

# Vascular endothelial growth factor induces brain erythropoietin expression?

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## Summary

To investigate whether the formation of vascular endothelial growth factor (VEGF) influences erythropoietin (EPO) expression in physiological conditions, we injected into the left lateral cerebral ventricle of the Mongolian gerbil an adeno-associated virus (AAV) vector capable of expressing the 165-amino-acid isoform of VEGF (VEGF<sub>165</sub>). Twelve and 18 days after AAV vector injection, the experimental animals were sacrificed and expression of EPO was evaluated through immunohistochemical analysis of both the hippocampus and the frontal cortex.

We observed that VEGF<sub>165</sub> induces EPO expression in the hippocampal pyramidal layers and in the frontal cortex of the gerbil, particularly after the 18<sup>th</sup> day following treatment with the vector, which suggests that VEGF<sub>165</sub> could act as a hypoxic-like signal for EPO production. This finding could help to clarify the functional role of EPO and the molecular mechanisms by which VEGF might mediate its effects in the brain.

**KEY WORDS:** adeno-associated virus, brain, erythropoietin, VEGF<sub>165</sub>.

## Introduction

It is well known that vascular endothelial growth factor (VEGF) is primarily involved in angiogenesis (1), particularly following brain hypoxia; moreover its expression, triggered within a few hours in the experimental model of brain ischemia induced by middle cerebral artery occlusion, persists for days to weeks (2). Accordingly, after an ischemic stroke, brain tissue has been reported to show up-regulation of VEGF mRNA in neurons, astrocytes and endothelial cells and marked expression of VEGF receptors in blood vessels (3).

This appears to be supported by previous experimental observations showing that brain ischemia induces expression of HIF-1 $\alpha$  and consequently of VEGF and its receptors (5).

Erythropoietin (EPO) is a primary regulator of erythropoiesis and is fundamentally produced by the kidney in adults and by hepatocytes in the foetus in response to hypoxia (6).

However, previous experimental findings demonstrated that EPO and its receptor are constitutively expressed in the brain of a wide variety of mammals, including humans, suggesting a direct role for this hormone in the central nervous system (7).

Interestingly, it has been shown that both VEGF and EPO exert neuroprotective effects in both *in vitro* and *in vivo* experimental models (8-12), probably through the common oxygen-sensing pathway represented by HIF-1 $\alpha$  (13).

These findings, taken together, could indicate a possible cross-talk between VEGF and EPO in the regulation of physiological and pathological events in the brain.

However, to date, the exact molecular mechanisms by which VEGF might mediate its actions in the central nervous system, and the possible implications for EPO in this regard, are not well understood.

In view of these preliminary considerations, we injected into the gerbil brain, without hypoxic stimulus and at different time points, an adeno-associated virus (AAV) vector capable of expressing the 165-amino-acid isoform of VEGF (VEGF<sub>165</sub>). Our aim was to investigate, in dis-

crete brain regions, namely the hippocampus and frontal cortex, whether VEGF can induce EPO production. It was felt that the chosen approach (gene transfer through an AAV vector) may better show the direct molecular effects of VEGF in the brain and help to clarify its mechanism of action.

### Materials and methods

Two recombinant AAV (rAAV) vectors were obtained expressing the LacZ reporter gene (control gene) and the cDNA for VEGF<sub>165</sub> under the control of the strong and constitutive cytomegalovirus immediate early promoter. The purified viral preparations used in this work had particle titres of  $1 \times 10^{12}$  viral genomes per ml. Male Mongolian gerbils, weighing 60-70 g, housed four to a cage at a constant room temperature of 21-22°C under a light/dark cycle of 12/12 h (7.00 a.m./7.00 p.m.) with free access to food and water *ad libitum*, were used.

Adaptation and experiments were carried out in accordance with internationally accepted principles and with Italian laws concerning the care and the use of laboratory animals, as well as with the European Communities Council Directive 86/609/EEC, of 24 November 1986. Twelve and 18 days before decapitation under general anaesthesia, gerbils were anesthetized with chloral hydrate (2.5%), placed in a stereotaxic apparatus and a guide cannula was implanted into the left lateral cerebral ventricle according to the Thiessen and Yahr atlas (14). The animals were then randomized to treatment with rAAV-LacZ (control) or with rAAV-VEGF<sub>165</sub> (0.5-25 U) in 10 µl of artificial cerebrospinal fluid injected into the left lateral cerebral ventricle.

