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14. ABSTRACT:

The goal of this project is to examine whether grafting of human medial ganglionic eminence (hMGE)-like precursor cells generated from the human induced pluripotent stem cells (hiPSCs) into the hippocampus of chronically epileptic rats (CERs) would: (1) diminish the frequency and intensity of spontaneous recurrent seizures (SRS, Specific Aim 1, SA1); and (2) ameliorate learning and memory impairments and depression (Specific Aim 2, SA2). Studies performed so far have focused on rigorously addressing the seizure-modulating effects of grafts in SA1. Three weeks of continuous EEG recordings have been collected and compared between the four groups of epileptic rats: CERs receiving hMGE-like cell grafts and cyclosporine (an immunosuppressant to promote the survival of human cell grafts in the rat brain), CERs receiving sham-grafting surgery, CERs receiving cyclosporine only and CERs receiving no treatment. The results showed that, in comparison to all three control CER groups, CERs receiving hMGE-like cell grafts displayed 75-78% reduction in the frequency of all SRS, 77-80% reduction in the frequency stage V seizures and 71-75% reduction in the total time spent in seizure activity. Notably, the robust suppression of seizures was associated with an excellent yield, pervasive migration and robust differentiation (~70%) of graft-derived cells into gamma-amino butyric acid positive (GABA-ergic) interneurons in the hippocampus. Thus, hMGE-like cell grafting into the hippocampus in a rat model of temporal lobe epilepsy greatly suppresses seizures with the addition of a large number of new GABA-ergic interneurons.

15. SUBJECT TERMS

Temporal lobe epilepsy, Human induced pluripotent stem cells, GABA-ergic precursor cells, Medial Ganglionic eminence, Memory dysfunction, Depression, Spontaneous Recurrent Seizures

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1. INTRODUCTION

Epilepsy, affects ~3 million Americans and ~60 million people globally. Military personnel are at a greater risk for developing temporal lobe epilepsy (TLE) because of increased incidences of traumatic brain injuries (TBIs). While antiepileptic drug (AED) therapy is effective for controlling seizures in many patients, ~35% of patients with TLE have chronic seizures that are resistant to AEDs. Besides, AED therapy has adverse side effects and does not reduce cognitive and mood impairments associated with TLE. Hence, alternative therapies having potential for both restraining spontaneous seizures and easing cognitive and mood impairments in chronic TLE are needed. Spontaneous recurrent seizures (SRS) in TLE originate from the temporal lobe foci and are associated with multiple epileptogenic changes in the hippocampus. Scarcity of inhibitory gamma-amino butyric acid (GABA)-ergic interneurons stands out among epileptogenic changes because higher levels of GABA are known to suppress the occurrences of seizures. Hence, the idea of restraining SRS in the epileptic hippocampus via grafting of cells that release the inhibitory neurotransmitter GABA has received much attention. The goal of this project is to examine whether grafting of medial ganglionic eminence (MGE)-like precursors generated from human induced pluripotent stem cells (hiPSCs) into the hippocampus of rats exhibiting chronic temporal lobe epilepsy (TLE) would: (1) diminish the frequency and intensity of spontaneous recurrent seizures (SRS, Specific Aim 1); and (2) ameliorate learning and memory impairments and depressive-like behavior (Specific Aim 2).

2. KEYWORDS

Cognitive dysfunction
Cell therapy Depression
Epilepsy
Spontaneous recurrent seizures (SRS)
GABA
GABA-ergic precursor cells
Hippocampus
Hippocampal neurogenesis
Human induced pluripotent stem cells
Medial ganglionic eminence (MGE)
Memory dysfunction
Stem cell differentiation
Stem cell proliferation
Stem cell therapy Temporal
lobe epilepsy

3. ACCOMPLISHMENTS

3.1. Major Goals: The major goals of this project are to examine whether grafting of MGE precursors generated from hiPSCs into the hippocampus of chronically epileptic rats (CERs) would: (i) diminish the frequency and intensity of SRS (Specific Aim 1 studies); and (ii) ameliorate learning and memory impairments and depressive-like behavior (Specific Aim 2 studies).

Accomplishment of objectives in Specific Aim 1 would require the following experiments: (1) Generating rats exhibiting chronic TLE, typified by SRS: This is done through induction of status epilepticus (SE) via graded kainic acid injections (3 mg/Kg/hour for 2-5 hours), termination of acute seizures 2 hours after SE onset via an injection of antiepileptic drug diazepam (5-10 mg/Kg) and measurement of behavioral SRS via direct observations at 2-3 months after SE. (2) Generation of MGE precursors from hiPSCs in culture: This is accomplished through a directed differentiation method, which is performed in the laboratory of our collaborator (Dr. Su-Chun Zhang (Univ of Wisconsin, Madison) and then shipped to our lab for further culturing and use in grafting studies. (3) Preparation of cell suspension of human MGE precursor cells and transplantation into the hippocampus of rats exhibiting chronic TLE (i.e. CERs). This involves bilateral injections of MGE precursor cells into the hippocampus (3 sites/hippocampus) using stereotactic neurosurgery and daily cyclosporine injections (10 mg/Kg/day) thereafter until the collection of tissues through euthanasia. (4) Sham-grafting surgery: This involves bilateral injections of the culture medium into the hippocampus of rats exhibiting chronic TLE (3 sites/side). (5) Surgical implantation of epidural electrodes for electroencephalographic (EEG) recordings (survival surgery). (6) Continuous EEG recordings for three weeks from CERs belonging to

grafted, sham-surgery, cyclosporine alone and epilepsy-only groups. (7) Analyses of the survival, migration and differentiation of graft-derived cells and measurement of GABA and glutamate in the epileptic hippocampus belonging to different groups.

Accomplishment of goals in Specific Aim 2 would require the following experiments: experiments 1-4 described in Specific Aim 1 above plus the following experiments: (5) Injections of 5'-bromodeoxyuridine into CERs receiving grafts at 2.5 months after grafting (equivalent to 5th month after SE) to label newly born cells and neurons in the hippocampus. The control groups also receive BrdU injections at comparable time-points after SE. (6) Measurement of functions such as learning, memory and mood using a series of behavioral tests. (7) Quantification of hippocampal neurogenesis and measurement of neurotrophic factors.

3.2. Studies Accomplished:

Duration of study: 02/04/2015 to 09/29/2016 (~20 months)

On February 3, 2015, we received a notification from the Department of the Army that the protocol PR130086 entitled, Human induced pluripotent stem cell (hiPSC)-derived GABA- ergic precursor cell therapy for chronic epilepsy", IACUC protocol number 2014-005, Protocol Principal Investigator Ashok Shetty, is approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 30-JAN-2015 for the use of rats and will remain so until its modification, expiration or cancellation. Upon receiving this notification, we commenced animal experiments for this study.

