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Erythropoietin protects hippocampal neurons during epileptogenesis

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Abstract

Objective: Despite the fact that basal brain erythropoietin (Epo) is neuroprotective, Epo system reactivity during neurodegenerative processes remains largely unknown. During the period of intensive neuronal loss occurring in a model of mesial temporal lobe epilepsy (MTLE), we determined Epo system reactivity in the hippocampus and examined whether exogenous recombinant human Epo (rHuEpo) could support neuronal survival.

Methods: Epo and Epo-receptor (Epo-R) expression was analyzed after pilocarpine-induced status epilepticus (Pilo-SE) in the hippocampus of adult rats at both messenger RNA and protein levels. Potential protective effects of rHuEpo (Eprex[®], Janssen-Cilag; 5,000 IU/kg) administered immediately after, 1 and 3 days after Pilo-SE was investigated 2 weeks later.

Results: By contrast to Epo, which is detected in few neurons in the adult rat hippocampus, Epo-R is expressed by a large 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. Therapeutic administration of rHuEpo reduces considerably neuronal degeneration in the hippocampus.

Interpretation: Exogenous rHuEpo may support astroglial Epo to promote hippocampus neuronal survival during epileptogenesis and encourages future studies aimed at evaluating beneficial effects of rHuEpo in patients susceptible to develop MTLE.

Introduction

Erythropoietin (Epo) was originally described for its role in hematopoiesis, which consists of promoting proliferation, differentiation and survival of erythroid progenitors to increase red blood cells.¹ Epo exerts its erythropoietic functions by binding with homodimers of Epo receptor (Epo-R), which belongs to class 1 superfamily of cytokine receptors.² The original evidence that Epo binding sites are largely distributed throughout the central nervous system in rodents³ and that neurons and astrocytes express both Epo and Epo-R in human brain tissue^{4, 5} 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.⁶⁻⁸

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,⁹ and in models of traumatic injury in both the spinal cord¹⁰ and the peripheral nervous system.^{11, 12}

To date, Epo system reactivity has not been investigated after chemically-induced *status epilepticus* (SE), a widely used model of human mesial temporal lobe epilepsy (MTLE) associated with neuronal death in the hippocampus.¹³ This neurodegenerative process has been suggested as one of multiple mechanisms causing drug resistance in a large part of patients with MTLE,¹⁴ and most of the apoptosis pathways involved in animal models of MTLE¹⁴ are those targeted by Epo.^{7, 8}

Our study is the first to investigate in the hippocampus the basal expression of endogenous Epo system and its reactivity in the rat pilocarpine model of MTLE. Here, we show that Epo-R is expressed by almost all neurons of the hippocampus in basal conditions, and rarely by astrocytes. Following pilocarpine-induced SE (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 demonstrate that administration of recombinant human Epo (rHuEpo) following Pilo-SE significantly protects hippocampal neurons, suggesting that rHuEpo may be therapeutically useful to counteract neurodegeneration in patients susceptible to develop MTLE.

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 $21 \pm 1^\circ\text{C}$ 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 favour recovery until sacrificed.

Pilo-SE.

Scopolamine methylnitrate (1 mg/kg, subcutaneously; Sigma) was administered 30 min prior to pilocarpine hydrochloride (300-375 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 2-3 hours by diazepam administration (Valium[®], 10 mg/kg, intraperitoneally; Roche). Rats were then hydrated with 2 ml 0.9% NaCl (subcutaneously). Pilocarpine dose used and duration of SE are mentioned below for each set of experiments.

Experimental design.

Two sets of experiments have been conducted.

In the first set, expression of Epo system both in basal conditions and following Pilo-SE has been examined. Rats experienced 3 hours of SE induced by 300 mg/kg pilocarpine hydrochloride. To determine the levels of mRNAs encoding key proteins of the Epo system (i.e. Epo, Epo-R, and HIF-1 α), 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). The hippocampus was 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 and Epo-R 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.

In the second set of experiments, we examined whether exogenous recombinant human Epo (rHuEpo) administered after Pilo-SE could protect vulnerable neurons of the hippocampus from neurodegeneration. Rats experienced 2 hours of SE induced by 375 mg/kg pilocarpine hydrochloride (n=25) and some (n=10) received injections of 5,000 IU/kg of rHuEpo (Eprex[®], Janssen-Cilag, France) 30 min, 1 day and 3 days after SE stopped. 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. Naïve rats (controls; n=5) were also included in this study.

