GCL and hilus of sham-kindled and kindled rats (see **Supplemental Table 2** for percentages of Iba-1⁺ cells expressing the activation marker CD11b). This number varied

by group ($F_{(1,8)} = 10.70$; $p < 0.05$), region ($F_{(1,8)} = 11.43$; $p < 0.01$), the interaction between activation state and group ($F_{(1,8)} = 28.27$; $p < 0.001$) and the overall interaction ($F_{(1,8)} = 29.20$; $p < 0.001$), but not by activation state or other interactions between the independent variables (all p values ≥ 0.07). More microglia were found in the hippocampi of kindled versus sham-kindled rats ($p < 0.05$) and in the hilus versus GCL of both groups combined ($p < 0.01^{**}$). More activated microglia were found in the GCL and hilus of kindled versus sham-kindled rats ($p < 0.01^{**}$ and $p < 0.001^{***}$, respectively) and fewer resting microglia were found in the hilus of kindled versus sham-kindled rats ($p < 0.05$). In kindled rats, more activated microglia were found in the hilus versus GCL ($p < 0.001^a$) and in the hilus, activated microglia outnumbered resting microglia ($p < 0.001^b$). In these rats, the ratio of activated versus resting microglia was 1.21 in the GCL and 2.13 in the hilus and the ratio of activated microglia in the hilus versus GCL was 1.52. In sham-kindled rats, fewer activated than resting microglia were found in the GCL ($p < 0.01^c$) and hilus ($p < 0.001^c$) and more resting microglia were found in the hilus versus GCL ($p \leq 0.05^d$). In these rats, the ratio of activated versus resting microglia was 0.50 in the GCL and 0.37 in the hilus and the ratio of activated microglia in the hilus versus GCL was 0.92. The data demonstrate that increased numbers of activated microglia can be detected in the hippocampi of kindled rats for at least 4 weeks after they exhibit their final Grade 5 seizure and that the effects are most robust in the hilus.

Total mature new neuron number correlated positively with total new neuron number ($r_{(10)} = 0.96$; $p < 0.0001$) and total new neuron number correlated positively with the total number of activated hilar microglia ($r_{(10)} = 0.65$; $p < 0.05$) and negatively with the total number of resting hilar microglia ($r_{(10)} = -0.74$; $p < 0.05$). Since similar fold-increases in surviving new neurons and dividing Type-1 NSCs were detected in the dentate gyri of kindled versus sham-kindled rats, the relationship between persistently activated hilar microglia and mature new neuron numbers could instead reflect the relationship between activated hilar microglia and dividing Type-1 NSC numbers.

3.2. Sustained SST expression neither affected neurogenesis nor microglial activation in a small cohort of kindled rats

Experiment 2 tested whether vector-mediated sustained SST expression could alter the effects of amygdala kindling on the number and phenotype of dividing EdU⁺ cells, surviving BrdU⁺ cells and Iba-1⁺ microglia in the hippocampi of rats. Kindled rats were injected with BrdU 48 h after reaching criterion and then infused intrahippocampally with eGFP or SST-eGFP vector. Three weeks later, they were given a test stimulation followed by post-test stimulations. The rats were injected with EdU 48 h after the final post-test stimulation and perfused 4 h later (see **Figure 1**).

Before assignment to vector treatment groups, eGFP- and SST-eGFP vector-treated rats exhibited similar AD threshold currents (eGFP: $200.0 \pm 35.4 \mu\text{A}$ and SST-eGFP: $137.5 \pm 12.5 \mu\text{A}$; $t_{(6)} = -1.67$; $p = 0.15$), exhibited their first Grade 5 seizure after a similar number of sessions (eGFP: 16.3 ± 1.1 sessions and SST-eGFP: 12.5 ± 2.9 sessions; $t_{(6)} = -1.21$; $p = 0.27$), exhibited similar numbers of high-grade seizures (eGFP: 5.8 ± 0.6 seizures and SST-eGFP: 5.0 ± 1.1 seizures; $t_{(6)} = -0.60$; $p = 0.57$) and required similar numbers of sessions to achieve criterion (eGFP: 21.3 ± 1.8 sessions and SST-eGFP: 16.3 ± 4.2 sessions; $t_{(6)} = -1.08$, $p = 0.32$). Although 2/4 SST-eGFP vector-treated rats exhibited Grade 0 seizures on the test session administered 3 weeks after vector injection, both groups exhibited similar average seizure grades on the test session (eGFP 3.5 ± 1.2 and SST-eGFP: 1.3 ± 1.0 ; $U = 3.50$, $n_1 = n_2 = 4$, $p = 0.25$) and similar average seizure grades across post-test sessions (eGFP: 2.8 ± 1.1 and SST-eGFP: 3.6 ± 0.7 ; $U = 8.00$, $n_1 = n_2 = 4$, $p = 1.00$). Although we did not observe an effect of SST-eGFP vector treatment on seizure behavior in *Experiment 2*, we were interested in testing whether SST-eGFP vector treatment produced independent effects upon neurogenesis and microglial activation in kindled rats.

Figure 5A shows a representative microphotograph of DAB-stained EdU⁺ cells and the inset shows a cluster of EdU⁺ cells under 63x magnification. **Figure 5B** shows a representative confocal image of dividing EdU⁺ cells (in green) expressing the Type-1 NSC marker GFAP (in cyan) and the Type-2 NPC marker SOX2 (in pink) counted 4 h after EdU injection. The insets show examples of

Type-1 GFAP⁺/SOX2⁺ NSCs (yellow arrow) and Type-2 GFAP⁺/SOX2⁺ NPCs (white arrows). **Figure 5C** shows that the total number of dividing EdU⁺ cells was similar in the hippocampi of SST-eGFP versus eGFP vector-treated rats ($t_{(6)} = 0.92$; $p = 0.39$). **Figure 5D** shows the mean ($\pm$ S.E.M.) total number of dividing EdU⁺ Type-1 NSCs and Type-2 NPCs in the hippocampi of kindled rats treated with eGFP or SST-eGFP vector (see **Supplemental Table 1** for the percentages of EdU⁺ cells expressing each phenotype). While more Type-1 NSCs were observed than Type-2 NPCs ($F_{(1,6)} = 14.25$; $p < 0.01^{**}$), these numbers neither varied by group ($F_{(1,6)} = 1.16$; $p = 0.32$) nor the overall interaction ($F_{(1,6)} = 0.02$; $p = 0.88$). **Figure 5E** shows that the mean ($\pm$ S.E.M.) total number of surviving BrdU⁺ cells was similar in the hippocampi of eGFP- and SST-eGFP vector-treated rats ($t_{(6)} = -1.95$; $p = 0.10$). **Figure 5F** shows the mean ($\pm$ S.E.M.) net numbers of surviving BrdU⁺ neurons and astrocytes in the hippocampi of eGFP and SST-eGFP vector-treated rats. These numbers varied by phenotype ($F_{(3,18)} = 317.14$; $p < 0.0001$), but not by group ($F_{(1,6)} = 0.57$; $p = 0.48$) or the overall interaction ($F_{(3,18)} = 1.80$; $p = 0.18$). In both groups combined, the majority of surviving BrdU⁺ cells were mature neurons ($p < 0.001^{***}$ versus all other phenotypes). Taken together, these data show that there was no effect of SST-eGFP vector treatment on surviving BrdU⁺ cells or on dividing EdU⁺ cells in the dentate gyri of kindled rats. However, dependent t-tests confirmed that while numbers of surviving BrdU⁺ and dividing EdU⁺ cell numbers were related in eGFP vector-treated rats ($t_{(3)} = 8.68$; $p < 0.01$), they were unrelated in SST-eGFP vector-treated rats ($t_{(3)} = 1.68$; $p = 0.19$). In combination with our *Experiment 1* observation that similar fold increases in dividing Type-1 NSCs and surviving neurons are detected in kindled rats (**Figure 3D and 4C**), these data suggest that SST-eGFP vector treatment increased within-subjects variability enough to mask the relationship between the number of dividing cells labeled with BrdU before and EdU after SST-eGFP vector treatment.

Figure 5G shows the mean ($\pm$ S.E.M.) total number of microglia in the GCL and hilus of eGFP- and SST-eGFP-vector-treated rats. Although more Iba-1⁺ microglia were found in the hilus versus GCL ($F_{(1,6)} = 30.55$; $p < 0.01^{**}$), this number neither varied by group ($F_{(1,6)} = 0.13$; $p = 0.73$) nor the

overall interaction ($F_{(1,6)} = 1.04$; $p = 0.35$). **Figure 5H** shows the mean ($\pm$ S.E.M.) numbers of resting and activated Iba-1⁺ microglia in the GCL and hilus of eGFP- and SST-eGFP vector-treated rats (see **Supplemental Table 2** for percentages of Iba-1⁺ microglia that expressed the activation marker CD11b). These numbers varied by region ($F_{(1,6)} = 30.55$; $p < 0.01$), activation state ($F_{(1,6)} = 319.26$; $p < 0.0001$) and the interaction between region and activation state ($F_{(1,6)} = 21.89$; $p < 0.01$) but not by group or other interactions between the independent variables (all p values ≥ 0.35). In both groups combined, hilar microglia outnumbered GCL microglia ($p < 0.01^{**}$), activated microglia outnumbered resting microglia ($p < 0.001$), the number of activated microglia outnumbered resting microglia in the GCL ($p < 0.01^a$) and the hilus ($p < 0.001^a$) and the number of activated microglia was higher in the hilus versus GCL ($p < 0.01^b$). The ratio of activated versus resting microglia was 2.05 in the GCL and the 3.86 in the hilus and the ratio of activated microglia in the hilus versus GCL was 1.47. These data show that SST-eGFP vector treatment did not reverse the kindling-induced activation of microglia.

In vector-treated rats, the total number of activated hilar microglia correlated positively with the numbers of resting ($r_{(8)} = 0.86$; $p < 0.01$) and activated GCL microglia ($r_{(8)} = 0.74$; $p < 0.05$) but negatively with mature new neuron numbers ($r_{(8)} = -0.75$; $p < 0.05$). In the GCL, numbers of activated and resting microglia were correlated positively ($r_{(8)} = 0.87$; $p < 0.01$). These correlations suggest that an inflammatory response persists most profoundly in the dentate gyri of kindled rats and that the severity of microglial activation may influence the survival of new neurons.

3.3 Sustained SST expression reverses kindling-induced changes in NSC proliferation and potentiates activated microglia numbers in behavioral responders.

Since we previously demonstrated that subsets of rats respond behaviorally to sustained SST expression (Natarajan et al., 2017), we kindled a larger cohort of rats to test whether sustained SST expression differentially impacts neurogenesis and neuroinflammation in behavioral responders and non-responders. Rats were kindled to criterion, treated with eGFP or SST-eGFP vector and then given a test session followed by post-test sessions after a 3-week recovery. Rats were injected with BrdU 48 h after their final post-test session and perfused 4 h later (see **Figure 1**).

A subset of rats in the SST-eGFP vector-treated group responded to treatment by exhibiting Grade 0 seizures on the test session while others exhibited high grade seizures. We therefore divided the SST-eGFP vector-treated group into SST-eGFP vector-treated responder ($n = 5$) and SST-eGFP vector-treated non-responder ($n = 8$) groups. Prior to vector treatment, the eGFP vector-treated controls, SST-eGFP vector-treated responders and non-responders exhibited similar AD threshold currents (eGFP vector-treated controls: $187.5 \pm 12.5 \mu\text{A}$, SST-eGFP responders: $230.0 \pm 33.9 \mu\text{A}$, SST-eGFP non-responders: $212.5 \pm 32.4 \mu\text{A}$; $F_{(2,14)} = 0.34$; $p = 0.72$), numbers of sessions before exhibiting their first Grade 5 seizure (eGFP vector-treated controls: 20.0 ± 3.1 sessions, SST-eGFP responders: 18.4 ± 2.3 sessions, and SST-eGFP non-responders: 16.5 ± 1.8 sessions; $F_{(2,14)} = 0.60$; $p = 0.56$), numbers of high-grade seizures (eGFP vector-treated controls: 9.3 ± 2.0 seizures and SST-eGFP responders: 5.4 ± 0.9 seizures and SST-eGFP non-responders: 7.1 ± 1.0 seizures; $F_{(2,14)} = 1.85$; $p = 0.19$) and numbers of sessions to achieve criterion (eGFP vector-treated controls: 27.0 ± 2.1 sessions, SST-eGFP responders: 23.0 ± 2.3 sessions, and SST-eGFP non-responders: 22.6 ± 1.7 sessions; $F_{(2,14)} = 1.18$, $p = 0.35$). We found a significant effect of vector treatment on test session seizure grade (eGFP vector-treated controls: 3.3 ± 0.9 , SST-eGFP responders: 0.0 ± 0.0 and SST-eGFP non-responders: 4.9 ± 0.1 ; $H_{(2,17)} = 12.72$; $p < 0.01$), such that SST-eGFP responders exhibited a lower seizure grade than either eGFP vector-treated controls ($p < 0.05$) and SST-eGFP non-responders ($p < 0.01$). The effect size of the difference between the eGFP vector-treated and SST-eGFP responder groups on test session seizure grade ($d = 2.47$) far exceeded Cliff's convention for a large effect size (Cliff, 1993). We tested average post-test session grade prior to stepping up the AD current as a measure of therapeutic resilience and found a significant effect of vector treatment (eGFP vector-treated controls: 4.6 ± 0.3 , SST-eGFP responders: 1.6 ± 0.9 , SST-eGFP non-responders: 4.8 ± 0.1 ; $H_{(2,17)} = 6.73$; $p < 0.05$), such that SST-eGFP responders exhibited a lower average seizure grade across post-test sessions than SST-eGFP non-responders ($p < 0.05$). Group differences were not detected at higher stimulation currents.