3.2.1. Major Activities:

The following narrative describes studies accomplished so far for Specific Aim 1 (SA1; Task 1) and Specific Aim 2 (SA2; Task 2).

The major goal of SA1 is to determine whether grafting of hMGE-like precursor cells generated from hiPSCs into the hippocampus of CERs (Group 1) would diminish the frequency and intensity of SRS, in comparison to SRS in three control CER groups. The grafted group also received daily injections of cyclosporine to promote the survival of human cell grafts in the rat brain. The control groups comprise: CERs receiving sham-grafting surgery (sham grafted group; Group 2), CERs receiving cyclosporine alone (cyclosporine only group; Group 3), CERs receiving no treatment (epilepsy-only group; Group 4). We have now completed seizure analyses for all four groups of CERs. This involved collection of three-weeks of continuous video-EEG recordings, commencing in the 3rd month after grafting or sham-grafting surgery (equivalent to ~6 months after SE in all groups). Furthermore, we collected and statistically compared data on various parameters using EEG traces from these four CER groups.

The major goal of SA2 is to evaluate whether grafting of hMGE-like precursor cells generated from hiPSCs into the hippocampus of CERs (Group 1) would ameliorate learning and memory impairments and depressive like behavior, in comparison to the four control groups described above. Changes in learning, memory and mood will also be compared with age-matched naïve control rats. We have performed behavioral testing of rats belonging to different groups of CERs. However, data analyses and interpretation are pending, which will be completed in the coming year.

3.2.2. Specific Activities:

(1) Kainic acid injection was given to a total of 163 rats to induce SE. Among these, 148 rats developed the required SE and survived the procedure.

(2) Rats that survived SE procedure (n=148) were evaluated for occurrences of behavioral spontaneous recurrent seizures (SRS) in the third month after SE through direct observations for 48 hours over a period of 3-4 weeks (~12 hrs/week). From this larger cohort, 69 CERs having similar range of SRS were assigned to different groups in SA1 studies (see Table 1), 32 CERs having similar range of SRS were assigned to different groups in SA2 studies (Table 2) and 29 rats having comparable range of SRS were assigned to a mechanistic study of grafts (see Table 3). The mechanistic studies on grafts were added later as an amendment to the original animal protocol, with approvals from both IACUC and the USAMRMC Animal Care and Use Review Office (ACURO). The remaining 18 CERs were euthanized and brain tissues were harvested.

3.2.2.1 Specific activities related to SA1 studies:

Seventy CERs (n=69) were randomly assigned to different groups in SA1 (see Table 1). The sequence of experiments, waiting periods between experiments, exclusions/inclusions and data collection are described below:

(1) CERs in the grafted group (Group 1) received MGE-like, GABA-ergic precursor cells generated from hiPSCs into the hippocampus (100,000 live cells/grafts, 3 sites/hippocampus) – first survival surgical procedure. This was performed in the 3rd month after SE. These rats also received daily subcutaneous injections of cyclosporine (10mg/Kg/day) until euthanasia, in order to prevent the rejection of human cell grafts in the rat brain. CERs in the sham grafted group (Group 2) received injections of the culture medium into the hippocampus (3 sites/hippocampus) – first survival surgical procedure. CERs assigned to cyclosporine only group (Group 3) received daily subcutaneous injections of cyclosporine (10 mg/Kg) until euthanasia. CERs in the epilepsy-only (Group 4) group did not receive any treatment.

(2) During the next 2 months (waiting period after grafting or sham grafting), some CERs died and some exhibited health issues (see Table 1 for details). CERs exhibiting health issues were excluded from the study.

(3) Some CERs in grafted and epilepsy-only groups (n=6/group) were used for harvesting of fresh tissues for biochemical studies.

(4) The remaining CERs in all groups were implanted with EEG electrodes in different batches (the second survival surgical procedure for grafted and sham-surgery groups and the first survival surgical procedure for cyclosporine only and epilepsy-only groups). This was done 2 months after grafting or sham-grafting surgery (i.e. in the 5th month after SE in all groups).

(5) Ten-fourteen days after the implantation of electrodes, each CER was connected to a tethered video-EEG system and continuous EEG recordings were taken for three weeks. These recordings were accomplished in the 5th and 6th month after SE in all groups.

(6) Some CERs lost head caps (EEG electrode unit cemented to the head) after the implantation surgery or during EEG recordings. Such CERs in all groups were excluded from the study.

(7) Three weeks of continuous video-EEG recordings were collected from a total of 30 CERs. These CERs comprised 9 in the grafted group, 7 in the sham-grafted group, 6 in the cyclosporine-only group, and 7 in the epilepsy-only group (Table 1).

(8) One CER in the grafted group had ectopic placement of grafts (when examined through histology following EEG recordings). Data from this CER was excluded for statistical analyses.

(9) Three weeks of continuous video-EEG recordings were available for statistical analyses from a total of 28 CERs. These CERs comprised 8 in the grafted group, 7 in the sham-grafted group, 6 in the cyclosporine-only group, and 7 in the epilepsy-only group (Table 1).

(10) After the completion of EEG recordings, the above rats were also employed for behavioral testing, using a battery of tests to examine cognitive, memory and mood. These were done in the 6th and 7th month after SE in all groups. The tests include novel object recognition test, object location test, pattern separation test, sucrose preference test and novelty suppressed feeding test. Following this, rats were perfused for histological studies.

Table 1: Numerical details of CERs used for Specific Aim 1

Sequence of events and exclusions in the duration of experiments	***Grafted Group***	***Sham Grafted Group***	***Cyclosporine-only group***	***Epilepsy-only group***
Number of CERs initially assigned	29	11	11	18
Mortality of CERs in the waiting period	3	1	0	1
Exclusion of CERs due to health issues	7	0	5	0
CERs used for harvesting tissues (for biochemical studies)	6	0	0	6
Exclusion of CERs due to loss of head caps (EEG recording unit)	4	3	0	4
Numbers of CERs from which EEG data were collected	9	7	6	7
Exclusion of CERs due to ectopic location of grafts	1	0	0	0
Final numbers of CERS employed for statistical analysis of EEG data	8	7	6	7

3.2.2.2 Specific Activities performed for SA2:

Thirty-two CERs (n=32) were randomly assigned to two groups in SA2 (see Table 2). The sequence of experiments, waiting periods between experiments and data collection are described below:

(1) CERs in the grafted group received procedures, as described above for SA1 studies.

