

## Effects of Visual Experience on Vascular Endothelial Growth Factor Expression during the Postnatal Development of the Rat Visual Cortex

Harkaitz Bengoetxea<sup>1,2</sup>, Enrike G. Argandoña<sup>3</sup> and José V. Lafuente<sup>1</sup>

<sup>1</sup>Laboratory of Clinical and Experimental Neuroscience (LaNCE), Department of Neuroscience, Faculty of Medicine and Odontology, Basque Country University, Barrio Sarriena, 48940 Leioa, Spain, <sup>2</sup>Institute of Neuroscience CNR, via G. Moruzzi 1, 56100 Pisa, Italy and <sup>3</sup>Laboratory of Clinical and Experimental Neuroscience (LaNCE), Department of Nursing I, Basque Country University, Barrio Sarriena, 48940 Leioa, Spain

**The development of the cortical vascular network depends on functional maturation. External inputs are an essential requirement in the modeling of the visual cortex, mainly during the critical period, when the functional and structural properties of visual cortical neurons are particularly susceptible to alterations. Vascular endothelial growth factor (VEGF) is the major angiogenic factor, a key signal in the induction of vessel growth. Our study focused on the role of visual stimuli on the development of the vascular pattern correlated with VEGF levels. Vascular density and the expression of VEGF were examined in the primary visual cortex of rats reared under different visual environments (dark rearing, dark-rearing in conditions of enriched environment, enriched environment, and laboratory standard conditions) during postnatal development (before, during, and after the critical period). Our results show a restricted VEGF cellular expression to astroglial cells. Quantitative differences appeared during the critical period: higher vascular density and VEGF protein levels were found in the enriched environment group; both dark-reared groups showed lower vascular density and VEGF levels, which means that enriched environment without the physical exercise component does not exert effects in dark-reared rats.**

**Keywords:** angiogenesis, critical period, dark-rearing, enriched environment, vascularization

### Introduction

Experience modulates the postnatal development of the rat visual cortex in order to shape functional and anatomical cortical architecture. Visual experience increases the number and size of synapses per neuron (Sirevaag et al. 1988), as well as neuronal activity (Gilbert 1998; Yao and Dan 2005). These changes lead to an increase of metabolic demand (Black et al. 1990; Harrison et al. 2002) that is satisfied by the adaptive remodeling of the vascular network (Sirevaag et al. 1988; Argandoña and Lafuente 1996; Tieman et al. 2004).

Most of the cortical changes induced by experience occur during a critical period early in postnatal life (Berardi et al. 2000; Hensch 2005). This time window is specific for each sensorial cortex and when experience-mediated reorganization of the cortex finishes, sensory functions reach maturity. In rats, for the visual system, this period starts at the end of the third postnatal week, peaks during the fourth week, and declines from the end of the sixth week (Fagiolini et al. 1994).

The study of experience-induced modifications in brain morphology and function has been performed by conducting studies in a laboratory setting where environmental conditions can be modified (Markham and Greenough 2004). Among others, 2 opposite paradigms have been widely employed.

Monocular deprivation and dark rearing (DR) are 2 classic paradigms for the study of experience-dependent changes in neural connectivity. The absence of visual experience from birth delays normal maturation and maintains visual cortex in an immature state (Fagiolini et al. 1994; Argandoña et al. 2003; Gianfranceschi et al. 2003). On the other hand, the effects of environmental enrichment (EE) have been studied since the 1960s, when quantitative neuroanatomical and neurochemical changes were reported (Bennet et al. 1964). More recent works have indicated that EE and exercise have strong effects on the plasticity of neural connections, especially in the visual cortex (Cancedda et al. 2004). EE increases the expression of several factors such as nerve growth factor (Pham et al. 2002), brain-derived neurotrophic factor (BDNF) (Sale et al. 2004; Franklin et al. 2006), or neurotrophin-3 (Ickes et al. 2000). The increase in neuronal activity induced by environmental stimuli triggers a series of important events for visual cortical plasticity, including an increase of the vascular network or an acceleration of visual system development at behavioral, electrophysiological, and molecular levels (Cancedda et al. 2004; Sale et al. 2004).

Cortical angioarchitecture correlates to metabolic demand from neuronal function. Brain vascularization occurs primarily by angiogenesis and is driven in part as a response to metabolic demands of the expanding nervous system. Adaptive changes during development are mediated by several cytokines such as epidermal growth factor and fibroblast growth factor, and are promoted by the vascular endothelial growth factor (VEGF) in response to hypoxia. VEGF is the major angiogenic factor whose expression has been shown to correlate with angiogenesis in the developing brain (Neufeld et al. 1999; Ferrara et al. 2003) and in pathological processes such as ischemia (Marti et al. 2000), brain injury (Lafuente et al. 2006), or tumor growth (Plate et al. 1992). In addition, recent works indicate that the role of VEGF in nervous tissue is even more extensive, because it exerts neuroprotective and neurotrophic effects (Rosenstein and Krum 2004; Storkebaum et al. 2004). VEGF-A belongs to the VEGF family that includes VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (Ferrara 2004). Alternative exon splicing of VEGF-A results in at least 6 isoforms, whose relative levels differed from organ to organ: VEGF<sub>164</sub> is the main in brain reaching 80% of the total amount, followed by 120 and 188 (Ng et al. 2001).

The relationship between different visual environments and vascular density has been previously described. Reports have detailed how deprivation of visual stimulation delays the maturation of the microvascular pattern and diminishes vascular density (Argandoña and Lafuente 1996, 2000). On the other hand, visual stimulation in an EE has been reported to increase the vascular density, as animals have larger and more

elaborately branched capillaries in the visual cortex (Black et al. 1987; Sirevaag et al. 1988; Markham and Greenough 2004; Argandoña et al. 2005). The aim of the present work was to correlate the expression and protein levels of VEGF during development with changes in the vascular network of rats reared under different visual environments.

## Experimental procedures

### Animals and Housing

Four series of pregnant Sprague-Dawley rats were raised in different rearing conditions:

1. *Standard laboratory environment (C)*: rats raised in a standard laboratory cage (500 mm × 280 mm × 140 mm) with 12-h light/dark cycle were sampled as controls.
2. *DR*: rats were raised in total darkness. Pregnant rats were placed in a dark room at the beginning of pregnancy. Litters were born in complete darkness in standard laboratory cages.
3. *EE*: rats were raised in a large cage (720 mm × 550 mm × 300 mm) furnished with colorful toys and differently shaped objects (shelters, tunnels) that were changed every 2 days (with 12-h light/dark cycle).
4. *DR-EE*: rats were raised in total darkness in enriched environment conditions.

