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Astroglial expression of erythropoietin as a potential support of neuronal

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Abbreviated title: Epo system reactivity during epileptogenesis

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Abstract

Brain erythropoietin (Epo) provides substantial trophic support to neurons during both development and mild injury in adults. Moreover, exogenously administered Epo has been shown to reduce neuronal degeneration in various models of brain disorders. However, endogenous Epo system in basal conditions and its reactivity during pathological processes 5 has received little attention. Here, we present for the first time the accurate *in situ* distribution of both Epo and Epo receptor (Epo-R) in the adult rat hippocampus in basal conditions, and reveal that Epo-R is expressed by a large majority of neurons, while Epo expression is restricted to some neuronal populations. Following pilocarpine-induced status epilepticus 10 (Pilo-SE), Epo is induced in numerous astrocytes. Hippocampal areas where astroglial Epo induction is the greatest exhibit a pattern of delayed neuronal death. The evidence that enhanced Epo-mRNA is preceded by an increase in transcript levels of HIF-1 α , the regulatory sub-unit of its transcription factor Hypoxia-Inducible Factor-1 (HIF-1), argues in favor of the hypothesis that hypoxic/ischemic insult occurs in the hippocampus following Pilo-SE and may reasonably be proposed as a cause of neurodegeneration during epileptogenesis. This in 15

situ analysis of both Epo/Epo-R and neuronal degeneration provides fundamental information on the ways Epo system may adapt to noxious microenvironment associated to epileptogenesis in order to support neuronal survival.

Introduction

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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 (Jelkmann, 1992). Epo exerts its erythropoietic functions by binding with homodimers of Epo receptor (Epo-R), which belongs to class 1 superfamily of cytokine receptors (Watowich et al., 1994). The original evidence that Epo binding sites are largely distributed throughout the central nervous system (Digicaylioglu et al., 1995) 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

(Campana and Myers, 2003; Maiese et al., 2004; Brines and Cerami, 2005).

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
15 expression, not only in basal conditions, but also in response to brain injury. In the adult human brain tissue, Epo and Epo-R are expressed by neurons mainly. However, following ischemic/hypoxic damage, both are strongly detected in reactive astrocytes (Juul et al., 1999; Sirén et al., 2001). To date, Epo system reactivity has been investigated in the neocortex in a model of cerebral ischemia (Bernaudin et al., 1999), and in both the spinal cord (Grasso et al., 2005) and peripheral nervous system (Campana and Myers, 2001; Li et al., 2005) in models

of traumatic injury.

In the hippocampus, neuron vulnerability has particularly been explored after *status epilepticus* (SE) induced either chemically or by sustained electrical stimulations of diverse brain regions (Morimoto et al., 2004), developed as models of human temporal lobe epilepsy. Our study is the first to investigate the basal expression of endogenous Epo system and its reactivity in a rat model of temporal lobe epilepsy. 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

30 of neuronal robustness, and a long-lasting induction of Epo-R, encouraging future studies aimed at investigating beneficial neuroprotective effects of exogenous Epo during epileptogenesis.

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

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Animals. Sprague-Dawley rats (Harlan, France) arrived at 5 weeks old in approved facilities, and housed at $21 \pm 1^{\circ}$ C under diurnal lighting conditions (lights on from 06:00 to 18:00).

10 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) was administered 30 min prior to pilocarpine hydrochloride (300 mg/kg, intraperitoneally). The onset of SE was

15 characterized by repetitive clonic activity of the trunk and limbs. SE was stopped after 3 hours by diazepam (Valium®, Roche; 10 mg/kg; intraperitoneally). Rats were then injected subcutaneously with 2 ml 0.9% NaCl.

Experimental design. To determine levels of mRNAs encoding key proteins of Epo system (i.e. Epo, Epo-R, aHIF, and HIF-1 α), animals were sacrificed following a lethal injection of

- 20 pentobarbital (250 mg/kg). Hippocampi were 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). Hippocampi were also collected in naive rats, sacrificed at t=0 (controls, n=5). To estimate neurodegeneration, either after NeuN-immunolabeling or Fluoro-Jade B staining, and both localize and characterize cells expressing Epo and Epo-R, rats were deeply
- 25 anesthetized with pentobarbital (250 mg/kg) at 1 (n=3), 3 (n=5), 4 (n=5) and 15 days (n=7) following Pilo-SE, and then transcardially perfused with a 4% paraformaldehyde solution. After cryoprotection in 25% sucrose, brains were frozen in isopentane and stored at -80°C. Naive rats (controls, n=4) were also included.

30 Methods

Reverse Transcriptase real time Polymerase Chain Reaction (RT-real time PCR). Total RNAs were extracted, purified and shown to be free of genomic DNA. Messenger RNAs, contained in 500 ng of hippocampal total RNAs, were then reverse transcribed and cDNAs of

targeted mRNAs were quantified by real time PCR. More details are available in supplemental material.

