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

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14. ABSTRACT stimulation at current levels initially without effect gradually produce a persistent state where severe seizures occur reliably. Animals tested during the reporting period establish that somatostatin gene delivery after development of maximal seizure susceptibility can produce complete amelioration of a seizure-prone state. The therapeutic effect is essentially all or nothing. The responder rate is 30-40%, below the 70% observed when gene delivery preceded kindling, but comparable to extant antiepileptic medication. Responder and non-responder cohorts cannot be explained by variation in injection placement, transduction efficiency, electrographic seizure variables, effects on seizure-stimulated brain stem cell division or differentiation, or obvious brain pathology. Kindling increased new cell generation in hippocampus, with a bias toward non-neuronal phenotypes, and somatostatin gene delivery uniformly suppressed this regardless of therapeutic efficacy against seizures.					
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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 synaptic receptor-effector cascades. Receptors on non-neuronal cells in brain, on macrophages, and peripheral interactions with classical inflammation mechanisms (TNFalpha, cytokines) implicate brain SST in inflammation and cellular proliferation controls. Synaptic function and brain cell proliferation are both are disrupted in epilepsy and may contribute to neuropathology and functional impairment beyond seizures per se. Brain neurons expressing somatostatin are especially vulnerable to seizure-related loss, but somatostatin gene transfer cannot mediate the same synaptic functions so efficacy is likely to be associated with other biological actions, possible related to anti-proliferation and anti-inflammatory properties recognized for SST outside the brain. Our initial tests demonstrated that intracranial somatostatin gene delivery prevented the evolution to high-level seizures in 70% of rats treated in an established electrical brain stimulation regimen. This impressive reduction in experimental epileptogenesis may provide strategies for new effective therapy for intractable epilepsy, but the efficacy mechanisms and parameters must be established prior to informed clinical development. We predict that somatostatin gene transfer will counteract the effects of epileptic development on the proliferation rate of hippocampal progenitor cells, their long-term phenotype, and their contribution to pro-convulsive networks. Second, hippocampal somatostatin gene delivery will reduce signs of inflammation stimulated by seizures that probably contribute 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. This induction of a seizure-prone state, called kindling, is measured by behavioral and electrographic severity, the number of stimulations required to produce maximally severe seizures, and the amount of damage displayed by cells in the brain post-mortem. The progressive hyperexcitability that develops in conjunction with lowered seizure thresholds is a therapeutic disease modification target distinct from seizures per se. Localized gene delivery using adeno-associated viral vectors for SST or inactive control protein will be tested for ability to suppress seizure susceptibility and severity during kindling. Histological markers for neurogenesis and for brain inflammation 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 will likely depend on other mechanisms. The evolution of epilepsy between an initial insult and recurrent spontaneous seizures is the most opportune time for therapeutic intervention, because loss of important neuronal populations is likely to have already occurred when these emerge, and because seizures tend to become more severe and/or frequent once recurrent seizures begin. Somatostatin gene delivery uses vectors currently performing well in human clinical trials, and could provide a new, safe, and effective way to interfere with this evolution, associated loss of brain tissue from pathology or resection, and functional impairment.

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?

Two specific aims were proposed:

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? (Tasks 1, 2, 3, 4).
2. Does SST gene transfer alter inflammation cascades over the time scale in which epileptogenesis occurs? (Tasks 1, 2, 5, 6)

What was accomplished under these goals?

1) Major activities

The major activities of the reporting period are consistent with the approved Statement of Work. Animals have been continuously generated and tested in the seizure kindling model, administered intracranial gene transfer vectors, evaluated for effects on seizures, and used for a variety of postmortem histological and biochemical analyses reflecting therapeutic mechanisms and safety. Effort during the reporting period has been increasingly devoted to neurogenesis quantification, estimating neural progenitor cell numbers stereologically, identifying by confocal microscopy dividing cells identified as Type 1, 2a or 2c, distinguishing proliferation from survival, and identifying neuronal and glial phenotypes, in relation to kindled seizures and somatostatin gene delivery.

2) Specific objectives

Statement of work (SOW) text is in *italicized* font.

Task 1. IACUC & ACURO (months 1-3)

Minor modifications have been approved for our IACUC protocol during the reporting year. Dr. Junli Zhou has been added to the personnel approved to conduct electrode implantation and vector injection surgery and Mr. Jeff Leibowitz's role has been expanded so that he is approved to conduct electrode implantation and vector injection surgery. The room locations for water maze behavioral testing have been changed and approved upon inspection by UF IACUC. Annual renewal was secured.

Task 2. Preparation and validation of gene transfer vectors (months 1-2)

New AAV5 vector preparations (1 each SST and GFP) were made in collaboration with the UF Vector Core facility. Plasmids for the somatostatin cassette were sequenced to verify correspondence with repository information. These data confirm the presence of the full coding sequence of the preprosomatostatin gene. Expression with both vectors appears equivalent to previously used preparations.

Task 3. Experiment 1: SST vector effects on dentate gyrus cell proliferation and integration after initial seizure (months 3-20)

Four experimental treatment groups of 10/group will be generated from rats that meet inclusion criteria after surgical implantation of indwelling stimulation and recording electrode headsets and gene transfer. These 40 rats will undergo behavioral testing before and after surgery. After exhibiting the first kindled seizure they will be labeled for proliferating cells, and 1 day later the brains will be

harvested for anatomical analyses of multiple cell phenotype markers. Behavioral, seizure, and anatomical data will be analyzed, interpreted, and written for peer-reviewed publication. The design and time constraints require sequential testing of small numbers of rats at a time, so that new animals will be generated continuously until the treatment groups have been filled. Completion of the described subtasks for 40 rats is estimated to require 68 weeks (17 months).

3a. Presurgical alternation behavior (months 3-19)

3b. Surgery for gene delivery, kindling electrode stimulation and recording electrode placement (months 3-19)

3c. Post-surgical alternation behavior (months 3-19)

3d. Pre-kindling BrDU administration (months 3-19)

3e. Kindling (months 3-19)

3f. Euthanasia and histological processing (months 4-20)

3g. Histometry (months 4-20)

3h. Statistical analysis (months 18-20)

3i. Manuscript preparation and review (months 20-21)

Task 4. Experiment 2: SST vector effects on dentate gyrus cell proliferation and integration after repeated seizures (months 18-35)

This task is similar to Task 2, evaluating the same measures in rats that have been kindled to reach maximal seizure severity, and will succeed Task 1 although a short overlap is possible.

4a. Presurgical alternation behavior (months 18-34)

4b. Surgery for gene delivery, kindling electrode stimulation and recording electrode placement (months 18-34)

4c. Post-surgical alternation behavior (months 18-34)

4d. Pre-kindling BrDU administration (months 18-34)

4e. Kindling (months 18-35)

4f. Euthanasia and histological processing (months 19-35)

4g. Histometry (months 19-35)

4h. Statistical analysis (months 33-35)

4i. Manuscript preparation and review (months 35-36)

As part of SOW Tasks 3 and 4 we have continued to characterize vector effects on kindled seizures in rats that have not received BrdU. These establish essential baseline kindling and vector response characteristics in a control population for potential BrdU effects. Results obtained during the reporting period have confirmed several important properties of SST therapeutic efficacy against an established seizure-susceptible state:

1) There are responders and nonresponders among subjects given the SST vector.

We now have 12 subjects in the GFP vector control group in which kindling generated a stable

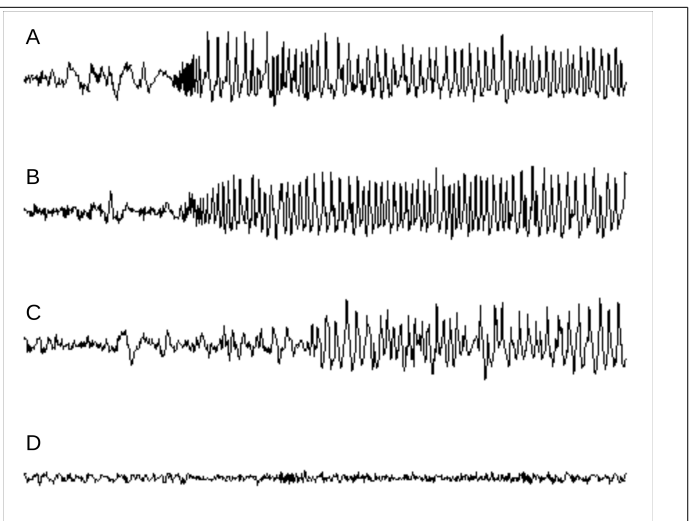


Figure 1: Electroencephalographic recordings from fully kindled rats exhibit high-amplitude synchronous activity during Racine grade 5 seizures (A, C). Intrahippocampal expression of GFP vector for 4 weeks does not alter evoked seizure intensity (B), in contrast to SST vector that can completely suppress the induced seizures (D).

seizure-prone state reflected in multiple measures of seizure severity (Figures 1, 3-7). Increasing the number of SST vector animals included based on confirmed electrode and vector placement to 13 has made it clear that only a subgroup (5/13) shows a clear therapeutic response on the primary outcome measure (Racine seizure grade).

2) The percentage of responders is lower when vector is administered after, rather than before a seizure-prone state is established.

The 38% responder rate in our present data set is lower than the 70% of rats that showed beneficial effects of SST gene delivery prior to kindling in our initial study, but is comparable to epilepsy therapeutics in clinical use.

3) Responders consistently show ~100% efficacy.

In animals that showed a therapeutic response, stimulation current levels that produced 3 consecutive Racine grade 5 seizures prior to SST gene transfer failed to induce any seizures (23/24 tests in 4 animals)(Figures 1,2). In contrast, nonresponders were not significantly different from GFP vector animals or their own pre-vector kindling susceptibility.

4) Full therapeutic efficacy is possible with expression restricted to specific subregions.

Careful reconstruction of inter-animal variability in vector placement has identified responders with transgene expression expression limited to either CA1 or dentate hilus subregions (Figure 3, Table 1).

5) No specific subregion appears necessary for full therapeutic efficacy.

We have confirmed examples of SST vector animals demonstrating full efficacy despite essentially zero expression in either CA1, CA3, or dentate gyrus (Figure 3).

6) Unilateral transduction can suffice to mediate maximal therapeutic efficacy.