Immunohistochemistry was performed in animals sacrificed with light chloral hydrate anaesthesia. Brains were removed and 40-µm thick brain coronal sections were cut using a freezing microtome and stored at 4°C. A set of sections, regularly spaced through the brain, was mounted and stained with thionin 0.25% for histological identification of nervous structures, drawing analogies with the rat brain according to the Thiessen and Yahr atlas (14). The remaining sections were used for immunohistochemical experiments. Biotinylated goat anti-rabbit primary antiserum for EP was used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Free-floating sections through the brain were rinsed in tris-buffered saline of pH 7.6 (TBS) and incubated overnight with the primary antibodies diluted in TBS containing 2% normal goat serum (NGS). Sections were then rinsed and incubated for 60 min with biotinylated goat anti-rabbit serum (diluted 1:200 in TBS containing 1% NGS); 60 min with avidin-biotinylated peroxidase complex (diluted 1:100) in TBS; and 4-8 min with a solution of 3,3'-diaminobenzidine tetrahydrochloride 0.5 M. Sections were finally mounted, air-dried and cover-slipped for microscopic observation. No immunostaining was observed in control tissue. Computer-assisted densitometry was used for quantitative estimation of immunostaining. For this purpose, the optical density of images was analyzed in a range of 256 grey level values. Microscope illumination and video-amplifier parameters were carefully adjusted to avoid saturation of images. These conditions were kept constant.

### Statistical analysis

All statistical procedures were performed using SPSS statistical software package, release 6.1.3 (SPSS Inc., Chicago, IL, USA). Data analysis was performed using one-way analysis of variance (ANOVA) with Scheffé post-hoc test for multiple comparisons. Each value represents the mean ± SD of six animals. Statistical significance was set at  $p \leq 0.05$ .

### Results

The animals challenged with rAAV-LacZ (controls) showed very low immunopositivity for EPO in the CA1 and CA3 areas of the left hippocampus and in the left frontal cortex (Fig.s 1, 2, 3, 4A, 4C).

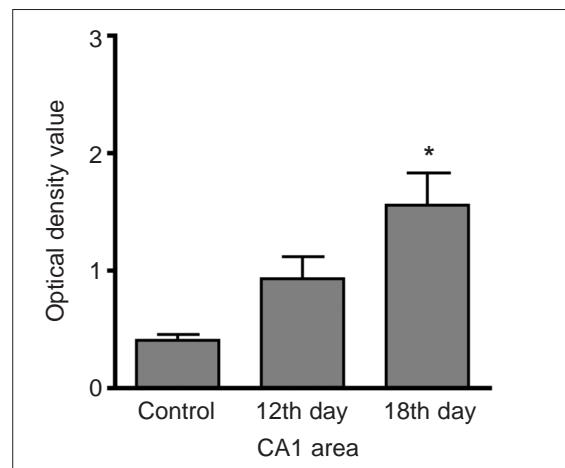


Figure 1 - Effects on immunostaining for EPO in the CA1 area of gerbils: (i) following rAAV-LacZ treatment (controls), (ii) 12 days after rAAV-VEGF<sub>165</sub> administration (iii) 18 days after rAAV-VEGF<sub>165</sub> administration. Each column represents the mean ± SD of five to six animals. \* $p < 0.01$  vs sham + rAAV-LacZ.