Histochemistry. Single colorimetric and fluorescent dual-labeling were performed on free floating sections selected at IA 5.40 (Paxinos and Watson, 1998) from paraformaledhyde-

- 5 fixed tissue, using as primary antibodies, rabbit polyclonal antibodies raised against either Epo (sc-7956; Santa Cruz) or Epo-R (sc-697; Santa Cruz) and mouse monoclonal antibodies raised against either NeuN (MAB-377; Chemicon), Ox-42 (CBL1512Z; Chemicon), or GFAP (G3893; Sigma) as described in more details in supplemental material. Biotin-conjugated secondary antibodies were from Jackson Immunoresearch, and Alexa-488 or Alexa-633
- 10 conjugated secondary antibodies were from Molecular Probes. Fluoro-Jade B (Chemicon) was used to stain specifically degenerating neurons after Pilo-SE in rats (Poirier et al., 2000). *Neuronal counts*. Sections selected at IA 5.40 and 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).
- 15 Neuronal counts were performed as described in more details in supplemental material. 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.

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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 (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 (Fig. 1). However, not all neurons expressed
30 Epo-R, especially in the hilus (Fig. 1*B*; see white arrows). Epo-R labeling was mainly distributed in neuronal cell bodies, except within CA1 where a strong signal was revealed in the dendritic field (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 (Fig. 2A) and never detected in resting microglial cells (data not shown).

Colorimetric immunohistochemistry evidenced the presence of Epo protein in the pyramidale layer of CA2 (data not shown), in the perikarya and processes of some hilar (Fig. 3A) and CA4 (data not shown) neurons, and in granule cells (data not shown). The labeling obtained in the other hippocampal neuronal layers 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), rarely by astrocytes in the hilus (Fig. 3*E*), and never detected in resting microglial cells (data not shown).

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Reactivity of Epo-R expression in the hippocampus during epileptogenesis. Following Pilo-SE, Epo-R-mRNA was robustly increased in the hippocampus from 1 through 7 days post-SE, with a peak observed at 3 days post-SE (Fig. 2*B*). Immunohistochemical studies performed at 4 days post-SE evidenced a reduction of the neuronal staining in the hilus and

- 15 CA4 (Figs. 2*C*, *E*). By contrast, additional glial-like cells expressed Epo-R within all hippocampal areas, as illustrated in the hilus (Figs. 2*C*, *E*) and CA2 (Figs. 2*D*, *F*; see black arrow). Double GFAP/Epo-R fluorescent immunolabeling identified these glial cells as astrocytes (Fig. 2*A*).
- 20 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) (Fig. 3*F*). 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 (Figs. 3*A*, *C*). By contrast, in hippocampal subfields that were clearly devoid of Epo-expressing cells
 25 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 (Figs. 3*B*, *D*). Such perikarya were also seen within the pyramidale layer of CA1 and CA2, the molecular layer of dentate gyrus (data not shown), and the hilus (Figs. 3*A*, *C*). In all hippocampal subfields mentioned, numerous of these "newly-detected" cells were identified
- 30 as GFAP-immunoreactive astrocytes (Fig. 3*E*; see white arrows). The scattering of the Epoexpressing 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) (Fig. 3*F*) and was accompanied by a reduced expression (-40%) of the natural HIF-1 α anti-sens RNA (aHIF) that was significant and long-lasting (Fig. 3*F*).

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 (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 (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% (Fig. 4B). In CA1 and CA2, Fluoro-Jade B staining was observed at 1 day post-SE, increased at 3 (Fig. 4A) and 4 days post-SE. Neuronal loss was evidenced at 3 days post-SE and reached ~ 50% by 15 days

15 post-SE (Fig. 4B). The SLMo exhibited no Fluoro-Jade B staining from 1 to 4 days post-SE (Fig. 4A). However, at 15 days post-SE, neuronal loss reached ~ 40% (Fig. 4B).

Discussion

20 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 expressed Epo and Epo-R in every area of the hippocampus. By contrast, Epo and Epo-R were induced in numerous astrocytes following Pilo-SE, and hippocampal areas where astroglial Epo induction was the greatest exhibited a pattern of neuronal death that was significantly delayed. This *in situ* analysis of both Epo/Epo-R and neuronal degeneration in non mutant animals provides fundamental information on the ways Epo system may adapt to noxious microenvironment associated to epileptogenesis in order to support neuronal survival.

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Numerous *in vitro* studies have evidenced that Epo-R is expressed by cultured hippocampal neurons (Kawakami et al., 2001; Chong et al., 2003; Leist et al., 2004; Ehrenreich et al., 2005). While both Epo binding sites (Digicaylioglu et al., 1995) and Epo-R (Ehrenreich et al., 2005) have been shown to be abundant in adult rodent hippocampus in

basal conditions, a single *in situ* study illustrated succinctly the presence of Epo-R expressing cells in the adult rat hippocampus (Morishita et al., 1997). Surprisingly, in another study, hippocampal presence of Epo-R was demonstrated after hypoxia exposure, but not in basal conditions (Lewczuk et al., 2000). Therefore, to fully discern cells which are influenced by 5 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 10 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 (Ehrenreich et al., 2005). Our in situ study agrees with a prior work (Chung et al., 2004) 15 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 (Sakanaka et al., 1998).