15 **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-1 α , 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-1 α** (GenBank Y09507) forward 5' CTC AGA GGA AGC GAA AAA TGG 3', reverse 5' AAT TCT TCA CCC TGC AGT AGG 3' (307 bp). All primer pairs were designed using "Primer 3" software (NIH; www.basic.nwu.edu).

10 *Colorimetric immunohistochemistry assays.*

Free floating sections (40 μ m 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- β c 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; Santa Cruz), 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).

25 *Fluorescent dual-labeling immunohistochemistry.*

Free floating sections (40 μ m 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.

5 *Neuronal degeneration.*

Fluoro-Jade B (Chemicon) was used to stain specifically degenerating neurons after Pilo-SE in rats.¹⁵

Neuronal counts.

10 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[®] 6.0; 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
15 selected at IA +5.40 mm.¹⁶ 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, 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 pyramidal layers of
20 areas CA1 and CA2, 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 \pm SEM of the different variables analyzed (mRNA level, neuron
25 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 all neuronal layers of the hippocampus (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 pyramidal layer of CA2 (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 neuronal layers 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).

Reactivity of Epo-R expression in the hippocampus during epileptogenesis.

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). 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 CA2 (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).

Reactivity of Epo expression in the hippocampus during epileptogenesis.

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 *stratum 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 pyramidal layer of CA1 and CA2, 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-1 α 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-1 α anti-sense RNA (aHIF) that was significant and long-lasting (see Fig 3F).

20

Time-course of neuronal loss in the dorsal hippocampus.

Animals undergoing Pilo-SE exhibited a pattern of neuronal loss that varied in the different subfields of the dorsal hippocampus analyzed (see Fig 4). 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 4A). Neuronal loss, determined from NeuN-immunostained sections, reached ~ 45% by 1 day post-SE, and then stabilized by 3 days post-SE at ~ 65% (see Fig 4B). In CA1 and CA2, Fluoro-Jade B staining was observed at 1 day post-SE, and increased at 3 (see Fig 4A) and 4 days post-SE. Neuronal loss was evidenced at 3 days post-SE and reached ~ 50% by 15 days post-SE (see Fig 4B). The SLMo exhibited no Fluoro-Jade B staining from 1 to 4 days post-SE (see Fig 4A). However, at 15 days post-SE, neuronal loss reached ~ 40% (see Fig 4B).

30

Therapeutic administration of rHuEpo rescues vulnerable mossy cells from degeneration after Pilo-SE.

In the hippocampus, a significant neuroprotective effect of rHuEpo was observed in all subfields of the hippocampus, as illustrated in CA1 (see Fig 5A to C) and the hilus (see Fig 5D to F). Quantitative analysis of the neuronal population stained for NeuN revealed that neuroprotection elicited by rHuEpo was modest but significant in CA1 and the hilus, while full protection of hippocampal neurons was attained in CA2 and the SLMo (see Fig 5D). Compelling data evidenced that glutamatergic mossy cells are the most vulnerable neurons of the hippocampus.¹⁷⁻¹⁹ Analysis of GAD65/67 immunohistolabeling in the hilus indicated that GABAergic interneurons were preserved following Pilo-SE (see Fig 6A to C). By contrast, the number of glutamatergic mossy cells, detected in the hilus following CGRP-immunohistochemistry²⁰ (see Fig 6D to E), dramatically decreased in rats which experienced Pilo-SE ($29 \pm 3\%$ of controls ; $p < 0.001$, see Fig 6G). However, in rats treated with rHuEpo after Pilo-SE, the number of CGRP-immunopositive cells detected (see Fig 6D to G) greatly increased ($71 \pm 4\%$ of controls; $p < 0.001$).

Discussion

This study presents for the first time the accurate distribution of both Epo and Epo-R in the rat hippocampus in basal conditions, and reveals that Epo-R is expressed in a large majority of neurons, while Epo expression is much more discrete and restricted to some neuronal populations. Few astrocytes express Epo and Epo-R in every area of the hippocampus. By contrast, Epo and Epo-R are induced in numerous astrocytes following Pilo-SE, and hippocampal areas where astroglial Epo induction is the greatest exhibit a pattern of neuronal death that is significantly delayed. 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 in an animal model of MTLE.

