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RESEARCH ARTICLE

Transplantation of neurogenic-fusionogenic embryonic stem cells modified to overexpress GABA into a model of temporal lobe epilepsy: Promises and potential pitfalls.

Kerry Thompson[1,2,3*], Michelle Kanemori[4], Emily Kimes[5], Evthokia Hobbs[6], Jennifer Phan[7], Travis Wilson[8], Alex Werthheimer[9], Taylor Yoshida[1], Diana Flores-Barnett[1], Minji Kang[1], Cameron Auyang[1].

[1]Occidental College, 1600 Campus Rd, Los Angeles, CA 90041, USA.
[2]VA Greater Los Angeles Healthcare System
[3]University of California Los Angeles
[4]University of Hawai'i, John A. Burns School of Medicine
[5]University of Missouri School of Medicine
[6]Providence Cancer Center Oncology & Hematology Care Clinic
[7]Keck Medicine of University of Southern California
[8]Emory School of Medicine
[9]Kaiser Permanente Sports Medicine

[*]kthompson@oxy.edu

ABSTRACT

Cell-based therapy is likely to become a clinically useful tool to treat severe neurological disease. Disease states such as intractable temporal lobe epilepsy will be candidates for this type of therapeutic approach. Embryonic stem cells are pluripotent, and are considered a viable source for cell replacement strategies. Embryonic stem cells can be genetically manipulated to express reporter molecules, and they can be forced into neural differentiation by controlling the expression of key transcription factors. One strategy for the treatment of temporal lobe epilepsy is to transplant enriched populations of transplantable GABAergic neurons into the damaged, epileptogenic, hippocampus. The goal would be to suppress seizures, and to replace and repair damaged circuits. We genetically engineered an embryonic stem cell -derived neurogenic, fusionogenic cell line with GAD65 and transplanted them into an animal model of temporal lobe epilepsy. We found some evidence of seizure suppression, and also evidence of widespread dispersion from the transplantation site. The pattern of staining of embryonic stem cell reporter molecule that was detected, suggested a pathotropism, and selective involvement of reportedly fusionogenic cell populations within the host. We feel that the evidence suggests that embryonic stem cell-derived neural cells might have a significant capacity to fuse with host neurons, which can cloud the interpretation of cellular replacement and circuit repair. This study, and other investigations that have reported stem cell fusion events in the transplanted host, reveal embryonic stem cell capacities that may not be fully appreciated. More research is needed to fully reveal the potential of cellular therapies using stem cells.

Introduction

The central nervous system (CNS) has a relatively limited capacity for neurogenesis and self-repair. As a result, trauma and diseases that produce neural injury and losses are particularly devastating. The greater the amount of neural tissue damaged or lost, the greater the impact on the patient. Cell loss or damage to limbic areas of the brain can lead to cognitive deficits and pharmacoresistant seizures[1]. Seizures are a common indication of neuronal dysfunction and can present after multiple types of insults including traumatic brain injury[2], metabolic disruptions like stroke and ischemia[3], and severe seizures, that lead to cell losses in epileptogenic areas of the brain[4-9]. The aging brain is particularly vulnerable to neuronal damage, because the added impact of age-related senescence and dysfunction amplifies the effects of CNS injury and disease[10].

Conventional therapies for epilepsy are typically designed to enhance CNS inhibition or to lessen excitatory drive. The pharmacological approach to seizure management is palliative and not disease modifying or curative, and, in a significant percent of cases of temporal lobe epilepsy, seizures are refractory to treatment[1,11]. In these refractory cases, surgical resection of the epileptogenic tissue may be an alternative, provided that the seizures do not originate in an eloquent area and there is not bilateral onset. Temporal lobe resection has a high success rate leading to manageable seizures, but there can be significant complications, and there is a cost with the loss of adjacent healthy brain tissue[12-14]. Additionally, in patients that are not candidates for resection, there is a critical need for alternative therapeutic approaches.

The development of cell-based therapies to enhance inhibition in epileptogenic regions of the brain is an active area of research[14-16]. The development of this type of therapy may potentially result in a clinically viable means to replace lost, or damaged, brain tissue with functional neurons and/or glia[17] as an alternative to resection. We have reported some success in suppressing seizures in animal models using genetically modified neuronal[16,18-20]and glial[21]cell lines with tetracycline-regulatable GABA production, but those cells do not consistently survive for extended periods of time[19,20], and there is little evidence of functional integration into the epileptogenic circuit. Since cell replacement, with the reformation of lost, or damaged, neural circuits, is a stated goal of cell-based therapies using stem cell therapy[22,23], using stem cells as a cell source may be preferable for brain tissue repair.

A number of preclinical studies have shown that embryonic stem cells (ESCs) can be transcriptionally reprogrammed using transcription factors that force neural and GABAergic phenotypic enrichment[23-25]. Neuralized ESCs, and stem cells from other sources, have been shown to survive transplantation, cross the blood brain barrier, adopt appropriate electrophysiological responses within the hippocampus, and modify disease progression[26,27,28]. These reports raise hope that selective replacement of interneurons, derived from stem cells, will develop into clinical therapies for epilepsy[15]. However, many challenges remain in determining the best cell source, the appropriate cocktail of transcription

factors/transgenes, and the best strategies of differentiation and cell grafting[23] for treating CNS diseases. Additionally, a fuller understanding and consideration of the biological properties of engrafted ESC derivatives within the host may reframe interpretations of transplantation outcomes. For example, a number of studies, including studies using ESC-derived neural cells, have revealed the capacity of stem cells to fuse with host cells following transplantation[29-34].

We, and others, have shown that ESC-derived neurogenic, or neuralized, cells can undergo fusion events that can confound the interpretation of cell replacement, and circuit reformation[35-37]. Fusionogenic capacity, both partial and complete, has been reported using multiple stem cell sources, and across multiple brain regions[29-32,34]. Importantly, for the purposes of this discussion, cell injury has been reported to enhance stem cell fusion events within the host brain[31,38,39]. An important question for the continued development of transplantation therapies for epilepsy is how stem cell dispersion, and, fusion capacity, might affect the outcomes following transplantation of ESC-derived GABAergic cells. We pursue this issue by transplanting GABAergic- neurogenic-fusionogenic ESCs using an established model of temporal lobe epilepsy. This model induces reproducible patterns of cell death, circuit reorganization, and spontaneous seizures. We found that the dispersion of cells transplanted into the hippocampus extends well beyond the injection site within the temporal lobes, and that the cells enter the general circulation. The distribution of transgene-expressing cells shows signs of pathotropism with selective involvement of vulnerable cell populations. We looked for staining patterns that might suggest stem cell fusion and found a number of positive indicators. We consider these results as cautionary evidence that stem cell transplantation studies should consider, and intentionally exclude, stem cell fusion as a possible contributor to transplantation outcomes that would otherwise suggest neural replacement and circuit integration.

Methods

CELL LINE GENERATION AND CULTURE

The ZHTc6 line was previously created using a bicistronic transgene that contains the reporter molecule β-galactosidase fused to the neomycin resistance gene as well as an additional transgene that replaced one of the Oct 3/4 alleles with an introduced version of Oct 3/4[40,41]. Both of the transgenes are under the control of a tTA-sensitive promoter in the ZHTc6 line that can be suppressed with doxycycline. We engineered the coding region for GAD65 using the LinGAD65 genetic construct developed previously[42] into this cell line using Lipofectamine 2000 according to the manufacturer's recommendations. Using similar methods to those that we have published previously[21,42,43], we generated clonal cells lines by selection in high concentrations of hygromycin (120 µg/ml). Clonal cell lines were isolated with glass cloning cylinders, and the resulting cell lines were propagated and evaluated for GABA production.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Cultured cells were trypsinized and plated 500K cells/well in six-well culture plates 24 h

prior to extraction. GABA released over 60 min was examined using basal medium (144 mM NaCl, 1 mM MgCl2, 4 mM KCl, 1.8 Mm CaCl2, 5 mM glucose, and 10 mM Hepes, pH 7.1) and high K+ medium (same as basal except 94 mM NaCl and 53 mM KCl). Total GABA content was measured by lysing the cells with 0.4 M perchloric acid. GABA content of filtered samples was analyzed using reverse-phase HPLC and fluorescence detection of o-phthalaldehyde-derivatized adducts. All experiments were performed in triplicate.

PILOCARPINE STATUS EPILEPTICUS AND SEIZURE MONITORING

Studies were approved by the Occidental College Institutional Animal Care and Use Committee. Status epilepticus (SE) was induced by lithium pretreatment followed 24hrs later with pilocarpine in 2-3-month-old rats. The animals experienced two hours of continuous seizure before they were treated with 10 mg/kg diazepam to stop the convulsive activity. The animals were injected with dextrose (5% body weight) twice a day for the next three days, they were given fresh orange halves daily, and they were continuously monitored for health and hand fed Nutri-cal (CSI Chemical Corporation, Bondurant, IA) if they showed signs of dehydration or severe weight loss.

UNILATERAL 6-HYDROXYDOPAMINE LESION

Dopaminergic neurons were unilaterally targeted by focal administration of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle in two animals as previously described[35]. Rats were anesthetized with a mixture of xylazine:ketamine (10mg/kg:85mg/kg) or with isoflurane vapor using a vaporizer. Rats were injected with 25 mg/kg (i.p.) desipramine, 30 minutes before 6-OHDA injection, in order to prevent uptake of 6-OHDA by noradrenergic neurons. Stereotaxic surgery was performed to deliver 6-OHDA unilaterally at two locations within the MFB: 2.5µl (0.5µl/2min) AP –4.4, ML –1.2, DV –7.8, and 2.0 µl (0.5 µl/2min) AP –4.0, ML -0.8, DV –8.0, relative to bregma[44-46]. The 6-OHDA was administered as 3 µg/µl in a 1% ascorbic acid solution. To minimize oxidation, 6-OHDA solutions were freshly made, kept on ice, and light-protected. Animals were kept on a 37.0°C warming pad during surgery and in a 37.0°C recovery chamber after the surgery. A solution of 5% dextrose was injected subcutaneously after surgery at 10% body weight.

BEHAVIORAL TESTING: ROTOMETRY

To confirm the lesion status of the animals they were behaviorally screened five to six days after surgery using the amphetamine rotation test. Rats were placed in a Rotomax rotometer (AccuScan Instruments, Inc., Columbus OH) which consists of a 12" diameter Plexiglas cylinder on a flat surface. A harness extends down and permits the animals to touch the wall of the cylinder. Animals were injected with 5 mg/kg amphetamine sulfate and the number turns/5 minutes were calculated from net ipsilateral turns over 30 minutes following a five-minute habituation period.

SURGERY AND TRANSPLANTATION

Both the parental ZHTc6 line and the GAD65-engineered Z6GAD65-4 line express the tTA, and β-galactosidase, and overexpress Oct 3/4 in the absence of doxycycline. These cells were transplanted into the hippocampi of animals that had experienced SE.

