VEGF reverts the cognitive impairment induced by a focal traumatic brain injury during the development of rats raised under environmental enrichment

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The role of VEGF in the nervous system is extensive; apart from its angiogenic effect, VEGF has been described as a neuroprotective, neurotrophic and neurogenic molecule. Similar effects have been described for enriched environment (EE). Moreover, both VEGF and EE have been related to improved spatial memory. Our aim was to investigate the neurovascular and cognitive effects of intracerebrally-administered VEGF and enriched environment during the critical period of the rat visual cortex development. Results showed that VEGF infusion as well as enriched environment induced neurovascular and cognitive effects in developing rats. VEGF administration produced an enhancement during the learning process of enriched animals and acted as an angiogenic factor both in primary visual cortex (V1) and dentate gyrus (DG) in order to counteract minipump implantation-induced damage. This fact revealed that DG vascularization is critical for normal learning. In contrast to this enriched environment acted on the neuronal density of the DG and V1 cortex, and results showed learning enhancement only in non-operated rats. In conclusion, VEGF administration only has effects if damage is observed due to injury. Once control values were reached, no further effects appeared, showing a ceiling effect. Our results strongly support that in addition to neurogenesis, vascularization plays a pivotal role for learning and memory.

1. Introduction

Growth factors and its receptors are key regulators for central nervous system development as well as for homeostasis maintenance. These molecules exert effects over all components of the neurogliovascular unit (NVU), such as vessels, neurons and glia, and some authors refer to these molecules as angioglioneurins [1,2]. Vascular Endothelial Growth Factor (VEGF) is the archetypal angioglioneurin and acts on both vascular and neural development.

The role of VEGF in the nervous system is extensive. Apart from its angiogenic effect in developmental and pathological angiogenesis [3], it has been described as a neuroprotective, neurotrophic and neurogenic molecule [4–7]. The neuroprotective function of VEGF appears to be due to a combination of direct neuroprotective effects and the stimulation of angiogenesis. In addition, previous studies have described its neurogenic effect in the subventricular zone (SVZ) [8] and in the subgranular zone (SGZ) of the dentate gyrus [9]. It has been postulated that VEGF promotes the proliferation and differentiation of neuronal precursors, releasing neurotrophic factors

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[10] or exerting a direct mitogenic action on neural precursors [11]. Further studies described VEGF as a direct mediator of improved cognition in rodents [12–14] and as inducing long-term synaptic enhancement in hippocampal neurons [15]. Nonetheless, a recent study has shown that VEGF promotes hippocampus-dependent memory independently of its effects on neurogenesis and angiogenesis, by increasing the synaptic strength [16].

Enriched environment (EE) is an experimental paradigm defined as "a combination of complex and inanimate social stimulation" [17] that induces cellular, molecular and behavioural effects, not only in standard conditions but also in pathological ones [18,19]. Whereas initial studies showed that EE modified cortical weight [20,21], subsequent studies described an increase of nuclear and soma size in neurons, dendritic branching or synapsis size [22–24] among other effects. Moreover, EE also increases neurogenesis in the hippocampus [13] as it increases the expression of angioglioneurins such as VEGF or BDNF [25–27]. Therefore, EE has strong effects on the plasticity of neural connections, especially in the visual cortex [28].

The postnatal development of the visual cortex is modulated by environmental experience. Sensory modifications during an early critical period result in substantial plasticity and are a crucial factor in establishing the mature circuitry [29]. In rats, the critical period for the visual system is located between the third and the fifth postnatal weeks and the maximum peak of experience-induced changes occurs during the fourth and the fifth weeks [30,31].

The aim of the present study was to investigate the neurovascular and cognitive effects of VEGF infusion and enriched environment during the critical period of the visual cortex in developing rats.

2. Material and methods

2.1. Animals and housing

10 Long–Evans rats (Harlan, Barcelona, Spain) were used for each experimental group. Animals were raised in different rearing conditions:

1. *Standard conditions* (SC): rats raised in a standard laboratory cage (500 mm × 280 mm × 140 mm) with 12-h light/dark cycle (lights on at 8 a.m.)
2. *Enriched environment* (EE): rats raised in a large cage (720 mm × 550 mm × 330 mm) furnished with colourful toys and differently shaped objects (shelters, tunnels) that were changed every 2 days (with 12-h light/dark cycle; lights on at 8 a.m.).

2.2. Minipump implantation

Experiments were performed on Long–Evans rats 72 h prior to the beginning of the critical period (P18, 30–40 g). Animals were anaesthetized with avertin (1 ml/100 g) and a sagittal incision was made midway between the eyes. Firstly, the skin was retracted, and then the periosteum. Subsequently a subcutaneous pocket was opened in the animal's back into which the osmotic minipump (Mod. 1004, Alzet, Cupertino, CA, USA) was inserted. A brain infusion kit (Mod. Alzet Brain Infusion Kit III, Alzet) was fixed to the skull with cyanoacrylate. Minipumps were connected via PVC tubing to the infusion kit and the cannula was implanted 1 mm lateral to the sagittal suture and 1 mm anterior to lambda into the left hemisphere. Total operating time was approximately 25 min.

Different experimental groups were used:

a) A group of non-operated rats as control
b) PBS infusion
c) VEGF infusion

VEGF (Ref: sc-4571, Santa Cruz Biotechnology Inc, Germany) was administered (100 ng/ml) at a delivery rate of 0.11 μl/h with the cannula placed in the middle cortical layers. Within each group, the following visual stimulation conditions were studied:

- SC-SC: Rats raised in standard conditions (SC) before and after minipump implantation.
- SC-EE: Rats raised in standard conditions (SC) until minipump implantation (P18) and in an enriched environment (EE) after implantation until P46.

After surgery had been completed, food and water were provided ad libitum. Minipumps were left in position for four weeks until P46 (150–200 g).

2.3. Ethics statements

All animal experiments were performed in accordance with the European Community Council Directive (2010/63/EU) and approved by the Ethics Committee for Animal Welfare (CEBA) of the University of the Basque Country.

2.4. Morris water maze

Spatial learning and memory were assessed in a Morris water maze task during the last 11 days of the infusion period. In this task, animals learned to locate the position of the submerged platform using extra-maze spatial cues.

The water maze consisted of a circular swimming pool, 170 cm diameter and 0.6 m height, filled with water (22 ± 1 °C) made opaque with non-toxic white paint. Visual cues were fixed on the walls constantly visible from the pool, which was conceptually divided into four quadrants and had four points designed as starting points (north, south, east and west). During five days (days 1–5), animals were tested for place-learning acquisition with the escape platform (11 cm diameter, 47 cm height) located in the middle of the southeast quadrant, 2 cm below water surface. Four trials per day were performed (30 min. inter-trial interval), introducing the rats randomly from each of the four starting positions while facing the wall, and allowing them to swim until they located the platform. Animals that failed to find the platform within 120 s were guided to the platform and left there for 60 s, as were the successful ones. On the 7th day, the platform was removed and a single probe trial was performed (120 s) to assess spatial memory retention for the platform location. Time spent in the target area as well as in the other quadrants was measured. Next day, a session with a cued visible platform was carried out in which the platform was marked by a black flag as a control procedure to discard motor or motivational sensory deficits between experimental groups. Finally, during days 9–11, animals were subjected to a reversal test. The location of the hidden platform was changed to northeast and animals were tested for place-learning acquisition of the new location. All the trials were recorded and traced with an image tracking system (SMART, Panlab SL, Barcelona, Spain) connected to a video camera placed above the pool. The latency and travelled path length to find the platform were measured in each trial and the mean value for each day was calculated.