The following age groups along the postnatal development were investigated and used in these experiments: P14, P21, P28, P35, P42, P49, P56, and P63. On the basis of the time window of postnatal development called critical period described and characterized in the introduction, age groups were grouped into 3 periods: precritical period (P14 and P21), critical period (P28, P35, and P42), and postcritical period (P49, P56, and P63). All animals had access to food and water ad libitum. Every effort was made to minimize animal suffering and to reduce the number of animals used. All procedures in the present study were performed in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC).

### Fixation and Tissue Processing

Rats were anesthetized with 6% chloral hydrate (performed under dim red light for DR and DR-EE groups), transcardially perfused with a fixative containing 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), and stored overnight at 4 °C in fresh fixative. Following day, samples to histochemical analysis were removed coronally with a Rodent Brain Matrix (Electron Microscopic Sciences, Hatfield), rinsed in 0.1 M PBS for 4 h, embedded in paraffin, serially cut with a microtome into sections of 4 μm, and mounted on slides coated with 3-aminopropyltriethoxysilane. Samples to immunohistochemical analysis were stored in 30% sucrose in 0.1 M PBS until the tissues sank, cut using a cryostat microtome in 60-μm sections, and stored in free-floating chambers in 0.1 M PBS.

### Histochemistry and Histology

Paraffin was removed from the tissue through xylene immersion and was rehydrated. Endogenous peroxidase activity was blocked by incubation in 4% H<sub>2</sub>O<sub>2</sub>/methanol for 20 min. Sections were washed in 0.1 M Tris buffer at pH 7.4 and incubated overnight with biotinylated lectin from *Lycopersicon esculentum* (tomato lectin LEA, Ref: L-0651, working dilution 5

μg/ml, Sigma-Aldrich, Spain) at 4 °C. Sections were rinsed with 0.1 M Tris buffer and incubated with avidin-biotin-peroxidase complex (Elite ABC kit, Vector Laboratories, Burlingame, CA). Reaction product was detected using 3,3'-diaminobenzidine (DAB 0.25 mg/ml) and hydrogen peroxide solution (0.01%). Slides were lightly counterstained with hematoxylin, dehydrated, and covered. Also included in each staining run were negative controls in which the lectin was omitted. Parallel sections were processed for hematoxylin-eosin for the identification and localization of the primary visual cortex.

### Immunohistochemistry

Free-floating sections were washed in 0.1 M PBS before inhibition of endogenous peroxidase activity using 4% H<sub>2</sub>O<sub>2</sub> in 0.1 M PBS. Sections were washed in 0.1 M PBS prior to the incubation with blocking solution (5% bovine serum albumin [BSA] in 0.1 M PBS) for 1 h. Polyclonal anti-VEGF primary antibody (Ref: sc-152, working dilution 1:200, Santa Cruz Biotechnology Inc, Germany) was added overnight at 4 °C in 5% BSA in 0.1 M PBS containing 0.3% Triton X-100. Three washes in 0.1 M PBS were followed and a biotinylated anti-rabbit secondary antibody (Elite ABC kit, Vector Laboratories) was added for 2 h at room temperature. After rinsing, the sections were incubated with avidin-biotin-peroxidase complex (Elite ABC kit, Vector Laboratories). Reaction product was detected using DAB (0.25 mg/ml) and hydrogen peroxide solution (0.01%). Sections were rinsed, mounted on gelatin-coated slides, dehydrated, and covered. Also included in each staining run were negative controls in which the primary antibody was omitted.

### Double Immunofluorescence Labeling

To assess the cellular localization of VEGF, we carried out double immunofluorescence on free-floating sections, using different markers: NeuN for neurons, glial fibrillary acidic protein (GFAP) for astrocytes, and endothelial barrier antigen (EBA) for endothelial cells. Sections were washed in 0.1 M PBS and were incubated with blocking solution (5% BSA in 0.1 M PBS) for 1 h followed by overnight incubation with a cocktail of primary antibodies in 5% BSA in 0.1 M PBS containing 0.3% Triton X-100. For neuronal, astroglial, and endothelial localization of VEGF, the antibodies used were polyclonal rabbit anti-VEGF (Ref: sc-152, working dilution 1:200, Santa Cruz Biotechnology, Inc., Germany) and monoclonal mouse anti-NeuN (Ref: MAB377, working dilution 1:100, Chemicon International, Inc., Spain) or monoclonal mouse anti-GFAP (Ref: G-3893, working dilution 1:400, Sigma-Aldrich) or monoclonal mouse anti-EBA (Ref: SMI-71, working dilution 1:2000, Sternberger Monoclonals, Inc., Lutherville, Maryland). After rinsing, sections were incubated for 2 h with fluorescein isothiocyanate conjugate anti-mouse IgG (Ref: F-9137, working dilution 1:100, Sigma-Aldrich) and TRITC conjugate anti-rabbit IgG (Ref: T-6778, working dilution 1:100, Sigma-Aldrich) in 5% BSA in 0.1 M PBS containing 0.3% Triton X-100. Sections were rinsed, mounted on gelatin-coated slides, and cover-slipped in aqueous medium. Also included in each staining run were negative controls in which the primary antibody was omitted. Images were acquired for confocal fluorescence microscopy with an Olympus Fluoview FV500 confocal microscope using sequential acquisition to avoid overlapping of fluorescence emission spectra.

### **Sample Preparation for Western Blotting and Enzyme-Linked Immunoassay**

Primary visual cortices from both hemispheres were excised from decapitated anesthetized rats. The tissue was manually homogenized at 4 °C with 200 µl of homogenization lysis buffer containing 10 mM PB (pH 7.4), 5 mM ethylenediaminetetraacetic acid, 5 mM ethyleneglycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 1 mM dithiothreitol, and the protease inhibitor cocktail (Ref: P-8340, Sigma-Aldrich). The homogenate was incubated for 30 min on ice, centrifuged at 13 000 rpm = 15 500g for 15 min at 4 °C, and the supernatant was collected and used as soluble protein extract. Protein concentrations were determined using Bio-Rad Protein Assay reagent (Ref: 500-0006, Bio-Rad Laboratories SA, Spain), with BSA as standard.