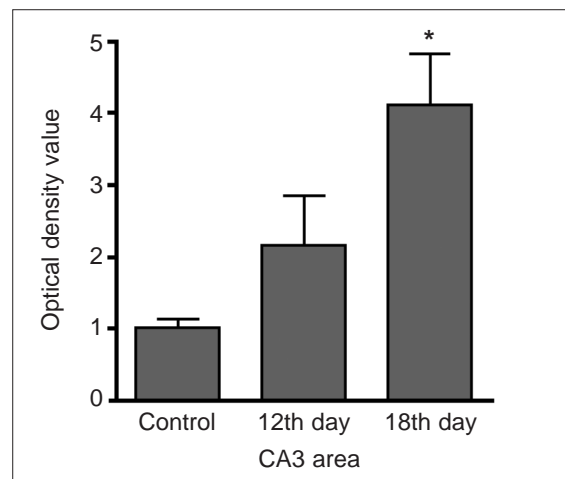


Figure 2 - Effects on immunostaining for EPO in the CA3 area of gerbils: (i) following rAAV-LacZ treatment (controls), (ii) 12 days after rAAV-VEGF<sub>165</sub> administration, (iii) 18 days after rAAV-VEGF<sub>165</sub> administration. Each column represents the mean ± SD of five to six animals. \* $p < 0.01$  vs sham + rAAV-LacZ.

By contrast, in gerbils 12 days and 18 days after rAAV-VEGF<sub>165</sub> administration, the computer-assisted densito-

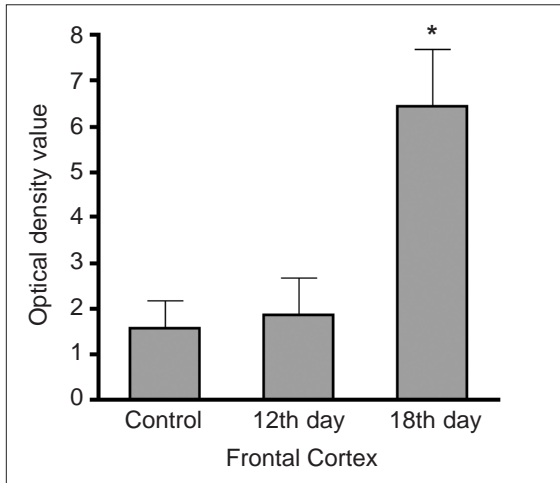


Figure 3 - Effects on immunostaining for EPO in the frontal cortex of gerbils: (i) following rAAV-LacZ treatment (controls), (ii) 12 days after rAAV-VEGF<sub>165</sub> administration (iii) 18 days after rAAV-VEGF<sub>165</sub> administration. Each column represents the mean ± SD of five to six animals. \*p<0.01 vs sham + rAAV-LacZ.

metry of microscopical sections revealed an immunopositivity for EPO in the left frontal cortex and the left hippocampus (Fig.s 1, 2, 3).

In particular, the strongest immunopositivity was observed 18 days after rAAV-VEGF<sub>165</sub> treatment in pyramidal cell layers of the left hippocampus (namely CA1 and CA3 areas) and in the left frontal cortex (Fig. 4B, 4D).

### Discussion

There is experimental evidence suggesting that VEGF could have beneficial effects on ischemia-induced brain injury (15,16); however, cerebral ischemia caused by severe hypoxia occurs in a series of steps, suggesting the possible involvement of other mediators in the neuroprotective effects of VEGF.

In this regard, acute hypoxic brain damage has been associated with increased brain EPO expression (17); indeed, EPO is strategically modulated in many cell types (neurons, endothelial and glial cells) as a function of the duration of the brain ischemia (18) and its administration prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery (19). Accordingly, different findings showed