2.5. Fixation and tissue processing

Rats were anaesthetized with 6% chloral hydrate. After anaesthesia, the cannula was removed and the animals were transcardially perfused with a saline solution followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate-buffered saline solution (PBS). After perfusion, brains were stored overnight at 4 °C in fresh fixative. The next day, the brain was stored in 30% sucrose in 0.1 M PBS until the tissues sank. Then, samples were coronally cut at 50 μm with a cryotome and serially-collected sections were kept in 0.1 M PBS to be histochemically and immunohistochemically processed using the free-floating method.

2.6. Butyryl cholinesterase histochemistry

Sections were histochemically processed for Butyryl Cholinesterase to visualize the vascular pattern. Sections were washed twice in 0.1 M Tris-maleate buffer (TMB) (pH 6), acetylcholinesterase was inhibited in a BW284CS1 (1,5-bis(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide) (Ref: A-9013, Sigma–Aldrich, Spain) 0.05 M 20 min bath and sections were incubated overnight in the following incubation solution: butyryl thiocholine iodide (Ref: 108150250, Acros Organics, Barcelona, Spain) 1 mg/ml, 5% sodium citrate 0.1 M, 10% copper sulphate 30 mM, 10% BW284CS1 0.05 mM, 10% potassium ferricyanide 5 mM and 65% TMB 0.1 M. The next day, sections were washed in TMB, mounted on gelatine-coated slides, dehydrated and covered.

2.7. Immunohistochemistry

Sections were immunohistochemically processed for NeuN (Ref: MAB377, Chemicon International Inc, Spain, 1:400). Firstly, sections were washed in 0.1 M PBS and then incubated with 4% H_2O_2/methanol for 20 min in order to quench endogenous peroxidase activity. Afterwards, non-specific sites were blocked by adding BSA 5% in the incubation medium for 1 h. Sections were then incubated overnight at 4 °C with primary antibody. The following day, sections were washed in 0.1 M PBS and incubated with biotinylated secondary antibodies (Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). The immunohistochemical reaction was revealed by the avidin-biotin complex (Elite ABC kit, Vector Laboratories) using diaminobenzidine as a chromogen. Sections were mounted on gelatine-coated slides, dehydrated and covered.

2.8. LEA lectin staining and double-label immunofluorescence

Free-floating sections of primary visual cortex and the dentate gyrus were used for both LEA lectin staining and double-label immunofluorescence. For microvessel staining, *Lycopersicon esculentum* (tomato lectin LEA) histochemistry

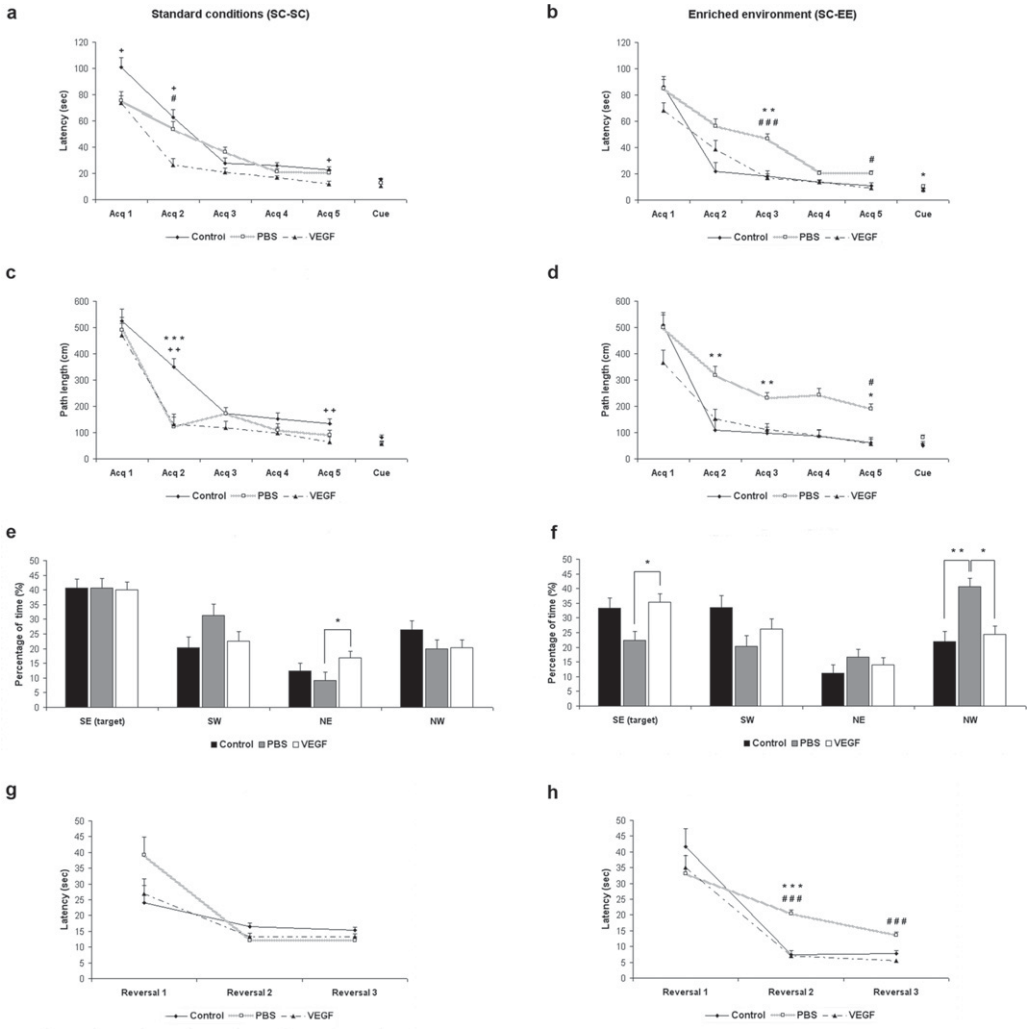

Fig. 1. Effects of VEGF administration on spatial learning and memory in the Morris Water Maze task of animals reared in standard conditions (SC-SC) and in enriched environment (SC-EE). 5 acquisitions, 1 cued, 1 removal and 3 reversal sessions were performed in different experimental groups (Control, PBS and VEGF). Escape latencies (a, b) and travelled path length (c, d) to reach the platform during the learning process. Percentage of time spent in each quadrant when platform was removed (e, f). Escape latency (g, h) to reach the platform during the reversal test. Mean ± SEM, $n = 10$. $^{+}$Significance between VEGF and Control ($^{+}p < 0.05$; $^{++}p < 0.01$). *Significance between Control and PBS ($^{*}p < 0.05$; $^{**}p < 0{,}01$; $^{***}p < 0.001$). $^{\#}$Significance between VEGF and PBS ($^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$).

was used. Sections were washed in 0.1 M PBS (pH 7.4). Then, non-specific sites were blocked by adding BSA 4% in the incubation medium for 1 h followed by overnight incubation with LEA-TRICT antibody (Ref: L9511, Sigma–Aldrich, Spain, 1:200) in 1% BSA in 0.1 M PBS containing 0.5% Triton X-100. Afterwards, sections were washed in PBS. Double immunolabelling using GFAP (glial fibrillary acidic protein), NeuN and LEA as markers for astrocytes, neurons and endothelial cells, respectively, and for VEGF were carried out to determine co-localization with each cell type. 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 1% BSA in 0.1 M PBS containing 0.5% Triton X-100. The antibodies used were monoclonal mouse anti-GFAP (Ref: G-3893, Sigma–Aldrich, Spain, 1:400), monoclonal mouse anti-NeuN (Ref: MAB377, Chemicon International Inc, 1:400), and polyclonal rabbit anti-VEGF (Ref: sc-152, Santa Cruz Biotechnology, Inc., USA, 1:400). After rinsing, sections were incubated for 2 h with the following fluorochrome conjugated secondary antibodies (goat antimouse Alexa Fluor® 488, goat antirabbit Alexa Fluor® 568; Invitrogen, Spain, 1:400) in 1% BSA in 0.1 M PBS containing 0.3% Triton X-100. Hoechst-33258 was added to counterstain the nuclei. Sections were rinsed, mounted on gelatine-coated slides, and cover-slipped in aqueous medium. For all methods, negative controls in which the primary antibodies were omitted were included in each staining run. Images were acquired for confocal fluorescence microscopy with an Olympus Fluoview FV500 confocal microscope using sequential acquisition to avoid overlapping of fluorescence emission spectra.