### **Western Blot Analysis**

Protein (50 µg) was boiled at 100 °C in sodium dodecyl sulfate sample buffer for 5 min, loaded onto a 12.5% polyacrylamide gel, and separated by electrophoresis (125 V for 90 min; Mini-Protean 3 Electrophoresis Cell, Bio-Rad Laboratories SA). The gel was blotted onto a polyvinylidene difluoride transfer membrane (Ref: RPN303F, Amersham Biosciences Europe, Spain) with a semidry blotter (Trans-Blot SD Semy-Dry Transfer Cell, Bio-Rad Laboratories SA) for 30 min at 15 V. This was then blocked for 2 h in 5% nonfat milk in TBS-T buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.1% Tween-20). Afterward the membranes were incubated at 4 °C with the primary anti-VEGF antibody (Ref: sc-152, working dilution 1:200, Santa Cruz Biotechnology, Inc., Germany). After washes in TBS-T buffer, the blots were incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies (Ref: A-6154, working dilution 1:10 000, Sigma-Aldrich). Following washing, the immunoblots were detected using an enhanced chemiluminescent Western blotting detection chemiluminescent reagent (Ref: RPN2106, Amersham Biosciences Europe). To ensure equal loading of samples, membranes used to measure VEGF protein expression were stripped in stripping buffer (100 mM glycine, pH 2.3) at 55 °C for 30 min and then probed with anti-actin antibody (Ref: A-2066, working dilution 1:500, Sigma-Aldrich).

### **VEGF Immunoassay**

The lysates obtained from both visual cortices (described above) were used to quantify the amount of VEGF using a commercially available enzyme-linked immunosorbent assay (ELISA) (Ref: MMV00 Quantikine M, R&D Systems, Germany). In brief, 50 µl of lysate was applied in duplicate to each well of a 96-well microtiter plate and incubated for 2 h at room temperature. The plate was washed 5 times with wash buffer. Anti-VEGF antibody (100 µl) was added to each well, mixed, and allowed to incubate at room temperature for 2 h. The plate was washed 5 times followed by incubation with substrate solution for 30 min at room temperature protected from light. Then the stop solution was added and the plate was read in an automatic ELISA reader (Stat Fax 2100, Fisher Bioblock Scientific, France) at 450 nm (correction wavelength set at 540 nm). A total of 128 animals aged between P14 and P63 were employed ( $n = 4$  per age and condition). Two independent values were determined from each sample and the mean value per animal was calculated for statistical analysis.

### **Morphometric Procedures**

For vascular density measurement, a blind morphometric study was performed, where the individual who measured the sections did not know the features of each case. All sections were incubated for histochemistry (LEA) and positive density was estimated by counting the number of positive vascular profiles per area of primary visual cortex. This was selected with the aid of the Paxinos and Watson atlas (Paxinos and Watson 1998). Layers were differentiated using parallel sections stained with hematoxylin-eosin as a reference. To estimate the number of profiles per area, the number of positive elements present in an area delimited by a grid fixed in the eyepiece was counted at 20× optical magnification, excluding those intersected by both the  $x$  and  $y$  axes. The grid was a square 250 µm in length (total surface was 62 500 µm<sup>2</sup>) and was randomly placed between cortical layers III and V, ensuring that layer IV was included. A total of 256 animals aged between P14 and P63 were employed ( $n = 8$  per age and condition). Measurements of each slice of the cortex were taken in both hemispheres for each of the 10 slices taken per animal (i.e., 60 fields per animal) and the mean value per animal was calculated for statistical analysis.

### **Image Analysis**

Autoradiographic films were quantified by digitizing the film images with an Epson Perfection 4990 Photo Scanner and processing them with computer-based Mercator image analysis software (Explora Nova, La Rochelle, France). The relative optical density (ROD) was measured for VEGF and actin and values were expressed as densitometric units relative to the standard (VEGF ROD divided by actin ROD). A total of 128 animals aged between P14 and P63 were employed ( $n = 4$  per age and condition). Two independent values were determined from each sample and the mean value per animal was calculated for statistical analysis.

### **Statistical Analysis**

All statistical analysis were performed using SPSS statistical software (version 13.0 for Mac, SPSS, Inc., Chicago, Illinois). Prior to analysis, data were examined for normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variances using Levene's test. Firstly, a 1-way ANOVA analysis was performed to corroborate that data from animals of different age pooled in the same age group do not differ. Post hoc tests used the Bonferroni correction for equal variances or Tamhane's T2 correction for unequal variances. The effects of age period, experimental condition, and their interaction were determined by 2-way ANOVA with post hoc analysis (Bonferroni or Tamhane's T2 corrections were used when appropriate), and to deeper explore the effects of experimental conditions, differences between them within age periods were evaluated using the 1-way ANOVA analysis with the previously mentioned post hoc studies. Data are described as mean ± SEM. Significance was declared at  $P < 0.05$ .

## **Results**

### **LEA Lectin Expression**

LEA histochemistry showed a consistent staining of the vascular network, which was homogeneously present on all cortical layers, from I to VI; however, vessels were more

densely packed on layer IV (Fig. 1). Within the cerebral cortex, there was no regional variability in labeling, and strong staining of all vessels was observed. The same expression pattern and distribution were present for all ages and experimental conditions.

### Quantitative Results

One-way ANOVA with Bonferroni correction showed no differences between the ages pooled into each of the 3 periods in controls. In contrast, 1-way ANOVA with Tamhane's T2 correction showed significant intraperiod differences within the precritical period in all experimental groups (DR, EE, DR-EE). On other hand, whereas both light reared groups (controls and EE) showed significant differences between precritical and critical periods, dark-reared groups (DR and DR-EE) did not.

Two-way ANOVA revealed a significant interaction between age and experimental condition ( $P < 0.0001$ ), as well as age ( $P < 0.0001$ ) and condition effects ( $P < 0.0001$ ). Therefore, a 1-way ANOVA analysis with corresponding post hoc tests was done to study the differences between experimental conditions at each age period.