(2) During the next 2-3 months (waiting period after grafting), some CERs died and some exhibited health issues (see Table 2). CERs exhibiting health issues were excluded from the study.

(3) Subgroups of CERs in both groups (n=6/group) were injected with 5'bromodeoxyurine (BrdU) at 2.5 months after grafting (equivalent to the 5th month after SE in both groups).

(5) The remaining CERs in both groups (n=12) were used for behavioral testing using a battery of tests to examine cognitive, memory and mood function, in the 5th and 6th months after grafting (equivalent to 7th and 8th months after SE in both groups). These include novel object recognition test, object location test, pattern separation test, sucrose preference test and novelty suppressed feeding test.

(6) Following the completion of behavioral tests (>6 months after grafting), animals in a subgroup of rats (n=6/group) were euthanized and brain tissues harvested for biochemical studies. Animals in another subgroup of rats (n=6/group) were perfused through the heart for analyses of neurogenesis and related histological studies.

Table 2: Numerical details of CERs used for specific Aim 2

Sequence of events and exclusions in the duration of experiments	***Grafted Group***	***Epilepsy-only Group***
Number of CERs initially assigned	20	12
Mortality of CERs in the waiting period	4	0
Exclusion of CERs due to health issues	4	0
Number of CERs used for behavioral testing	12	12
CERs used for harvesting tissues (for biochemical studies)	6	6
CERs perfused for neurogenesis and other histological studies (These rats received BrdU injections in the 5th month after SE)	6	6

3.2.2.3 Specific Activities performed for additional mechanistic studies

To evaluate whether GABA-ergic interneurons derived from transplanted hMGE progenitor cells are functional, we employed hMGE-like precursor cells transduced with pharmacologically selective designer Gi-protein-coupled receptor hM4D (DREADDs) as donor cells for grafting. We tried two different approaches. The first approach employed grafting of hMGE progenitors (obtained from hPSCs expanded from human embryonic stem cells) that were transduced with DREADDs through CRISPR/Cas9 technology (CRISPR/Cas9 DREADDs). These cells were obtained from our collaborator, Dr. Su-Chun Zhang, University of Wisconsin. The second approach employed grafting of hMGE progenitors (obtained from hiPSCs) that were transduced with AAV-hSyn-hM4D(Gi)-mCherry (mCherry-DREADDs). These vectors were procured from the vector core laboratory at the University of North Carolina, Chapel Hill, NC (Dr. Bryan Roth's laboratory). Injections of Clozapine-N-Oxide (CNO) were used to activate DREADDs at certain periods of EEG recordings. Our hypothesis is that GABA-ergic cell grafting suppresses seizures in epileptic animals through inhibition of excitatory neurons in the hippocampus. This effect can be ascertained from the reduced frequency of SRS during baseline recordings. If this is truly the mechanism, suppression of activity of hM4di transduced GABA-ergic cells through CNO would result in increased number of seizures specifically during the treatment period and reduced number of seizures during the washout period. Table 3 shows assignment of CERs to different groups. The sequence of experiments and exclusions are described below:

(1) CERs grafted with MGE-like precursor cells transduced with CRISPR/Cas9 DREADDs or mCherry DREADDs received daily subcutaneous injections of cyclosporine to prevent graft rejection. CERs assigned to CNO control group did not receive any treatment.

(2) During the next 2 months (waiting period after grafting), some CERs died and some exhibited health issues (see Table 3). CERs exhibiting health issues were excluded from the study.

(3) The remaining CERs were next implanted with EEG electrodes in the 3rd month after grafting.

(4) Two weeks later, rats were connected to the tethered video-EEG system and baseline EEG recordings were taken for a week. Following this, CERs received injections of CNO (3 mg/Kg, once every 8 hours for 2-3 days) to activate DREADDS. CNO control group also received CNO to test the effect of CNO

alone on seizures. Baseline recordings were taken again for 4-7 days after the washout of CNO. EEG tracings from all of these groups will be quantified in the next year.

Table 3: Numerical details of CERs used for DREADDs study

Sequence of events and exclusions in the duration of experiments	CRISPR/Cas9 DREADDs group	mCherry-DREADDs group	CNO control group
Number of CERs initially assigned	10	10	9
Mortality of CERs in the waiting period	1	2	2
Exclusion of CERs due to health issues	3	2	0
Exclusion of CERs due to loss of head caps (EEG recording unit)	0	0	1
Numbers of CERs from which EEG data were collected (with or without CNO administration)	6	6	6

3.2.3. Progress Details:

3.2.3.1. Results from behavioral SRS studies:

We analyzed the frequency of all SRS/h, frequency of stage V-SRS/h, duration of individual seizures (seconds) and total time spent in seizure activity (% of recorded time) for 148 CERs used in this project so far. These data, based on 48 hours of observations, were collected to confirm the presence of chronic epilepsy (i.e. SRS) in the 3rd month after SE, prior to the assignment of rats to different groups.

Behavioral SRS Scores

(i) Frequency of all SRS 0.24 ± 0.01/hour (~6.0 seizures/day)
(ii) Frequency of Stage V-SRS 0.2 ± 0.01/hour (~5.0 seizures/day)
(iii) Duration of individual SRS 36.75 ± 0.56 seconds
(iv) Time spent in seizure activity 0.24 ± 0.01(%)

3.2.3.2. Extent of seizure activity prior to grafting or treatment in CERs assigned to different groups in SA1 studies:

CERs assigned to different groups for EEG recordings demonstrated similar frequency of SRS prior to grafting or treatment (i.e. in the 3rd month after SE). Comparison of the frequency of SRS in CERS belonging to the four groups prior to grafting or treatment with one-way ANOVA showed no differences between CER groups ($p>0.7$)

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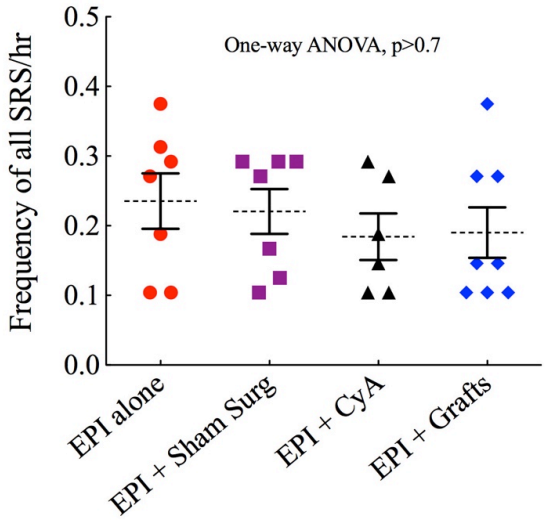


