Erythropoietin protects hippocampal neurons following status epilepticus

Jérémie Nadam, Fabrice Navarro, Pascal Sanchez, Colette Moulin, Béatrice Georges, Aël Laglaine, Jean-Marc Pequignot, Anne Morales, Philippe Ryvlin, Laurent Bezin

Erythropoietin protects hippocampal neurons following *status epilepticus*

Jérémie Nadam,[1, 2] Fabrice Navarro,[1, 2] Pascal Sanchez,[1, 2] Colette Moulin,[1, 2] Béatrice Georges,[1, 2] Aël Laglaine,[1, 2] Jean-Marc Pequignot,[1, 2] Anne Morales,[1, 2] Philippe Ryvlin,[3, 4, 5] and Laurent Bezin,[1, 2]

[1]Laboratory of Integrative Cellular and Molecular Physiology, Centre National de la Recherche Scientifique and Université Claude Bernard Lyon 1 UMR5123, 69622 Villeurbanne; [2]IFR 41; [3]Department of Experimental Epilepsy, Université Claude Bernard Lyon 1 EA1880, 69008 Lyon; [4]Hôpital Neurologique P. Wertheimer, Lyon, and [5]IFR 19, France

Corresponding author : Laurent Bezin ; e-mail: laurent.bezin@univ-lyon1.fr

Abstract

Objective: Neuroprotective functions of brain-derived erythropoietin (Epo) are thought to involve both Epo receptor (Epo-R) and common β chain (βc). Here, we measured the response of Epo system (Epo, Epo-R and βc) during neurodegenerative processes occurring following status epilepticus (SE), and examined whether recombinant human Epo (rHuEpo) could support neuronal survival.

Methods: We analyzed Epo, Epo-R and βc expression at both messenger RNA and protein levels after pilocarpine-induced SE (Pilo-SE) in the adult rat hippocampus. Potential protective effect of rHuEpo (Eprex®, Janssen-Cilag; 5,000 IU/kg) was investigated 2 weeks after SE.

Results: By contrast to Epo and βc, detected in few neurons only in the hippocampus, Epo-R is expressed by a majority of neurons. Following Pilo-SE, Epo is induced in numerous astrocytes and hippocampal areas where astroglial induction of Epo is the greatest exhibit a pattern of delayed neuronal death. βc is induced in reactive microglia only. Therapeutic administration of rHuEpo reduces considerably neuronal degeneration in the hippocampus.

Interpretation: rHuEpo may support astroglial Epo to promote hippocampus neuronal survival following SE, likely through a mechanism involving Epo-R mainly. Paucity of βc in the hippocampus rules out the possibility that βc can play a major role in rHuEpo neuroprotective effects following SE.

Introduction

Erythropoietin (Epo) was originally described for its role in hematopoiesis, which consists of increasing red blood cells[1] by protecting erythroid progenitors against apoptosis[2]. The original evidence that Epo and its receptor (Epo-R) are expressed in rodent and human brain tissue,[3-6] in cultured neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells [7] has encouraged the discovery of additional biological roles of Epo. Rapidly, exogenous administration of Epo revealed considerable neuroprotective properties, in both *in vitro* and *in vivo* models of central and peripheral neuronal injury occurring in contexts of trauma, stroke and inflammation.[8-10]

Recombinant human Epo (rHuEpo) peripherally-administered has been shown to cross the blood brain barrier (BBB),[11-14] and to mediate neuroprotection in various *in vivo* models of neurological pathologies associated with neuronal damage.[10, 13] Pioneer studies performed in rodent models of temporal lobe epilepsy (TLE) revealed significant blocking effects of

rHuEpo on the development of *status epilepticus* (SE), but did not analyze whether rHuEpo decreased neuronal death,[11, 15] which is a characteristic feature of SE induced epileptogenesis and likely one of multiple mechanisms causing drug resistance in a large part of patients with TLE.[16] It is noteworthy that most of the apoptosis pathways involved in animal models of TLE [16] are those targeted by Epo.[9, 10]

Some clinical issues have been raised regarding the unwanted side effects linked to chronic administration of rHuEpo, such as increase in blood pressure, thrombosis, tumor expansion, and finally mortality,[9, 10] justifying the development of Epo derivatives that are neuroprotective but not erythropoietic.[17] Carbamylated Epo (CEPO), which is one of such derivatives, has a low affinity for Epo-R homodimer as compared to native Epo and exerts neuroprotective effects both in the neocortex[17] and the spinal cord.[17, 18] While some groups claim that Epo-mediated neuroprotection can occur via Epo-R homodimers,[19] CEPO- and rHuEpo-mediated neuroprotection in the spinal cord involves a heteroreceptor complex, comprising 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 βc-knockout mice, which express normal levels of Epo-R.[10, 18, 20] Thus, the cumulating data obtained in both *in vitro* and *in vivo* models of neuronal degeneration suggest that various Epo binding sites may serve neuroprotection, either through Epo-R homodimers or Epo-R/βc heteroreceptors. Unfortunately, *in situ* description of βc expression in brain of wild-type animals was still lacking.

The high number of studies which evidenced neuroprotective effect of exogenous Epo contrasts with the little attention that the endogenous Epo system has received, particularly to define with precision *in situ* modalities (tissue level and cell types) of both Epo and Epo-R expression, not only in basal conditions, but also in response to brain injury. Epo system reactivity has been investigated in a model of cerebral ischemia within the neocortex,[21] and in models of traumatic injury in both the spinal cord[22] and the peripheral nervous system.[23, 24] To date, Epo system reactivity has not been investigated after chemically-induced SE, which is widely used to induce a series of changes leading to a state of chronic seizures that has some resemblance to TLE.[25]

Our study is the first to investigate in the adult rat hippocampus the expression of Epo system (Epo, Epo-R and the βc) both in basal conditions and in response to pilocarpine-induced SE (Pilo-SE). Here, we show that Epo-R is expressed by almost all neurons of the hippocampus in basal conditions, and rarely by astrocytes. Following Pilo-SE, we evidence a transient induction of Epo in hippocampal astrocytes, which is particularly strong in regions

exhibiting the greatest degree of neuronal robustness, and a long-lasting induction of Epo-R. We also report that hippocampal βc subunit expression is low as compared to the spinal cord in basal conditions, whereas it dramatically increases after Pilo-SE in reactive microglia. We finally demonstrate that administration of rHuEpo following Pilo-SE significantly protects hippocampal neurons, suggesting that rHuEpo may be therapeutically useful to counteract neurodegeneration in patients susceptible to develop TLE.

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 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 hours by diazepam administration (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/mL 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/mL), previously determined as the optimal neuroprotective dose with this route of administration.[26] Naïve rats received saline only.

Experimental design.

First set of experiments: response of hippocampal Epo system following Pilo-SE. Rats experienced 3 hours of SE induced by 300 mg/kg pilocarpine hydrochloride. In this study, we used the minimal dose of pilocarpine needed to induce a sustained SE in a large majority of rats without compromising long term survival. 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 hours (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 both 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 made in 0.1M 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.

Second set of experiments: uptake of rHuEpo in brain tissue of control rats and rats undergoing Pilo-SE

Plasma and brain tissue concentration of rHuEpo was determined at different times following its peripheral administration in rats (n=20), pre-treated or not with the same dose of rHuEpo 24 hours before, and subjected or not 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.

Third set of experiments: can rHuEpo modify the development of Pilo-SE and rescue vulnerable hippocampal neurons? In this set, rats experienced 2 hours of SE induced by 375 mg/kg pilocarpine hydrochloride, which is the dose previously used to characterized neuronal degeneration.[25] Three series of experiments have been 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 hours 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 only when rHuEpo was injected 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.[27] 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, 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 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 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 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 D13566) forward 5' CCA GCT CTA AGC TCC TGT GC 3', reverse 5' CTT CAG GTG AGG TGG AGT GG 3' (68 bp); ***HIF-1α*** (GenBank 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 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 x g for 20 min at 4°C) and blood samples (760 x 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 ImmunoResearch), 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.[14, 18, 28]. In addition, the specificity of sc-697 antibody used to detect Epo-R in rodent brain tissue has already been demonstrated.[29]

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 specifically degenerating neurons after Pilo-SE in rats.[30] 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 allows to scan at magnification

20X 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.[31] 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 ± SEM 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 (see Fig 1A). Dual fluorescent labeling of Epo-R together with specific markers of either neurons (NeuN), astrocytes (GFAP) or microglial cells (OX-42) evidenced that Epo-R was mostly expressed by neurons (see Fig 1). However, not all neurons expressed Epo-R, especially in the hilus (see white arrows on Fig 1B). Epo-R labeling was mainly distributed in neuronal cell bodies, except within CA1 where a strong signal was revealed in the dendritic field (see 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 (see Fig 2A) and was never detected in resting microglial cells (data not shown).

Colorimetric immunohistochemistry evidenced the presence of Epo protein in the pyramidale layer of CA3 (data not shown), in the perikarya and processes of some hilar (see Fig 3A) and CA4 (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 by neurons mainly (data not shown) and rarely by astrocytes in the hilus (see Fig 3E). Epo was never detected in resting microglial cells (data not shown).

