Subventricular Zone-Derived Neural Stem Cell Grafts Protect Against Hippocampal Degeneration and Restore Cognitive Function in the Mouse Following Intrahippocampal Kainic Acid Administration

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ABSTRACT

Temporal lobe epilepsy (TLE) is a major neurological disease, often associated with cognitive decline. Since approximately 30% of patients are resistant to antiepileptic drugs, TLE is being considered as a possible clinical target for alternative stem cell-based therapies. Given that insulin-like growth factor I (IGF-I) is neuroprotective following a number of experimental insults to the nervous system, we investigated the therapeutic potential of neural stem/precursor cells (NSCs) transduced, or not, with a lentiviral vector for overexpression of IGF-I after transplantation in a mouse model of kainic acid (KA)-induced hippocampal degeneration, which represents an animal model of TLE. Exposure of mice to the Morris water maze task revealed that unilateral intrahippocampal NSC transplantation significantly prevented the KA-induced cognitive decline. Moreover, NSC grafting protected against neurodegeneration at the cellular level, reduced astrogliosis, and maintained endogenous granule cell proliferation at normal levels. In some cases, as in the reduction of hippocampal cell loss and the reversal of the characteristic KA-induced granule cell dispersal, the beneficial effects of transplanted NSCs were manifested earlier and were more pronounced when these were transduced to express IGF-I. However, differences became less pronounced by 2 months postgrafting, since similar amounts of IGF-I earlier and were more pronounced when these were transduced to express IGF-I. However, differences became less pronounced by 2 months postgrafting, since similar amounts of IGF-I were detected in the hippocampi of both groups of mice that received cell transplants. Grafted NSCs survived, migrated, and differentiated into neurons—including glutamatergic cells—and not glia, in the host hippocampus. Our results demonstrate that transplantation of IGF-I producing NSCs is neuroprotective and restores cognitive function following KA-induced hippocampal degeneration. Stem Cells Translational Medicine 2013;2:185–198

INTRODUCTION

Epilepsy is a common neurological disease, affecting 50 million people globally. With a worldwide annual incidence rate of 24–53 per 100,000, it is a major health and economic burden to society. Given that nearly 30% of epileptic patients do not respond to existing medications, it becomes obvious that there is great need for new therapeutic modalities. Temporal lobe epilepsy (TLE), seen in approximately one-third of epileptic patients, is typically characterized by partial seizures and hippocampal degeneration and is associated with learning and memory impairments [1]. Notably, TLE is among the types of epilepsy that are often drug-resistant. During the last decade, transplantation of neural stem/precursor cells (NSCs), in their native state or genetically modified to express particular molecules with neuroprotective potential, including various growth factors, has emerged as a possible therapeutic strategy for neurological diseases, such as Parkinson’s or spinal cord injury [2–5]. This approach has recently received attention as an alternative prospective therapy for TLE [6–9].

Neurotrophic factors appear to be promising candidates as therapeutic agents. Among them, insulin-like growth factor I (IGF-I) has been shown to act as a neuroprotectant in a number of experimental systems of neurodegeneration, such as ischemia [10], penetrating brain injury [11], or stroke [12], and in animal models of neurological diseases, such as amyotrophic lateral sclerosis [13], multiple sclerosis [14], Alzheimer’s...
disease [15], Parkinson’s disease [16], and cerebellar ataxia [17]. In previous work from our laboratory [18, 19] we have shown that IGF-I can also ameliorate the cellular degenerative processes following kainic acid administration in the rat and mouse hippocampus, a well-characterized model of TLE in rodents.

Based on the above, in the present work we assessed the regenerative potential of subventricular zone (SVZ)-derived mouse NSCs either transduced or not with a lentiviral vector for overexpression of IGF-I following kainic acid-induced hippocampal degeneration in mice. Of relevance, in a previous study we have shown that ex vivo IGF-I transduction of postnatal SVZ-derived NSCs can enhance their differentiation toward a neuronal phenotype [20]. This characteristic suggested that these cells may be useful as potential therapeutic agents in neurodegenerative processes.

In this study, NSCs were generated from the SVZ of 5-day-old transgenic mice expressing the green fluorescent protein (GFP) under control of the ubiquitous β-actin promoter (actin-GFP mice) and were transplanted into the hippocampus of adult mice that had received kainic acid intrahippocampally. The transplanted NSCs were either transduced ex vivo prior to grafting with a lentiviral vector driving the expression of IGF-I, or not. We then followed up to 2 months their fate in vivo regarding survival, migration, and differentiation into neurons and glial cells. Furthermore, we assessed their therapeutic potential at both (a) the functional, behavioral level by monitoring their performance in the Morris water maze, a hippocampus-dependent task, and (b) the cellular level by evaluating hippocampal degeneration, granule cell dispersal, and degree of astrogliosis, as well as (c) hippocampal neurogenesis, which is known to increase following kainic acid (KA) administration. It should be noted that the KA-induced increase in neurogenesis is considered to be aberrant and possibly pathogenic [21, 22]. Our experimental hypothesis was that the transplanted NSCs, particularly those transduced with the IGF-I lentiviral vector, would survive, differentiate into neurons, and exert protective effects on the host hippocampus, ameliorating the degenerative processes and the accompanying cognitive decline.

