Supporting Information

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SI Materials and Methods

In Vivo Experimental Design. *Animals.* Male Sprague–Dawley rats (Harlan) were used throughout the experiments. They arrived at 5 weeks old (experiments 1, 3, and 4) or at 2 weeks old with a dam (exeriment 2) in approved facilities, and were housed at 21 °C under diurnal lighting conditions (lights on from 06:00 AM to 06:00 PM). They were maintained in groups of 6 in plastic cages (type E cages; Charles River) with free access to food and water. After 2 weeks of acclimatization, rats underwent 3 weeks of experimental procedure. After pilocarpine-induced status epilepticus (Pilo-SE), rats were housed individually to support recovery until being killed. Control rats were housed in groups of 6 throughout all of the experiment to avoid deleterious effects related to stress isolation. Control rats received systematically corresponding injections of saline solution.

Experiment 1. Brain expression of *Epo* and *EpoR* genes was determined in the hippocampus (Hi) and the ventral limbic region (VLR) of control rats ($n = 10$) and rats subjected to either 1 ($n = 13$) or 3 ($n = 22$) hypoxic (H) exposure(s). Expression profiles of erythropoietin (Epo) and Epo receptor (EpoR) mRNAs were measured by RT-qPCR in control rats ($n = 6$), and at various reoxygenation times after either 1H (0 h, $n = 4$; 1 day, $n = 3$; 2 days, $n = 3$; 8 days, $n = 3$) or 3H (0 h, $n = 4$; 1 day, $n = 4$; 2 days, $n = 4$; 8 days, $n = 4$). Also, immunostaining on brain sections of Epo and EpoR was realized in combination or not with neuronal (NeuN) and astrocytic (GFAP) markers at 1 ($n = 3$) and 3 ($n = 3$) days after 3H ($n = 6$). Degenerative processes were also assessed after reoxygenation in all groups. Control rats ($n = 4$) were included in this analysis.

Experiment 2. Rats were housed in MARLAU enriched cages (EC), developed in our group to standardize environmental enrichment, or 2 standard cages (SC, type E, Charles River). Six and 12 rats were grouped together in SC and EC, respectively, from weaning until completion of the experiment. Six weeks after weaning, half of the rats of each SC and EC conditions (6/12 rats in each condition) were subjected to 3H. Levels of *EpoR* and *Epo* transcripts were measured by RT-qPCR in the dorsal Hi (HiD), the ventral Hi (HiV), the neocortex (NC), and the VLR of 4 rats in each group. The remaining rats ($n = 2$ per group) were used for EpoR immunostaining.

Experiment 3. To determine whether 3 hypoxic exposures could enhance recombinant human (rh)Epo neuroprotective effects after Pilo-SE, rats were subjected to 3H ($n = 12$) or not ($n = 12$) before Pilo-SE. In each group, half of the rats that underwent Pilo-SE were treated or not with rhEpo at 5,000 international units/kg i.p. immediately after cessation of SE, and then 1 and 3 days later. This dose of rhEpo corresponds to the optimal neuroprotective dose with this route of administration (1). Neuronal protection was evaluated 6 days after Pilo-SE by measuring the density of NeuN+ cells in the VLR. Control rats ($n = 6$) received the same injections as rats subjected to SE, except that saline was substituted for pilocarpine.

Experiment 4. Tissue concentration of rhEpo in the VLR and the HiD was determined at different times (1 and 4 h) after its peripheral administration in control rats ($n = 6$) and in rats subjected to Pilo-SE ($n = 6$). In these latter, rhEpo was administered just after cessation of SE.

RT-qPCR. Sequences of the different primer pairs used are as follows: *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); *EpoR* (GenBank D13566) forward 5′-CCA GCT CTA AGC TCC TGT GC-3′, reverse 5′-CTT CAG GTG AGG TGG AGT GG-3′ (68 bp), *IGF-1* (GenBank NM_178866.2) forward 5′-ATG CCC AAG ACT CAG AAG GA-3′, reverse 5′-CGT GGC ATT TTC TGT TTC TC-3′ (110 bp), *Tpo* (GenBank D32207) forward 5′-CCC AAG CAG AAC CTC TCA AC-3′, reverse 5′-TCA GGT ATC CAG GGA TTT GG-3′ (200 bp), *TpoR* (2) forward 5′-AGA ACC CAC AGA GTG GTG TG-3′, reverse 5′-TCA CAC CAT CCA GGA GCA AGA AT-3′ (220 bp). All primer pairs were designed by using Primer 3 software (National Institutes of Health; www.basic.nw-u.edu).