2.9. Morphometric procedures

Vascular and neuronal densities were measured by the optical dissector method in Butyryl Cholinesterase histochemistry and NeuN immunohistochemistry, respectively, with the aid of the Mercator Image Analysis system (Explora Nova, La Rochelle, France). For this purpose, probes of 50 μm × 50 μm separated by 120 μm were launched into a previously-delimited area corresponding to layer IV of the primary visual cortex and the granular cell layer of the dentate gyrus. Positive cells and vessels were counted if found to be present inside the probe and if they did not touch the forbidden *X* and *Y* axes. 10 animals and 10 histological sections per animal were used for each experimental group, and 2 measurements were taken for each section (ipsilateral and contralateral hemisphere), making a total of 200 measurements for each group. Measurements of each slice of the cortex were taken and the mean value per animal was calculated.

2.10. Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 19.0, IBM, Spain). Prior to analysis, data were examined for normal distribution using the Kolmogorov–Smirnov test and for homogeneity of variances using Levene's test. The effects of experimental conditions were evaluated using the one-way ANOVA analysis with the Bonferroni correction for equal variances or Tamhane's T2 correction for unequal variances. Data are described as mean ± SEM. Significance was declared at $p < 0.05$.

3. Results

3.1. Effects of VEGF administration in learning and memory

3.1.1. Standard conditions (SC-SC)

Spatial memory acquisition testing showed that the escape latency as well as total path length of the VEGF-infused group were

Table 1
Effect of enriched environment on spatial learning and memory in each experimental group (Control, PBS, VEGF). Mean value of escape latency and travelled path length ± SEM and *p* value between environmentally-enriched (SC-EE) and standard condition (SC-SC) reared groups within each experimental group.

	Control		PBS		VEGF	
	SC-EE vs. SC-SC		SC-EE vs. SC-SC		SC-EE vs. SC-SC	
	Latency (s)	*p*	Latency (s)	*p*	Latency (s)	*p*
Acq 1	86 ± 7 vs. 101 ± 7	0.863	84 ± 7 vs.75 ± 7	0.988	68 ± 5 vs. 73 ± 5	1.000
Acq 2	22 ± 6 vs. 62 ± 6	0.000	56 ± 5 vs. 53 ± 6	1.000	38 ± 7 vs. 26 ± 5	0.886
Acq 3	18 ± 4 vs. 27 ± 4	0.227	46 ± 3 vs. 36 ± 3	0.940	17 ± 3 vs. 20 ± 3	0.951
Acq 4	13 ± 2 vs. 26 ± 2	0.003	20 ± 2 vs. 21 ± 2	1.000	13 ± 1 vs. 16 ± 1	0.795
Acq 5	11 ± 2 vs. 22 ± 2	0.001	20 ± 2 vs. 20 ± 2	1.000	9 ± 1 vs. 12 ± 2	0.795
Cue	7 ± 1 vs. 15 ± 1	0.002	10 ± 1 vs. 12 ± 1	0.999	8 ± 1 vs. 10 ± 1	0.510
Rev 1	41± 5 vs. 24 ± 5	0.427	33 ± 5 vs. 39± 5	0.994	35 ± 4 vs. 27 ± 4	0.096
Rev 2	7 ± 1 vs. 16 ± 1	0.000	20 ± 1 vs. 12 ± 1	0.035	7 ± 1 vs. 13 ± 1	0.033
Rev 3	7 ± 1 vs. 15 ± 1	0.000	13 ± 1 vs. 12 ± 1	0.784	5 ± 0.8 vs. 13 ± 1	0.000
	Control		PBS		VEGF	
	SC-EE vs. SC-SC		SC-EE vs. SC-SC		SC-EE vs. SC-SC	
	Latency (s)	*p*	Latency (s)	*p*	Latency (s)	*p*
Acq 1	508 ± 48 vs. 525 ± 46	1.000	501 ± 46 vs. 490 ± 48	1.000	367 ± 46 vs. 471 ± 46	0.169
Acq 2	110 ± 37 vs. 350 ± 30	0.000	319 ± 33 vs. 122 ± 38	0.008	153 ± 36 vs. 131 ± 38	1.000
Acq 3	98 ± 23 vs. 139 ± 22	0.494	232 ± 21 vs. 173 ± 22	0.931	112 ± 21 vs. 118 ± 25	1.000
Acq 4	86 ± 25 vs. 153 ± 23	0.013	244 ± 24 vs. 110 ± 24	0.246	89 ± 22 vs. 97 ± 24	1.000
Acq 5	61 ± 21 vs. 133 ± 19	0.001	192 ± 18 vs. 90 ± 19	0.225	57 ± 19 vs. 64 ± 20	0.998
Cue	49 ± 9 vs. 82 ± 9	1.000	82 ± 8 vs. 58 ± 10	1.000	57 ± 8 vs. 57 ± 9	0.812

decreased compared to non-operated control groups and the group infused with PBS during the learning process (Fig. 1a, c). The VEGF-infused group showed statistically-significant differences in escape latency compared to non-operated control group (Acq 1, $p = 0.005$; Acq 2, $p = 0.001$; Acq 5, $p = 0.003$) and the PBS-infused group (Acq 2, $p = 0.009$) during the acquisition phase. The VEGF-infused group also showed a diminished total path length compared to the non-operated control group (Acq 2, $p = 0.001$; Acq 5, $p = 0.002$), as did the non-operated control group compared to the PBS-infused group (Acq 2, $p = 0.001$). The probe trial was performed 24 h after the final day of training and no differences between experimental groups were observed in the target quadrant (40% in the control, PBS-infused and VEGF-infused groups; $p = 1.000$) (Fig. 1e). In addition, when the platform location was changed, no statistical differences were found in escape latencies between the studied groups (Fig. 1g).

3.1.2. Enriched environment (SC-EE)

The VEGF-infused group showed statistically-decreased escape latency and total path length compared to the PBS-infused group during the learning process (Fig. 1b, d). Between the third and fifth days of acquisition, the VEGF-infused group presented lower latency compared to the PBS-infused group ($p = 0.000$ and $p = 0.010$ respectively). Differences in path length were also statistically significant (Acq 3, $p = 0.037$ and Acq 5, $p = 0.022$). In addition, the non-operated control group also showed statistically-significant lower latency than the PBS-infused group in the third day of acquisition ($p = 0.001$), whereas the differences in path length were significant during the second ($p = 0.002$), third ($p = 0.009$) and fifth day ($p = 0.029$) of the learning process. On the cued visible platform day, the PBS-infused group also showed a statistically-significant difference compared to the control group in escape latency ($p = 0.026$) but no differences were observed in total path length. Furthermore, in the probe trial performance, the PBS-infused group spent less time in the target quadrant than the VEGF-infused group and the non-operated control group (33% control, 22% PBS-infused and 35% VEGF-infused group; $p = 0.043$ and $p = 0.295$ respectively) (Fig. 1f). When the platform location was changed, the VEGF-infused and control groups also presented significantly lower escape latency than the PBS-infused group ($p = 0.000$ in both cases) (Fig. 1h).

