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Embryonic Stem Cell Derived Neural Precursor Grafts for Treatment of Temporal Lobe Epilepsy

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Abstract

Complex partial seizures arising from mesial temporal lobe structures are a defining feature of Mesial Temporal Lobe Epilepsy (TLE). For many TLE patients, there is an initial traumatic head injury that is the precipitating cause of epilepsy. Severe TLE can be associated with neuropathological changes, including hippocampal sclerosis, neurodegeneration in the dentate gyrus, and extensive reorganization of hippocampal circuits. Learning disabilities and psychiatric conditions may also occur in patients with severe TLE for whom conventional anti-epileptic drugs are ineffective. Novel treatments are needed to limit or repair neuronal damage, particularly to hippocampus and related limbic regions in severe TLE and to suppress temporal lobe seizures. A promising therapeutic strategy may be to restore inhibition of dentate gyrus granule neurons by means of cell grafts of embryonic stem cell (ES)-derived GABAergic neuron precursors. “Proof-of-concept” studies show that human and mouse ES-derived neural precursors can survive, migrate and integrate into the brains of rodents in different experimental models of TLE. Additionally, studies have shown that hippocampal grafts of cell lines engineered to release GABA or other anticonvulsant molecules can suppress seizures. Furthermore, transplants of fetal GABAergic progenitors from the mouse or human brain have also been shown to suppress the development of seizures. Here, we review these relevant studies and highlight areas of future research directed toward producing ES-derived GABAergic interneurons for cell-based therapies for treating TLE.

INTRODUCTION

Complex partial seizures arising from mesial temporal lobe structures are a defining feature of Mesial Temporal Lobe Epilepsy (TLE). Traumatic brain injury (TBI), in addition to viral infections, high fevers, or genetic mutations, can lead to TLE.¹ Estimates suggest as many as 1.4 million Americans sustain traumatic brain injuries each year and that 5.3 million Americans are coping with long-term, severe disabilities resulting from brain injuries. Closed head injuries, such as those caused by high pressure “shock waves” from close-range detonation of...
hand-grenades or “Improvised Explosive Devices” are the signature trauma experienced by American Iraq war veterans. These alarming statistics suggest that TBI-induced epilepsy is a significant medical problem. Because this form of epilepsy or TLE with prolonged status epilepticus (SE) may be associated with neurodegenerative changes and abnormal hyperexcitability localized to the dentate gyrus, conventional anti-epileptic drugs (AEDs) are often ineffective for suppressing seizures and there is an unmet need for more effective treatments.

Many studies suggest that the hyperexcitable state of the dentate gyrus is at least partly due to degeneration of hippocampal GABAergic interneurons in the polymorphic region of the dentate gyrus. These inhibitory neurons are especially prone to injury and are lost in the subset of TLE patients who have TBI or prolonged status epilepticus. Similar to patients with prolonged SE, rodents developing SE after TBI or systemic injections of the chemoconvulsant pilocarpine, exhibit hilar interneuron loss and other neuroplastic changes that are thought to contribute to hyperexcitability of the dentate gyrus and epileptogenesis. Despite extensive evidence from experimental animal models showing an association between the loss of hippocampal GABAergic neurons and epileptogenesis, many patients with TLE do not show evidence of hippocampal cell death or sclerosis. Therefore, the causal relationships between neuropathological changes in the dentate gyrus and the development of TLE in human patients is still controversial.

The current treatments for TLE include anticonvulsant medications, neurosurgery, vagus nerve stimulation, and the ketogenic diet. Antiepileptic drugs are the most conventional means to treatment epilepsy. However, none of the available treatments is free of side effects, among which cognitive and behavioral disturbances are common. Seizures can be difficult to control in TLE patients with conventional anti-epileptic drugs (AEDs), and poorly-controlled epilepsy may be associated with learning disabilities and psychiatric conditions. In some patients, surgical removal of the epileptic focus is one of the few effective therapies but if the seizure focus is located within temporal lobe regions controlling language or memory, surgery can result in severe cognitive impairment or other undesired complications. A special restricted, high-fat diet is another option for children with epilepsy, but this diet is often not well-tolerated and can be difficult to implement. New and more effective treatments are needed to limit or repair neuronal damage, particularly to the hippocampus and related limbic regions. This review focuses on the potential for novel cell-based therapies with mouse and human embryonic stem cells (ES) cells. We highlight some of the promising approaches that are being developed to drive ES-derived neural precursors (ESNPs) toward GABAergic neuron fates and potential uses of these cells for hippocampal repair and seizure suppression in TLE.

Reorganization of Temporal Lobe Circuits in TLE

In addition to kindling models that are discussed elsewhere in this volume, the chemoconvulsant models using systemic injections of kainic acid or pilocarpine are widely-used experimental paradigms leading to the development of spontaneous seizures. Additional, newer models are also being developed that may be more equivalent to milder forms of human TLE. Our laboratories have utilized the kainic acid and pilocarpine models in mice to study the therapeutic potential of ES-derived neural precursors for repairing the pyramidal cell layer and the dentate gyrus, and for suppressing seizures. Our working hypothesis is that, due to extensive degeneration of GABAergic interneurons in the dentate gyrus, replacing hippocampal inhibitory interneurons with ES-derived GABAergic precursors may be efficacious for treating TLE.