Figure 6A shows that the mean ($\pm$ S.E.M.) total number of dividing BrdU⁺ cells differed in the hippocampi of eGFP vector-treated controls, SST-eGFP responders and SST-eGFP non-responders ($F_{(2,14)} = 7.84$; $p < 0.01$), such that SST-eGFP responders had fewer dividing BrdU⁺ cells than either eGFP vector-treated controls ($p < 0.01^a$) or SST-eGFP non-responders ($p < 0.05^b$). **Figure 6B** shows the mean ($\pm$ S.E.M.) total number of dividing BrdU⁺ Type-1 NSCs and Type-2 NPCs in the hippocampi of eGFP vector-treated controls, SST-eGFP non-responders, and SST-eGFP responders (see **Supplemental Table 1** for the percentages of BrdU⁺ cells expressing each phenotype). Generally, more Type-1 NSCs were observed than Type-2 NPCs ($F_{(2,14)} = 20.54$; $p < 0.001$), but these numbers varied by group ($F_{(2,14)} = 7.45$; $p < 0.01$) and by the overall interaction ($F_{(2,14)} = 23.67$; $p < 0.0001$). Fewer dividing BrdU⁺ cells were found in the dentate gyri of SST-eGFP responders versus eGFP vector-treated controls and SST-eGFP non-responders ($p < 0.01$ and $p < 0.05$, respectively). This effect was primarily due to the reduced number of dividing Type-1 NSCs found in the dentate gyri of SST-eGFP responders versus eGFP vector-treated controls and SST-eGFP non-responders (both p values $< 0.001^{***}$). Moreover, fewer dividing Type-2 NPCs than dividing Type-1 NSCs were found in the dentate gyri of eGFP vector-treated controls ($p < 0.05^a$) and SST-eGFP non-responders ($p < 0.001^a$), but similar numbers of dividing Type-1 NSCs and Type-2 NPCs were observed in SST-eGFP-responders. These data suggest that sustained SST transgene expression may block the proliferation of Type-1 NSCs in behavioral responders.

Figure 6C shows the mean ($\pm$ S.E.M.) total number of microglia in the GCL and hilus of eGFP vector-treated controls, SST-eGFP non-responders, and SST-eGFP responders. More Iba-1⁺ microglia were found in the hilus versus GCL ($F_{(2,14)} = 15.10$; $p < 0.01$), but the numbers neither varied by group ($F_{(2,14)} = 0.44$; $p = 0.66$) nor the overall interaction ($F_{(2,14)} = 1.09$; $p = 0.36$). **Figure 6D** shows the mean ($\pm$ S.E.M.) numbers of resting and activated Iba-1⁺ microglia in the GCL and hilus of eGFP vector-treated controls, SST-eGFP non-responders and SST-eGFP responders (see **Supplemental Table 2** for percentages of Iba-1⁺ cells expressing the activation marker CD11b).

These numbers varied by region ($F_{(2,14)} = 15.10$; $p < 0.01$), activation state ($F_{(2,14)} = 207.97$; $p < 0.0001$) and the interaction between region and activation state ($F_{(1,6)} = 52.80$; $p < 0.0001$) but neither by group nor other interactions between the independent variables (all p values ≥ 0.09). Generally, hilar microglia outnumbered GCL microglia ($p < 0.01$) and activated microglia outnumbered resting microglia ($p < 0.001$). In fact, activated microglia outnumbered resting microglia in both the GCL ($p < 0.01^{**}$) and hilus ($p < 0.001^{***}$) and the number of activated microglia was higher in the hilus versus GCL ($p < 0.001^a$). The ratio of activated versus resting microglia was 1.54 in the GCL and the 2.89 in the hilus and the ratio of activated microglia in the hilus versus GCL was 1.54.

The total number of dividing Type-1 NSCs correlated positively with the total number of activated GCL ($r_{(17)} = 0.62$; $p < 0.0001$) and hilar ($r_{(17)} = 0.43$; $p < 0.05$) microglia and negatively with the total number of resting GCL ($r_{(17)} = -0.37$; $p < 0.05$) and hilar ($r_{(10)} = -0.44$; $p < 0.01$) microglia. These data suggest that microglial activation and Type-1 NSC proliferation may be related processes in the hippocampi of kindled rats.

Discussion

In this study, we tested whether sustained vector-mediated expression of a SST gene could alter the effects of kindling on neurogenesis and neuroinflammation in the dentate gyri of adult male rats. We found that the proliferation of Type-1 NSCs was stimulated in the hippocampi of kindled rats and observed a microglial response that persisted in the hilus for several weeks. These effects were also observed in the hippocampi of kindled rats that did not respond behaviorally to SST vector treatment. However, numbers of dividing Type-1 NSCs (but not activated microglia) were normalized in the hippocampi of rats that did respond behaviorally to SST vector treatment by exhibiting Grade 0 seizures on the test session. Our data are consistent with a role for aberrant neurogenesis in seizure maintenance and suggest the value of optimizing SST-based treatment strategies or alternatives that target upregulated Type-1 NSC division in models of temporal lobe epilepsy.

In agreement with studies employing single or repeated electrical stimulation protocols we found that kindling upregulated the number of dividing cells in the hippocampi of adult rats (Bengzon et al., 1997; Nakagawa et al., 2000; Parent et al., 1998; Parent et al., 1997). Dividing cells in the hippocampal dentate gyrus can include relatively quiescent Type-1 NSCs and more commonly proliferative uncommitted Type-2a and neuronal lineage-committed Type -2b NPCs (Filippov et al., 2003; Kronenberg et al., 2003; Seri et al., 2001; Steiner et al., 2006; Suh et al., 2007). We add to the picture of how electrical kindling affects neurogenesis by showing that the number of dividing Type-1 NSCs (but not Type-2 NPCs) is elevated ~3-fold even 48 h after rats experience their 3rd consecutive Grade 5 seizure in twice daily sessions (**Figure 3E**) or after rats that had already achieved criterion weeks earlier experience their final intermittent seizure in post-test sessions administered every 2-3 days (**Figures 5D** and **6B**). We expect that these increases may be more robust in this model at earlier time points (for example, 0-48 h) after the final seizure, as the neurogenic effect may degrade over time after an induced seizure (Nakagawa et al., 2000). Our finding is consistent with studies describing increased numbers of dividing Type-1 NSCs in the dentate gyri of rodents after electroconvulsive seizures or status epilepticus induced by the chemoconvulsants kainic acid and pilocarpine (Borges et al., 2006; Hüttmann et al., 2003; Segi-Nishida et al., 2008; Steiner et al., 2008; Zhu et al., 2005). Whether the abnormal upregulation of Type-1 NSC division in kindling models directly contributes to the production of ectopic granule neurons with aberrant morphologies and connectivity across epilepsy models is unclear.

The observation that supraphysiological numbers of new neurons with aberrant morphologies and connectivity are produced in the hippocampi of animals kindled electrically or chemically led to the hypothesis that aberrant neurogenesis may underlie seizure development and maintenance in epilepsy models (Jessberger and Parent, 2015). Several studies have shown that ablating neurogenesis with targeted γ -irradiation, anti-proliferative drug or genetic ablation strategies does not eliminate seizures but can reduce the number of seizures exhibited and their duration (Chen et al.,

2001; Cho et al., 2015; Jung et al., 2004; Jung et al., 2006; Maesawa et al., 2000; Mori et al., 2000). Importantly, quiescent Type-1 NSCs are neither susceptible to these ablation strategies nor to doses of ^3H -thymidine or thymidine analogues that are lethal to dividing cells (Barazzuol et al., 2017; Cho et al., 2015; Morshead et al., 1994) rendering the ablation of neurogenesis using these strategies incomplete. Therefore, the development of novel strategies that specifically ablate quiescent Type-1 NSCs may be necessary to test the role that aberrant neurogenesis plays in the development and maintenance of seizures. Future work employing a complete ablation strategy would be necessary for testing whether SST vector-mediated changes in neurogenesis impacted seizure behavior or whether SST vector-mediated changes in seizure behavior impacted neurogenesis in SST-responder rats.

New neurons produced in the adult rodent hippocampus become morphologically and electrophysiologically indistinguishable from mature granule neurons after about a month (Cameron and McKay, 2001; Speisman et al., 2013a; Speisman et al., 2013b; van Praag et al., 1999a). Consistent with these data, we found that most 4 week-old cells expressed a mature neuronal phenotype in the dentate gyri of both sham-kindled and kindled rats. We did not specifically test whether new neurons matured and integrated more quickly in the dentate gyri of kindled versus sham-kindled rats (Overstreet-Wadiche et al., 2006), but we did observe similar ratios of dividing and surviving neurons between groups, suggesting that neuronal differentiation and the survival of new neurons was unaffected by the physiological changes that accompany the fully-kindled seizure susceptible state. Note that thymidine analogues are metabolically active for ~2h after injection, that NSCs/NPCs complete division in ~ 24h and divide several times and that a large number of progeny die within 2 weeks of birth in the adult hippocampus (Cameron and McKay, 2001; Cameron et al., 1993). The similar numbers of dividing cell numbers quantified 4h after and surviving new neuron numbers quantified weeks after thymidine analogue injection in sham rats and in kindled rats in *Experiment 1* (**Figure 3** versus **4**) and in control vector and in SST-eGFP vector-treated rats in *Experiment 2* (**Figure 5**) also supports the notion that new neuron survival was not impacted by the

seizure susceptible state, additional imposed seizures, vector injection or sustained SST or GFP expression. In fact, these results are consistent with other studies that quantify labeled cells in cage control animals at similar time points after a single thymidine analog injection (Cameron et al., 1993; Gould et al., 1999) and with the natural decline reported in surviving versus dividing numbers quantified at similar time points after multiple thymidine analog injections (Kempermann et al., 1997). Seizures can induce mossy fiber sprouting and ectopic migration in young neurons but more profoundly impact neurons born during or just after the seizure (Kron et al., 2010). Testing whether Type-1 NSCs that are stimulated to divide in the kindled brain produce Type-2a NPCs, Type-2b NPCs and/or neurons with more aberrant morphologies, placement or maturation rates than dividing Type-2 NPCs may provide insight into whether and how aberrant neurogenesis supports the development and maintenance of seizures.

Once fully kindled, rats continue to reliably produce robust evoked seizures across time and multiple tests (Brandt et al., 2004, Kalynchuk, 2000, Pinel and Rovner 1978). Thus, any subsequent low-grade evoked seizure could be considered a response, whereas a prolonged reduction in evoked seizure grade may be considered therapeutic resilience. The key distinction lies in the question being asked. Our responder criterion based on evoked seizure grade upon the first re-stimulation after vector is clinically relevant and is often used in the preclinical study of anti-epileptic drugs. An interesting aspect of the SST vector treatment was the all-or-none response, which we observed in prior work as well (Natarajan et al., 2017). We took advantage of this observation to probe differences in neurogenesis and in markers of inflammation between treatment responders versus non-responders.

Since SST vector blocked seizures in a subset of fully kindled rats and SST is anti-proliferative in other tissues, we hypothesized that SST may produce behavioral effects by modulating neurogenesis in the dentate gyri of kindled rats. We found that sustained SST transgene expression normalized the division of Type-1 NSCs in dentate gyri of kindled rats that responded behaviorally to

SST treatment. This effect is interesting because inhibitory hilar SSTergic neurons may selectively die early in epileptogenesis (Botterill et al., 2017; Buckmaster and Dudek, 1997; Robbins et al., 1991; Sloviter, 1987; Sun et al., 2007). Our data raise the interesting possibility that vector-mediated SST expression may potentiate the anti-proliferative effects that surviving SSTergic neurons may exert on dividing Type-1 NSCs. Although SST expressing cells are found in the dentate gyrus SGZ/GCL region (Billova et al., 2007; Natarajan et al., 2017), whether NSCs and NPCs express SSTRs in adult animals is unknown. SST does modulate neuronal migration during embryogenesis and embryonic NSCs/NPCs express SSTRs (Maubert et al., 1994; Yacubova and Komuro, 2002). In addition, SSTR₂ agonists suppress the adrenalectomy-induced proliferation of pituitary cells and TGF- β works through SST to suppress the expansion of neuroendocrine tumor cells (Leu et al., 2008; Nolan et al., 2007). Future experiments that identify the mechanisms through which SST modulates Type-1 NSC proliferation may lead to better SST responder rates, since SSTR expression could differ between SST responder and non-responder rats. In addition, the higher responder rates observed when vector-mediated SST expression is initiated before the onset of kindling sessions (Zafar et al., 2012), suggests that SST may work through transient mechanisms with timing that varied between responder and non-responder rats. Although we detected similar infection intensities and overall SST responder rates between Experiment 2, which employed a SST-eGFP vector with a medium titer (1.0×10^{13} vg/ml; 2/4 responders) and Experiment 3, which employed SST-eGFP vectors with lower (6.1×10^{12} vg/ml; 1/7 responders) or higher (4.9×10^{13} vg/ml; 4/6 responders) titers, SST responder ratios suggest that responder rates may increase with increasing titers. However, differences in design between Experiments 2 and 3 and low rat numbers within each titer group precluded direct comparison of the effects of titer dose on dividing Type-1 NSCs and on seizure severity. Future work should experimentally test whether SST vector titer dose impacts these variables.