3.1.3. Enriched environment (SC-EE) vs. standard conditions (SC-SC)

During the learning process, animals reared in enriched environment showed statistically-decreased latency compared to standard-reared animals in the non-operated control group (Acq 2, $p = 0.000$; Acq 4, $p = 0.003$; Acq 5, $p = 0.001$ and Cue, $p = 0.002$). Path length was also statistically diminished in enriched animals compared to the control group that was standard-reared during the learning process (Acq 2, $p = 0.000$; Acq 4, $p = 0.013$; Acq 5, $p = 0.001$). In the PBS and VEGF-infused groups, no differences in the escape latency between SC-EE and SC-SC reared rats were found. In contrast, in the PBS-infused group, the path length for enriched rats was significantly longer than in standards during the second day of the acquisition phase ($p = 0.008$). In addition, whereas in the PBS-infused group the probe trial performance showed statistically-significant lower time spent in the target quadrant of SC-EE reared rats (40% in SC-SC vs. 22% in SC-EE; $p = 0.002$), no significant differences were found in non-operated controls (40% in SC-SC and 33% in SC-EE, $p = 1.000$) or the VEGF-infused groups (40% in SC-SC and 35% in SC-EE; $p = 1.000$). Furthermore, in the non-operated control group, SC-EE reared rats also showed lower latency during the reversal test compared to SC-SC reared rats ($p = 0.000$). The same results were obtained in the VEGF-infused group ($p = 0.033$ and $p = 0.000$). In contrast, in the PBS-infused group, lower escape latency was observed in the SC-SC reared group during the reversal learning process ($p = 0.035$) (Table 1).

3.2. Effects of VEGF administration in the dentate gyrus

3.2.1. Standard conditions (SC-SC)

The vascular density of the dentate gyrus showed no differences among the studied experimental groups. In the ipsilateral cortex, the PBS-infused group showed 3% lower density compared to the non-operated group, while in the VEGF-infused group, observed density was 3% higher ($p = 1.000$ in both cases). The difference between the PBS-and VEGF-infused groups was 7% ($p = 1.000$), being higher in the latter one. Similar results were found in the contralateral hemisphere. Whereas the PBS-infused group presented 5% ($p = 1.000$) lower vascular density, the VEGF-infused group

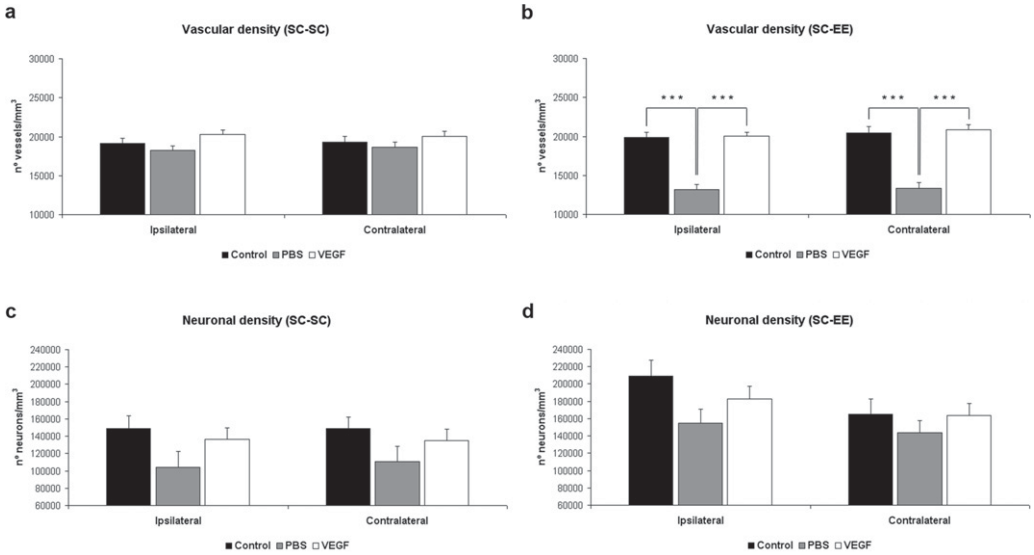

Fig. 2. Effects of VEGF administration on vascular and neuronal densities in the dentate gyrus. Quantitative study between different experimental groups (Control, PBS and VEGF) in animals reared in standard conditions (SC-SC) and in enriched environment (SC-EE). Horizontal axes show the ipsilateral and contralateral hemispheres. Vertical axes show number of vessels of the dentate gyrus (a, b) and NeuN positive cells (c, d) per mm^3 of the granular cell layer of the dentate gyrus. Mean ± SEM. $^{***}p < 0.001$.

showed 6% ($p = 1.000$) higher density than the non-operated control group. Finally, the VEGF-infused group showed 11% ($p = 0.162$) higher vascular density than the PBS-infused group (Fig. 2a).

Neuronal density was quantified in the granular cell layer of the dentate gyrus. Although no statistically-significant differences were found, in the ipsilateral hemisphere, the PBS-infused group showed 30% ($p = 0.131$) lower neuronal density compared to non-operated animals. On the other hand, the VEGF-infused group showed 31% ($p = 0.843$) higher density compared to the PBS-infused group and 8% ($p = 1.000$) lower than the non-operated group. The observed trend was maintained in the contralateral hemisphere. Whereas the PBS-infused group presented 26% less vessels than the control group, the VEGF-infused group showed 22% higher and 9% lower density than the PBS-infused and non-operated control groups, respectively ($p = 1.000$ for all the experimental groups) (Fig. 2c).

3.2.2. Enriched environment (SC-EE)

In animals reared in enriched environment, vascular density showed statistically-significant differences between the studied experimental groups. The PBS-infused group presented 34% ($p = 0.000$) lower vascular density than the non-operated control group, whereas the VEGF-infused group showed 56% ($p = 0.000$) higher and 2% lower ($p = 1.000$) density than the PBS-infused and non-operated control groups, respectively. In the contralateral cortex results were similar. The PBS-infused group showed 33% ($p = 0.000$) lower density than the control group. On the other hand, the VEGF-infused group presented 52% ($p = 0.000$) and 1% ($p = 1.000$) higher vascular density than the PBS-infused and non-operated control groups, respectively (Fig. 2b).

Neuronal density of the granular cell layer showed no statistical differences among the experimental groups although similar results compared to standard reared animals were found. In the ipsilateral cortex, the PBS-infused group presented 26% ($p = 0.513$) lower neuronal density than the control group, whereas in the VEGF-infused group, the observed neuronal density was 18% ($p = 0.989$) higher and 12% ($p = 0.998$) lower than in the PBS-infused and non-operated control groups, respectively. In the contralateral hemisphere, the PBS-infused and VEGF infused groups showed 13% and 1% lower density, respectively, compared to the non-operated control group ($p = 1.000$ in both cases). Finally, the VEGF-infused group presented 14% ($p = 1.000$) higher neuronal density than the PBS-infused one (Fig. 2d).