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

Recent investigations by others have suggested in murine models of spinal cord trauma that the neuroprotective effect of Epo required the presence of a heteroreceptor comprising both Epo-R and βc, the signal-transducing subunit shared by the GM-CSF, and the IL-3 and IL-5 receptors.[18] Our results in control rats, after quantification by real-time PCR following reverse transcription of 500 ng of tissue total RNA, evidences that the transcript encoding βc is expressed at low levels in the hippocampus (702 ± 80 copies) and the neocortex (718 ± 80 copies) as compared to the spinal cord (1,830 ± 153 copies; $p<0.001$) (see Fig 4A). By contrast, Epo-R mRNA is abundantly expressed in the hippocampus (991 ± 59 copies) and the spinal cord (849 ± 151 copies) as compared to the neocortex (592 ± 31 copies; $p<0.001$) (see 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 (see Fig 4B). Detection of βc protein in the hippocampus was restricted to the hilus (see Fig 5). Comparison of Epo-R and βc protein presence in the dentate gyrus and the neocortex indicates that the number of cells expressing Epo-R is greater than that of cells expressing βc (see Fig 5).

Epo-R expression in the hippocampus after Pilo-SE.

Following Pilo-SE, mRNA encoding Epo-R was robustly increased in the hippocampus from 1 day through 7 days post-SE, with a peak observed at 3 days post-SE (see Fig 2B). This effect was specific to Pilo-SE and was not consequent to scopolamine/diazepam (SD) treatments used to stop seizures, since no difference was observed between naive rats and rats which received scopolamine/diazepam treatments (control/SD rats) only at all time points examined (data not shown). Immunohistochemical studies performed at 4 days post-SE evidenced a reduction of the neuronal staining in the hilus and CA4 (see Figs 2C and E). By contrast, additional glial-like cells expressed Epo-R within all hippocampal areas, as illustrated in the hilus (see Figs 2C and E) and CA3 (see Figs 2D and black arrow on 2F). Double GFAP/Epo-R fluorescent immunolabeling helped to identify these glial cells as astrocytes (see Fig 2A).

Epo expression in the hippocampus after Pilo-SE.

Following Pilo-SE, mRNA encoding Epo was transiently enhanced in the hippocampus at 2 days post-SE (+196%; $p<0.001$) (see Fig 3F). Immunohistochemical studies performed at 1, 3 and 4 days post-SE revealed that neuronal labeling disappeared in the hilus, as shown at 3 days post-SE (see Figs 3A and C). By contrast, in hippocampal subfields that were clearly devoid of Epo-expressing cells in basal conditions, such as the s*tratum lacunosum moleculare* (SLMo) and the *stratum radiatum* of CA1, Epo was detected in numerous round-shaped perikarya at 3 days post-SE (see Figs 3B and D). Such perikarya were also seen within the pyramidale layer of CA1 and CA3, the molecular layer of dentate gyrus (data not shown), and the hilus (see Figs 3A and C). In all hippocampal subfields mentioned, numerous of these "newly-detected" cells were identified as GFAP-immunoreactive astrocytes (see white arrows on Fig 3E). The scattering of the Epo-expressing cells in both the SLMo and the hilus at 3 days post-SE made it easier to characterize them all as being astrocytes. Epo was never detected in OX-42-immunopositive activated microglial cells (data not shown).

Increased levels of the transcript encoding HIF-1α were observed in the hippocampus at 8 hours following the onset of Pilo-SE (+61%; $p<0.001$). This induction was maintained until 2 days post-SE (+97%; $p<0.001$) (see 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 (see Fig 3F).

Altered levels of Epo mRNA, HIF-1α mRNA and aHIF RNA were specific to Pilo-SE and were not consequent to scopolamine/diazepam (SD) treatments used to stop seizures, since no difference was observed between naive rats and control/SD rats at all 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 was dramatically and transiently increased in the hippocampus, peaking at 1 day post-SE (+9,897%; $p<0.001$) (see Fig 6A). This effect was specific to Pilo-SE and was not consequent to scopolamine/diazepam (SD) treatments used to stop seizures, since no difference was observed between naive rats and control/SD rats at all time points examined (data not shown). Immunohistochemical studies performed at 3 days post-SE evidenced a reduction of the neuronal staining in the hilus (data not shown) and an increased staining throughout the hippocampus, as illustrated in CA1 (see Fig 6B), within

cells bearing the morphological features of activated microglial cells, as previously shown in rats after lipopolysaccharide injection and middle cerebral artery occlusion.[32]

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

Fluoro-Jade B was used to determine 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.[31] Animals undergoing Pilo-SE exhibited a pattern of neuronal loss that varied in the different subfields of the dorsal hippocampus analyzed (see Fig 7). In the hilus, intense Fluoro-Jade B staining was observed at 1 day post-SE and was maintained throughout the time period examined, as shown at 3 days post-SE (see Fig 7A). Neuronal loss reached ~ 45% by 1 day post-SE, and then stabilized by 3 days post-SE at ~ 65% (see Fig 7B). In CA1 and CA3, Fluoro-Jade B staining was observed at 1 day post-SE, and increased at 3 (see Fig 7A) and 4 days post-SE. Neuronal loss was evidenced at 3 days post-SE and reached ~ 50% by 15 days post-SE (see Fig 7B). The SLMo exhibited no Fluoro-Jade B staining from 1 to 4 days post-SE (see Fig 7A). However, at 15 days post-SE, neuronal loss reached ~ 40% (see Fig 7B).

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

rHuEpo was known to cross efficiently the BBB after systemic injection and to accumulate in the cerebro-spinal fluid in rats and humans.[11, 12] Whether rHuEpo concentrates evenly within the brain parenchyma still needed to be determined. In control rats, after the injection of 5,000 IU/kg rHuEpo intraperitoneally, we evidenced 4 hours later that rHuEpo reached a ~ 3-fold greater tissue concentration in the neocortex as compared to the hippocampus (see Fig 8, Group 1). When control rats were pre-treated with rHuEpo 24 hours before the second rHuEpo injection, brain uptake of rHuEpo was considerably affected in the neocortex only: rHuEpo was not detected 1 hour after the injection, and the levels detected at 4 hours were ~ 7-fold lower than that measured without pre-treatment (see Fig 8, Group 2). In pilocarpine-treated rats which were not pre-treated with rHuEpo, tissue uptake of rHuEpo administered 2.5 hrs post-SE onset was 2-fold greater than that found in controls in the hippocampus (see Figs 8 and 9, Group 1). When pilocarpine-treated rats were pre-treated with rHuEpo, the profile of tissue rHuEpo uptake (see Figs 8 and 9, Group 2) was: i) quite similar to that found in control rats in the hippocampus, and ii) no more inhibited as in controls in the neocortex, peaking 2 hours post rHuEpo injection at levels comparable to that found in non rHuEpo-treated controls 4 hours post-injection. Plasma levels of rHuEpo measured in all rats tested

were not different from each other throughout 1-7 hours 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.

Prior studies by others have evidenced that rHuEpo administered either to mice or rats prolongs the latency and reduces the severity of chemically-induced seizures.[11, 15] Our results demonstrated that pre-treatment with 5,000 IU/kg rHuEpo (24 hours plus 30 min prior to pilocarpine administration) significantly reduced (-40%; $p<0.05$) the number of rats entering into SE. In rHuEpo pre-treated rats which developed a SE, the onset of continuous convulsions was similar to that recorded in rats which 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 hrs of SE.

rHuEpo rescues vulnerable mossy cells from degeneration when administered immediately after Pilo-SE.

Previous studies in rats have shown in a model of ischemic stroke that rHuEpo treatment at 5,000 IU/kg 24 hours before transient middle cerebral artery occlusion reduced the infarct volume significantly, the beneficial effect of systemic Epo being maintained when rHuEpo was applied up to 3 hours after the onset of ischemia.[11] Similar tissue protection was obtained in a model of brain trauma, with rHuEpo applied as in the model of ischemic stroke, with the difference that rHuEpo was also administered once daily for 4 additional days following the injury.[11] Repeated rHuEpo administrations appear to be well tolerated in rats,[11, 33, 34] even when administered during 7 consecutive days despite the increase in hematocrit levels.[33] Therefore, to increase the chance for rHuEpo to exert a neuroprotective effect following Pilo-SE, we have decided to administer it repetitively within two different treatment paradigms: in the first, rHuEpo was administered both preventively (24 hours and 30 min before SE onset) and therapeutically (1 and 3 days after SE), in the second, rHuEpo was administered therapeutically only, 2.5 hours, 1 and 3 days after the onset of SE. Only rats which developed a sustained SE were included in this part of the study.