**Materials and Methods**

**Neurosphere Culture and Lentiviral Transduction**

Neurosphere cultures were prepared from the SVZ area of postnatal day 5 (P5) transgenic C57BL/6J mice expressing GFP (GFP-NSCs) under control of the ubiquitous β-actin promoter (actin-GFP mice; Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) and were maintained in a serum-free medium containing 20 ng/ml recombinant human basic fibroblast growth factor (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) and 20 ng/ml epidermal growth factor (R&D Systems) [20, 23]. The lentiviral vector Trip.IGF-I used for IGF-I expression under control of the cytomegalovirus promoter has been previously described [20]. For lentiviral transduction, at least third-passage neurospheres were single-cell dissociated, grown in suspension for 2–3 days, and then exposed for 6–8 hours to nonconcentrated Trip.IGF-I viral supernatant. Three or four viral copies of the transgene were integrated in the genome of Trip.IGF-I-transduced NSCs as estimated by quantitative polymerase chain reaction using a unique sequence within the pTrip vector [20]. The levels of IGF-I secretion by Trip.IGF-I-transduced NSCs were 1,104 ± 275 pg of IGF-I per 10^6 cells (n = 5) as determined by enzyme-linked immunosorbent assay (ELISA) 4 days post-transduction, whereas IGF-I was not detectable in the culture supernatant of nontransduced NSCs or NSCs transduced with a control lentiviral vector driving the expression of GFP [20].

**Stereotaxic Kainic Acid Injection for Induction of Hippocampal Degeneration and Cell Transplantation**

All animals were handled according to European Union (E.U.) legislation for animal rights (86/906/EEC and 2007/526/EU). Experiments were performed on adult male C57BL/6J mice 2.5–3 months old housed in groups of three and maintained under controlled temperature (24 ± 1°C) and lighting (12:12 hour light-dark cycle) conditions. The number of animals used in each experiment is shown in the respective figure legends.

Mice were deeply anesthetized by intraperitoneal injection of 300 mg/kg of body weight chloral hydrate and positioned in a stereotaxic apparatus. The dorsal surface of the skull was exposed through a midline incision and a burr hole was drilled at the following coordinates: antero-posterior, −1.6 mm caudal to bregma; lateral, 1.8 mm to the midline on the right side. A 5-μl Hamilton syringe (Hamilton, Bonaduz, Switzerland, http://www.hamiltoncompany.com) fitted with a 26-gauge needle was slowly inserted into the brain 2.4 mm from the surface (ventral). KA (Sigma-Aldrich, Steinheim, Germany, http://www.sigmaaldrich.com) in a total volume of 0.4 μl (1 μg/μl freshly prepared solution in phosphate buffered saline, pH 7.4) was administered intrahippocampally [24]. The needle was left in place for an extra 15 minutes and then withdrawn gently. The skin was sutured, a local anesthetic containing 25 mg/g lidocaine and 25 mg/g pilocarpin was applied, and the animals were kept warm until they were fully awake. As intrahippocampal KA administration induces seizure activity, mice were videorecorded for the next 5 hours following injection, and their behavior was analyzed and scored off-line for signs of seizure activity [25]. Behavioral manifestation of seizure activity was evident within 5 minutes from awakening and was maintained throughout the 5 hours of observation with a severity similar to that previously described, documenting the epileptogenic efficacy of kainic acid [18]. Sham-operated (control [CTR]) animals were anesthetized, operated as described above, and injected intrahippocampally with 0.4 μl of saline.

Four days after unilateral KA administration, two groups of animals were injected unilaterally with 1 μl of freshly dissociated GFP-NSCs from actin-GFP mice (10^6 cells), either transduced with Trip.IGF-I or not. Injection was performed within the anterior part of the hippocampus, 600 μm rostrally to the site of KA injection. Cells were injected slowly over 5 minutes, the syringe was left in place for an extra 5 minutes and then withdrawn gently, and the skin was sutured. Two additional groups of animals (control-saline injected and KA-injected) received an intrahippocampal injection of 1 μl of Hanks’ balanced saline solution. After surgery, all mice were kept on a heated cushion before being returned to their home cages. Animals were sacrificed 8, 30, and 60 days after transplantation surgery.

**Behavioral Analysis of Learning and Memory, Histology and Immunohistochemistry, Tissue Homogenization for ELISA, Cell Quantification, and Statistical Analysis**

Detailed methods are presented in the supplemental online Materials and Methods.
Severe cognitive impairment and extensive bilateral hippocampal degeneration were induced following unilateral intrahippocampal KA injection, in agreement with our previous results [18]. Four days after KA injection, NSCs derived from actin-GFP mice were transplanted into the right anterior hippocampus, 600 μm rostrally to the site of KA injection. Prior to grafting, NSC were either transduced with the Trip.IGF-I lentiviral vector to express IGF-I (Trip.IGF-I-GFP-NSCs) or not (GFP-NSCs). We have previously obtained evidence that the in vitro proliferation/differentiation characteristics of NSCs from wild-type mice—transduced or not with a control lentiviral vector that drives expression of GFP—as well as the properties of NSCs derived from actin-GFP mice are indistinguishable [20, 26]. Therefore, in the present study we generated two groups of animals that received cell transplants after KA treatment: one group that received Trip.IGF-I-GFP-NSCs (KA-Trip.IGF-I-GFP group) and one that received nontransduced GFP-NSCs (KA-GFP group). Two additional groups consisted of animals that received no cells: one that received only KA treatment (KA group) and one that was sham-operated and received only saline (CTR group).

**Effect of NSC Transplantation in the Morris Water Maze**

Performance of KA-Treated Animals

Intrahippocampal administration of kainic acid resulted in a severe cognitive deficit in performance in the hippocampus-dependent task of the Morris water maze, affecting both learning and memory, as previously described [18]. NSC transplantation exerted a beneficial effect and restored cognitive abilities. Although all groups of animals managed to learn the task, showing a decrease in the latency to find the hidden platform during the 5 days of training ($p < .001$), a significant treatment × day effect was observed on latency ($p = .023$; Fig. 1A). Further analysis showed that on the first and second days of training the CTR group of animals performed better than the other three experimental groups ($p < .05$ for control vs. all other groups). Most interestingly, during the next days of training, animals transplanted with cells (either Trip.IGF-I-transduced or not) performed equally well as those in the CTR group, whereas animals in the KA group that had received no transplants kept on showing learning deficits ($p < .05$ for KA vs. all other groups). To ensure that the group differences observed were not due to differences in motor ability, swim speed was also determined. All mice, irrespective of group (KA, KA-Trip.IGF-I-GFP, KA-GFP, or CTR), appeared to swim normally and climbed normally on the hidden platform provided. No difference was observed in the animals’ swim speed during the Morris water maze task (data not shown).

