Research Report

The combined therapy of intrahippocampal transplantation of adult neural stem cells and intraventricular erythropoietin-infusion ameliorates spontaneous recurrent seizures by suppression of abnormal mossy fiber sprouting

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ABSTRACT

Adult neural stem cells (NSCs) possess the potentials to self-renew and exert neuroprotection. In this study, we examined whether adult NSCs had anti-epileptic effects in rats with status epilepticus (SE) induced by kainic acid (KA) and whether co-administration of erythropoietin (EPO) enhanced anti-epileptic effects or cell survival. Adult NSCs were transplanted into KA-lesioned hippocampus with or without intracerebroventricular EPO infusion. Electronic encephalography (EEG) was recorded for 3 weeks after transplantation. The frequency of abnormal spikes in rats with NSC transplantation decreased significantly compared to those of rats without NSC transplantation. Most of the transplanted NSCs differentiated into GFAP-positive astrocytes. EPO infusion significantly enhanced the survival of NSCs, but not neuronal differentiation or migration. NSC transplantation increased the number of neuropeptide Y (NPY) and glutamic acid decarboxylase 67 (GAD67)-positive interneurons. NSC transplantation also suppressed mossy fiber sprouting into the inner molecular layer with subsequent reduction of hippocampal excitability, which finally prevented the development of spontaneous recurrent seizures in adult rats after KA-induced SE. This study might shed light on the cytoarchitectural mechanisms of temporal lobe epilepsy as well as clarify the effect of adult NSC transplantation with intracerebroventricular EPO infusion for temporal lobe epilepsy.

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1. Introduction

Temporal lobe epilepsy (TLE) is one of the most common forms of acquired refractory epilepsy. Healthy individuals may acquire TLE as the consequence of brain insults such as stroke, trauma and neurodegenerative disease. TLE is characterized by the progressive expansion of spontaneous recurrent motor seizures stemming from the limbic system...
regions, especially the hippocampus (Dudek and Sutula, 2007).

Epileptogenesis is the process that occurs during the period from initial acute seizures or head injury to the onset of chronic epilepsy. Numerous changes have been associated with the development of epileptogenesis and subsequent seizure manifestation, including neuronal loss, increased excitation, altered inhibition, circuitry rearrangements and individual synapse abnormalities (Tremblay and Ben-Ari, 1984; Babb et al., 1991; Sarkisian et al., 1997; Rao et al., 2006).

Among the several hypothetical mechanisms responsible for epileptogenesis in TLE, two anatomical and functional changes after acute seizure are of particular interests: the loss of vulnerable interneurons in the hilus and the formation of new recurrent excitatory circuits after mossy fiber sprouting.

The loss of inhibitory gamma amino butyric acid-ergic (GABAergic) interneurons in hilus is extensively observed in different kinds of animal TLE models (Dudek and Sutula, 2007). Many hilar neurons degenerate and are replaced by ectopic granule cells. As a result, normal granule cells and hilar ectopic granule cells become synaptically connected by recurrent mossy fibers forming a repetitive network. The network is unique to epileptic brain thus reducing the threshold for granule cell synchronization.

Hippocampal mossy fibers are the axons of dentate granule cells projecting to hippocampal CA3 pyramidal cells and mossy cells of the dentate hilus as well as a number of interneurons in these two areas (Babb et al., 1991; Rao et al., 2006). The appearance of Timm’s staining in the inner molecular layer of dentate gyrus is generally described as “recurrent mossy fibers” and thought to be due to the formation of new recurrent excitatory circuits. Furthermore, neuropeptide Y (NPY) is expressed de novo in the abnormal mossy fiber pathway. Immunohistochemical investigations revealed that a dense band of NPY was recognized in the inner molecular layer of TLE animals, which was consistent with mossy fiber sprouting in epileptic animals (Schwarzer et al., 1995). Abnormal mossy fiber sprouting into the inner molecular layer and the formation of new functional recurrent excitatory circuits are clearly detectable for at least 8 weeks from 2 weeks after completion of SE model (Rao et al., 2006). There is strong association between the development of progressive epilepsy and the degree of mossy fiber sprouting after limbic epilepsy (Lemos and Cavaiheiro, 1995; Buckmaster, 2004).

Then it emerged that the strategies to reform the hippocampus during the periods when pathological changes progressed by cell transplantation. In order to avoid the progress of epileptogenesis, cell transplantation using stem cells might compensate the lost inhibitory interneurons, promote the intrinsic neurogenesis of interneurons or secret various neurotrophic factors for neuroprotective effects. Historically, many kinds of cells have been transplanted to treat the epilepsy model of animals with subsequent demonstration of therapeutic and neurogeneic potentials (Zaman and Shetty, 2000; Turner and Shetty, 2003; Chu et al., 2004; Ruschenschmidt et al., 2005; Thompson, 2005; Thompson and Suchomelova, 2004; Güttinger et al., 2005; Shetty et al., 2005; Hattiangady et al., 2006).

Erythropoietin (EPO) is a glycoprotein produced primarily in the kidney but with localized production also in neural and non neural tissues. EPO was originally characterized as the principal regulator of erythropoiesis by inhibiting apoptosis and by stimulating the proliferation and differentiation of erythroid precursor cells. For over the last decade, a wide variety of experimental studies showed that EPO exerted a remarkable neuroprotection in both cell cultures and animal models of nervous system disorders, accompanied by the effects on neurovascular remodeling and neuronal replacement (Shingo et al., 2001; Lu et al., 2005; Iwai et al., 2007; Chen et al., 2008). Recently EPO is also considered as a neuroprotetant during the process of SE. Studies in rodent models of TLE revealed that EPO significantly antagonized the development of SE (Uzüm et al., 2006; Nadam et al., 2007; Yang et al., 2007).

