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ORIGINAL PAPER

Cellular distribution of vascular endothelial growth factor A (VEGFA) and B (VEGFB) and VEGF receptors 1 and 2 in focal cortical dysplasia type IIB

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Abstract Members of the vascular endothelial growth factor (VEGF) family are key signaling proteins in the induction and regulation of angiogenesis, both during development and in pathological conditions. However, signaling mediated through VEGF family proteins and their receptors has recently been shown to have direct effects on neurons and glial cells. In the present study, we immunocytochemically investigated the expression and cellular distribution of VEGFA, VEGFB, and their associated receptors (VEGFR-1 and VEGFR-2) in focal cortical dysplasia (FCD) type IIB from patients with medically intractable epilepsy. Histologically normal temporal cortex and perilesional regions displayed neuronal immunoreactivity (IR) for VEGFA, VEGFB, and VEGF receptors (VEGFR-1 and VEGFR-2), mainly in pyramidal neurons. Weak IR was observed in blood vessels and there was no notable glial IR within the grey and white matter. In all FCD specimens, VEGFA, VEGFB, and both VEGF receptors were highly expressed in dysplastic neurons. IR in astroglial and balloon cells was observed for VEGFA and its receptors. VEGFR-1 displayed strong endothelial staining in FCD. Double-labeling also showed expression of VEGFA, VEGFB and VEGFR-1 in cells of the microglia/macrophage lineage. The neuronal expression of both VEGFA and VEGFB, together with their specific receptors in FCD, suggests autocrine/paracrine effects on dysplastic neurons. These autocrine/paracrine effects could play a role in the development of FCD, preventing the death of abnormal neuronal cells. In addition, the expression of VEGFA and its receptors in glial cells within the dysplastic cortex indicates that VEGF-mediated signaling could contribute to astroglial activation and associated inflammatory reactions.

Keywords Vascular endothelial growth factor · Receptors · Dysplastic neurons · Astrocytes · Immunocytochemistry · Epilepsy

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Introduction

The vascular endothelial growth factor (VEGF) family includes seven members which are structurally homologous, but display molecular and functional diversity [58, 81]. VEGFA, the most well known member of the VEGF family, is a crucial regulator of angiogenesis and vascular permeability in both physiological and pathological conditions such as tumor growth, chronic inflammation, and ischemia [10, 16, 19]. In addition to the unquestioned role in angiogenesis, it has recently been shown that VEGFA

has direct trophic effects on neuronal and glial cells in the central nervous system [10, 22, 25, 53].

VEGFB is most closely related to VEGFA [46, 81]; however, the biological function of VEGFB is less well characterized than the function of VEGFA. VEGFB is expressed early during development and appears to have prominent expression in the central nervous system [1, 36]. Additionally, VEGFB has been shown to function as an angiogenic and neuroprotective protein [29, 63, 68] and recent evidence suggests a role for VEGFB in neurogenesis [69, 70].

The diverse functions of VEGF proteins can be explained by their differential binding to signaling VEGF receptors [VEGFRs; VEGFR-1 (Flt-1), VEGFR-2 (Flk1/KDR), and VEGFR-3 (Flt-4) [50, 81]]. VEGFA binds to VEGFR-1 and VEGFR-2, whereas VEGFB binds specifically to VEGFR-1 and not to VEGFR-2 [81].

The VEGF-signaling pathway, involving both neuronal and glial cells, has been implicated in several neurological disorders, including neurodegeneration, stroke, and cerebral and spinal trauma [10]. In addition, expression of VEGFA is upregulated in neuronal and glial cells after epileptic seizures in rats [13], suggesting a role for VEGFA in seizure disorders. A recent study points to a neuroprotective role for VEGFA following status epilepticus [45]. The relevance of these findings in animal models to human epileptic disorders is uncertain. Using serial analysis of gene expression (SAGE), we recently identified the *VEGFB* gene to be upregulated in human tissue from a patient with focal cortical dysplasia (FCD) and intractable epilepsy compared to control cortex (Boer et al., unpublished observations). Upregulation of VEGFA and its receptor has also been recently shown in the hippocampus of cases of human temporal lobe epilepsy (TLE) [56]. However, the distribution of VEGFA, VEGFB, and VEGFRs in epilepsy-associated human malformations of cortical development has not yet been defined.

In the present study, we investigated the expression of both VEGFA and VEGFB and their receptors (VEGFR-1 and VEGFR-2) in patients with FCD, which is a developmental disorder known to be a major cause of intractable epilepsy [73]. We report the specific cellular distribution, including both the neuronal and the glial components of the dysplastic cortex, and we discuss the potential role of VEGFA, VEGFB, and their receptors in the histogenesis and epileptogenesis of this developmental lesion.

Materials and methods

Subjects

The cases included in this study were obtained from the databases of the Departments of Neuropathology of the Academic Medical Center (University of Amsterdam; UVA) in Amsterdam and the University Medical Center in Utrecht (UMCU). We examined surgically resected tissue from nine patients undergoing epilepsy surgery for focal cortical dysplasia. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. The tissue was obtained and used in a manner compliant with the Declaration of Helsinki.

The classification system proposed by Palmini et al. [48] was used for grading the degree of FCD and only patients with FCD type IIB located in the temporal lobe were included. The clinical characteristics derived from the patient's medical records are summarized in Table 1. The predominant type of seizure pattern was that of complex partial seizures, which were resistant to maximal doses of antiepileptic drugs (AEDs; carbamazepine, valproic acid, phenytoin, levetiracetam, oxcarbazepine, and clonazepam). Information concerning the exact time of last seizure occurrence prior to surgical resection was not available. However, all the patients included in our series did not have seizure activity in the last 24 h before surgery. The patients underwent presurgical evaluation [74]. Intraoperative ECoG was performed routinely in all operations for tailoring of surgery and we classified the post-operative seizure outcome according to Engel [17]. