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Neuroprotective effects of erythropoietin in the rat hippocampus after pilocarpine-induced *status epilepticus*

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Neuroprotective functions of erythropoietin (Epo) are thought to involve a heteroreceptor composed of both Epo receptor (Epo-R) and common β chain (βc). Here, we measured the response of hippocampal Epo system components (Epo, Epo-R and βc) during neurodegenerative processes following pilocarpine-induced *status epilepticus* (SE), and examined whether recombinant human Epo (rHuEpo) could support neuronal survival. We evidence that Epo is induced in astroglia following SE, in particular within areas displaying delayed neuronal death. In addition, we demonstrate for the first time that rHuEpo reduces considerably hippocampal neurodegeneration following SE. rHuEpo may thus supplement astroglial induction of Epo to promote enhanced hippocampal neuronal survival following SE. We also show that Epo-R is expressed by neurons and astrocytes mainly, while βc is barely detectable in basal conditions and induced in reactive microglia exclusively following SE. Altogether, our results suggest that Epo/rHuEpo exerts neuroprotection, through Epo-R signaling and independently of βc, and, therefore, may be anti-epileptogenic.

Keywords: Hippocampus; Mossy cells; CGRP; HIF-1 alpha; aHIF; Blood–brain barrier; Neocortex; Spinal cord; Neuroprotection

Introduction

Erythropoietin (Epo) was originally described for its role in hematopoiesis, which consists of increasing red blood cells (Jelkmann, 1992) by protecting erythroid progenitors against apoptosis (Ghezzi and Brines, 2004). Epo and its receptor (Epo-R) are expressed in rodent and human brain, in cultured neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells (Marti et al., 1996; Juul et al., 1999; Chin et al., 2000; Siren et al., 2001; Hasselblatt et al., 2006), which has led to studies of additional biological roles of Epo. Exogenous administration of Epo revealed neuroprotective activity *in vitro* and *in vivo* in models of central and peripheral neuronal injury occurring in the contexts of trauma, stroke and inflammation (Juul et al., 2004; Campana and Myers, 2003; Maiese et al., 2004; Brines and Cerami, 2005).

Recombinant human Epo (rHuEpo)-administered peripherally crosses the blood–brain barrier (BBB) (Brines et al., 2000; Ehrenreich et al., 2004; Juul et al., 2004; Gorio et al., 2005). Studies in rodent models of temporal lobe epilepsy (TLE) revealed significant effects of rHuEpo in antagonizing the development of *status epilepticus* (SE), but did not determine whether rHuEpo was neuroprotective (Brines et al., 2000; Uzum et al., 2006). Interestingly, many of the programmed cell death pathways involved in animal models of TLE (Henshall and Simon, 2005) are those targeted by Epo (Maiese et al., 2004; Brines and Cerami, 2005).

Possible adverse effects of rHuEpo, such as increase in blood pressure, thrombosis, tumor expansion, and mortality (Maiese et al., 2004; Brines and Cerami, 2005) have spurred the development of Epo derivatives that are neuroprotective, but not erythropoietic (Leist et al., 2004). Carbamylated Epo (CEPO),

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which is one such derivatives, has a low affinity for Epo-R homodimer compared to native Epo, and is neuroprotective *in vivo* in the neocortex (Leist et al., 2004) and the spinal cord (Brines et al., 2004; Leist et al., 2004). Although it has been suggested that Epo-mediated neuroprotection occurs via Epo-R homodimers (Maiese et al., 2005), CEPO- and rHuEpo-mediated neuroprotection in the spinal cord may involve a heteroreceptor complex, consisting of a single Epo-R and a homodimer of the common β chain (βc) of the Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF), Interleukin-3 (IL-3) and IL-5 receptors, as demonstrated in âc-knockout mice, which express normal levels of Epo-R (Brines et al., 2004; Genc et al., 2004; Brines and Cerami, 2005). Thus, available data suggest that several Epo binding sites may mediate neuroprotection, either through Epo-R homodimers or Epo-R/βc heteroreceptors.

Although exogenous Epo appears to be neuroprotective, little is know about the expression patterns of Epo system constituents under basal and induced conditions. Epo system expression has been investigated after cerebral ischemia (Bernaudin et al., 1999) and traumatic injury in the spinal cord (Grasso et al., 2005) and peripheral nervous system (Campana and Myers, 2001; Li et al., 2005). However, Epo system expression has not yet been assessed after chemically-induced SE, which initiates the process of epileptogenesis (Turski et al., 1983).

In this study, we have investigated the expression of Epo system constituents (Epo, Epo-R and the βc) both in basal conditions and in response to pilocarpine-induced SE (Pilo-SE). Our results show that Epo-R is expressed constitutively by most neurons of the hippocampus, but rarely by astrocytes. Following Pilo-SE, we report the transient induction of Epo in hippocampal astrocytes and a long-lasting induction of Epo-R. We also report that constitutive expression of βc subunit is low in the hippocampus compared to the spinal cord, whereas βc subunit expression dramatically increases after Pilo-SE in reactive microglia. Finally, we demonstrate that administration of rHuEpo following Pilo-SE significantly protects hippocampal neurons, suggesting that rHuEpo may be both neuroprotective and anti-epileptogenic.

Procedures and methods

All animal procedures were in compliance with the guidelines of the European Union (directive 86/609), taken in the French law (decree 87/848) regulating animal experimentation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Procedures

Animals

Sprague–Dawley male rats (Harlan, France) were used throughout the experiments. They arrived at 5 weeks old in approved facilities, and housed at 21±1°C under diurnal lighting conditions (lights on from 06:00 to 18:00). They were maintained in groups of 5 in plastic cages with free access to food and water. After 2-week acclimatization, rats (180–200 g) underwent Pilo-SE as described below, and were then housed individually to support recovery until sacrificed. Control rats were housed in groups of 5 throughout the experiment to avoid the effect of isolation stress.

Pilo-SE

Scopolamine methylnitrate (1 mg/kg, subcutaneously; Sigma) was administered 30 min prior to pilocarpine hydrochloride (300 or 375 mg/kg, intraperitoneally; Sigma). After an initial period of immobility, the onset of SE was characterized by repetitive clonic activity of the trunk and limbs, occurring following repeated rearing with forelimb clonus and falling. SE was stopped after 2 or 3 h by a single administration of diazepam (Valium®, 10 mg/kg, intraperitoneally; Roche). Rats were then hydrated with 2 mL 0.9% NaCl (saline; subcutaneously). Pilocarpine dose used and duration of SE are mentioned below for each set of experiments.

Treatment with rHuEpo

Injected solution of rHuEpo (Eprex®, generous gift from Janssen-Cilag, France) was prepared at the concentration of 2.5 IU/μL by diluting the source solution (40,000 IU/mL; i.e. 336 μg) with saline. Rats were then treated intraperitoneally with 5,000 IU/kg (i.e. 42 μg/kg), previously determined as the optimal neuroprotective dose with this route of administration (Calapai et al., 2000). Naïve rats received saline only.

Experimental design

Experiment 1: hippocampal Epo system expression in normal rats and following Pilo-SE

Rats experienced 3 h of SE induced by 300 mg/kg pilocarpine hydrochloride. In this study, we used the minimal dose of pilocarpine needed to induce sustained SE in a majority of rats (55%; 48/87) without compromising long-term survival. With this concentration of pilocarpine, onset of SE was observed 22.3± 0.9 min (mean±S.E.M., $n=48$) after pilocarpine administration, and rats exhibited an average of 4.49±0.21 stage-4/5 seizures (mean±S.E.M., $n=48$) during the 3-h period of SE.

To determine the levels of mRNAs encoding key proteins of the Epo system (i.e. Epo, Epo-R, βc and HIF-1α), animals were sacrificed following a lethal injection of pentobarbital (250 mg/kg). The hippocampus was rapidly removed and frozen at different times (t) after the onset of SE: $t=8$ h (0.3 day) ($n=5$), $t=1$ day ($n=6$), $t=2$ days ($n=6$), $t=3$ days ($n=8$) and $t=7$ days ($n=3$). Control scopolamine- and diazepam-treated rats (control/SD rats) received scopolamine and diazepam injections exactly as pilocarpine-treated rats and were sacrificed at the same time points ($n=5$ per time point examined). The hippocampus, the neocortex and the spinal cord (at the vertebrate level T2/T4) were also collected in naive rats, sacrificed at $t=0$ (controls, $n=5$).

To estimate neuronal death, either after NeuN-immunolabeling or Fluoro-Jade B staining, and to localize and characterize cells expressing Epo, Epo-R and βc in the hippocampus, rats were deeply anesthetized (lethal intraperitoneal injection of pentobarbital at 250 mg/kg) at 1 day ($n=3$), 3 days ($n=5$), 4 days ($n=5$) and 15 days ($n=7$) following Pilo-SE, and then transcardially perfused with chilled 4% paraformaldehyde in 0.1 M phosphate buffer. After cryoprotection in 25% sucrose, the brains were frozen in isopentane and stored at −80°C. Naive rats (controls, $n=4$) were included in this study.