The neurons in the hilus are heterogeneous, consisting of the excitatory mossy cells, which are glutamatergic interneurons that express high levels of the GluR2 subunit of the AMPA receptor, and inhibitory GABAergic interneurons identified by the expression of calcium-binding
protein parvalbumin, or the neuropeptides somatostatin and neuropeptide Y (NPY). Although some of the dying cells in the hilus correspond to mossy cells, many are GABAergic interneurons. In the pilocarpine and kainate models of TLE in rodents, the hilar mossy cells and the GABAergic interneurons of the hilus and CA1 subfield are among the first cell types to undergo degeneration.13, 14, 15 The subtype of hilar interneuron that is somatostatin-positive is particularly vulnerable to seizures.13, 16, 17 Somatostatin-positive interneuron cell death contributes to the majority of GABAergic cell death after seizures, and somatostatin-deficient mice exhibit exaggerated seizure severity.17, 18 These interneurons provide inhibitory synapses onto the dendrites of granule cells in the outer two thirds of the molecular layer of the hippocampus, thereby gating the excitatory entorhinal inputs onto dentate granule cells. 19, 20 A subtype of somatostatin-positive interneuron also expresses NPY, a neuropeptide that has anticonvulsant effects.21 NPY-positive interneurons also degenerate after seizures, and the death of these neurons is thought to contribute to epilepsy induction.22 In accordance with the loss of interneurons induced by seizures, GABA and NPY receptor expression are also altered.21, 22, 23, 24 Epileptic mice show decreased dentate gyrus granule cell inhibition measured by the frequency of monosynaptic inhibitory postsynaptic potentials, which is thought to be a direct consequence of hilar interneuron cell death and the subsequent changes to the hippocampal microenvironment.15

The role of interneuron cell death in granule neuron hyperexcitability is complex, because additional studies suggest that the parvalbumin-expressing basket cells can survive SE, but lose their excitatory afferent inputs, and become dormant.25, 26 Additional research found that inhibition to the dentate granule cells may actually increase during the chronic phase due to an increase in excitatory inputs from mossy fibers to dentate gyrus inner molecular interneurons27 or a change in the pharmacological properties of GABA<sub>A</sub> receptors of dentate granule cells.28

Studies in rodent models of TLE and postmortem studies of human autopsy material have shown that the extent of hilar neuron degeneration positively correlates with the degree of mossy fiber sprouting.29,30,31 The recurrent excitatory synapses formed by mossy fibers onto granule neurons are thought to form an abnormal reverberating network that reduces the threshold for granule neuron synchronization.30,32 Just as the link between interneuron death and epileptogenesis has not been proven, the connection between mossy fiber sprouting and epileptogenesis is also controversial, because spontaneous seizures can develop in the absence of mossy fiber sprouting.33 Once epilepsy develops however, recurrent seizures may also induce hippocampal sclerosis and further damage to anatomically interconnected limbic brain regions, including the amygdala,34 thalamus, entorhinal cortex, and piriform cortex, which may develop into seizure foci.35

In addition to neurodegenerative changes, there are other pathological changes after seizures including gliosis, microglia activation, and upregulation of neurotrophic factors.22-36 Reservoirs for endogenous neural stem cell populations are found in the subventricular zone (SVZ) lining the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus, and both of these regions have been studied in rodent and human brains.37 Although neurogenesis increases shortly after seizures (days to several weeks), it is suppressed in the dentate gyrus during the chronic phase when seizures occur spontaneously.36 Although the link between altered neurogenesis and the etiology of chronic seizures is poorly understood, hippocampal neurogenesis during adulthood has been hypothesized to play an important role in learning and memory, and if it is impaired by chronic seizures, the deficit in neurogenesis could contribute to the cognitive deficits that are seen in patients with TLE.38 As discussed later, transplantation of neural stem cells into the dentate gyrus could partially compensate for this deficit, as suggested by studies showing that ES-derived neural progenitors transplanted into more anterior levels of the dentate gyrus tend to migrate into and colonize the subgranular zone.39
In studies of the stem cell niche in the hippocampus of experimental rodent models of TLE, aberrant neurogenesis leads to the production of displaced populations of granule neurons in the hilus. Adult-born granule neurons also may form aberrant connections that are hypothesized to contribute to hyperexcitability within hippocampal networks, although recent work contests this hypothesis.40

The progenitor cells within the SGZ are categorized into three distinct subsets, each with varying differentiation potentials and replication capacities.37 These distinctive progenitor classes show different responses to external stimuli and seizures.41 Jessberger and colleagues found that seizures increase neurogenesis mainly in the DCX-positive, late developmental stage progenitor cells, indicating that progenitors vary in their sensitivity to seizures.42 By using BrdU birthdating in a pilocarpine-induced seizure mouse model, Walter and colleagues discovered that neurons born after the seizure insult migrate aberrantly and extend their basal dendrites into the dentate hilus.43 These abnormal basal dendrites receive excitatory inputs from dentate granule cells. As a consequence, new excitatory circuits are formed,30,5 and this is thought to increase excitability of the dentate gyrus. The re-introduction of inhibitory GABAergic interneurons into the epileptic dentate gyrus has the potential to counter-balance hyper-excitation, as discussed in the following sections.