Immunohistochemistry. Free-floating sections (40 μm thick) from paraformaldehyde-fixed tissue were incubated either with a rabbit polyclonal anti-Epo antibody (sc-7956; Santa Cruz), or with a rabbit polyclonal anti-EpoR antibody (sc-697; Santa Cruz), or with a mouse monoclonal anti-NeuN antibody (MAB-377; Chemicon) or a GFAP antibody (G3893; Sigma). For colorimetric immunolabeling, sections were then incubated with a biotinylated donkey antibody raised against mouse IgG (715-065-151; Jackson ImmunoResearch). Sections were incubated with avidin-biotin-peroxidase (1:500; Vectastain Elite ABC kit, Vector) and reacted with 0.4 mM 3′,3-diaminobenzidine (DAB, Vector). For fluorescent dual immunolabeling, sections were exposed to an Alexa Fluor-488-conjugated donkey anti-rabbit IgG antibody (A-21206; Molecular Probes) and to an Alexa Fluor-633-conjugated goat anti-mouse IgG antibody (A21052; Molecular Probes). Sections were then mounted on Superfrost-Plus slides and coverglassed with Prolong Gold Antifade reagent (Molecular Probes). Images were captured by a TCS SP2 confocal microscopy system (Leica). Images were then imported into Adobe Photoshop 8.0.1 (Adobe Systems) for further editing.

Antibody Characterization. For Epo-R, a polyclonal rabbit antibody [Santa Cruz no. sc-697 (M-20)] raised against the C-terminal cytoplasmic domain of the precursor form of mouse EpoR was used. This antibody reveals in rat brain homogenates a single band slightly above the expected 66-kDa size (3, 4). Brain mapping of EpoR protein using this antibody has been a matter of intensive debate due to the production of diverging results (4). Its specificity has then been questioned in a report showing immunolabeling in *EpoR*-knockout mice ($EpoR^{-/-}$) (5). However, conclusions raised from these $EpoR^{-/-}$ mice must be taken with caution, because the *EpoR* gene, which is composed of 8 exons, has been silenced by deletion of exons 1–6 only (3, 6). In the eventuality of a chimeric protein, partly encoded by the remaining exons 7–8, a signal may be obtained with the M-20 antibody, which is directed against an epitope encoded by exons 7–8 of the *EpoR* gene. A complete abolition of Epo-R immunohistofluorescent labeling in rat brain tissue sections has been reported, when this antibody was preincubated with a 5-fold excess of the M-20-immunizing peptide (sc-697P) (7, 8).

For Epo, a polyclonal rabbit antibody [Santa Cruz no. sc-7956 (H-162)] raised against amino acids 28–189 from mature human Epo was used. This antibody stains a single 33-kDa band in Western blot analysis of Epo expression in normal rat kidney cell line transformed by the Kirsten murine sarcoma virus (KRNK cells) (manufacturer's technical information).

For the detection of neuronal cell type in brain slices, we used the monoclonal mouse antibody (Chemicon no. MAB-377) raised against undetermined nuclear proteins of neurons (NeuN). This antibody recognizes 2 or 3 bands in the 46- to

48-kDa range, and possibly another band at ≈66 kDa (manufacturer's technical information). Even if the sequence of the immunizing antigen(s) has not been established yet, this antibody has been shown to stain neurons exclusively, both in vivo and in vitro, recognizing most of neuronal cell types throughout the central nervous system of numerous vertebrates (9).

Mouse anti-GFAP (Sigma no. G3893, monoclonal, immunogen: whole GFAP protein from pig spinal cord) reacts specifically with GFAP in immunoblotting assays and labels astrocytes in brain tissue sections (manufacturer's technical information).

Neuronal Density Measurement. Sections immunostained for NeuN were observed under a light microscope (Diaplan; Leitz) by 2 independent investigators who were blind to the groups from which the sections were taken. Images were captured with a video camera 3CCD (DXC-930P; Sony) coupled to an image analysis system (Visilog 6.3; Noesis). The system allows one to scan at magnification 10× adjacent fields throughout the whole ventral region, and to reconstruct a single image composed of a mosaic of the digitized adjacent fields. Mosaics of the ventral region, composed of the amygdala, the piriform cortex, and the insular agranular cortex, were captured at both interaural (IA) +6.44 and +5.40 mm. To obtain an index of the neuronal density, the surface area occupied by NeuN+ cells within the VLR or within areas CA1 and CA3 of the HiD was divided by the total surface area.