Experiment 2: uptake of rHuEpo in brain tissue of control rats and rats undergoing Pilo-SE

Plasma and brain tissue concentrations of rHuEpo were determined at different times following its peripheral administration in rats ($n=20$), pre-treated or not with the same dose of rHuEpo 24 h before, and subjected to Pilo-SE. After deep anaesthesia of the rats (lethal intraperitoneal injection of pentobarbital at 250 mg/kg), blood samples were obtained by cardiac punction and collected in heparinized tubes. Following 1 min transcardiac perfusion with chilled 0.9% NaCl, both the hippocampus and the neocortex were dissected, weighted, frozen in liquid nitrogen, and stored at −80°C until further use. Controls were treated identically except for pilocarpine injection.

Experiment 3: can rHuEpo modify the development of Pilo-SE and rescue vulnerable hippocampal neurons?

Rats experienced 2 h of SE induced by 375 mg/kg pilocarpine hydrochloride, which is the dose previously used to produce neuronal degeneration (Turski et al., 1983). Three series of experiments were conducted, each including rats of the three following groups: 1) rats receiving injections of rHuEpo ($n=10$) following Pilo-SE only, i.e. 30 min, 1 day and 3 days after SE stopped; 2) rats receiving 4 injections of rHuEpo ($n=20$): 24 h and 30 min prior to pilocarpine administration, 1 day and 3 days after SE stopped; 3) rats receiving no rHuEpo ($n=20$). Naive control rats ($n=5$) were also included in this study and received saline when rHuEpo was given to rats having experienced Pilo-SE. In the three groups of rats, we monitored different variables characterizing the development of SE (continuous tonic activity), i.e.: 1) latency to develop SE; 2) time of appearance and number of stage 4 (rearing accompanied by bilateral forelimb clonus) and stage 5 (rearing, with loss of balance and falling, accompanied by generalized clonus) seizures, as previously described (Racine, 1972). To evaluate neuronal protection and characterize rescued neurons, rats were deeply anesthetized 15 days following Pilo-SE, and their brains were fixed, cryoprotected, frozen and stored at −80°C.

Methods

Reverse Transcriptase real time Polymerase Chain Reaction (RT-real time PCR)

Total RNAs were extracted with Tri-reagent LS (Euromedex) and genomic DNA was removed after DNase I digestion (RNAse Free DNAse Set, Qiagen). After column purification (RNeasy kit, Qiagen) and prior to reverse transcription, total RNA from all samples were shown to be free of genomic DNA contamination by a PCR amplification of the exon V of the gene encoding brain-derived neurotrophic factor (BDNF) (see below for details). Messenger RNAs, contained in 500 ng of hippocampal total RNAs, were then reverse transcribed with the reverse transcriptase RNase H minus (Promega) using oligod $(T)_{15}$, in the presence of 80 pg of a synthetic external and non-homologous poly(A) Standard RNA (SmRNA) used to normalize the reverse transcription of mRNAs of biological samples (Morales and Bezin, patent WO2004.092414). cDNAs obtained from the reverse transcription of targeted mRNAs were quantified by real time PCR performed on the LightCycler® System (Roche Diagnostics) using the QuantiTect SYBR®Green PCR Kit (Qiagen) for Epo, Epo-R, SmRNA, and HIF-1α, or the FastStart DNA Master SYBR Green I kit (Roche Diagnostics) for aHIF and BDNF. All PCR fragments were confirmed by sequencing. Results obtained for the targeted mRNAs were normalized against the SmRNA. Sequences of the different primer pairs used are: *aHIF* (GenBank accession no.U85044.1) forward 5′ TTT GTG TTT GAG CAT TTT AAT AGG C 3′, reverse 5′ CCA GGC CCC TTT GAT CAG CTT 3′ (279 bp); *BDNF(exV)* (GenBank accession no.X67108) forward 5′ AAA TTA CCT GGA TGC CGC AA 3′, reverse 5′ CGC CAG CCA ATT CTC TTT TT 3′ (345 bp); *Epo* (GenBank accession no.NM_017001) forward 5′ GCT CCA ATC TTT GTG GCA TC 3′, reverse 5′ ATC CAT GTC TTG CCC CCT A 3′ (66 bp); *Epo-R* (GenBank accession no.D13566) forward 5′ CCA GCT CTA AGC TCC TGT GC 3′, reverse 5′ CTT CAG GTG AGG TGG AGT GG 3′ (68 bp); HIF-1á (GenBank accession no.Y09507) forward 5′ CTC AGA GGA AGC GAA AAA TGG 3′, reverse 5′ AAT TCT TCA CCC TGC AGT AGG 3′ (307 bp); βc (GenBank accession no.NM_133555.1) forward 5′ CCT GGT GGC TCT CTG CTG 3′, reverse 5′ GCA GAG TCT TCA GAG GGA CAG T 3′ (68 bp). All primer pairs were designed using "Primer 3" software (NIH;www.basic.nwu.edu).

Quantitative determination of rHuEpo using Elisa

Frozen hippocampus and neocortex were homogenized in 150 μL and 300 μL of chilled 0.9% NaCl, respectively. After centrifugation of both homogenates (12,000×*g* for 20 min at 4°C) and blood samples (760×*g* for 15 min at 4°C), rHuEpo was measured in 100 μL of the supernatant or plasma using an Elisa kit (R&D Systems) following manufacturer's instructions. The sensitivity of the assay was ~1.0 mIU/mL. Results are expressed as mIU per gram of wet tissue (mIU/gt) for both the hippocampus and the neocortex, and as mIU/mL for the plasma.

Colorimetric immunohistochemistry assays

Free floating sections (40 μm thick) from paraformaldehyde-fixed tissue were incubated either with a rabbit polyclonal anti-Epo antibody diluted at 1:250 (sc-7956; Santa Cruz), a rabbit polyclonal anti-Epo-R antibody diluted at 1:500 (sc-697; Santa Cruz), a rabbit polyclonal anti-βc antibody diluted at 1:100 (sc-678; Santa Cruz), a mouse monoclonal anti-NeuN antibody diluted at 1:1000 (MAB-377; Chemicon), a mouse monoclonal anti-GAD65/67 diluted at 1:10 000 (GC-3108; BIOMOL), or a goat polyclonal anti-CGRP antibody diluted at 1:500 (1720-9007; Biogenesis). After washes, the sections were then incubated with a biotinylated donkey antibody diluted at 1:1000, either raised against rabbit IgG antibody (711-066-152; Jackson Immuno-Research), mouse IgG (715-065-151; Jackson ImmunoResearch), or goat IgG (705-066-147; Jackson ImmunoResearch). After washes, sections were incubated with avidin biotin peroxydase (1:500; Vectastain Elite ABC kit, Vector) and reacted with 0.4 mM 3′,3-diaminobenzidine (DAB, Sigma Fast). They were then mounted, dehydrated and coverslipped in DPX (Fluka). Rabbit polyclonal antibodies from Santa-Cruz have already been successfully used by others (Chong et al., 2003; Brines et al., 2004; Gorio et al., 2005). In addition, the specificity of sc-697 antibody used to detect Epo-R in rodent brain tissue has already been demonstrated (Grimm et al., 2002).

Fluorescent dual-labeling immunohistochemistry

Free floating sections (40 μm thick) from paraformaldehyde-fixed tissue were co-incubated with a rabbit polyclonal anti-Epo antibody diluted at 1:250 (sc-7956; Santa Cruz) or a rabbit

polyclonal anti-Epo-R antibody diluted at 1:500 (sc-697; Santa Cruz) and a mouse monoclonal antibody raised against NeuN diluted at 1:1000 (MAB-377; Chemicon), Ox-42 diluted at 1:2000 (CBL1512Z; Chemicon) or GFAP antibody diluted at 1:2500 (G3893; Sigma). After washes, sections were exposed to an Alexa-488-conjugated donkey anti-rabbit IgG antibody (A-21206; Molecular Probes) and to an Alexa-633-conjugated goat anti-mouse IgG antibody (A-21052; Molecular Probes), both diluted at 1:500. Sections were then mounted on SuperFrost®Plus slides and coverglassed with Prolong Gold Antifade reagent (Molecular Probes). Images captured using a TCS SP2 confocal microscopy system (Leica) were imported into Adobe Photoshop 8.0.1 (Adobe Systems) for further editing.

Neuronal degeneration

Fluoro-Jade B (Chemicon) was used to stain degenerating neurons after Pilo-SE in rats (Poirier et al., 2000; Schmued and Hopkins, 2000). Bright fluorescence observed in degenerating neurons made it easy to distinguish them from background fluorescence, which was more diffuse.

Neuronal counts

Sections immunostained for NeuN were observed under a light microscope (Diaplan; Leitz), and images were captured with a video camera 3CCD (DXC-930P; Sony) coupled to an image analysis system (Visilog® 6.3; Noesis). The system permits magnification at 20× of adjacent fields throughout the whole dorsal hippocampus and to reconstruct a single image composed of a mosaic of the digitized adjacent fields. Dorsal hippocampus was selected at IA +5.40 mm (Paxinos and Watson, 1998). Due to the morphological diversity of the neuronal populations composing the hippocampus, we could not establish a standard "neuronal" profile to perform an automated neuronal count. Therefore, NeuN-immunopositive neurons were counted manually by two independent observers from the reconstructed images, within the hilus and the *stratum lacunosum moleculare*. To evaluate the density of neurons expressing NeuN in the pyramidale layers of areas CA1 and CA3, we measured within a 282,440 μm^2 window the surface area occupied by NeuN-immunopositive cell bodies within the field delineated by pyramidal neurons.

Statistical analysis

Data are expressed as mean±S.E.M. of the different variables analyzed (mRNA level, neuron number) and were compared among groups by using one- or two-way ANOVA followed by Fisher's protected Least Significance Differences (LSD) test.