Fetal Neural Stem Cells May Provide Neurons and Glia for Transplantation into Patients With Drug-Resistant Forms of Epilepsy

Mature neurons lose the ability to undergo cell division once they fully differentiate; therefore, cell replacement is recognized as a promising approach to treat neurodegenerative diseases.44-45,46,47 The migration and functional integration of GABAergic progenitors harvested from the fetal brain medial ganglionic eminence (MGE) was tested following transplantation into the adult or neonatal brain.48,49 The migration of MGE precursors in the adult brain was substantial – up to 1.5 mm from the injection site. Their ability to migrate in the neonatal brain is even more extensive, with dispersion distances of up to 5 mm, several months after transplantation.49 The efficacy of cell replacement therapies, based on transplanting various embryonic cell types and cell lines, has been studied extensively in rodent seizure models.47,50 Fresh fetal neurons from various brain areas such as noradrenaline-rich locus coeruleus,51,52 hippocampal regions,51,53,54 or the GABAergic neuron-rich fetal striatum, were transplanted to the hippocampus or substantia nigra to test their efficacy in suppressing seizures. Transplantation of these fetal cells reduced abnormal electrical activities in response to electrical stimulation of the perforant path and after discharges in kindling models,51,55 prevented mossy fiber sprouting,54 and reduced calbindin-immunoreactive neuron cell death.53

In another study, neural precursors, derived from 15-week-old human fetal brain ventricular zone were delivered to the brain via tail injection, following pilocarpine-induced seizures in adult rats. Surprisingly, the transplanted cells found their way to the brain regions involved in seizure circuits, including the hippocampus, piriform cortex and the amygdala and few were detected in the striatum, thalamus and cerebellum.56 In the piriform cortex, approximately 26% of the transplanted cells were positive for GABA and 31% were positive for parvalbumin. Field excitatory postsynaptic potentials (EPSPs) exhibited reduced amplitudes in the rats receiving human fetus-derived neural precursor grafts, compared to epileptic rats without grafts. However, the percentage of the excitatory neurons versus GABAergic inhibitory neural progenitors in the transplants was not quantified in this study, making it hard to interpret their observations of the reduced amplitude of field EPSPs. Studies have not yet determined how many GABAergic progenitors must be transplanted to achieve seizure suppression. Better characterization of the cell types being transplanted and the proportion that are GABAergic are also important issues that future work should address. Despite these promising findings,
most clinics have limited access to human fetal tissue for transplantation in TLE patients, and medical use of aborted fetuses is fraught with ethical concerns. Xenografts of fetal tissue from other species have been tested as an alternative approach with fewer ethical roadblocks, however, the feasibility and safety of grafting non-human fetal tissue into patients with TLE has not been extensively tested.\textsuperscript{46}

**Transplantation of Genetically-Modified Cells for Treating Epilepsy**

Another approach for seizure suppression has relied on modified cell lines. Some have been engineered to produce GABA or adenosine. Transplantation of modified GABA-producing cells decreased seizure duration and severity,\textsuperscript{57} reduced behavioral seizures,\textsuperscript{58} or suppressed spontaneous seizures in rodents.\textsuperscript{59} In some of these studies, GABA was released into the host brain in a controlled manner by exogenous factors such as doxycycline.\textsuperscript{58} Another group has derived neuron progenitors from the neural cell line RN33B and obtained functional integration and differentiation into pyramidal neurons when they were transplanted into the cerebral cortex or hippocampus of neonatal rats.\textsuperscript{60}

Adenosine is an endogenous anticonvulsant and inhibitory neuromodulator expressed by astrocytes, and studies show that it can decrease neuronal excitability.\textsuperscript{61} However, within several hours of a seizure, adenosine kinase down-regulates adenosine within the hippocampus. Seizure-induced reductions in adenosine have been hypothesized to play a decisive role in epileptogenesis.\textsuperscript{61} In accordance with this theory, transplantation of adenosine kinase-deficient neural progenitors derived from modified ES cells abolished spontaneous seizures measured by EEG in KA-treated rodents.\textsuperscript{62} Moreover, when adenosine was upregulated using lentiviral RNAi against adenosine kinase in human mesenchymal stem cell transplants, it led to a dramatic rescue of host brain hippocampal neurons following KA-induced seizures.\textsuperscript{53} In conclusion, engineered stem cells deficient for adenosine kinase, cell lines releasing GABA, or cells engineered to express short hairpin RNAs directed against adenosine kinase show promise for suppressing seizures (further studies in this area are discussed elsewhere in this issue). However, a limitation of these approaches is that they do not allow neuronal activity-dependent regulation of the anticonvulsant molecules. These transplants are suboptimal for focal epilepsy therapy due to their inability to increase inhibition during the preictal phase before a seizure begins. Furthermore, the limited survival of these cell lines in the adult nervous system and their failure to incorporate into host brain circuits, limits their utility for neural replacement or activity-dependent seizure control.\textsuperscript{58}

**Technology for Generating Neural Progenitors from ES Cells**

ES cell transplantation may provide another avenue for treating TLE.\textsuperscript{64} ES cells can be easily propagated in vitro and generate different cell types, including neurons and glia. Studies directly comparing adenosine-releasing engineered cell lines with ES-derived neural progenitors have found that the ES-derived cell grafts show more extensive integration to the host brain environment.\textsuperscript{65} Despite the advantages, two significant barriers for using ES based cell therapies are the tendency for cells to remain tightly packed together at the core of the graft site and for them to generate tumors. Immature brains appear to allow more extensive migration and integration. Since the majority of human brain diseases are modeled in adult animals, increasing the survival, migration and functional integration of transplanted ES-cell derived progenitors in the adult brain will greatly facilitate our understanding of stem cell therapy. To address the problem of tumor formation, protocols have been developed for eliminating tumor-causing cell types, such as residual undifferentiated ES cells, particularly by the use of fluorescence-activated cell sorting (FACS).\textsuperscript{66}

In the past few years numerous protocols have been developed to derive neural stem cells from ES cells.\textsuperscript{67} These protocols can be broadly classified into two approaches. One approach
produces an embryoid body (EB) followed by neural lineage selection. Another differentiates ES cells at a low density in adhesive cultures under nutrient-poor conditions. In these approaches, co-culturing with another cell line, such as PA6, or other modifications can be introduced to promote neurogenesis or to derive specific cell types, as described below.