The animals were maintained in a vivarium on a 12 hr light/dark cycle and were given food and water ad libitum. Approximately 18 months after the initial seizure, eight animals with chronic seizures were randomly placed in two groups. One group was transplanted with ZHTc6 cells and the other group was transplanted with Z6GAD65 GABA-overexpressing stem cells. Cells were targeted to the hippocampus using the strategy depicted in Figure 1.

The cells were grown in the absence of doxycycline for at least three days, they were then trypsinized, washed and resuspended at 150K cells/µl in sterile PBS. Cells were drawn into a Hamilton microliter syringe and injected into two hippocampal sites dorsally, followed by injections into the ventral hippocampus at a 40° angle (dorsal site at AP: -3.8, ML 3.5 and DV: 3.0 and the ventral site at AP: -5.3, ML: .75 (40° from vertical in the coronal plane) and DV: 7.9, 5.4, and 2.5). The animals were then implanted with an electrode connected to skull screws with one serving as a ground. After surgery, animals were injected with dextrose and placed into a 37° recovery chamber until they were ambulatory. They were then transferred to their home cages. One animal was found dead the morning after surgery.

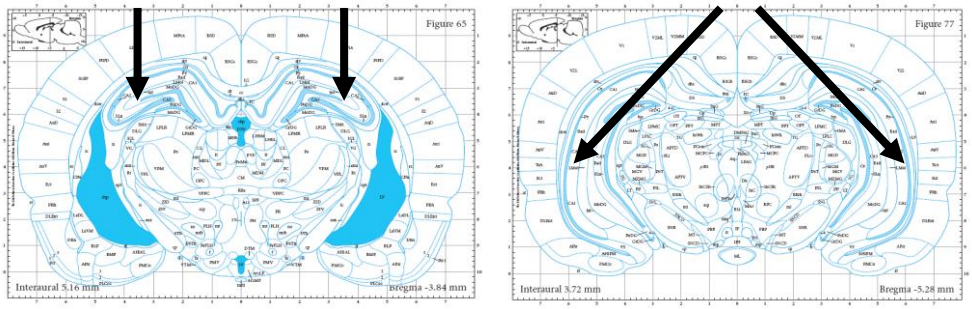


Figure 1. Transplantation scheme for hippocampal transplants of stem cells. The cells were injected at four sites within the hippocampus, bilaterally. Each site received 150K cells over two minutes with a 2 min pause before needle extraction from the injection site.

The hippocampal grafting was modified slightly for the 6-OHDA-lesioned animals to limit dorsal hippocampal damage created by the injection needle. In these two animals, three injections into the ventral hippocampus were made unilaterally on the unlesioned side using the coordinate system described above. This represents many fewer cells than the SE-injected animals, but it is the same number of cells transplanted in our previous study which revealed widespread dispersion[35]. These animals were evaluated 10 days after transplantation using β-galactosidase histochemistry and immunohistochemistry in combination with standard light microscopy. This is a timepoint, that we previously established, when injected cells are still present at the injection site, but evidence of dispersion and fusion can be demonstrated[35].

TELEMETRY

One week to ten days after transplantation surgery, the pilocarpine treated animals were housed in dedicated environmental rooms and placed in observation chambers. Cortical electrodes were connected to a commutator that fed an amplified signal to a seizure monitoring system with EEG and video detection capabilities (Stellate Harmonie, Quebec, Canada). Using methods that we have described previously[20], the animals were monitored for three-day periods for spontaneous seizures. Three of the four animals that were transplanted with GAD-engineered cells were then given doxycycline in their drinking water and monitoring was continued at the end of another ten-day period. Finally, the animals were taken off of doxycycline, and then another three-day monitoring session was conducted seven to ten days later.

HISTOLOGY

At the end of our behavioral studies the animals were transcardially perfused and the brains were cryoprotected for subsequent sectioning using a cryostat. Histological analyses were carried out using several methods to identify the fate of transplanted stem cells and the grafted brain tissue. We used conventional nissl stains to evaluate cell morphology, cell loss, ongoing cell death, and we used histochemistry, and immunohistochemistry, to study stem cell reporter transgene expression/presence, and ectopic gene expression in dentate granule cells which can also indicate synaptic reorganization in the chronic seizure model employed in this study.

Histological methods have been reported earlier[35]. Briefly, animals were transcardially perfused with cold 4% paraformaldehyde and the brains (and liver samples) were removed and cryoprotected in 30% sucrose. Coronal sections of the brain, encompassing the entire hippocampus, were serially sectioned using a cryostat at 20 and 40 µ. Adjacent sections were used for hematoxylin and eosin (H&E) and X-gal histochemistry (each 20 µ) and 40 µ sections taken for immunohistochemistry. For H&E staining and X-gal histochemistry, sections were cut onto glass slides. For immunohistochemistry sections were floated into PBS using 24 well dishes.

X-GAL HISTOCHEMISTRY

β-gal positivity was used to analyze and locate the β-gal-positive cells in the brain and liver tissue sections. Glass-mounted sections of both brain and liver, and 60µ free-floating sections of the brain, were pretreated by permeabilization with 0.01% deoxycholic acid and 0.02% Igepal in PBS at RT. The slides were then thoroughly washed and the mounted sections were encircled using a PAP pen or grease, so that each of the sections could be incubated in a filtered X-gal solution containing 5 mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM MgCl2, and 250 µg/ml X-gal in PBS (pH 7.4 or 8.5 to discriminate bacterial β-gal from endogenous enzyme sources) within a dark box placed within a humidified chamber and 37°C. The sections were left to incubate overnight. Sections were then rinsed, mounted onto glass slides if necessary, and coverslipped the next morning. Tissue sections were left to dry and then evaluated with conventional light microscopy. Negative controls were incubated for equivalent periods.