Results

Basal expression and distribution of both Epo-R and Epo in the hippocampus

In control rats, 983±133 copies of Epo-R cDNA and 224±55 copies of Epo cDNA were quantified by real time PCR following reverse transcription of 500 ng of hippocampal total RNA.

Colorimetric immunolabeling showed that Epo-R was expressed in the principal cell layers of the dentate gyrus and Ammon's horn (Fig. 1A). Dual fluorescent labeling of Epo-R together with specific markers of either neurons (NeuN), astrocytes (GFAP) or microglial cells (OX-42) demonstrated that Epo-R was expressed primarily by neurons (Fig. 1B, white arrows). However, not all neurons expressed detectable Epo-R, especially within the hilus (Fig. 1B, asterisks). Epo-R labeling was mainly present in neuronal cell bodies, except within CA1, where intense immunoreactivity was present in the dendritic field as well (Fig. 1). Immunofluorescence labeling made it possible to detect in the whole hippocampus a punctuated and intensive signal, which may reflect the presence of Epo-R in numerous neuronal varicosities. Epo-R was rarely found in astrocytes under basal conditions (Fig. 2A) and was not detectable in resting microglial cells (data not shown).

Colorimetric immunohistochemistry indicated the presence of Epo protein in the CA3 pyramidal layer (data not shown), in the perikarya and processes of some hilar (Fig. 3A) and CA3c (data not shown) neurons, and in granule cells (data not shown). The labeling obtained in the other parts of the hippocampus was close to detection threshold. Dual fluorescent labelings of Epo together with either NeuN, or GFAP, or OX-42 revealed that Epo was expressed primarily by neurons (data not shown) and rarely by astrocytes in the hilus (Fig. 3E). Epo was not detectable in resting microglial cells (data not shown).

Basal expression and distribution of the common β-subunit in the rat brain

Recent studies in mice suggest that the neuroprotective effect of Epo after spinal cord trauma requires the presence of a heteroreceptor comprising both Epo-R and βc, the signal-transducing subunit shared by the GM-CSF, IL-3 and IL-5 receptors (Brines et al., 2004). Our results in control rats, after quantification by real-time PCR following reverse transcription of 500 ng of tissue total RNA, indicate that the transcript encoding βc is constitutively expressed at low levels in the hippocampus (702±80 copies) and the neocortex (718±80 copies), compared to the spinal cord (1830±153 copies) (Fig. 4A). In contrast, Epo-R mRNA is more abundantly expressed in the hippocampus (991±59 copies) and spinal cord (849±151 copies), than in the neocortex (592±31 copies) (Fig. 4A). As a consequence, the Epo-R mRNA/βc mRNA ratio in the hippocampus is 1.6- and 3.0-fold greater than that found in the neocortex and the spinal cord, respectively (Fig. 4B). Detection of βc protein in the hippocampus was restricted to the dentate hilus (Fig. 5). Comparison of Epo-R and βc protein presence in the dentate gyrus and the neocortex indicates that the number of cells expressing detectable Epo-R is greater than that of cells expressing βc (Fig. 5).

Epo-R expression in the hippocampus after Pilo-SE

Following Pilo-SE, mRNA encoding Epo-R was increased significantly in the hippocampus 1–7 days post-SE, with an apparent peak observed 3 days post-SE (Fig. 2B). This effect was specific to Pilo-SE and was not produced by scopolamine/diazepam (SD) treatments (data not shown). Immunohistochemical studies of Epo-R protein performed at 4 days post-SE indicated a reduction of the neuronal staining in the hilus and CA3c (Figs. 2C, E). By contrast, additional glial-like cells expressed Epo-R within all hippocampal areas, as illustrated in the hilus (Figs. 2C, E) and CA3 (Figs. 2D, F; see black arrow). Double GFAP/Epo-R fluorescent immunolabeling identified these glial cells as astrocytes (Fig. 2A).

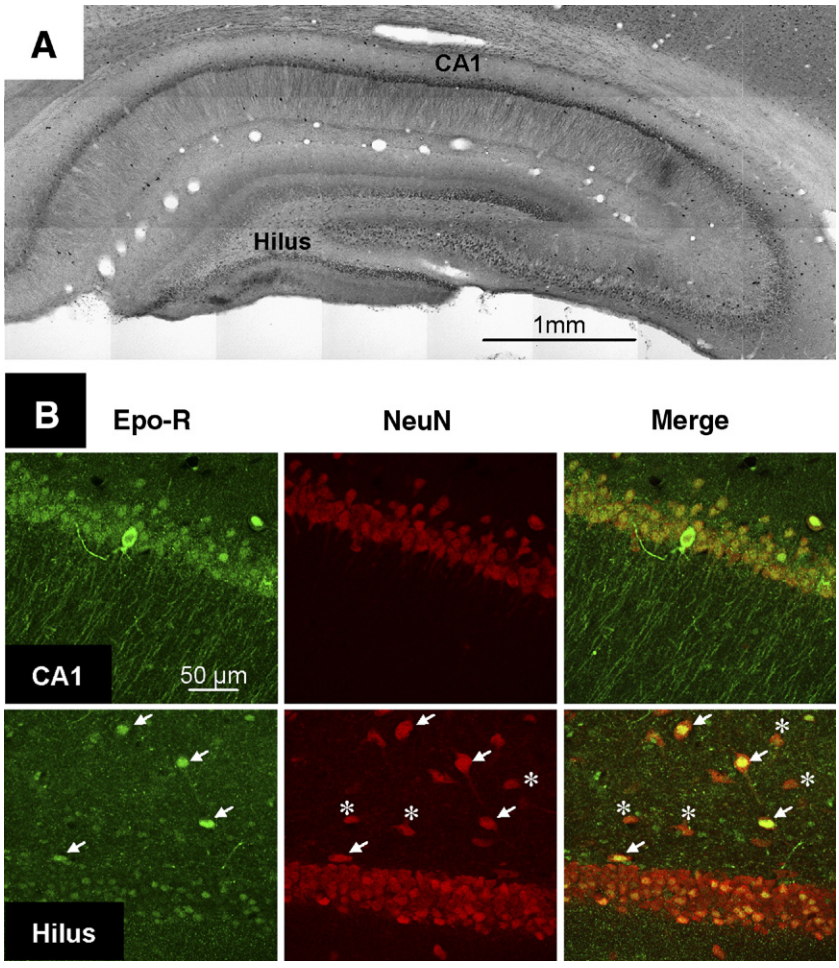

Fig. 1. Hippocampal distribution of Epo-R under basal conditions. (A) Colorimetric staining demonstrates that Epo-R is expressed throughout the rat hippocampus. (B) Dual fluorescent labeling evidences that Epo-R (green) is strictly expressed by neurons (NeuN, red) in particular in vulnerable areas of the hippocampus, such as CA1 and the hilus (arrows). However, not all hilar neurons express Epo-R (asterisks). Scale bars: A, 1 mm; B, 50 µm.

Epo expression in the hippocampus after Pilo-SE

Following Pilo-SE, mRNA encoding Epo was transiently enhanced in the hippocampus 2 days post-SE (+196%; $P<0.001$) (Fig. 3F). Immunohistochemical studies of Epo protein performed at 1, 3 and 4 days post-SE revealed that neuronal labeling disappeared in the hilus, as illustrated at 3 days post-SE (Figs. 3A, C). By contrast, in hippocampal subfields devoid of Epo-expressing cells under basal conditions, such as the *stratum lacunosum moleculare* (SLMo) and the *stratum radiatum* of CA1, Epo was detected in numerous round-shaped perikarya at 3 days post-SE (Figs. 3B, D). Similar perikarya were also seen within the pyramidal layer of areas CA1 and CA3, the molecular layer of dentate gyrus (data not shown), and the hilus (Figs. 3A, C). In all hippocampal subfields mentioned, numerous "newly-detected" cells were identified as GFAP-immunoreactive astrocytes (Fig. 3E, white arrows). The scattering of the Epo-expressing cells in both the SLMo and the hilus at 3 days post-SE made it possible to characterize them all as being astrocytes. Epo was not detectable in OX-42-immunopositive activated microglial cells (data not shown).

Increased levels of the transcript encoding HIF-1α were observed in the hippocampus 8 h after the onset of Pilo-SE (+61%; $P<0.001$). This induction was maintained at 2 days post-SE (+97%; $P<0.001$) (Fig. 3F), and was accompanied by a reduced expression (−40%) of the natural HIF-1α anti-sense RNA (aHIF) that was significant and long-lasting (Fig. 3F).

Altered levels of Epo mRNA, HIF-1α-mRNA and aHIF-RNA were specific to Pilo-SE and were not produced by SD treatments used to stop seizures, since no difference was observed between naive rats and control/SD rats at any time points examined (data not shown).

Common β-subunit is exclusively expressed by activated microglial cells after Pilo-SE

Following Pilo-SE, levels of mRNA encoding βc were dramatically and transiently increased in the hippocampus, peaking at 1 day post-SE (+9897%; $P<0.001$) (Fig. 6A). This effect was specific to Pilo-SE and was not produced by SD treatments (data not shown). Immunohistochemical studies of βc protein performed at 3 days post-SE revealed a reduction of the neuronal staining in the hilus (data not shown) and an increased staining throughout the hippocampus, as illustrated in area CA1 (Fig. 6B), within cells bearing the morphological features of activated microglial cells, as previously shown in rats after either lipopolysaccharide injection or middle cerebral artery occlusion (Appel et al., 1995).