Measurement of the Intensity of Epo- or EpoR-Immunofluorescent Labeling in Neurons. Sections used were all processed together for the dual immunofluorescent labeling of Epo or EpoR with NeuN. They were then analyzed at the same conditions of photomultiplier gain, offset, and pinhole aperture by using a TCS SP2 confocal microscopy system (Leica), allowing the comparison of fluorescence intensity in all neurons contained in the image by using an image analysis system (Visilog 6.3; Noesis).

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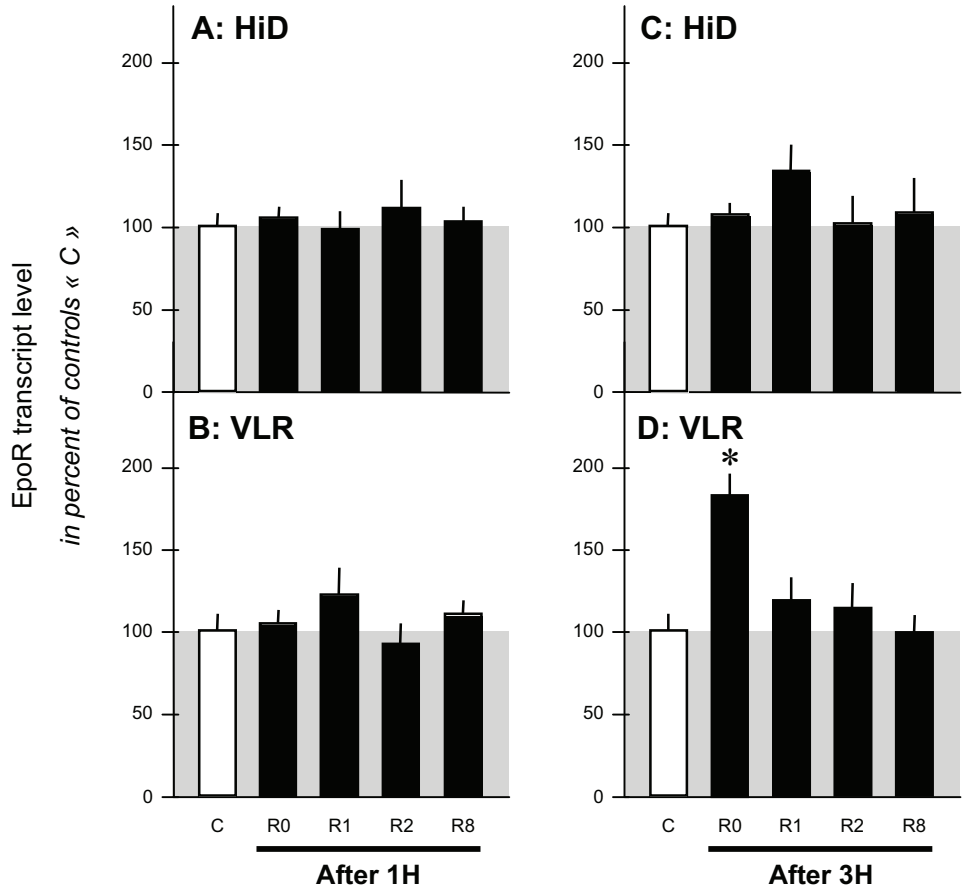

Fig. S1. Time course of *EpoR* transcript level after 1H and 3H in the HiD and the VLR. Controls and rats subjected either to 1H or to 3H were killed immediately after (R0), 1 (R1), 2 (R2), and 8 (R8) days after the last hypoxic exposure. Levels of *EpoR* transcript were measured by RT-qPCR. Each bar represents the mean ± SEM ($n = 4$ in each group); *, $P < 0.05$, compared to controls (C).