Here we intended to explore whether transplantation of adult neural stem cells (NSCs) into hippocampus could ameliorate SE-induced electrophysiological, pathological alterations using an experimental animal model of SE. We also explored synergistic effects of EPO administration and transplantation of NSCs for SE model of rats.

2. Results

2.1. Characteristics of NSCs in vitro

Most of the NSCs were nestin positive (93±4.5%) after two passages. NSCs were differentiated mainly into GFAP-positive cells (62±7.4%) at 4 days after adding fetal bovine serum into the medium, although β-tubulin-positive cells were few (5.3±2.2%).

2.2. Transplanted NSCs alleviate abnormal spikes induced by KA injection as observed by EEG monitoring

After KA-lesion, all animals presented different degrees of abnormal spikes as manifested by high and sharp waves on the EEG monitoring (asterisks, Fig. 1A–F). During the first week after transplantation, no statistic differences of spike frequencies were found between the NSC+veh group and the other four control groups (43.4±7.8/min versus 50.6±1.2/min, 45.3±7.0/min, 60.3±6.3/min and 59.0±6.2/min as for NSC+veh, Fib+veh, Sal+veh and Sal+EPO and Sal+veh groups, respectively; P>0.05). However, rats in NSC+EPO group presented significant lower frequency of abnormal spikes (23.7±4.7/min) than the other 5 groups including the NSC+veh group (P<0.05; Fig. 1G, week 1). In the second week after transplantation, although no statistic difference was found between the two NSC-transplanted groups (28.1±2.4/min versus 32.6±6.0/min for NSC+EPO and NSC+veh groups, respectively; P=0.6318; Fig. 1G, week 2), they presented

Fig. 1 – NSC transplantation decreases the number of abnormal spikes induced by SE. Representative EEG recordings from NSC+veh (A), NSC+EPO (B), Fib+EPO (C), Fib+EPO (D), Sal+veh (E) and Sal+EPO (F) groups at 3 weeks after the electrode implantation showed the typical abnormal spikes (asterisks). (G) The graph showed the changes of abnormal spikes with time post transplantation among different groups. ’P<0.05 versus all the other five groups (week 1) and the four control groups (week 2 and week 3), **P<0.05 versus Sal+veh, Sal+EPO, Fib+veh and Fib+EPO groups.
lower frequencies of abnormal spikes, compared with the four control groups (56.6 ± 4.9/min, 49.5 ± 5.8/min, 61.3 ± 1.0/min and 57.7 ± 8.4/min for Fib+EPO, Fib+veh, Sal+EPO and Sal+veh groups, respectively; P < 0.05; Fig. 1G, week 2). In the third week after transplantation, the two NSC-transplanted groups still presented lower frequencies of spikes than the four control groups (27.4 ± 3.6/min and 30.9 ± 3.2/min versus 59.0 ± 5.8/min, 58.1 ± 6.0/min, 72.1 ± 14.7/min and 71.0 ± 9.6/min as for NSC+EPO, NSC+veh, Fib+EPO, Fib+veh, Sal+EPO and Sal+veh groups, respectively; P < 0.05; Fig. 1G, week 3). No significant difference was found between the two NSC-transplanted groups (P = 0.514).

2.3. Effects of EPO on the survival, migration and differentiation of transplanted NSCs

Consistent with previous reports, unilateral i.c.v injection of KA produced degeneration of ipsilateral CA3 pyramidal neurons and hippocampal atrophy compared with the contralateral side (Figs. 2A, B). All principal cell layers were nearly intact in the contralateral hippocampus as revealed by NeuN immunostaining.

Examination of the KA-lesioned hippocampus in NSC transplantation rats at 5 weeks after transplantation revealed graft survival in particular subregions. Transplanted NSCs survived in the CA3 region (Fig. 2C) and arrayed along the subgranular zone (Fig. 2D) indicating the migration of transplanted NSCs. Compared with NSCs, very few transplanted fibroblasts survived in the host brain expressing weak GFP and confined to the injection site forming clusters (Figs. 2E, F).

In previous reports, diverse differentiation of transplanted NSCs into astrocyte, neurons and oligodendrocyte could be found. In vitro experiments of this study, almost half of NSCs differentiated into GFAP-positive cells at 4 days after serum addition into the culture medium. In vivo experiments of the
Fig. 3 – Differentiation of transplanted NSCs. Representative images from the NSC+EPO group showing the differentiation of transplanted NSCs in the host hippocampus as visualized under a confocal microscope. Most of the grafted GFP-positive cells (B) differentiated into red GFAP-positive astrocytes (A). (C) Merged image of panels A and B. (D-F) Very few transplanted cells differentiated into red NeuN-positive mature neurons. (F) Merged image of panels D and E revealing that GFP/NeuN double-positive cells were located adjacent to dentate granular zone. Scale bars: 20 μm.
study, immunohistochemical investigations demonstrated that many transplanted NSCs with or without EPO administration expressed astrocytic marker GFAP with morphological changes (Figs. 3A–C) and few differentiated into mature neurons as positive for NeuN (Figs. 3D–F) implying that under the present condition, EPO may have little effects on the differentiation of transplanted cells in vivo. Additionally no transplanted cells were found double-labeled with doublecortin and GFP in either group in this experiment (data not shown).

We transplanted NSCs into CA3 region but many of the cells were found adjacent to DGZ at 5 weeks post-transplantation, so the evaluation of cell survival and migration was thoroughly performed. In the NSC+EPO group, more transplanted cells survived, compared with the NSC+veh group (9717.8±1759.7 cells/rat versus 3131.8±327.9 cells/rat; *P*<0.05; Fig. 4A) suggesting that EPO has potentials to enhance the survival of transplanted NSCs. Despite the fact that more transplanted cells migrated adjacent to the DGZ in the NSC+EPO group than in the NSC+veh group (5584.0±768.8 cells/rat versus 1678.8±289.6 cells/rat; *P*<0.05; Fig. 4A), there was no significant difference of migrating rate between the two groups (60.2±8.1% versus 56.4±12.7%; *P*>0.05; Fig. 4B) suggesting that EPO might had little effect on the migration ability of transplanted NSCs. The fact that higher numbers of transplanted cells migrated to the DGZ in the NSC+EPO group could be explained by that more cells survived in this group after transplantation.

As mentioned previously in this section, in two NSC transplantation groups, the evaluation of abnormal spikes displayed significant differences in the number of spikes, compared with the other four control groups. Subsequently, we explored if there were correlations between the numbers of overall abnormal spikes and surviving NSCs in each hippocampal subregion. Linear analyses revealed that the frequency of abnormal spikes had negative correlations with the number of surviving cells (Figs. 5A, B). There were tendencies that rats with more surviving cells displayed lesser spikes especially when grafts are located in the DGZ as calculated by linear regression (*r* = −0.536, *P* = 0.0135, for the relationship of the number of total surviving cells and average spikes; *r* = −0.902, *P*<0.0001, for the relationship of the number of cells migrating

![Fig. 4](image1.png)

**Fig. 4** – EPO enhanced the survival of transplanted NSCs but not cell migration. (A) The graph demonstrated that the surviving cell number of transplanted NSCs with EPO administration was significantly more than that in the NSC+veh group. (B) Percentage of transplanted cells migrating to the subgranular zone clarified no significant differences between NSC+EPO and NSC+veh groups. *P*<0.05 versus the NSC+veh group.

![Fig. 5](image2.png)

**Fig. 5** – Correlation between the average abnormal spikes and the numbers of surviving NSCs. (A) The number of total surviving cells and abnormal spikes was negatively correlated (*r* = −0.536, *P* = 0.0135). (B) The number of abnormal spikes and cells migrated near the dentate granular zone also has highly negative correlation (*r* = −0.902, *P*<0.0001).
presented a low density of Timm’s staining in the inner molecular layer of the lesioned hippocampus indicating aberrant MFS in these regions (Figs. 6C, D). There were also dense Timm’s stained cells in the dentate hilus of rats receiving NSC transplantation (Figs. 6E, F). However, the densities in the inner molecular layer were lesser than those of the four control groups (6.5±1.3 and 11.1±2.3 particles/μm² for NSC+EPO and NSC+veh versus 37.9±8.8, 30.1±4.8, 43.0±7.6 and 31.0±8.6 for Fib+EPO, Fib+veh, Sal+EPO and Sal+veh groups, respectively, P<0.05; Fig. 6G). Rats in the NSC+EPO group presented a low density of Timm’s staining in the inner molecular layer when comparing with the NSC+veh group, but no statistic significance was found (6.5±1.3 particles/μm² versus 11.1±2.3 particles/μm², P=0.1008).

In order to validate the results of Timm’s staining, we assessed the occurrence of aberrant MFS in representative sections of epileptic animals with NPY immunostaining. This also confirmed the presence of significant aberrant MFS in the inner molecular region in all rats at 5 weeks post-SE. NPY-stained particles were not detected in the inner molecular layer of normal control rats but some NPY-positive cells could be found in the hilus (Figs. 6H, I). In contrast, heavy NPY staining could be found in the inner molecular layer of all 6 KA-lesioned groups (Figs. 6J–M). The densities of NPY staining at the inner molecular region of two NSC-transplanted groups were lower than the four control groups (9.8±0.8 and 7.9±2.4 particles/μm² for NSC+EPO and NSC+veh groups versus 25.5±2.9, 22.9±3.2, 27.2±3.1 and 19.6±1.0 particles/μm² for Fib+EPO, Fib+veh, Sal+EPO and Sal+veh groups, respectively, P<0.05; Fig. 6N). There was no significant difference between two NSC-transplanted groups (9.8±0.8 particles/μm² versus 7.8±2.4 particles/μm² for NSC+EPO and NSC+veh groups, respectively, P=0.5372).

2.4. Changes in the densities of mossy fiber sprouting with NSC transplantation and EPO administration

We investigated the extent of aberrant mossy fiber sprouting (MFS) in all groups by using Timm’s histochemical staining and morphometric analyses. Compared with intact hippocampus (Figs. 6A, B), Timm’s staining demonstrated heavily stained cells both in the dentate hilus and inner molecular layer of the lesioned hippocampus indicating aberrant MFS into these regions (Figs. 6C, D). There were also dense Timm’s stained cells in the dentate hilus of rats receiving NSC transplantation (Figs. 6E, F). However, the densities in the inner molecular layer were lesser than those of the four control groups (6.5±1.3 and 11.1±2.3 particles/μm² for NSC+EPO and NSC+veh versus 37.9±8.8, 30.1±4.8, 43.0±7.6 and 31.0±8.6 for Fib+EPO, Fib+veh, Sal+EPO and Sal+veh groups, respectively, P<0.05; Fig. 6G). Rats in the NSC+EPO group presented a low density of Timm’s staining in the inner molecular layer when comparing with the NSC+veh group, but no statistic significance was found (6.5±1.3 particles/μm² versus 11.1±2.3 particles/μm², P=0.1008).