In the hippocampus, the neuroprotective effect of rHuEpo in of two protocols of rHuEpo administration was observed, that was dependant on both the regimen of rHuEpo treatment and the neuronal populations. This is illustrated in CA1 and the hilus (see Fig 10A to H): a similar neuroprotective effect was found in CA1 between both treatment paradigms

(see Fig 10A to D), whereas hilar neuronal protection was observed when rHuEpo was administered after SE only (see Fig 10E to 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 CA1, while full protection of hippocampal neurons was attained in CA3 and the SLMo (see Fig 10I). By contrast, only therapeutic injection of rHuEpo (post Pilo-SE) promoted a significant neuroprotective effect on vulnerable hilar neurons (see Fig 10I).

Compelling data evidenced that glutamatergic mossy cells are the most vulnerable neurons of the hippocampus.[35-37] Analysis of GAD65/67 immunohistolabeling in the hilus indicated that GABAergic interneurons were preserved following Pilo-SE (see Fig 11A to C). By contrast, the number of glutamatergic mossy cells, detected in the hilus following CGRP-immunohistochemistry[38] (see Fig 11D to E), dramatically decreased in rats which experienced Pilo-SE (29 ± 3% of controls ; $p<0.001$, see Fig 11G). However, in rats treated with rHuEpo after Pilo-SE, the number of CGRP-immunopositive cells detected (see Fig 11D to G) greatly increased (71 ± 4% of controls; $p<0.001$).

Discussion

This study presents for the first time the accurate distribution of both Epo, Epo-R and common β chain (βc) in the rat hippocampus in basal conditions. Epo-R is expressed in a large majority of neurons, while Epo and βc expression are much more discrete and restricted to some neuronal populations. Few astrocytes express Epo and Epo-R in every area of the hippocampus. Following Pilo-SE, Epo and Epo-R are induced in numerous astrocytes and hippocampal areas where astroglial Epo induction is the greatest exhibit a pattern of neuronal death that is significantly delayed. βc expression is restricted to microglial cells following Pilo-SE. We also show that rHuEpo, when administered therapeutically following Pilo-SE, provides a robust protection of hippocampal neurons. Our study thus indicates that exogenous rHuEpo may act in synergy with astroglial induction of Epo to enhance neuronal survival in the hippocampus following SE.

Numerous *in vitro* studies have evidenced that Epo-R is expressed by cultured hippocampal neurons.[17, 28, 39, 40] While both Epo binding sites[41] and Epo-R[40] have been shown to be abundant in adult rodent hippocampus in basal conditions, a single *in situ* study illustrated succinctly the presence of Epo-R expressing cells in the adult rat hippocampus.[42] Surprisingly, in another study, hippocampal presence of Epo-R was demonstrated after

hypoxia exposure, but not in basal conditions.[43] Therefore, to fully discern cells which are influenced by the endogenous Epo system (and which might be the target of exogenous Epo), we absolutely needed to expand our knowledge of the accurate distribution of Epo-R in the adult rat hippocampus. One major result of this study is that Epo-R is mainly expressed by neurons in the hippocampus, and that almost all neurons of the hippocampus express Epo-R, except within the hilus. Neuronal Epo-R immunolabeling is concentrated within cell bodies and varicosities, except in 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 plays an important role in neuronal homeostasis. Epo is barely detectable in the adult hippocampus by western blot analysis in basal conditions.[40] Our *in situ* study agrees with a prior work[44] showing that Epo is mainly localized in pyramidal neurons, hilar neurons and granule cells. Here, we also evidence that few astrocytes express Epo. This low hippocampal steady-state level of Epo has been demonstrated to play an important role in counteracting death of vulnerable neurons following weak brain insults, as evidenced following short-time brain ischemia in gerbils.[45]

When the insult is robust or long-lasting, the widespread presence of Epo-R in neurons of the hippocampus may increase the probability to bind efficiently Epo induced locally in the injured tissue. Here, we show that Epo-mRNA increases rapidly after Pilo-SE, followed by the detection, 24 hours later, of Epo protein in numerous astrocytes, especially within the SLMo. The induction of Epo gene in most tissue is regulated by hypoxia-inducible factor-1 (HIF-1), which is activated by a variety of stressors, including hypoxia.[46] 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.[46] Here we show that enhanced hippocampal Epo-mRNA is preceded by an increase in transcript levels of HIF-1α, and accompanied by the downregulation of aHIF-RNA, the natural anti-sense RNA complementary to the 3' UTR of HIF-1α-mRNA and likely involved in the regulation of its degradation.[47] 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 expansion of blood vessels originating from capillaries of the hippocampal fissure[48] may explain the astroglial induction of Epo in the SLMo. Indeed, astroglial expression of Epo is greatly enhanced by hypoxia[4, 49] and by agents which mimic hypoxic insults.[50] Concomitantly

to Epo gene activation, 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 evidence that both Epo and Epo-R gene expression are dramatically increased in neurons and astrocytes in a model of severe neuronal damage occurring in highly vulnerable areas of the adult rat hippocampus, namely CA1 and the hilus. The profiles of Epo and Epo-R induction are in line with most observations noted in mouse cortex after brain ischemia,[21] in rat spinal cord after trauma injury,[14, 22] and in rat peripheral nerve injury,[24] 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 which may trigger the release of yet unknown astroglial factors involved in neuronal survival.

Neurodegenerative process and neuronal loss have been carefully examined in this study in the short term (1-4 days) after Pilo-SE in the different subfields of the hippocampus using Fluoro-Jade B staining and NeuN-immunohistochemical detection, respectively. We noted that neurodegeneration occurred rapidly in the hilus, corroborating prior studies which qualified hilar neurons as the most vulnerable neurons of the hippocampus.[35-37] By contrast, the neurons located in the SLMo appeared to be more robust than those located in the hilus and Ammon's horn, since no Fluoro-Jade B staining was seen throughout the 1-4 day period following Pilo-SE. It can thus be concluded that 1) basal neuronal expression of Epo in the hilus is not capable to maintain the integrity of hilar neuronal population in the short-term (24 hours) following Pilo-SE, and 2) the greater robustness of SLMo neurons cannot be attributed to local presence of Epo in 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 is only transient following Pilo-SE, a result which may be explained in the hippocampus by the activation of proinflammatory cytokines,[51, 52] known to impair Epo expression.[14, 53, 54] 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 at 15 days post-SE.

Neuroprotection induced by rHuEpo applied systemically requires that it crosses the BBB efficiently. 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,[11] as well as in the human epileptic tissue.[55] By using In-111-labeled rHuEpo injected intravenously, rHuEpo has been shown to penetrate the human brain with intact BBB.[12] Here, we measured 4 hours 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 rat brain tissue were consistent with those previously measured in the cerebro-spinal fluid (~ 100 mIU/mL measured 3.5 hours post-injection) using the same administration protocol.[12] 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 hours 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 hours 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,[12] since rHuEpo pre-treatment has been shown to prevent BBB breakdown both *in vitro*[56] and *in vivo*[15]. 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 hour post-rHuEpo injection may be explained by SE-induced BBB breakdown,[15] 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 hours before the onset of ischemia or brain trauma significantly protected the injured brain areas.[21, 57] 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 hours 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 rats.[11, 15] In rats which developed a sustained SE, we found 15 days post-SE that rHuEpo treatment preserved the neuronal population in CA1, CA3 and the SLMo but had no effects on the hilus. 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.[58] Neuroprotection in the hilus concerned mossy cells, considered as the most vulnerable neurons of the hippocampus.[35-37] Indeed, we noted a significant protection of CGRP-immunolabeling, attributed to mossy cells in the hilus.[38] It is now impossible to explain why neuronal population in the *stratum pyramidale* of CA1, which exhibits the greatest Epo-R immunoreactivity, was only modestly protected by rHuEpo in the two treatment approaches tested. In the hilus, while rHuEpo administered post-SE rescued a great percentage of mossy cells, numerous neurons still degenerated (~ 30% of hilar neurons). We hypothesize that neurons which are not rescued by rHuEpo are those which were found not to express Epo-R in basal conditions in the hilus. If that it the case, their extreme vulnerability may partly result from their incapability to respond to endogenous Epo and, hence, to rHuEpo.