### Dark Rearing versus Control

DR rats showed lower vascular density than C rats during development. Vascular density in the DR group was 7.54% lower than C in the precritical period. In the critical period, this difference was 8.28% and in the postcritical period was 9.18%. In the precritical period, statistical analysis showed that the difference was not significant, whereas in the critical and postcritical periods, the differences were significant (Fig. 2, Table 1).

### Dark Rearing in Conditions of Enriched Environment versus Control

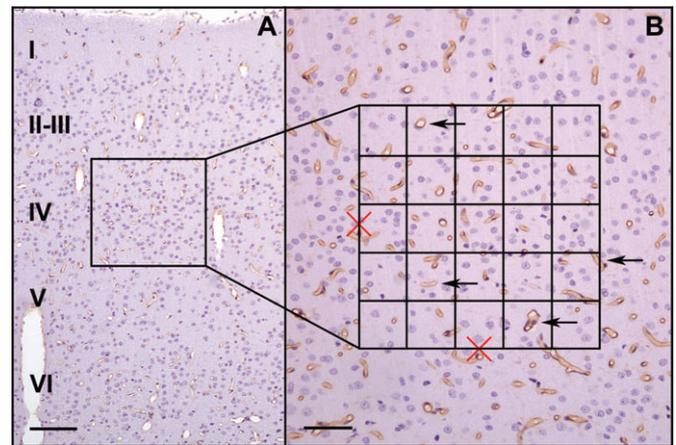
DR-EE rats showed lower vessel density per area in the 3 periods examined. During the precritical period the difference was 10.35% and statistical analysis showed that the difference was not significant. In the critical period, this difference decreased to 7.52% but was statistically significant. In the postcritical period, the difference was 11.50% and was statistically significant (Fig. 2, Table 1).

### Enriched Environment versus Control

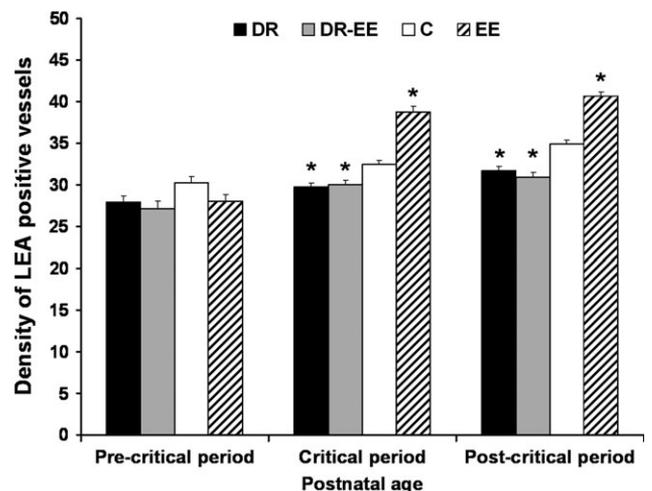
Although the vascular profiles per area were lower in EE rats in the precritical period, during development the situation reverted and the density was higher in the EE group in the critical and postcritical periods. The difference was 7.37% in the precritical period and was not statistically significant. In the critical period, EE rats showed higher vascular density than C rats and the difference was 19.28%. And in the postcritical period, the density was 16.38% higher in EE rats. In critical and postcritical periods differences between the 2 groups were significant (Fig. 2, Table 1).

### Dark Rearing in Conditions of Enriched Environment versus Dark Rearing

Vascular density during postnatal development showed similar values in the 2 experimental groups reared in darkness. The differences were minimal in the 3 periods examined and were statistically nonsignificant. The density was 3.04% lower in DR-EE group in the precritical period, 0.84% higher in the critical period, and 2.55% lower in the postcritical period (Fig. 2, Table 1).



**Figure 1.** Photographs of the primary visual cortex stained by LEA lectin histochemistry and counterstained by hematoxylin. (A) Histochemical vascular profile staining throughout the rat primary visual cortex in layers I–VI. Scale bar = 100  $\mu\text{m}$ . (B) Vascular density was measured by counting the number of positive vascular profiles per area delimited by an overlying grid, excluding those intersected by both the x and y axes (some of the profiles are marked with arrows). Grid side = 50  $\mu\text{m}$ . Grid area = 62 500  $\mu\text{m}^2$ . Scale bar = 50  $\mu\text{m}$ .



**Figure 2.** Comparison of average measurements between DR, DR-EE, EE, and C groups in each of the periods considered. Horizontal axes show the age of the animals. Vertical axes show number of LEA-positive vascular profiles per 62 500  $\mu\text{m}^2$  of primary visual cortex. Mean  $\pm$  SEM. \*Experimental group versus control significance ( $P < 0.05$ ) (1-way ANOVA test with post hoc correction).

### Cellular Expression of VEGF

Immunohistochemistry against VEGF was performed to visualize the cellular expression (Fig. 3A–C). Immunohistochemical staining demonstrated that only the astroglial structures expressed this growth factor in the whole of the primary visual cortex (Fig. 3A). We observed VEGF-positive glial cells (Fig. 3B) and strong staining of glial cell fibers surrounding large blood vessels (Fig. 3C). The same cellular expression pattern was present for all ages and experimental conditions examined. Next, to better assess whether VEGF expression of the visual cortex was restricted to reactive astroglial cells, we used double immunofluorescence staining for VEGF and glial fibrillary acidic protein (GFAP; a marker protein for astroglial cells). Double labeling for GFAP (Fig. 3D) and VEGF (Fig. 3E)