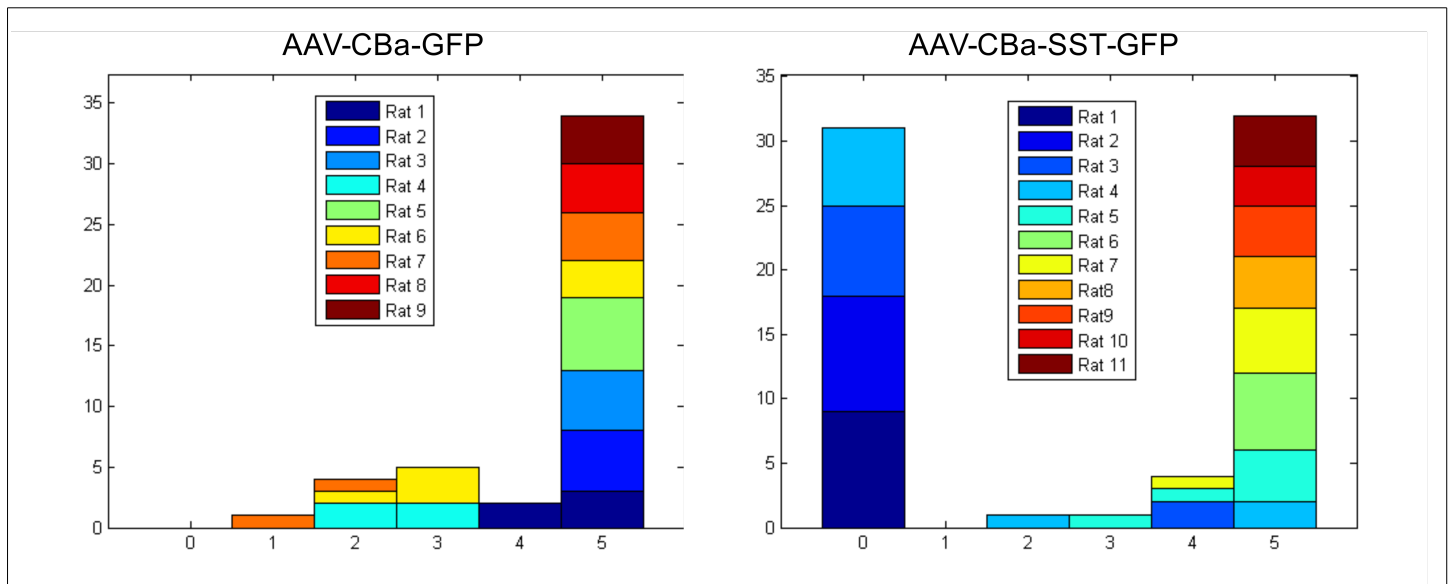


Figure 2: Efficacy of somatostatin gene delivery after establishment of a fully kindled state. Left, in control GFP vector rats tested 3 weeks after infusion most kindling stimulation episodes continued to produce Racine grade 5 seizures; there was no significant effect of treatment ($P= 0.5$, $h=0$, Wilcoxon sign rank test). Right, among all fully kindled rats that received SST vector, seizure grade was reduced only in a subset of responders ($P= 0.1250$, $h=0$ excluding Rat 1 and Rat 2, Wilcoxon sign rank test). However in responders most often no seizures could be induced even when a) subjected to more test episodes than GFP controls, or b) stimulated at current levels that previously induced maximal grade 5 seizures. Rat 1 and Rat 2 were subject to repeated interval testing of the fully kindled state and were injected with vector 1 month following fully kindled state.

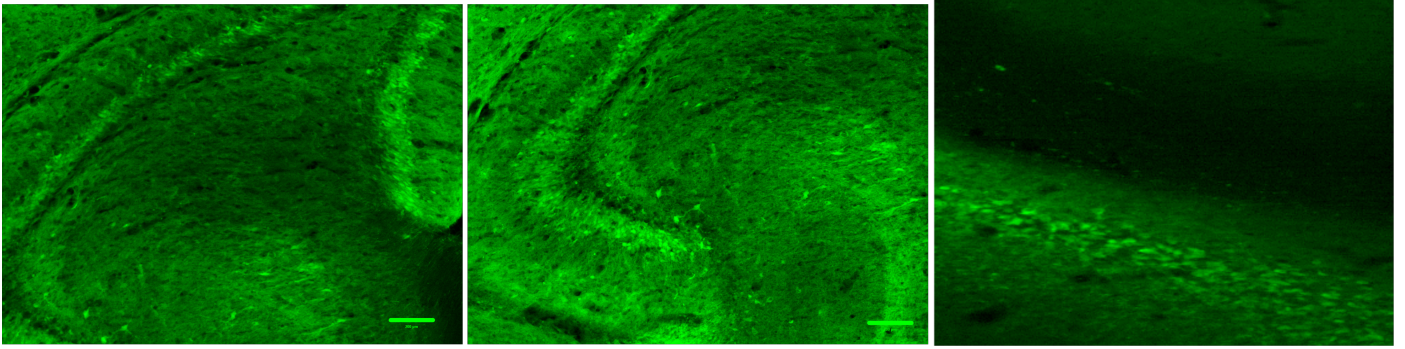


Figure 3: Left, center frames: pAAV-CBa-SST-GFP transduction and expression in the DG of the hippocampus of a rat that produced grade 0 seizures after SST gene transfer. Putative SST cells were predominantly found in the DG region (a,b) and in the CA2/CA3 region unilaterally. Right frame, pAAV-CBa-SST-GFP transduction and expression in the CA1 subfield of the hippocampus of a rat that produced grade 0 seizures after SST gene transfer. Expression of putative SST cells was restricted to the CA1 region, rostral along the A-P axis of the hippocampus, unilaterally. 50 μ M coronal sections were viewed under the 3035-B OMF green fluorescent protein filter using the Olympus 1X71 fluorescent microscope. Scale bar, 200 μ M.

It does not appear necessary to transduce the hippocampus bilaterally. For patients with disease exhibiting highly unilateral pathophysiology this may translate into not having to subject both temporal lobes to gene delivery.

Results 4-6 support the concept that therapeutic efficacy is more closely related to effects on distributed synaptic networks than on particular neuronal subregions. An ability to impose therapeutic effects on distributed networks by overexpressing SST at any of several brain loci suggests that the approach may work even when neuronal loss in certain populations is advanced.

7) Some EEG characteristics prior to vector treatment may have predictive value with respect to whether animals will show therapeutic efficacy.

After careful analysis determined that SST vector expression location and extent could not explain therapeutic response differences, we looked retrospectively at a variety of

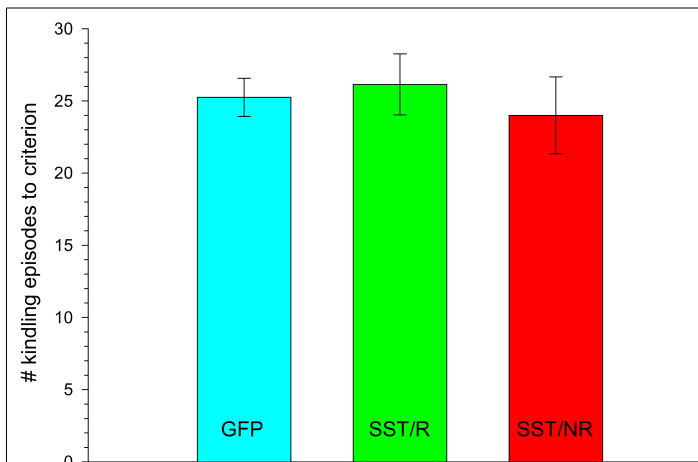


Figure 4: There were no differences in the number of kindling episodes necessary to reach 3 consecutive Racine grade 5 seizures ($p < 0.7688$, ANOVA).

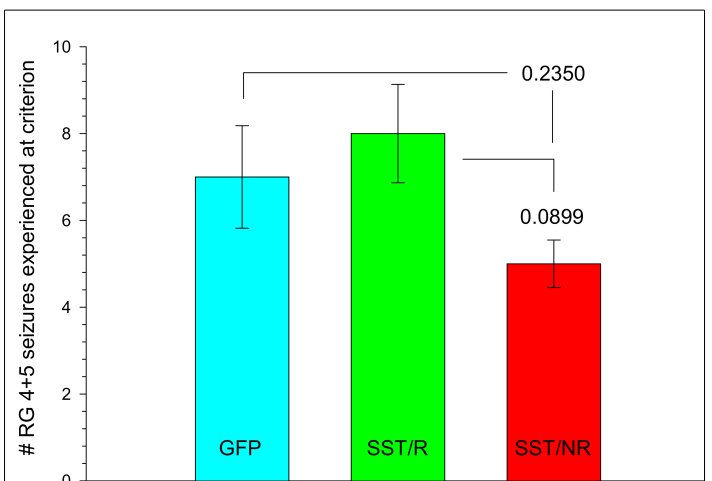


Figure 5: There was a trend for animals that showed a therapeutic response to SST vector ($N=5$) to have experienced fewer high-grade seizures than nonresponders ($N=7$) when they reached a fully kindled state. GFP vector animals ($N=11$) and SST vector animals did not differ. P values by least-means post-hoc comparisons after 1 way ANOVA.

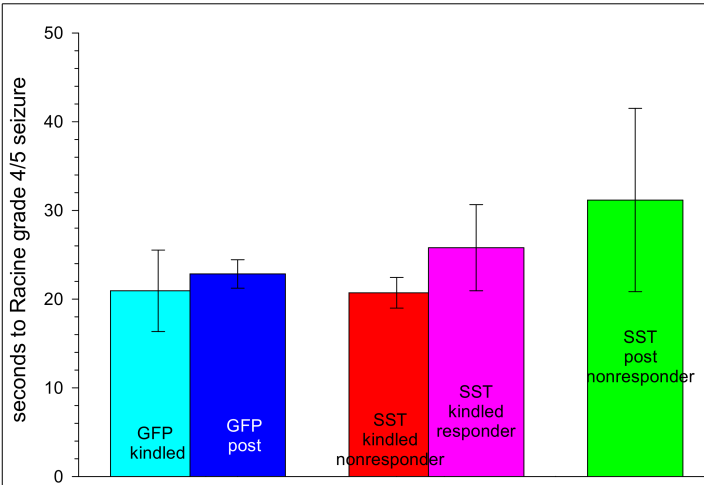


Figure 6: GFP and SST vector group animals had equivalent development of individual kindled seizures to a behavioral grade 4 stage at initial full kindling. Animals that did go on to respond to vector ("SST kindled responder") may have had seizures develop more slowly than animals that will be nonresponders. Increased variability among SST 'nonresponders' reflects how some animals showed an increase in time to reach grade 4 severity after SST vector ("SST post nonresponder"), whereas GFP vector animals ("GFP post") did not.