Fig. 1: comparison of the frequency of SRS in chronically epileptic rats belonging to the four groups prior to grafting or treatment. EPI alone, Epilepsy alone group; EPI + Sham Surg, epileptic rats receiving sham grafting surgery; EPI + CyA, epileptic rats receiving cyclosporine alone; EPI + Grafts, epileptic rats receiving hMGE-like precursor cell grafts

3.2.3.3. Effects of hMGE precursor cell grafting on SRS:

We have performed detailed quantification of video-EEG tracings collected for three continuous weeks from CERs belonging to the 4 groups: (1) CERs receiving no treatment (EPI alone group, n=7, 439 hrs/rat, total = 3,073 hrs of recordings). (2) CERs receiving sham-grafting surgery (EPI + Sham Surg group, n=7, 485 hrs/rat, total = 3,395 hrs of recordings). (3) CERs receiving cyclosporine alone (EPI + CyA group, n=6, 453 hrs/rat, total = 2,718 hrs of recordings). (4) CERs receiving hMGE-like precursor cell grafts (EPI + grafts group, n=8, 498 hrs/rat, total = 3,984 hrs of recordings. **Thus, the overall seizure analysis in this project is robust, as it involved quantification of seizures in video-EEG tracings recorded for 13,170 total hours.** Figure 2 illustrates examples of EEG traces from four different groups.

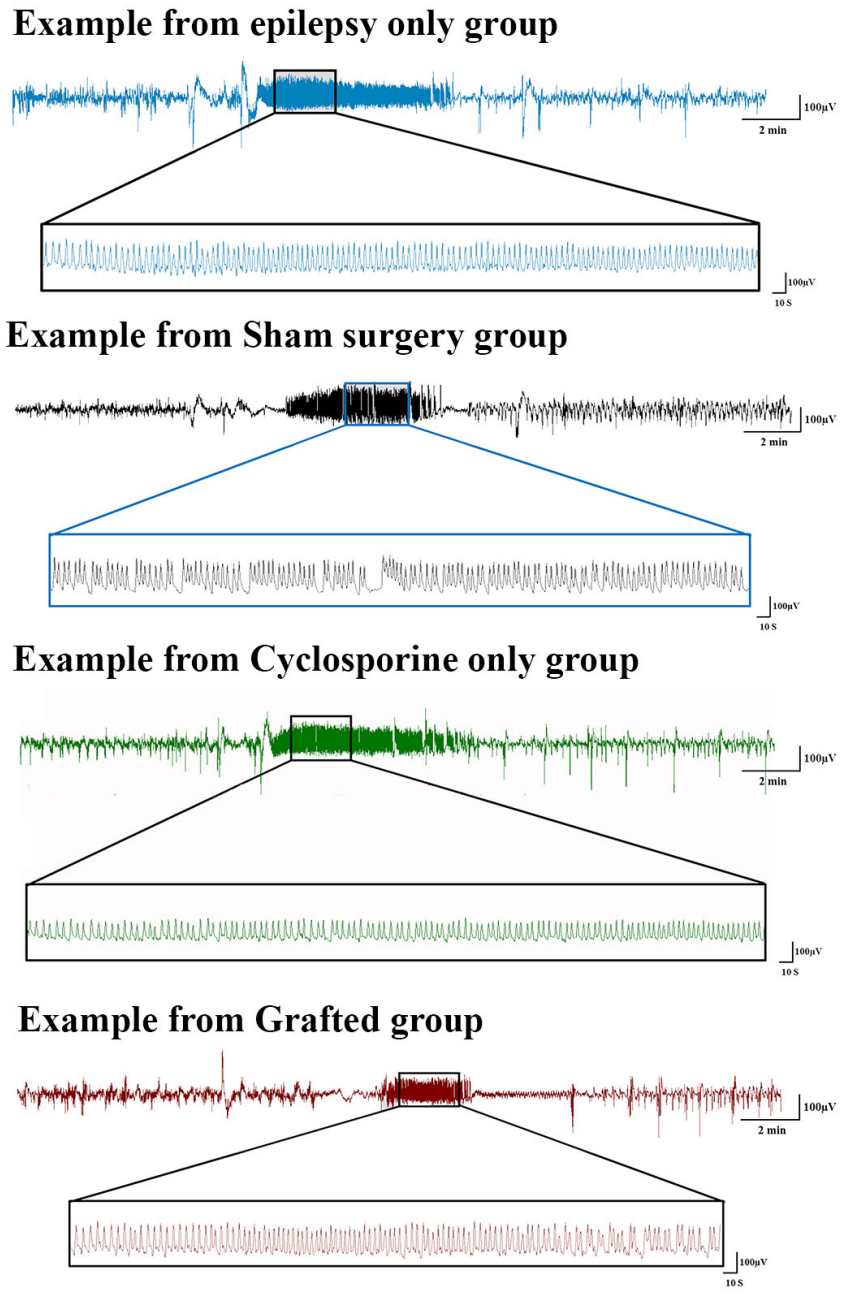


Fig. 2: Examples EEG traces during spontaneous recurrent seizures (SRS) from epilepsy alone (top), sham surgery (second from above), cyclosporine alone (third from above) and grafted (bottom) groups. Magnified views illustrate a large number of polyspikes in traces associated with seizures.

(a) Extent of all SRS/hr: We compared the extent of all SRS across the four groups using one-way ANOVA with Newman-Keuls multiple comparison post-hoc tests. This revealed highly significant ANOVA p-value ($p<0.0001$). Post-hoc tests revealed a significant difference between the grafted group and each of the other three control groups (epilepsy alone, sham grafted and cyclosporine alone groups, $p<0.001$). The overall reductions in the grafted group, in comparison to the three control groups, varied from 75-78% (Fig.3 [upper left panel]).

(b) Extent of Stage-V SRS/hr: We compared the extent of Stage-V SRS (the most intense type of SRS typified by bilateral forelimb clonus with rearing and falling) across the four groups using one-way ANOVA with Newman-Keuls multiple comparison post-hoc tests. This revealed highly significant ANOVA p-value ($p<0.0001$). Post-hoc tests revealed a significant difference between the grafted group and each of the other three control groups (epilepsy alone, sham grafted and cyclosporine alone groups, $p<0.001$). The overall reductions in the grafted group, in comparison to the three control groups, varied from 77-80% (Fig.3 [upper right panel]).