While neuroinflammation is typically reported to compromise neurogenesis (Chen et al., 2011; Ekdahl et al., 2003; Lee et al., 2013; Monje et al., 2003; Ormerod et al., 2013), neurogenesis and

neuroinflammation are both potentiated in the hippocampi of kindled rats (Bengzon et al., 1997; Khurgel and Ivy, 1996; Parent et al., 1998; Parent et al., 1997; Plata-Salamán et al., 2000). In the current study, we found an increased number of activated microglia immediately after rats exhibited their 3rd Grade 5 seizure that persisted most robustly in the hilus for at least 4 weeks. The increased number of dividing Type-1 NSCs found in the dentate gyri of kindled rats after they exhibited their 3rd Grade 5 seizure both correlated positively with activated microglia number and translated into a similar increase in new neuron number 4 weeks later. Given that complex stimuli can produce variable microglial activation states (Butovsky et al., 2006; Siddiqui et al., 2016), kindling could stimulate a microglial activation state that differs from that which ablates neurogenesis in other models. For instance, seizures can induce the microglial production of insulin-like growth factor-1, which stimulates hippocampal cell proliferation (Aberg et al., 2003; Beck et al., 1995; Choi et al., 2008; O'Kusky et al., 2000; Trejo et al., 2001) and reducing microglial activation after status epilepticus by blocking microglial CX3CR₁ signaling normalizes subgranular zone cell proliferation (Ali et al., 2015). We are currently profiling hippocampal protein samples of control, kindled and vector-treated kindled rats to identify which cytokines vary in concentration across these contexts. Alternatively, kindling may produce a neuroinflammatory response similar to that which ablates neurogenesis that is initially superseded by another physiological effect produced by kindling and/or kindled seizures. The observation that hippocampal neurogenesis is severely diminished in chronic epilepsy models or in individuals with chronic epilepsy (D'Alessio et al., 2010; Hattiangady et al., 2004) suggests that either the chronic neuroinflammatory response eventually compromises neurogenesis or possibly that the progenitor pool becomes exhausted by the initial supraphysiological upregulation of Type-1 NSC division. Identifying the mechanisms through which neurogenesis is modulated in the kindled brain may provide insights into novel therapeutic strategies.

Conclusions

We demonstrated that the number of dividing Ty NSCs is potentially increased 48 h after kindled rats exhibit their 3rd consecutive Grade 5 seizure and that sustained vector-mediated SST expression normalizes this number in the subset of rats that respond behaviorally to SST treatment. This finding encourages the refinement of strategies that more specifically target SST signaling and their timing with the intention of improving response rates in future work. Consistent with previous work, the increase in dividing Type-1 NSCs that we observed was paradoxically associated with an increase in the number of activated microglia in the GCL and a more robust and persistent increase in the hilus of kindled rats and this neuroinflammatory response was unaffected by vector treatment. Testing whether or how the neuroinflammatory or other physiological responses that accompany epilepsy or that are stimulated by kindling in animal models of epilepsy may uniquely render neurogenesis aberrant may provide insight into the mechanisms that drive the development and maintenance of seizure behavior. Collectively, our results suggest that Type-1 NSCs may be a novel target for epilepsy treatments and that aberrant neurogenesis *could be* a cellular mechanism of seizure development and maintenance. Of course, future work employing complete neurogenesis ablation strategies (i.e. targeting resistant Type-1 NSCs) would be required to test whether aberrant neurogenesis (through the upregulation of Type-1 NSCs) is a cellular mechanism for epilepsy or physiological response to seizures. In the latter case, aberrantly connected young neurons could still provide a cellular substrate for seizure maintenance.

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Figure Captions

Figure 1. Schematic of experiment designs. *Experiment 1* tested the effects of kindling on the number of dividing Type-1 neural stem cells and Type-2 neural progenitor cells and the survival of their progeny. Rats were kindled to criterion or sham-kindled and then injected with BrdU (100 mg/kg) 48 h later. Kindled and sham-kindled rats were perfused 4 h later to quantify the number and phenotype of dividing cells or 4 weeks later (n = 5 per group) to quantify the number and phenotype of their progeny. *Experiment 2* tested the effects of sustained SST gene expression on the number and phenotype of surviving cells 6 weeks after kindling and on the number of dividing Type-1 NSCs and Type-2 NPCs. Rats were kindled to criterion and then injected with BrdU (100 mg/kg) 48 h later and then injected intrahippocampally with either eGFP or SST-eGFP vector. They received one test stimulation 3 weeks later followed by periodic post-test stimulations for an additional 3 weeks to test persistence of kindling and vector effects. eGFP- and SST-eGFP vector-treated rats were injected with EdU (100 mg/kg) 48 h after their last post-test stimulation and perfused 4 h later (n = 4 and n = 4, respectively) to quantify surviving and dividing cells. *Experiment 3* tested the effects of sustained SST gene expression on the number of dividing Type-1 NSCs and Type-2 NPCs. Rats were kindled until criterion and then injected intrahippocampally with either eGFP or SST-eGFP vector. They received one test stimulation 3 weeks later followed by periodic post-test stimulations for an additional 3 weeks to test persistence of kindling and vector effects. eGFP- and SST-eGFP vector-treated rats were injected with BrdU (100 mg/kg) 48 h after their last post-test session and perfused 4 h later (n = 4 and n = 13, respectively) to quantify the number and phenotype of dividing cells.

Figure 2. Schematic showing vector target regions in the hippocampus and a confocal image of eGFP expression in a hippocampal section of a SST-eGFP vector-treated rat. Vector was injected bilaterally into the dentate gyrus and CA1 region (2 μ l per injection site). Bilateral eGFP expression was detected through the rostral-caudal extent of at least 1 major hippocampal subfield (the CA1 region, CA3 region and/or dentate gyrus) of all rats and moderate to strong bilateral eGFP

expression was found through the CA1 and CA3 region of most rats. eGFP expression was detected in the cortex dorsal to and thalamic regions ventral to the hippocampus of some rats. No overt differences in eGFP expression were found between eGFP vector-treated, SST-eGFP vector-treated responder and SST-eGFP vector-treated non-responder groups.

Figure 3. The number of dividing Type-1 neural stem cells and activated microglia was increased in the hippocampi of kindled rats. Representative microphotographs of DAB-stained dividing BrdU⁺ cells (in brown) in the dentate gyrus of a kindled **(A)** and sham-kindled **(B)** rat. **(C)** Representative fluorescent image of dividing BrdU⁺/Sox2⁺/GFAP⁺ Type-1 NSCs (yellow arrowheads) and a BrdU⁺/Sox2⁺/GFAP⁻ Type-2 NPC (white arrowhead). **(D)** Stereological estimates of BrdU⁺ cells within the DG revealed a ~2-fold increase in proliferation ($p < 0.001^{***}$) 48 h after rats reached kindling criterion. **(E)** More BrdU⁺ Type-1 NSCs were found in the dentate gyri of kindled versus sham-kindled rats ($p < 0.001^{***}$) and in kindled rats, more dividing Type-1 NSCs were detected than Type-2 NPCs ($p < 0.001^{***}$). **(F)** Representative microphotograph of DAB-stained Iba-1⁺ microglia (in brown) in the dentate gyrus. **(G, H)** Representative fluorescent images of a resting Iba-1⁺/CD11b⁻ microglia **(G)** and activated Iba-1⁺/Cd11b⁺ microglia **(H)**. **(I)** Stereological estimates revealed similar Iba-1⁺ microglia numbers in the granule cell layer and hilus of kindled and sham-kindled rats but more Iba-1⁺ microglia were found in the hilus versus granule cell layer of all rats ($p < 0.05^*$), **(J)** however, fewer resting microglia were found in the GCL ($p < 0.01^{**}$) and hilus ($p < 0.001^{***}$) of kindled versus sham-kindled rats that had a greater number of activated microglia in the GCL ($p < 0.001^{***}$) and hilus ($p < 0.001^{***}$). In sham-kindled rats, more resting than activated microglia were found in both the GCL ($p < 0.01^a$) and hilus ($p < 0.001^a$). In kindled rats, more activated than resting microglia were found in the GCL ($p < 0.001^b$) and hilus ($p < 0.001^b$) and the number of activated microglia was elevated in the hilus versus GCL ($p < 0.05^c$).

Figure 4. Kindling increases the number of surviving neurons and leads to persistent microglial activation. Representative fluorescent images of a new adult-born BrdU⁺/NeuN⁺ granule

neuron **(A)** and BrdU⁺/GFAP⁺ astrocyte **(B)**. **(C)** Stereological estimate of surviving (BrdU⁺) cells revealed a ~2-fold increase in the number of surviving cells in kindled rats ($p < 0.001^{***}$) at 4 weeks after reaching kindling criterion and BrdU injection. **(D)** Quantification of the number of surviving immature (BrdU⁺/DCX⁺/NeuN⁻), transitioning (BrdU⁺/DCX⁺/NeuN⁺), mature (BrdU⁺/DCX⁻/NeuN⁺) neurons, and astrocytes (BrdU⁺/GFAP⁺) revealed that kindling increased the number of mature neurons relative to sham-kindling ($p < 0.001^{***}$) and consistent with other reports and our timeline, the majority of newly-born cells differentiated into neurons with most having reached the mature phenotype ($p < 0.001^a$ versus all other phenotypes, followed by transitioning neurons ($p \leq 0.05^b$ versus immature neurons and astrocytes). **(E)** Stereological estimates for total microglia (Iba-1⁺) within the GCL and hilus revealed more microglia in both regions in kindled rats versus sham-kindled and more microglia in the hilus versus GCL of all rats ($p \text{ values} < 0.05^*$). **(F)** Quantification of resting and activated microglia in the GCL and hilus revealed no difference in the number of resting microglia in the GCL between groups but fewer resting in the hilus of kindled versus sham-kindled rats ($p < 0.05^*$). There were more activated microglia in both the GCL ($p < 0.001^{**}$) and the hilus ($p < 0.001^{***}$) of kindled rats versus sham-kindled. There were more microglia in the hilus versus GCL of all rats ($p < 0.05^*$). In kindled rats, more activated microglia were found in the hilus versus GCL ($p < 0.001^a$) and in the hilus, activated microglia outnumbered resting microglia ($p < 0.001^b$). In sham-kindled rats, fewer activated than resting microglia were found in the GCL ($p < 0.01^c$) and hilus ($p < 0.001^c$) and more resting microglia were found in the hilus versus GCL ($p \leq 0.05^d$).

Figure 5. Sustained SST transgene expression did not impact dividing, surviving cells, or microglial response in kindled rats. **(A)** Representative photomicrograph of DAB-stained dividing EdU⁺ cells under a 10X objective with an inset of a cluster of newly dividing/divided cells under 63X magnification. **(B)** Representative fluorescent image of newly divided EdU⁺/Sox2⁺ Type-2 NPCs in the GCL of kindled rats. **(C)** Stereological estimates for newly divided (EdU⁺) cells revealed no significant difference in dividing cell number between eGFP- and SST-eGFP vector-treated rats. **(D)**

Quantification of the number of newly divided Type-1 NSCs and Type-2 NPCs revealed no differences between eGFP- and SST-eGFP vector-treated rats 2d after their last post-test stimulation but more Type-1 NSCs than Type-2 NPCs in both groups combined ($p < 0.01^{**}$) **(E)** Stereological estimates for surviving (BrdU⁺) cells revealed no difference 6 weeks after vector-treated rats achieved kindling criterion and received their respective vector injections. **(F)** Quantification of the phenotype (immature DCX⁺/NeuN⁻ neuron, transitioning DCX⁺/NeuN⁺ neuron, mature DCX⁻/NeuN⁺ neuron, or GFAP⁺ astrocyte) of surviving cells revealed no difference between treatment groups 6 weeks after treated rats reached kindling criterion and received their respective vector injections, but more mature neurons were found versus all other cell types in both groups combined ($p < 0.001^{***}$). **(G)** Stereological estimates of total microglia (Iba-1⁺) within the GCL and hilus revealed no differences between treatment groups on microglia phenotype in the GCL or hilus but more microglia were found in the hilus versus GCL of both groups combined ($p < 0.01^{**}$). **(H)** In both groups combined, hilar microglia outnumbered GCL microglia ($p < 0.01^{**}$), the number of activated microglia outnumbered resting microglia in the GCL ($p < 0.01^a$) and the hilus ($p < 0.001^a$) and the number of activated microglia was higher in the hilus versus GCL ($p < 0.01^b$). These data show that SST-eGFP vector-treatment did not reverse the kindling-induced activation of microglia.

Figure 6. Kindling-stimulated changes in Type-1 division were diminished in responders to SST gene transfer. **(A)** Stereological estimates of dividing BrdU⁺ cell number revealed no difference between eGFP- and SST-eGFP vector-treated non-responders, but a significant decrease in SST-eGFP vector-treated responders versus eGFP vector-treated rats ($p < 0.01^a$) and SST-eGFP vector-treated non-responders ($p < 0.05^b$). **(B)** Quantification of the number of newly divided Type-1 NSCs and Type-2 NPCs revealed a decrease in the proliferation of BrdU⁺ Type-1 NSCs in SST-eGFP vector-treated responders, but not in SST-eGFP vector-treated non-responders, versus eGFP vector-treated rats ($p < 0.001^{***}$) and fewer BrdU⁺ Type-2 NSCs in eGFP vector-treated ($p < 0.05^a$) and SST-eGFP vector-treated non-responders ($p < 0.001^a$) than BrdU⁺ Type-1 NPCs, but not in SST-eGFP

vector-treated responders. **(C)** Stereological estimates of Iba-1⁺ microglia revealed no differences in microglia number between groups in either the GCL or hilus hippocampal subregions but more microglia in the hilus versus GCL of all groups combined ($p < 0.001^{**}$). **(D)** Activated microglia outnumbered resting microglia in both the GCL ($p < 0.01^{**}$) and hilus ($p < 0.001^{***}$) and the number of activated microglia was higher in the hilus versus GCL ($p < 0.001^a$).