Table 2
Effect of enriched environment on vascular and neuronal densities of the dentate gyrus in the ipsilateral (IL) and contralateral (CL) hemispheres of animals reared in standard conditions (SC-SC) and in enriched environment (SC-EE). Average measurements of studied groups (Mean value ± SEM), and percentage of difference and *p* value between SC-EE and SC-SC groups of each experimental group (Control, PBS, VEGF).

		Control		PBS		VEGF	
		SC-SC	SC-EE	SC-SC	SC-EE	SC-SC	SC-EE
Vascular density	IL	19,307 ± 726	20,452 ± 839	18,651 ± 649	13,352 ± 750	20,027 ± 634	20,896 ± 619
	CL	19,172 ± 635	19,871 ± 658	18,238 ± 564	13,180 ± 658	20,289 ± 550	20,030 ± 537
Neuronal density	IL	148,552 ± 15,315	209 278 ± 18,471	104,213 ± 18,471	155,138 ± 15,818	136,763 ± 13,061	182,811 ± 14,439
	CL	149,081 ± 13,363	165,371 ± 17,563	110,607 ± 17,563	143,400 ± 14,127	134,844 ± 13,025	163,835 ± 13,363

		Vascular density (SC-EE vs. SC-SC)			Neuronal density (SC-EE vs. SC-SC)		
		Control	PBS	VEGF	Control	PBS	VEGF
% Dif	IL	5	−28	4	41	48	33
	CL	3	−28	1	10	30	22
p	IL	1.000	0.000	1.000	0.199	0.261	0.607
	CL	1.000	0.000	1.000	1.000	1.000	1.000

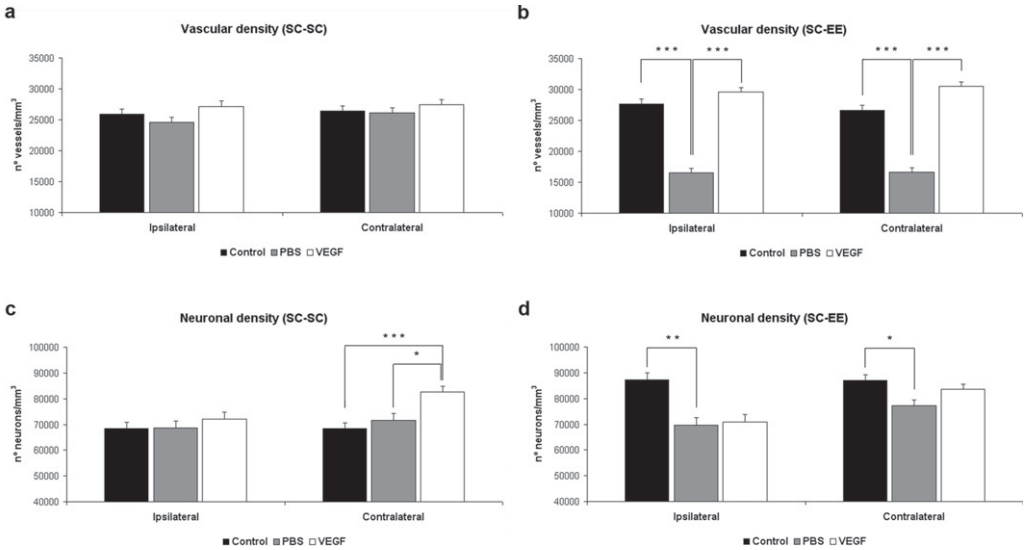

Fig. 3. Effects of VEGF administration on vascular and neuronal densities in the primary visual cortex. Quantitative study between different experimental groups (Control, PBS and VEGF) in animals reared in standard conditions (SC-SC) and in enriched environment (SC-EE). Horizontal axes show the ipsilateral and contralateral hemispheres. Vertical axes show number of vessels (a, b) and NeuN positive cells (c, d) per mm^3 of the primary visual cortex. Mean ± SEM. $^*p < 0.05$; $^{***}p < 0.001$.

3.2.3. Enriched environment (SC-EE) vs. standard conditions (SC-SC)

In animals reared in enriched environment, vascular density was 5% ($p = 1.000$) and 3% ($p = 1.000$) higher in the non-operated control group (ipsilateral and contralateral hemispheres, respectively). The PBS-infused group presented statistically significant-differences compared to SC-SC reared rats in both hemispheres (28%, $p = 0.000$ in the ipsilateral cortex; 28%, $p = 0.000$ in the contralateral cortex). In the VEGF-infused group, no effects of enriched environment were found, as SC-EE reared rats showed 4% ($p = 1.000$) and 1% ($p = 1.000$) higher vascular density than SC-SC reared rats in the ipsilateral and contralateral cortex, respectively (Table 2).

Although no statistical differences were found in the neuronal density of the granular cell layer in the dentate gyrus, rats raised in SC-EE conditions presented higher neuronal density in all experimental groups. In the ipsilateral hemisphere, 41%, 48% and 33% higher neuronal density was found in SC-EE reared animals in the non-operated controls ($p = 0.199$), the PBS-infused ($p = 0.261$) and the VEGF-infused ($p = 0.607$) groups, respectively. In the contralateral hemisphere, SC-EE reared rats also showed 10%, 30% and 22% higher neuronal density in the control ($p = 1.000$), PBS ($p = 1.000$) and VEGF-infused ($p = 1.000$) groups (Table 2).

3.3. Effects of VEGF administration in V1 cortex

3.3.1. Standard conditions (SC-SC)

No significant differences were observed in vascular density in animals reared in standard conditions. In the ipsilateral hemisphere, the PBS-infused group showed 5% ($p = 1.000$) less vessels than non-operated controls. The VEGF-infused group showed a 5% ($p = 1.000$) and 10% ($p = 0.464$) higher vascular density than non-operated controls and the PBS-infused group. In the contralateral hemisphere, similar results were found. Whereas the PBS-infused group presented 1% ($p = 1.000$) less vessels than non-operated controls, the VEGF-infused group presented a 4% ($p = 1.000$) and 5% ($p = 0.998$) higher vascular density compared to the control and PBS groups, respectively. None of the differences were statistically significant (Fig. 3a).

On the other hand, neuronal density in the ipsilateral hemisphere showed no difference between PBS-infused and non-operated controls (0.38%; $p = 0.977$). The VEGF-infused group presented 5% higher neuronal density than both the control and PBS groups ($p = 0{,}998$ and $p = 1.000$ respectively). Nonetheless, the VEGF group showed statistically-significant more neurons than the non-operated controls (20%; $p = 0.000$) and the PBS-infused (15%; $p = 0.030$) group in the contralateral hemisphere. The PBS-infused group also showed 5% more neurons than controls ($p = 1.000$) (Fig. 3c).

3.3.2. Enriched environment (SC-EE)

In animals reared in enriched environment after minipump implantation, significant differences were found in the vascular density of the primary visual cortex. In the ipsilateral hemisphere, the PBS-infused group showed 40% ($p = 0.000$) lower vascular density than non-operated controls and 79% ($p = 0.000$) lower than the VEGF-infused group. Thus, 7% higher density was observed in the VEGF-infused group compared to the non-operated control group, resulting in a statistically-insignificant difference. Similar results were found in the contralateral hemisphere. Whereas the PBS-infused group showed 37% ($p = 0.000$) lower vascular density than non-operated controls, the VEGF-infused group showed a significantly higher density than both the control (15%; $p = 0.014$) and PBS-infused groups (83%; $p = 0.000$) (Fig. 3b).