In vitro studies have revealed that neuroprotective effects of Epo involved multiple intracellular pathways, suggested to be initiated by homodimer Epo-R mediated transphosphorylation of Janus kinase 2, leading to the activation of different cellular pathways implicated in neuronal protective signal.[10, 19, 59] The mechanism by which Epo prevents degeneration unquestionably requires Epo-R *in vivo*.[45, 60] However, carbamylated Epo (CEPO), a derivative of Epo molecule devoid of hematopoietic activity, confers neuroprotective effects comparable with that of Epo.[17] As neither Epo nor CEPO are neuroprotective in the injured spinal cord of mice lacking βc, it has been suggested that neuroprotection conferred by both Epo and CEPO requires the recruitment of a heteroceptor comprising a single Epo-R monomer and a βc homodimer.[10, 18] In the hippocampus of control rats, we found that βc was concentrated within the hilus exclusively, indicating that the role of βc cannot be prevalent over Epo-R in the neuroprotective effects demonstrated here in both the SLMo and Ammon's horn. EPO-R/βc mRNA ratio in the hippocampus, which is 1.6- and

3.0-fold greater than that found in the neocortex and the spinal cord, respectively, suggests that the composition of rHuEpo binding sites are not homogenously composed throughout the central nervous system. If that is the case, CEPO-induced neuroprotection should be greater in the spinal cord than in the hippocampus. Thus, the potential existence of various binding sites for Epo, present in distinct areas of the central nervous system, encourages diverse forms of protective molecules to be engineered, devoid of the adverse effects activated by the homodimeric Epo-R.[10] Such molecules already exist, as CEPO, described above, which may be used in situation of injuries affecting selectively βc-expressing neurons, e.g. mossy cells in the hippocampus, some cortical neurons, and a majority of neurons in the spinal cord. Because brief exposure to Epo is required to trigger neuroprotective pathways,[42] other molecules, such as asialo-erythropoietin, with short half-live *in vivo*,[10] may be used to protect hippocampal neurons expressing homodimeric Epo-R, e.g. pyramidal neurons of CA1. Indeed, asialo-erythropoietin binds efficiently to CA1 neurons,[61] and protects cultured hippocampal neurons against excitotoxic damages.[17]

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 SE leading to spontaneous seizures resembling TLE. 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 supports astroglial-derived Epo to promote enhanced neuronal survival following SE. A successful proof-of-concept clinical trial using rHuEpo has been preformed in stroke patients.[62] Our accurate analysis of the neuroprotective effects of rHuEpo after Pilo-SE opens new possibilities to improve the neurological outcome following a severe episode of SE.

Acknowledgements

This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS) and the Université Claude Bernard Lyon 1 (UCBL1). We thank B. Smatti, D. Ressnikoff and Y. Tourneur from the Centre Commun de Quantimétrie (CCQ) of the UCBL1 for their excellent technical assistance in confocal microscopy studies.

References

1. Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol. Rev. 1992;72:449-489
2. Ghezzi P, Brines M. Erythropoietin as an antiapoptotic, tissue-protective cytokine. Cell Death Differ. 2004;11 Suppl 1:S37-44
3. Juul SE, Yachnis AT, Rojiani AM, Christensen RD. Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. Pediatr Dev Pathol. 1999;2:148-158
4. Siren AL, Knerlich F, Poser W et al. Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. Acta Neuropathol (Berl). 2001;101:271-276
5. Marti HH, Wenger RH, Rivas LA et al. Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci. 1996;8:666-676
6. Chin K, Yu X, Beleslin-Cokic B et al. Production and processing of erythropoietin receptor transcripts in brain. Brain Res Mol Brain Res. 2000;81:29-42
7. Hasselblatt M, Ehrenreich H, Siren AL. The brain erythropoietin system and its potential for therapeutic exploitation in brain disease. J Neurosurg Anesthesiol. 2006;18:132-138
8. Campana WM, Myers RR. Exogenous erythropoietin protects against dorsal root ganglion apoptosis and pain following peripheral nerve injury. Eur J Neurosci. 2003;18:1497-1506
9. Maiese K, Li F, Chong ZZ. Erythropoietin in the brain: can the promise to protect be fulfilled? Trends in Pharmacological Sciences. 2004;25:577-583
10. Brines M, Cerami A. Emerging biological roles for erythropoietin in the nervous system. Nature Reviews Neuroscience. 2005;6:484-494
11. Brines ML, Ghezzi P, Keenan S et al. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proceedings of the National Academy of Sciences of the United States of America. 2000;97:10526-10531
12. Ehrenreich H, Degner D, Meller J et al. Erythropoietin: a candidate compound for neuroprotection in schizophrenia. Mol. Psychiatry. 2004;9:42-54
13. Juul SE, McPherson RJ, Farrell FX et al. Erytropoietin concentrations in cerebrospinal fluid of nonhuman primates and fetal sheep following high-dose recombinant erythropoietin. Biol Neonate. 2004;85:138-144
14. Gorio A, Madaschi L, Di Stefano B et al. Methylprednisolone neutralizes the beneficial effects of erythropoietin in experimental spinal cord injury. Proc Natl Acad Sci U S A. 2005;102:16379-16384
15. Uzum G, Sarper Diler A, Bahcekapili N, Ziya Ziylan Y. Erythropoietin prevents the increase in blood-brain barrier permeability during pentylentetrazol induced seizures. Life Sci. 2006;78:2571-2576
16. Henshall DC, Simon RP. Epilepsy and apoptosis pathways. J Cereb Blood Flow Metab. 2005;25:1557-1572
17. Leist M, Ghezzi P, Grasso G et al. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science. 2004;305:239-242
18. Brines M, Grasso G, Fiordaliso F et al. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc. Natl. Acad. Sci. U. S. A. 2004;101:14907-14912
19. Maiese K, Li F, Chong ZZ. New avenues of exploration for erythropoietin. JAMA. 2005;293:90-95
20. Genc S, Koroglu TF, Genc K. Erythropoietin as a novel neuroprotectant. Restorative Neurology and Neuroscience. 2004;22:105-119
21. Bernaudin M, Marti HH, Roussel S et al. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. Journal of Cerebral Blood Flow and Metabolism. 1999;19:643-651
22. Grasso G, Sfacteria A, Passalacqua M et al. Erythropoietin and erythropoietin receptor expression after experimental spinal cord injury encourages therapy by exogenous erythropoietin. Neurosurgery. 2005;56:821-827; discussion 821-827
23. Campana WM, Myers RR. Erythropoietin and erythropoietin receptors in the peripheral nervous system: changes after nerve injury. Faseb J. 2001;15:1804-1806
24. Li X, Gonias SL, Campana WM. Schwann cells express erythropoietin receptor and represent a major target for Epo in peripheral nerve injury. Glia. 2005;51:254-265
25. Turski WA, Cavalheiro EA, Schwarz M et al. Limbic seizures produced by pilocarpine in rats: behavioural, electroencephalographic and neuropathological study. Behav. Brain Res. 1983;9:315-335
26. Calapai G, Marciano MC, Corica F et al. Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. Eur. J. Pharmacol. 2000;401:349-356
27. Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol. 1972;32:281-294
28. Chong ZZ, Kang JQ, Maiese K. Erythropoietin fosters both intrinsic and extrinsic neuronal protection through modulation of microglia, Akt1, Bad, and caspase-mediated pathways. British Journal of Pharmacology. 2003;138:1107-1118
29. Grimm C, Wenzel A, Groszer M et al. HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. Nat Med. 2002;8:718-724
30. Poirier JL, Capek R, De Koninck Y. Differential progression of Dark Neuron and Fluoro-Jade labelling in the rat hippocampus following pilocarpine-induced status epilepticus. Neuroscience. 2000;97:59-68
31. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 4th ed: Academic Press, 1998:256
32. Appel K, Buttini M, Sauter A, Gebicke-Haerter PJ. Cloning of rat interleukin-3 receptor beta-subunit from cultured microglia and its mRNA expression in vivo. J Neurosci. 1995;15:5800-5809
33. Wang L, Zhang Z, Wang Y et al. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke. 2004;35:1732-1737