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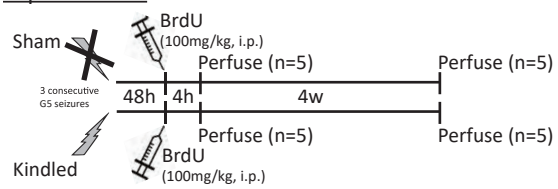
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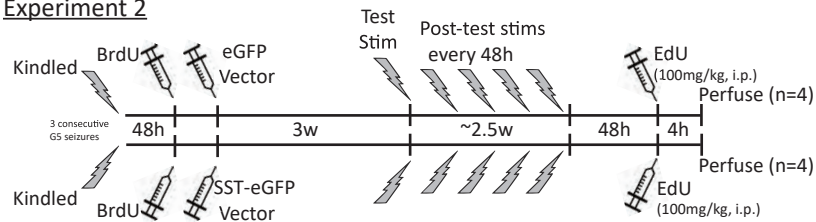
Figure 1

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Experiment 1



Experiment 2



Experiment 3

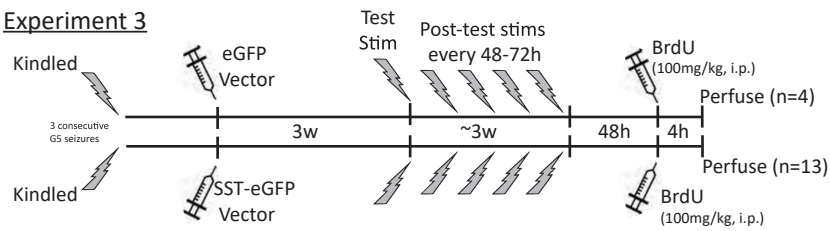


Figure 2

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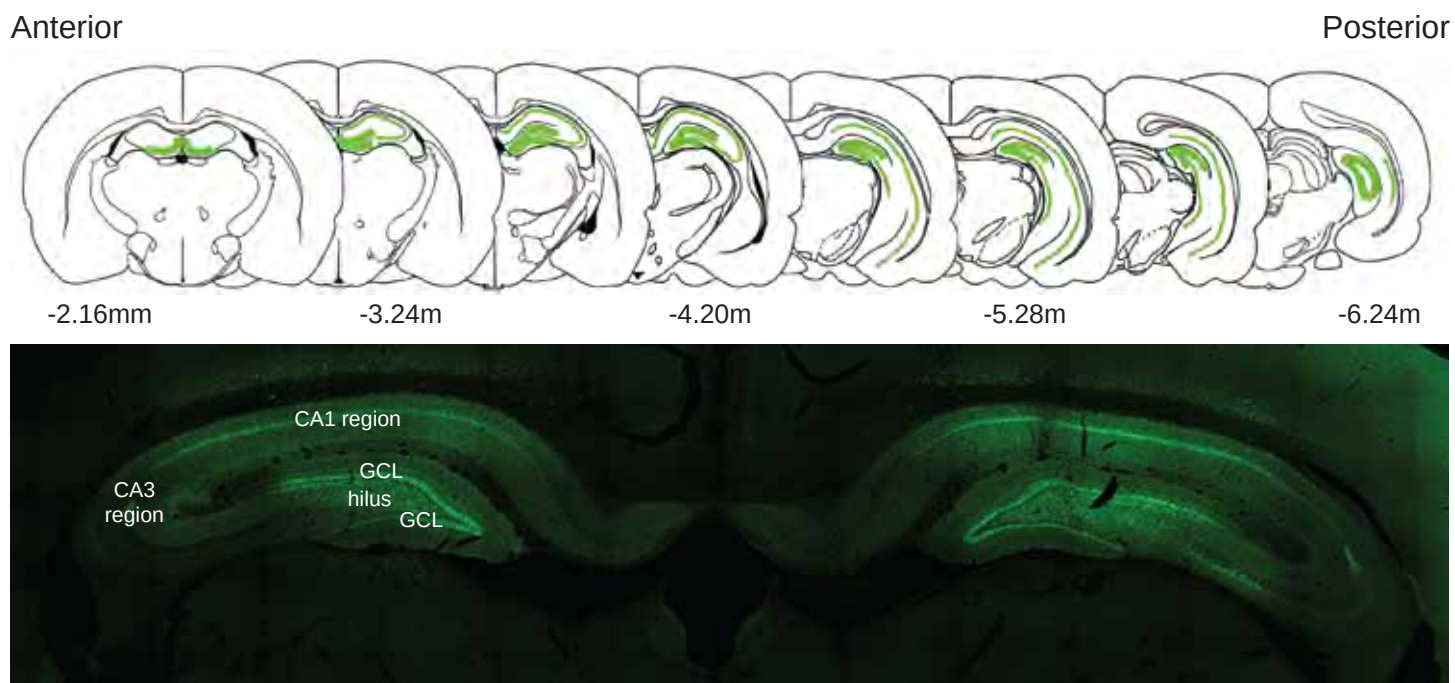


Figure 3
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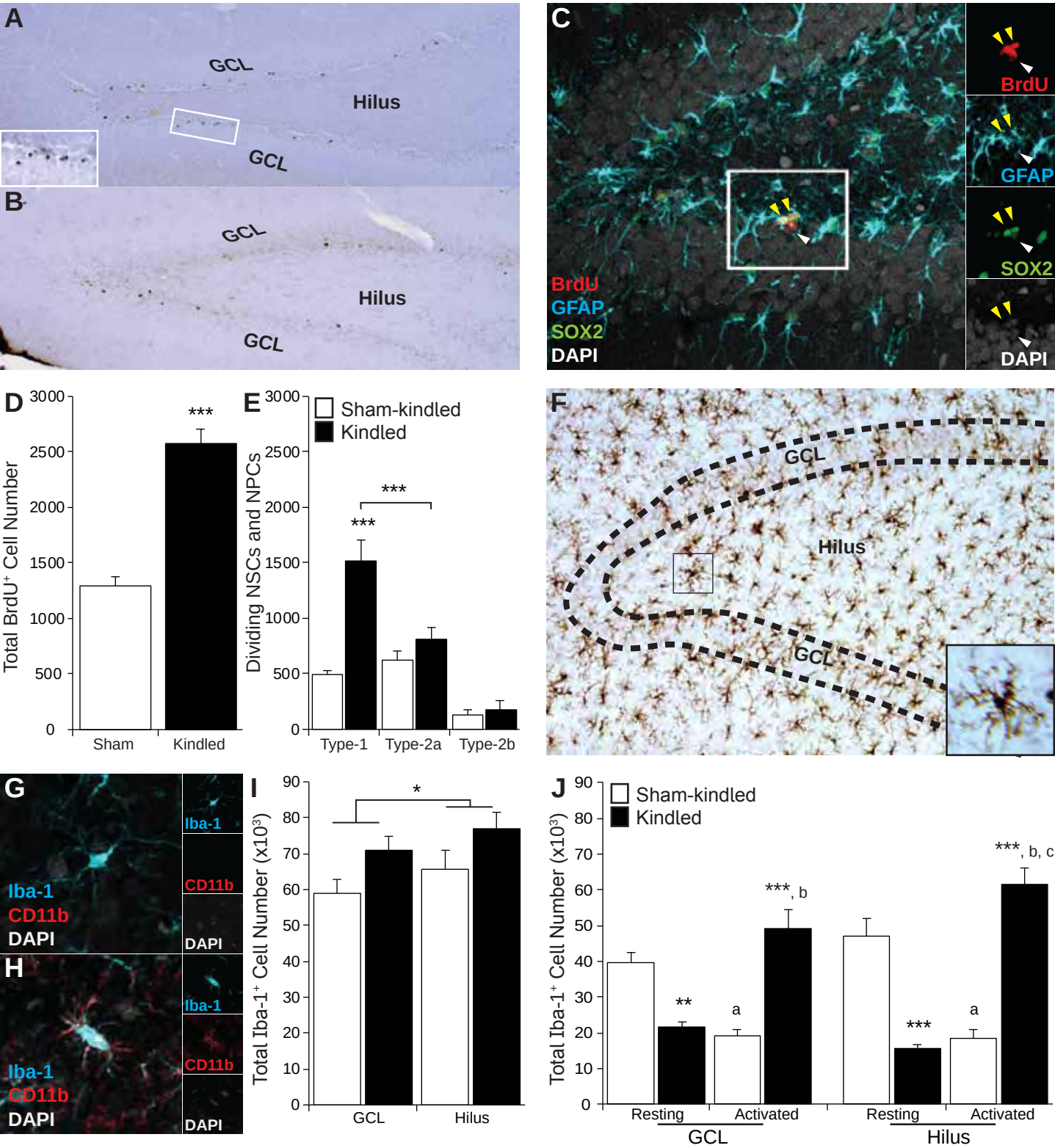


Figure 4
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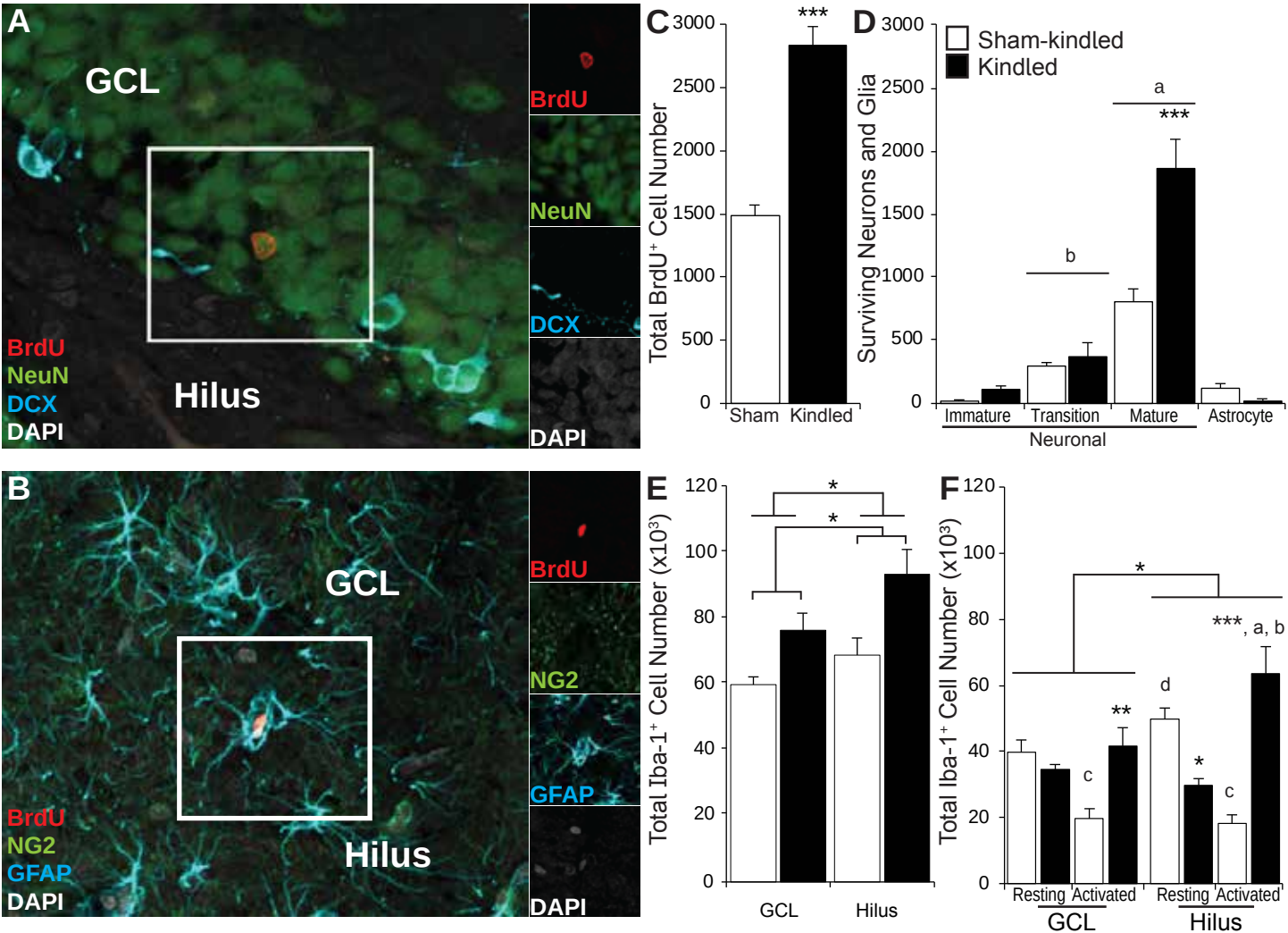