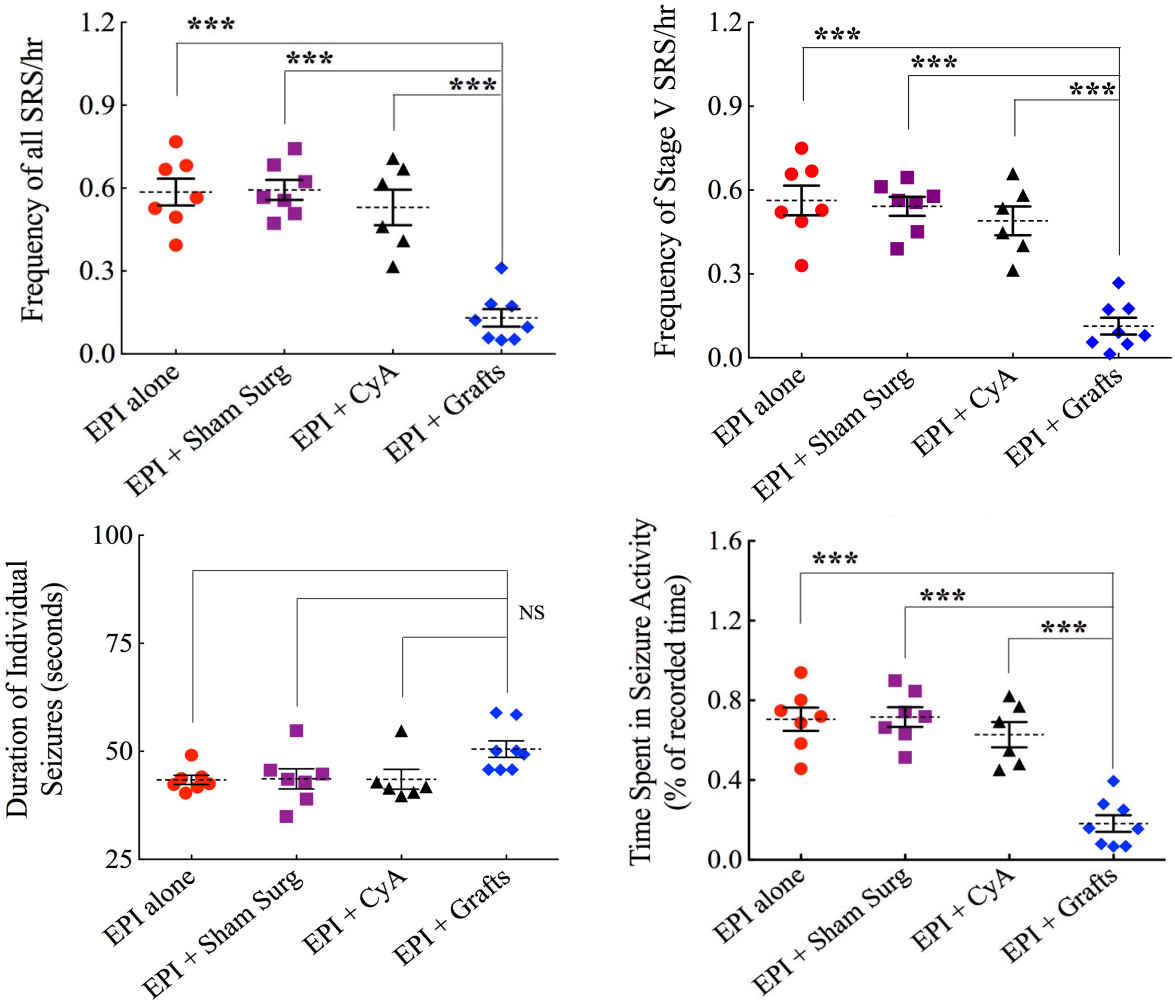


Fig. 3: Comparison of various parameters of spontaneous recurrent seizures (SRS) across CERs belonging to epilepsy alone (EPI alone), sham surgery (EPI + Sham Surg), cyclosporine alone (EPI + CyA), and grafted (EPI + Grafts) groups. Note that CERs receiving hMGE precursor cell grafts display greatly reduced frequencies of all SRS (upper left panel) as well as stage-V SRS (upper right panel). The overall time spent in seizure activity is also greatly reduced in the grafted group (lower right panel).

(c) Duration of Individual SRS: We compared the duration of individual SRS across the four groups using one-way ANOVA with Newman-Keuls multiple comparison post-hoc tests. This revealed no significant differences between different groups ($p>0.05$; Fig. 3 [lower left panel]). This finding suggests that, when seizures occur in the grafted group, they last for similar duration as seizures in other control groups, although the frequency of all seizures and stage-V seizures was dramatically reduced.

(d) Time Spent in SRS activity: We compared the time spent in SRS activity (% of the recorded time) across the four groups using one-way ANOVA with Newman-Keuls multiple comparison post-hoc tests. This revealed highly significant ANOVA p-value ($p<0.0001$). Post-hoc tests revealed a significant difference between the grafted group and each of the other three control groups (epilepsy alone, sham grafted and cyclosporine alone groups, $p<0.001$). The overall reductions in the grafted group, in comparison to the three control groups, varied from 71-75% (Fig. 3 [lower right panel]).

Thus, grafting of hMGE-like precursor cells from hiPSCs into the hippocampus greatly reduces the frequency of all SRS, frequency of Stage-V SRS and the overall time spent in SRS activity, in comparison to all three control groups. There were no differences between the three control groups, implying that neither sham grafting nor cyclosporine injections have any effect on the occurrences of SRS. This also supports the conclusion that grafts rather than surgery or cyclosporine injections mediate greatly reduced SRS activity in the grafted group.

3.2.3.4. Survival and migration of graft-derived cells in the hippocampus:

The total number of cells grafted into each hippocampus was 300,000 cells (100,000 cells into each of the 3 sites). To visualize cells derived from hMGE-like precursor cell grafts in the hippocampus, we performed human nuclear antigen (HNA) immunostaining (Fig. 4), using serial sections through the entire hippocampus. We next quantified the yield of graft-derived cells using stereology (optical fractionator method). This revealed a recovery of 555,664 ± 64,779 HNA+ cells per hippocampus, which is equivalent to ~185% of injected cells. Increased number of graft-derived cells than originally implanted suggests that some grafted cells have undergone 1-2 divisions after grafting. Graft-derived cells also migrated pervasively into different regions and cell layers of the hippocampus, which include the subgranular zone and the dentate hilus in the dentate gyrus, and strata oriens and radiatum of CA1 and CA3 subfields (Fig. 4).