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3. Discussion

Here we have demonstrated that adult neural stem cells transplanted early into the lesioned hippocampus induced a significant and long-term suppression of abnormal spikes after KA-induced SE. Simultaneous EPO co-administration strongly enhanced the survival of transplanted NSCs but had little effect on the migration and differentiation. The mechanisms of ameliorating abnormal spikes might be involved in the inhibition of aberrant mossy fiber sprouting, augmented survival of inhibitive interneurons or alteration in the expression of GAD67 and NPY in neurons.

3.1. EPO augmented the survival of transplanted NSCs

Cell therapy is a prominent area of investigation in the biomedical field, particularly for the treatment of other refractory CNS disorders (Yasuhara et al., 2006). But the intrinsic factors regulating the survival, migration and differentiation of grafts are still not understood clearly and so become obstacles for further extensive application. EPO, as an autocrine-paracrine factor, exerts remarkable functions of neuroprotection, neurovascular remodeling, neuronal replacement and functional recovery in both cell cultures and animal models of CNS disorders (Shingo et al., 2001; Lu et al., 2005; Iwai et al., 2007; Chen et al., 2008; Grant et al., 2008). Recent studies have shown that neurogenesis is enhanced after hypoxia through EPO upregulation as the intrinsic response. Thus we ask if co-administration of EPO can modulate the survival, migration and differentiation of exogenous NSC-transplant in the injured adult hippocampus.

In this research, more transplanted cells survived at 5 weeks after transplantation with co-administration of EPO. This result suggests that EPO possesses the ability to enhance
the survival of transplanted NSCs. After transplantation, grafted cells should face the hypoxia and immune response. EPO exerts anti-apoptotic, anti-inflammatory, anti-oxidative, angiogenic, immunomodulating and neurotrophic properties to protect transplanted cells from harmful factors in micro-environment (Shingo et al., 2001; Lu et al., 2005; Uzüm et al., 2006; Iwai et al., 2007; Nadam et al., 2007; Yang et al., 2007; Chen et al., 2008). Our other series of experiments show that EPO can ameliorate host immune reaction around the grafts, such as GFAP- and Iba1-positive cell infiltration (data not show). Under multiple neuroprotective functions induced by EPO co-administration, grafted NSCs overcame detrimental conditions with subsequent long-term survival.

Although many cells survived after transplantation with co-administration of EPO, our data show little effect of EPO on the migration of grafted cells. In our previous study, in vivo data showed that EPO infusion resulted in a decrease in the number of intrinsic NSCs in the subventricular zone, an increase in the number of newly generated cells migrating to the olfactory bulb and new interneurons in the olfactory bulb (Shingo et al., 2001). In consideration of previous study and presented data in this study, we supposed that different signals might participate in modulating the migration of endogenous and exogenous NSCs. Additionally, EPO might act on separate pathways for regulating the proliferation of exogenous NSCs.

### 3.2. Cytodendritic mechanisms of TLE

The pathogenesis of TLE has been investigated intensively by human studies and animal models, which have provided several explanations for the increased excitability of neurons in the epileptic hippocampus. Some mechanisms are related with the hyper-excitability of the hippocampus during epilepsygenesis. Potential mechanisms include the sprouting of new axon collaterals resulting in the formation of recurrent excitatory connections on granule cells, the changes in the intrinsic excitability of neurons and the altered expression of neurotransmitter receptors, such as NMDA and GABA receptors (Sarkisian et al., 1997; Grunwald et al., 1999). There are animal models that reflect some features of partial complex seizures and may anatomically resemble mesial temporal sclerosis. Pathology of animal models in the chronic phase often correlates with the human situation, that is, aberrant mossy fiber sprouting and loss of GABA-synthesizing enzyme GAD in most of the interneurons (Shetty and Turner, 2000).

### 3.3. NSC transplantation attenuates aberrant mossy fiber sprouting into the inner molecular region

In serial studies (Shetty and Turner, 1997; Zaman and Shetty, 2003; Shetty et al., 2005; Rao et al., 2006), transplants of embryonic fetal hippocampal cells suppress the development of aberrant circuits in hippocampus by attracting sufficient host mossy fibers. In our research, the decreased density of Timm’s staining and NPY staining in the inner molecular layer of rats receiving NSC transplantation reflects suppression of aberrant mossy fiber sprouting. However, no proof was observed to indicate that grafted NSCs attracted aberrant mossy fiber sprouting.