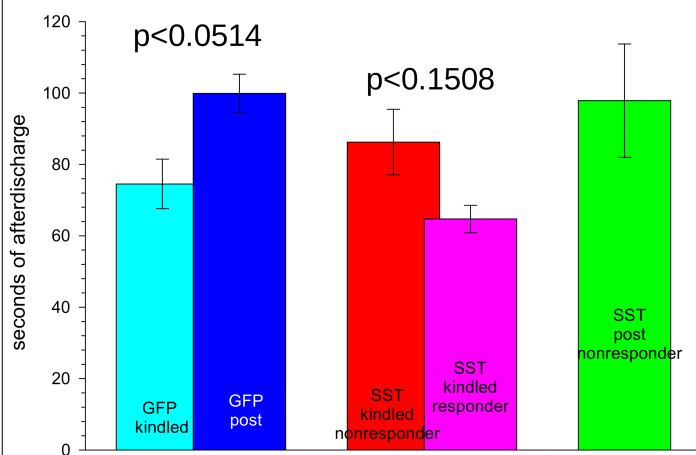


Figure 8: GFP and SST groups had equivalent afterdischarge (AD) durations at initial full kindling. ADs became longer during the 3 week GFP expression interval, but this trend was obscured in SST vector 'nonresponders', consistent with a possible antiepileptogenic effect. Animals that became 'responders' after SST vector initially had a tendency for shorter AD durations than animals that would become 'nonresponders'.

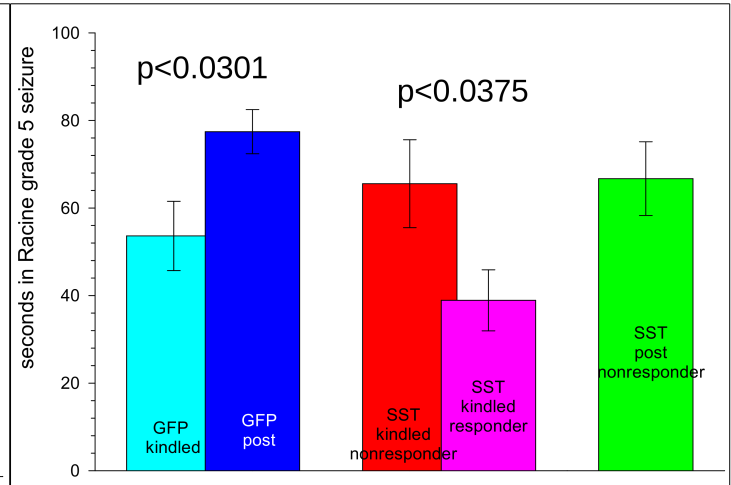


Figure 7: GFP (N=8) and SST (N=12) groups had equivalent durations in Racine grade 5 seizure at initial full kindling. GFP animals spontaneously progressed to exhibit more severe stimulated seizures 3 wks. after vector delivery. This was not observed in SST 'nonresponders'. SST responders (N=5) had few grade 5 seizures (thus no 'responder post' bar) 3 wks. after vector delivery, unless stimulation currents were increased 50-100 uA. Animals that would become responders had shorter grade 5 durations prior to vector delivery than animals that did not show a therapeutic effect. P values from least-squares means post-hoc tests after ANOVA.

electroencephalographic properties of seizures recorded during the initial acquisition of a kindled state and after prolonged transgene expression. Because the probability being refractory to treatment is linked in human temporal lobe epileptics with the number of seizures prior to initiation of therapy (Kwan & Brodie '00), we first looked at whether nonresponders had more seizures than responders. This was not the case when only the number of kindling episodes was examined (Figure 4), but we did detect a trend for the number of severe seizures experienced before experiencing 3 consecutive grade 5 (i.e. fully kindled) to be higher in nonresponders (Figure 5). Probing further into the electroencephalographic characteristics of seizures during kindling and after vector delivery, examination of the time spent in grade 5 seizures revealed that duration in responders was less than half of what nonresponders endured (Figure 5). Responders also tended to have spent less time showing afterdischarges (Figure 6).

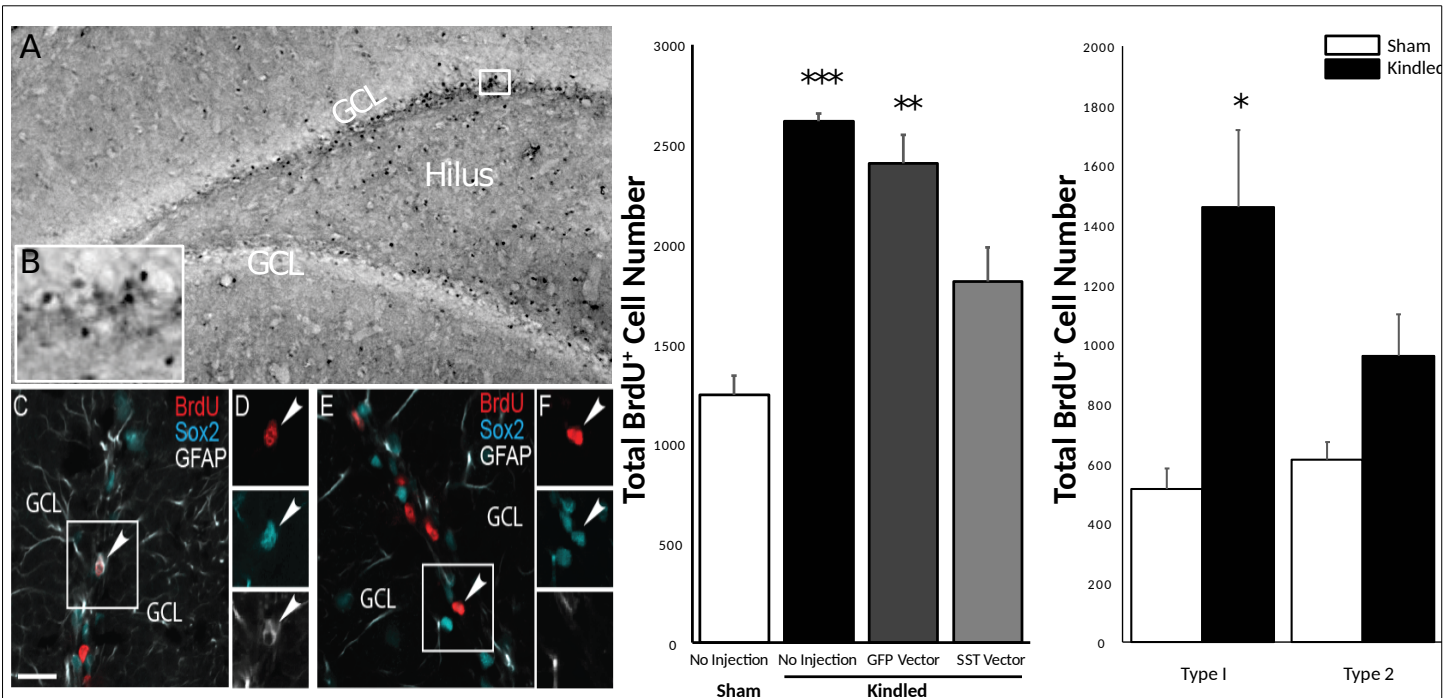


Figure 9: Kindling-induced increases in neurogenesis are reversed by somatostatin. A: Representative photomicrograph of BrdU+ dividing progenitor cells in dentate subgranular zone (inset, B). Confocal images of Type 1 (C and inset) and Type 2a (E and inset) progenitor cells. Significantly more BrdU+ dividing progenitor cells are found in the dentate gyri of kindled versus sham rats (G; $p < 0.05$) unless SST vector is delivered intrahippocampally (H; $p < 0.05$). Kindling upregulates the division of normally quiescent Type 1 progenitor cells (I; $p < 0.05$) and fewer Type 1 progenitor cells divide in the dentate gyri of kindled rats treated with SST versus a control vector (J; $p < 0.05$).

Statistical testing of our original hypothesis is confounded by the existence of responder and nonresponder subgroups. Based on consultation with professional biostatisticians, and power analyses of the current data, we determined that about 10 more SST vector rats would be necessary to resolve a therapeutic effect without *post-hoc* separation of responders and nonresponders. Of 14 animals committed to this effort in process, 2/14 have been given AAV-SST-GFP and are now in testing phase. The remainder are in the process of kindling and will be distributed to increase SST and GFP vector group size.

In addition to the rats that did not receive BrdU, during the reporting period we have generated animals for SOW Tasks 3 & 4, evaluating kindling effects on brain cytogenesis, and whether SST gene delivery alters kindling-induced cytogenesis effects. To test whether the efficacy of somatostatin gene delivery could be related to effects of somatostatin on the production, survival and/or placement of new neurons and glia stimulated by repeated seizures, the experimental design included sham and kindled rats and kindled rats that were treated with control vector or somatostatin vector to test whether seizures altered the number and/or type of cells labeled with the cell birth date markers bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) that is incorporated into DNA of cells that divide 2-4h after either has been injected systemically.

During the reporting period we performed electrode implantation surgery and kindling on 27 rats, followed by vector injection surgery, 3 week expression interval, seizure retesting, BrdU administration 48 hours after the last stimulation, and euthanasia 4 hours after that to examine proliferation *per se*. Six SST vector and 4 GFP vector rats satisfied inclusion criteria and were used for cell count studies. In addition, 2 cohorts of animals have been generated with proliferation marker BrdU administered 48 hours after kindling to criteria, followed by 4 week survival to evaluate *surviving* (as opposed to generated) cell numbers. The first cohort consisted of 5 rats that received kindling but