34. Lu D, Mahmood A, Qu C et al. Erythropoietin enhances neurogenesis and restores spatial memory in rats after traumatic brain injury. J. Neurotrauma. 2005;22:1011-1017
35. Sloviter RS, Zappone CA, Harvey BD et al. "Dormant basket cell" hypothesis revisited: relative vulnerabilities of dentate gyrus mossy cells and inhibitory interneurons after hippocampal status epilepticus in the rat. J. Comp. Neurol. 2003;459:44-76
36. Sloviter RS. The functional organization of the hippocampal dentate gyrus and its relevance to the pathogenesis of temporal lobe epilepsy. Ann Neurol. 1994;35:640-654
37. Ratzliff AH, Santhakumar V, Howard A, Soltesz I. Mossy cells in epilepsy: rigor mortis or vigor mortis? Trends Neurosci. 2002;25:140-144
38. Freund TF, Hajos N, Acsady L et al. Mossy cells of the rat dentate gyrus are immunoreactive for calcitonin gene-related peptide (CGRP). Eur. J. Neurosci. 1997;9:1815-1830
39. Kawakami M, Sekiguchi M, Sato K et al. Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. Journal of Biological Chemistry. 2001;276:39469-39475
40. Ehrenreich H, Hasselblatt M, Knerlich F et al. A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain. Proceedings of the National Academy of Sciences of the United States of America. 2005;102:862-867
41. Digicaylioglu M, Bichet S, Marti HH et al. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proceedings of the National Academy of Sciences of the United States of America. 1995;92:3717-3720
42. Morishita E, Masuda S, Nagao M et al. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience. 1997;76:105-116
43. Lewczuk P, Hasselblatt M, Kamrowski Kruck H et al. Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin. Neuroreport. 2000;11:3485-3488
44. Chung YH, Kim SI, Joo KM et al. Age-related changes in erythropoietin immunoreactivity in the cerebral cortex and hippocampus of rats. Brain Research. 2004;1018:141-146
45. Sakanaka M, Wen TC, Matsuda S et al. In vivo evidence that erythropoietin protects neurons from ischemic damage. Proceedings of the National Academy of Sciences of the United States of America. 1998;95:4635-4640
46. Marti HH. Erythropoietin and the hypoxic brain. Journal of Experimental Biology. 2004;207:3233-3242
47. Rossignol F, de Laplanche E, Mounier R et al. Natural antisense transcripts of HIF-1alpha are conserved in rodents. Gene. 2004;339:121-130
48. Sloviter RS. The neurobiology of temporal lobe epilepsy: too much information, not enough knowledge. Comptes Rendus Biologies. 2005;328:143-153
49. Masuda S, Okano M, Yamagishi K et al. A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. Journal of Biological Chemistry. 1994;269:19488-19493
50. Bernaudin M, Nedelec AS, Divoux D et al. Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. Journal of Cerebral Blood Flow and Metabolism. 2002;22:393-403
51. Rizzi M, Perego C, Aliprandi M et al. Glia activation and cytokine increase in rat hippocampus by kainic acid-induced status epilepticus during postnatal development. Neurobiol Dis. 2003;14:494-503
52. Voutsinos-Porche B, Koning E, Kaplan H et al. Temporal patterns of the cerebral inflammatory response in the rat lithium-pilocarpine model of temporal lobe epilepsy. Neurobiol Dis. 2004;17:385-402
53. Frede S, Fandrey J, Pagel H et al. Erythropoietin gene expression is suppressed after lipopolysaccharide or interleukin-1 beta injections in rats. Am J Physiol. 1997;273:R1067-1071
54. Nagai A, Nakagawa E, Choi HB et al. Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. J Neuropathol Exp Neurol. 2001;60:386-392
55. Eid T, Brines ML, Cerami A et al. Increased expression of erythropoietin receptor on blood vessels in the human epileptogenic hippocampus with sclerosis. J Neuropathol Exp Neurol. 2004;63:73-83
56. Martinez-Estrada OM, Rodriguez-Millan E, Gonzalez-De Vicente E et al. Erythropoietin protects the in vitro blood-brain barrier against VEGF-induced permeability. Eur J Neurosci. 2003;18:2538-2544
57. Bernaudin M, Bellail A, Marti HH et al. Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. Glia. 2000;30:271-278
58. Cavalheiro EA. GAD-immunoreactive neurons are preserved in the hippocampus of rats with spontaneous recurrent seizures. Braz. J. Med. Biol. Res. 1990;23:555-558
59. Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature. 2001;412:641-647
60. Prass K, Scharff A, Ruscher K et al. Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. Stroke. 2003;34:1981-1986
61. Erbayraktar S, Grasso G, Sfacteria A et al. Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. Proc Natl Acad Sci U S A. 2003;100:6741-6746
62. Ehrenreich H, Hasselblatt M, Dembowski C et al. Erythropoietin therapy for acute stroke is both safe and beneficial. Molecular Medicine. 2002;8:495-505

Figure legends

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. However, not all hilar neurons express Epo-R (white arrows). Scale bars: A, 1 mm; B, 50 µm.

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.

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.

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.

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: Hi, hippocampus; NCx, neocortex. Scale bars: Hilus, 100 µm; NCx, 33 µm.

Fig 6. Dramatic increased 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.

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^{31} following Pilo-SE. NS, not statistically significant; †, $p<0.05$; ††, $p<0.01$; †††, $p<0.001$ 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 hours following its last systemic administration (5,000 IU/kg intraperitoneally) (n=8). rHuEpo was applied once in Group 1 (n=4) and twice, 24 hours 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 hour after the last intraperitoneal injection.

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).

Fig 10. Systemic administration of rHuEpo (5,000 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.

Fig 11. rHuEpo (5,000 IU/kg intraperitoneally) 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.

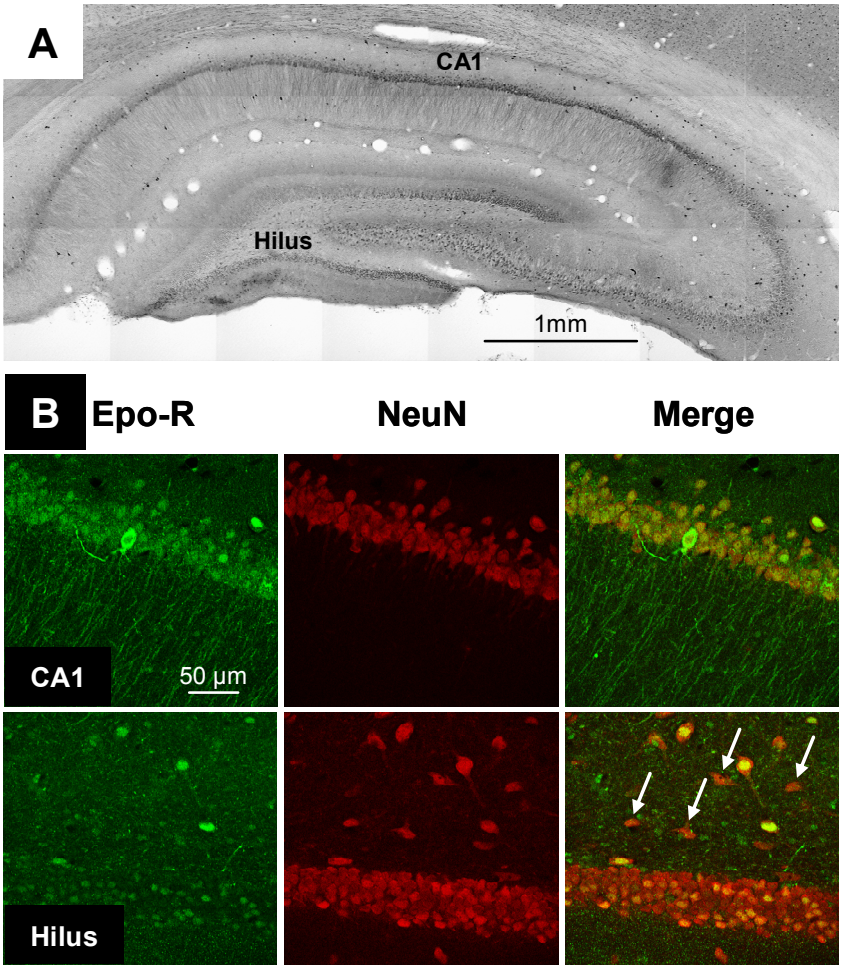

Figure 1

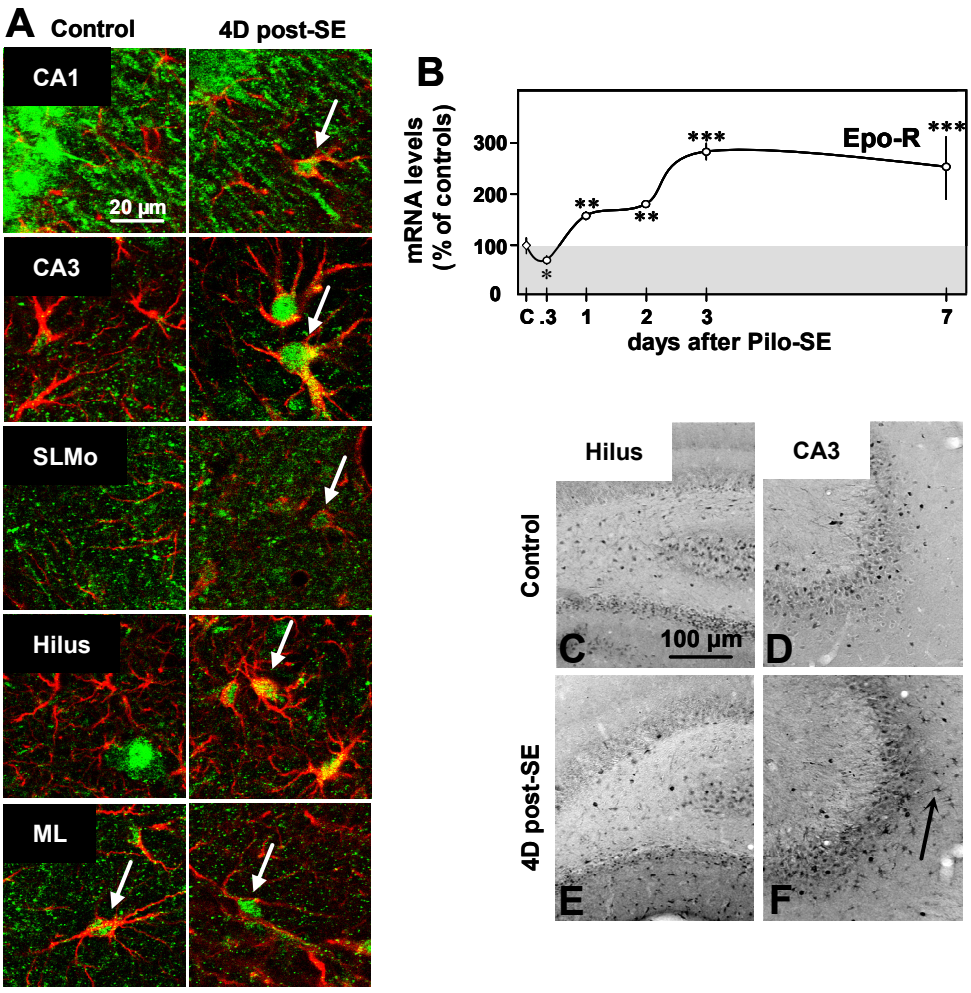
A
Control
4D post-SE
CA1
20 µm
CA3
SLMo
Hilus
ML
B
mRNA levels
(% of controls)
300
200
100
0
C .3 1 2 3 7
days after Pilo-SE
Epo-R
***
**
**
*
***
Hilus
CA3
Control
C
100 µm
D
4D post-SE
E
F