To determine whether ES cells could generate neurons and glial cell types, a number of studies have tested the effects of treatment with signaling molecules such as retinoic acid. When applied during the EB stage, ES cells generated neurons with normal polarity including axons expressing growth-associated protein, GAP-43, and dendrites expressing the microtubule-associated protein, MAP2. Molecular markers involved in synapse formation were also expressed in culture. ES-derived neurons primarily formed excitatory synapses, as shown by electrophysiological studies. However, a second study found that the majority of the connections generated from ES-derived neurons derived from embryoid bodies were inhibitory. ES-derived neurons also developed voltage-gated ion channels such as K+, Na+, and Ca+ channels, and neurotransmitter receptor-mediated channels, suggesting that ES-derived neurons formed in vitro, develop in a manner comparable to their in vivo counterparts.

Upon exposure to appropriate conditions and growth promoting molecules, ES cells have been shown to be capable of differentiating into the three major cell lineages in the nervous system - neurons, astrocytes and oligodendrocytes. The sequence of signals required for generating these cell types from ES cells, and the process and pattern of differentiation toward neural lineages, resembles that required for neurogenesis during embryonic development. Therefore, improving our understanding of the signals that promote fetal neural development will facilitate the development of protocols for deriving specific neural types/subtypes from ES cells.

ES cell differentiation can be directed toward different neural or glial pathways using specific signaling molecules, cell-substrate interactions, and extracellular signaling molecules. Using sequential combinations of mitogens or growth factors, ES cells were directed towards oligodendrocyte and astrocyte fates by Brustle and colleagues, in a protocol that combined treatments with fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), and platelet derived growth factor (PDGF). Once transplanted into the spinal cord of newborn myelin-deficient rats, these cells formed myelin sheaths and extended long distances inside the host nervous system, demonstrating that ES-derived glial cells can be used for myelin-replacement therapy.

A number of studies have examined the ability of transplanted neural precursors derived from ES cells to differentiate into specific types of neurons, as defined by their neurotransmitter phenotypes. Following transplantation into the lateral ventricles of mouse embryos, one study showed that ES cell-derived neural precursors (ESNPs) survived, migrated and differentiated into various types of neurons in different brain regions including cerebral cortex, hippocampus, and amygdala. This study also found that the cells differentiated into glutamatergic (44.7%), GABAergic (14.6%), tyrosine hydroxylase-positive neurons (5.3%), and occasionally serotonergic neurons, adopted appropriate morphologies, and displayed normal membrane properties. The transplanted cells received synaptophysin-positive terminals from host brain cells and made contacts with host brain cells. Despite their apparent functional integration, however, the transplanted cells did not adopt the correct fates for the host brain regions that they were grafted into, based on mis-expression of embryonic brain-region specific markers such as the distal-less class homeobox gene, dlx.

Similar to the findings from mouse ES cell transplantation studies, human ESNPs grafted in the lateral ventricles of newborn rodents were shown to migrate extensively into the gray and white matter of the host brain after several weeks. Donor cell-derived glia were frequently
observed in the white matter and expressed glial lineage specific markers such as oligodendrocyte progenitor marker NG-2, 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase), and glial fibrillary acidic protein (GFAP). Neurons labeled with antibodies against beta-3 tubulin were only observed in the olfactory bulb, a region undergoing neurogenesis into adulthood. Further studies found that motor neurons enriched from ES cells were also able to incorporate into the chick embryonic spinal cord. These studies show that ES cells can differentiate into functionally distinct types of neurons and glia in culture and respond to the environment of the host brain after transplantation, demonstrating the broad applicability of ES derived cell therapy for brain repair in neurological disorders such as epilepsy.

Transplantation of ES-Derived Neural Precursors into Rodent Models of Temporal Lobe Epilepsy

The fate of ESNPs transplanted into adult seizure rodent models has not been studied extensively. Ruschenschmidt and colleagues transplanted GFP+ ESNPs bilaterally into mouse hippocampi one month after pilocarpine-induced seizures. Thirty to 34 days later, cells formed clusters at the transplantation site and projected extensive processes invading the host brain. Electrophysiological examinations found that more than 80% of transplanted cells received synaptic input and exhibited non-NMDA and GABA_A receptor-mediated currents as well as ion channel-mediated currents, indicating the viability and normal functionality of ESNPs after transplantation into rodents with established seizures. However, the differentiation profiles of the transplanted cells and the behavioral improvement of the animals were not examined.

Strategies to enrich forebrain excitatory neurons and GABAergic inhibitory interneurons from ES cultures are being perfected. In our laboratories, we have used defined media protocols to derive neural precursors from mouse and human ES cells, and tested the ability of these cells to survive transplantation in two different models of TLE, the kainic acid model and the pilocarpine model. As shown in Figures 1–3, we have generated neural precursors from the YC5 mouse ES line and the human H1 ES line and tested their ability to incorporate into the brains of mice subjected to SE. We first transplanted YC5 cells into the CA3 region of the hippocampus, one week after a single administration of kainic acid to induce seizures. When transplanted into the CA3 region, the ESNPs showed a strong tendency to migrate into the dentate gyrus. In a second series of experiments, we transplanted ESNPs derived from the YC5 cell line directly into the dentate gyrus two weeks following pilocarpine-induced seizures, a time when spontaneous seizures first develop. As shown in Figure 1 (A–G), the ESNPs transplanted into the chronic seizure model remained within the hilus, often aligned with the upper blade of the dentate gyrus and many retained immature stem cell markers such as nestin (Fig. 1 A-E) or GFAP and nestin (Fig. 1 D-G). In kainate treated mice, grafted ESNPs express immature neuronal marker PSA-NCAM shortly after transplantation. With longer survivals of 4–8 weeks, some of the grafted cells show region-appropriate differentiation as shown by expression of Prox1, an early marker for granule neurons (Fig. 1 H-J). Many of the grafted neurons received synaptophysin-positive synaptic terminals eight weeks after transplantation, suggesting that transplanted cells incorporated successfully into the dentate gyrus. The maturation of ESNPs into appropriate cell types appears to be influenced by the site they are transplanted into the host brain because we also found that when transplanted into the fimbria of kainate-treated mice, ESNPs differentiated into oligodendrocytes rather than neurons.

We have recently extended these studies to include human ESC-derived ESNPs and observed a similar fate following transplantation to the CA3 region of the hippocampus, as shown in Figure 2. ESNPs were derived from the H1 human ES cell line modified to constitutively express GFP. ESNPs were derived using a modification of the EB-based defined medium
To isolate enriched populations of ESNPs, colonies with appropriate morphology were manually picked for further passage and expansion on laminin-coated dishes. At the time of transplantation, an average of 87% of the cells were nestin-positive ESNPs. On average, 27% of these cells expressed the neuronal marker βIII tubulin and less than 1% were positive for the ES marker Oct4 (Figure 2).

Transplantation of the H1 ESNPs into control and KA-treated mice was performed 7 days following seizures, as previously described. The brains were analyzed 4 or 8 weeks following grafting. As observed with mouse ESNPs, the human ESNPs tended to migrate to the upper blade of the dentate gyrus in mice that had experienced seizures, but not in control mice. In experimental mice subjected to KA-induced seizures, ESNP-derived cells in the dentate gyrus expressed the neuroblast markers doublecortin or PSA-NCAM (Fig. 2), but the expression of the granule cell marker Prox-1 was restricted to endogenous granule neurons. These data suggest that the human ESNPs were able to initiate, but not complete, differentiation to granule neurons, perhaps because 8 weeks was insufficient to promote this extent of differentiation.

Unlike mouse ESNPs, the human ESNPs did not form teratocarcinomas when transplanted into the mouse hippocampus. Some transplanted cells failed to incorporate into the brain and remained as discrete, proliferating cell aggregates, but these cells continued to express only neural markers. Again, unlike the mouse ESNP experiments, we found that the formation of these unincorporated aggregates was not prevented by prior seizure experience.

In recent studies, we made electrophysiological recordings one month after transplanting murine ESNPs into the dentate gyrus in mice subjected to kainic acid-induced seizures. We determined that the transplanted neurons show normal electrophysiological properties, receive synaptic inputs, and have the ability to fire spontaneous action potentials (Figure 3). In summary, after transplantation into the rodent seizure models, mouse or human ESNPs survive, migrate and show spontaneous electrical activity, suggesting that they have incorporated into host brain circuitry. The next step in this line of investigation is to generate specific subtypes of GABAergic neurons and test their efficacy for suppressing seizures. Several promising strategies are discussed further in the following sections.

Methodology for Genetically Manipulating ES Cells for Transplantation into the Nervous System

Viral vectors have been widely used to both transiently and stably transfer genes into ES cells. Among these viral-mediated approaches, lentivirus-based gene delivery into ES cells is one of the most efficient and more likely to result in stable gene expression over long intervals. Detailed protocols have been developed for lentiviral vector-based gene transfer both in mouse ES cells and human ES cells. In one study, lentiviral transduction of ES cells with a vector containing GFP driven by an internal promoter (rather than the LTR), resulted in reliable transgene expression, even after ES cells differentiated into hematopoietic lineages. Southern blot analyses confirmed that the foreign gene was incorporated into the ES cell genome. In the same study, transduction mediated by a retrovirus resulted in loss of transgene expression upon differentiation, most likely due to the fact that the retroviral vector used the LTR to drive expression. Although it is often concluded that long-term gene expression from lentiviral vectors is superior to retroviral vectors, it depends on whether the LTR or another promoter is used to drive transgene expression, as well as a number of other factors. If transient expression is desired, however, retroviral vectors may be more advantageous.

Non-viral mediated gene transfer to derive specific cell types from ES cells can be accomplished by means of lipophillic reagents or electroporation. The choice of gene delivery method depends on multiple factors such as target cell lines, the gene of interest, and promoters to be used. The common agents for gene transfections are lipids, polymer complexes, or...
particulates that enhance DNA uptake by membrane fusion, endosomal release, or nuclear targeting. When a panel of gene transfection methods was compared, Exgen was the most effective lipophillic reagent for transfecting hES cells with GFP. However, the transfection efficiency was low and associated with considerable toxicity. Liew et al. also found that Exgen showed the highest level of transgene expression compared to other methods. Lipofection has also been found to be an efficient approach. Electroporation and nucleofection are methods to open pores in cell surface plasma membranes to allow DNA uptake. The most recent studies used transfection with the Amaxa nucleoporator or BTX’s electroporator. Nucleofection is a technique that is gaining popularity for use in gene therapy and for forcing gene expression in ES cells, due to high transfection efficiency, low toxicity, and ease of use, compared to viral approaches.