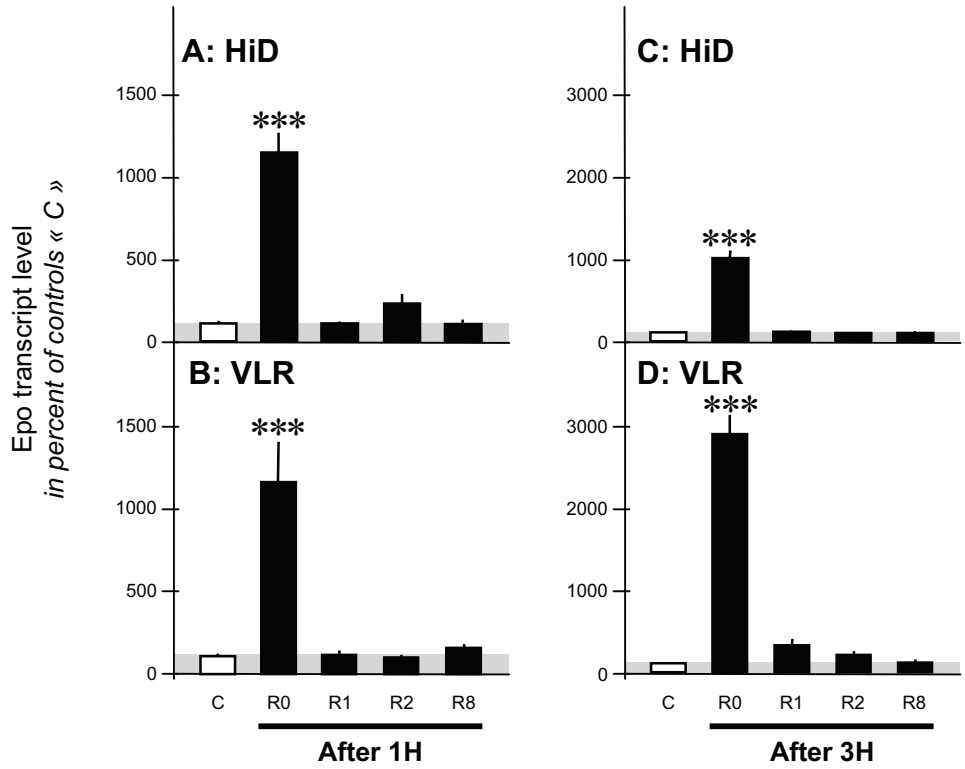

Fig. S2. Time course of *Epo* transcript level after 1H and 3H in the HiD and the VLR. Controls and rats subjected either to 1H or to 3H were killed immediately after (R0), 1 (R1), 2 (R2), and 8 (R8) days after the last hypoxic exposure. Levels of *Epo* transcript were measured by RT-qPCR. Each bar represents the mean ± SEM ($n = 4$ in each group); ***, $P < 0.001$, compared to controls (C).

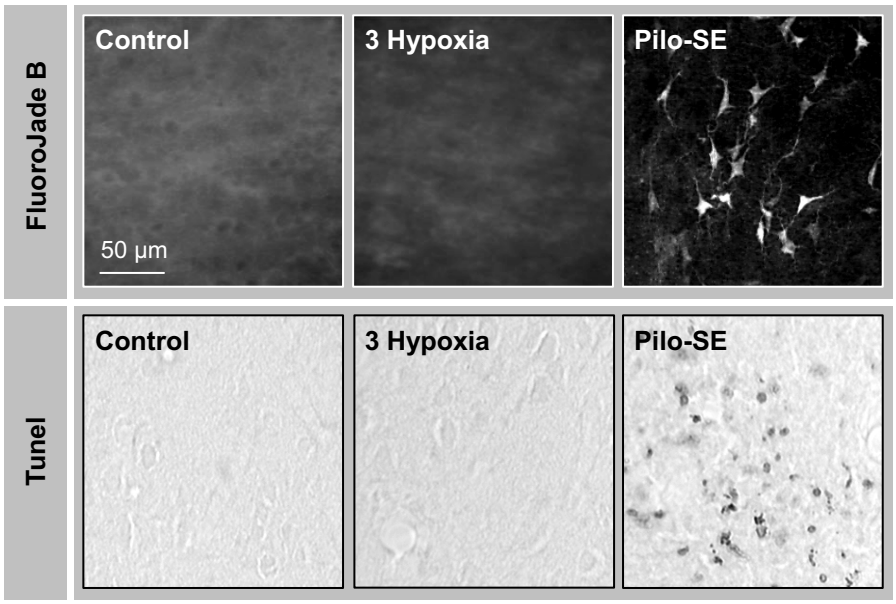

Fig. S3. Repetition of hypoxia exposures does not induce detectable degenerative processes. Fluorojade B staining or TUNEL was used to detect neuronal suffering or cell death associated with DNA breaks, respectively. No signal was obtained in controls and 3H rats with both methods, as shown here in the HiD 3 days after reoxygenation. By contrast, both Fluorojade B and TUNEL+ cells were detected in the Hi 1 day after Pilo-SE.