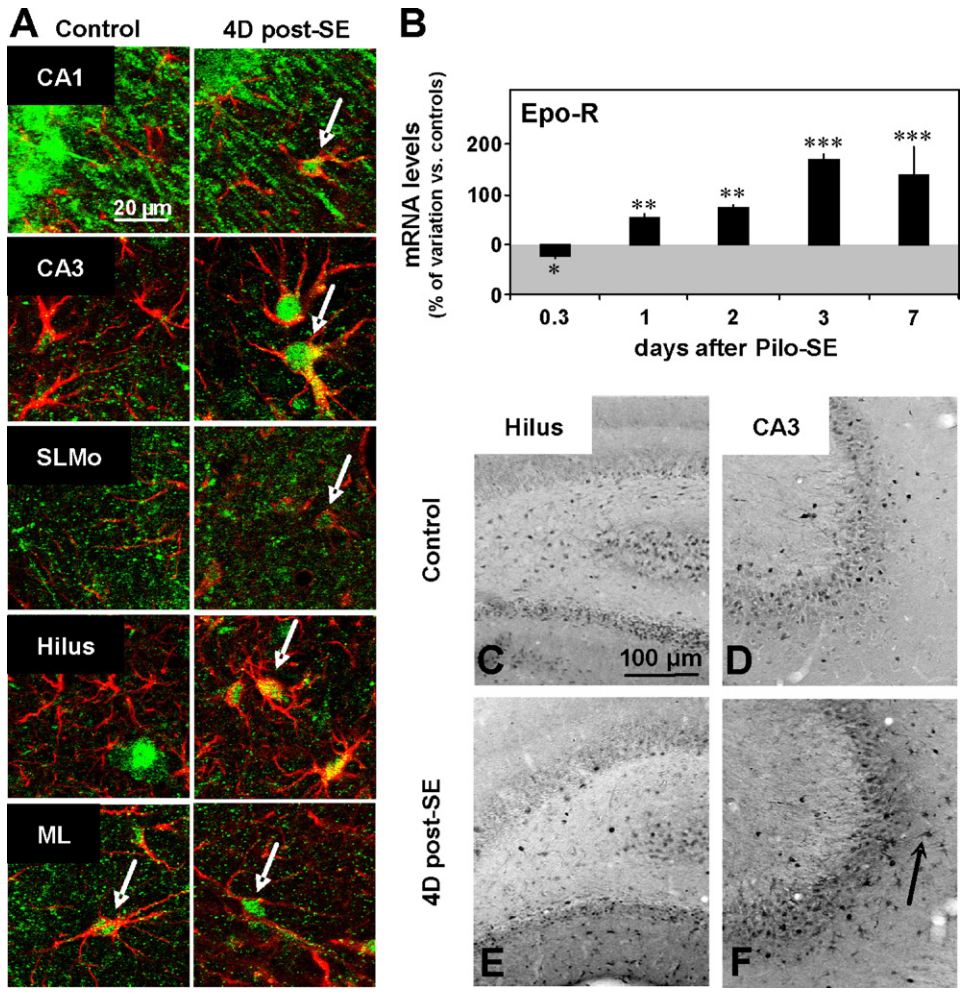

Fig. 2. Epo-R expression is increased in hippocampal astrocytes following Pilo-SE. (A) Dual fluorescent labeling evidences that astrocytes (GFAP, red) express Epo-R (green) at 4 days post-SE in all hippocampal areas (white arrows). (B) RT-real time PCR demonstrates that Epo-R-mRNA level is significantly induced throughout the 7 days following Pilo-SE. *$P<0.05$; **$P<0.01$; ***$P<0.001$ as compared to controls. (C–F) Colorimetric staining evidences that neuronal expression of Epo-R is reduced in the hilus following Pilo-SE (C, E), while "newly" Epo-R expressing cells bearing morphological features of astrocytes are detected in all hippocampal areas, as illustrated in CA3 (black arrow) (D, F). Abbreviations: D, day; ML, molecular layer; SLMo, stratum lacunosum moleculare. Scale bars: A, 20 µm; C–F, 100 µm.

Time-course of neuronal loss in the dorsal hippocampus after Pilo-SE

Fluoro-Jade B was used to identify the times at which neurons were degenerating. NeuN-immunostaining was used to provide a quantitative evaluation of neuronal loss in CA1 (proximal to CA2), CA3, the SLMo and the hilus at IA +5.40 mm (Paxinos and Watson, 1998). Animals undergoing Pilo-SE exhibited a pattern of neuronal loss that varied in the different subfields of the dorsal hippocampus analyzed (Fig. 7). In the hilus, intense Fluoro-Jade B staining was observed 1 day post-SE and was maintained throughout the time period examined, as shown 3 days post-SE (Fig. 7A). Neuronal loss reached ~45% by 1 day post-SE, and then stabilized by 3 days post-SE at ~65% (Fig. 7B). In areas CA1 and CA3, Fluoro-Jade B staining was observed 1 day post-SE, and increased 3 (Fig. 7A) and 4 days post-SE. Neuronal loss was present 3 days post-SE and reached ~50% cell loss by 15 days post-SE (Fig. 7B). The SLMo exhibited no Fluoro-Jade B staining 1–4 days post-SE (Fig. 7A). However, at 15 days post-SE, neuronal loss reached ~40% (Fig. 7B).

Brain tissue uptake of rHuEpo under basal conditions and after Pilo-SE

rHuEpo is known to cross the BBB after systemic injection and to accumulate in the cerebro-spinal fluid in adult rats and humans (Brines et al., 2000; Ehrenreich et al., 2004). We determined whether rHuEpo concentrates evenly within the brain parenchyma. In control rats, we found that rHuEpo reached a ~3-fold greater tissue concentration in the neocortex than that in the hippocampus 4 h after the injection of 5,000 IU/kg rHuEpo intraperitoneally (Fig. 8, Group 1). When rHuEpo was administered twice, 24 h apart, neocortical uptake of rHuEpo was considerably reduced after the second administration (Fig. 8, Group 2). However, this inhibition was not observed in rats undergoing Pilo-SE (Fig. 9, Group 2). Plasma levels of rHuEpo measured in all rats tested were not different from each other

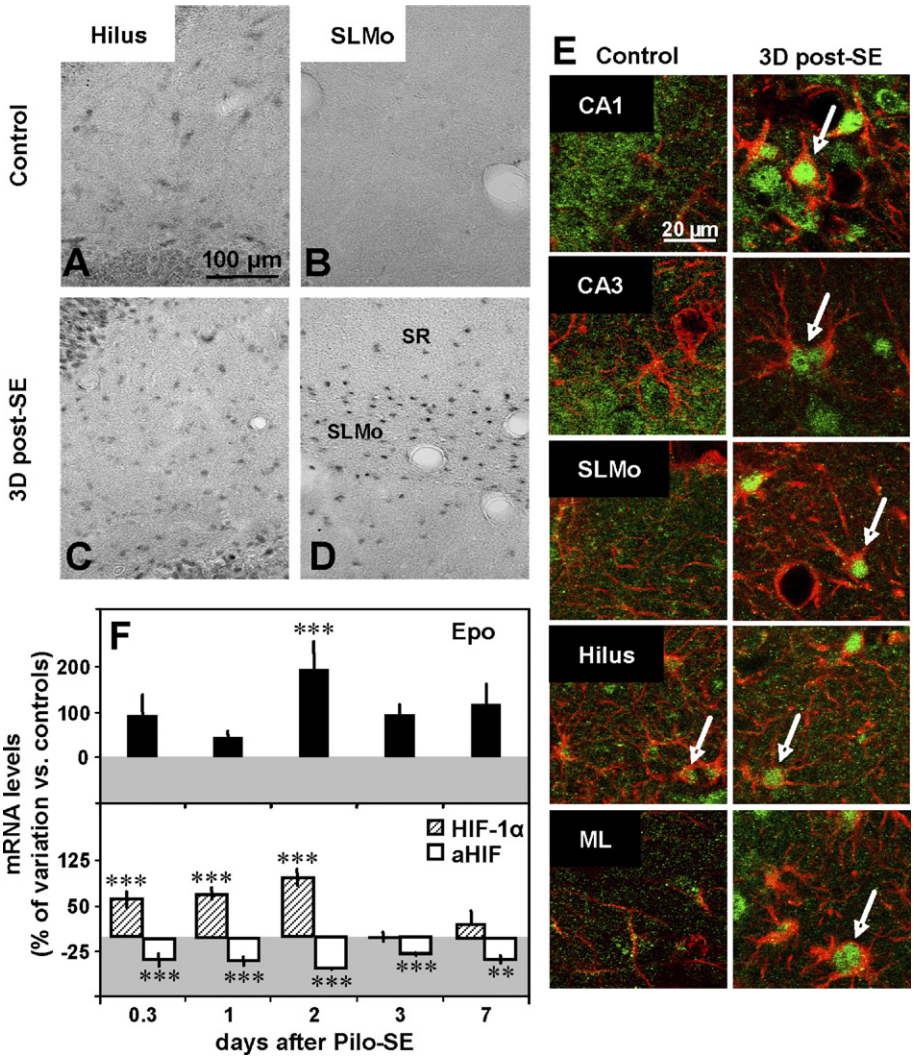

Fig. 3. Transient astroglial induction of Epo in the hippocampus following Pilo-SE. (A–D) Colorimetric staining evidences expression of Epo in neurons of the hilus in controls (A) and in "newly" detected cells bearing morphological characteristics of astrocytes at 3 days post-SE (C, D). (E) Dual fluorescent labelings confirm that the "newly" detected cells expressing Epo (green) are astrocytes (GFAP, red). (F) RT-real time PCR reveals that Epo-mRNA is transiently enhanced in the hippocampus, peaking at 2 days post-SE; this induction is preceded by increased HIF-1α-mRNA levels accompanied by a long-lasting inhibition of its natural anti-sens RNA aHIF. *$P<0.05$; **$P<0.01$; ***$P<0.001$ as compared to controls. Abbreviations: as in Fig. 2; SR, stratum radiatum. Scale bars: A–D, 100 µm; E, 20 µm.