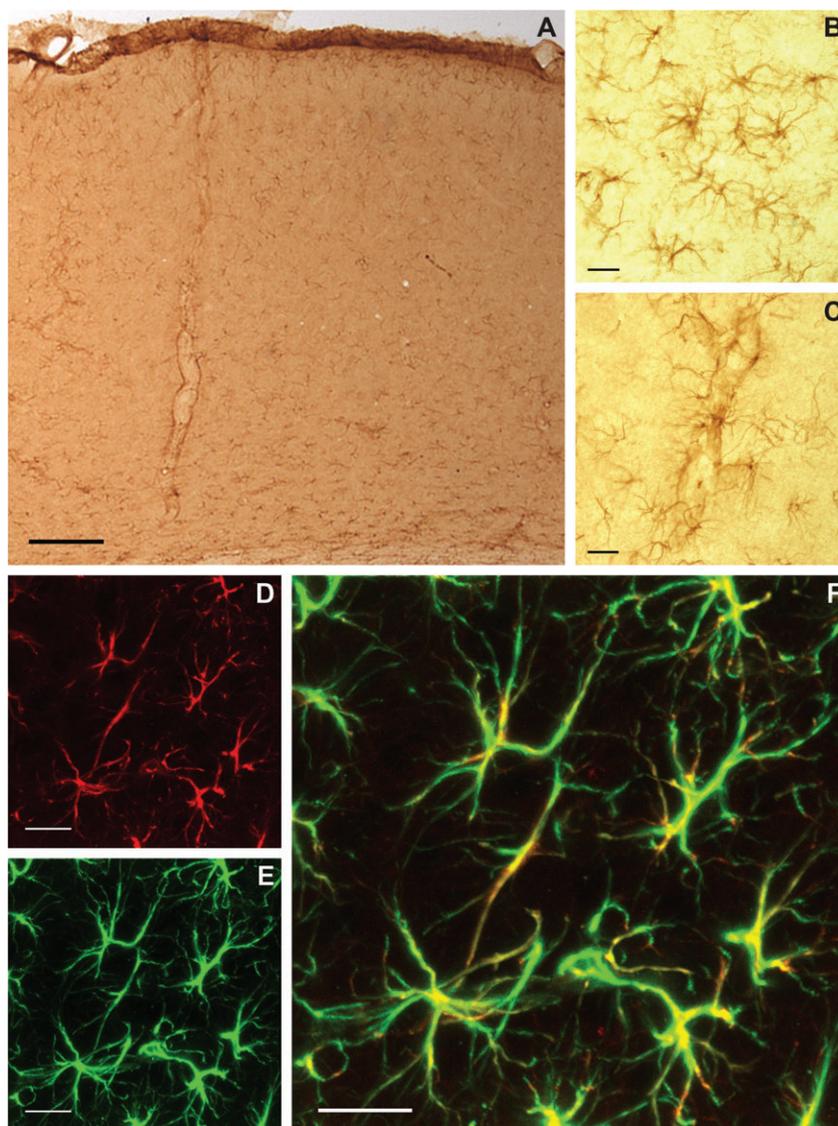
**Table 1**

LEA-positive vessels density at various ages of postnatal development divided into 3 periods (precritical period, critical period, postcritical period) for C, DR, DR-EE, and EE rats (mean number of LEA-positive vessels per 62 500  $\mu\text{m}^2$  of primary visual cortex  $\pm$  SEM; percentage of difference and statistical significance, *P* value; *n* = 16 for each of the experimental and control condition in precritical period; *n* = 24 in critical period; *n* = 24 in postcritical period) (1-way ANOVA test with post hoc correction)

Age (period)	C	DR	DR-EE	EE
Precritical	30.23 $\pm$ 0.79	27.95 $\pm$ 0.72	27.10 $\pm$ 0.96	28.00 $\pm$ 0.88
Critical	32.47 $\pm$ 0.50	29.78 $\pm$ 0.48	30.03 $\pm$ 0.56	38.73 $\pm$ 0.71
Postcritical	34.93 $\pm$ 0.46	31.72 $\pm$ 0.47	30.91 $\pm$ 0.58	40.65 $\pm$ 0.51

Age (period)	C versus DR		C versus DR-EE		C versus EE		DR versus DR-EE	
	% Diff.	<i>P</i>	% Diff.	<i>P</i>	% Diff.	<i>P</i>	% Diff.	<i>P</i>
Precritical	-7.54	0.330	-10.35	0.054	-7.37	0.310	-3.04	0.510
Critical	-8.28	0.001	-7.52	0.007	19.28	0.000	0.84	0.740
Postcritical	-9.18	0.000	-11.50	0.000	16.38	0.000	-2.55	0.170



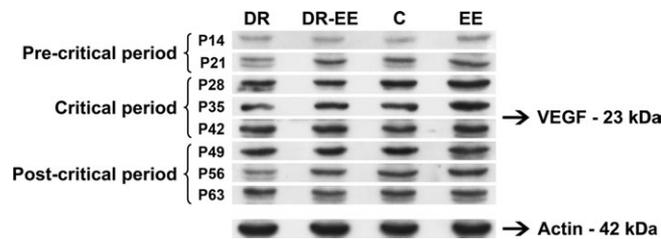
**Figure 3.** Photographs to assess the cellular expression of VEGF. (A) VEGF immunohistochemistry of the primary visual cortex. VEGF labeling is restricted to astrocytes throughout layers I–VI. Scale bar = 75  $\mu\text{m}$ . (B) VEGF-positive astrocytes. Scale bar = 20  $\mu\text{m}$ . (C) VEGF-positive astrocyte fibers surrounding a large blood vessel. Scale bar = 25  $\mu\text{m}$ . (D–F) Confocal images of double immunofluorescence of VEGF (red) and GFAP (green) to show the localization in astrocytes of VEGF expression. Red- and green-positive signals display yellow color in the merged figure (F). Scale bar = 20  $\mu\text{m}$ .

clearly confirmed that almost all reactive astrocytes were VEGF positive, indicated by a yellow color in the merged confocal images (Fig. 3F). In contrast, no VEGF positivity was found in neurons (NeuN positive) or in endothelial cells (EBA positive) (not shown).

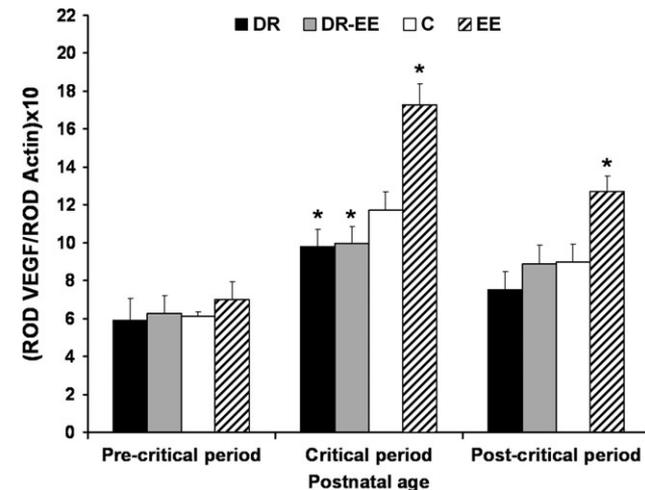
#### VEGF Western Blot

Immunoblot analysis revealed that immunoreactive bands corresponding to VEGF displayed a stronger expression in the EE group than in the C group (Fig. 4) in the periods examined. Quantification of the bands revealed a significantly higher expression of VEGF in rats reared under EE conditions (Fig. 5, Table 2).