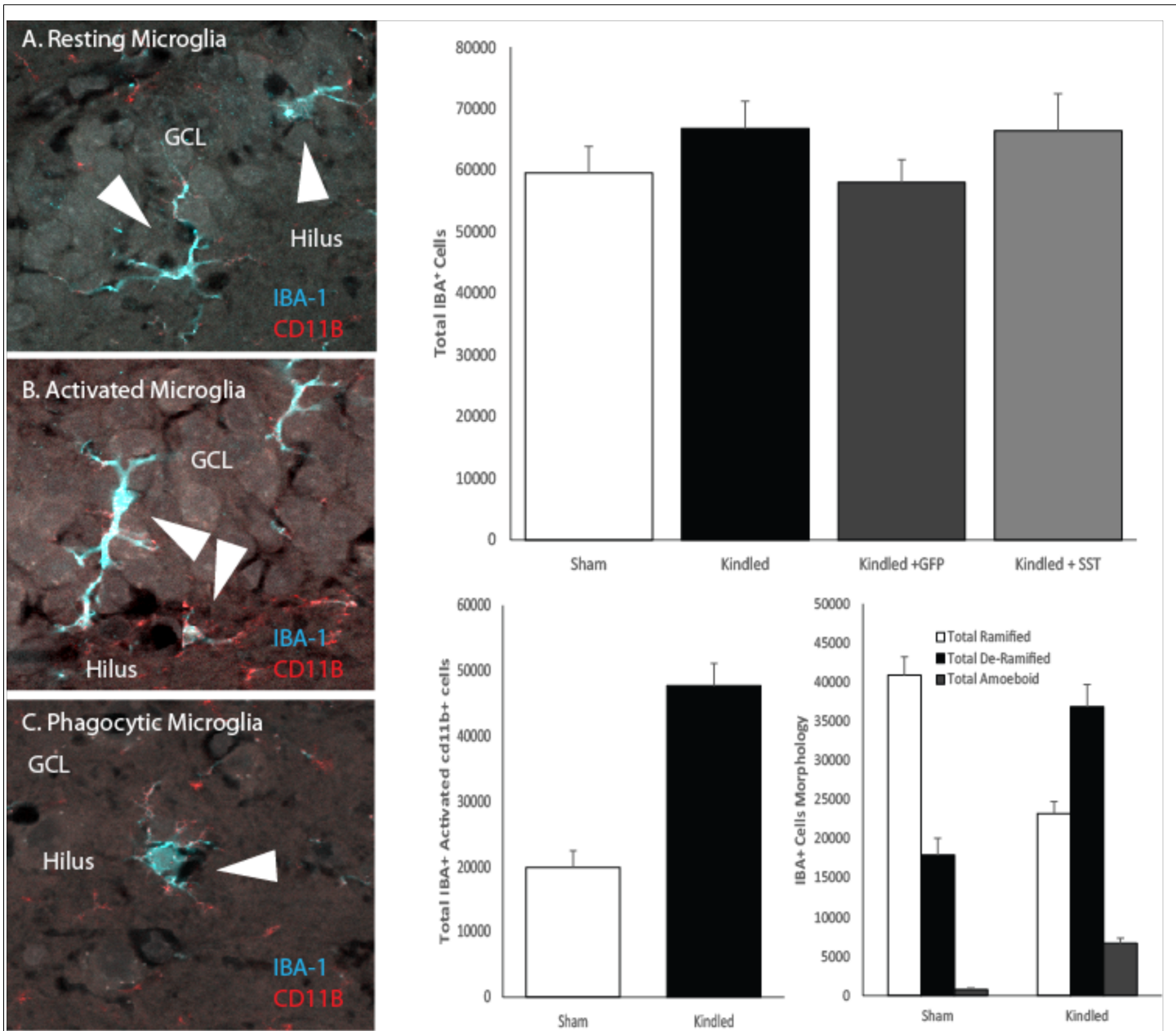


Figure 10: Kindling elicits an inflammatory response. (A-C) Confocal images of a resting IBA-1+/CD11b- microglia, an activated IBA-1+/CD11b+ microglia and a highly activated IBA-1+/CD11b+ microglia. (D) Kindling with and without somatostatin treatment did not change the absolute number of IBA-1+ microglia in the dentate gyrus of adult rats. (D) A combined stereological and confocal analysis of IBA-1+/CD11b- resting and IBA-1+/CD11b+ activated microglia revealed significantly more activated microglia in the dentate gyri of kindled rats ($p < 0.001$). (E) A morphological analysis revealed significantly more activated ($p < 0.001$) and highly activated (phagocytic; $p < 0.05$) microglia in the dentate gyri of kindled rats.

no vector, 5 rats that underwent sham kindling and received no vector, and 1 rat that failed to kindle. The second cohort is in progress and currently consists of 2 rats that received BrdU after kindling and EdU 48 hours after the last testing 3 weeks after GFP control vector delivery, to look at proliferation from the second and survival from the first labeling. Two additional rats failed to kindle to criteria. Animals on order (N=8) will be used to generate SST vector counterparts and increase the size of the GFP vector group.

New data reveal effects of kindling and SST gene delivery. We stereologically estimated the total number of dividing neural progenitor cells in the subgranular zones of rats treated with BrdU 4h prior to perfusion. The total number of dividing BrdU⁺ neural progenitor cells was higher in the dentate gyri of kindled versus sham-kindled rats (Figure 9), replicating and extending the established effects of seizures on the proliferation of neural progenitor cells. Kindled rats that received GFP vector had equivalent increases to those that received no vector and the effect was at least partially reversed in SST-treated rats. In the dentate gyrus, three types of neural progenitor cells (cells capable of generating neurons and glia) have been identified. Relatively naïve Type 1 (GFAP⁺/Sox2⁺) neural progenitor cells can generate neurons and glia and are relatively quiescent in adulthood while Type 2a (GFAP⁻/Sox2⁺) neural progenitor cells exhibit neuronal commitment and divide more frequently in the adult dentate gyrus. A significant finding of this work is that division of the more naïve Type 1 cells is stimulated by kindling (Figure 9I) and that this effect is also partially reversed by somatostatin treatment (Figure 9J). These data suggest that the stimulation of more naïve and typically quiescent Type 1 progenitor cells may contribute to the maintenance of seizure behavior and that SST may reverse that effect. Ongoing analyses are testing whether these effects differ in SST non-responder versus responder rats and whether the localization of these dividing progenitor cells plays a role in either seizure behavior or the effects of SST treatment. We have collected sections from rats with longer survival times after BrdU injection to quantify whether the cells produced by these dividing progenitor cells acquire neuronal or glial phenotypes and whether their localization and morphologies resembles those observed in control rats following kindling with and without somatostatin treatment.

We next examined the total number of Iba⁺ microglia (Figure 10A) that were mildly (CD11b⁺; red in Figure 10B and C) or strongly (CD68⁺) activated following kindling with and without somatostatin treatment. We found that the total number of microglia did not differ between these groups. We undertook a combined stereological and confocal analysis of the total number of Iba-1⁺ microglia that expressed the activation marker CD11B and found that total number of activated microglia was significantly higher in the dentate gyri of kindled versus control rats. Because we did not see evidence of highly activated microglia using this approach we employed a highly reliable morphological analysis of activated microglial phenotypes. In this scenario, resting microglia have highly ramified process with small cell bodies (Figure 10A). Activated microglia exhibit less ramified processes and larger nuclei (Figure 10B) and phagocytic microglia exhibit an amoeboid morphology with short processes (Figure 10C). This analysis replicated our finding that the total number of activated microglia significantly increases in the dentate gyri of kindled rats along with the total number of highly activated or phagocytic microglia. We are currently completing these analyses in kindled rats treated with and without somatostatin and will conduct these analyses on rats with longer survival times after BrdU injection to test the persistence of this inflammatory response, whether it responds to somatostatin treatment and how activated microglial numbers relate to measures of neurogenesis. We are also conducting this analysis in the CA3 and CA1 regions of the hippocampus in these rats as these areas exhibit pathological changes and could be sites that are impacted therapeutically by somatostatin treatment.

A second set of animals that received BrdU, kindling, and vectors has been committed for biochemical analyses (SOW Tasks 5 &6). Tissue harvested from these animals is being accumulated and stored frozen for planned inflammation marker assays.

We have made qualitative examination of the effects of kindling and gene delivery on the abundance and localization of SST receptors viewed by immunohistochemistry. Neither kindling nor gene delivery have obvious effects.

3) Significant results/key outcomes/major findings, developments, or conclusions (positive and negative)

Responders and non-responders: With additional animals it is now clear that somatostatin vector delivery can interfere with a seizure-susceptible state. Responders make up 30-40% of tested rats. Pretreatment seizure history characteristics may have predictive value for discriminating responders.

All-or-nothing response: Almost every animal that shows an effect of somatostatin vector delivery exhibits full resistance to electrical stimulation. Stimulus current levels that consistently elicited maximal seizures prior to gene delivery fail to induce even the lowest grade of behavioral or electrographic seizures.

Multiple therapeutic delivery sites: Maximal efficacy can be achieved with SST vector delivery into distinct hippocampal or thalamic subregions, and even with unilateral overexpression.

Kindling effects on neurogenesis: Hippocampal SST vector delivery can suppress the elevation of progenitor cell proliferation, differentiation to glial phenotype, and survival provoked by seizure kindling.

4) Other achievements

Stated goals not met

Tissue for Task 5 & 6 examination of inflammation markers is being generated for collective analyses in the upcoming year. Behavioral testing was deferred due to staffing limitations but is set to begin in October 2015.

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

During the reporting year graduate student Gowri Natarajan has advanced to candidacy for the Ph.D. degree based on passing qualifying examinations. She has worked closely with all 3 PIs and provided all the experimental animals used to date and is training graduate student Jeffrey Leibowitz in electrode implantation and vector delivery surgery. Jeffrey Leibowitz has completed several of the neurogenesis and inflammation experiments and has trained Gowri Natarajan in several imaging and histological techniques. Gowri and Jeffrey are currently being trained to implement behavioral testing methods. UF undergraduate Junior Medical Honors Program student Shahrukh Bengali conducted a project supervised by PIs Carney and King, investigating somatostatin expression in the hippocampus resected from a young temporal lobe epilepsy patient. Post-baccalaureate student Andrew Moss has since expanded this project to begin to characterize somatostatin and receptor distribution in a cohort of similar patients, who might represent potential candidates for clinical somatostatin gene delivery. UF undergraduate Yang Zhao has participated as a volunteer lab assistant. Under the supervision of PI King and Ms. Natarajan he has scored video and EEG seizure records, behavioral data, and mapped histological reconstructions of electrode placement and vector delivery location and expression.

How were the results disseminated to communities of interest?

A poster presentation was made at the 2014 Society for Neuroscience Annual Meeting. PI King presented an invited talk "Intracranial Somatostatin Gene Delivery for Epilepsy" to students and faculty at Louisiana State University Health Sciences Center May 21, 2015. Two abstracts have been submitted for presentations at professional society meetings (Biomedical Engineering, Society for Neuroscience) later this year.

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

- Neurogenesis will be quantified in rats with longer survival times after BrdU to quantify new neuron and glia numbers and their localization and how they are impacted by kindling and somatostatin treatment to determine whether aberrant neurogenesis could be a mechanism of kindling-induced seizure behavior.
- Neurogenesis will be quantified in more somatostatin responders and non-responders to determine whether the therapeutic effect of somatostatin may be mediated by reversing the effects of kindling on the number of new neurons or their localization.
- Microglial counts and activation states will be conducted in rats with longer survival time after BrdU to determine the persistence of the inflammatory response in kindled rats and whether the effect of kindling is reversed by somatostatin.
- Microglial counts and activation states will be conducted in more somatostatin responder and non-responder rats to determine if the therapeutic effects of somatostatin by changing the inflammatory response.
- Microglial counts and activation states will be conducted in the hilar, CA3 and CA1 regions of the hippocampus as these regions could mediate therapeutic effects of somatostatin on seizure behavior.
- Measures of neurogenesis and neuroinflammation will be associated with one another and with measures of seizure severity and resilience to further explore their robustness as mechanisms of seizure behavior and mechanisms through which somatostatin may mediate therapeutic effects. We will finish collecting protein samples from all groups so that concentrations of inflammatory proteins can be quantified using bioplex technology. This powerful technology requires that all collected samples be run simultaneously to minimize cost.
- Water maze and alternation behavior will be measured as potential indices of therapeutic effect or safety.
- EEG characteristics will be examined more closely for variables that might predict responders and indicate relevant disease mechanisms.