Numerous *in vitro* studies have evidenced that Epo-R is expressed by cultured hippocampal neurons.²¹⁻²⁴ While both Epo binding sites³ and Epo-R²⁴ 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.²⁵ Surprisingly, in another study, hippocampal presence of Epo-R was demonstrated after hypoxia exposure, but not in basal conditions.²⁶ 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.²⁴ Our *in situ* study agrees with a prior work²⁷ 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.²⁸

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.²⁹ HIF-1 α 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.²⁹ Here we show that enhanced hippocampal Epo-mRNA is preceded by an increase in transcript levels of HIF-1 α , and accompanied by the downregulation of aHIF-RNA, the natural anti-sense RNA complementary to the 3' UTR of HIF-1 α -mRNA and likely involved in the regulation of its degradation.³⁰ In addition to the required post-translational stabilization of HIF-1 α , the coordinated regulation of both HIF-1 α -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 fissure³¹ may explain the astroglial induction of Epo in the SLMo. Indeed, astroglial expression of Epo is greatly enhanced by hypoxia^{5, 32} and by agents which mimic hypoxic insults.³³

Concomitantly to Epo gene activation, transcript levels of Epo-R are also increased following Pilo-SE and maintained at a high level during epileptogenesis. This Epo-R gene induction is associated with the detection of Epo-R in numerous astrocytes, particularly in areas CA1 and CA2, the SLMo, and the hilus. This study is the first to evidence that Epo and Epo-R gene expression are dramatically increased, particularly in 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,⁹ in rat spinal cord after trauma injury,^{10, 34} and in rat peripheral nerve injury,¹² 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. The observation that both Epo and Epo-R are expressed by astrocytes in the SLMo 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.

In this study, neurodegenerative process and neuronal loss have been carefully examined during the early period of epileptogenesis (1-4 days post-SE) in the different subfields of the hippocampus using Fluorojade-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.¹⁷⁻¹⁹ 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 Fluorojade-B staining was seen throughout the 1-4 day period following Pilo-SE in the SLMo. 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 cannot exclude the possibility, however, that the induction of Epo in the SLMo contributed to prolong the survival of SLMo neurons following Pilo-SE. The 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,^{35, 36} known to impair Epo expression.^{34, 37, 38} 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.

Considering the wide distribution of Epo-R at the surface of hippocampal neurons, it was fundamental to determine whether therapeutic administration of exogenous Epo could significantly reduce hippocampal neurodegeneration following Pilo-SE. Therefore, we administered rHuEpo three times after SE (30 min, 1 and 3 days post-SE). Injection of rHuEpo 30 min after the end of SE was justified by the fact that degeneration occurs rapidly in the hilus, since neuronal loss was significant as soon as day 1 post-SE. Here we show 2 weeks after Pilo-SE that therapeutic rHuEpo treatment preserved completely the neuronal population in CA2 and the SLMo, and significantly enhanced neuronal survival in the hilus and the *stratum pyramidale* of CA1. Neuroprotection in the hilus was assigned to mossy cells, since we noted a significant protection of CGRP-immunolabeling, which has been attributed to mossy cells in the hilus.²⁰ It is now impossible to explain why neuronal population in the *stratum pyramidale* of CA1, which exhibits the greatest Epo-R immunoreactivity, is only modestly protected by exogenous Epo. In the hilus, while rHuEpo administered post-SE rescues a great percentage of mossy cells, numerous neurons still degenerated (~ 30% of hilar neurons). Our data indicate that GABAergic interneurons, identified by *in situ* detection of

GAD65/67, survived to Pilo-SE. 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 is the case, their extreme vulnerability may partly result from their incapability to respond to endogenous Epo and, hence, to exogenous rHuEpo.

5

In conclusion, this study is the first to evidence in the hippocampus a potential neuroprotective effect of endogenous Epo in a model of MTLE and to demonstrate beneficial effects of rHuEpo administration on neuronal survival. A successful proof-of-concept clinical trial using rHuEpo has been performed in stroke patients.³⁹ Our accurate analysis of neuroprotective effects of rHuEpo in the pilocarpine model of MTLE encourages rHuEpo therapeutic treatment in patients prone to develop MTLE, but translation into clinical application requires further work. Indeed, we need now to extend the neuroprotective effects of rHuEpo to other animal models of SE displaying more subtle neuronal loss, e.g. selective degeneration of mossy cells.¹⁸ This should help to evaluate, in the long term, how enhanced selective neuronal survival in the hippocampus modifies the occurrence and the severity of seizures.