Figure 5
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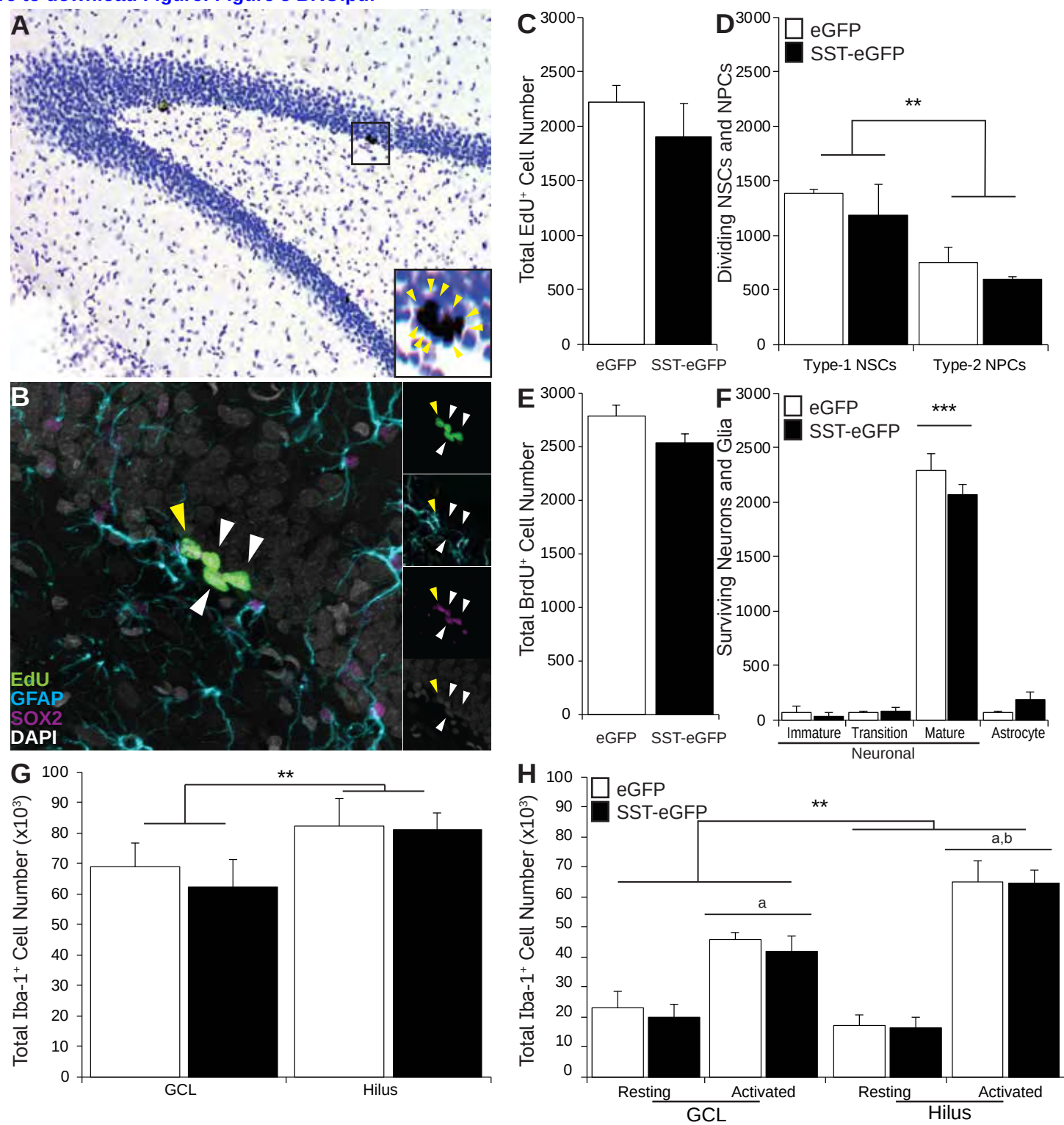
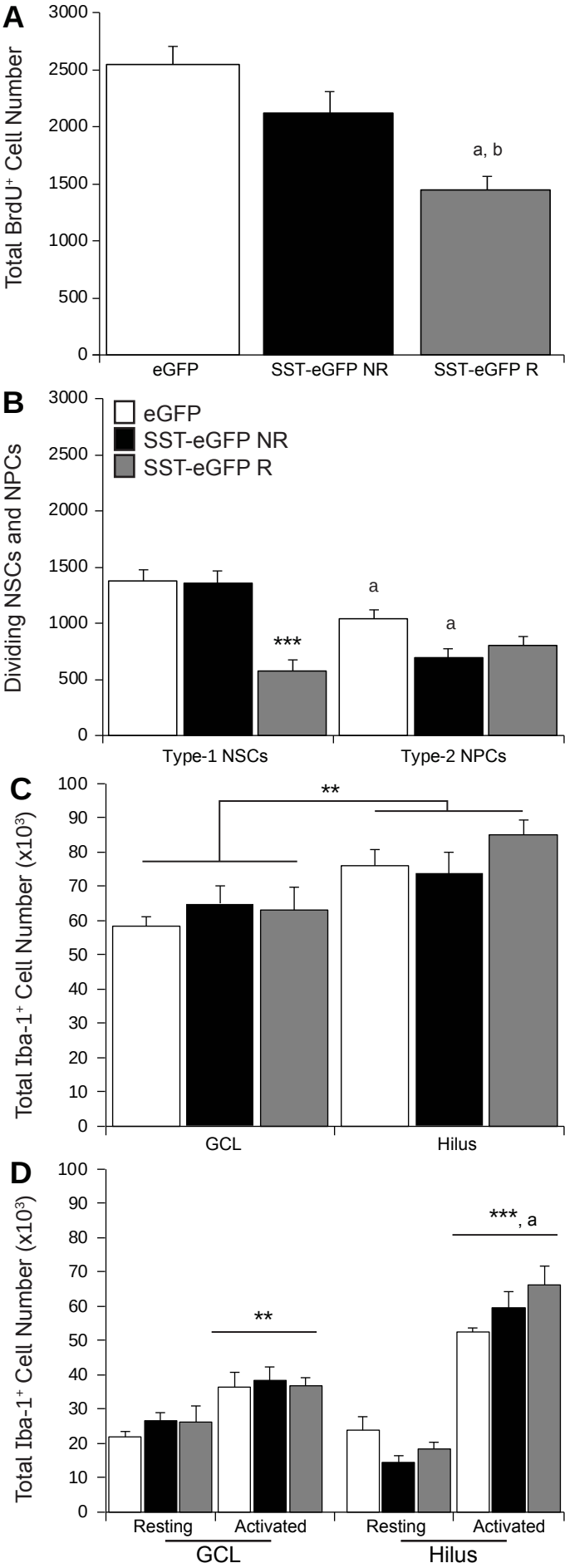


Figure 6
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Supplemental Table 1

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Supplemental Table 2

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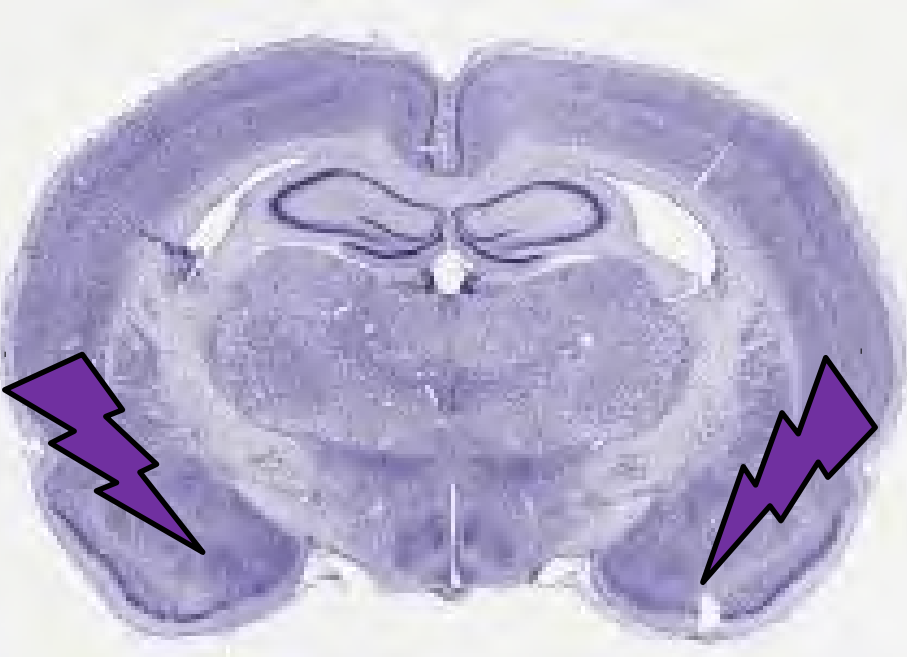
consecutive Racine Grade 5 seizures. Subsequently, AAV serotype 5 vector driving eGFP (AAV5-CBa-eGFP) or rat preproSST and eGFP (AAV5-CBa-preproSST-eGFP) gene expression was injected bilaterally into the hippocampus dentate gyrus and CA1 region. Three weeks later, rats were re-tested at periodic intervals 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 temporal lobe epilepsy. 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 somatostatin maybe an effective and safe therapeutic candidate for temporal lobe epilepsy. 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 intracranial preproSST gene transfer has putative antiepileptic effects *in vivo* in a rat model of TLE – **therapeutic efficacy**.
Aim 2: Determine the effects of preproSST gene transfer *in vivo* on hippocampus dependent learning and memory – **safety**.