IMMUNOHISTOCHEMISTRY

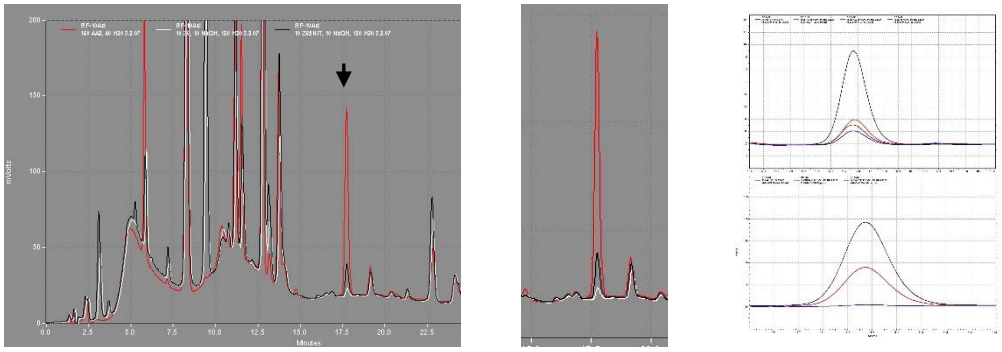
Indirect immunofluorescence and immunoperoxidase histochemical strategies were performed on free-floating sections. A polyclonal anti-β-galactosidase antibody (Chemicon International) was used at [1:1000], an anti-GFAP antibody (Millipore) was used at [1:1200] an anti-NeuN (Millipore) antibody was used at [1:500] and an anti-neuropeptide-Y ((NPY), Invitrogen) antibody was used at [1:1000]. For immunoperoxidase staining, an ABC kit (Vector Labs) was used in accordance with the manufacturer's instructions. In all cases, tissue sections were incubated in the primary antibodies overnight at 4°C. Immunohistochemical controls included the omission of the primary antibodies, the secondary antibodies, and, in some cases, the inclusion of an endogenous biotin-blocking procedure (Vector Labs).

Results

Our methods allowed for the development of transplantable ESC-derived GABA-expressing neurogenic cell lines. The resultant cell lines produced GABA, suppressed seizures, and survived for at least one month, post transplantation. Transplanted cells demonstrated extensive migration, and pathotropic fusion behavior, in our pilocarpine status epilepticus model of temporal lobe epilepsy.

CELLS

The ZHTc6 line has been characterized molecularly in previous reports[40,41,47]. The ZHTc6 line has been genetically modified to overproduce Oct3/4 (relative to the normal biallelic levels) in the absence of doxycycline, which makes them neurogenic in the absence of LIF and growth factors. We have reported neuronal phenotypes and nestin expression, along with consistent transgene expression of the β-galactosidase reporter gene under differentiating cell culture conditions in an earlier study[35]. Lipofection of the ZHTc6 cell line with a doxycycline-regulatable, GAD65-containing, construct produced several clones that were evaluated for GABA production (Fig. 2). When we analyzed the GABA content and release of both the ZHTc6 cells and the GAD-transfected cells, we found that both cell lines produced GABA (Fig 2 A&B). The basal GABA produced by the parental ZHTc6 cells accounted for as much as 50% of the GABA produced by the GABA-overexpressing cells, and it was released spontaneously into the media. The level of GABA production in the GAD-engineered stem cell lines is at the low end of the amount of GABA produced by our GAD-engineered neural cell lines (Fig. 2 C&D) which is in the range of 0.6-10.5μM[19]. This may be related to the capacity of neuralized stem cells to endogenously produce GABA, and also, efficiently metabolize GABA[48] . In our work with neural cell lines, control cells (cells not engineered with GAD) produced no GABA (Fig. 2 C&D). It is for this reason that we refer to the GAD-engineered stem cells as GABA-*overexpressing* stem cells. The GAD-engineered cells could be provoked to release more GABA when exposed to depolarizing conditions (50mM K+) (data not shown) which is an attractive feature in transplantation scenarios.



A B C (top) D (bottom)

Figure 2. ZHTc6 and ZGAD65-4 cells produce GABA. Lysate from GAD-engineered and ZHTc6 cell lines was analyzed by HPLC. (A) shows amino acid standards (red trace) overlaid with GAD-engineered cells (black trace) and ZHTc6 cells (white trace). Arrowhead shows the GABA peak. (B) Isolated overlaid GABA peaks. (C) The ZHTc6 cell line (blue trace) makes a significant amount of GABA. (D) Comparison with the non-GABAergic neural cell line CN1.4[42]. Black trace in (C) and (D) is the GABA standard.

SEIZURES

One GAD-engineered cell line, Z6GAD65-4, was chosen for transplantation studies based on relatively higher GABA production, and the parental ZHTc6 line was used as control. When spontaneously seizing animals were transplanted with these neurogenic-fusionogenic- GABAergic stem cells, there were indications of seizure suppression in all cases (Table 1). The parental (control) ZHTc6 cells suppressed seizures and epileptiform EEG activity completely in two out of three animals, but this is not surprising considering that they produce, and release, GABA in culture. The evidence that regulatable GABA production could show additional influence on seizure incidence was demonstrated when three animals that received GABA-overexpressing cells were then given doxycycline in their drinking water. Two of the three animals demonstrated increased seizure frequency during doxycycline administration. One animal showed a dramatic onset of seizures after being seizure free before doxycycline administration. The other showed a doubling of seizures while on doxycycline. One animal however did not show a change in seizure expression while on doxycycline and remained seizure-free during those 10 days. Importantly, the two animals that did show an increase in seizures while on doxycycline both became seizure-free one week after doxycycline was removed from the drinking water. These data demonstrate that transgene regulation with genetically modified stem cells can translate to regulation of a therapeutic effect (in this case, seizure suppression).