Figure 2

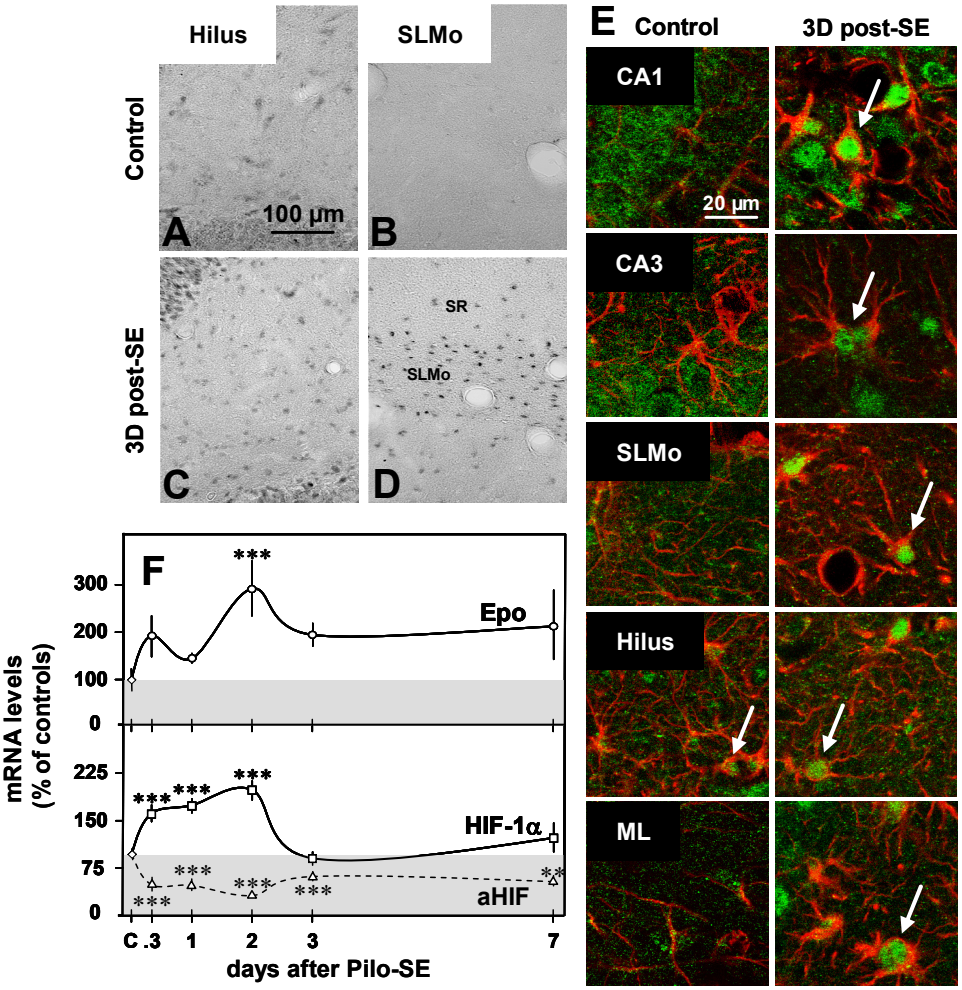
Hilus
SLMo
Control
3D post-SE
100 µm
A
B
C
D
SR
SLMo
E
Control
3D post-SE
CA1
CA3
SLMo
Hilus
ML
20 µm
F
mRNA levels
(% of controls)
Epo
HIF-1α
aHIF
C .3 1 2 3 7
days after Pilo-SE

Figure 3

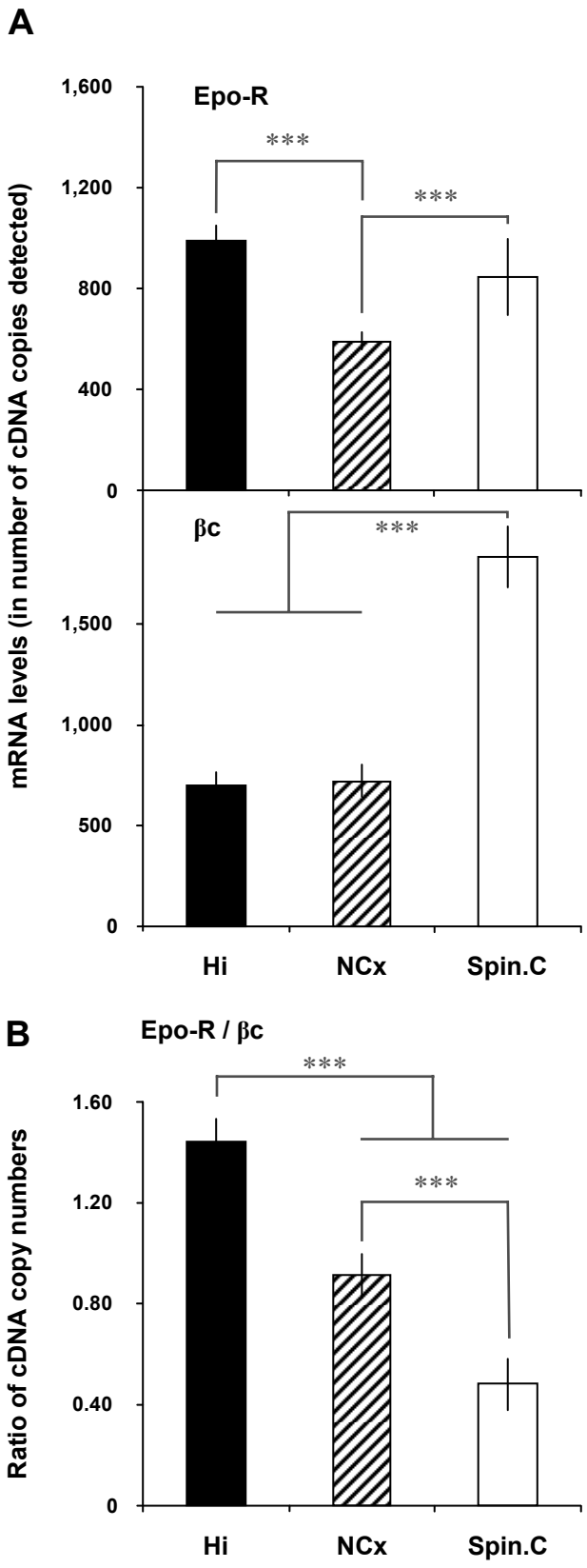
A
Epo-R
***
***
1,600
1,200
800
400
0
βc
***
1,500
1,000
500
0
mRNA levels (in number of cDNA copies detected)
Hi
NCx
Spin.C
B
Epo-R / βc
***
***
1.60
1.20
0.80
0.40
0
Ratio of cDNA copy numbers
Hi
NCx
Spin.C