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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 in the hilus, CA2 and 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 (Marti, 2004). HIF-1α protein, the regulatory sub-unit of HIF-1, is known to be degraded under normoxic conditions, and its accumulation, observed in hypoxic tissues, requires post-translational modifications (Marti, 2004). 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 (Rossignol et al., 2004). 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 fissure (Sloviter, 2005) may explain the astroglial induction of Epo in the SLMo. Indeed, astroglial expression of Epo is greatly enhanced by hypoxia (Masuda et al., 1994; Sirén et al., 2001) and by agents

which mimic hypoxic insults (Bernaudin et al., 2002).

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- Concomitantly to Epo gene activation, transcript levels of Epo-R are also increased 10 following Pilo-SE and maintained at a high level during epileptogenesis. This Epo-R gene induction is associated to 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 15 CA1 and the hilus. These results are in line with most observations noted in mouse cortex after brain ischemia (Bernaudin et al., 1999), in rat spinal cord after trauma injury (Gorio et al., 2005; Grasso et al., 2005), and in rat peripheral nerve injury (Li et al., 2005), with the notable exception that Epo and Epo-R are detected neither in resting or reactive microglial cells, nor in endothelial cells following Pilo-SE. The present study evidences that 20 neurodegeneration in the hilus precedes the astroglial induction of Epo, whereas neurodegeneration is delayed in the SLMo which exhibits the greatest astroglial induction of Epo. Thus, our results together with data published by others support strongly the hypothesis that Epo-mediated neuronal survival depends on both the severity of the damage and the state of the endogenous Epo system. Basal activity of Epo system, which involves neuronal 25 interactions mainly, appears to be sufficient to counteract the progression toward neuronal death following mild injuries. By contrast, in situations of severe brain damage, efficient neuronal protection may require Epo system reactivity, involving a pronounced astroglial induction of Epo. However, such an induction appears to be not fast enough to protect the
- 30 Epo-R has been evidenced in human brain tissues following hypoxic damage (Sirén et al., 2001). Here, the observation that Epo-R is mainly expressed by astrocytes in the SLMo following Pilo-SE when astroglial induction of Epo is the greatest underscores the possibility that astroglial Epo is an autocrine signaling molecule which may trigger the release of yet unknown astroglial factors involved in neuronal survival.

most vulnerable neurons present in the hilus. A strong astroglial reactivity of both Epo and

Epo induction is only transient following Pilo-SE, a result which may be explained in the hippocampus by the activation of proinflammatory cytokines (Rizzi et al., 2003; Voutsinos-Porche et al., 2004), known to impair Epo expression (Frede et al., 1997; Nagai et

5 al., 2001; Gorio et al., 2005). This transient up-regulation of Epo appears to be insufficient to support long-term survival of vulnerable neurons in such a deleterious tissue microenvironment, since neuronal loss was observed at 15 days post-SE in the SLMo. Therefore, considering the wide distribution of Epo-R at the surface of hippocampal neurons, it will be interesting in future studies to determine whether therapeutic administration of

10 exogenous Epo can significantly reduce hippocampal neurodegeneration following Pilo-SE.

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Figure legends

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Figure 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 μm.

- Figure 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 μm; *C*-*F*, 100 μm.
- Figure 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.

Figure 4. Diverse patterns of neuronal loss in various areas of the dorsal hippocampus 30 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 (Paxinos and Watson, 1998) 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 µm.





Figure 1



Figure 2



Figure 3



Figure 4

Supplemental material

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 5 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 10 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 (2004); 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-15 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' 20 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
 25 3' (68 bp); *HIF1a* (GenBank Y09507) forward 5' CTC AGA GGA AGC GAA AAA TGG
- 3' (68 bp); *HIF1α* (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; <u>www.basic.nwu.edu</u>).

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), or a rabbit polyclonal anti-Epo-R antibody diluted at 1:500 (sc-697; Santa Cruz), or a mouse monoclonal anti-NeuN antibody diluted at 1:1000 (MAB-377; Chemicon). After washes, the sections were then incubated with a biotinylated donkey antibody diluted at 1:1000, either raised against rabbit IgG (711-066-152; Jackson ImmunoResearch), or mouse IgG (715-065-151; 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).

- 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 either against NeuN
 (MAB-377; Chemicon, diluted at 1:1000), or Ox-42 (CBL1512Z; Chemicon, diluted at
 1:2000) or GFAP (G3893; Sigma, diluted at 1:2500). After washes, sections were exposed to
- 10 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
- 15 Systems) for further editing.

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® 6.0; Noesis). The system allows to scan at magnification 20X adjacent fields throughout the whole dorsal hippocampus and to

20 reconstruct a single image composed of a mosaic of the digitized adjacent fields. Dorsal hippocampus was selected at IA 5.40 mm (Paxinos and Watson, 1998). Due to the morphological diversity of the neuronal populations composing the hippocampus, we could not establish a standard "neuronal" profile to perform an automated neuronal count. Therefore, neurons were counted manually by two independent observers from the 25 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 CA2, we measured within a 282,440 µm² window the surface area occupied by NeuN-immunopositive cell bodies within the field delineated by pyramidal neurons.

30