In the ipsilateral cortex, lower neuronal density compared to enriched-environment reared controls was found in both the PBS and VEGF-infused groups, (20%, $p = 0.001$ and 19%, $p = 0.074$ respectively). Therefore, the PBS and VEGF-infused groups showed similar neuronal density to each other (2%; $p = 1.000$). In the contralateral hemisphere, the PBS-infused group also showed a statistically-significant lower neuronal density than non-operated controls (11%; $p = 0.044$). On the contrary, the VEGF-infused group presented 3% less neurons than the control group, resulting in a non-significant difference ($p = 1.000$). Finally, the VEGF-infused group showed 8% ($p = 0.477$) higher density than the PBS-infused group (Fig. 3d).

3.3.3. Enriched environment (SC-EE) vs. standard conditions (SC-SC)

In the vascular density of non-operated controls, no statistical differences were found in rats reared in enriched environment compared to rats reared in standard conditions ($p = 1.000$ in both hemispheres). In the VEGF-infused group, SC-EE reared rats also presented 9% (ipsilateral; $p = 0.564$) and 10% (contralateral;

Table 3
Effect of enriched environment on vascular and neuronal densities of the primary visual cortex in the ipsilateral (IL) and contralateral (CL) hemispheres of animals reared in standard conditions (SC-SC) and in enriched environment (SC-EE). Average measurements of studied groups (Mean value ± SEM), and percentage of difference and *p* value between SC-EE and SC-SC groups of each experimental group (Control, PBS, VEGF).

		Control		PBS		VEGF	
		SC-SC	SC-EE	SC-SC	SC-EE	SC-SC	SC-EE
Vascular density	IL	25,940 ± 798	27,677 ± 798	24,600 ± 788	16,496 ± 724	27,153 ± 872	29,572 ± 758
	CL	26,432 ± 794	26,591 ± 850	26,124 ± 863	16,623 ± 765	27,404 ± 850	30,497 ± 716
Neuronal density	IL	68,281 ± 2465	87,284 ± 2662	68,547 ± 2772	69,523 ± 3170	71,966 ± 2732	70,787 ± 2903
	CL	68,505 ± 2111	86,998 ± 2369	71,650 ± 2629	77,157 ± 2338	82,571 ± 2297	83,573 ± 2102

		Vascular density (SC-EE vs. SC-SC)			Neuronal density (SC-EE vs. SC-SC)		
		Control	PBS	VEGF	Control	PBS	VEGF
% Dif	IL	2	−33	9	28	1	−1
	CL	0.006	−36	10	27	7	1
p	IL	1.000	0.000	0.564	0.001	1.000	1.000
	CL	1.000	0.000	0.179	0.000	1.000	1.000

$p = 0.179$) higher vascular density. In contrast, in the PBS-infused group SC-EE reared rats showed statistically lower vascular density compared to SC-SC reared rats in both hemispheres (33% in the ipsilateral hemisphere, $p = 0.000$; 36% in the contralateral hemisphere, $p = 0.000$) (Table 3).

Neuronal density of non-operated controls presented a statistically-significant increase in rats reared in SC-EE conditions (28% in the ipsilateral hemisphere, $p = 0.001$; 27% in the contralateral hemisphere, $p = 0.000$). No effects of enriched environment were found in the PBS and VEGF-infused groups. In the PBS-infused group, SC-EE reared rats showed 1% (p= 1.000) and 7% ($p = 1.000$) higher neuronal density compared to SC-SC reared rats in the ipsilateral and contralateral hemispheres respectively. The VEGF-infused group presented 1% lower ($p = 1.000$) and 1% higher ($p = 1.000$) neuronal density in rats reared in an enriched environment, this being a statistically-insignificant difference (Table 3).

3.4. VEGF co-expression in DG

VEGF expression was similar in the dentate gyrus of all experimental groups and most of the VEGF-positive cells co-localized with GFAP (Fig. 4a-c). VEGF/NeuN immunofluorescence did not show differences among the experimental groups, although some VEGF-positive neurons were found (Fig. 4d–f). No co-localization with LEA was observed (Fig. 4g–i). Therefore, VEGF expression corresponds to astrocytes in the dentate gyrus at P46.

3.5. VEGF co-expression in V1 cortex

Double immunofluorescence of VEGF/GFAP showed a significantly lower expression of both antigens in non-operated rats compared to the PBS and VEGF groups, higher expression being observed in the latter (Fig. 5a–c). Most of the VEGF-positive cells corresponded to astrocytes, as they co-localized with GFAP. In addition, VEGF/NeuN immunolabelling confirmed that co-localization with NeuN positive cells is lower in both infused groups (PBS and VEGF groups) compared to the control group (Fig. 5d–f). No co-localization with LEA was observed in any of the experimental groups (Fig. 5g–i). Thus, the majority of VEGF expression corresponds to astrocytes in the primary visual cortex at P46.

4. Discussion

4.1. Effects of VEGF administration

VEGF administration during the critical period of the visual cortex shows different responses depending on the rearing environment. Angiogenic, neuroprotective and neurogenic properties have been described for VEGF in the brain, as well as its influence over hippocampus-dependent memory [12,14,15].

Our results revealed an improved trend during the learning process induced by VEGF administration compared to non-operated and PBS-infused animals raised in standard conditions although no beneficial effects were found in long-term spatial memory and reversal learning. Since the dentate gyrus is the primary afferent into the hippocampus, we carried out a quantitative analysis of vascular and neuronal densities of DG. Results did not show statistically-significant differences in vascular and neuronal densities of the dentate gyrus in the studied experimental groups reared in standard conditions. Nevertheless, in the VEGF-infused group, an increase in neuronal density of the granular cell layer of DG was observed. Recently, it has been shown that VEGF promotes hippocampus-dependent memory, increasing the synaptic strength, independently of its effects on neurogenesis and angiogenesis [16], which could explain the lack of neurovascular changes found in the dentate gyrus.

In animals reared in enriched environment, data also showed a significant improvement during learning acquisition in the VEGF-infused group compared to the PBS-infused and non-operated control groups. Moreover, whereas PBS-infusion impaired long-term spatial memory, VEGF administration counteracted this negative effect and showed an improved reversal test as well. It has been previously described that VEGF improves cognition in rodents and our data revealed similar results when administered intracortically in developing rats. Furthermore, quantitative results showed that PBS-infusion induced a negative effect on vascular density in the dentate gyrus, which was reverted with VEGF administration in enriched animals. Consistent with previous studies that demonstrated that angiogenesis was critical to normal learning and memory [32], our data also suggest that the vascular component of DG could be determinant, as increased vascular density was found in the VEGF-infused group. VEGF can activate neurogenesis by targeting neurons directly or protecting them indirectly through its angiogenic effect [14,33]. Nonetheless, in addition to augmenting the number of newborn cells in the brain, newly formed vessels are needed for their metabolic and trophic demands [34]. Thus, several studies have indicated that neurogenesis is not sufficient by itself for memory enhancement [16,35,36]. Our results suggest that vascularization is related to normal learning although VEGF could also increase connectivity between mature neurons to enhance learning as well.