The disparity between ours and other studies might be due to the differentiation and distribution of transplanted cells. Cells used in previous studies were embryonic fetal hippocampal cells and possessed some properties of host mature

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**Fig. 6** - NSC transplantation ameliorated sprouting of aberrant mossy fibers. (A–F) Timm’s staining: Although Timm’s staining of the hippocampus of age-matched adult rats demonstrated no mossy fiber sprouting in the inner molecular layer (A, B), densely stained fibers were recognized in the inner molecular layer and DGZ of Sal+veh group (C, D). (E, F) However, in the NSC+veh group, Timm’s staining demonstrated less stained fibers in the inner molecular layer and DGZ, than those of the Sal+veh group. (B, D, F) Enlarged parts in panels A, C and F, respectively. (H–M) Although in age-matched adult rats, NPY staining demonstrated no densely stained cells in the dentate gyrus, but some slightly positive cells (H, I) (NPY immunostaining and DAPI staining), intense NPY-positive cells were found in the inner molecular layer of the Sal+EPO group (J, K) (NPY immunostaining). (L, M) NPY immunostaining staining and GFP fluorescence: On the contrary, NPY staining revealed sparsely stained cells in the inner molecular layer with GFP-positive NSCs migrating along DGZ in the NSC+EPO group. (I, K, M) Enlarged parts in panels H, J and L, respectively. *P<0.05 compared with Fib+EPO, Fib+veh, Sal+EPO and Sal+veh groups. Scale bars: A, C, E, H and L, 500 μm; B, D, F, I, K and M, 50 μm.
hippocampal cells (Shetty and Turner, 1997; Zaman and Shetty, 2003; Shetty et al., 2005). Additionally, many of the grafted cells differentiated into mature neurons and attracted mossy fiber sprouting. On the contrary, most of our transplanted NSCs differentiated into GFAP-positive astrocytes but not mature neurons. Over half of the grafted cells migrated
and arrayed along DGZ. After KA-induced SE, due to the degeneration of CA3 pyramidal cells and interneurons in dentate hilus, axons of the dentate granule cells lose their normal connecting targets and sprouted abnormally. In consideration of the results of correlation between the numbers of cells in the subgranular zone and abnormal spikes, it maybe supposed that migrated cells arraying along DGZ serve as a barrier blocking sprouting of aberrant mossy fiber into the inner molecular region.

3.4. NSC transplantation enhances the number of inhibitive interneurons in dentate gyrus

The loss and degeneration of somatostatin and NPY-immunoreactive interneurons in the hilus of dentate gyrus participated in the development of TLE (Thompson and Suchomelova, 2004; Thompson, 2005). Degeneration of hilar interneurons is with subsequent diminished inhibitory synaptic transmission onto distal dendrites of dentate granular cells (Lothman and Collins, 1981; de Lanerolle et al., 1989; Represa et al., 1989; Houser et al., 1990; Arabadzisz et al., 2005).

Although epilepsy is fundamentally a circuit phenomenon, the most basic manifestation of the hyper-excitability of epilepsy must be evident at the level of a single neuron. Furthermore, in the future, manipulations of surviving neurons within the epileptic focus will constitute one of the best therapeutic targets for intervention to cure this devastating disease. Therefore, the more we learn about epileptogenic alterations in this population of surviving inhibitive interneurons, the more potential avenues for therapeutic intervention will emerge.

Somatostatin and/or NPY-immunoreactive neurons are especially vulnerable for KA-induced SE or repeated perforant pathway stimulation. NPY/GABA interneurons in the hippocampus were selectively ablated in rat epilepsy models (Sloviter, 1991) and in epilepsy patients (de Lanerolle et al., 1989).

In this research, we used two markers, NPY and GAD67, for estimating the extent of inhibitive interneurons in the lesioned hippocampus. From the observation of our results, GAD67- or NPY-positive interneurons sparsely distributed in the control lesioned hippocampus with subsequent deterioration of epilepsy represented by higher frequencies of abnormal spikes. On the contrary, more GABAergic interneurons could be found in the lesioned hippocampus associated with lower frequencies in rats with NSC transplantation. KA i. c.v. injection caused permanent loss of GAD67 expression in a significant fraction of interneurons, rather than widespread degeneration of interneurons (Shetty and Turner, 2001). Furthermore, in our experiments, most of the transplanted NSCs differentiated into GFAP-positive astrocytes. Transplanted NSCs might upregulate GAD67 and NPY expression in a major fraction of endogenous interneurons through neurotrophic potentials. This may be the explanation that more GAD67- or NPY-positive interneurons presented in the dentate gyrus of rats receiving NSCs. Simultaneously, endogenous interneurons newly developing from intrinsic neural progenitor cells promoted by NSC transplantation may also be due to neuroprotective/neurogenic potentials.

NSCs have splendid application prospects for neurodegenerative diseases due to their capacity to differentiate into appropriate cell types in accordance to the microenvironment. Many researchers explore mechanisms underlying the CNS regeneration achieved by NSC transplantation. However, the precise mechanisms are poorly understood. It remains to be solved which is the most important mechanism to replace the degenerative cells or to secrete nutrient factors for degenerating neurons. Until now there is not so much evidence that newly transplanted neurons can actively and spontaneously replace dead neurons by leading to functional recovery in an injured brain. Therefore, neurotrophic potencies of NSC transplantation attract attention now (Yasuhara et al., 2006).

From our results, it seems paradoxical that more NPY-positive interneurons appeared in NSC-transplanted groups in the dentate gyrus with less immunofluorescence activity of NPY in the inner molecular layer. Reduced NPY expression in the inner molecular layer might be due to the decrease of aberrant mossy fiber sprouting. On the contrary, the existence of more NPY-positive cells in the dentate gyrus might be caused by the mechanism that NSC upregulates NPY in some interneurons or affects neural progenitor cells to differentiate into NPY-positive interneurons. In the NSC+EPO group, a higher density of NPY staining in the inner molecular layer was found compared with the NSC+Sal group with more NPY-positive cells in the dentate gyrus, in spite of no significant differences.