Lineage specific or fluorescent reporter genes can allow selection of specific populations and provide a means for tracking transplanted cells in the brain. One of the advantages of genetic manipulations of ES cells is to enrich for a certain lineage of differentiated cells by introducing drug-resistant genes and/or fluorescent markers to track the ESNPs once they have been transplanted. In one study, the researchers introduced a marker gene β-geo into the sox2 locus, a neural marker gene. Addition of G418 to the differentiating cultures was toxic to the pluripotent stem cells, whereas the Sox2-expressing neural precursors were resistant. This method has been used to enrich Sox2-positive neural lineages. An alternative approach has been to drive GFP expression from the early neural progenitor marker Sox1. Prior work suggested that sox1 is widely expressed in cells adopting neural lineages. When the cells were FACS-sorted, using GFP expression, it was possible to achieve much higher enrichment of neural progenitors. These studies in ESNPs demonstrate that neuronal fate determination can be monitored in vitro using cell lineage-specific promoters to drive fluorescent proteins or enzymes. Furthermore, cell lineage-specific promoters can be used to express drug resistance-conferring genes or fluorescent markers in differentiating neural precursors to allow enrichment of neural precursor populations using drug selection or FACS. Genetic approaches are invaluable for selecting particular cell types for further propagation and transplantation, and they also provide a means for eliminating pluripotent, and potentially tumorigenic, stem cells from those that will be transplanted.

**Strategies for Identifying GABAergic Progenitors from ES cells**

The use of genetic modifications to mark or enrich ES-derived GABAergic progenitor cells is not a technique that has been used extensively to date. The default expression of glutamic acid decarboxylase-67 (GAD67), the rate-limiting synthetic enzyme for GABAergic interneurons, occurs in approximately 30-50% of ES-derived neurons. A stem cell line was created in which the lacZ gene was placed under the Gad1 promoter. However, in that study, GABAergic cell lineages were not selected. The further development of reliable methods for detecting earlier stages of fate specification for ES cells-derived GABAergic progenitor cells and following their integration in vivo, will greatly facilitate efforts to test their efficacy for cell-based treatments for epilepsy.

**Enrichment Strategies for Producing Forebrain GABAergic Progenitors from ES cells by Modified Culture Conditions**

A number of approaches are being explored for producing forebrain GABAergic neural progenitors for transplantation in epilepsy and other neurological disorders. Investigations of differentiating ES cells through EB stage toward GABAergic lineage revealed that approximately half of the population expressed GAD67 protein, a gene product required for GABA synthesis. Barberi and colleagues published the first study showing enrichment of forebrain GABAergic neurons from ES cells in a monolayer culture system. Their major finding was that sequential exposure to combinations of patterning cues could efficiently direct
ES cells toward multiple lineages, including GABAergic neurons. Sequential treatments enriched the percentage of GABAergic neurons in the cultures to ~70% of the total neuronal population; the first treatment combined N2 media (N2) and fibroblast growth factor (FGF2), followed by sequential treatments with: N2+FGF2, N2+SHH+FGF8, and a final step in N2+ neurotrophin 4 (NT4) and brain derived neurotrophic factor (BDNF). Recently, Gaspard and colleagues cultured mouse ES cells at low density in defined medium with Sonic hedgehog (Shh) inhibitor; they could generate a highly enriched population of cortical pyramidal neurons. Without cyclopamine, they found a higher proportion of GABAergic interneurons in these mixed cultures. Taken together, these studies show that it is feasible to manipulate ES culture conditions to favor neurogenesis of either dorsal forebrain cortical pyramidal neurons or basal forebrain inhibitory interneurons. Despite rapid progress in this area, methods for generating specific pure populations of GABAergic progenitors from ES cells are not yet available. Combinations of growth factors, signaling molecules (or inhibitors such as cyclopamine), or conditioned media from cultures of embryonic GABAergic precursors are among some of the possible approaches that may be effective for generating forebrain GABAergic neurons, as illustrated schematically in Figure 4. Additionally transfecting ES cells with genes that drive anterior GABAergic cell fates is a promising approach (Fig. 4).

To derive forebrain ESNPs, serum-free growth of embryoid bodies treated with Wnt and Nodal antagonists (Dkk1 and LeftyA) has been used. This strategy increased expression of the forebrain marker Bf1 to up to ~35% of the total population. Fifteen percent of Bf1+ cells were Nkx2.1+ compared to 36% Pax6+ cells, suggesting that basal forebrain lineage comprised only a small portion of the enriched Bf1+ population. However, by adding SHH at the later stage of serum-free EB (SFEB) culture, the expression of ventral markers Nkx2.1 and/or Islet1 increased to 60%-70% of the Bf1+ cells, at the expense of dorsal forebrain markers. Later studies from the same group further improved the SFEB culture system so it reliably increased Bf1+ cells to 65%-75% of the total population. After replating these cells onto adhesive culture dishes, they aggregated and formed a two-dimensional polarized neuroepithelium. They could also respond to signaling molecules such as FGF8. These findings suggest that when patterning signals are delivered to ES cultures at the appropriate times, both mouse and human ES cells can be efficiently directed toward forebrain neurons. Rapid advances in our understanding of the molecular mechanisms of basal forebrain neurogenesis in the embryo are also providing insights into candidate genes and signaling molecules that may be required for deriving GABAergic progenitor cells from ES cells in vitro. Although it is reasonable to assume that derivation of neural progenitors from either mouse or human ES will require similar factors, the timing is likely to be very different due to species differences in the length of the cell cycle and fetal brain development.