1–7 h post-injection, reaching 538±8 mIU/mL (4520±67 pg/mL) during that time period.

rHuEpo either prevents SE development or reduces stage-4/5 seizure number

Previous studies indicated that rHuEpo administered to mice or rats prolongs the latency and reduces the severity of chemically-induced seizures (Brines et al., 2000; Uzum et al., 2006). Our results demonstrate that pre-treatment with 5000 IU/kg rHuEpo (24 h plus 30 min prior to pilocarpine administration) significantly reduced (−40%; $P<0.05$) the number of rats that developed SE. In rHuEpo pre-treated rats that developed SE, the onset of continuous convulsions was similar to that recorded in rats that did not receive rHuEpo prior to pilocarpine administration (28.6±2.3 min; $P=0.962$). However, rHuEpo pre-treated rats exhibited a reduced number of stage 4/5 seizures (pilocarpine only: 6.00±0.84 seizures, rHuEpo+pilocarpine: 2.00±0.58 seizures; $P=0.015$) during the 2 h of SE.

rHuEpo prevents degeneration of vulnerable neurons, even when administered immediately after Pilo-SE

Previous studies in rats after ischemic stroke indicate that rHuEpo treatment at 5000 IU/kg 24 h before transient middle cerebral artery occlusion significantly reduced infarct volume, with the beneficial effect of systemic Epo being maintained when rHuEpo was given up to 3 h after the onset of ischemia (Brines et al., 2000). Similar tissue protection was obtained in a trauma model, when rHuEpo was given 24 h before and then for 4 days following the injury (Brines et al., 2000). Repeated rHuEpo injections were reportedly well tolerated in rats (Brines et al., 2000; Wang et al., 2004; Lu et al., 2005), even when administered during 7 consecutive days, despite an increase in hematocrit levels (Wang

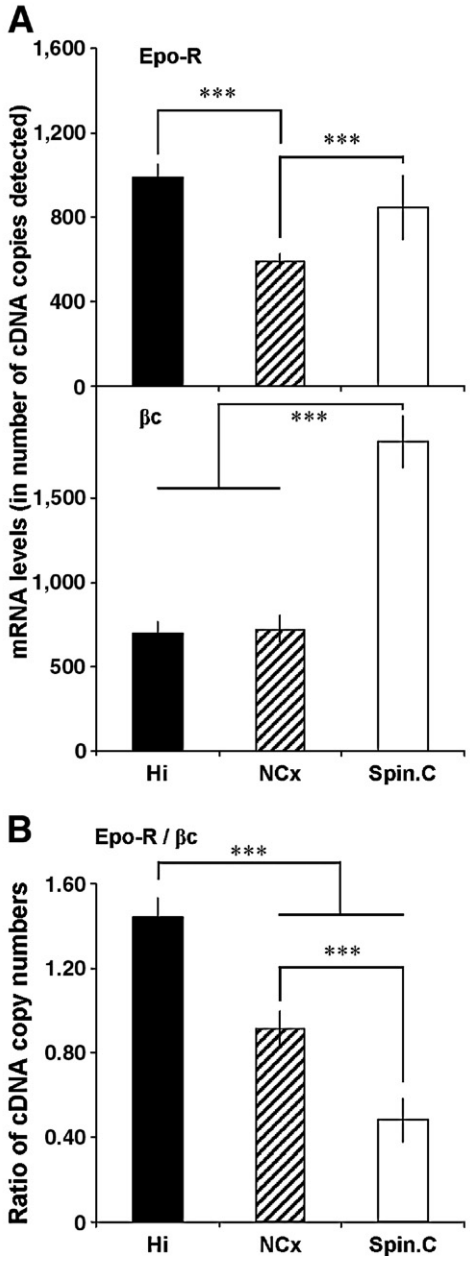

Fig. 4. Epo-R versus βc expression is structure-dependant within the rat central nervous system. In control rats, RT-real time PCR demonstrates that Epo-R mRNA is abundantly expressed in the hippocampal formation and the spinal cord whereas βc mRNA concentration is the greatest within the spinal cord as compared to the hippocampus and the neocortex. Abbreviations: Hi, hippocampus; NCx, neocortex; Spin.C, spinal cord. ***$P<0.001$ as compared to the other structure expression levels.

et al., 2004). To increase the chance of detecting a neuroprotective effect by rHuEpo following Pilo-SE, we administered it repetitively within two different treatment paradigms: 1) rHuEpo was administered preventively (24 h and 30 min before SE onset), and therapeutically (1 and 3 days after SE), and 2) rHuEpo was administered therapeutically only, 2.5 h, 1 and 3 days after the onset of SE. Only rats that developed sustained SE were included in this part of the study.

In the hippocampus, neuroprotective effects of rHuEpo were observed in both protocols of rHuEpo administration, and were dependent on both the regimen of rHuEpo treatment and the neuronal population. This is illustrated in area CA1 and in the hilus (Figs. 10A–H). A similar neuroprotective effect was found in area CA1 between both treatment paradigms (Figs. 10A–D), whereas hilar neuronal protection was only observed when rHuEpo was administered after SE (Figs. 10E–H). Quantitative analysis of the neuronal population stained for NeuN revealed 15 days post-SE that neuroprotection elicited by both rHuEpo treatment paradigms was moderate but significant in area CA1, while full protection of hippocampal neurons was attained in area CA3 and in the SLMo (Fig. 10I). By contrast, only therapeutic injection of rHuEpo (post Pilo-SE) promoted a significant neuroprotective effect on vulnerable hilar neurons (Fig. 10I).

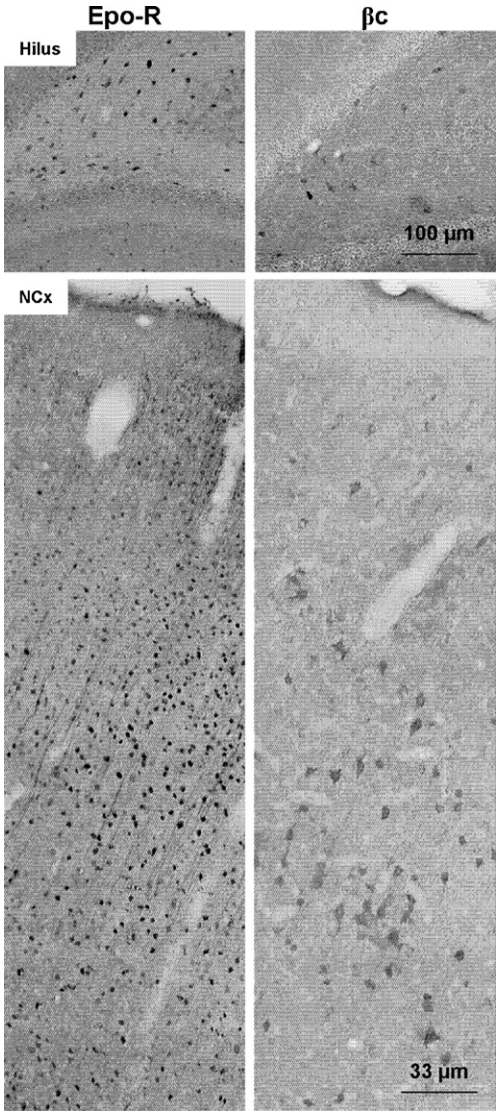

Fig. 5. Localization of Epo-R and βc proteins in the hippocampus and the neocortex. In the hippocampus, βc protein expression is restricted to the hilus, and the number of βc-labeled cells is less than that of Epo-R labeled cells. In the neocortex, a dense Epo-R labeling is observed as compared to βc immunolabeling. Abbreviations: as in Fig. 4. Scale bars: Hilus, 100 μm; NCx, 33 μm.

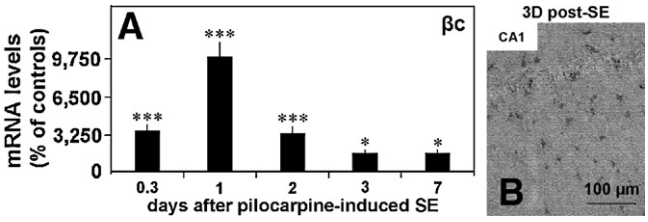

Fig. 6. Dramatic increase in βc expression within microglia after Pilo-SE. (A) RT-real time PCR demonstrates that βc-mRNA level is significantly induced throughout the 7 days following Pilo-SE with a dramatic peak observed at 1 day. *$P<0.05$; ***$P<0.001$ as compared to controls. (B) Colorimetric staining, performed at 3 days post-SE, evidenced an increased staining in all the hippocampal layers, as illustrated in CA1, within cells bearing the morphological features of activated microglial cells. Scale bars: B, 100 µm.