4. Conclusions

NSC transplantation can prevent the development of spontaneous recurrent seizures in adult rats after KA-induced SE through suppression of aberrant mossy fiber sprouting and augmentation of the number of inhibitive interneurons. EPO infusion can enhance the survival of grafted NSCs, but not affect neuronal differentiation or migration of NSCs. Transplantation with EPO administration might be beneficial for epilepsy patients because it might enhance the self-repairing system by inhibitive interneurons as well as suppress the abnormal development of the neuronal circuit leading to epilepsy, although we need to explore the relationship between each phenomena for anti-epilepsy.

Fig. 7 – NSC transplantation showed neuroprotective effects on surviving interneurons. Transplanted NSCs increased the number of inhibitive interneurons in the host dentate gyrus. GAD 67 (A) and NPY (B) immunostaining in the DG of age-matched normal rats showed the existence of GABAergic and NPY-positive interneurons (A, E). GAD67 and NPY staining of the NSC+EPO group (C, G) demonstrated enhanced distribution and the increase in number of positive inhibitive interneurons, although GAD67- and NPY-positive cells were sparsely recognized in the Sal+EPO group (B, F). Scale bars: A–F, 100 mm. (D, H) Graphs of the statistical analysis of inhibitive interneurons in the dentate gyrus. *P < 0.05 compared with Sal+veh, Sal+EPO, Fib+veh and Fib+EPO groups.
5. Experimental procedures

5.1. Animals

Male F344 rats were used (n=34, 190–210 g at the beginning of the experiment, Japan SLC, Inc.) according to the approved guidelines of institutional animal care and use committee of Okayama University. They were housed two per cage in a temperature and humidity controlled room, maintained on a 12-h light/dark cycle, and they had free access to food and water.

5.2. Cell culture

GFP-expressing transgenic adult Sprague–Dawley rats were used for cell culture as described previously (Shingo et al., 2001; Muraoka et al., 2006; Kameda et al., 2007). Briefly, the rats were decapitated under deep anesthesia using pentobarbital. Tissue was dissected from the defined subventricular zone, passaged and cultured in the following mixed hormone media. Cells were seeded in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen Corporation, Grand Island, NY) containing 0.6% glucose, 2 nM NaHCO3, 0.5 mM HEPES, 100 μg/ml human apo-transferrin, 60 μM putrescine, 20 nM progesterone, 30 nM selenium chloride, 25 μg/ml human insulin, 2 μM l-glutamine (all from Sigma Chemical Co., St. Louis, MO) and EGF (human recombinant, PeproTech EC, London, UK). Cells were grown in uncoated plastic flasks during the primary culture as free-floating clusters (neurospheres) at 37 °C in a humidified 5% CO2 atmosphere. The spheres were collected then passaged by mechanical disassociation every 10 days and reseeded on poly-2-hydroxyethyl methacrylate (Sigma)-coated flasks as a single-cell solution at a density of 1×10⁵ cells/ml into the culture media. Neural stem/progenitor cell populations were obtained from neurospheres after at least two passages. Before differentiation and at 4 days after addition of fetal bovine serum into the medium, immunocytochemical investigations were performed.

Fibroblasts were also cultured from subcutaneous tissues of GFP transgenic adult Sprague–Dawley rats as mentioned previously (Kameda et al., 2007). Simply, fibroblasts were isolated from the dermis of the back of sacrificed adult male rats, and the cells were cultured for 2 weeks in Dulbecco’s modified Eagle’s medium containing 10% FBS and antibiotics. After the second passage, cells were transplanted into rat models, which served as a control group.

Before transplantation, all the cell cultures were prepared into single cell suspension.

5.3. Administration of KA

Animals were anesthetized with 3% halothane in a mixture of 70% N2O and 30% O2. Following induction of anesthesia, 1.5% halothane was maintained. The rats were fixed into a stereotactic apparatus (Narishige, Tokyo, Japan) with the plane of incision bar set at 3.3±0.33 mm below the interaural line. After midline incision, the skull was exposed and one burr hole was drilled according to the coordinates: anterior-posterior (AP)=0.5 mm posterior to bregma, lateral (L)=1.4 mm right lateral to the midline and ventral (V)=3.5 mm from the surface of brain ( Paxinos and Watson, 1986). KA (0.4 μg in 0.4 μl saline, Sigma-Aldrich) was injected into the right lateral ventricle using a 5 μl Hamilton syringe. The injection rate was 0.05 μl/2 min and the syringe was withdrawn slowly at 15–20 min after the injection. And then a piece of bone wax (Ethicon, Sommerville, NJ) was used to seal the skull defect to prevent cerebrospinal fluid leakage and the skin was closed with surgical thread.

5.4. Behavior monitoring

After KA injection, the rats were monitored under video camera continuously for 4 h to evaluate the behavior changes. The severity of epilepsy was ranked according to Lothman scale (Lothman and Collins, 1981). The onset of SE was defined as the onset of the first stage 4-seizure that did not abate after several minutes. To reduce the mortality rate, if SE continued for over 1 h, diazepam (5 mg/kg, i.p) was injected. These rats with prolonged seizures and those presented no SE were omitted out of the experiment. Only animals presented initial SE were used in the following experiment.