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Figure legends

Fig 1. Hippocampal distribution of Epo-R under basal conditions. A, Colorimetric staining demonstrates that Epo-R is expressed in all neuronal layers of 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 μ m.

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 CA2 (black arrow) (D, F). D, day; ML, molecular layer; SLMo, stratum lacunosum moleculare. Scale bars: A, 20 μ m; C-F, 100 μ m.

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-1 α -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 μ m; E, 20 μ m.

Fig 4. 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 mm¹⁶ following Pilo-SE. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ as compared to the prior time point. Abbreviations: as in Fig 3. Scale bar: A, 100 μ m.

- Fig 5. Systemic administration of rHuEpo (5,000 IU/kg) administered immediately, 1 and 3 days after Pilo-SE protects hippocampal neurons against degeneration. A-F, NeuN staining at 15 days post-SE evidences protective effects of rHuEpo in CA1 and the hilus. G, Therapeutic administration of rHuEpo rescued completely neurons in the SLMo and in pyramidal neurons in CA2, and partially hilar neurons and pyramidal neurons in CA1 from degeneration induced by SE. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$, 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 .
- 10 Fig 6. Systemic administration of rHuEpo (5,000 IU/kg) administered immediately, 1 and 3 days protects vulnerable hilar mossy cells against cell death generated by Pilo-SE. A-C, GABAergic interneurons, detected following GAD65/67 immunohistochemistry, have their population maintained 15 days following Pilo-SE. D-F, Glutamatergic hilar mossy cells (black arrows), detected following 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 cell rescued by rHuEpo following Pilo-SE. †††, $p < 0.001$ as compared to rats which underwent Pilo-SE and did not receive rHuEpo. Abbreviations: as in Fig 2. Scale bar: A-F, 100 μm .
- 15