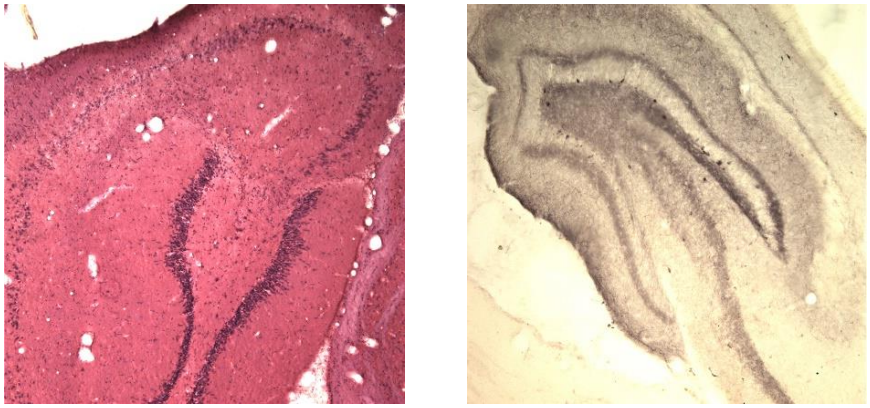
Table 1. GABA producing stem cells suppress spontaneous seizures. Animals were monitored for the occurrence of spontaneous seizures (sz) in three sessions. SE 1,2, and 5 (top 3 rows) received the GABA-producing line ZHTc6. SE 3,6, 7, and 8 (bottom 4 rows) received the GABA-overexpressing line Z6GAD65-4. Doxycycline was delivered in their drinking water.

Animal	7-10 days post ESC transplant	3-10 days on dox	7-10 days off dox
SE 1**	2 sz/day	NA	NA
SE 2*	Seizure free	NA	NA
SE 5	Seizure free***	NA	NA
SE 3*	Seizure free	6.6 sz/day	Seizure free
SE 6*	.3 sz/day	NA	NA
SE 7**	Seizure free	Seizure free	Seizure free
SE 8*	.6 sz/day	1.3 sz/day	Seizure free

*Animals were demonstrating stage 3 seizures in the 48 hrs preceding transplantation. **Animals were expressing multiple stage 5 seizures in the 48 hrs preceding transplantation. ***SE 5 showed epileptiform bursting but it was not detected as seizure activity and no video was captured in the 48 hrs preceding transplantation.

HISTOLOGY

Animals that experience two hours of pilocarpine-initiated SE, which then develop spontaneous recurrent seizures, typically demonstrate severe neuronal losses within limbic and extra-limbic cortices. In the chronic seizure period, selective cell losses within the hilar region of the dentate gyrus and the pyramidal cell layers can be shown with classic nissl stains (Fig. 3A), and circuit reorganization (mossy fiber sprouting) can be shown with Timm stains and/or ectopic peptide expression of granule cell projections following seizures[49]. We found evidence of damage in animals that experienced SE and evidence of circuit reorganization (mossy fiber sprouting) using NPY staining in two of the four animals that experienced recorded seizures during the study. (Fig.3B). We wanted to track stem cell survival and distribution under these pathological conditions. Therefore, we used histochemical, and immunohistochemical, methods, concurrently, to track the presence of the stem cell reporter molecule β-galactosidase in these key regions of transplanted animals. We found that the overall patterns of staining were the same for both methods, but that indirect immunohistochemistry was more sensitive in detecting the reporter molecule in the cell processes of positively-stained cells (Fig. 4).



A B

Figure 3. Animals that experienced 2hrs of pilocarpine induced status epilepticus have severe hippocampal damage and circuit reorganization. Hematoxylin and eosin staining (A) showed severe cellular dropout within the pyramidal cell layers and hilus, but relatively resistant cells remain in the granule cell layer. (B) Mossy fiber sprouting into the inner molecular layer was revealed through ectopic neuropeptide Y expression

When stem cell injections were targeted to the dorsal and ventral hippocampus, staining for the reporter molecule β-galactosidase revealed neuronal profiles consistent with widespread dispersion into all areas of the hippocampus. Within the hippocampus, in the pilocarpine-treated animals, labeled cells were detected in areas that typically suffer the most severe cell losses in this model of TLE (the CA3 and CA1 pyramidal cell layers and the hilus). Labeled cells were present more densely in the ventral aspects of the hippocampus, and within typically vulnerable cell layers. Some, but fewer, labeled cells, could be detected in areas that are reportedly not as severely affected (dentate granule cells) (Fig. 4 A-D). Within labeled areas β-gal-positive cells demonstrated alignment within preexisting cell layers, and this could be in large numbers (e.g. pyramidal cell layers, and the inner border of granule cell layers (Fig. 4 A-D& F). They also were found in areas where GABAergic interneurons typically reside (e.g. hilar region of the dentate gyrus (Fig. 4 E) and stratum oriens Fig. 4C). When IHC was used to detect the cells, the neuritic profiles showed mature neuronal phenotypes, and projections into the areas that were appropriate for the cell layer (Fig. 4 E&F).