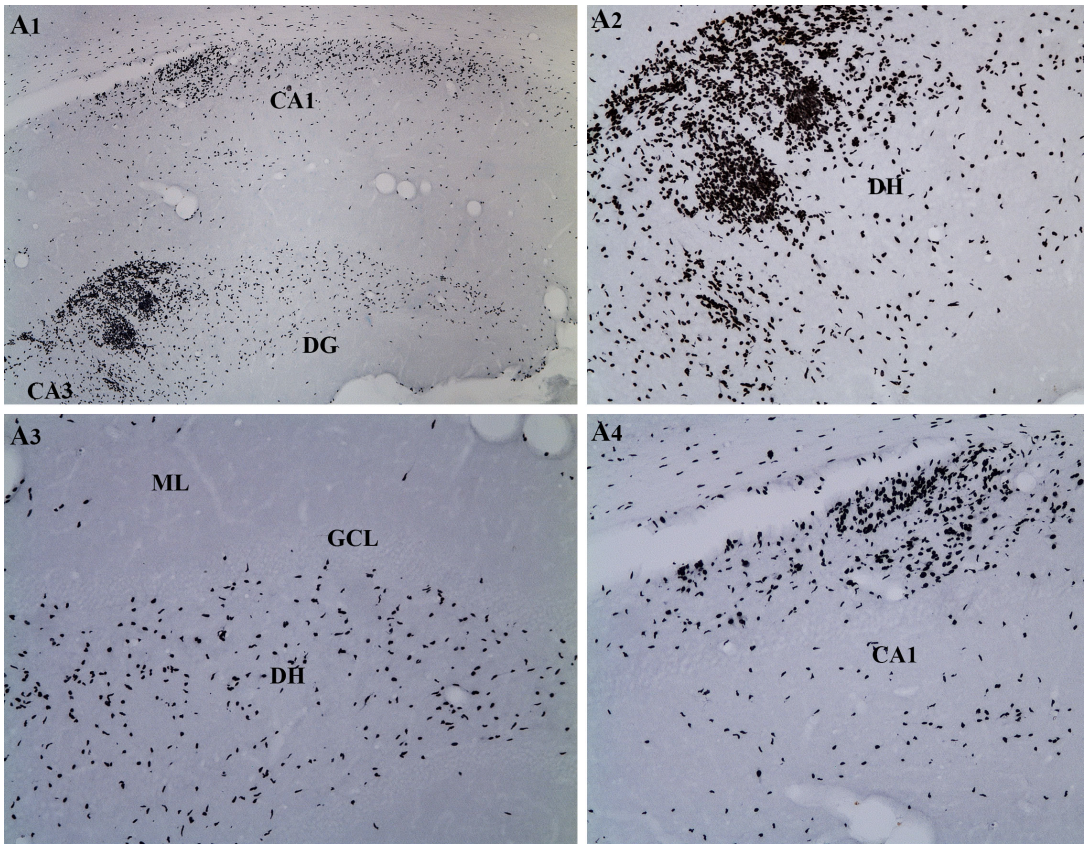


Fig. 4: Distribution of cells from an hMGE precursor cell graft placed in the hippocampus, visualized with human nuclear antigen (HNA) immunostaining. The graft core is located at the lateral end of the upper blade of the dentate gyrus (a larger mass of cells A1). An enlarged view of this mass is illustrated in A2. Figures A3 and A4 show cells that have migrated extensively in the dentate hilus (A3) and into different layers of the CA1 subfield. DG, dentate gyrus; DH, dentate hilus; GCL, granule cell layer; ML, molecular layer.

3.2.3.5. Neuronal differentiation of graft-derived cells in the hippocampus:

Dual immunostaining for HNA and neuron-specific nuclear antigen (NeuN, a marker of mature neurons) and confocal microscopic analysis showed that 88.2 ± 1.1 % of the graft-derived cells differentiated into mature neurons (**Fig. 5**). A vast majority of graft-derived cells (~70%) appear to differentiate into GABA-positive neurons. Several other dual immunofluorescence and confocal microscopic analyses are currently in progress to quantify the percentages of different types of interneurons. These include HNA-GABA, HNA-glia fibrillary acidic protein (GFAP, a marker of astrocytes), HNA-S100β (a marker of mature astrocytes), HNA-neuron-glia proteoglycan 2 (NG2, marker of oligodendrocytes progenitors), and HNA-Ki67 (a marker of cell proliferation). In addition, we plan to carefully analyze graft-derived cells with pluripotent stem cell markers and tumor-specific markers, to rule out the presence of pluripotent stem cells and tumor-generating cells within grafts. The results from these analyses will be reported in the coming year.