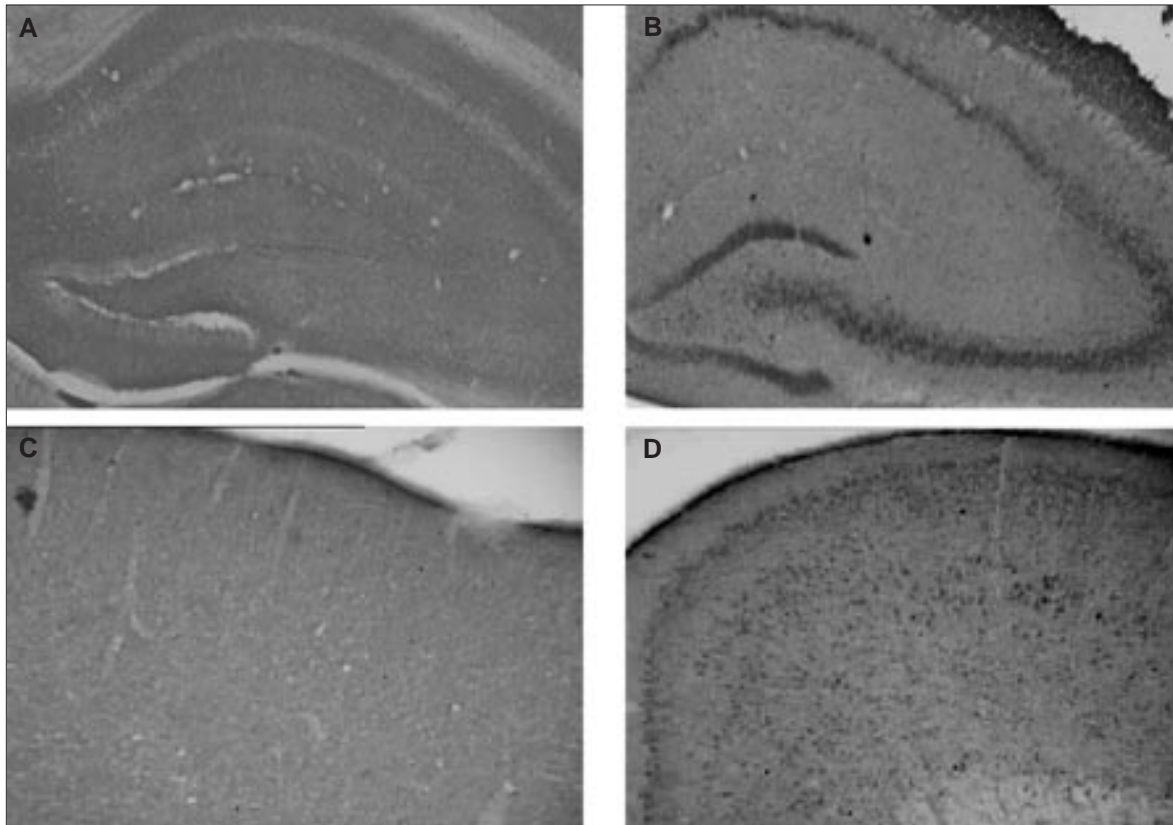


Figure 4 - A) Slide of left hippocampus in a Mongolian gerbil sacrificed 18 days after injection of rAAV-LacZ into the left lateral cerebral ventricle. Absence of immunopositive EPO-elements. B) Immunostaining for EPO in section of left hippocampus in a Mongolian gerbil sacrificed 18 days after injection of rAAV-VEGF<sub>165</sub> (x10) into the left lateral cerebral ventricle. Several EPO neurons are clearly marked black, denoting immunopositivity. C) Slide of left frontal cortex of a Mongolian gerbil sacrificed 18 days after injection of rAAV-LacZ into the left lateral cerebral ventricle. No EPO-positive elements are displayed. D) Immunostaining of left frontal cortex of a Mongolian gerbil sacrificed 18 days after injection of rAAV-VEGF<sub>165</sub> (x10) into the left lateral cerebral ventricle. Several neurons (black stained) display clear EPO positivity.

constitutive presence of EPO and its receptor in neurons, glial and endothelial cells (19), providing new insights into a possible role for this hormone in the mammalian brain, even in the absence of hypoxic stimulus (20,21).

Thus, we decided to evaluate, only in physiological conditions (and thus without hypoxic stimulus), EPO expression in the Mongolian gerbil following the injection of the AAV-VEGF vector capable of expressing VEGF<sub>165</sub>. In this regard, VEGF presents four different isoforms, VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, VEGF<sub>206</sub>, which are generated by alternative splicing of mRNA (22).

Currently promising new strategies for gene delivery to the brain (23) include the use of AAV vectors. These vectors are derived from a non-pathogenic and widespread defective *Parvovirus* and are able to transduce both dividing and nondividing cells (24-26). Since rAAV vectors are devoid of any viral genes, they elicit virtually no inflammatory or immune response in the sites of injection (27).