Consistent with the widespread dispersion and fusion reported earlier[35], cells that were originally injected into the hippocampus could be found throughout the brain. Labeled neurons were found within the thalamus, the cortex, the midbrain, the cerebellum, and the circumventricular organs. Evidence that the cells intravasated into the vasculature was indicated by positive staining of cells within blood vessels, and the choroid plexus (Fig.4 G&H). As was the case within the hippocampus, β-galactosidase expressing cells were found positioned appropriately for both principal cells, and interneurons, in cortical areas.

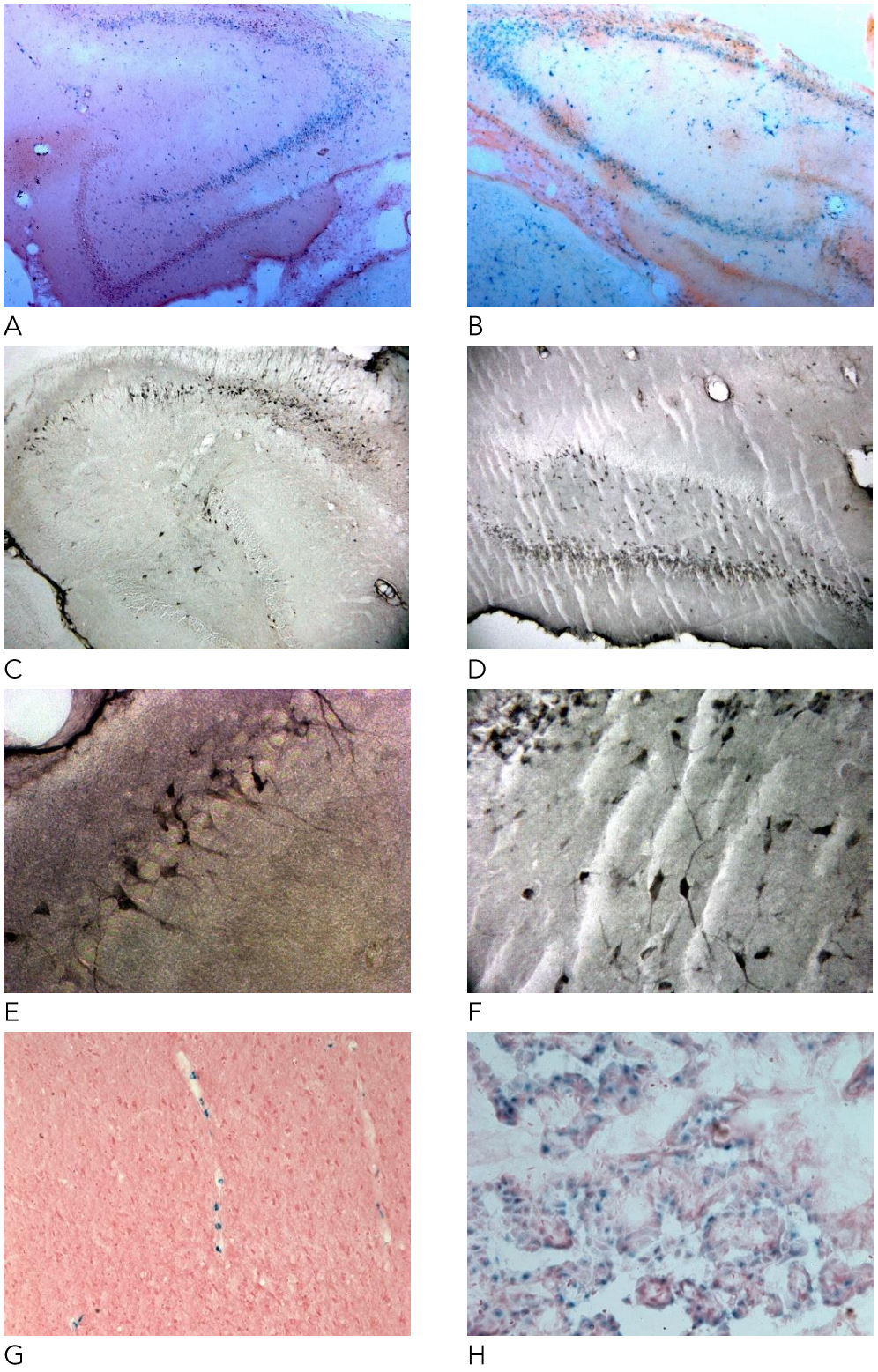


Figure 4. X-gal positive Z6GAD65-4 cells transplanted into chronically seizing animals have the ability to distribute into damaged hippocampal cell layers. (A&B (most extreme case). IHC showed neuronal morphologies (C-F) and region-specific phenotypes (E, F). Areas CA1, CA3, CA4 and hilus were disproportionately stained. Resistant cell layers contained fewer β-galactosidase-containing cells (D). Appropriate neuritic projections were appropriately stained (E, CA3 region, F hilus). Undifferentiated cells were identified in vessels (G) and in the choroid plexus (H).

We tested whether the stem cells would produce the same pattern of staining within the hippocampus of animals with a chronic neurological deficit, but without obvious hippocampal damage. We transplanted cells into the ventral hippocampus in a model of unilateral medial forebrain bundle lesioning. In these two animals, the pattern of staining was qualitatively different following the hippocampal injections. Most of the cells within the hippocampus were in the needle track, and, when the needle track was associated with the principal cell layers within the hippocampus, the staining was mostly ectopic (Fig. 5). There was very little evidence of the hippocampal region-appropriate cell alignment that was detected in chronically seizing animals (Fig. 5 A&B, and D). There was however, still evidence of widespread dispersion. Despite a lack of obvious staining in neuronal profiles within the hippocampal pyramidal cells (Fig. 5C), cerebellar Purkinje cells had β-galactosidase positivity in a subset of cells (Fig. 5D).