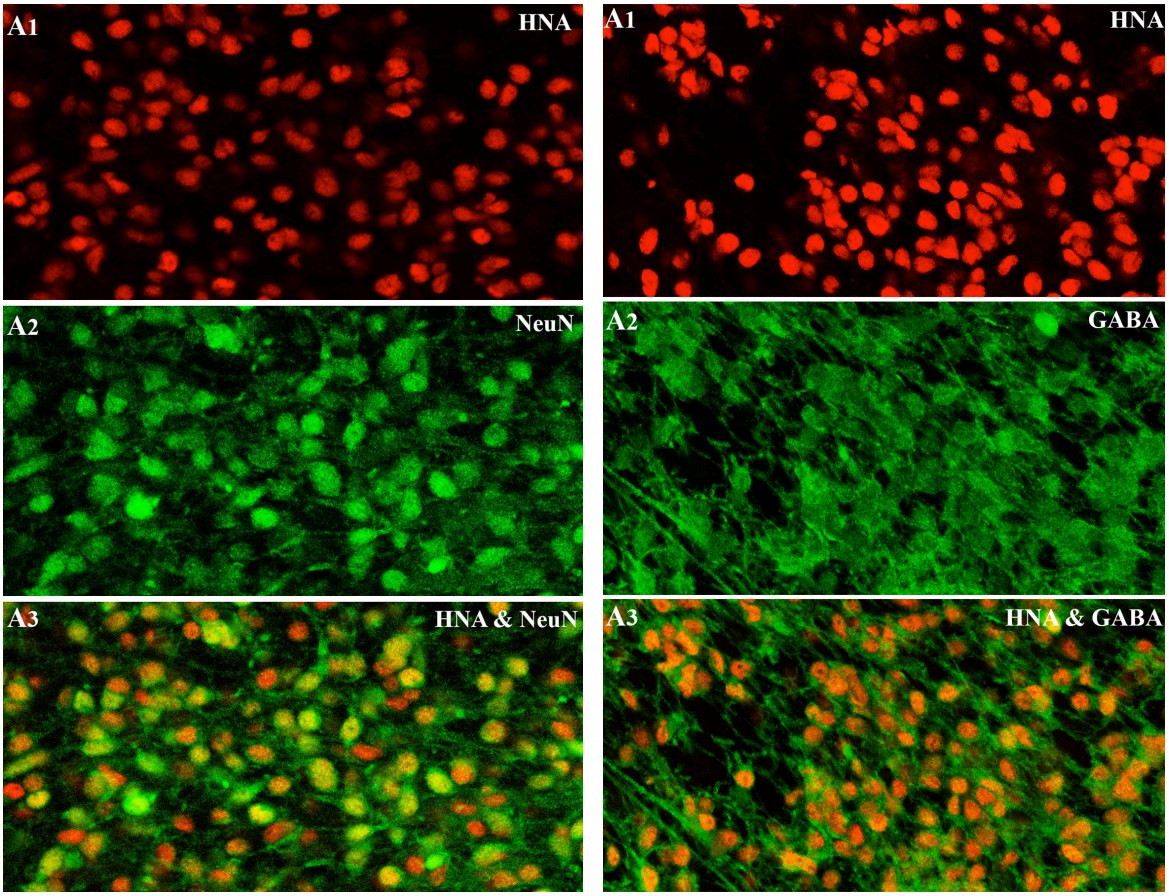


Fig. 5: Left panels show that a vast majority of graft-derived cells differentiate into NeuN+ mature neurons. Right panels illustrate that a majority of graft-derived cells differentiate into GABA+ interneurons. Upper panels: human nuclear antigen (HNA) positive cells; middle panels: GABA+ neurons; lower panels: cells positive for both HNA and GABA.

3.3. Opportunities for Training and Professional Development:

The full time Senior Research Associate (Dr. Dinesh Upadhya) working on this project has received considerable training for the various specialized experimental approaches and techniques from the PI (A. K. Shetty, Professor) and/or a senior researcher (Dr. B. Hattiangady, Assistant Professor) working for this project. These include induction of SE, measurement of behavioral SRS, Culturing and characterization of human iPSCs and MGE precursors derived from them, transplantation neurosurgery, implantation of EEG electrodes and chronic EEG recordings using a tethered EEG system, EEG data collection and analyses. Professional development activity for key research personnel in this project mainly comprised participation in departmental seminars and journal clubs, and discussion on epilepsy and stem cell therapy research advances with the PI on a regular basis. In addition, Dr. Upadhya attended American Society

for Neural Therapy and Repair (ASNTR) meeting at Clear Water beach, Florida in April (28-30) 2016. Furthermore, he will be attending the American Epilepsy Society (AES) meeting to be held in Houston in December 2016.

3.4. Dissemination of Results to Communities of Interest:

This will be done through an original article publication in the coming year. The PI (Dr. Ashok K. Shetty) presented some of the results of this project during his keynote address at the Arkansas Stem Cell Consortium Meeting on October11, 2016.

3.5. Plans for the Next Reporting Period:

In the third year, we will be working on pending experiments of Task 1 (SA1 studies) and Task 2 (SA2 studies). These include the following topics: (1) dual immunofluorescence and confocal microscopic analyses of grafts for measuring the percentages of different subclasses of interneurons and types of proliferating cells using a variety of markers. (2) Investigation of the possible presence of pluripotent stem cells and tumor-generating cells within grafts using specific antibodies and dual immunofluorescence and confocal microscopic methods. (3) Additional detailed analyses of EEG data from SA1 studies for EEG power (spectral analysis). (4) Statistical analyses of data from the various behavioral tests to discern the effects of hMGE precursor cell grafting on cognitive, memory and mood function, once data from all groups have been validated with inclusion and exclusion criteria. (5) Biochemical assays to measure the levels of glutamate, GABA and neurotrophic factors in freshly harvested hippocampal tissues from different groups of CERs. (6) Analyses of neurogenesis using BrdU immunostaining, BrdU-NeuN dual immunofluorescence, DCX immunostaining and stereological cell counting methods. (7) Collection and analyses of seizure data from EEG tracings in DREADDs study. (8) Analyses of graft cell survival and differentiation in DREADDs study. (9) Effects of grafting on the survival of host interneurons in the hippocampus.

4. IMPACT:

The findings obtained so far suggest that hMGE precursor cell grafting into CERs can greatly restrain SRS activity. This is a high impact finding, as it has implications for treating chronic TLE, particularly the type that is resistant to antiepileptic drugs.

5. CHANGES AND PROBLEMS:

(i) Changes in approach:

Since our Institution (Institute for Regenerative Medicine) was scheduled to move from the Temple campus of the TAMU College of Medicine to the College Station campus of the TAMU College of Medicine in August 2016, a greater emphasis was placed on getting the animal component of the work done during the past year. Now, our Institute has moved to the College Station campus and the labs are functional in the new location. Animal Use protocol has been submitted to the IACUC in the College Station campus for approval. Once approved by the IACUC, the protocol will be submitted to the ACURO for further review and approval. Because of a major focus on animal studies during the past year, the coming year will have a strong focus on studying tissues that have been already harvested. This will include multiple biochemical assays, immunofluorescent and immunohistochemical studies on brain tissue sections and quantification of various parameters.

(ii) Actual or Anticipated Problems or Delays and Plans to Resolve them:

As per the notice of grant award, this project commenced from 09/30/2014. Nonetheless, since the ACURO approval was received on 02/03/2015, the research work for this project actually commenced from 02/04/2015. Thus, the experiments for this project commenced from the 2nd month of 2nd quarter in the first year. Hence, ~20 months of work has been performed for this project during the past two years. It is anticipated some additional time (beyond the scheduled 3 years) will be required to complete all studies proposed in this project.

(iii) Changes that had a significant impact on expenditures:

Nothing to Report

(iv) Significant Changes in the use of vertebrate animals or biohazards:

The changes requested to the animal protocol during the past year have already been approved by the ACURO. Since our Institute has now moved to the College Station campus from the earlier Temple campus, Animal Use protocol has been submitted to the IACUC in the College Station campus for approval. Once approved by the College Station IACUC, the protocol will be submitted to the ACURO for further review and approval.

(v) Significant Changes in the Care of Vertebrate Animals:

Nothing to Report

6. PRODUCTS:

Publications:

1. **Shetty AK**, Upadhya D. GABA-ergic cell therapy for epilepsy: Advances, limitations and challenges. ***Neurosci Biobehav Rev.*** 2016; 62: 35-47.