Furthermore, AAV vectors boast several important features, including the capacity to promote long-term expression of the gene transferred.

Our experimental data indicate that VEGF<sub>165</sub> administration in the left lateral cerebral ventricle of the gerbil, in the absence of hypoxic stimulus, triggers EPO production in the left hippocampus and the left frontal cortex. Interestingly, we observed that EPO immunoreactivity was also present in brain regions (i.e., right hippocampus, thalamus) remote from the left cerebral ventricle, although it was clearly and significantly higher in the brain regions near the site of injection. These data are very much in line with our previous finding that the process of VEGF gene expression after gene delivery was not limited to the left hippocampus, but extended to the contralateral hippocampus and thalamus (16).

In order to clarify this issue, vector expression in cerebrospinal fluid in similar experiments investigating leptin expression in brain tissue, as well as in the ependymal cells lining the ventricular system, has been hypothesized (28).

In the present study, the animals that received rAAV-LacZ (controls) showed very low immunopositivity for EPO in all the brain regions observed (Fig.s 1, 2, 3, 4A, 4C), while clear immunostaining for EPO was found in the left frontal cortex and left hippocampus of animals treated with VEGF<sub>165</sub> (Fig.s 1, 2, 3, 4B, 4D), suggesting that this isoform could, itself, act as an EPO synthesis inducing factor.

Furthermore, our data showed that EPO presents marked expression 18 days after rAAV-VEGF injection, predominantly in the pyramidal layers of the hippocampus (CA1 and CA3 areas). Obviously, further experiments are needed to test, in the same experimental model, the duration of EPO expression after rAAV-VEGF injection.

As mentioned above, we observed that EPO expression was most evident within the hippocampus and the frontal cortex. These are, in fact, regions vulnerable to ischemia (especially the hippocampus) and, although our experiments were carried out in the absence of hypoxic stimuli, we suggest that it is likely that VEGF, at least in this isoform and in physiological conditions, could act as a hypoxic-like signal for EPO production only in brain regions that are impaired in pathological

conditions of hypoxia (i.e., stroke) (29-31). In addition, the presence of specific EPO binding sites in these areas (32) further supports this idea suggesting that EPO may play a crucial role as a polyfunctional rather than as a monofunctional growth factor in the mammalian brain. Again, our observations agree with a large body of literature indicating that the hypoxic stimulus/HIF-1 $\alpha$  plays a crucial role in pathological conditions of reduced availability of oxygen through the mutual functional activation of VEGF and EPO (summarized in Fig. 5).

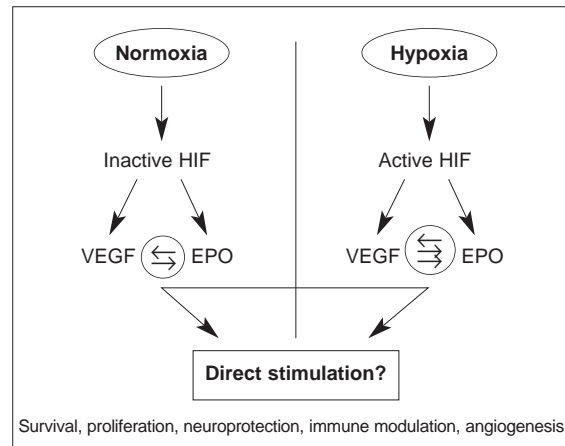


Figure 5 - Hypothetical scheme showing the interaction between VEGF and EPO in different environmental conditions.

In the light of these observations, it is possible to speculate that also in conditions where the hypoxic stimulus is not present, the increased EPO expression in the hippocampus and/or in other brain regions following VEGF treatment could be directly related to the effects of VEGF (Fig. 5).

In conclusion, our results show, for the first time, that in the absence of hypoxic stimuli, the production of VEGF obtained through gene transfer can be associated with EPO formation. These data, taken together, might help to clarify a new possible synergic mechanism that could explain possible protective properties of VEGF and a polyfunctional role of EPO in different brain pathological conditions.

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