In both the short-term and long-term memory probe trials (1 and 24 hours after the last training trial; Fig. 1B and 1C, respectively), mice in the KA group exhibited a marked mnemonic deficit showing no quadrant preference (treatment × quadrant interaction: for the short-term probe trial, $p = .045$, and for the long-term probe trial, $p < .001$, post hoc $p = .297$ and $p = .434$, respectively), whereas the other three groups of animals preferred the target quadrant (post hoc $p < .001$ for all three groups of animals). We conclude that KA-treated animals are characterized by a severe cognitive impairment that can be reversed by transplantation of NSCs either transduced or not with Trip.IGF-I.

**KA-Induced Hippocampal Degeneration and Effect of NSC Transplantation on Hippocampal Cytoarchitecture**

We then investigated the kainate-induced hippocampal degeneration underlying cognitive deficits [18] and the therapeutic effects of NSC transplantation at the cellular level. Extensive bilateral hippocampal degeneration was observed with cresyl violet staining 8, 30, and 60 days following unilateral intrahippocampal KA administration (Figs. 2, 3). In area 1 of cornus ammonis (CA1 area), KA induced a marked cell loss at all three time points studied, both ipsi- and contrateralateral to the injection site (side × treatment effect: $p < .001$; post hoc $p < .001$, for both sides). Interestingly, at all time points transplantation of cells, either Trip.IGF-I-transduced or not, partially protected against the KA-induced decrease in the number of cresyl violet cells in both the ipsi- and the contralateral CA1 (post hoc $p < .001$ for both the comparisons with CTR and KA groups of animals).

In the CA3 area, KA significantly reduced the number of stained cells in both hemispheres at all three time points (side × treatment × time effect: $p < .001$; post hoc $p < .001$, for both sides). Transplantation of either Trip.IGF-I-transduced cells or nontransduced cells fully protected against the KA effects on cell numbers in the contralateral CA3 at all time points studied (post hoc $p < .005$ for the comparisons with the KA group of animals). Notably, in the ipsilateral CA3 area, transplantation of Trip.IGF-I-transduced cells had different effects than the transplantation of nontransduced cells: at the earliest time point studied (8 days following transplantation), Trip.IGF-I-transduced cells partially prevented the KA-induced decrease in cell numbers, whereas nontransduced cells had no protective effect (post hoc $p = .042$ vs. CTR, $p < .001$ vs. KA or KA-GFP group). Interestingly, both types of cells acted neuroprotectively at later time points (30 and 60 days after transplantation), partially inhibiting cell loss (post hoc $p < .001$ vs. either CTR or KA groups).

Similarly to the CA3 area, in CA4 kainic acid administration caused a dramatic decline in the number of stained cells in both hemispheres at all three time points (side × treatment × time effect: $p < .018$; post hoc $p < .001$, for both sides). Cell transplantation could not protect against this KA-induced neurodegeneration in the ipsilateral CA4 at any of the time points examined (post hoc $p < .001$ for both the KA-Trip.IGF-I-GFP group and the KA-GFP group). Notably, though, in the contralateral CA4 cell transplantation of either Trip.IGF-I-transduced or nontransduced cells) partially protected against the KA-induced cell loss at 30 and 60 days after transplantation (post hoc $p < .001$ vs. CTR and $p < .05$ vs. the KA group; data not shown).

In the dentate gyrus (DG), KA injection led to bilateral decrease in the number of cresyl violet-stained cells at all three time points (side × treatment × time effect: $p = .004$; post hoc $p < .01$, for both hemispheres). In the contralateral DG, cell transplantation had no effect on the number of stained cells, whereas in the ipsilateral side only transplantation of Trip.IGF-I-transduced cells partially ameliorated the effects of KA-injection on cell numbers at all time points studied (post hoc $p < .05$ for KA-Trip.IGF-I-GFP group vs. control as well as vs. KA or KA-GFP; Figs. 2, 3). Moreover, in the ipsilateral DG the characteristic KA-induced granule cell dispersal was evident, along with a marked hypochromicity of the remaining granule cells in the overwhelming majority of both the KA-injected animals (83% of the cases) and the KA-GFP ones (78%). These phenomena were prevented to a significant extent when Trip.IGF-I-transduced cells were transplanted, as only 39% of the animals in
this group showed granule cell dispersal (Fig. 2). These results indicate that transplantation in KA-treated animals of NSCs, whether Trip.IGF-I-transduced or not, has a significant neuroprotective effect, with the Trip.IGF-I-transduced cells in some cases acting earlier (i.e., in the CA3 area) and in other cases (i.e., in the DG) being more beneficial.