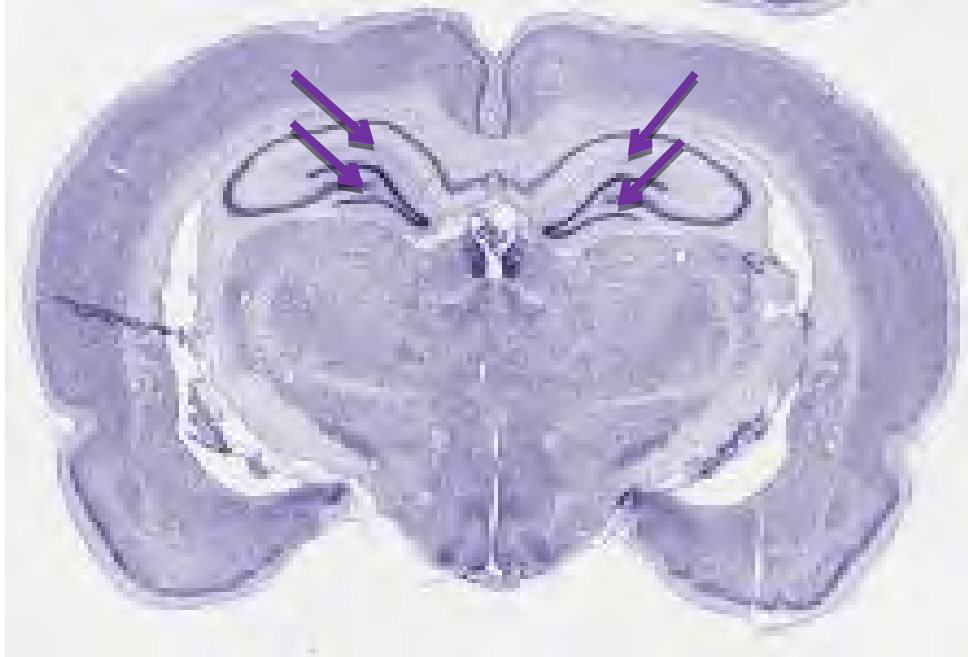
SURGICAL PROCEDURES

Surgery 1: Electrode implantation surgery



AP: -2.2mm, ML: ±4.8mm , DV: -8.3mm

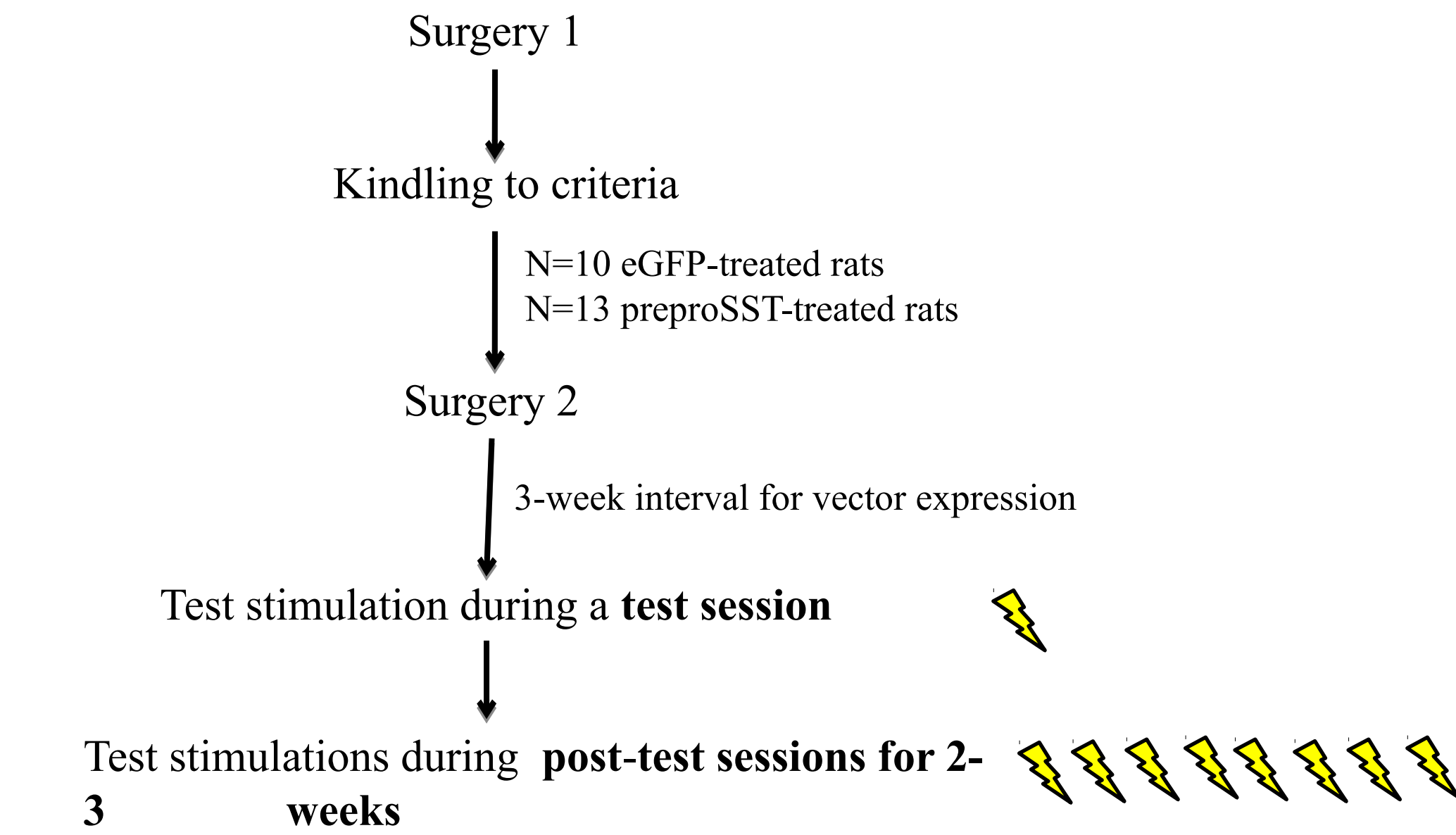
Surgery 2: Vector injection surgery



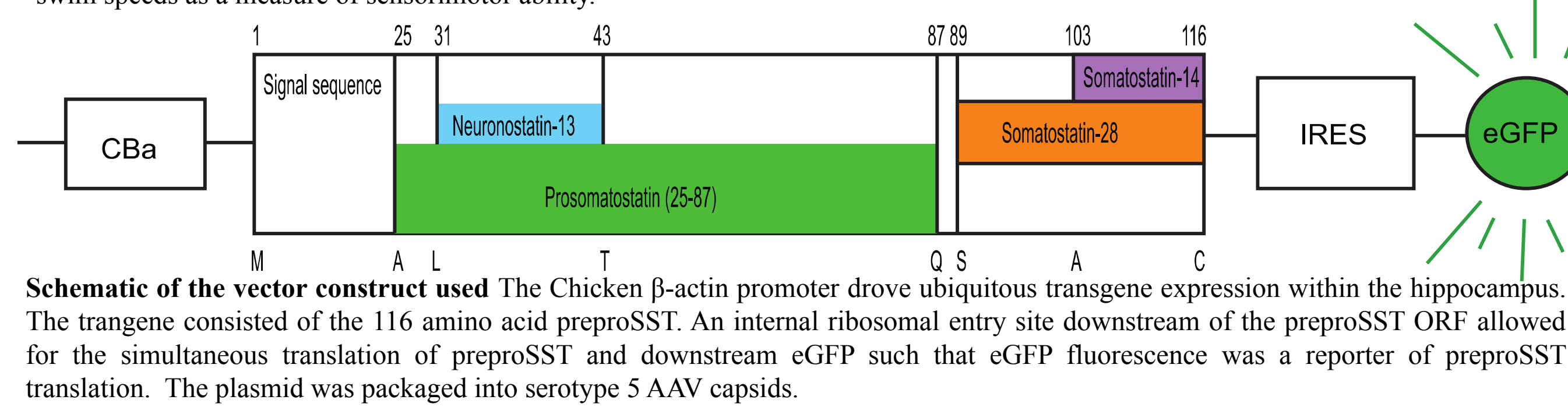
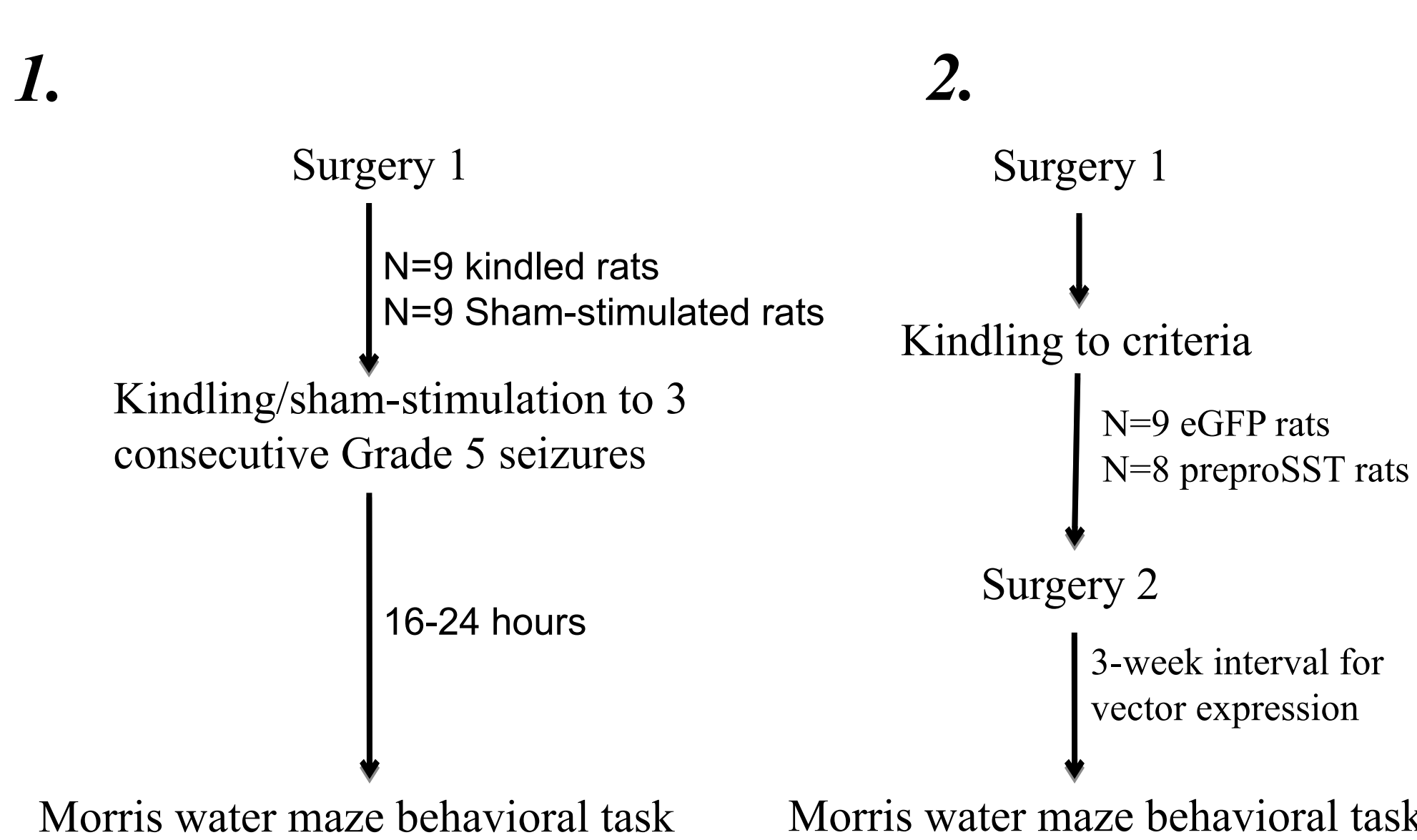
AP: -3.8mm, ML: ±1.8mm , DV: -3.4mm
dentate gyrus, -2.4mm CA1 region

EXPERIMENT DESIGN

Aim 1

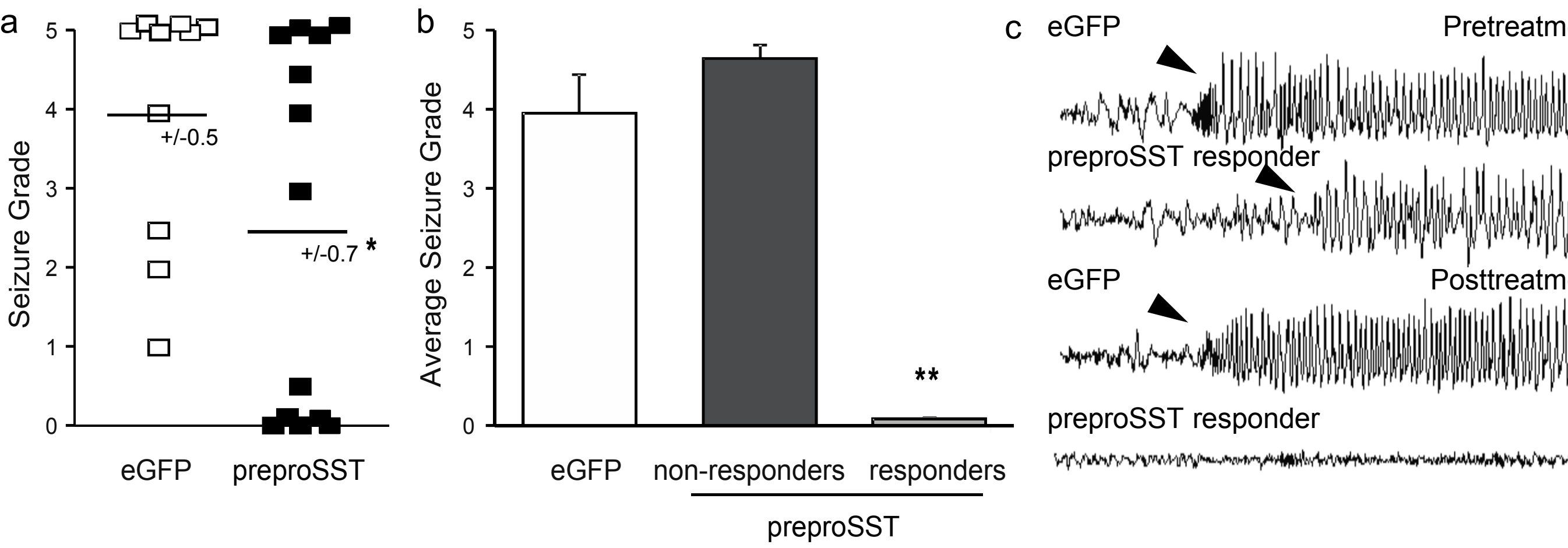


Aim 2



RESULTS

1) Sustained preproSST expression produced seizure resistance in a subset of rats



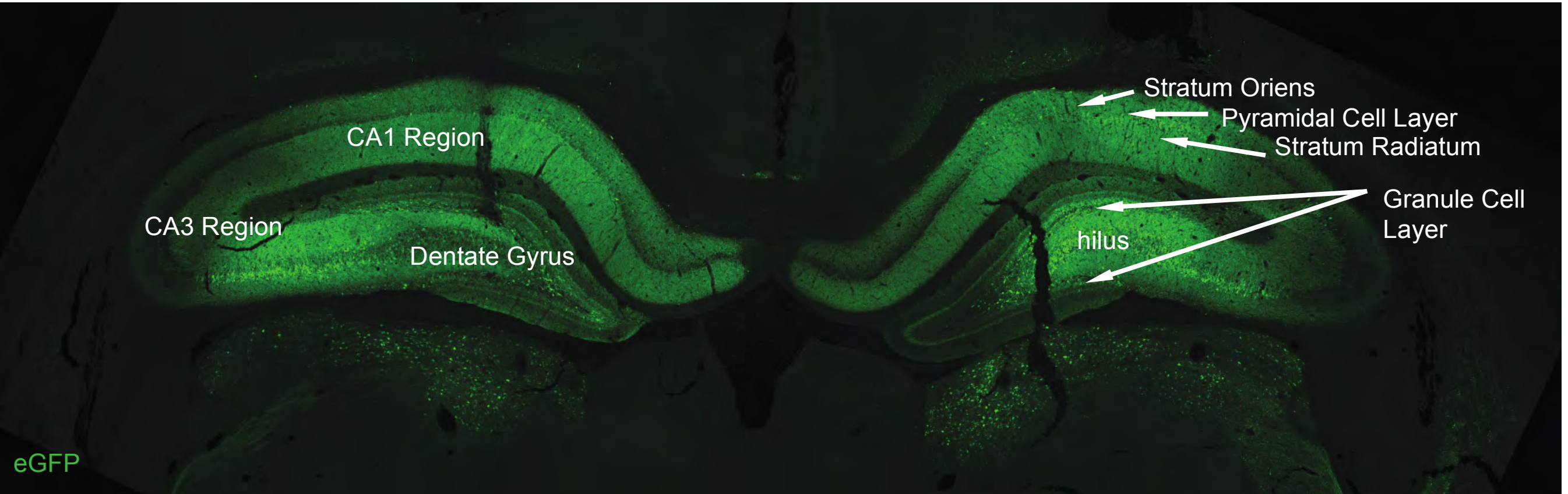
Sustained preproSST expression prevented induced seizures in a subset of kindled rats a) The average seizure grade produced by the preproSST-treated rats (n=13) was significantly lower than the average seizure grade produced by eGFP-treated rats [(n=10), * $p < 0.05$, one-tailed]. b) The average seizure 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 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.

2) Vector effects were stable and persistent

	PTS-1	PTS-2	PTS-3	PTS-4	PTS-5	PTS-6 (+50 μ A)	PTS-7 (+50 μ A)	PTS-8 (+100 μ A)
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	0	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.