**The Combinatorial Code of Transcription Factors for GABAergic Neurons**

GABAergic interneurons represent ~20% of the total neuronal population in neocortex and hippocampus. However, within this relatively small population, interneurons show enormous diversity morphologically and physiologically. During mouse embryonic development, GABAergic interneuron precursors are generated in the ganglionic eminence (GE) between E9.5 (embryonic day 9) and E15.5 and migrate tangentially to the cortex and the future striatum. The GE is subdivided into three zones – the medial, lateral and caudal ganglionic eminences (MGE, LGE and CGE, respectively) and each of these zones may be further subdivided, based on transcription factor expression and anatomical landmarks, such as grooves, along the ventricles.

The transcriptional factor expression profiles, migration routes, and neuropeptide and calcium binding protein expression patterns vary for each functional subpopulation derived from the GE. Parvalbumin (PV) and somatostatin (SST) interneurons are mostly generated from

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MGE, while calretinin (CR) interneurons are predominantly generated from CGE. The LGE is associated with the striatal SST and cholinergic neurons in the adult. Interestingly, even within the MGE, subtypes of interneurons are generated from different sectors, with PV interneurons predominately generated from the ventral MGE, SST and NPY interneurons mostly born from the dorsal MGE.\textsuperscript{104}

The regional differences within the GE and the subsequent interneuron diversity are the consequences of the interplay between transcriptional factors. By using in situ hybridization, immunohistochemistry, and transplantation, Flames et al. showed that there are nearly 20 domains within the rodent embryonic basal forebrain.\textsuperscript{105} At E13.5, Dlx2 is expressed broadly in the ventricular zone (VZ) and subventricular zone (SVZ) of both MGE and LGE; in contrast, Gsh2 is expressed only in SVZ. Nkx2.1 is expressed exclusively in MGE while Nkx6.2 is confined to the MGE and LGE border in the SVZ. Lhx6 and Lhx7, which are involved in interneuron migration\textsuperscript{106} and cholinergic neuron specification,\textsuperscript{107,108} respectively, are both predominantly expressed by MGE.

The complexity of GABAergic interneuron determination and the underlying molecular pathways are only beginning to be appreciated. Studies comparing Mash1 and Dlx1/2 mutants suggested that Mash1 is involved in early neurogenesis in GE while Dlx1/2 regulates slightly later neurogenesis.\textsuperscript{109} They both influenced Notch signaling; Mash1 down-regulated the signal, while Dlx1/2 significantly expanded the expression domains of Notch signaling. Mash1 mutations caused VZ cells to express SVZ genes prematurely in the LGE, and the loss of progenitor cells in the SVZ of MGE,\textsuperscript{110} suggesting that one of the functions of Mash1 is to regulate ventral neurogenesis.

In our laboratories, we are investigating whether driving the expression of particular transcription factors in human and mouse ES cells can be used to generate GABAergic precursors. This approach is diagrammed in Figure 4. Additionally it may be important to use selection protocols to enrich for GABAergic precursors, based on the expression of glutamic acid decarboxylase-67 (GAD67), the rate-limiting synthetic enzyme for GABAergic interneurons.\textsuperscript{96} Marking and enriching the ES cells-derived GABAergic progenitor cells is becoming feasible, and the realization of this technique will tremendously facilitate cell therapies for treating relevant neurodegenerative diseases.

Recent findings have also shown that environmental signals may be manipulated to optimize the production of forebrain neural progenitors for transplantation. Growth factor treatments or ganglionic eminence-conditioned media are two putative approaches that may be used to generate GABAergic precursors; therefore, exposing ESNPs to modified cell culture conditions could be another powerful tool for directing differentiation into GABAergic progenitors. Several notable examples showed that adding growth factors or hormones to media vastly improves neurogenesis and survival of neural precursors. Allopregnanolone is a natural steroid hormone produced in the mammalian brain that promotes neural precursor proliferation in the adult hippocampus. Addition of allopregnanolone to rodent and human neural stem cells in culture promotes proliferation of neural progenitor cells,\textsuperscript{111} but it is not yet known whether adding this hormone to ES cultures enhances GABAergic neural progenitor production. Myocyte enhancer factor 2C (MEF2C), a molecule required for embryonic development, not only promotes the production of neural precursors from ES cells, but also enhances their survival after transplantation by up-regulating pro-survival genes.\textsuperscript{112} Additionally, overexpression of the neural cell adhesion molecule L1 enhanced the production of neural precursors from ES cells and promoted survival and integration of ES-derived GABAergic neurons after transplantation into the striatum in an excitotoxic lesion model.\textsuperscript{113}
Protocols to promote the derivation of specific cell types from ES cells have also utilized cocultures with other cell types or conditioned the media with embryonic cells. The idea of coculturing ES cells with other cell types is not entirely new given the original report of growing ES cells on feeder cells. The rationale for enriching specific cell lineages by means of coculturing with relevant cell types is that soluble factors from the co-culture can stimulate ES cells or their derivatives to initiate lineage specific gene expression, even when the identities of the soluble factors are not known. We illustrate this approach for deriving GABAergic neurons from ESNPs in Figure 4.