One-way ANOVA with Tamhane's correction showed no differences between the ages pooled into the precritical periods and postcritical periods in all groups. In contrast, significant intraperiod differences were recorded within the critical period in EE rats, differences that were located between the fourth and the fifth weeks ( $P = 0.001$ ). On other hand, significant differences between precritical and critical periods were recorded in all experimental groups.



**Figure 4.** Comparison of VEGF protein level of the primary visual cortex of rats reared under different visual environments (DR, DR-EE, EE, C) during the postnatal development. Animal primary visual cortex was homogenized and analyzed by Western blot using anti-VEGF polyclonal antibody. Actin protein was used to control the protein loaded. Molecular weight for VEGF is 23 kDa and the molecular weight for actin is 42 kDa.



**Figure 5.** Comparison of average measurements between DR, DR-EE, EE, and C groups in each of the periods considered. Horizontal axes show the age of the animals. Vertical axes show ROD VEGF over ROD actin (10 $\times$ ). Mean  $\pm$  SEM. \*Experimental group versus control significance ( $P < 0.05$ ) (1-way ANOVA test with post hoc correction).

Two-way ANOVA did not reveal a significant interaction between age and experimental condition ( $P = 0.069$ ), but it revealed both age ( $P < 0.001$ ) and condition ( $P < 0.001$ ) significant effects. Therefore, a 1-way ANOVA analysis with corresponding post hoc tests (as appropriate) was done to study the differences between experimental conditions at each age period.

#### Dark Rearing versus Control

Semiquantitative analysis of VEGF revealed that in the precritical period, DR rats showed lower values than C with a difference of 3.10%. The critical period showed a significant difference between both groups, of 16.21%. In the postcritical period, the difference decrease to 16.01% and was statistically not significant.

#### Dark Rearing in Conditions of Enriched Environment versus Control

DR-EE rats showed lower optical density values than C in the 3 periods examined. The difference was minimal in the precritical period (2.61%). This difference increased to 14.93% in the critical period and decreased to 1.33% in the postcritical period where the values for DR-EE rats are higher than C rats. During the postnatal development, the differences were only statistically significant for the critical period.

#### Enriched Environment versus Control

At all periods examined, the values were higher in EE groups with statistically significant differences in the critical and postcritical periods. In the precritical period, the difference was 14.22%. The difference increased in the critical period and was 47.53%. And in the postcritical period was 41.16%.

#### Dark Rearing in Conditions of Enriched Environment versus Dark Rearing

During postnatal development, the 2 experimental groups reared in darkness showed similar values, and differences were minimal and statistically nonsignificant. In the precritical period, the ROD value was 5.90% lower in the DR group. In the critical period, the difference decreased to 1.53% and DR-EE rats showed higher values. And in the postcritical period, the difference was 17.48% and the optical density was higher in DR-EE rats.

#### VEGF Enzyme-Linked Immunoassay

We analyzed the concentration of VEGF with respect to the total amount of protein in the visual cortex. The results showed a similar expression pattern to the Western blot analysis (Fig. 6, Table 3).

One-way ANOVA with Tamhane's T2 correction showed no differences between the ages pooled into the precritical periods in all groups, and significant differences between critical and postcritical periods. In contrast, it showed significant intraperiod differences within the critical period in EE rats between the fourth and the fifth weeks ( $P = 0.001$ ).

Two-way ANOVA did not reveal a significant interaction between age and experimental condition ( $P = 0.193$ ), but it revealed both age ( $P < 0.014$ ) and condition ( $P < 0.001$ ) significant effects. Therefore, a 1-way ANOVA analysis with corresponding post hoc tests (as appropriate) was done to study the differences between experimental conditions at each age period.

**Table 2**

Western blot VEGF: relative units of ROD VEGF over ROD actin ( $10\times$ ) at various ages of postnatal development divided into 3 periods (precritical period, critical period, postcritical period) for C, DR, DR-EE, and EE rats (mean value of ROD  $10\times \pm$  SEM; percentage of difference and statistical significance,  $P$  value;  $n = 8$  for each of the experimental and control condition in precritical period;  $n = 12$  in critical period;  $n = 12$  in postcritical period) (1-way ANOVA test with post hoc correction)

Age (period)	C	DR	DR-EE	EE
Precritical	6.12 $\pm$ 0.23	5.93 $\pm$ 1.13	6.28 $\pm$ 0.93	6.99 $\pm$ 0.95
Critical	11.72 $\pm$ 0.96	9.82 $\pm$ 0.90	9.97 $\pm$ 0.90	17.29 $\pm$ 1.10
Postcritical	8.99 $\pm$ 0.92	7.55 $\pm$ 0.92	8.87 $\pm$ 1.01	12.69 $\pm$ 0.84

Age (period)	C versus DR		C versus DR-EE		C versus EE		DR versus DR-EE	
	% Diff.	$P$	% Diff.	$P$	% Diff.	$P$	% Diff.	$P$
Precritical	-3.10	0.218	2.61	0.887	14.22	0.507	5.90	0.890
Critical	-16.21	0.026	-14.93	0.009	47.53	0.003	1.53	0.838
Postcritical	-16.01	0.084	-1.33	0.909	41.16	0.022	17.48	0.222

### Dark Rearing versus Control

VEGF amount was higher in the C group in the 3 periods and differences were statistically significant for the critical and postcritical periods. VEGF concentration was 12.14% lower in DR rats in the precritical period. The difference increased to 39.59% in the critical period and in postcritical period this difference was 43.34%.

### Dark Rearing in Conditions of Enriched Environment versus Control

The comparison between DR-EE and C rats showed statistical differences only in the critical period where the highest difference between VEGF concentrations was found. In the precritical period DR-EE rats showed 5.58% higher values. In the critical period, the difference reverted and the value was 46.68% lower in the DR-EE group. And in the postcritical period, the difference decreased to 22.11% and DR-EE showed less concentration.