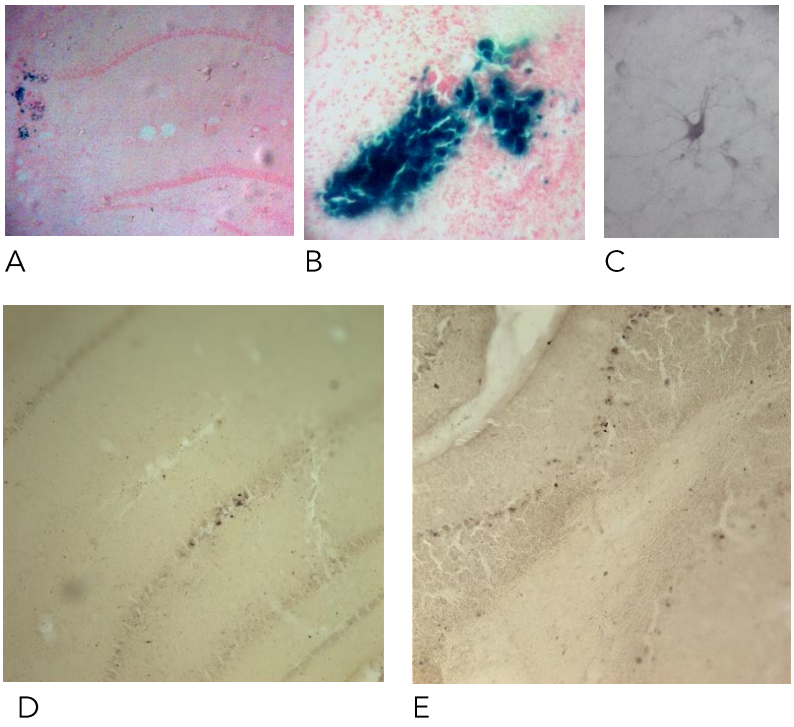


Figure 5. Neurogenic ESCs injected into the hippocampus of 6-OHDA-treated animals show a different pattern of staining than animals with severe hippocampal damage. (A) Within the hippocampus, cells expressing the transgene stained in the needle track, but principal cell layer staining was rare. (B) Higher magnification of needle track, and little staining elsewhere (D, IHC). Cells infiltrated other areas such as the midbrain (C), and the fusionogenic cerebellar Purkinje cell layer at this timepoint (E).

Discussion

The neurogenic potential of ESCs can be enhanced by controlling the expression of key transcription factors[24,25,47] and ESC-derived GABAergic phenotypes can be further enriched with protocols that attempt to recreate the developmental programs that produce interneurons[28]. Here we demonstrate an alternative strategy of producing GABAergic neurons by producing regulatable GAD65 expression in neurogenic ESCs. These cells over-express GABA in standard culture

conditions in the absence of doxycycline. Previously, we successfully generated, and characterized, neural cell lines with regulatable GABA production for transplantation into models of temporal lobe epilepsy[42]. Neural cell lines raise hippocampal GABA concentrations focally in a regulated manner and suppress epileptiform activity generated within the hippocampus[19], and they can suppress spontaneous seizures[16,20]. Developing ESC-derived neurons using a similar strategy avoids some of the of limitations of using cell lines that carry oncogenes. Embryonic stem cell derivatives are anticipated to lead to a clinically viable cell-type[17]. Here we attempted to further explore the potential of this strategy by transplanting GABA-producing neurogenic ESC into an accepted animal model of temporal lobe epilepsy.

The results of the present study revealed important differences from our previous studies using genetically engineered neural cell lines, and may inform the interpretation of the present, and similar studies[50]. The biological capacities of the ESC lines used in this study affected the measured outcomes in a manner that distinguishes them from our transplantable GAD65 engineered immortalized cell lines[21,42]. For example, we found that both the parental cell line, and clonal cell lines generated by transfection with our doxycycline-regulatable construct, produce GABA. We found that up to 50% of the basal GABA-production in our GAD65-engineered ESCs, could be accounted for in non-GAD65-engineered ESCs. Regulation of the transgene is desirable, as it provides an important internal control, and it can lead to individualized production of the therapeutic molecule. Endogenous GABA production limits the control provided by the tetracycline-regulated system used in this study.

Based on our previous reports, and other studies using ESC-derived neurons[51], we assumed that delivery of these cells into the damaged, seizure-prone hippocampus would focally increase GABA with new neurons that could potentially integrate into hippocampal circuits. What we likely underestimated is both the baseline GABA production of neurogenic ESC cells, and the capacity for ESC dispersion, and fusion, that has been reported for these[35] and other stem cell derivatives[31,52,53]. Viewing our results through this lens led us to a necessary pause, and to a re-consideration of our interpretation that the seizure-reduction and transgene expression pattern were direct evidence of cell replacement and circuit integration, within the damaged hippocampus.