On the other hand, the absence of VEGF effects in animals reared in standard conditions but not in enriched ones could be due to enhanced experience after minipump implantation, as these animals were reared in an enriched environment only after the surgery. Interaction of experience with time sensitive

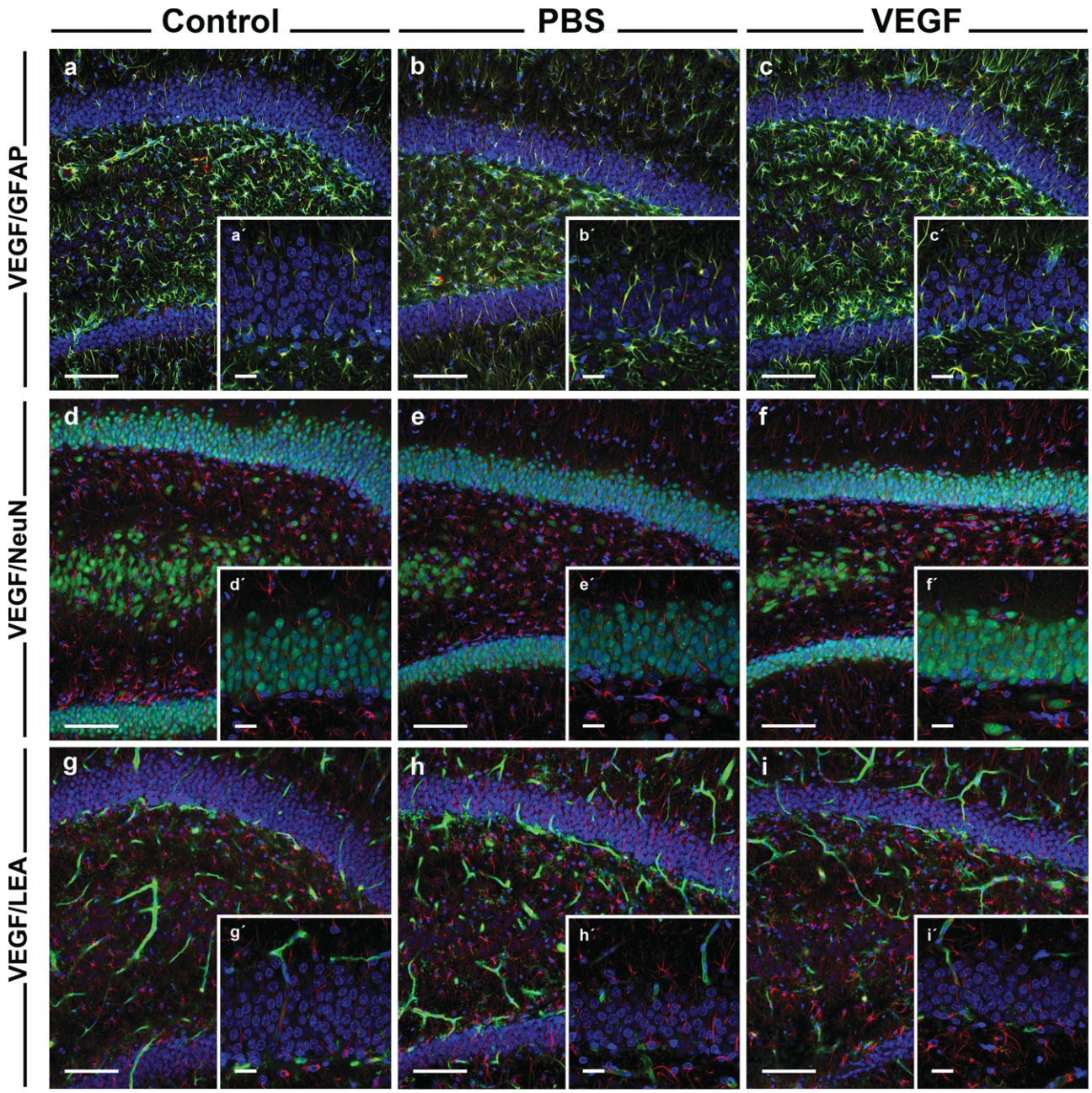

Fig. 4. VEGF/GFAP, VEGF/NeuN and VEGF/LEA co-expression in the dentate gyrus. Confocal images of double immunolabelling of VEGF and GFAP (a–c), VEGF and NeuN (d–f), and VEGF and LEA (g–i) to determine if VEGF positivity corresponds to astrocytes, neurons or endothelial cells respectively. Blue corresponds to Hoeschst 33258 positive nuclei. Scale bar = 100 μm (a–i) and 20 μm (a′–i′).

injury-induced changes has been described as shaping brain reorganization in a long-lasting manner, including areas proximal to the injury that might be particularly important for functional outcome [37]. Therefore, our results suggest that VEGF infusion might aid recovery from minipump implantation-induced-lesions.

4.2. DG-V1 cortex connection

Whereas most studies to determine VEGF function in learning and memory used intracerebroventricular [8,38,39] or intrahippocampal administration [40], in our work, VEGF has been administered immediately anterior to the primary visual cortex and a direct arrival to the DG could be discarded. The visual cortex provides a crucial sensory input to the hippocampus and is a key component for the creation of visuospatial memories [41]. Diverse studies have suggested that experience-dependent synaptic plasticity occurs in the adult primary visual cortex, which could constitute a key element in neocortico-hippocampal transfer of information [41,42]. This relationship between learning and visual cortical changes has also been observed in rodents [43,44]. In addition to the hippocampus and the parahippocampal region, the enthorinal cortex [45] and postrhinal cortex [46] also have a reciprocal connection with the visual system. On the other hand, the retrosplenial cortex has also been demonstrated to contribute to memory and navigation, and being anatomically close to V1, a direct effect of VEGF should not be discarded [47].

Our data demonstrate that effects of VEGF administration in the DG are also present in the visual cortex, near to the cannula