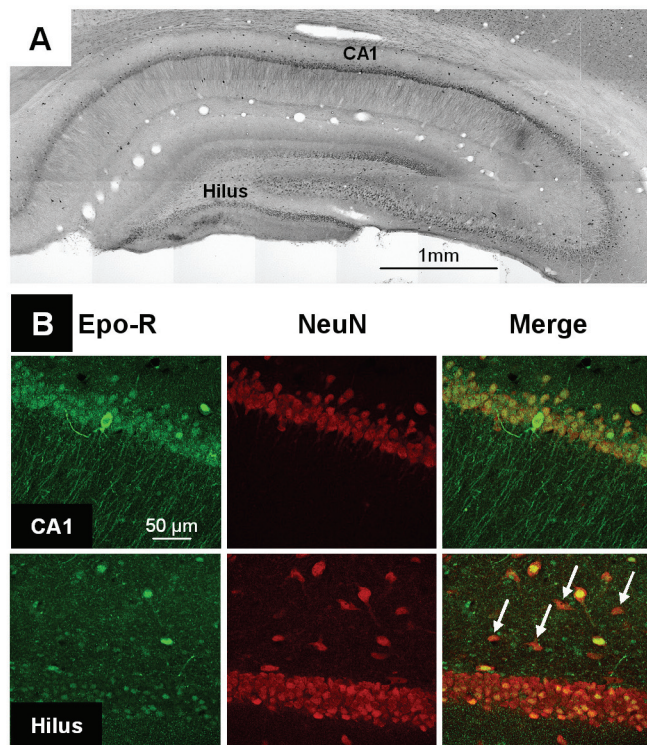


Figure 1

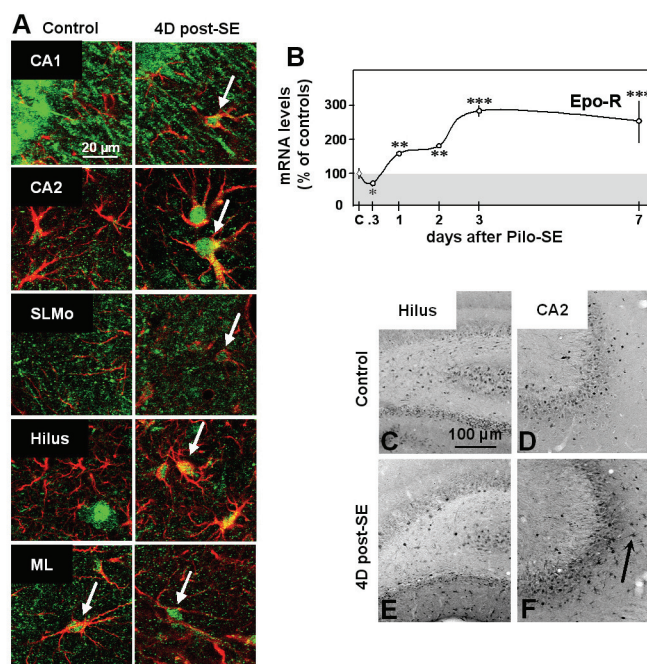


Figure 2

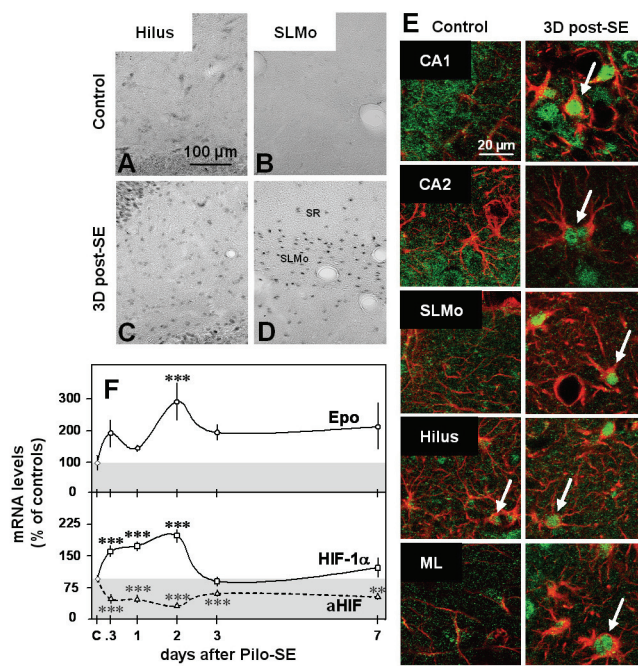


Figure 3