Our seizure study suggests that both the parental cell line of neurogenic ESCs, and the GABA-overexpressing cell line, have the capacity to suppress spontaneous seizures. Although no strong conclusions can be made in such a limited study, the relatively rare seizure occurrence, in all groups after transplantation, is consistent with our earlier reports[16,18,42,54]. The fact that the parental cell line produces GABA, and seemed to suppress seizures in this small study, suggests that non-engineered ESC-derived neural stem cells can influence seizure behavior, which has been shown in previous studies[51]. We forced GABA production in a clonal ESC cell line by engineering the GABA synthesizing enzyme GAD65 using a regulatable promoter. There were few seizures recorded in the three-day

analysis period post-transplantation using these cells, and the seizure rate increased in two of three rats when they were given doxycycline in their drinking water. Additionally, when doxycycline was again omitted, detected seizure rates went to zero in all three animals one month after transplantation. Regulation of therapeutic transgene expression would serve a vital role in individualized dosing and potentially controlling unwanted side-effects in cell-based therapies.

An extremely important issue in this study is the survival, and fate, of transplanted stem cells within the diseased, seizure-damaged, and seizure-prone, brain. After transplantation into models of epilepsy, stem cells have been reported to remain primarily undifferentiated[55], partially differentiated[27], and fully differentiated with region appropriate phenotypes found exclusively in the seizure-affected limbic regions[28]. However, a conventional means of transplanted ESC identification, using the presence of reporter molecules expressed by only the ESC genome, may not reveal a complete picture. Stem cell fusion with neurons has been reported to occur at a very low rate following transplantation with hematopoietic stem cells[29,34,56], isolated adult neural stem cells[30], and ESC-derived neural stem cells[36,37]. Two types of fusion have been well studied in Purkinje cells, complete fusion with binucleation[56] and partial cell fusion without binucleation[34]. The former case leads to stable heterokaryon and nuclear reprogramming, and the latter case leads to the sharing of biologically active cytoplasmic constituents (including transgene products), likely through the formation of transient nanotubes[57].

We evaluated the histological outcomes following transplantation of these ESC-derived neurogenic-fusionogenic cells, and found indicators of fusion which we have reported on earlier[35]. Reporter molecule-expressing ESCs can be found within a bolus of cells at the injection site within the first two weeks[35] of injection, but the reporter molecule is more commonly seen in mature neuronal profiles within the host brain at later timepoints. The cells disperse widely throughout the brain after targeted injection (even with unilateral injection). Undifferentiated cells expressing the reporter molecule can be found in blood vessels, the choroid plexus, and in peripheral tissues (liver in this case). Finally, some neuronal cell types seem to be more fusionogenic (more likely to fuse with circulating stem cells) than others, including Purkinje cells of the cerebellum, and large pyramidal neurons of the cortex (both seen in this study).

An interesting finding in this study, that may be related to enhancement of fusionogenicity, was the apparent pathotropism of the cells. Positive β-galactosidase staining was revealed in the principal cell layers of the hippocampus of rats with severe damage in the region, but not in the hippocampi of animals with a striatal lesion but no hippocampal damage. Previous reports have shown that fusion events are increased by cellular damage, pathophysiology, and age[32,38,39,58]. All of these variables are components of the model used here, and can be found in other similar investigations[59]. In fact, all studies using ESC-derived neural stems cells, or stem cells from other sources, that transplant into damaged tissue, might miss fusion events if they are not actively investigated[60]. Models of chronic disease like the pilocarpine induced status

epilepticus model of temporal lobe epilepsy, and the unilateral 6-OHDA striatal lesioning model of hemiparkinsonism used here, are not uncommonly used in preclinical studies using stem cells. A careful examination of transgene expression in neural phenotypes, and an interrogation of possible fusion events, would be reasonable in these and similar neurodegenerative disease models.

One of the biggest challenges in detecting cell fusion is discriminating between complete and partial fusion events. In a previous study we used transmission electron microscopy to evaluate over 100 β-galactosidase positive cells. None were found to be binucleated[35], and only two cortical pyramidal cells showed TEM evidence of binucleation, despite very many β-galactosidase-positive cells being detected. Confocal microscopic analysis used in that study showed that most of the β-galactosidase positive cortical cells had one large dispersed nucleus, and a cell-adjacent, relatively condensed, satellite nucleus which matches the profile of stem cell nuclei in other fusion studies[56]. Elegant methods of detecting partial cell fusion have been devised such as using transplanted cells to deliver cre recombinase to the genomes of neural cells containing lox sites in order to drive reporter gene expression[34], and the use of sex-mismatched transplanted cells with Y chromosome detection[61]. More accessible methods like BrdU pre-labeling may be able to confirm cell fate, and/or exclude binucleation, but cells undergoing continuous cell divisions can dilute the marker, and still lead to confusion. A detailed genetic analysis may be the best way to answer these questions[60].

Conclusions

This study suggests that ESC-derived neural cells may have the potential to treat neurological diseases like intractable temporal lobe epilepsy. The use of GABAergic-neurogenic-fusionogenic ESCs has desirable, but also potentially undesirable, features that should be more fully explored. Many questions remain: 1) What are the most reasonable strategies for confirming, or excluding, fusion events in similar studies? 2) Can fusion be controlled/prevented/enhanced in a targeted manner? 3) How long do undifferentiated stem cells circulate and continue to be fusionogenic within the host? 4) Are there negative side-effects produced by fusion events? and 5) Can fusion be used as a means to deliver therapeutic genes to compromised cells in other disease models? Here, we desired focal inhibition but likely produced effects outside of our target area of the temporal lobe, although this was not strictly studied. In many cases however, global delivery of therapeutic transgene products may be desirable, and more investigation in this area is warranted.

Conflict of Interest Statement:

The authors have no conflicts of interest to declare.

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