4. IMPACT:

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

The results support the possibility that gene therapy might be an appropriate intervention for progressive epilepsy. They encourage further investigation of mechanisms of action, optimal application, alternate transgenes and delivery systems, as well as translational efforts to advance the approach to clinical acceptability. They provide new insight into potential roles and actions of somatostatin in the brain, and into the understanding of epilepsy as a network disorder.

What was the impact on other disciplines?

The results contribute to a growing body of evidence that intracranial gene transfer using AAV vectors is a safe and effective approach to treating neurological disease.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Changes that had a significant impact on expenditures

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:

Presentations:

Localized intracranial somatostatin gene delivery persistently reduces seizure severity in a rat model of temporal lobe epilepsy, Gowri Natarajan, Jessica Anne McElroy, Rabia Zafar, Michael A. King, Junli Zhou, Paul R. Carney; Society for Neuroscience Annual Meeting poster 314.18, Nov 17, 2014. (appended)

Abstracts:

Leibowitz JA, Natarajan G, Asokan A, King MA, Carney PR, Ormerod BK: Kindling increases Type-1 progenitor cell division in the dentate gyrus of adult rats. Biomedical Engineering Society (BMES) Annual Meeting, October 7-10, 2015 in Tampa, Florida: Abstract ID: 1252.

Leibowitz JA, Natarajan G, King MA, Carney PR, Ormerod BK: Somatostatin reverses kindling-induced increases Type-1 progenitor cells in the dentate gyrus of adult rats. Society for Neuroscience Annual Meetings 2015.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Michael A. King, Ph.D.
Project Role:	PI
Research Identifier:	

Nearest person month worked	3
Contribution to Project:	kindling; histological processing, microscopy, stereology; behavioral testing; statistical analysis
Funding Support:	

Name:	Paul R. Carney, M.D.
Project Role:	PI
Research Identifier:	
Nearest person month worked	3
Contribution to Project:	Oversight of electrode implantation & vector delivery surgery, euthanasia, kindling, seizure data analysis, and behavioral testing
Funding Support:	

Name:	Brandi K. Ormerod, Ph.D.
Project Role:	PI
Research Identifier:	0000-0003-0982-0788
Nearest person month worked	3
Contribution to Project:	Oversight of behavioral, neurogenesis and neuroinflammation data collection and analyses
Funding Support:	

Name:	Gowri Natarajan, M.S.
Project Role:	Graduate student
Research Identifier:	
Nearest person month worked	12
Contribution to Project:	Electrode implantation & vector delivery surgery, euthanasia; kindling; histological processing, microscopy, stereology; behavioral testing
Funding Support:	

Name:	Jeffrey Leibowitz
Project Role:	Graduate Student
Research Identifier:	
Nearest person month worked	12
Contribution to Project:	Histologies, quantification of neurogenesis, histological quantification of neuroinflammation and bioplex quantification of cytokines
Funding Support:	

Name:	Junli Zhao
Project Role:	Postdoctoral associate
Research Identifier:	
Nearest person month worked	2
Contribution to Project:	Electrode implantation & vector delivery surgery, euthanasia; kindling
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?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

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

APPENDICES: SFN2014Natarajan.pdf

314.18 Localized intracranial somatostatin gene delivery persistently reduces seizure severity in a rat model of temporal lobe epilepsy

Gowri Natarajan^{1,2,3,4,5,6}, Jessica.A.McElroy^{1,3,4}, Rabia Zafar^{1,3,4,5,6}, Junli Zhou^{1,3,4}, Michael.A.King^{7,8}, Paul.R.Carney^{1,2,3,4,5,6}

¹Wilder Center of Excellence for Epilepsy Research, ²J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Departments of ³Pediatrics and ⁴Neurology, University of Florida, ⁵Department of Neuroscience, ⁶McKnight Brain Institute, University of Florida, ⁷Department of Pharmacology and Therapeutics, University of Florida, ⁸NF/SG VA Medical Center, Gainesville, Florida

INTRODUCTION

In the brain, the neuropeptide somatostatin is co-expressed with the inhibitory neurotransmitter GABA in neurons within the dentate gyrus (DG) and in the CA1 and CA3 regions of the hippocampus. We have previously demonstrated that overexpression of SST in the hippocampus significantly reduces the occurrence of seizures during early epileptogenesis (Zafar et al., 2012). These results suggest that localized, persistent intracranial SST supplementation could be an effective, non-destructive therapeutic intervention, with a specific and immediate therapeutic potential administered prior to last-resort neurosurgical resections in pharmacoresistant cases of TLE. **The objective of the current study** was to test whether intracranial delivery of SST vector could similarly suppress seizures when delivered after a stable seizure state was established. **We tested the hypothesis** that the serotype 5 AAV vector-mediated overexpression of SST *in vivo* reduces the severity of seizures in the rodent amygdala electrical kindling model of TLE.

METHODS

1)Electrode implantation surgery

- Bilateral bipolar twist electrodes implanted in the left and the right amygdala for stimulating and recording respectively
- Two screw electrodes for ground and reference points implanted rostral to the bregma and caudal to the lambda respectively
- Placeholder cannula screws for injection of the vector in the second surgery

2) Amygdala kindling model to generate seizures

- Electrical kindling of the amygdala twice a day by administration of a standard 2s, 1ms pulse duration, 50Hz biphasic square wave pulse at a specific current intensity
- Current intensity determined prior to kindling for each rat
- Behavior changes associated with seizure progression during kindling classified into 5 Racine seizure grades; EEG activity reflecting seizure development and progression simultaneously recorded from the contralateral amygdala
- Kindling terminated after attainment of “fully kindled state” (3-5 consecutive grade 5 seizures)

3) Vector injection surgeries

- **pAAV-CBa-SST-GFP injection OR pAAV-CBa-GFP injection (n=z)**
- Vector injection of the precursor pre-pro-SST or GFP in the DG and CA1 of the bilateral hippocampus (2 μ l per injection site; 8 μ l total volume per animal)
- Three week waiting period for optimal expression of the vector

4) Post injection testing

- Periodic administration of test stimulations at the kindled current intensity for all vector injected animals after optimal gene expression

5) Immunohistochemistry

- Animals perfused through the ascending aorta
- 40-50 μ M coronal sections cut using a freezing microtome
- Representative sections containing the hippocampus along the entire rostro-caudal axis for each animal mounted and observed under a green fluorescent protein filter (GFP-3035B-OMF, Semrock) for evidence of GFP in the hippocampus
- SST14, 28 and 25 immunostaining performed in 2 rats that were fully kindled to 3 grade 5 seizures and perfused 48 hours after the last grade 5 seizure

RESULTS

- Persistent intracranial SST supplementation reduced the severity of kindled seizures in 50% of our kindled rats transduced with the precursor SST pre-pro-peptide (4/8=grade 0, 5/8 < grade 5, p=0.0625, h=0 at 5% significance level, comparison to fully kindled state);
- In the grade 0 rats additional stimulation episodes at and also +/- 100 μ A above the kindled current intensity did not increase the severity of kindled seizures
- Testing in all rats was carried out over a 3-week period

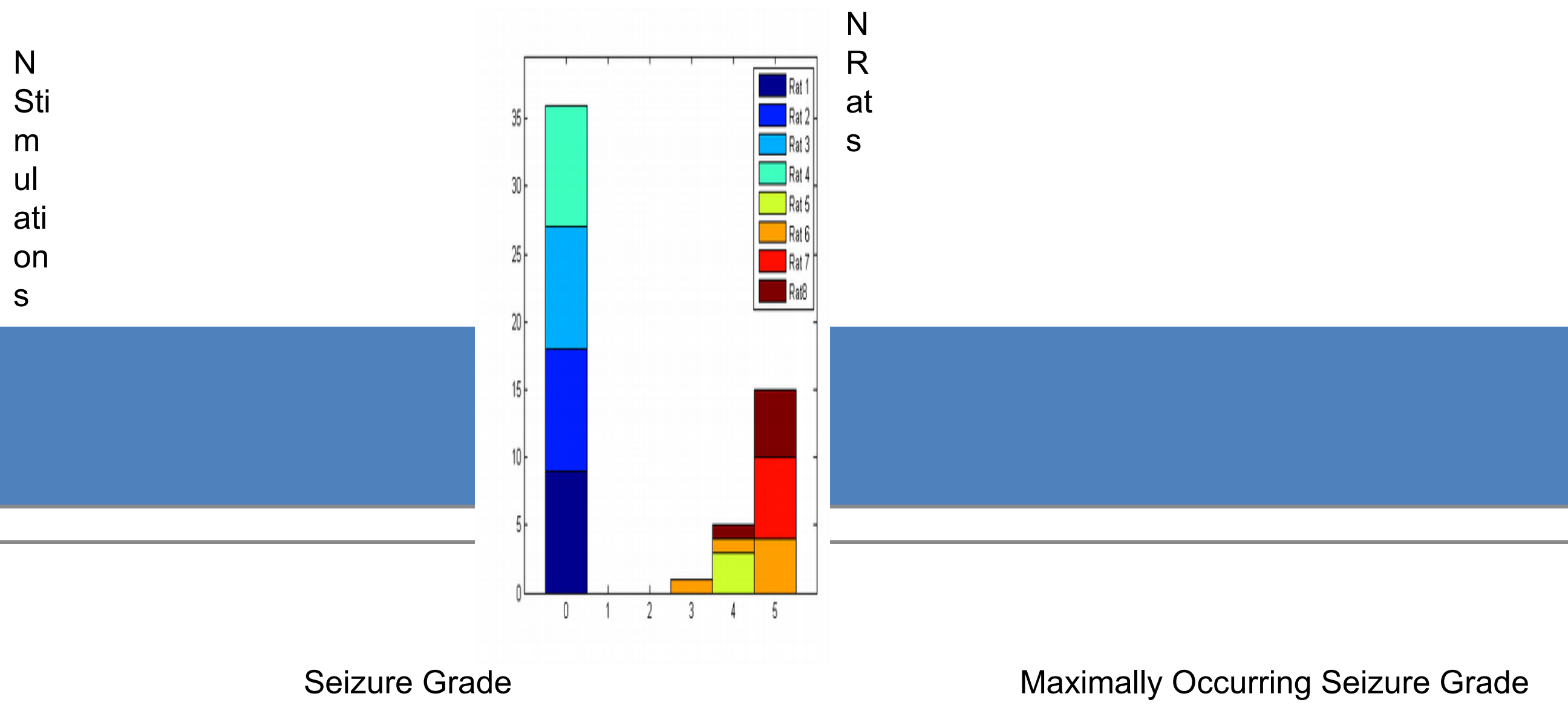


Fig.1 Histograms representing the seizure grade produced by each rat (R1-R8) at each test stimulation administered after SST injection. Rats that initially produced grade 0 seizures (4/8, Rat 1, Rat 2, Rat 3, Rat 4) were tested for upto 9 test stimulations over the 3 week testing period. Rat 5 was subjected to 3 test stimulations within 1 week. Rat 6,7 and 8 were subject to 6 test stimulations over a 2-3 week testing period