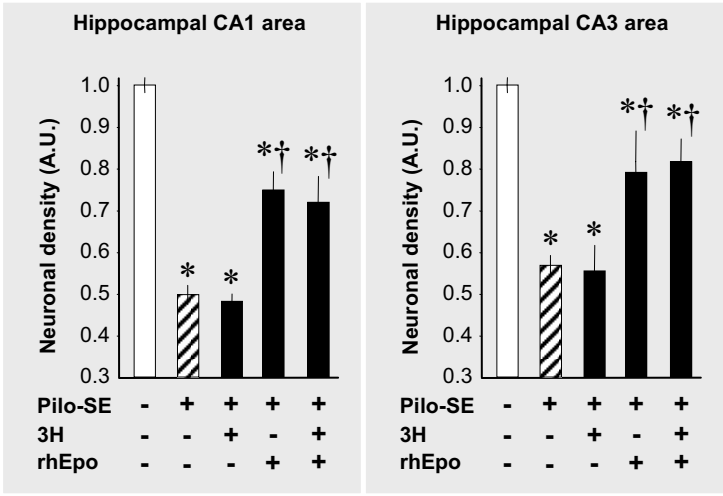

Fig. S4. Neuroprotective effects of rhEpo in the HiD 6 days after Pilo-SE. Neuronal density in CA1 and CA3 areas was measured at anatomical planes corresponding to IA +6.44 and +5.40 mm, according to ref. 10. Because the anatomical plane itself had no effect, and no significant interaction was found between ''anatomical plane'' and ''treatment condition,'' results for neuronal density were collapsed over the anatomical plane factor; 3H alone did not induce any neuroprotective effects in the 2 hippocampal areas after Pilo-SE. By contrast, rhEpo administered alone or in rats subjected to 3H significantly protected pyramidal neurons of CA1 and CA3 areas against neurodegeneration after Pilo-SE. However, no greater neuroprotective effect of rhEpo was observed in rats subjected to 3H. ∗, $P < 0.001$, compared with controls; †, $P < 0.001$, compared to ''Pilo-SE'' or ''Pilo-SE + 3H'' ($n = 6$ in each group).

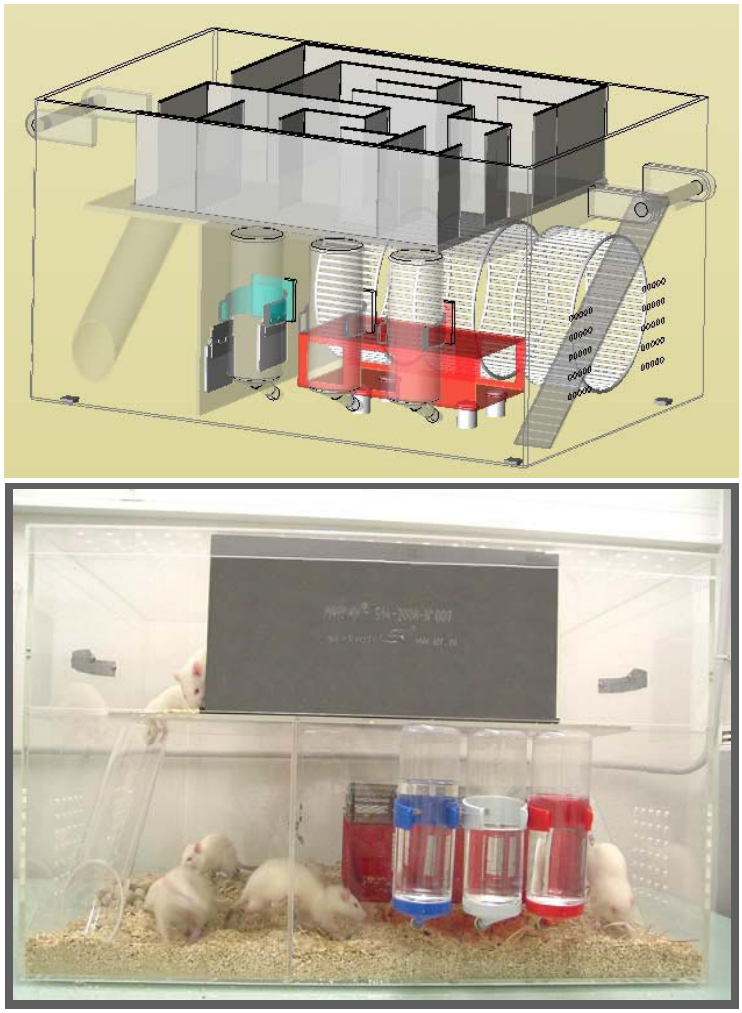

Fig. S5. MARLAU cage (patent no. FR09/00544), a cage to calibrate environmental enrichment in rodents. This cage allows increased social interactions (12 rats per cage), increased voluntary exercise (large surface area and presence of 3 running wheels), ''diverting'' activities (red tunnel, ladder, and toboggan slide), and cognitive stimulations using labyrinths, the configuration of which is changed 3 times a week.