**Effect of NSC Transplantation on Astroglial Reaction to the KA-Induced Hippocampal Lesion**

The KA-induced hippocampal lesion was accompanied by the emergence of large numbers of reactive astrocytes. Glial fibrillary acidic protein (GFAP) immunohistochemistry stained both...
hypochromicity and granule cell dispersal in the contralateral CA3 transplanted with cells (either Trip.IGF-I-transduced or not) the degeneration in the ipsilateral CA3 area was markedly reduced. IP injection of Trip.IGF-I-transduced cells (either Trip.IGF-I-transduced or not) partially protected the CA1 area both ipsilaterally (B, C, I, J, P, Q), KA plus transplantation of GFP cells (D, E, K, L, R, S), and KA plus transplantation of Trip.IGF-I-transduced-GFP cells (F, G, M, N, T, U). Administration of KA resulted in degeneration of the CA1 area both ipsilaterally (C) and contralaterally (B) to the injection site (compare with sham-operated animal in [A]). Transplantation of cells (either Trip.IGF-I-transduced or not) partially protected the CA1 area both ipsilaterally (I) and contralaterally (J), respectively. Following KA injection, the CA3 area was severely affected ipsilaterally (I) and to some extent contralaterally (J). In animals transplanted with cells (either Trip.IGF-I-transduced or not) the degeneration in the ipsilateral CA3 area was markedly reduced (N, L), whereas in the contralateral CA3 (M, K) it was totally prevented. The DG was severely disrupted by KA administration mainly ipsilaterally, showing hypochromicity and granule cell dispersal (Q), whereas in the contralateral hemisphere degeneration was of a much smaller extent (P). Cell transplantation had no influence on the effects of KA in the DG of the contralateral hemisphere (R, T), whereas in the ipsilateral side only transplantation with Trip.IGF-I-transduced cells had a beneficial effect, partially preventing degeneration ([U], compare with [S]). Scale bar = 30 μm. Abbreviations: CA, cornus ammonis; CTR, control; DG, dentate gyrus; GFP, green fluorescent protein; IGF-I, insulin-like growth factor I; KA, kainic acid.

Dentate Gyrus Subgranular Zone Neurogenesis

We next examined the fate of grafted NSCs in the two groups of animals that received cell grafts following KA administration at 8, 30, and 60 days after transplantation (Fig. 6A, 6B). Survival of normal and reactive astrocytes [27]. However, reactive astrocytes were easily detected based on their characteristic morphology with increased cell size as well as enlarged and branched processes (Fig. 4). In all KA-injected animals, at all three time points examined (8, 30, and 60 days) the appearance of reactive astrocytes and the intensity of GFAP labeling were increased, with a more intense activation in the ipsilateral hemisphere (treatment × side of the brain: p < .001; post hoc p < .001, vs. control for both hemispheres). Most interestingly, administration of cells, either Trip.IGF-I-transduced or not, partially prevented the KA-induced astroglial reaction, decreasing GFAP immunoreactivity (post hoc p < .002 for both comparisons vs. KA and for both hemispheres), without, however, reaching control levels (post hoc p < .006 for both comparisons vs. control and for both hemispheres). Therefore, it appears that NSC transplantation contributes to a reduction of astroglial activation, which may be beneficial for enhancing regeneration.

Effect of NSC Transplantation on KA-Induced Increase in Dentate Gyrus Subgranular Zone Neurogenesis

KA administration led to enhanced precursor cell proliferation, as assessed by the number of Ki67-immunopositive cells in the dentate gyrus subgranular zone, compared with the levels of cell proliferation in control animals, at both the early and intermediate time points examined (8 and 30 days), whereas no difference was observed following more prolonged survival (60 days) (Fig. 5A–5C, 5E, 5F; treatment × time effect: p = .020; post hoc KA vs. control p < .005 for both 8 and 30 days). Interestingly, after transplantation of NSCs (either Trip.IGF-I-transduced or not-transduced) the number of Ki67-immunopositive cells at 30 days was restored to almost normal levels as compared with that following KA injection (Fig. 5C–5F; post hoc KA vs. KA-GFP or vs. KA-Trip.IGF-I-GFP group p < .05).

To examine whether KA-induced cell proliferation was associated with increased generation of newborn neurons from endogenous precursor cells, we estimated the number of proliferating precursor cells expressing the transcription factor prospero-related homeobox 1 (Prox1), which is a marker for proliferating neuroblasts in the dentate gyrus of the hippocampus, being expressed earlier than doublecortin (DCX) [28], whereas its expression is also evident in differentiated granule neurons (Fig. 5G) [28]. We found that the Ki67+/Prox1 double-immunopositive cells were significantly increased in the KA-group at 8 days as compared with the control group (Fig. 5H, 5I; post hoc KA vs. control p = .007), whereas the increase was significantly smaller in the two groups that had received cell transplants (either Trip.IGF-I-transduced or not-transduced NSCs; Fig. 5H, 5I; post hoc KA-Trip.IGF-I-GFP group vs. control p = .126; KA-GFP group vs. control p = .213). At later time points, 30 and 60 days, the levels of Ki67+/Prox1 double-immunopositive cells were similar in all groups of mice (Fig. 5H, 5I; post hoc KA vs. control p = .839 for 30 days and p = .182 for 60 days; KA-Trip.IGF-I-GFP group vs. control p = .304 for 30 days and p = .243 for 60 days; KA-GFP group vs. control p = .488 for 30 days and p = .150 for 60 days).

NSC Fate After Transplantation in the Hippocampus of KA-Treated Mice

Next we examined the fate of grafted NSCs in the two groups of animals that received cell grafts following KA administration at 8, 30, and 60 days after transplantation (Fig. 6A, 6B). Survival of