Figure 4

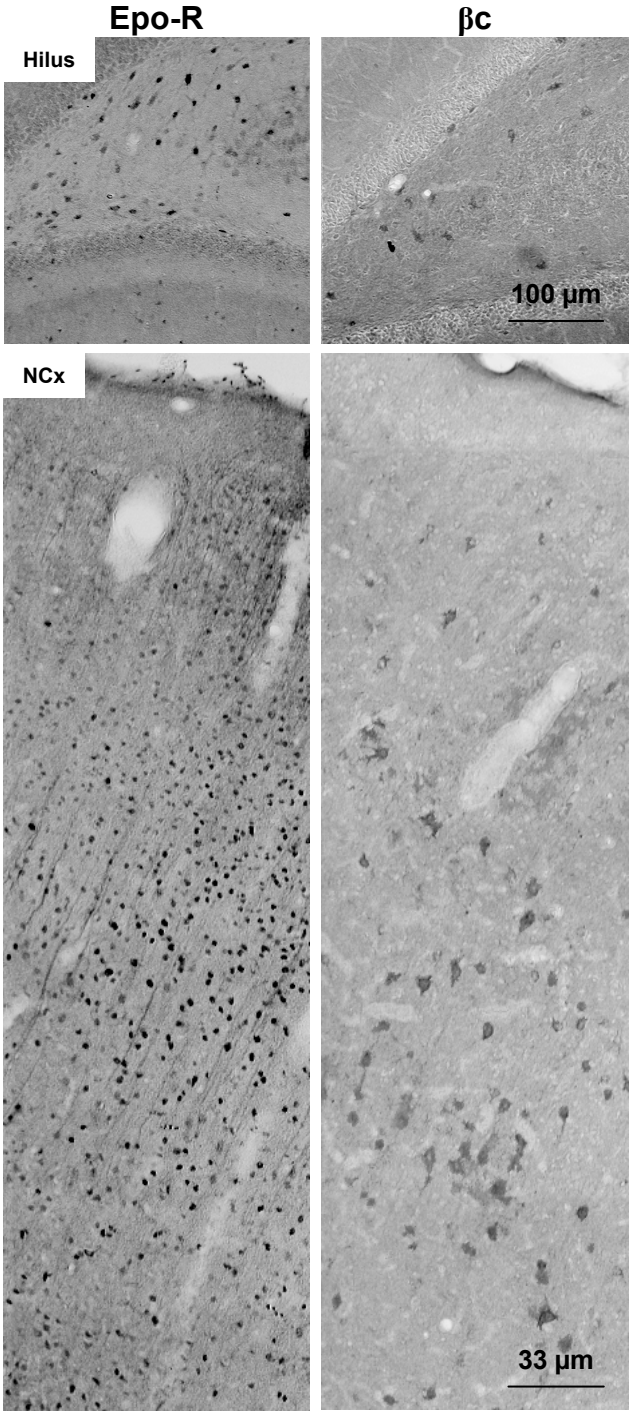

Figure 5

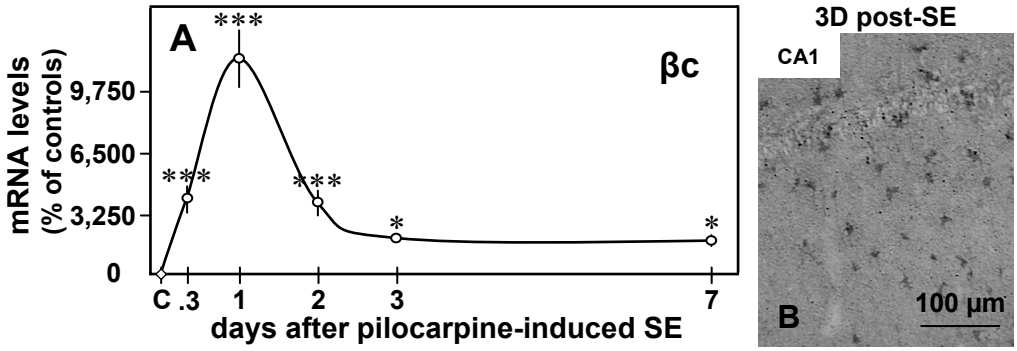
A
βc
mRNA levels
(% of controls)
9,750
6,500
3,250
0
C .3 1 2 3 7
days after pilocarpine-induced SE
***
***
***
*
*
3D post-SE
CA1
B
100 µm

Figure 6

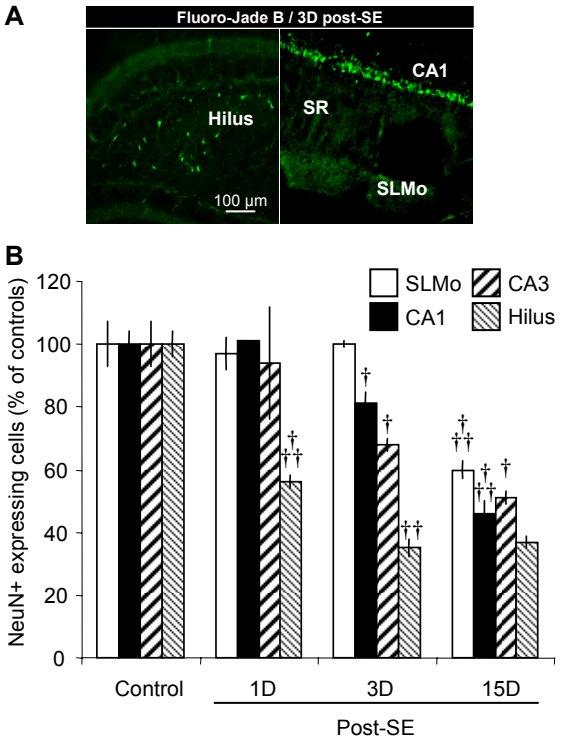
A
Fluoro-Jade B / 3D post-SE
Hilus
SR
CA1
SLMo
100 μm
B
NeuN+ expressing cells (% of controls)
120
100
80
60
40
20
0
SLMo
CA3
CA1
Hilus
Control
1D
3D
15D
Post-SE

Figure 7

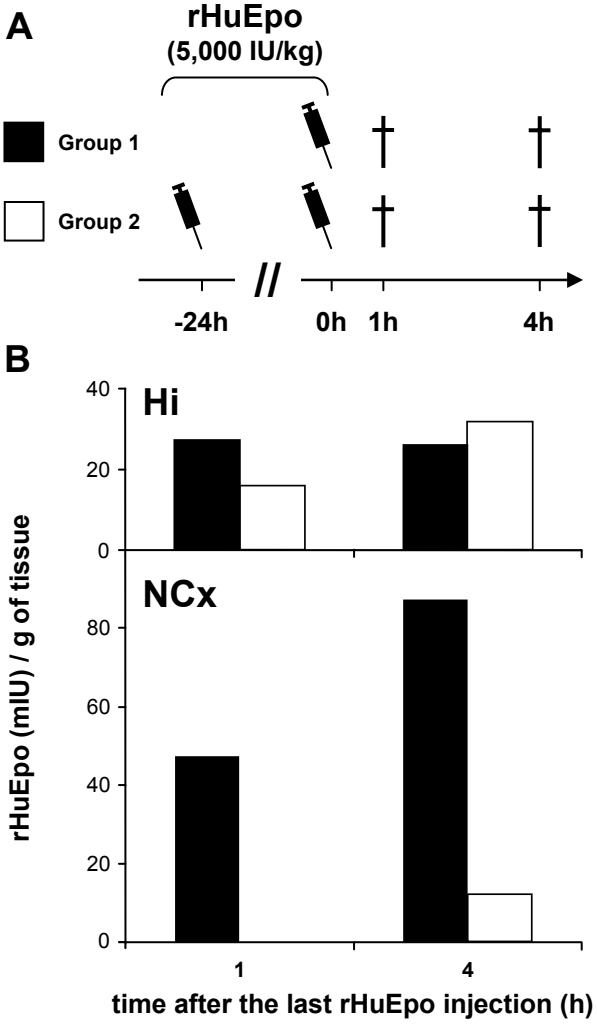
A
rHuEpo
(5,000 IU/kg)
Group 1
Group 2
†
†
†
†
//
-24h
0h
1h
4h
B
Hi
NCx
40
20
0
80
60
40
20
0
rHuEpo (mIU) / g of tissue
1
4
time after the last rHuEpo injection (h)

Figure 8

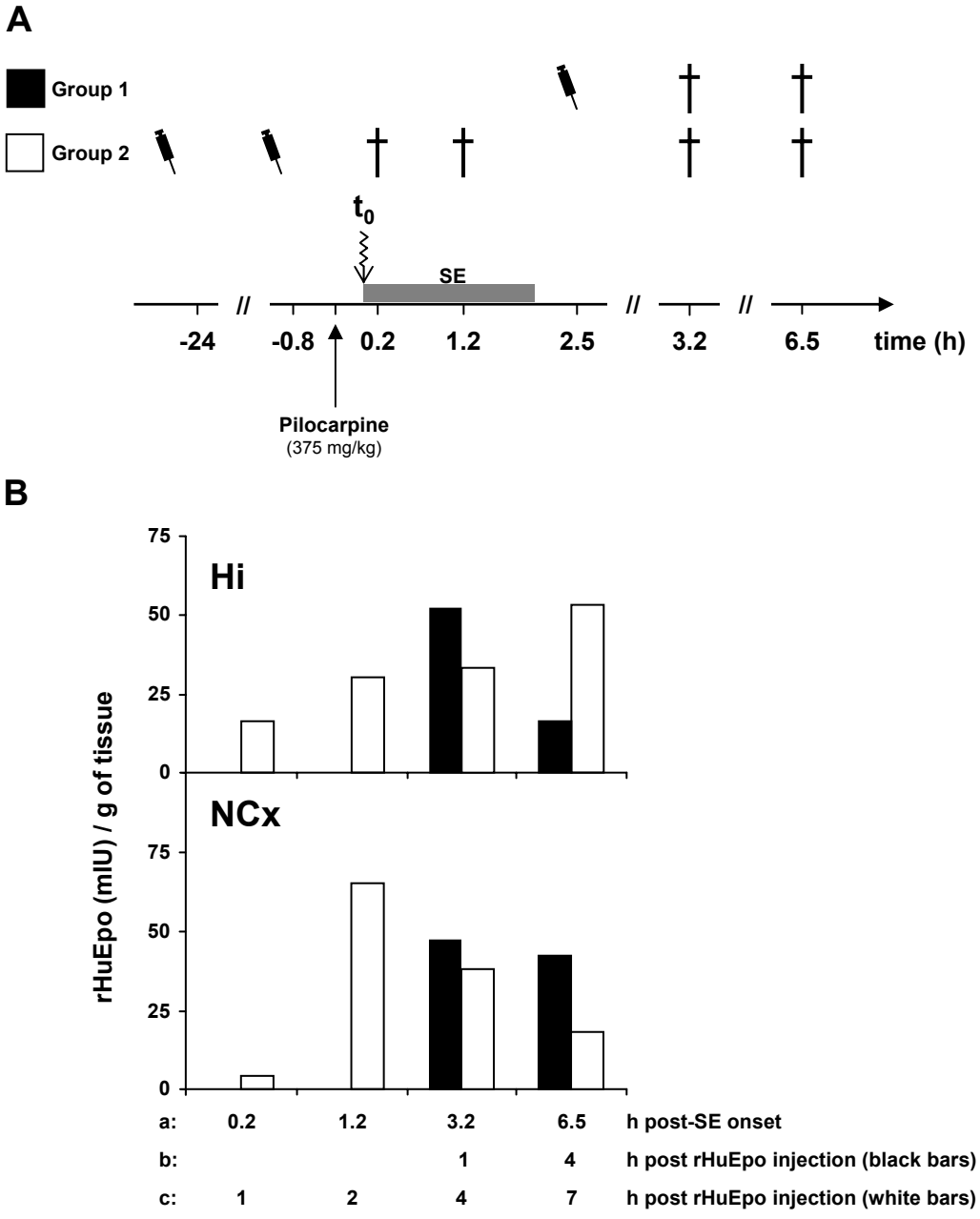
A
Group 1
Group 2
t0
SE
-24
-0.8
0.2
1.2
2.5
3.2
6.5
time (h)
Pilocarpine
(375 mg/kg)
B
Hi
NCx
rHuEpo (mIU) / g of tissue
75
50
25
0
a: 0.2 1.2 3.2 6.5 h post-SE onset
b: 1 4 h post rHuEpo injection (black bars)
c: 1 2 4 7 h post rHuEpo injection (white bars)