- 80% of our fully kindled rats (4/5) injected with a control vector (AAV-GFP) continued to consistently elicit grade 5 seizures upon repeated testing with 48 hour, 72 hour, 1 week and 1 month time intervals of testing (p=1, comparison to fully kindled state)

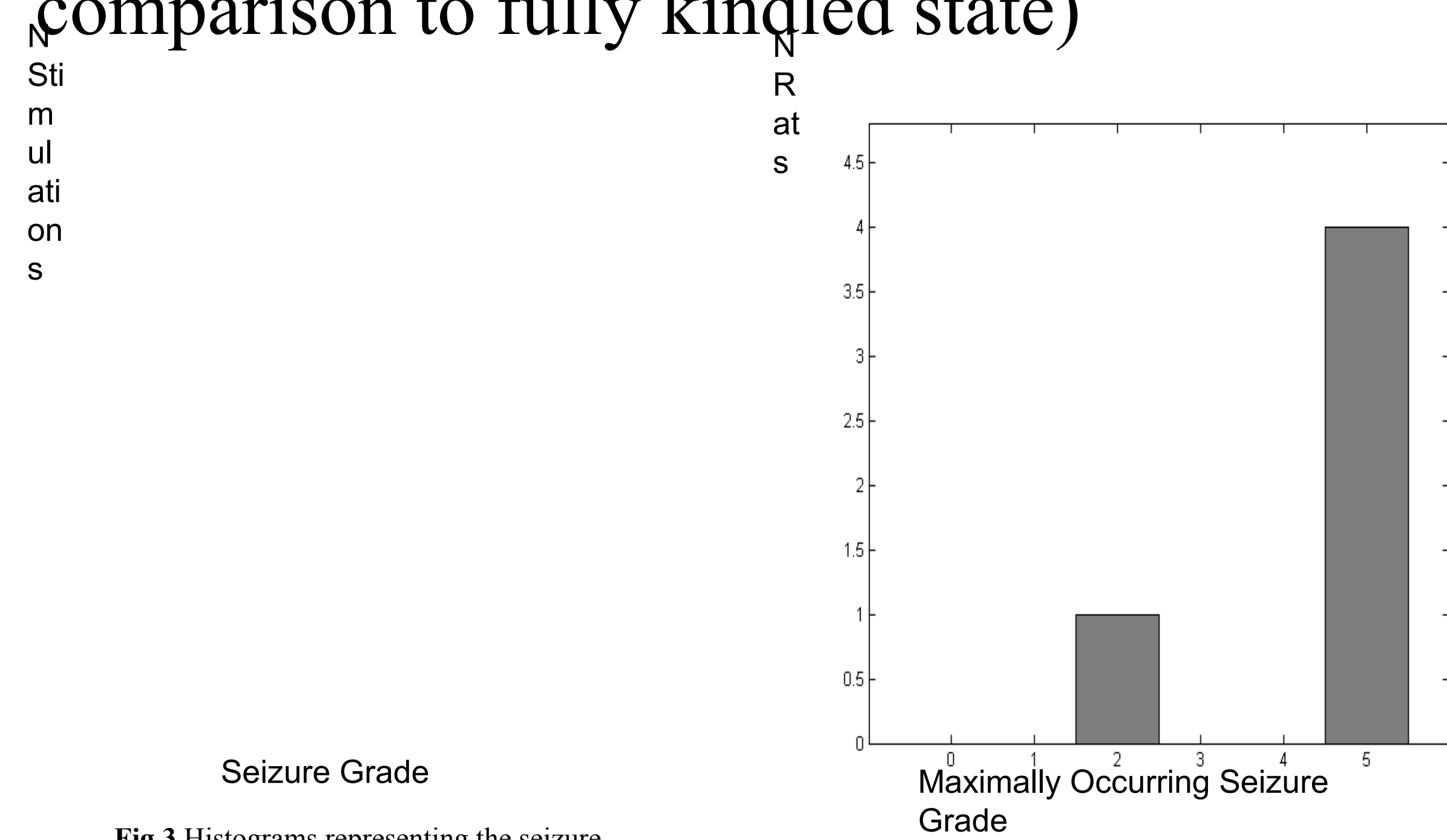


Fig.3 Histograms representing the seizure grade produced by each rat (R1-R5) at each test stimulation administered after GFP injection. Following the test stimulation 1, additional test stimulations in 4/5 rats were administered at intervals of 48 hours, 72 hours, 1 week and 1 month

Fig.4 The Wilcoxon signed-rank test was used to compare the maximally occurring seizure grade for each rat after GFP injection to its standard fully kindled pre-injection grade five seizure (p=1)

- Representative EEG recordings from the SST treated cohort further corroborated the observed behavior changes after SST gene transfer and the refractoriness to kindled seizures during the time period of testing

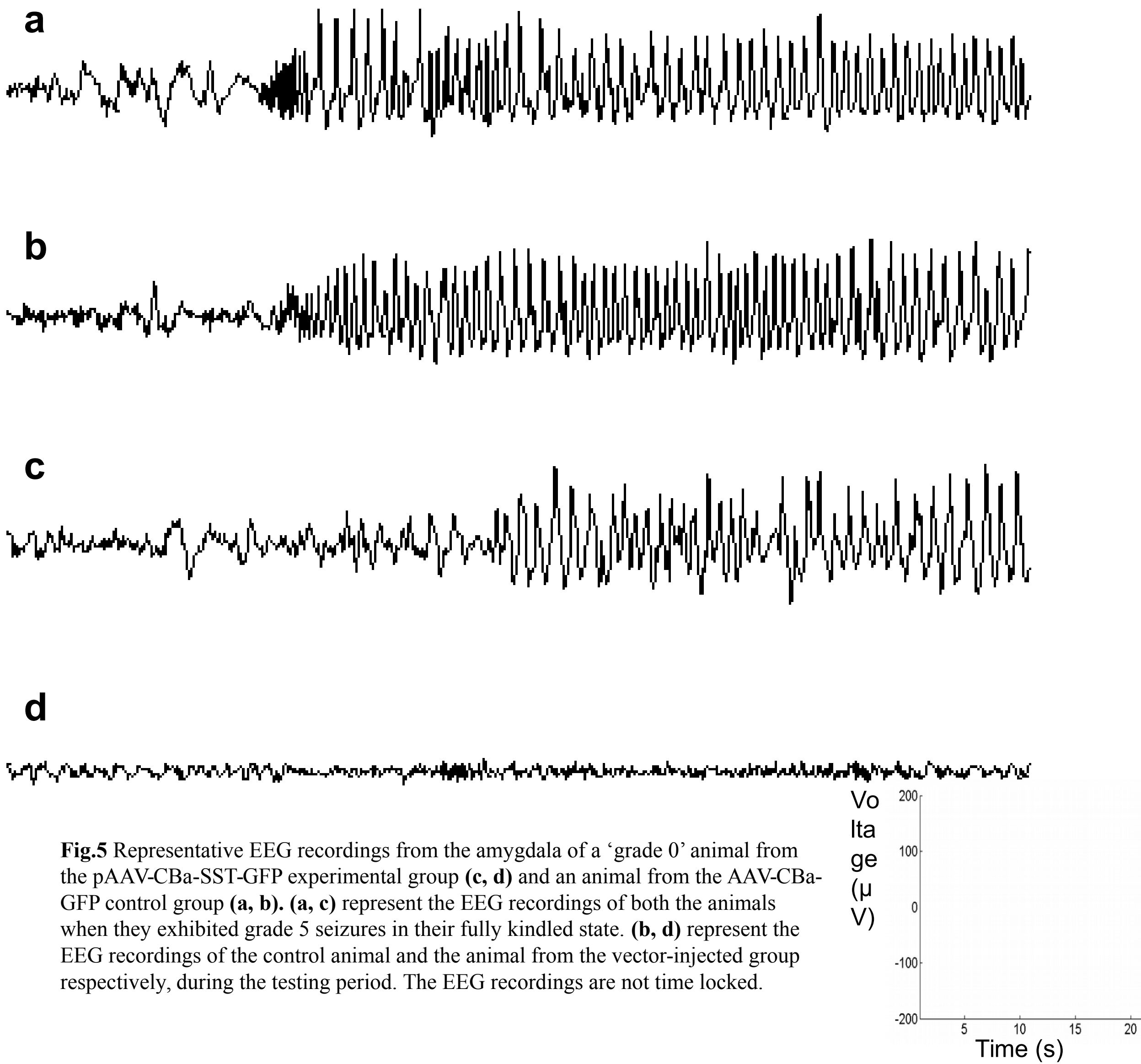


Fig.5 Representative EEG recordings from the amygdala of a 'grade 0' animal from the pAAV-CBa-SST-GFP experimental group (c, d) and an animal from the AAV-CBa-GFP control group (a, b). (a, c) represent the EEG recordings of both the animals when they exhibited grade 5 seizures in their fully kindled state. (b, d) represent the EEG recordings of the control animal and the animal from the vector-injected group respectively, during the testing period. The EEG recordings are not time locked.