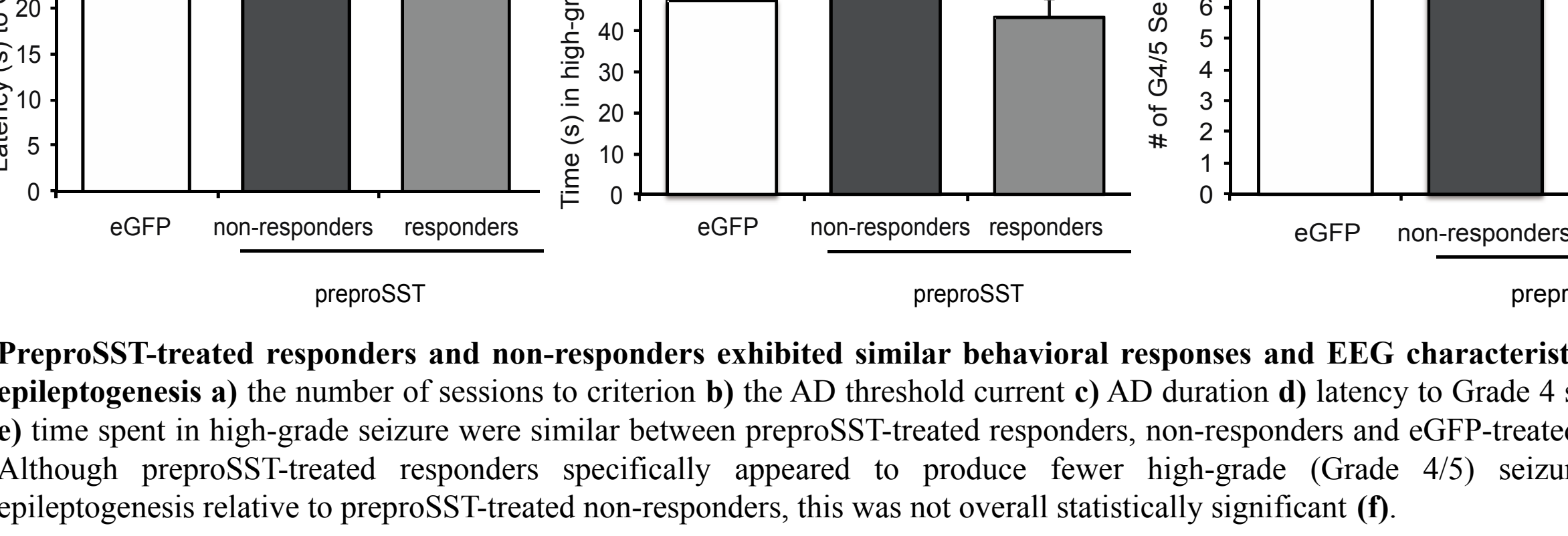
3) eGFP expression was similar in preproSST-treated responders and non-responders



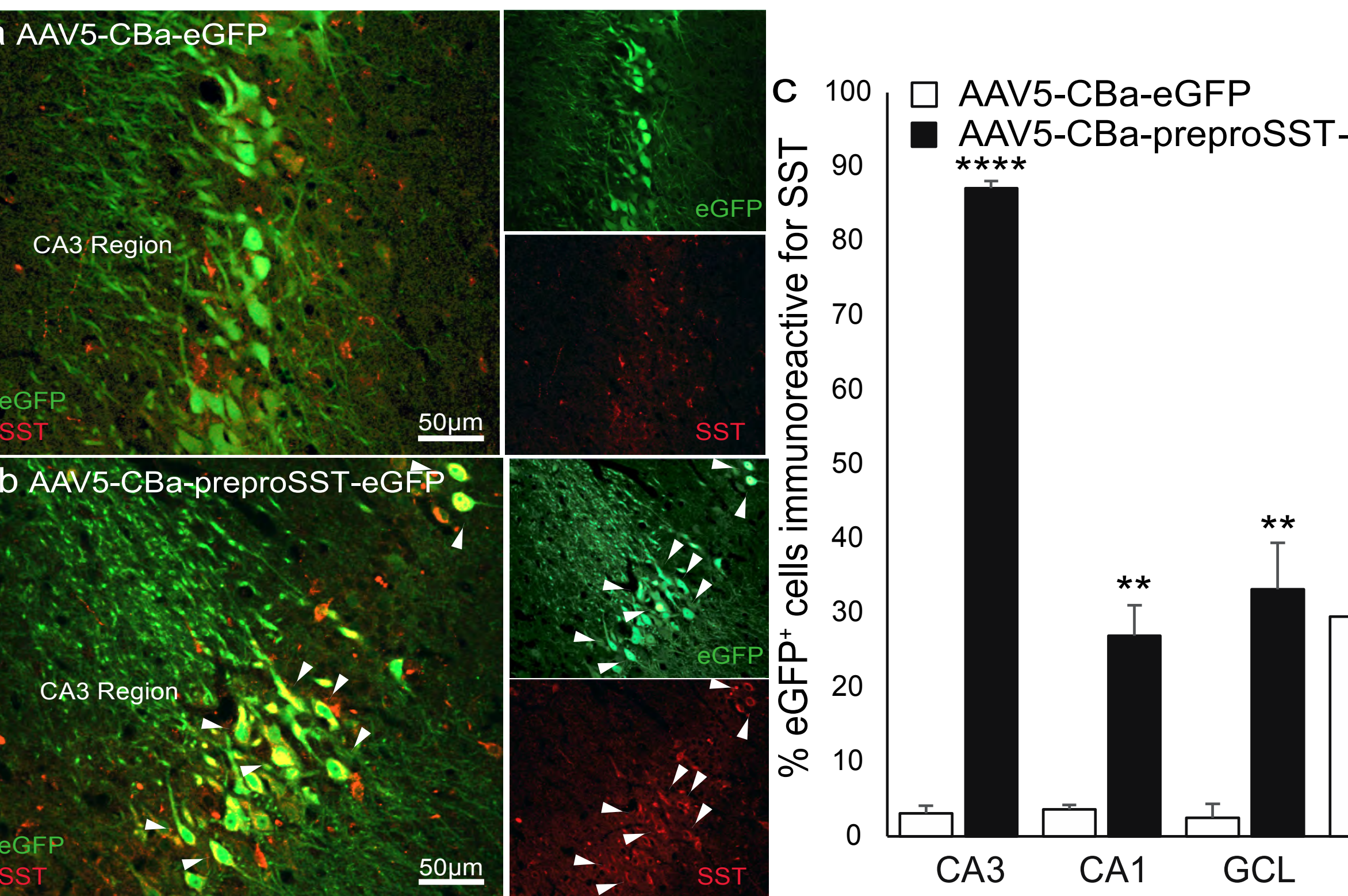
AAV5-CBa-preproSST-eGFP transduction within the hippocampus of a representative rat in the preproSST vector-treated group Robust eGFP expression was observed bilaterally within the dentate hilus, granule cell layer, CA3 region and CA1 region of the hippocampus. In this rat a relatively small number of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

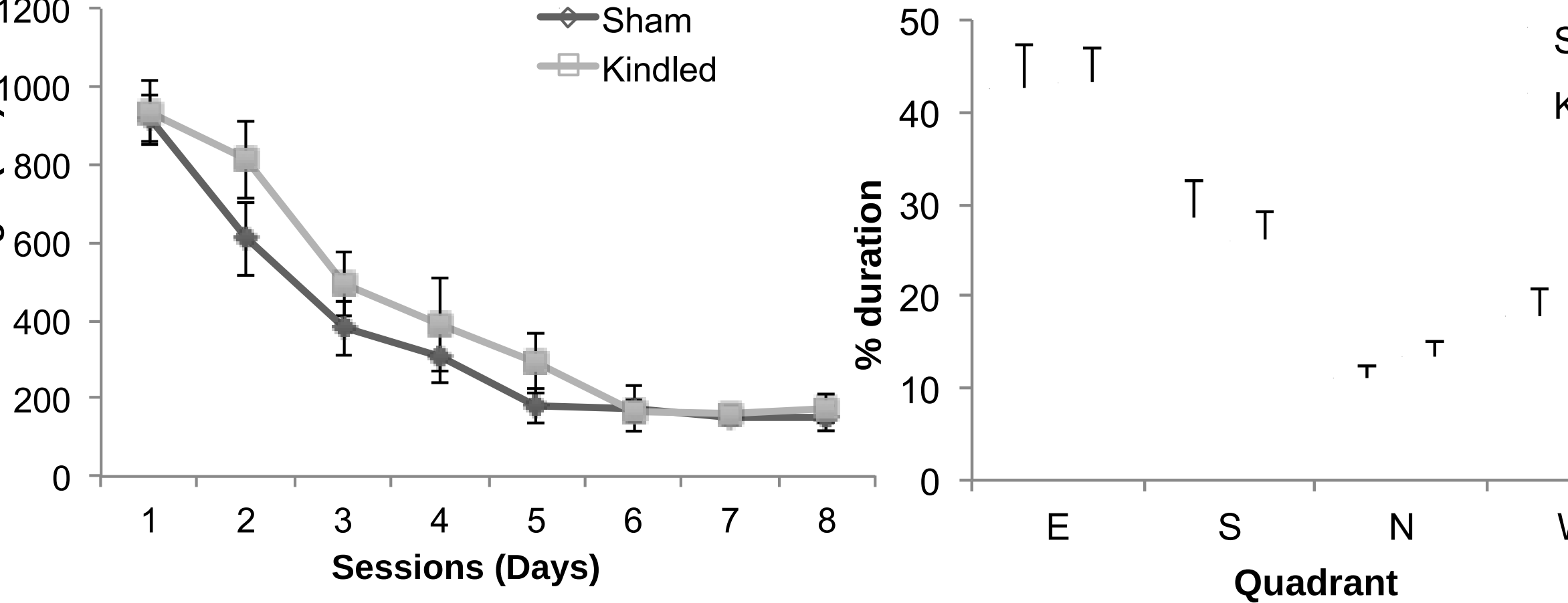


Co-labeling for eGFP and SST was consistent with vector transduction in hippocampal principal neurons



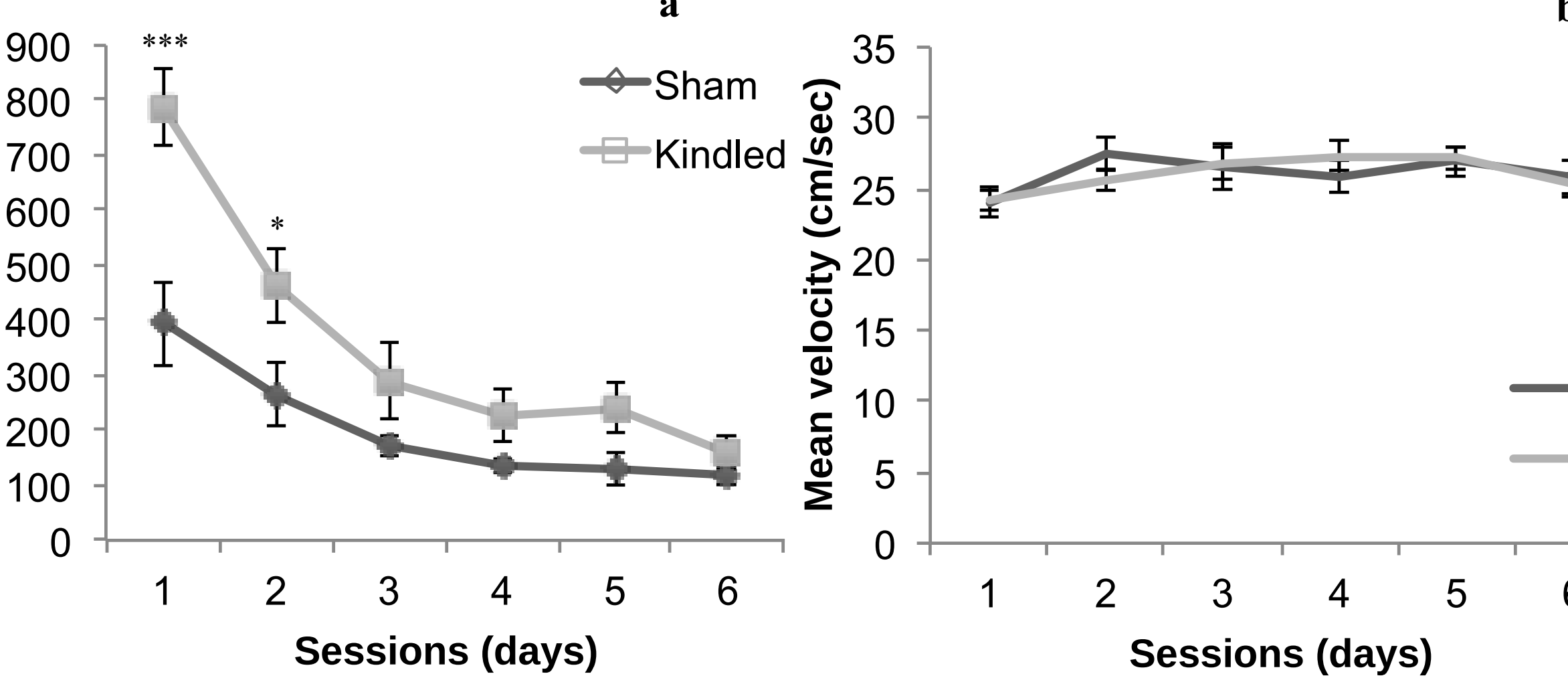
Coincident eGFP and SST localization was consistent with vector transduction of hippocampal principal neurons Examples of native eGFP+ neurons (in green), eGFP+/SST+ (both green and red) and SST+ (in red) neurons found in hippocampal region of rats treated with the control (a) or preproSST (b) vector. 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 (kindled 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 $\times$ training sessions: $F_{(7,112)} = 0.67$; $p = 0.696$) to locate the hidden platform b) Percent duration in each quadrant did not differ significantly 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 for learning a) kindled rats travelled longer distances to locate the hidden platform relative to sham-stimulated rats (kindled sham; $F_{(1,16)} = 13.63$; $p = 0.002$) across training sessions (days 1-6; $F_{(5,80)} = 29.64$; $p = 0.000$ and interaction effect: seizures $\times$ training sessions: $F_{(5,80)} = 4.08$; $p = 0.002$). Specifically, kindled rats travelled significantly longer distances relative to sham-stimulated rats on day 2. * Represents $p < 0.05$ and ***represents $p < 0.0005$ b) There were no significant effects of kindling epileptogenesis (kindled 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 $\times$ training sessions: $F_{(5,80)} = 1.244$; $p = 0.297$) 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 (2 μ l/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.