Compelling data indicate that glutamatergic mossy cells are among the most vulnerable neurons of the hippocampus (Sloviter, 1987, 1991, 1994; Blumcke et al., 2000; Sloviter et al., 2003). Analysis of GAD65/67 immunohistolabeling in the hilus indicated that GABAergic interneurons were preserved following Pilo-SE (Figs. 11A–C). By contrast, the number of glutamatergic mossy cells, detected in the hilus following CGRP-immunohistochemistry (Freund et al., 1997) (Figs. 11D–E), dramatically decreased in rats which experienced Pilo-SE (29±3% of controls; $P<0.001$, Fig. 11G). However, in rats treated with rHuEpo after Pilo-SE, the number of CGRP-immunopositive cells detected (Figs. 11D–G) was significantly higher (71±4% of controls; $P<0.001$), indicating that rHuEpo protected hilar mossy cells.

Discussion

The original findings of this study include description of Epo, Epo-R, and common β chain (βc) expression in the rat hippocampus under basal and induced conditions. Epo-R protein is constitutively expressed in a large majority of neurons, while Epo and βc protein expression are much more discrete, and restricted to specific neuronal populations. Few astrocytes constitutively express Epo and Epo-R in every area of the hippocampus. Following Pilo-SE, Epo and Epo-R are induced in numerous astrocytes, and in hippocampal areas where neuronal death has occurred, βc expression appears to be restricted to microglial cells following Pilo-SE. We also show that rHuEpo, when administered therapeutically following Pilo-SE, provides a robust protection of vulnerable hippocampal neurons. Our study thus indicates that exogenous rHuEpo may act in synergy with astroglial induction of endogenous Epo to enhance neuronal survival in the hippocampus following brain-damaging SE.

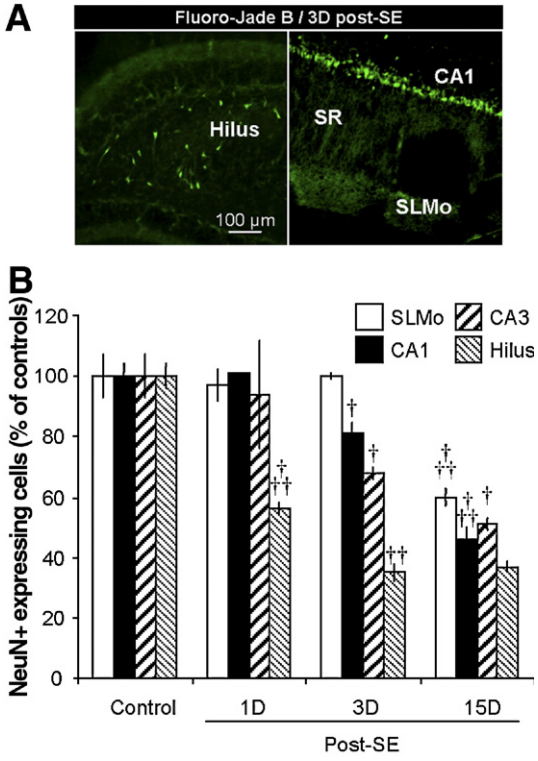

Fig. 7. Diverse patterns of neuronal loss in various areas of the dorsal hippocampus following Pilo-SE. (A) Fluoro-Jade B staining evidences that degenerating neurons are present in the hilus and CA1 but not in the SLMo at 3 days post-SE. (B) Time course of neuronal loss estimated in the hippocampus at IA +5.40 mm (Paxinos and Watson, 1998) following Pilo-SE. NS, not statistically significant; $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$ as compared to the prior time point. Abbreviations: as in Figs. 2 and 3. Scale bar: A, 100 µm.

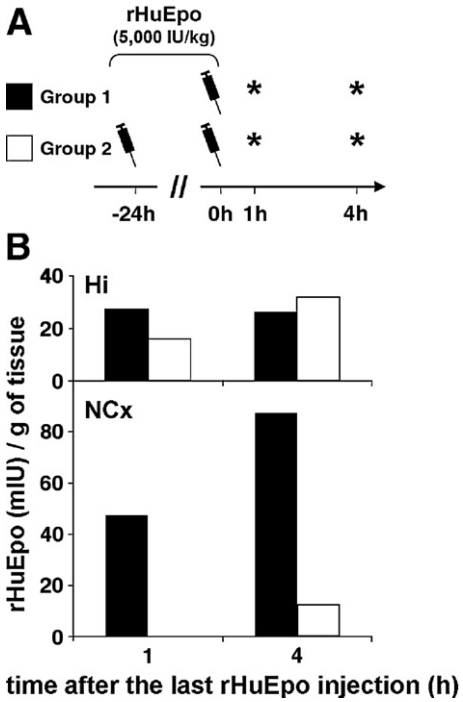

Fig. 8. rHuEpo accumulation in the rat cerebral parenchyma after its systemic administration in basal condition. (A) Experimental design: the hippocampal and neocortical tissue concentration of rHuEpo is determined in rats sacrificed 1 and 4 h following its last systemic administration (5000 IU/kg intraperitoneally) ($n=8$). rHuEpo was applied once in Group 1 ($n=4$) and twice, 24 h apart, in Group 2 ($n=4$). (B) Each bar represents the mean concentration of rHuEpo determined from the values of 2 rats providing similar results. Note in Group 2 that rHuEpo could not be detected within the neocortex of both rats 1 h after the last intraperitoneal injection. Abbreviations: as in Fig. 4; *: time of termination.

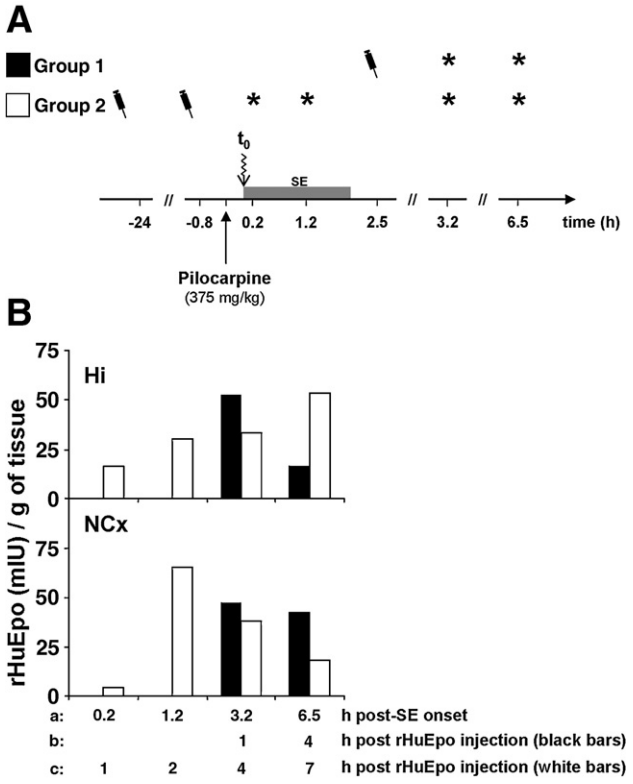

Fig. 9. rHuEpo accumulation in the cerebral parenchyma of rats submitted to Pilo-SE. (A) Experimental design: t_0 is the time at which rats entered into SE following pilocarpine administration. rHuEpo was injected only after (30 min, 1 and 3 days) the end of SE in Group 1 ($n=4$), and was injected both before (−24 h and −30 min) pilocarpine administration and after (1 and 3 days) the end of SE in Group 2 ($n=8$). (B) Each bar represents the mean concentration of rHuEpo determined from the values of 2 rats providing similar results. On the *x* scale, time (in hours) is referred to the onset of SE (a), or to the last injection of rHuEpo (b for Group 1; c for Group 2). Abbreviations: as in Fig. 4; *: time of termination.

Numerous *in vitro* studies have reported that Epo-R is expressed by cultured hippocampal neurons (Kawakami et al., 2001; Chong et al., 2003; Leist et al., 2004; Ehrenreich et al., 2005). While both Epo binding sites (Digicaylioglu et al., 1995) and Epo-R (Ehrenreich et al., 2005) have been shown to be abundant in adult rodent hippocampus under basal conditions, only one study has reported the presence of Epo-R expressing cells in the adult rat hippocampus (Morishita et al., 1997). Conversely, another study found that hippocampal Epo-R was expressed after hypoxia exposure, but not under basal conditions (Lewczuk et al., 2000). Therefore, we conducted the present study to resolve these discrepancies. One major finding of this study is that Epo-R is primarily expressed by neurons in the hippocampus, and that almost all neurons of the hippocampus express Epo-R constitutively, except within the hilus. Neuronal Epo-R immunolabeling is concentrated within cell bodies and varicosities, except in area CA1 where Epo-R is also found in basal dendrites of pyramidal neurons laying throughout the *stratum radiatum*. Such a massive basal expression of Epo-R in the hippocampus suggests that released Epo may play a role in neuronal homeostasis. Epo is barely detectable in the adult hippocampus by western blot analysis in basal conditions (Ehrenreich et al., 2005). Our *in situ* study agrees with a prior work (Chung et al., 2004) showing that Epo is mainly localized in pyramidal neurons, hilar neurons and granule cells. Here, we also show that few astrocytes express Epo. This low hippocampal steady-state level of Epo may play a role in counteracting death of vulnerable neurons after weak brain insults, such as short-time ischemia in gerbils (Sakanaka et al., 1998).