- In the fully kindled brain (prior to any vector injection), ENDOGENOUS SST containing neurons were predominantly found in the hilar region of the DG (polymorphic layer), neurons in the CA1 stratum oriens and in inhibitory neurons of the CA2 and CA3 regions
- Robust labeling along the entire A-P axis of the hippocampus

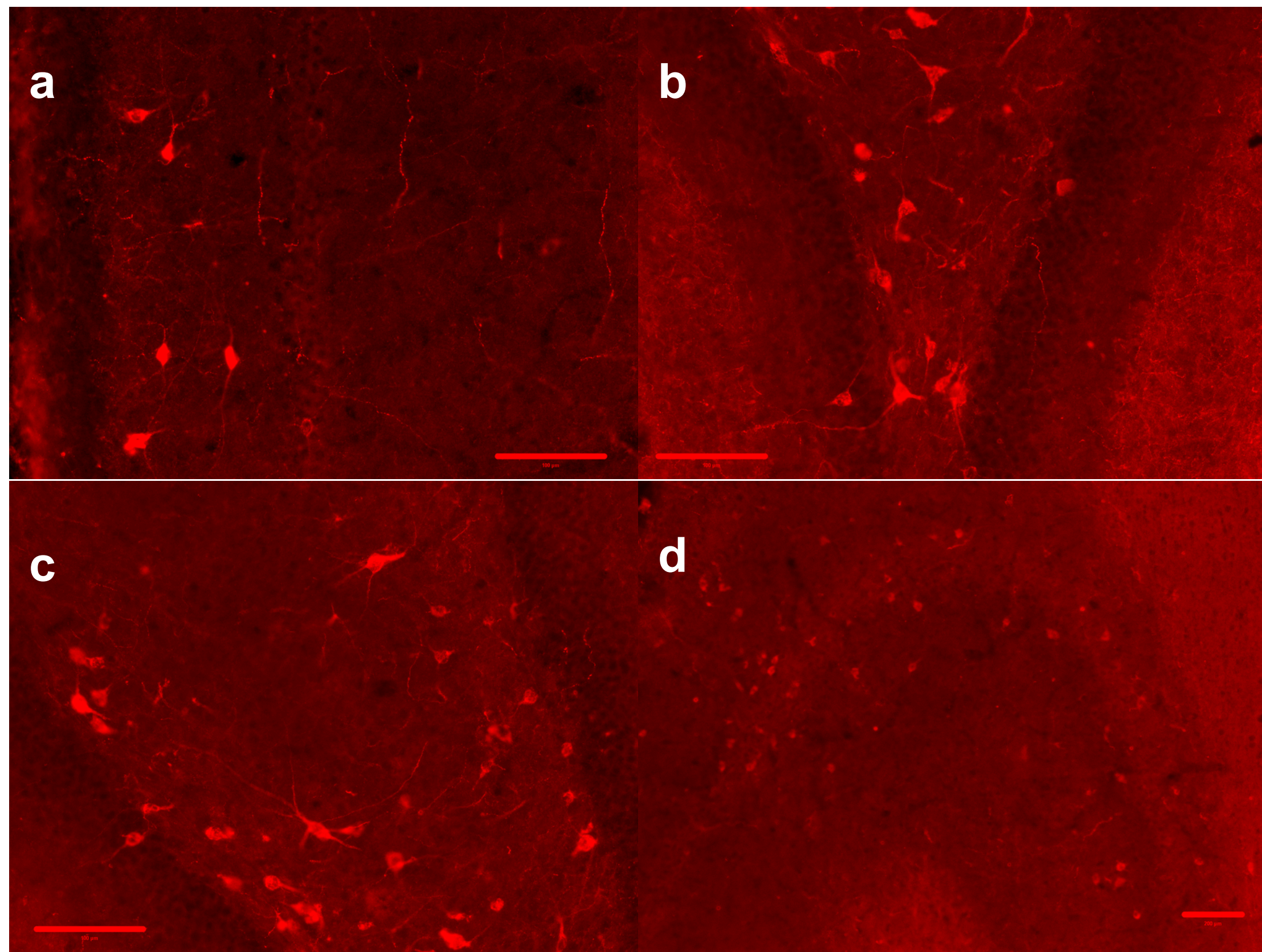


Fig.6 ENDOGENOUS SST expression in the hippocampus of a representative rat that was fully kindled to 3 consecutive grade 5 seizures and perfused 48 hours after the last grade 5. (a-d) Represent SST positive cells in the CA1 (a), dentate gyrus (b), and CA2/CA3 (c) subfields of the hippocampus. 50 μ M coronal sections were cut using a freezing microtome and viewed under the the Olympus IX71 fluorescent microscope. Horizontal scale bar is 100 μ M in a-c and 200 μ M in d.

- Therapeutic efficacy is possible with expression restricted to specific subregions
- No specific subregion appears necessary for therapeutic efficacy
- Unilateral transduction is sufficient for maximal therapeutic efficacy

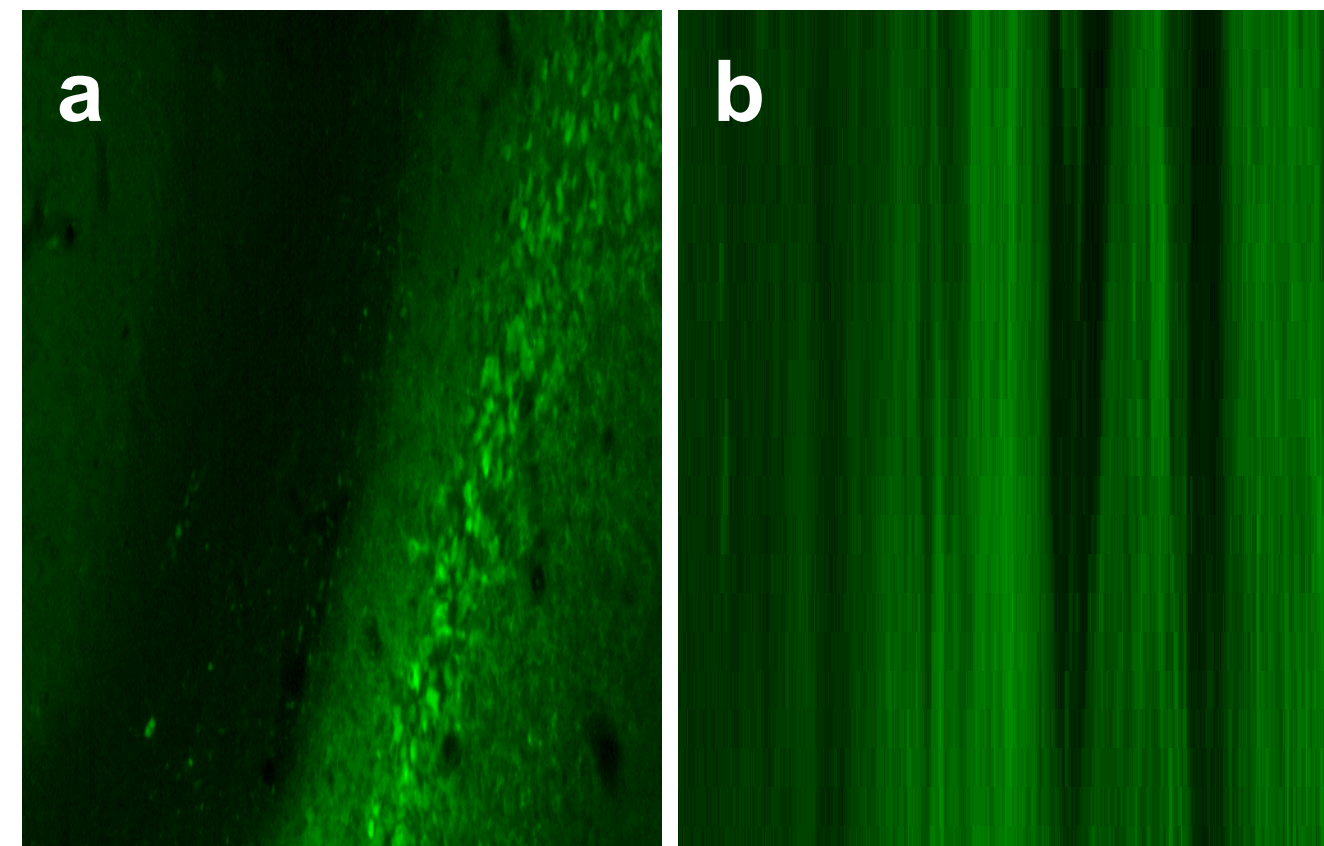


Fig.7 (a,b) pAAV-CBa-SST-GFP transduction and expression in the CA1 subfield of the hippocampus of a rat that produced grade 0 seizures after SST gene transfer. Expression of putative SST cells was restricted to the CA1 region, rostral along the A-P axis of the hippocampus, unilateral. 50 μ M coronal sections were viewed under the 3035-B OMF green fluorescent protein filter using the Olympus IX71 fluorescent microscope. Horizontal scale bar is 200 μ M.

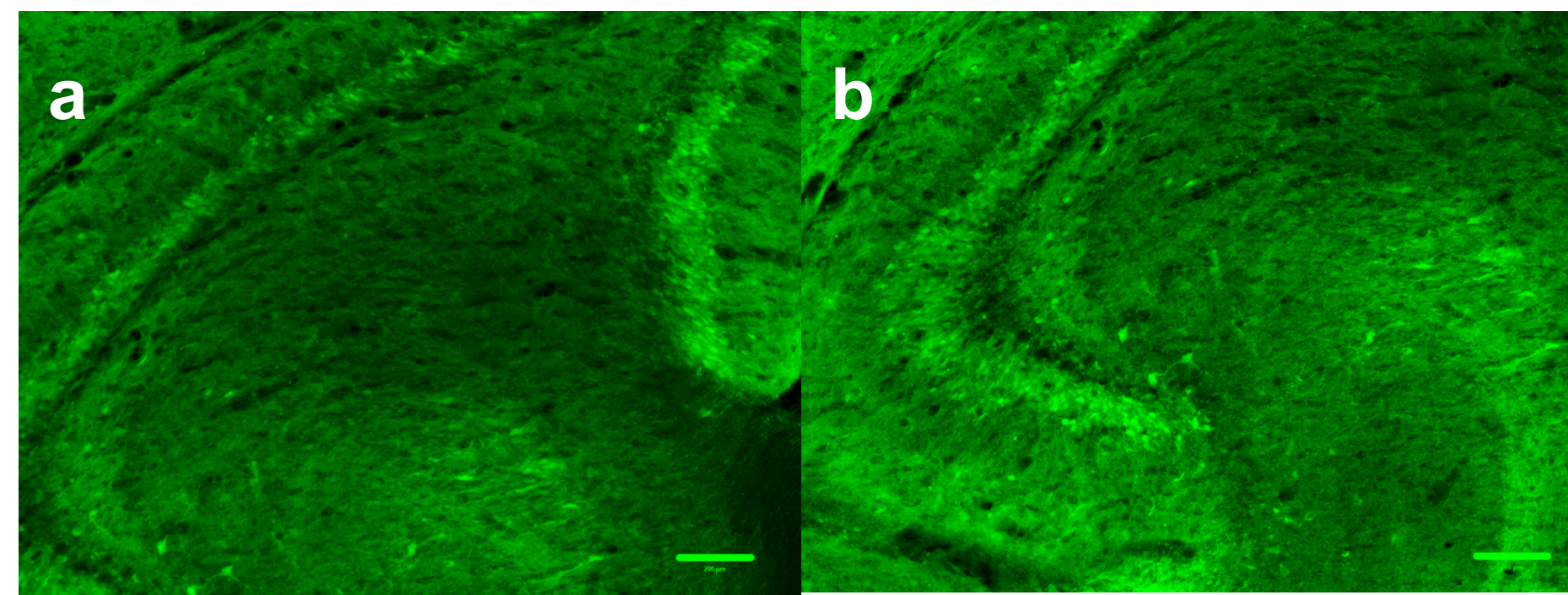


Fig.8 (a,b) pAAV-CBa-SST-GFP transduction and expression in the DG of the hippocampus of a rat that produced grade 0 seizures after SST gene transfer. Putative SST cells were predominantly found in the DG region (a,b) and in the CA2/CA3 region, unilateral. 50 μ M coronal sections were viewed under the 3035-B OMF green fluorescent protein filter using the Olympus IX71 fluorescent microscope. Horizontal scale bar is 200 μ M.