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Figure 3. Quantification of cresyl violet-stained cells in CA1 (A), CA3 (B), and DG (C) hippocampal areas 8, 30, and 60 days following cell transplantation in KA-injected animals. For the histological staining, six animals per experimental group and time point were used. Bars represent means ± SEM. At all three time points examined, KA injection resulted in reduced numbers of stained cells in the CA1 area both ipsi- and contralaterally to the injection site (KA vs. control: ‡‡‡, p < .001; KA vs. KA plus GFP cells: †††, p < .001; KA vs. KA plus Trip.IGF-I-transduced cells: ###, p < .001) although not capable of complete prevention (control vs. KA plus GFP cells: §§§, p < .001; control vs. KA plus Trip.IGF-I-transduced cells: †††, p < .001). In the CA3 area, KA significantly reduced the number of stained cells in both hemispheres at all three time points (KA vs. control: ‡‡‡, p < .001; KA vs. KA plus GFP cells: †††, p < .001; KA vs. KA plus Trip.IGF-I-transduced cells: ###, p < .001). In the ipsilateral CA3 area, 8 days following transplantation IGF-I-transduced cells partially prevented the KA-induced decrease in cell numbers (control vs. KA plus Trip.IGF-I-transduced cells: †, p < .05; KA vs. KA plus Trip.IGF-I-transduced cells: #, p < .05). In the contralateral DG, cell transplantation had no significant effect on the number of stained cells (control vs. KA plus GFP cells: §§§, p < .001; control vs. KA plus Trip.IGF-I-transduced cells: ††, p < .05). Notably, in the ipsilateral side transplantation of Trip.IGF-I-transduced cells partially ameliorated the effects of KA-injection on cell numbers at all time points studied (control vs. KA plus Trip.IGF-I-transduced cells: †, p < .05; ††, p < .01; †††, p < .001; KA vs. KA plus Trip.IGF-I-transduced cells: §, p < .05, §§§, p < .001). In the contralateral DG, cell transplantation had no significant effect on the number of stained cells (control vs. KA plus GFP cells: §§§, p < .001; KA vs. KA plus Trip.IGF-I-transduced cells: §§§, p < .001). Abbreviations: CA, cornus ammonis; contra, contralateral; CTR, control; DG, dentate gyrus; GFP, green fluorescent protein; IGF-I, insulin-like growth factor I; ipsi, ipsilateral; KA, kainic acid.
grafted cells was approximately 25% (Fig. 6C) in both groups and at all time points examined. A number of cells migrated posterior to the site of transplantation toward the lesioned area, similarly to the findings of Hartman et al. [29], although clusters of grafted cells remained at the site of transplantation. In some cases grafted cells could be found in the oriens layer or within the CA3 area of the hippocampus (Fig. 6A–6D).

IGF-I expression was detectable by immunofluorescence (data not shown) in a larger fraction of the grafted cells in the KA-Trip.IGF-I-GFP group as compared with the KA-GFP group 8 days after transplantation (66 ± 16% of cells were IGF-I+/GFP+ in the KA-Trip.IGF-I-transduced cells; ‡‡, p < .01; KA vs. KA plus Trip.IGF-I-transduced cells: ##, p < .01, for both hemispheres), whereas at 30 and 60 days no difference was observed between the two groups (75 ± 8% vs. 65 ± 4% at 30 days and 78 ± 7% vs. 67 ± 10% at 60 days, respectively). Therefore it appears that although IGF-I is not detectable in proliferating non-transduced NSCs in vitro [20], these cells gradually become IGF-I-positive in vivo. In agreement, as determined by ELISA, 60 days after transplantation hippocampal levels of IGF-I were similar in the two groups of animals that had received cell transplants, whereas they were higher compared with those in the KA or CTR group of animals (main effect of treatment p < .006, post hoc tests p < .05 for both comparisons; supplemental online Fig. 1).

To assess whether NSCs retained their proliferative potential 60 days after transplantation, the two groups of KA-treated animals that received cell grafts were given a 2-hour 5-bromo-2'-deoxyuridine (BrdU) pulse, after which the animals were sacrificed. No grafted cells, whether Trip.IGF-I-transduced or not, were found to incorporate BrdU at this time point, indicating that the grafted NSCs had lost their proliferative potential by 60 days postgrafting.

The expression of the NSC marker nestin was assessed in order to explore the neural stem/precursor cell identity of grafted cells. Nestin is highly expressed in cultured NSCs, and more than 70% of cells were nestin-positive 1 day prior to transplantation (data not shown). Eight days after transplantation, a
Figure 5. Effects of kainic acid (KA) or kainic acid plus neural stem/precursor cell (NSC) transplantation on proliferating cells (A–F) and proliferating neuronal precursors in the subgranular zone of the hippocampal dentate gyrus (G–I). Shown are representative photomicrographs of Ki67-immunopositive cells (red) in the ipsilateral hippocampus of animals sham-operated (A, CTR) or injected with either KA (B, 8 days; C, 30 days) or KA plus Trip.IGF-I-transduced cells (D, 30 days). Nuclei were stained with TO-PRO3 (blue). Scale bar = 80 μm. For quantification, four to six animals per experimental group and time point were used (E, F). Bars represent means ± SEM per brain section ipsilaterally (E) and contralaterally (F). KA resulted in increased cell proliferation at 8 days (B) and 30 days (C) in both the ipsilateral (F) and the contralateral (G) hippocampi compared with control animals (CTR) (A) (post hoc KA vs. control: **, p < .005 for both 8 and 30 days). Eight days...
significant fraction of the grafted cells still expressed nestin (37 ± 4% of Trip.IGF-I-GFP-NSCs vs. 20 ± 2% of GFP-NSCs, p = .002), but its expression was reduced at subsequent time points in both Trip.IGF-I-GFP-NSCs and control GFP-NSCs (at 30 days: 16 ± 3% vs. 7 ± 2%, p = .030; at 60 days 9 ± 2% vs. 7 ± 2%, p = .112, respectively; Fig. 6E–6H). Our data indicate that both types of grafted NSCs eventually downregulate nestin, indicating that they gradually lose their immature neural stem/precursor cell identity and differentiate.