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% H₂O₂ 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% H₂O₂. 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 Stereoinvestigator 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.

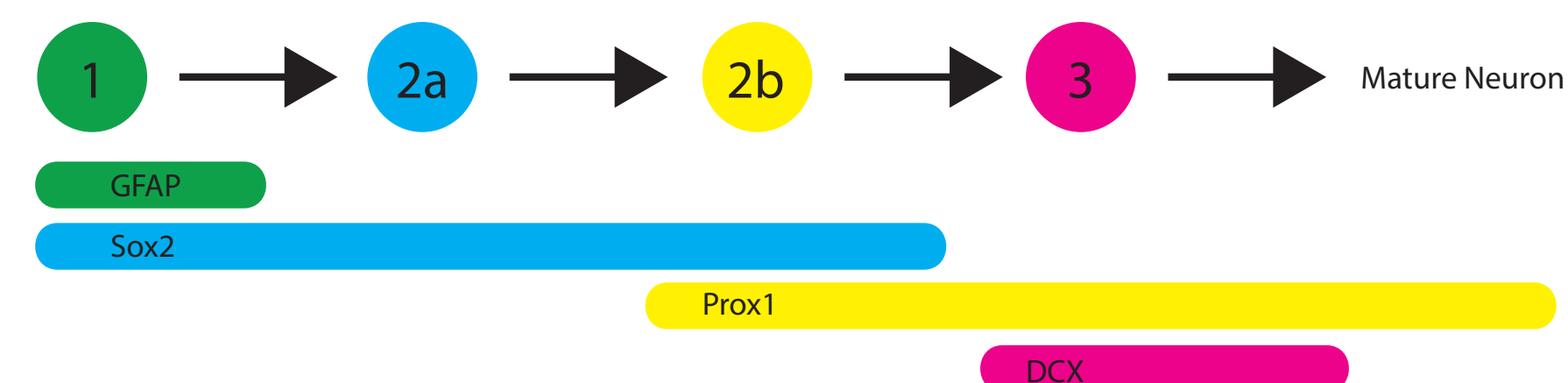


Figure 1. Schematic of stage stem/progenitor cell staging using stem/progenitor cell neuronal protein markers.

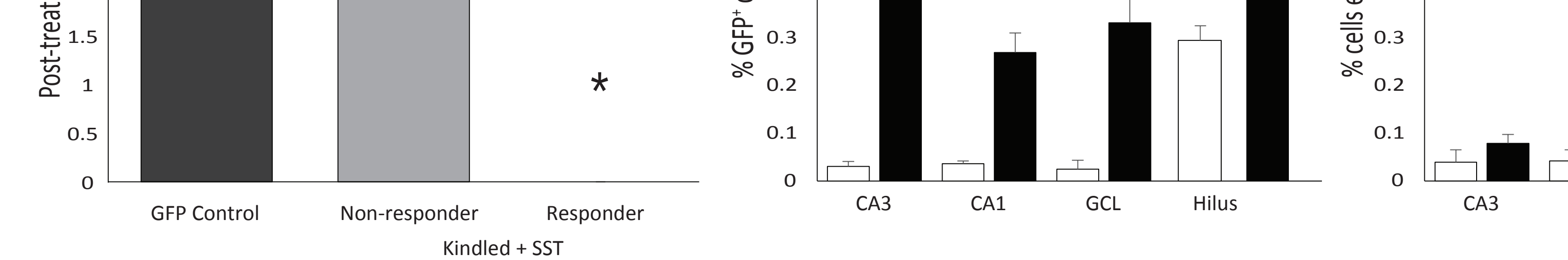


Figure 2 (A) Cresyl violet stained atlas figure [19] showing the bilateral placement of electrodes in the amygdala as well as AAV-Cba-GFP and AAV-Cba-SST-GFP vector revealed that SST-responders exhibited significantly lower seizure grades than GFP control rats and SST non-responders (p values < 0.05). (C,D) Representative confocal images of the dentate gyrus of adult rats 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 ($p < 0.0001$), GCL ($p < 0.0001$), and hilus ($p < 0.0001$) subregions of preproSST versus 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 hippocampus 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 progenitor

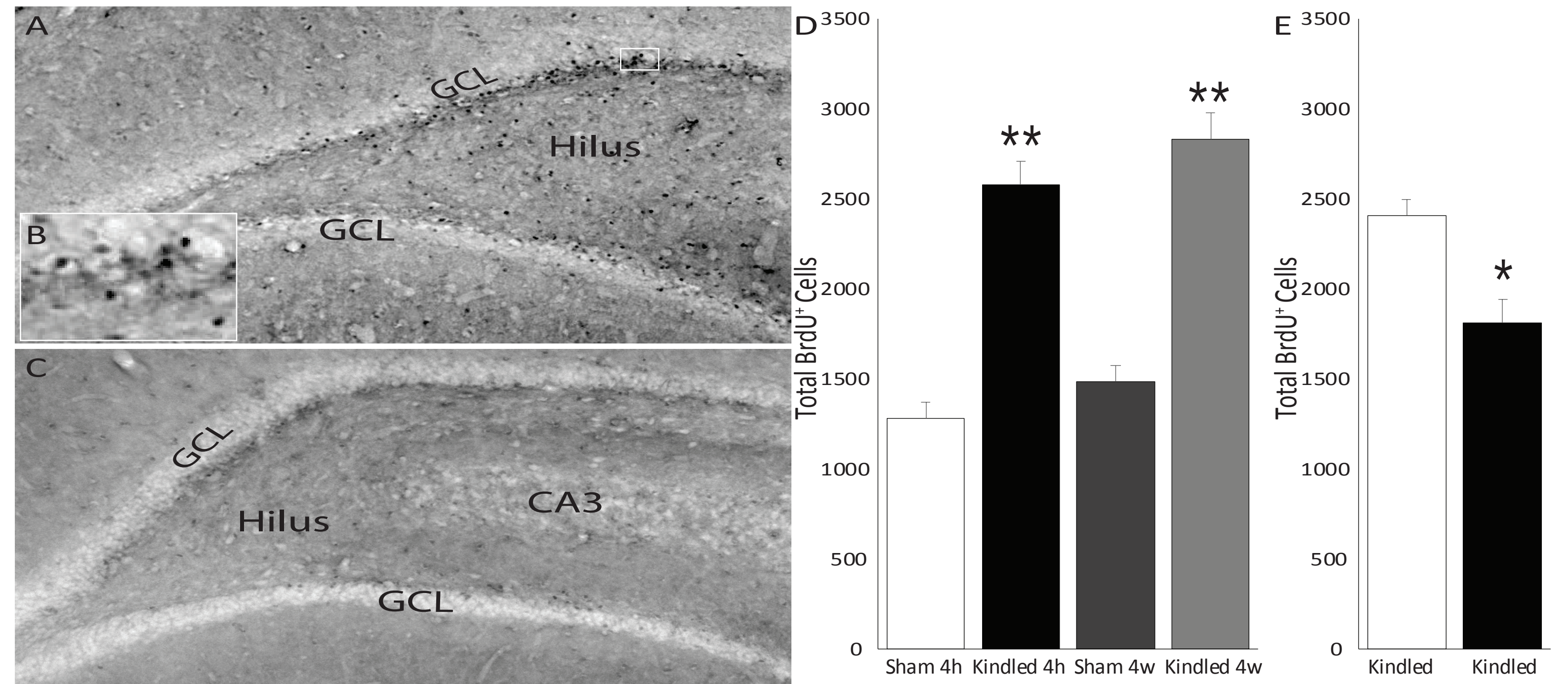


Figure 3. Kindled rats had higher levels of Type-1 progenitor cell division and SST gene delivery reversed this affect. (A-C) DAB stained BrdU⁺ cells are shown for kindled rats. (D) BrdU⁺ proliferation is increased in kindled rats ($p < 0.001$) versus control and an increase in surviving BrdU⁺ cells at 4w post-injection ($p < 0.001$). (E) There is a decrease in proliferation in kindled rats receiving preproSST versus control vector. (F) The percentage of GFP⁺ cells colabeled with SST was increased significantly in the CA3 ($p < 0.0001$), CA1 ($p < 0.0001$), GCL ($p < 0.0001$), and hilus ($p < 0.0001$) subregions of preproSST versus control vector treated rats. (G) 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 hippocampus 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.

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

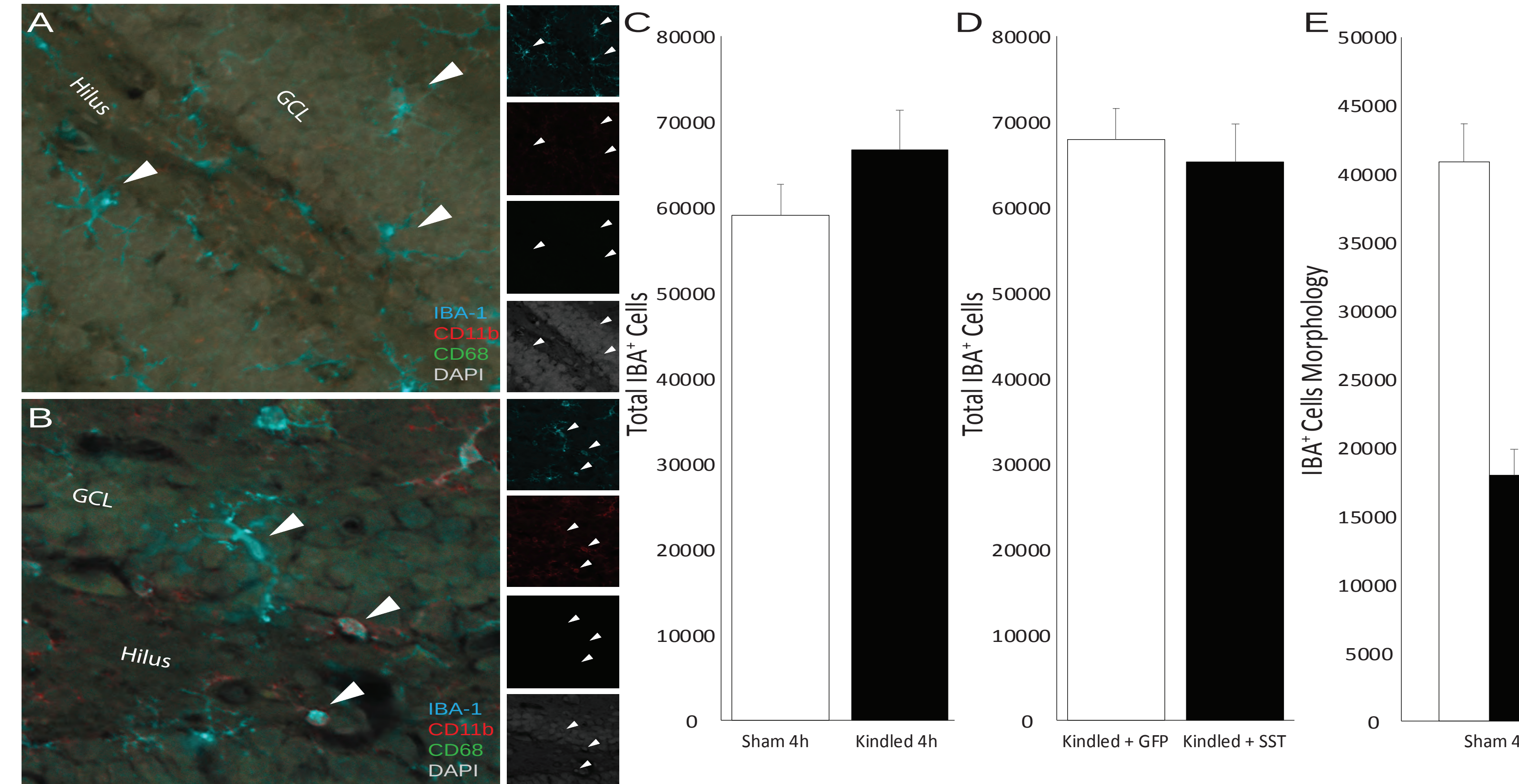


Figure 4. There is an inflammatory response to kindling. (A) Representative confocal images of IBA-1⁺/CD11b⁺ activated and amoeboid (B) and IBA-1⁺/CD11b⁺ resting microglia in the dentate gyrus of adult rats even when accompanied with vector mediated SST or GFP gene delivery. (E) A morphological analysis revealed significantly more activated 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 vector treated rats.