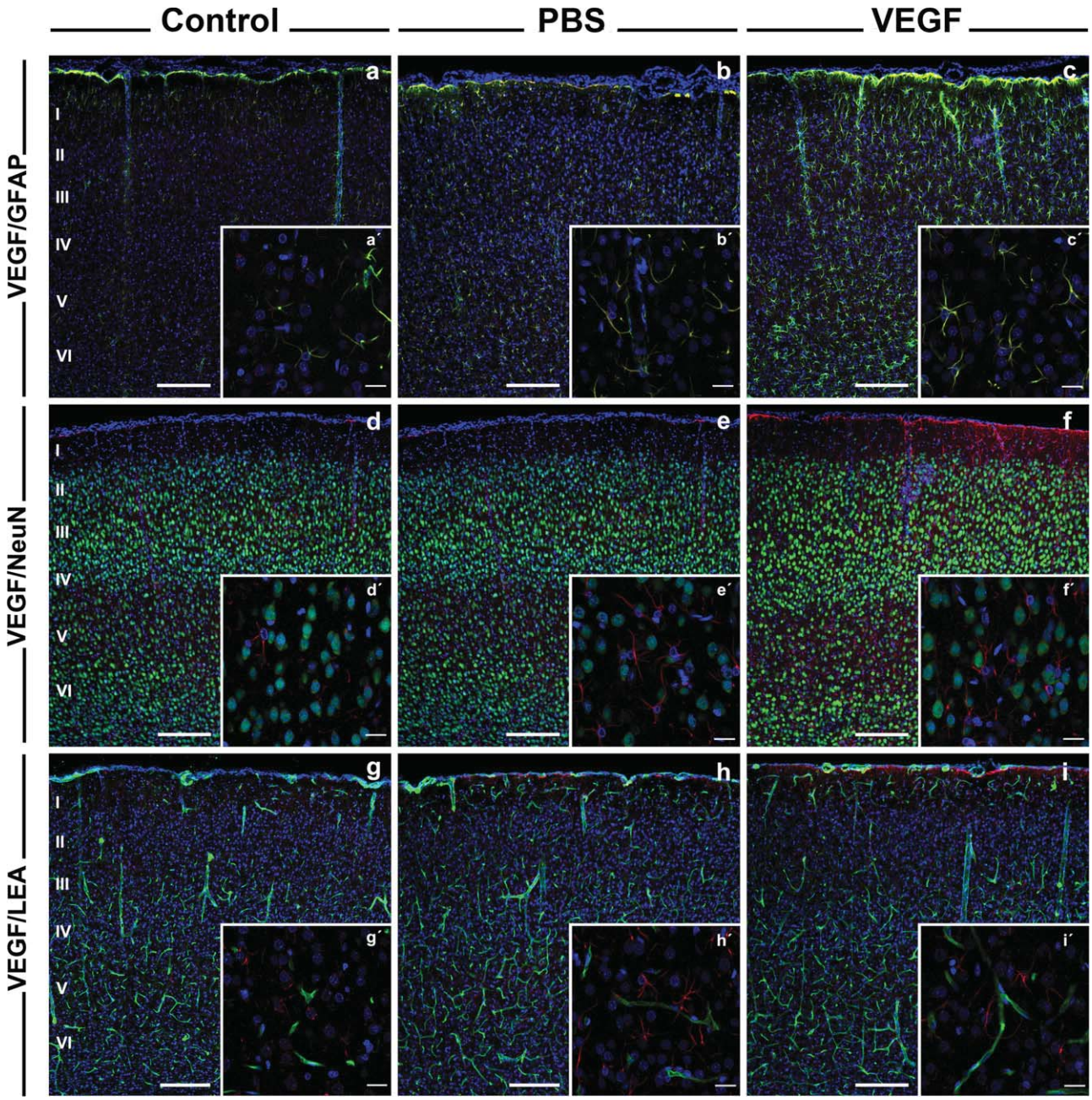

Fig. 5. VEGF/GFAP, VEGF/NeuN and VEGF/LEA co-expression in the primary visual cortex. Confocal images of double immunolabelling of VEGF and GFAP (a–c), VEGF and NeuN (d–f), and VEGF and LEA (g–i) to determine if VEGF positivity corresponds to astrocytes, neurons or endothelial cells respectively. Blue corresponds to Hoeschst 33258 positive nuclei. Scale bar = 200 μm (a–i) and 20 μm (a′–i′).

placement. As in the dentate gyrus, no effect of VEGF administration was observed in the neuronal and vascular densities of the primary visual cortex in standard conditions. Previous studies in our group demonstrated that minipump implantation produced negative effects in the V1 cortex that were reverted after 1 week of VEGF administration [48,49]. In this case, we carried out a chronic VEGF or vehicle administration and therefore a chronic minipump implantation during four weeks. This time lapse is enough to repair the surrounding tissue, thus recovering from injuries induced by minipump implantation. In addition, focal small lesions such as the one produced by cannula implantation can be repaired easily [50].

In contrast, in animals reared in enriched environment, the PBS-infused group showed a significantly lower vascular density, similar to that observed in DG. VEGF infusion counteracted this negative effect on microvascularization and reached non-operated control values. The PBS-infusion group also presented a significantly lower neuronal density compared to non-operated controls. VEGF administration did not revert to the neuronal density in the primary visual cortex as both the PBS and VEGF-infused groups presented values similar to the standard-reared control group. It has been proposed that there is a ceiling effect of VEGF and FGF-2 for inducing changes on neurovascular components after injury [39]. This fact could explain that VEGF acts only if the studied parameters are lower than physiological values.

Therefore, the connections described above could explain the fact that changes in DG are reflected in the primary visual cortex and vice versa.

4.3. Enriched environment vs. standard conditions

In addition to VEGF administration, an enriched environment by itself presents direct effects on studied neurovascular components and on the learning process. Exposure to an enriched environment has been shown to induce morphological changes in the brain, both in normal and pathological conditions [51,52]. These morphological changes include structural reorganization, especially in the visual cortex [53], which is associated with improved learning and memory and enhanced neural plasticity [54].

As has been observed before in the adult brain [53,54], our results have shown that enrichment exposure until P46 in the non-operated control group enhances the learning process and reversal learning compared to standard-reared rats. Enrichment-induced improvement of learning has been related to increased synaptic plasticity or hippocampal neurogenesis, among other effects [9,55,56]. Even if no differences in vascular density were observed, our results showed that enrichment induced an enhancement in the granular cell layer of the dentate gyrus. Although it has been reported that enrichment induces a survival-promoting effect in the dentate gyrus [9], the PBS and VEGF-infused groups did not show any improvement during the learning process. Nonetheless, the enriched environment enhances reversal learning in VEGF-infused animals whereas in PBS-infused ones, it impairs the performance. In fact, recent studies have suggested that neurogenesis is not the unique supporter of the enhancement induced by enrichment [35,36] since newly proliferated neurons need 3–4 weeks to be functionally incorporated into existing learning networks [57,58]. In agreement with these studies, our results showed that in the VEGF and PBS-infused groups, rearing in enriched environment also induced an increase in neuronal density of the granular cell layer in the dentate gyrus that was not accompanied by an improvement during the learning process.

On the other hand, previous studies have demonstrated that rearing in an enriched environment from birth to adulthood increases vascular density in the rat visual cortex, and that this is correlated with an increase in VEGF expression [25]. In addition, it was observed that exposure to EE from P18 onwards increased microvasculature at P25 [48]. Nevertheless, rearing in EE during the entire critical period (from P18 to P46) induced an increase in neuronal density. Enrichment elicits neuroprotective responses as it induces changes in genes related to neuroprotection [59], and expression of angioglioneurins such as BDNF [60] and VEGF [25] are increased as well. Therefore, enrichment elicits neurorescuer effects if applied throughout the critical period. In contrast, no beneficial effects were found in the V1 of the PBS and VEGF-infused groups, probably due to the minipump implantation-induced lesion. Enrichment-induced effects are different when applied in normal or pathological situations [61–63].

4.4. VEGF co-expression in V1 and DG

In the developing brain, VEGF is initially produced by neurons. At P13 neuronal expression of VEGF starts to diminish and astrocytic expression becomes more evident until localization of VEGF switches from being predominantly neuronal to glial at P24 [25]. However, in the hypoxic brain, high levels of neuronal and glial VEGF are maintained until P33 [63]. Our results showed that VEGF immunonoreactivity co-localized with astrocytes and neurons in the V1 cortex although most VEGF-positive cells corresponded to astrocytes in all experimental groups.

On the other hand, whereas our results showed that VEGF is expressed in astrocytes and neurons of the granular cell layer and the polymorphic layer of the dentate gyrus, as has been previously reported [12], no co-localization with endothelial cells has been found. Moreover, most VEGF expression corresponds to astrocytes. Some authors have proposed that astrocytes are an important niche for neurogenesis [64], and that astrocytes-derived VEGF expression could induce an increase in angiogenesis as they are localized adjacent to cerebral vessels [12]. Neuronal expression of VEGF has also been proposed to play an important role in the regulation of neurogenesis [12].

5. Conclusions

VEGF infusion as well as enriched environment induces neurovascular and cognitive effects in developing rats. VEGF administration produces an enhancement during the learning process and acts as an angiogenic factor in order to counteract minipump implantation-induced damage, revealing that DG vascularization is critical for normal learning. On the other hand, enriched environment acts on the neuronal component of DG and V1 cortex. Moreover, results showed learning enhancement only in non-operated rats, leading to the conclusion that in addition to neurogenesis, vascularization plays a pivotal role for learning and memory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version

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