2. Upadhya D, Hattiangady B, Shetty GA, Zanirati G, Kodali M, **Shetty AK**. Neural Stem Cell or Human Induced Pluripotent Stem Cell-Derived GABA-ergic Progenitor Cell Grafting in an Animal Model of Chronic Temporal Lobe Epilepsy. ***Curr Protoc Stem Cell Biol.*** 2016; 38:2D.7.1-2D.7.47

7. PARTICIPANTS AND OTHER COLLABORATIVE ORGANIZATIONS

The following research staff members from PI's laboratory were compensated from this grant (for the percentage of effort contributed to this project):

Personnel	Role	Percent Effort
Ashok K. Shetty	Principal Investigator	17%
Bharathi Hattiangady	Research Scientist	48%
Dinesh Upadhya	Senior Research Associate	90%
Xiaolan Rao	Research Assistant	50%

Other Collaborators:

Su-Chun Zhang Lab (University of Wisconsin, Madison)

Dr. Zhang's laboratory has provided the required donor cells (MGE-like cells derived from hiPSCs) for grafting studies in this project, as approved in the project. A sub award to the University of Wisconsin, Madison has been approved and implemented at the commencement of this project.

Changes in active other support of the PI or Key Personnel:

Dr. Bharathi Hattiangady (Research Scientist) has left our laboratory in September 2016. A new post-doc (Dr. Raghavendra Upadhya) will contribute 25% of his effort to this project effective October 1, 2016. The effort levels of PI (Dr. Ashok K. Shetty), Senior Research Associate (Dr. Dinesh Upadhya) and Ms. X. Rao (Research Assistant) will continue with no changes in the coming year.

Other Organizations Involved in this Project:

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHART is attached (Page 16 of this document)

9. APPENDICES

QUAD CHART is attached (Page 16 of this document)

Title: **Human iPSC-Derived GABA-ergic Precursor Cell Therapy for Chronic Epilepsy**
ERMS/Log Number and Task Title: CDMRP Log Number, **PR130086**
Insert Award Number: **W81XWH-14-1-0558**; Grants.gov ID Number: **GRANT11498566**

PI: Ashok K. Shetty, PhD **Org:** Texas A&M University System Health Science Center **Award Amount:** $908,423

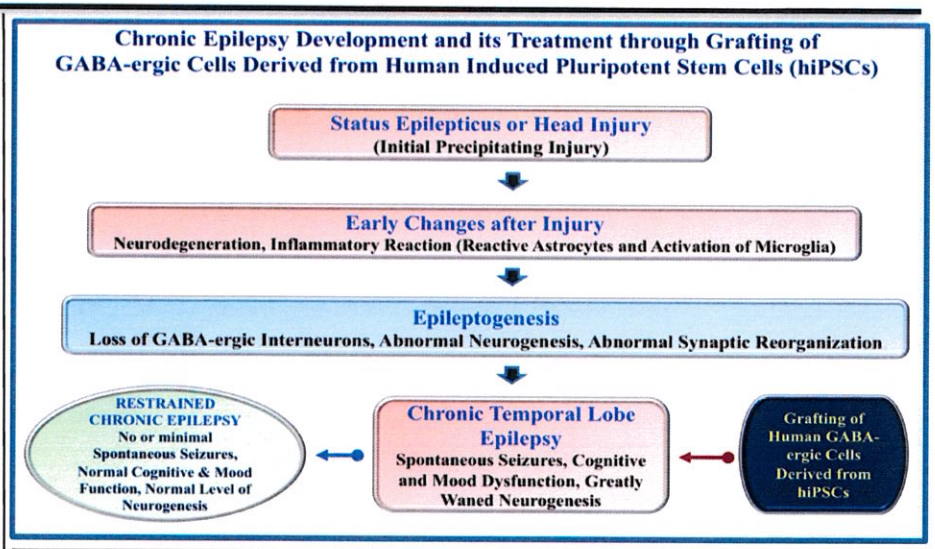
Study Aims

Specific Aim 1: Test the hypothesis, "Grafting of medial ganglionic eminence (MGE)-like gamma amino butyric acid (GABA)-ergic precursors from human induced pluripotent stem cells (hiPSCs) into the hippocampus of chronically epileptic rats (CERs) greatly diminishes the frequency and intensity of spontaneous recurrent seizures (SRS).

Specific Aim 2: Address the hypothesis, "Grafting of MGE-like GABA-ergic precursors from hiPSCs into the hippocampus of CERs greatly improves learning and memory function and reverses depressive-like behavior.

Approach

We will generate MGE precursors from hiPSCs in culture. We will then prepare a suspension of these cells and transplant into the hippocampus of rats exhibiting chronic epilepsy. In studies pertaining to Specific Aim 1, we will quantify the effects of grafts on the frequency, severity and duration of SRS via chronic video-electroencephalographic (video-EEG) recordings. In Specific Aim 2, we will quantify the effects of grafting on functions such as learning, memory and mood.



Accomplishment: ~70% or work related to Specific Aim 1 and 40% of work related to Specific Aim 2

Timeline and Cost

Activities / CY	14	15	16	17
Aim 1: Induction of seizures, Grafting of GABA-ergic cells, EEG recordings				
Aim 1: Grafting of GABA-ergic cells, EEG Recordings, Histology, Immunohistochemistry				
Aim 2: Induction of seizures, Grafting of GABA-ergic cells, Behavioral tests, Analyses of neurogenesis				
Estimated Budget, total cost ($K)	**$304K**	**$300K**	**$304K**	

Updated: (College Station, TX, October 25, 2016)

Goals/Milestones

CY14 Goals - Generate rats with chronic epilepsy
- ☑ Induction of acute seizures

CY15 Goals – Grafting cells into epileptic rats; testing its effect on seizures
- ☑ Induction of acute seizures; Monitoring of behavioral seizures
- ☑ Intracerebral grafting of human GABA-ergic cells
- ☑ EEG Recordings
- ☑ Histology; Immunohistochemistry (analyses of grafts)

CY16 Goals – Grafting cells into epileptic rats; testing its effect on seizures
- ☑ Intracerebral grafting of human GABA-ergic cells
- ☑ EEG Recordings
- ☑ Histology; Immunohistochemistry (analyses of grafts)
- ☑ Induction of acute seizures; Monitoring of behavioral seizures for Aim 2 studies

CY17 Goals – Grafting cells into epileptic rats; testing its effect on behavior
- ☐ Intracerebral grafting of human GABA-ergic cells
- ☐ Behavioral tests for Learning, Memory and Mood function
- ☐ Analyses of Hippocampal Neurogenesis

Budget Expenditure to Date: **$420,662 + 113,207 (sub-award), $533,869 total cost**