### Enriched Environment versus Control

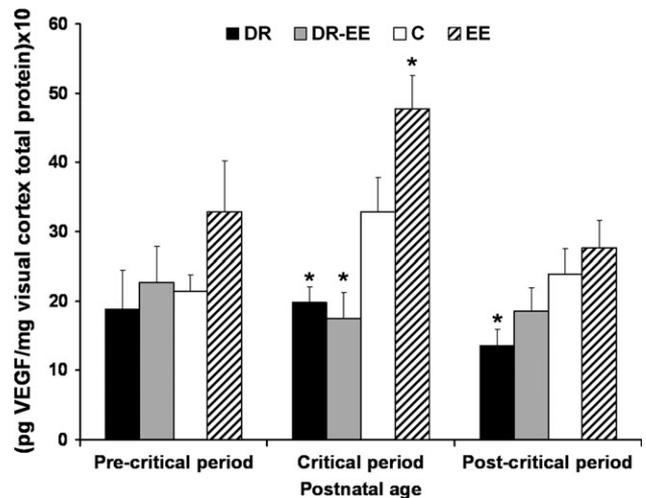
At all periods examined, the values were higher in the EE group, with statistically significant differences in the critical period and nonsignificant differences in the pre- and postcritical periods. In the first period examined, the difference was 52.82%. The difference varied slightly in the critical period and was 45.16%. In the postcritical period, the difference decreased to 15.91%.

### Dark Rearing in Conditions of Enriched Environment versus Dark Rearing

The comparison between both experimental groups reared in darkness showed statistically nonsignificant differences during the postnatal development. The difference was 20.18% in the precritical period, where DR-EE rats showed higher VEGF concentration. In the critical period, DR group showed higher values and the difference was 11.73%. And in the postcritical period it was the DR-EE group that showed higher VEGF concentration, the difference being 37.47%.

## Discussion

The postnatal development of cortical vascularization is established mainly by active angiogenesis and it supplies adapted metabolic support to neurons. Our data show a correlation between the complexity of visual experience, the density of capillary networks, and expression of VEGF. This finding could lead us to postulate that VEGF plays a relevant



**Figure 6.** Comparison of average measurements between DR, DR-EE, EE, and C groups at each of the periods considered. Horizontal axes show the age of the animals. Vertical axes show pg VEGF of the primary visual cortex over mg primary visual cortex total protein ( $10\times$ ). Mean  $\pm$  SEM. \*Experimental group versus control significance ( $P < 0.05$ ) (1-way ANOVA test with post hoc correction).

role on the vascular response to the increment of neural activity promoted by enhanced visual stimulation. These changes are parallel to the neuronal development of the visual cortex, as they correlate to the critical period that in rats is located between the third and the sixth postnatal weeks. Another main finding of our work is that EE without the physical exercise component does not compensate for the lack of visual experience promoted by DR on visual cortical vascular network development, suggesting that this rearing paradigm has mainly visual effects.

### Vascular Density

During the postnatal development of brain cortical vasculature, a dense capillary network dependent on the local function is established (Cavaglia et al. 2001; Fonta and Imbert 2002). In standard conditions, it is completed by the end of the fourth postnatal week and thereafter it remains essentially quiescent except for specific pathologies or modifications in the complexity of the environment like an enhance or deprivation of visual stimulus. The quantitative effects of both visual deprivation and increased visual experience during postnatal development have already been reported in previous works, and interestingly, analyses of a nonsensory area like the

**Table 3**

ELISA VEGF: pg VEGF of the primary visual cortex over mg primary visual cortex total protein ( $10\times$ ) at various ages of postnatal development divided into 3 periods (precritical period, critical period, postcritical period) for C, DR, DR-EE, and EE rats (mean value of ROD  $10\times \pm$  SEM; percentage of difference and statistical significance,  $P$  value;  $n = 8$  for each of the experimental and control condition in precritical period;  $n = 12$  in critical period;  $n = 12$  in postcritical period) (1-way ANOVA test with post hoc correction)

Age (period)	C		DR		DR-EE		EE	
Precritical	21.49 $\pm$ 2.31		18.88 $\pm$ 5.59		22.69 $\pm$ 5.10		32.84 $\pm$ 7.42	
Critical	32.86 $\pm$ 4.92		19.85 $\pm$ 2.14		17.52 $\pm$ 3.73		47.70 $\pm$ 4.79	
Postcritical	23.88 $\pm$ 3.67		13.53 $\pm$ 2.39		18.60 $\pm$ 3.30		27.68 $\pm$ 3.88	

Age (period)	C versus DR		C versus DR-EE		C versus EE		DR versus DR-EE	
	% Diff.	$P$	% Diff.	$P$	% Diff.	$P$	% Diff.	$P$
Precritical	-12.14	0.655	5.58	0.835	52.82	0.215	20.18	0.630
Critical	-39.59	0.035	-46.68	0.045	45.16	0.043	-11.73	0.570
Postcritical	-43.34	0.041	-22.11	0.347	15.91	0.485	37.47	0.223

retrosplenial cortex failed to show similar effects, suggesting that visual experience plasticity was specific to the visual cortex in the absence of other stimuli such as exercise (Black et al. 1990; Argandoña and Lafuente 1996; Swain et al. 2003). Our present work agrees with the above mentioned as significant differences were obtained in the critical period, when vascular density was higher in EE animals without physical exercise and lower in DR animals (Argandoña et al. 2005). However, our results show that the effects of an EE without physical exercise do not appear in DR-EE rats. This finding leads us to postulate that the effects of enhanced experience without exercise are mostly visual. Some authors have pointed that EE accelerates the beginning of the critical period (Cancedda et al. 2004). In previous works we have also reported that this acceleration is also correlated in vascular density (Argandoña et al. 2005); moreover, in this work we saw a lack of difference between precritical and critical periods in the DR and DR-EE groups showing that the lack of visual experience inhibits the beginning of the critical period with parallel vascular effects.