An important goal for an ES-based cell therapy for neurological diseases is to identify each component required for survival and directed differentiation of functionally distinct neural and glial cell types. Once these requirements are known, defined nutrients or factors can be applied to ES cells or their derivatives sequentially to generate specific GABAergic cell types. For large scale production needed for transplantation therapies in human patients, it will be necessary to use small molecular agonists, antagonists, or genetic modifications, to avoid contamination with products from other animal species. Human ES cells cultured without feeder cells and serum exhibit long-term pluripotency and can be directly differentiated in response to added molecules such as retinoic acid and purmorphamine, an agonist of SHH and potential ventralizing agent.

CONCLUSIONS AND FUTURE DIRECTIONS

The prerequisites for using ESNPs, or stem cells in general, for treating neurodegenerative diseases are that 1) cells for transplantation can be generated continuously and can consistently differentiate into functional neural types in vitro, preferentially specific cell types that are impaired by the disease; 2) after transplantation into the brain regions, transplanted cells should populate and survive in the damaged brain areas for extended periods of time; 3) cells should express appropriate ion channels and molecular markers such as neurotransmitter receptors, possess normal membrane properties, and demonstrate an ability to fire action potentials; 4) transplanted cells should receive and make synaptic connections with host brain cells and incorporate into the host circuitry with appropriate regional specification; 5) transplantation should improve behavioral defects, such as abnormal neuronal spiking recorded by electroencephalogram (EEG), without generating inflammation or initiating tumor formation. Directing the differentiation of ES cells toward particular cell lineages is now possible, and strategies to generate specific subtypes of neurons such as GABAergic neurons are now on the horizon.

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Figure 1.
Transplants of YC5 ESNPs into the pilocarpine model or kainate models of TLE. When transplanted into the hilus of pilocarpine-treated mice, YC5-derived neural precursors expressing EYFP (stained with an antibody against GFP) survived for at least 8 weeks and colonized the subgranular zone, a neural stem cell niche. These cells extended processes into the overlying granule cell layer (A) and retained expression of immature markers such as nestin (B) or GFAP. Some co-expressed GFAP (E) and nestin (F) demonstrating that cells within the SGZ had adopted the fates of the SGZ neural stem cells. Some cells in the grafts expressed markers of immature neurons, such as beta-III tubulin, shown using the TuJ-1 antibody (C). In mice treated systemically with kainic acid to induce SE, YC5 derived ESNPs showed extensive migration from the site of injection in CA3 into the dentate gyrus granule cell layer (GCL), where some differentiated into Prox-1-positive granule neurons (H-J).
Figure 2.
Transplantation of GFP-expressing human H1 ESNPs into the hippocampus of adult mice resulted in survival, migration and differentiation into neural lineages by 8 weeks. Human ESNPs in cell cultures before transplantation showed extensive expression of nestin (A, B), with only a small percentage of the cells expressing Tuj-1, a marker of immature neurons (C, D). GFP+ ESNPs migrated into the hilus of the dentate gyrus (DG) of the injected site, and were located predominantly in the subgranular zone (E), but did not incorporate into the granule cell layer. F-J, Additional examples of GFP-labeled human ESNPs in upper dentate gyrus. (E, H) Transplanted human cells survive and migrate to the dentate gyrus. (F, G). Many of the transplanted cells were migratory, as indicated by the expression of doublecortin (DCX) immunoreactivity. (I, J) Others expressed the immature neuronal marker, polysialated neural cell adhesion molecule (PSA-NCAM). Arrows indicate grafted cell bodies and processes. Scale bars: A-D, 100 μm; E-J, 100 μm.
Neurophysiological characterization of YC5 derived neural progenitors after transplantation into kainic acid model showed that some of the cells exhibited normal neurophysiological properties. (A) Differential Interference Contrast (DIC) view of the brain slice with a cluster of transplanted cells in the CA1 region. The patch clamp recording of the neuron was made with a glass microelectrode, indicated by the white dashed lines. (B) The epifluorescence view of the EYFP+ neuron from (A). (C) and (D), electrophysiological recordings indicate that the cell fired action potentials in response to threshold (C) and higher intensity stimuli (D). The membrane potential was held at $-74$ mV. (E) The neuron generated spontaneous action potentials in response to depolarized holding membrane potential. (F) In response to
hyperpolarizing stimulation, the neuron became hyperpolarized. (G) Spontaneous miniature end-plate potentials were recorded at a holding membrane potential $-69mV$, indicating that the neuron received functional synaptic inputs. (H) A magnified view of the boxed area in G showing a putative individual EPSP. Scale bars: C, D and F, 0.2 seconds; E, 2 seconds; G, 0.5 seconds; H, 0.02 seconds.
Figure 4.
Three approaches for generating or enriching populations of GABAergic neuron precursors from murine and human ES cell lines. ES cells are first differentiated into neural progenitor cells. Treatment of neural precursor cells with conditioned media, gene transfection of transcriptional factors into the neural progenitor cells, or sequential treatment with growth factors and signaling molecules may successfully guide progenitor cells toward GABAergic lineages and induce the expression of GABAergic cell-type specific genes, upon differentiation.