When the insult is robust or long-lasting, the widespread presence of Epo-R in hippocampal neurons may increase the probability that Epo receptors bind Epo induced locally in injured tissue. We observed that Epo-mRNA increased rapidly after Pilo-SE, followed by the detection of Epo protein 24 h later in numerous astrocytes, especially within the SLMo. The induction of the Epo gene in most tissues is regulated by hypoxia-inducible factor-1 (HIF-1), which is activated by a variety of stressors, including hypoxia (Marti, 2004). HIF-1α protein, the regulatory sub-unit of HIF-1, is known to be degraded under normoxic conditions, and its accumulation, observed in hypoxic tissues, requires post-translational modifications (Marti, 2004). Our results indicate that enhanced hippocampal Epo-mRNA expression is preceded by an increase in the transcript levels of HIF-1α, and is accompanied by the downregulation of aHIF-RNA, the natural anti-sense RNA complementary to the 3′ UTR of HIF-1α-mRNA, that may be involved in the regulation of its degradation (Rossignol et al., 2004). In addition to the required post-translational stabilization of HIF-1α, the coordinated regulation of both HIF-1α-mRNA and aHIF-RNA following Pilo-SE suggests that the hippocampus has been exposed to severe hypoxic damages. The reduced tissue perfusion (and thus oxygenation) reported 1–3 days post-SE and likely caused by the abnormal expansion of blood vessels originating from capillaries of the hippocampal fissure (Sloviter, 2005) may explain the astroglial induction of Epo in the SLMo. Indeed, astroglial expression of Epo is enhanced by hypoxia (Masuda et al., 1994; Siren et al., 2001) and by agents that mimic hypoxic insults (Bernaudin et al., 2002). Transcript levels of Epo-R are also increased and maintained at a high level after Pilo-SE. This Epo-R gene induction is associated with the detection of Epo-R in numerous astrocytes, particularly in areas CA1 and CA3, the SLMo, and the hilus.

This study is the first to demonstrate that both Epo and Epo-R gene expression are increased in neurons and astrocytes in a model of severe neuronal damage. The profiles of Epo and Epo-R induction are in line with most observations noted in mouse cortex after brain ischemia (Bernaudin et al., 1999), in rat spinal cord after trauma injury (Gorio et al., 2005; Grasso et al., 2005), and in rat peripheral nerve injury (Li et al., 2005), with the notable exception that Epo and Epo-R are detected neither in resting or reactive microglial cells, nor in endothelial cells following Pilo-SE. In the SLMo, the observation that both Epo and Epo-R are expressed by astrocytes following Pilo-SE underscores the possibility that astroglial Epo in this hippocampal area is an autocrine signaling molecule that may trigger the release of unknown astroglial factors involved in neuronal survival.

Neurodegeneration was examined 1–4 days and 15 days after Pilo-SE. We found that neurodegeneration occurred rapidly in the hilus, corroborating prior studies which showed that hilar neurons are among the most vulnerable hippocampal neurons (Sloviter, 1987, 1991, 1994; Blumcke et al., 2000; Sloviter et al., 2003). By contrast, the neurons located in the SLMo appeared to be more robust than those located in the hilus and in the Ammon's horn, since no Fluoro-Jade B staining was seen during the 1–4-day period following Pilo-SE. It can thus be concluded that i) basal neuronal expression of Epo in the hilus is not capable to maintain

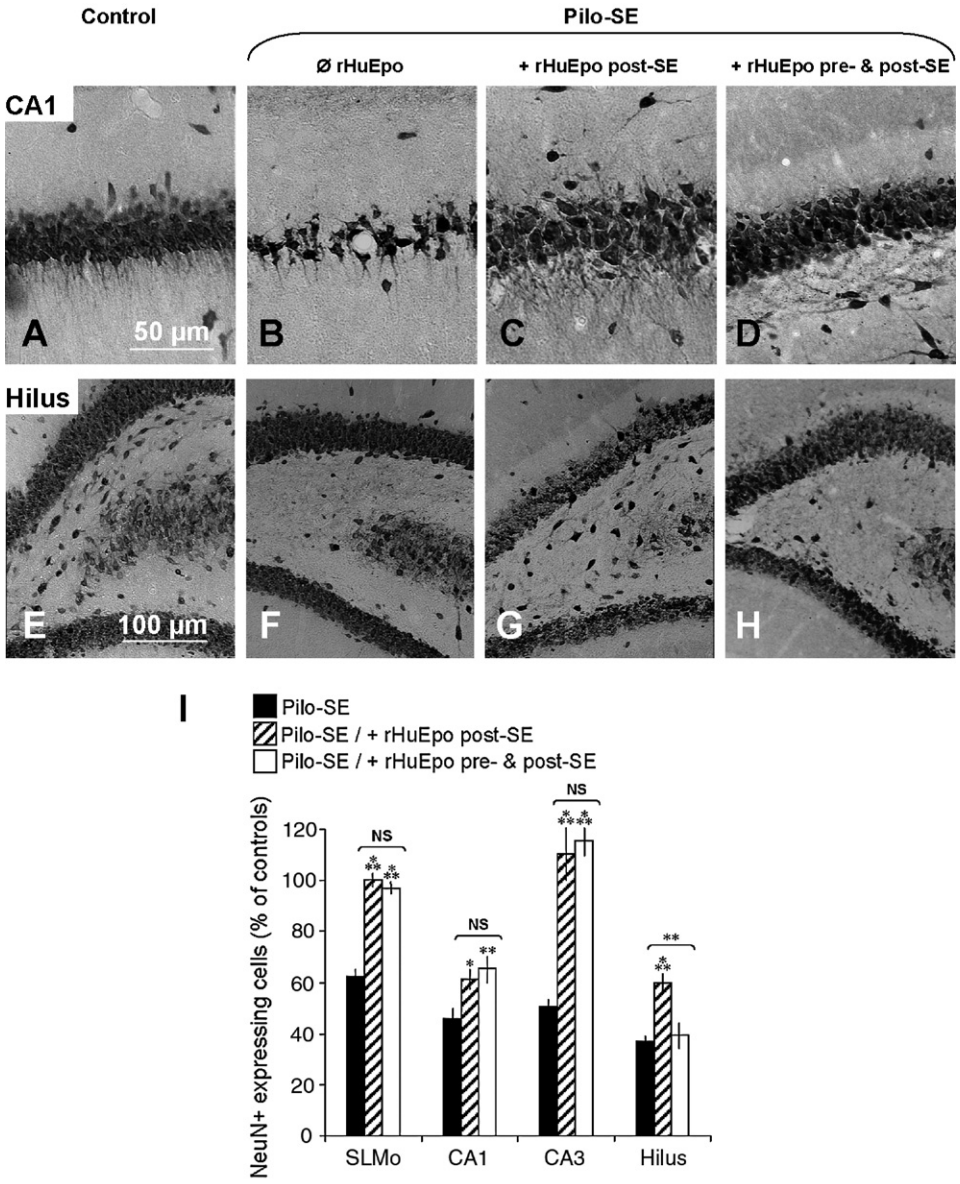

Fig. 10. Systemic administration of rHuEpo (5000 IU/kg) protects hippocampal neurons against degeneration. (A–F) NeuN staining at 15 days post-SE evidences that both pre/post- or and post-treatment of rHuEpo protects neurons in CA1 but only post-treatment have a neuroprotective action on hilar neurons. (G) Both protocols of rHuEpo administration rescued completely neurons in the SLMo and pyramidal neurons in CA3, and partially pyramidal neurons in CA1 from degeneration induced by SE, but only post-treatment of rHuEpo prevents hilar neurons death. NS, not statistically significant; $*P<0.05$; $**P<0.01$; $***P<0.001$, as compared to rats which underwent Pilo-SE and did not receive rHuEpo. Abbreviations: as in Fig. 2. Scale bars: A–C, 50 µm; D–F, 100 µm.

the integrity of hilar neuronal population in the short-term (24 h) following Pilo-SE, and ii) the greater robustness of SLMo neurons cannot be attributed to local presence of Epo under basal conditions, since Epo was not detected in the SLMo of control rats. One possibility to explain the delayed death of SLMo neurons is that the astroglial induction of Epo in the SLMo following Pilo-SE contributed to prolong neuronal survival. However, one major issue is that Epo induction was only transient following Pilo-SE, a result which may be explained in the hippocampus by the activation of proinflammatory cytokines (Rizzi et al., 2003; Voutsinos-Porche et al., 2004), known to impair Epo expression (Frede et al., 1997; Nagai et al., 2001; Gorio et al., 2005). This transient up-regulation of Epo appears to be insufficient to support long-term survival of SLMo neurons, since neuronal loss was also evidenced in that area 15 days post-SE.