**NSCs Generate Neurons and Not Glial Cells After Transplantation in the KA-Lesioned Hippocampus**

We then assessed the differentiation potential of transplanted cells in the two groups of animals that received either Trip.IGF-I-GFP-NSCs or GFP-NSCs after KA treatment. This analysis was performed at 30 and 60 days after transplantation, when differentiation was clearly detectable. Immunofluorescence labeling, which clearly detected host GFAP (Fig. 4; supplemental online Fig. 2) and O4 immunopositive cells (supplemental online Fig. 3), failed to reveal among the grafted cells practically any, expressing either the astrocytic marker GFAP or the oligodendrocytic marker O4 at these time points (supplemental online Figs. 2, 3). On the other hand, we found that practically all transplanted cells differentiated into the neuronal lineage in both groups of mice at 30 and 60 days. Analysis of the expression of DCX, which labels both neuronal progenitors and differentiated immature neurons, showed that a large proportion of the transplanted cells were DCX⁺ both at 30 and 60 days in both groups of mice. Moreover, the percentage of DCX⁺/GFP⁺ cells was higher in the KA-Trip.IGF-I-GFP group as compared with the KA-GFP group (at 30 days: 87 ± 2% vs. 57 ± 13%, p = .038; at 60 days: 89 ± 5% vs. 57 ± 5%, p = .004). Persistent DCX expression in a high percentage of graft-derived neurons was rather unexpected, suggesting that either graft-derived neurons remained immature for prolonged periods or that a fraction of the graft-derived DCX⁺ population was generated later, a few weeks before the animals were sacrificed.

Analysis of the expression of the neuronal marker NeuN revealed that the percentage of grafted cells that had differentiated into more mature neurons was significantly higher in the KA-Trip.IGF-I-GFP group as compared with the KA-GFP group 30 days after transplantation, whereas there were no significant differences between the two groups at 60 days (Fig. 7A–7C, 7G). Quantification indicated that the percentage of NeuN⁺/GFP⁺ cells in the KA-Trip.IGF-I-GFP group was 1.6-fold higher as compared with the KA-GFP group at 30 days (37 ± 4% vs. 23 ± 4%, p = .025), whereas the percentage of graft-derived NeuN⁺/GFP⁺ cells was similar in both groups at 60 days (37 ± 2% vs. 35 ± 4%, p = .640; Fig. 7G).

Finally, at 60 days post-transplantation, a considerable fraction of the grafted cells had differentiated into glutamatergic neurons, as revealed by immunofluorescence for the glutamate transporter EAAC1 (14 ± 3% in the KA-Trip.IGF-I-GFP group vs. 12 ± 2% in the KA-GFP group, p = .379; Fig. 7D–7F, 7H). Surprisingly, no differentiation into the GABAergic phenotype, as assessed by GFP⁺/GAD⁺ double-positive cells, was detected, although host hippocampal GAD⁺ interneurons were clearly identified, verifying the specificity of labeling. These results indicate that, after engraftment in the KA-treated hippocampus, NSCs differentiated efficiently into neurons, including glutamatergic cells. Notably, the Trip.IGF-I-transduced NSCs differentiated earlier into a neuronal phenotype, in agreement with their previously observed enhanced neuronal differentiation in vitro [20].

**DISCUSSION**

Temporal lobe epilepsy is a major neurological disease, which is still in need of new effective therapeutic approaches. Recently the use of cell therapies has been explored in animal models of TLE, in an effort to address this issue [6, 7, 30]. Stem cells, unmodified [31–34] or modified to secrete antiepileptic factors [35–37], have been shown to act neuroprotectively and to ameliorate TLE symptoms to a certain extent. Nevertheless, recovery of the cognitive deficit associated with TLE has not been demonstrated so far. In our present work we show for the first time that transplantation of neural precursor cells derived from the early postnatal SVZ into the adult KA-treated hippocampus ameliorated cellular changes and, most importantly, cognitive decline. Thus, cell transplantation reduced cell loss in the CA1 and CA3 hippocampal areas, and additionally, it decreased astroglial reaction, which is well documented to accompany neurodegeneration following a variety of insults [38–41]. In particular for epilepsy, the process of astrocyte proliferation and activation could participate in the etiopathogenic mechanisms, since astrocytes have been shown to contribute to hyperexcitability [42–45]. Furthermore, transplantation of NSCs contributed in maintaining the number of proliferating granule cells in the hippocampal subgranular zone at normal levels.

It should be pointed out that KA administration could drive cells into a state of chromatolysis and thus prevents their staining with cresyl violet, resulting in reduced numbers of cells. In the absence of any neuroprotection, the cells proceed on to die. The neuroprotective effects of the transplanted NSCs reverse this process by aiding the recovery of the cells from the state of chromatolysis [46] into a normal cresyl violet staining condition, resulting in the detection of increased number of cells.