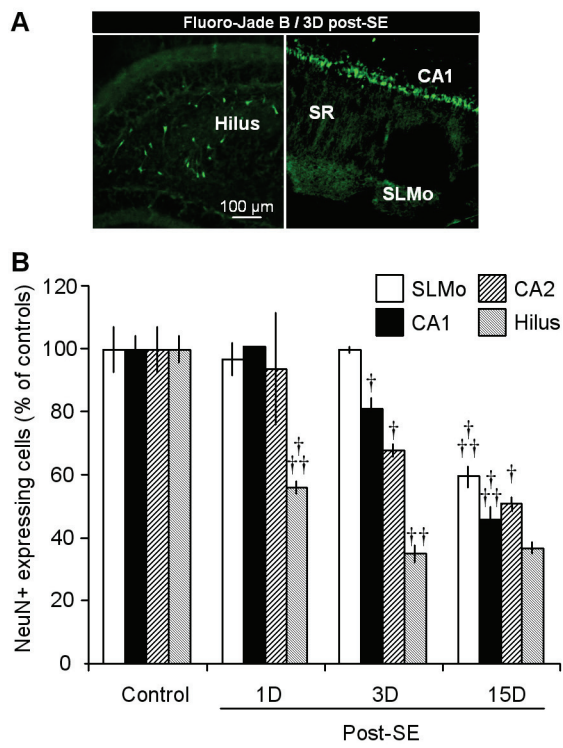


Figure 4

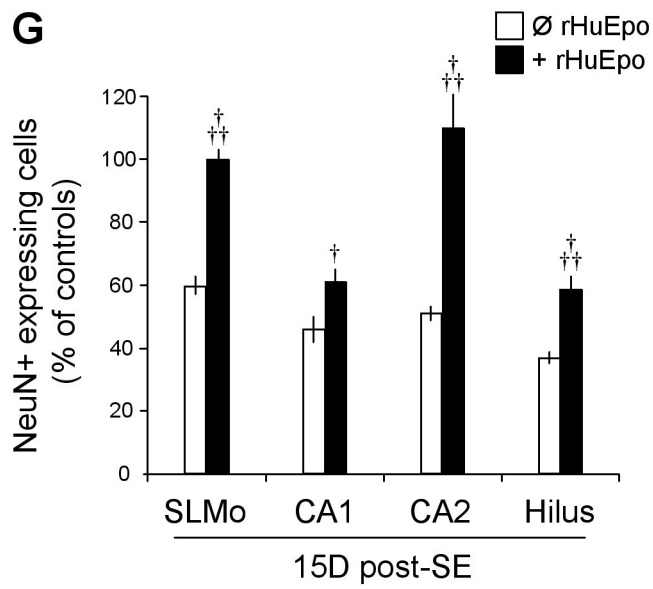
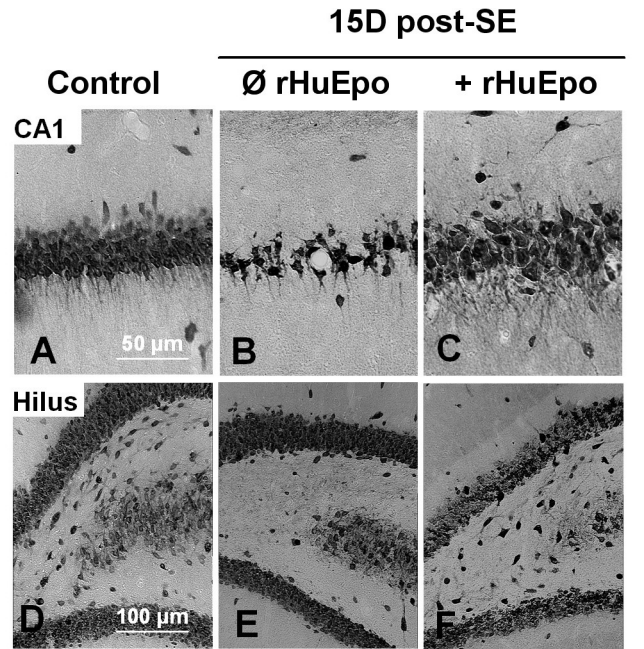


Figure 5

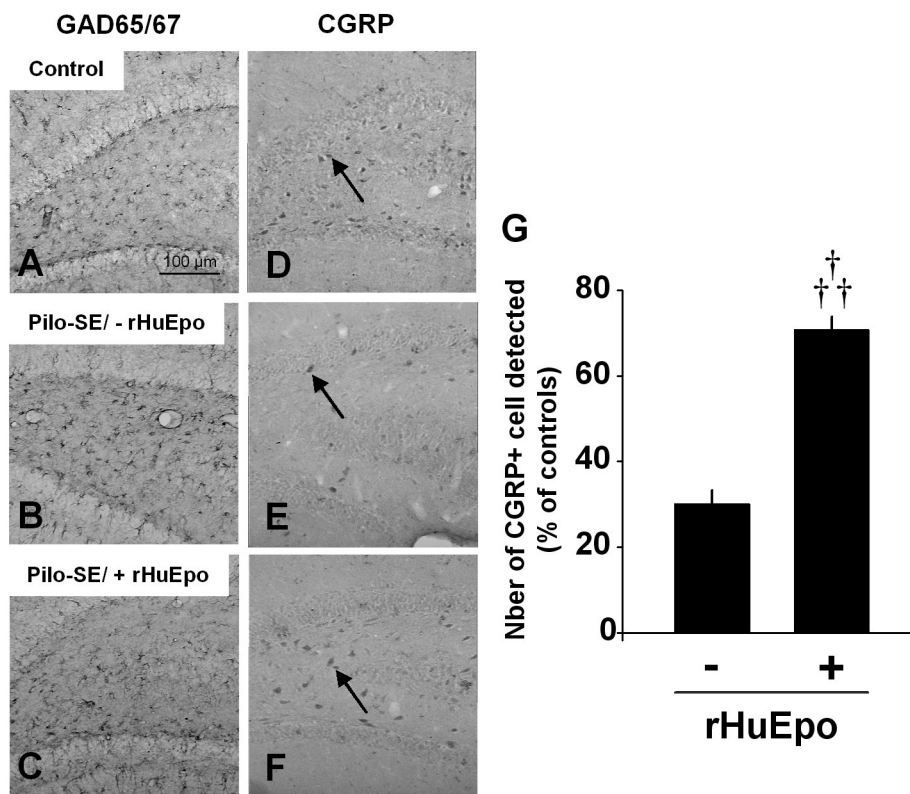


Figure 6