Neuroprotection induced by rHuEpo applied systemically requires that it crosses the BBB. The presence of Epo-R at the apical cell surface of brain endothelial cells has been proposed to mediate the transcytosis of circulating rHuEpo into healthy brain tissue (Brines et al., 2000), as well as in the human epileptic tissue (Eid et al., 2004). By using In-111-labeled rHuEpo injected intravenously, rHuEpo has been shown to penetrate the human brain with intact BBB (Ehrenreich et al., 2004). Here, we measured 4 h post-rHuEpo administration (5,000 IU/kg intraperitoneally) ~85 mIU/mg tissue in the healthy rat neocortex. Approximating that 1 mg tissue ≅ 1 mL, we estimated that levels of rHuEpo in the

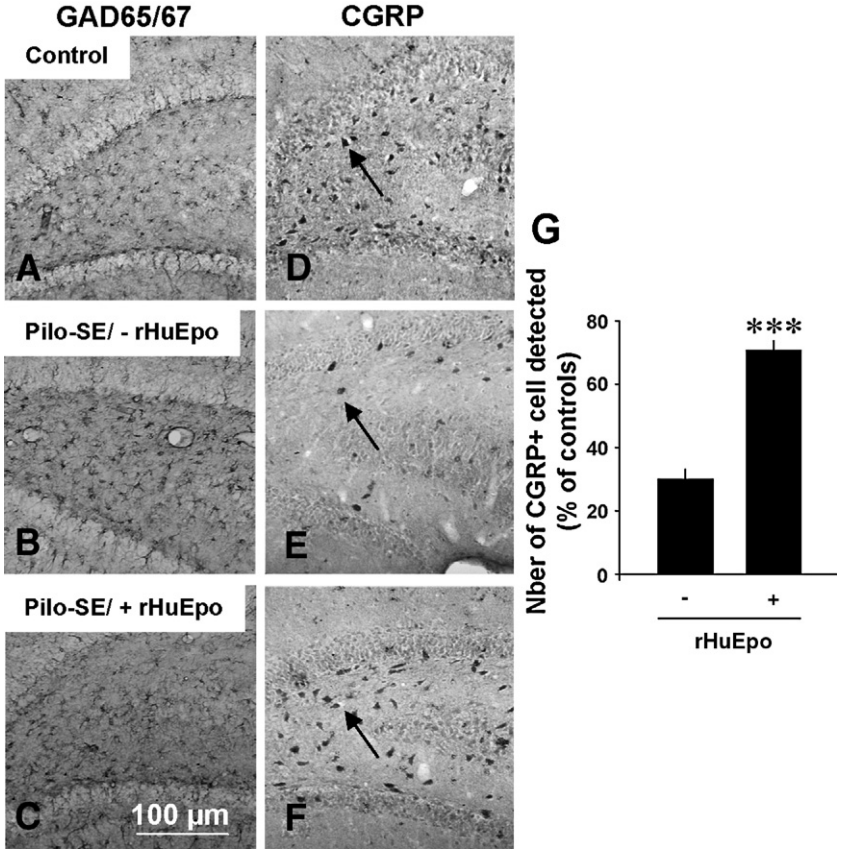

Fig. 11. rHuEpo (5000 IU/kg) administered immediately, 1 and 3 days post-SE protects vulnerable hilar mossy cells against cell death generated by Pilo-SE. (A–C) Population of GABAergic interneurons, detected by GAD65/67 immunohistochemistry, is maintained at 15 days post Pilo-SE. (D–F) Glutamatergic hilar mossy cells (black arrows), detected by CGRP-immunohistochemistry, are extremely vulnerable and die massively following Pilo-SE, but a substantial number of them are rescued by rHuEpo treatment. (G) Quantification of hilar mossy cells following Pilo-SE. ***$P<0.001$ as compared to rats which underwent Pilo-SE and did not receive rHuEpo. Scale bar: A–F, 100 µm.

rat brain tissue were consistent with those previously measured in the cerebro-spinal fluid (~100 mIU/mL measured 3.5 h post-injection) using the same administration protocol (Ehrenreich et al., 2004). We also showed that brain uptake of rHuEpo was not homogeneous throughout the healthy rat brain. Indeed, rHuEpo uptake in the neocortex was ~3-fold greater than that measured in the hippocampus. However, when rats were pre-treated with rHuEpo 24 h before the second injection of rHuEpo, brain uptake was almost abolished, but in the neocortex only. While the reasons which may explain the inhibition of rHuEpo uptake in the neocortex of healthy rats pre-treated with rHuEpo are still obscure, data obtained in pilocarpine-treated rats allow us to reject the hypothesis that rHuEpo pre-treatment caused a down-regulation of Epo-R at the apical surface of endothelial cells in the neocortex. Indeed, in rHuEpo pre-treated rats, we found 1.2 h after the onset of Pilo-SE that rHuEpo injected just prior to pilocarpine administration was present at high levels in the neocortex. This result cannot be explained by a facilitation of brain entry of circulating rHuEpo due to the BBB breakdown occurring during SE (Ehrenreich et al., 2004), since rHuEpo pre-treatment has been shown to prevent BBB breakdown both *in vitro* (Martinez-Estrada et al., 2003) and *in vivo* (Uzum et al., 2006). Thus, in rHuEpo pre-treated rats, brain penetration of rHuEpo administered just before Pilo-SE requires functional Epo-R at the surface of the endothelial cells. By contrast, when rHuEpo is applied post-SE only, the 2-fold increase in brain rHuEpo levels observed in the hippocampus 1 h post-rHuEpo injection may be explained by SE-induced BBB breakdown (Uzum et al., 2006), thus facilitating brain entry of circulating rHuEpo.

Considering the wide distribution of Epo-R at the surface of hippocampal neurons, it was fundamental to determine whether administration of rHuEpo could significantly reduce hippocampal neurodegeneration induced by Pilo-SE. Our first approach was to provide rHuEpo prior to the onset of SE, because earlier studies had reported that preventive rHuEpo treatment 24 h before the onset of ischemia or trauma significantly protected the injured brain areas (Bernaudin et al., 1999; Bernaudin et al., 2000). We also administered rHuEpo 30 min prior to, 1 day and 3 days after pilocarpine administration to increase the chance to protect neurons as explained in the result section. Our data show that rHuEpo administered 24 h and 30 min prior to pilocarpine administration decreased by 40% the number of rats entering into SE and reduced the severity of behavioral seizures, corroborating earlier studies using kainate- or pentylentetrazol to induce SE in adult mice or rats, respectively (Brines et al., 2000; Uzum et al., 2006). In rats that developed sustained SE, rHuEpo treatment preserved the neuronal population in areas CA1 and CA3, and in the SLMo 15 days post-SE, but had no effect on hilar neuron survival. In a second approach, which is more relevant for clinical practice, we administered rHuEpo after SE only (30 min, 1 and 3 days post-SE)

and noted that neuroprotection was further extended to hilar neurons. In the hilus, we found that GABAergic interneurons, identified by *in situ* detection of GAD65/67, survived to Pilo-SE, which is in accordance with previous work (Cavalheiro, 1990). Neuroprotection in the hilus concerned mossy cells, considered as the most vulnerable neurons of the hippocampus (Sloviter, 1987, 1991, 1994; Blumcke et al., 2000; Sloviter et al., 2003). Indeed, we noted a significant protection of CGRP-immunolabeling, attributed to mossy cells in the hilus (Freund et al., 1997). We hypothesize that neurons which are not rescued by rHuEpo are those which were found not to express Epo-R under basal conditions in the hilus. If that it the case, their extreme vulnerability may partly result from their inability to respond to endogenous Epo and, hence, to rHuEpo.

In the neocortex (Leist et al., 2004) and the spinal cord (Brines et al., 2004), rHuEpo-mediated neuroprotection may involve a receptor that is distinct from Epo-R homodimer necessary to stimulate hematopoiesis. *In vivo* studies in mice lacking βc have suggested that this receptor may consist of a single Epo-R monomer and a βc homodimer (Brines and Cerami, 2005). Here, we demonstrate that the relative abundance of βc compared to Epo-R is not homogeneous throughout the central nervous system. Indeed, we found that Epo-R/βc mRNA ratio in the hippocampus was 1.6- and 3.0-fold greater than that found in the neocortex and the spinal cord, respectively. These results suggest that diverse Epo binding sites may exist in the central nervous system, and that the probability to find neuronal Epo-R/$(\beta c)_2$ heteroceptor in the spinal cord is greater than in the hippocampus. The potential existence of various binding sites for Epo thus encourages diverse forms of protective molecules to be engineered, devoid of the adverse effects activated by the homodimeric Epo-R (Brines and Cerami, 2005). Molecules targeting receptors other than Epo-R homodimers already exist. Carbamylated Epo (CEPO), which has a low affinity for Epo-R homodimers, and its analogues are devoid of erythropoietic activity (Leist et al., 2004), and are neuroprotective in the neocortex and the spinal cord (Brines et al., 2004; Leist et al., 2004; Villa et al., in press). However, if CEPO indeed targets Epo-R/$(\beta c)_2$ heteroceptors, our results suggest that Epo may be more efficient than CEPO to protect hippocampal neurons, due to the paucity of βc in the hippocampus.

In conclusion, this study is the first to evidence in the hippocampus a transient astroglial increase in both Epo and Epo-R in a model of chemically-induced SE. We also demonstrate that rHuEpo administration is neuroprotective following SE. Thus, the observation that Epo administration has beneficial effects on neuronal survival strongly suggests that astroglial induction of Epo following SE is protective rather than deleterious. Hence, we propose that treatment with rHuEpo supplements astroglial-derived Epo to promote enhanced neuronal survival following SE. A successful proof-of-concept clinical trial using rHuEpo has been conducted in stroke patients (Sloviter, 1991; Ehrenreich et al., 2002). Our finding that rHuEpo is neuroprotective after Pilo-SE opens new possibilities to improve the neurological outcome following SE.

Acknowledgments

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