following NSC transplantation (either Trip.IGF-I-transduced or nontransduced) the number of Ki67-immunopositive cells did not differ from that following KA injection (E, F) (control vs. KA-GFP: §, p < .005; control vs. KA-Trip.IGF-I-GFP: ‡, p < .005), whereas at 30 days it was significantly reduced (E, F) compared with that following KA injection (C) (post hoc KA vs. KA-GFP: †, p < .05, or KA vs. KA-Trip.IGF-I-GFP group: ‡, p < .05), reaching almost normal levels. (G): Representative photomicrographs of Ki67 (red) and Prox1 (cyan) immunopositive cells (arrowheads) in the ipsilateral hippocampus of animals injected with KA (8 days). Colocalization is verified in orthogonal sections. Scale bar = 20 μm. (H, I): Quantification of the number of Ki67⁺/Prox1⁺ cells per section 8, 30, and 60 days after transplantation. Bars represent means ± SEM per brain section ipsilaterally (H) and contrateraterally (I). Ki67⁺/Prox1⁺ double-immunopositive cells were significantly increased in the KA group at 8 days as compared with the control group (post hoc HOA vs. control: #, p < .01), whereas the increase was significantly smaller in the two groups that had received cell transplants (either Trip.IGF-I-transduced or nontransduced NSCs): post hoc KA-Trip.IGF-I-GFP group vs. control: p = .126; KA-GFP group vs. control: p = .213). At later time points, at 30 and 60 days, the levels of Ki67⁺/Prox1⁺ cells were similar in all groups of mice (post hoc KA vs. control: p = .839 for 30 days and p = .182 for 60 days; KA-Trip.IGF-I-GFP group vs. control: p = .304 for 30 days and p = .243 for 60 days; KA-GFP group vs. control: p = .488 for 30 days and p = .150 for 60 days). Abbreviations: CTR, control; GFP, green fluorescent protein; IGF-I, insulin-like growth factor I; KA, kainic acid.
The neuroprotective effects of NSC transplantation following kainic acid-induced hippocampal degeneration were bilateral in the case of the CA1 and CA3 areas of the hippocampus, which receive strong, topographically organized projection from the opposite CA3 via the hippocampal commissure [47–49]. Thus the beneficial effects in the contralateral CA1 and CA3 could be due to transport of IGF-I from the site of NSC transplantation (close to the CA3 area) via these commissural fibers. It should be noted that in the CA3 area a fair number of cells remained in spite of the degeneration caused by kainic acid. In contrast, in the CA4 area practically all cells were eliminated, including the projection neurons to the contralateral DG [47]. Thus, IGF-I or other growth factors secreted by the transplanted NSCs would have no way to be transported and exert a neuroprotective effect in the contralateral DG. These phenomena could underline our finding that in the DG the neuroprotective effects were restricted to the side ipsilateral to transplantation.

It is noteworthy that the neuroprotective effects of NSC transplantation in the CA3 area, one of the most vulnerable to neurotoxic insults hippocampal areas [50], were more prominent in the hemisphere contralateral to the site of injection, indicating that NSCs ameliorated degenerative processes involved in “secondary” brain injury. It should also be remembered that the CA3 area greatly contributes to the processes underlying hippocampal-dependent learning and memory [51–53]. This finding is of potential clinical relevance, since in most patients secondary brain injury is the cause of debilitation in cognitive abilities and/or death [54, 55]. Most interestingly, NSC transplantation improved the performance of the animals in the Morris water maze. Notably, the neuroprotective effects occurred earlier and were more pronounced (as in the reduced cell loss and reversal of the characteristic kainic acid-induced granule cell dispersal observed in the DG) when the transplanted cells were genetically modified, using a lentiviral vector to express IGF-I.

An interesting and at first glance counterintuitive result that has been well documented recently is that KA administration increases adult hippocampal neurogenesis [28, 56]. However, these newborn neurons are considered aberrant and possibly even constitute foci of neuronal hyperexcitability [21, 22]. Indeed, in the present work we verify that KA administration led to increased hippocampal neurogenesis as assessed by estimating the number of proliferating cells in the subgranular zone of the hippocampus as well as the generation of Prox1+/Kit67+ newborn neurons; transplantation of NSCs suppressed these phenomena.

The neuroprotective effects of the transplanted cells could be due, at least in part, to the secretion of IGF-I, since even transplantation of unmodified NSCs resulted in elevated levels of

Figure 6. NSC transplantation in the hippocampus of KA-treated mice. (A): Diagram illustrating the sites of KA injection (a) and NSC transplantation (b) in the mouse hippocampus; the two coronal sections of mouse brain that are represented correspond to the site of KA administration and, 600 μm rostrally, to the site of NSC transplantation. Grafted cells migrated posteriorly from the site of transplantation toward the lesioned area (c). (B): Schematic representation of viral transduction of NSCs derived from the postnatal subventricular zone of actin-GFP mice, prior to transplantation. (C, D): Low-power confocal images of coronal sections through the rostral hippocampus, showing IGF-I-transduced grafted cells detected by GFP fluorescence (green), 8 days (C) and 30 days (D) after transplantation. Survival of grafted cells was similar (around 25%) in the two groups of animals that received cell transplants, either IGF-I-transduced or not, at all three time points examined (n = 8 per experimental group and time point). Similar micrographs were also obtained with non-transduced cells. Scale bar = 75 μm. (E–H): Nestin immunoreactivity in grafted NSCs. (E–G): Confocal images of coronal sections immunostained for the NSC marker nestin (red) 8 days after transplantation of Trip.IGF-I-GFP-NSCs (green). Scale bar = 50 μm. (H): Quantification of the percentage of nestin+ cells out of all GFP+ cells 8, 30, and 60 days after transplantation (n = 6 per experimental group and time point). A significant fraction of the GFP cells expressed nestin 8 days after transplantation (37 ± 4% of Trip.IGF-I-GFP-NSCs vs. 20 ± 2% of GFP-NSCs, p = .002), but its expression was reduced at subsequent time points in both Trip.IGF-I-GFP-NSCs and control GFP-NSCs (at 30 days: 16 ± 3% vs. 7 ± 2%, p = .030; at 60 days: 9 ± 2% vs. 7 ± 2%, respectively). Abbreviations: CA, cornus ammonis; GFP, green fluorescent protein; IGF-I, insulin-like growth factor I; KA, kainic acid; NSC, neural stem/precursor cell; Or, oriens layer of hippocampus; SLu, stratum lucidum.
IGF-I in the host hippocampus, as shown by our immunohistochemistry and ELISA results. IGF-I has been shown to be neuroprotective in a number of systems, acting through its type I receptor and the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase-3β/β-catenin pathway [20, 57]. Most important in neuroprotection is the metabolic role of IGF-I leading to increased tissue glucose availability [58], since it is known that a crucial factor in excitotoxicity-induced neurodegeneration is a deficiency in cellular energy. Notably, in previous work from our laboratory, we have shown that IGF-I is antiepileptogenic in the kainic acid model of TLE [18].