5.5. Cell transplantation and implantation of osmotic pumps

For rats receiving NSC transplantation, at 1 week after KA injection, 1.2×10⁶ live cells in 12 μl saline were injected into right hippocampus through 3 burr holes (for each site, 0.4×10⁶ cells in 4 μl saline) using a 5 μl Hamilton syringe (Zaman and Shetty, 2003) over a period of 8 min at the following coordinates: AP=−3.2 mm, L=2.0 mm, V=3.5 mm; AP=−3.8 mm, L=2.6 mm, V=3.5 mm; and AP=−4.5 mm, L=3.3 mm, V=3.5 mm according to the atlas (Paxinos and Watson, 1986). After transplantation, the needle was left in place for an additional 5 min before withdrawal in 5 min. Same procedures were performed on the rats of control groups substituted with fibroblast or saline for rats in control groups.

Just after cell transplantation, a single osmotic pump containing human EPO or vehicle was implanted into the right lateral ventricle using the same coordinate for KA injection. Osmotic pump (flow rate 1.0 μl/h for 7days, Alzet model 2001; Alza, Palo Alto, CA) was prepared as per manufacturer’s instructions mentioned before (Shingo et al., 2001). One osmotic pump contained 1500 U of recombinant human EPO (EPOGIN, Chugai Pharm, Japan) dissolved in 300 μl of 0.9% saline containing 1 mg/ml rat serum albumin. In the vehicle implantation group, only 300 μl of 0.9% saline containing 1 mg/ml rat serum albumin was injected through the pump. Thus 6 groups each containing 5 rats were included in the further experiments: NSC transplantation with EPO/vehicle co-administration, NSC+EPO/NSC+veh groups; fibroblasts-graft with EPO/vehicle co-administration, Fib+EPO/Fib+veh groups; saline injection with EPO/vehicle co-administration, Sal+EPO/Sal+veh groups. Four rats were used as age-matched control animals.

5.6. Electrode implantation

At 1 week after cell transplantation or saline injection, the rats were again anesthetized and fixed into stereotactic apparatus.
A bipolar electrode, consisting of two twisted strands of stainless steel wire, insulated except at the cross section of their tips, was stereotactically implanted into the right amygdala using the following coordinates: AP = −2.5 mm, L = 5.0 mm and V = 7.5 mm (Paxinos and Watson, 1986). A single stainless steel wire was inserted into the left frontal cortex acting as earth electrode. The electrodes were attached to male connector pins, which were inserted into a connector strip. Stainless steel screws were threaded into the right and left parietal cranium to help fix the electrode. The electrode assembly was then fixed to the skull with acrylic resin before the scalp was sutured.

5.7 EEG examination and analysis

At 3 days after electrode implantation, all rats underwent EEG examination 3 times a week (Nihon Kohden, Neuropack JB-441B). Recordings were performed in the afternoon to minimize potential circadian variations, following the habituation of the rats to the test cage. For spike evaluation, the amplitude threshold was set at 50 μV above the baseline. The data were collected to evaluate average spikes at 1st, 2nd and 3rd week post cells/saline injection.

5.8 Tissue processing and histological examination

At the end of the experiments, the rats were sacrificed using overdosed pentobarbital and perfused transcardially first with cold PBS followed by PFA in 0.1 M phosphate buffer (pH 7.2). The brains were rapidly removed and postfixed in 4% PFA overnight at 4 °C, before being transferred to 30% sucrose in 0.1 M phosphate buffer for 1 week. Brains were embedded in Tissue Tek O.C.T compound (Sakura Seiki Finetek, Tokyo, Japan) before they were cryosectioned coronally at 14 μm. Sections in the range between the levels of AP = −2.8 mm and −4.9 mm were collected for further analyses. Because between these two levels, the complete structures of dentate gyrus, CA1, CA2 and CA3 subareas can be clearly identified according to the atlas and also in the range of cell transplantation.

The following primary antibodies were used for immunohistochemical investigations: mouse anti-neuronal nuclei (anti-NeuN, 1:200, Chemicon), goat anti-doublecortin (1:500, Santa Cruz), mouse anti-GAD67 (1:1000, Chemicon), rabbit anti-glial fibrillary acidic protein (anti-GFAP, 1:500, Dako Denmark AS), rabbit anti-NPY (1:1000, Sigma). The second antibodies used were donkey anti-mouse IgG-Cy3, donkey anti-goat IgG-Cy3 and donkey anti-rabbit IgG Cy3 (1:200, all from Jackson ImmunoResearch).

For NeuN, doublecortin and GFAP immunostaining, sections were first incubated overnight at room temperature in primary antibody diluted in 0.1% TritonX-100 in PBS containing 10% normal horse serum (NHS). At the following day, after washing in PBS sections were incubated with regular Cy3 conjugated secondary antibodies and DAPI (1:500, Molecular Probes) for staining of nuclei for 1 h at 37 °C. After washing in PBS and in double-distilled water, all the slices were allowed to dry under cool air and coverslipped with Gelmount (Biomedical Corp., Foster City, CA). Control studies include exclusion of the primary antibodies substituted with 10% NHS in PBS. No immunoreactivity was observed in the controls.

As for NPY staining, the slides were washed as mentioned above followed by exposing in PBS containing 0.1% Triton X-100 three times for 10 min. And then incubate the slides with PBS containing 0.1% Triton X-100 and 10% NHS at room temperature for 90 min followed with incubation overnight at 4 °C with PBS containing 0.1% Triton X-100, 5% NHS and anti-NPY antibody. After rinsing several times, slides were incubated at room temperature for 1 h with PBS containing 0.1% Triton X-100, 2% NHS, corresponding secondary antibody and DAPI.