### VEGF Expression

We have examined the cellular expression of VEGF and VEGF protein levels in order to correlate morphological changes in cortical vasculature with the expression of the main angiogenic factor. The potential role of VEGF in rat postnatal brain development has been studied (Ogunshola et al. 2000; Adris et al. 2005) as well as the role in human postnatal brain development (Virgintino et al. 2003). However, the influence of different visual environments has yet to be studied in detail. Our results show a differential VEGF level throughout development that varies with visual environment and age, but the cellular expression of VEGF remains restricted to astroglial cells in all ages and experimental groups. In a number of periods described here, VEGF remains at similar levels in the precritical period in all groups, although it was higher in EE group. The expression changes in the critical period and differences were significant between the control and experimental groups. EE rats showed the highest values. This finding is in accordance with a previous study, which reported that analysis of VEGF expression by Western blot in the rat brain cortex shows high levels of protein at P33 in hypoxic rats compared with normoxic animals (Ogunshola et al. 2000). In both cases, EE and hypoxia, insufficient vascular supply, and the resultant reduction in tissue oxygen level often lead to greater VEGF secretion via hypoxia-inducible factor (HIF) (Shweiki

et al. 1992) and the consequent neovascularization in order to satisfy the needs of the tissue. And in the postcritical period, VEGF levels return to the values previously reported for the precritical period. To the contrary, other works indicate that VEGF messenger RNA levels increased 2-fold at P15 compared with P5 and remained at this level in the adult brain. Ng et al. (2001) showed that total VEGF and VEGF164 do not present changes during postnatal brain development. In DR and DR-EE rats, VEGF level remains at the same values during postnatal development, including the critical period, which is in accordance with the hypothesis that visual deprivation such as DR from birth delays the normal development of the visual cortex, which remains in an immature state, retaining cortical plasticity, and resulting in a prolongation of the critical period.

### Environmental Enrichment

This is the first study to correlate the developmental distribution pattern of microvasculature and VEGF expression in the rat visual cortex during development in DR-EE rats. Our results indicate that our experimental paradigm of enrichment without physical exercise is directly correlated with visual stimulation. The parameters defined for study in the microvascular bed of the visual cortex show no statistical differences between the 2 DR groups. The results we observed in the DR-EE group are particularly important and the characterization of the vascular pattern presented here is complementary to prior research that described vascularization throughout development. We show that 1) there are no vascular density differences between DR and DR-EE rats during postnatal development; 2) the ROD obtained by Western blot analysis showed that both groups exhibit similar VEGF expression; and 3) the VEGF fraction of the total protein amount of the visual cortex is similar in both groups.

There was only 1 previous study that has used DR-EE paradigm, which concluded that EE reverses the effect of DR (Bartoletti et al. 2004). Although our results are contradictory to this work, there was 1 great difference between the DR-EE groups of both studies: physical exercise. An important question about the so-called EE is physical exercise (Will et al. 2004). Including a running wheel as a component of the EE increases physical exercise and the possibilities for motor activity are enhanced. However, when the EE has been dissociated from exercise, it has demonstrated that exercise per se may induce behavioral and cerebral modifications. A recent study showed that exercise improves learning after kainic acid-induced hippocampal neurodegeneration in

association with an increase of BDNF but EE does not present the same effects (Gobbo and O'Mara 2005). In some studies, it has been observed that exercise takes part in modulating several cerebral factors in the plasticity of the brain, increasing the levels of BDNF (Cotman and Berchtold 2002) and the captation of insulin-like growth factor. These factors imply better health of the animal, greater protection against neuronal death (Carro et al. 2001), and increased neuronal proliferation (Cao et al. 2004; Matsumori et al. 2006).

Another point of interest is the coordinated response in the vascular and nervous systems; an overlap of growth factors that affect both the vascular and the nervous systems has been recently described (Raab and Plate 2007). Neurotrophins are the principal effectors of cortical plasticity, being responsible for changes produced by experience in the visual cortex (Caleo and Maffei 2002). Among other functions, VEGF has been also implied in neuroprotection, neurogenesis, and neurodegeneration (Storkebaum and Carmeliet 2004; Storkebaum et al. 2004; Raab and Plate 2007). However, recent studies have described a cross-talk between vessels and nerves in the opposite direction; the role played by BDNF in angiogenesis has been described in neuroblastoma cells via HIF-1 $\alpha$ , increasing VEGF release (Nakamura et al. 2006) or directly through direct actions on endothelial cells that express TrkB receptor (Kermani et al. 2005). In the neural stem cell-vascular niche, both factors are critical participants for vessel formation and neural stem cell self-renewal (Li et al. 2006). All these studies indicate the close relationship and cross-talk between neural plasticity and vascular system plasticity, in which both factors appear as a possible bridge between the 2 processes. The interactions between the 2 systems involving common growth factors suggest that both systems have evolved in an interconnected way (Ward and Lamanna 2004).

### Conclusions and Future Perspectives

Cortical angiogenesis, VEGF expression, and the rearing environment of the postnatal developing brain are closely correlated both temporally and spatially. The pattern of VEGF protein distribution is congruent with the role of VEGF as a mediator of angiogenesis, providing the signal for inducing and guiding the growth of newly forming vessels in response to visual stimuli and a major metabolic demand. We report angiogenesis and VEGF expression increase in the visual cortex that are modulated by an EE in absence of physical exercise, and delay in the maturation of vascular pattern in DR and DR-EE rats, especially during the critical period.

A deeper understanding of the basic mechanisms of vascular pattern formation will aid in the development of strategies to improve vessel regeneration in ischemic complications or specifically target neo-vessel formation in antitumor therapy. In this way, another point of interest is the role of externally administrated VEGF to improve the outcome of neurological diseases such as amyotrophic lateral sclerosis (Storkebaum et al. 2004), stroke (Zhang et al. 2000; Sun et al. 2003), or brain injury (Argandoña et al. 2006). Potentially detrimental effects, on the other hand, might compromise beneficial actions of VEGF. For example, VEGF has been involved in the development of vascular leakage after brain hypoxia as the causative factor (Schoch et al. 2002). Further studies are required to be able to dissociate the neuroprotective effects of VEGF from the permeability effects, in order to use it for

therapeutic purposes. In this direction, the relationship between VEGF and BDNF may play a key role, as BDNF lacks permeability effects and is able to stimulate angiogenesis in a VEGF-independent basis.

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Address correspondence to Harkaitz Bengoetxea, PhD Department of Neuroscience, Faculty of Medicine and Odontology, Basque Country University, Leioa 48940, Bizkaia, Spain. Email: hbengoetxea@gmail.com.

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