The transplanted cells survived, migrated, and differentiated into neurons, and not glial cells, in agreement with the results of Carpentino et al. [59]. Interestingly, NSCs transduced with the IGF-I gene differentiated earlier into neurons, in accordance with our previous in vitro findings showing that this genetic modification results in the generation of more mature neurons, possessing longer neurites and capable of higher motility and tissue penetration [20]. Relevant to our previous and present results is the report that IGF-I stimulates neuroblast exit from the SVZ and migration toward the rostral migratory stream and the olfactory bulb, suggesting that it participates in the differentiation process of these cells in vivo [60]. Interestingly, more than 10% of the transplanted cells differentiated into the glutamatergic phenotype 2 months following transplantation, in agreement with previous observations showing that SVZ cells can indeed differentiate into glutamatergic neurons in vivo [61].

Figure 7. Grafted neural stem/precursor cells (NSCs) differentiate into neurons in the kainic acid (KA)-lesioned hippocampus, in both groups of transplanted mice at 30 and 60 days after transplantation. (A–C): Expression of the neuronal marker NeuN (red) in grafted Trip.IGF-I-transduced GFP-NSCs (green), 30 days after cell transplantation. The framed area in (C) is presented at higher magnification. Scale bar = 50 μm. (D–F): A significant fraction of the grafted cells differentiated into glutamatergic neurons at 60 days post-transplantation, as revealed by immunofluorescence for the glutamate transporter (GluT, red), with no differences between the two groups of grafted animals. Shown are Trip.IGF-GFP-NSCs. The framed area in (F) is also shown at higher magnification. Scale bar = 50 μm. (G): Quantification of the percentage of NeuN+ out of all grafted GFP+ cells 30 and 60 days after transplantation (n = 6 per experimental group and time point). Analysis of the expression of the neuronal marker NeuN revealed that the percentage of grafted cells that differentiated into neurons was significantly higher (1.6-fold) in the KA-Trip.IGF-I-GFP group as compared with the KA-GFP group 30 days after transplantation (37 ± 4% vs. 23 ± 4% respectively, p = .025), whereas there were no significant differences between the two groups at 60 days (37 ± 2% vs. 35 ± 4%, p = .640). (H): Quantification of the percentage of GluT+ out of all grafted GFP+ cells, 60 days after transplantation (n = 6 per experimental group; KA-Trip.IGF-I-GFP group, 14 ± 3% vs. KA-GFP group, 12 ± 2%, p = .379). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; IGF-I, insulin-like growth factor I; KA, kainic acid.
An unexpected finding in our study was that, although initially—at 8 days postgrafting—IGF-I levels were significantly higher in the hippocampi of mice that had received IGF-I-transduced NSCs, by 30 and 60 days this difference was no longer detectable. The initial difference in IGF-I expression is likely to account for the early observations relating to differential expression of precursor or neuronal markers, which were leveled off at later time points. We have previously noted that nontransduced proliferating NSCs, cultured in the presence of epidermal growth factor and fibroblast growth factor-2, do not express IGF-I in vitro. However, when these cells are induced to differentiate by removal of the growth factors, endogenous IGF-I expression is also induced, although at lower levels as compared with IGF-I-transduced NSCs [20]. According to our present data, it seems that the grafted NSCs were stimulated to express significant levels of IGF-I in vivo under the influence of the lesioned host environment. However, we cannot rule out the possibility that host cells were also induced to produce IGF-I under the influence of the graft. An increase in endogenous IGF-I levels has been previously reported to occur within the lesioned brain, as a response to neurodegenerative insult [39]. A similar mechanism, possibly potentiated by reciprocal host-graft interactions, may have resulted in elevated levels of IGF-I in the lesioned hippocampus irrespective of whether the transplanted cells were transduced or not. This is an interesting finding from a possible prospective therapeutic point of view, as it alleviates possible complications associated with the use of viral vectors. Apparently, IGF-I may not be the only reason for the observed beneficial effects stemming from NSC transplantation in our study, as additional cellular and/or molecular interactions within the host tissue may have contributed synergistically to the outcome.

Our results showing an ameliorative effect on cognition are different from those of Waldau et al. [34], who did not observe any effect on cognition following transplantation of medial ganglionic eminence (MGE)-derived neural stem cells in the kainic acid model of TLE, although they noted seizure suppression. In the latter work, the transplanted cells differentiated into GABAergic neurons, as expected from their source, since the MGE is the origin during embryogenesis of GABAergic interneurons [64]. In our case, we did not observe the generation of graft-derived GABAergic neurons, but we could detect differentiation into the glutamatergic phenotype. The differences between our results and those of Waldau et al. [34] further support the idea—also documented by others [59]—that the brain area from which NSCs are derived plays a critical role in the differentiation path they will follow after transplantation, in addition to the host environment [61].

**CONCLUSION**

Taken together the results of the present study demonstrate that SVZ-derived NSCs producing IGF-I can survive, migrate, and differentiate, to also yield glutamatergic neurons, when transplanted into the degenerating hippocampus following kainic acid administration. Furthermore, such transplantation ameliorates cellular damage, suppresses aberrant hippocampal neurogenesis, and, most importantly, restores cognitive function following kainic acid-induced hippocampal degeneration. These results indicate that NSC transplantation could be considered in the future as part of the clinical management of hippocampal degeneration and potentially of drug-resistant TLE.

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**AUTHOR CONTRIBUTIONS**

P.M. and P.N.K.: collection and assembly of data, data analysis and interpretation; G.K. and A.S.: collection and assembly of data, data analysis and interpretation, manuscript writing; R.M. and F.S.: conception and design, financial support, data analysis and interpretation, manuscript writing.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

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