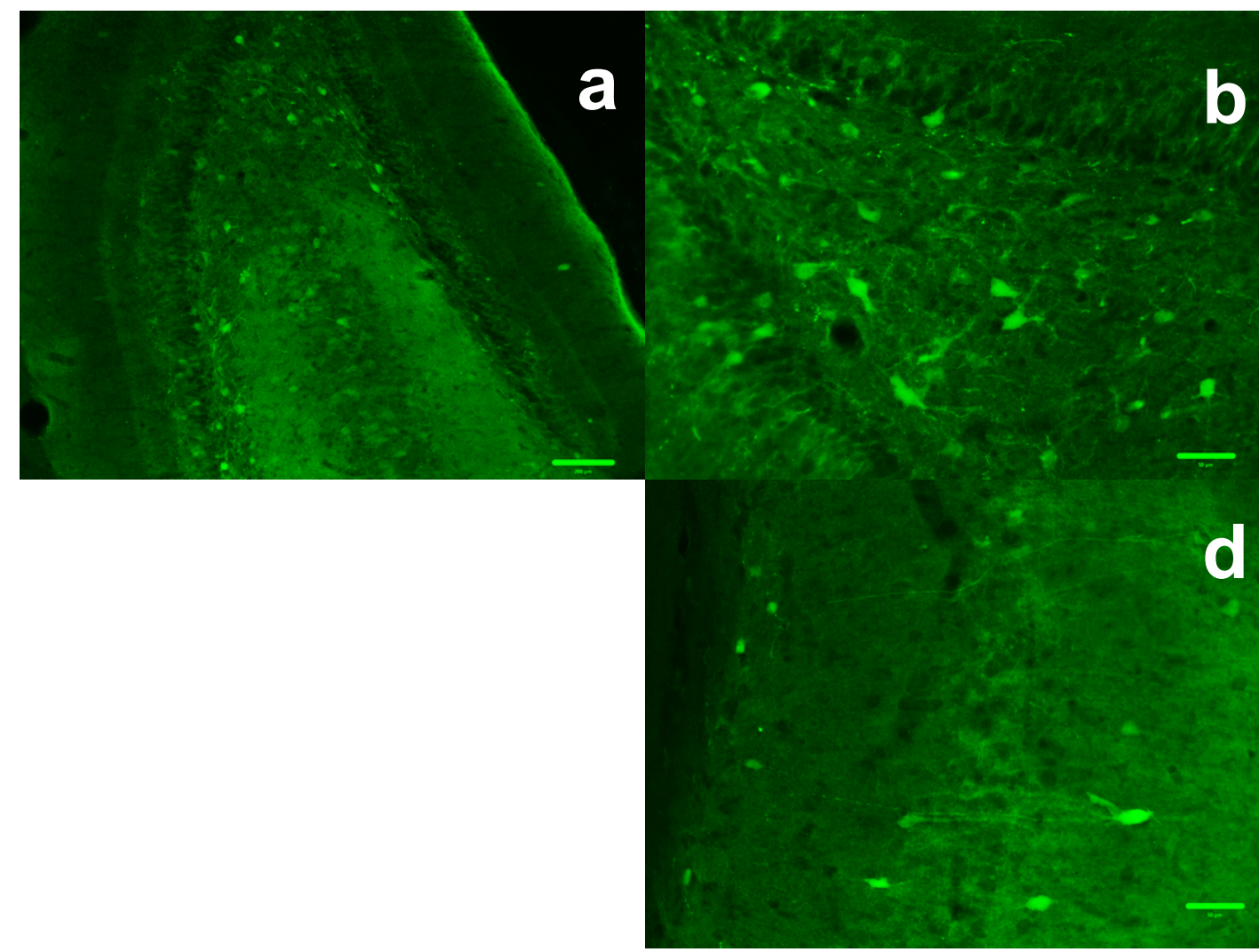


Fig.9 (a,b) pAAV-CBa-SST-GFP transduction and expression in the DG, CA2/CA3 and CA1 of the hippocampus of a rat that produced grade 0 seizures after SST gene transfer. Putative SST containing neurons were found in the DG region (a,b), in the CA2/CA3 region (c) and in the CA1 region (d). The expression in the CA1 and CA2/CA3 regions was bilateral. Transduction was observed along the entire A-P axis of the hippocampus in the DG and the CA1. 40 μ M coronal sections were viewed under the 3035-B OMF green fluorescent protein filter using the Olympus IX71 fluorescent microscope. Horizontal scale bar is 50 μ M in b-d and 200 μ M in a.

- All rats injected with pAAV-CBa-GFP (5/5) had expression of GFP in the hippocampus
- In 4/5 rats expression was restricted to the unilateral hippocampus
- Thalamic and cortical labeling present along with hippocampal labeling in some animals
- Robust GFP expression observed in the early, middle and late hippocampus in 4/5 rats
- GFP positive cells found in DG, CA1, CA2, CA3 and subiculum across rats

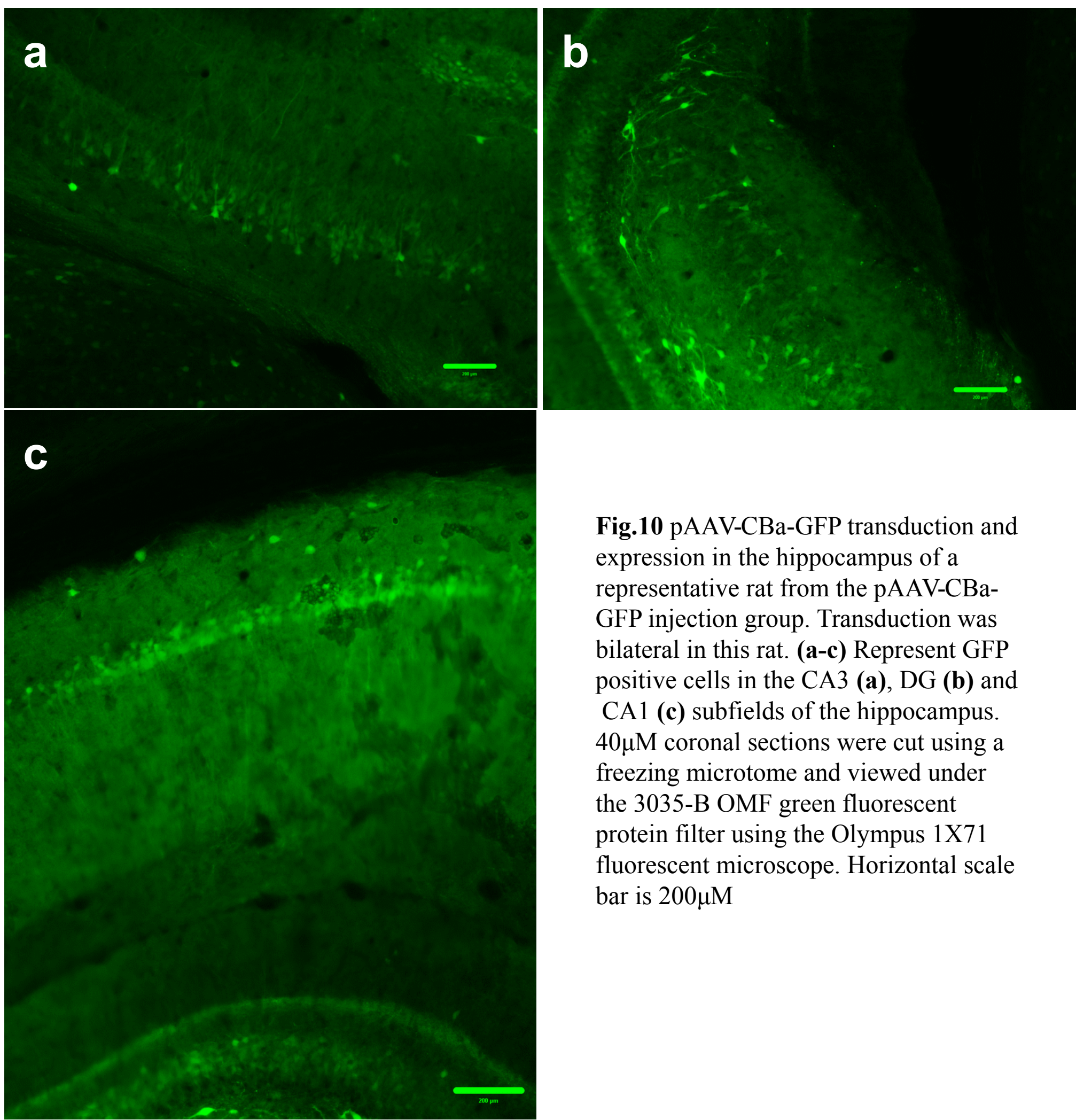


Fig.10 pAAV-CBa-GFP transduction and expression in the hippocampus of a representative rat from the pAAV-CBa-GFP injection group. Transduction was bilateral in this rat. (a-c) Represent GFP positive cells in the CA3 (a), DG (b) and CA1 (c) subfields of the hippocampus. 40 μ M coronal sections were cut using a freezing microtome and viewed under the 3035-B OMF green fluorescent protein filter using the Olympus IX71 fluorescent microscope. Horizontal scale bar is 200 μ M

CONCLUSIONS

- 50% of the animals with hippocampal expression of SST vector became persistently seizure-free even when tested repeatedly and with stronger stimulations
- Vector SST is probably not merely compensating for deficient inhibitory action owing to limited neuronal loss in the kindling model at our survival intervals
- The therapeutic efficacy of vector mediated SST gene therapy may depend in part on the final achievable levels of expression, distribution of the vector across the septo-temporal axis of the hippocampus and the specific sub-regions and possibly cell types within the hippocampus where high gene expression is achieved.
- Regardless of the source of extracellular SST (different phenotypes of cells transduced with the precursor peptide), transduction is likely to produce sustained agonist occupation at each of the five receptor subtypes present in rat hippocampus

ACKNOWLEDGEMENTS

This project is funded and supported by the Department of Defense Congressionally Directed Medical Research Programs (CDMRP) grant number PR121769, The Wilder Center of Excellence for Epilepsy Research, The Children's Miracle Network and North Florida/South Georgia Veterans Health System