For GAD67 staining, the slides were exposed to PBS containing 0.05% Triton X-100 and 10% NHS at room temperature for 30 min, and incubated for 36 h at 4 °C with PBS containing 0.05% Triton X-100, 5% NHS and anti-GAD67 antibody. After rinsing several times with PBS, slides were incubated at room temperature for 1 h with PBS containing 0.05% Triton X-100, corresponding secondary antibody and DAPI.

Timm’s staining was performed according to references with slight modifications (Chafetz, 1986; Danscher, 1996; Stoltenberg et al., 2005). Sections were dipped into 0.37% sulfide solution for 10 min (containing 2.75 g NaH₂PO₄·2H₂O plus 2.925 g Na₂S·9H₂O dissolved in 250 ml distilled water adjusted to pH 7.2). The slides were then washed with distilled water for 20 min. Subsequently, slides were placed in glass jars filled with the developer (containing 120 ml 50% gum Arabic, 20 ml 2 M citrate buffer, 60 ml 0.5 M hydroquinone, 1 ml of 17% AgNO₃ solution). The entire set-up was prepared in plain daylight on the laboratory bench, but covered with a dark hood throughout the actual development. After 60 min at room temperature under gentle shaking, the development was stopped by washing under a gentle stream of running tap water for 20 min followed by washing with distilled water. The slides were then dried under a cool air and to be counterstained with 0.1% cresyl violet, gradually dehydrated in a graded ethanol and finally mounted with coverslip.

For immunocytochemical investigations, in addition to the GFAP antibody, we used mouse-derived monoclonal anti-nestin antibody (1:200, Abcam), and mouse-derived monoclonal anti-β-tubulin antibody (1:500, Sigma) and DAPI.

5.9 Morphological analyses

5.9.1 Quantification of NSCs survival

Quantification of transplanted NSCs survival was performed as introduced (Shingo et al., 2001; Muraoka et al., 2006) with a computerized analysis system (Olympus Sp-1000, Japan). Before further immunological staining process, a one-in-seven series of coronal sections, were sampled for evaluation of GFP expressing NSCs survival. The number of surviving cells located in the CA1, CA3 region and those migrating along dentate granular zone (DGZ) were counted, respectively. The number of total surviving cells was calculated by multiplying the number of GFP-positive surviving cells by 7 to take into account that the sections were collected as a 1-in-7 series. The values for GFP-positive live cells were calculated separately for each animal using data from every section.

5.9.2 Differentiation of transplanted NSCs

For determining the differentiation of transplanted NSCs, double immunofluorescent images were acquired
using a Zeiss LSM510 confocal microscope (Oberkochen, Germany).

5.9.3 Evaluation of mossy fiber sprouting
In this research, two methods were used to evaluate the degree of mossy fiber sprouting into the inner molecular layer: Timm’s staining and NPY immunostaining. Another two series of every 7th section of each animal were used for calculating. Evaluation of Timm’s staining was performed according to the method introduced by Shetty and Turner (1997). Only minor changes in brightness and contrast were made for display. The densitometric analysis consisted of measuring the optical density of the inner and outer molecular layer in the representative sections. The Timm’s staining images from each section were taken under a ×40 objective lens and transferred into grayscale files as mentioned above. The upper and lower dentate blades were imaged for each section, respectively, with an area of 8760 μm² (40 μm × 219 μm rectangle region parallel with DGZ) and an average density value was determined for both the inner and outer molecular layer. The background of the inner molecular layer was very similar to that of the middle and outer molecular layers, if no supragranular sprouting was present. Thus, it was assumed that the more distal molecular layer was a control region for the inner molecular layer, and the difference in density value between the inner and outer molecular layers was determined for the corresponding regions of interest.

The extent of NPY staining densities in the inner molecular region was evaluated according to the method introduced by Arabadzisz et al. (2005). Alterations of NPY immunofluorescence intensity were assessed by semi-quantitative densitometry. These images were acquired from each animal with a ×40 objective lens. For each section, the image acquisition parameters were adjusted using the internal capsule as background. Inner molecular regions close to the upper and lower dentate blades were imaged for each section. For each blade also a 40 μm × 219 μm rectangle region parallel with DGZ was taken for evaluation. Average fluorescent densities of interest area were measured using Image J software (ImageJ 1.41o, NIH).

5.9.4 Quantification of NPY- and GAD67-positive interneurons in dentate gyrus
All countings were under a ×10 objective lens. NPY-positive cells were counted on the same series sections of NPY staining mentioned in the previous graph. A 500 μm × 500 μm area in dentate gyrus was randomly selected first under DAPI staining fluorescence (blue) and converted to NPY-stained field (red). The images of the same field under two optical filters were taken for evaluation. Each section was carefully examined and cross-checked. Only the NPY staining structures with blue karyon were counted.

On another series of only one in 7th sections, GAD67-positive cells were counted using the same method as of NPY-positive cell counting. The results were presented as immunoreactive cells/0.25 mm². All the number of counted cells was corrected by the Abercrombie formula to avoid over-estimation.

5.9.5 Statistics analyses
All data were presented as mean ± SEM. Repeated ANOVA and post-hoc Student–Newman–Keuls test were chosen for statistical analysis unless otherwise mentioned. The operations were performed using StatView software (version 5.0, SAS Institute Inc., Cary, NC, USA). Statistical significance was set at P < 0.05.

REFERENCES