Figure 9

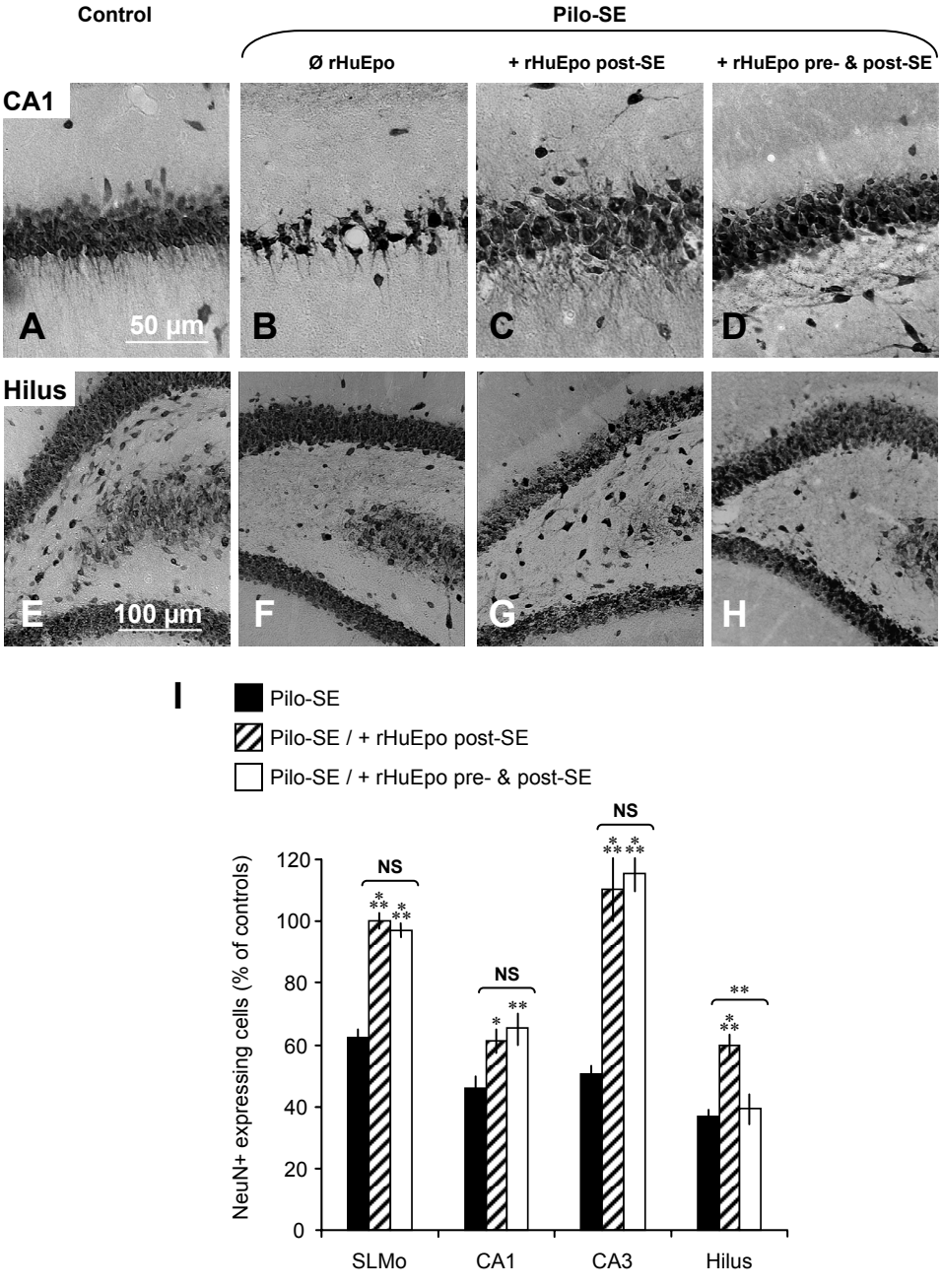
Control
Pilo-SE
Ø rHuEpo
+ rHuEpo post-SE
+ rHuEpo pre- & post-SE
CA1
A
50 µm
B
C
D
Hilus
E
100 µm
F
G
H
I
Pilo-SE
Pilo-SE / + rHuEpo post-SE
Pilo-SE / + rHuEpo pre- & post-SE
NeuN+ expressing cells (% of controls)
120
100
80
60
40
20
0
NS
NS
NS
**
SLMo
CA1
CA3
Hilus

Figure 10

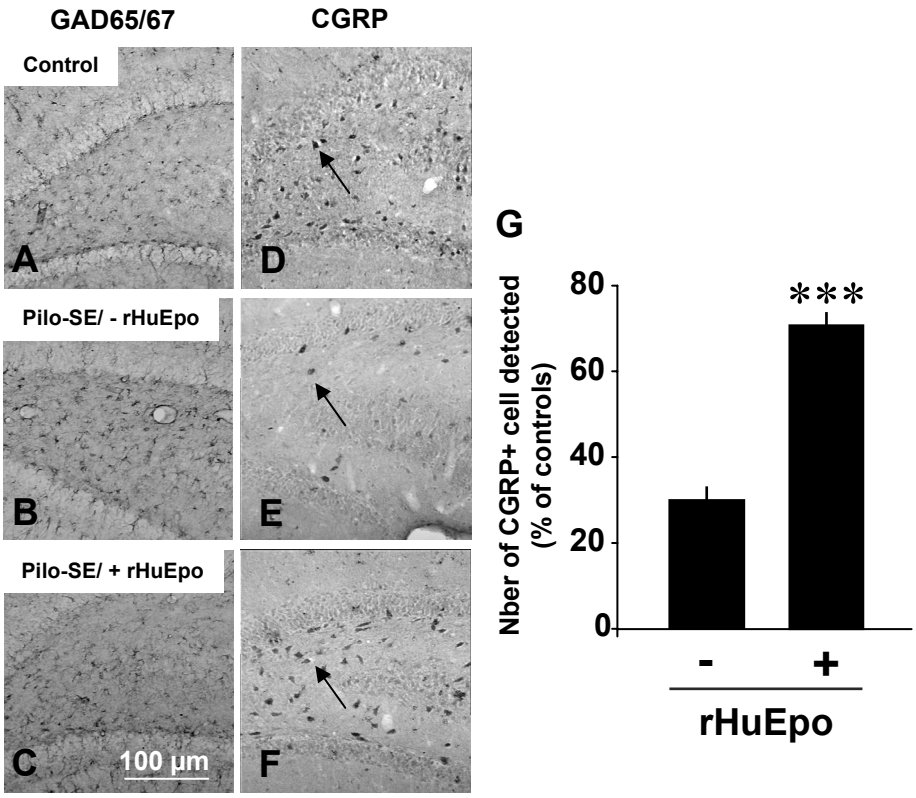
GAD65/67
CGRP
Control
A
D
Pilo-SE/ - rHuEpo
B
E
Pilo-SE/ + rHuEpo
C
100 µm
F
G
Nber of CGRP+ cell detected (% of controls)
80
60
40
20
0
***
-
+
rHuEpo

Figure 11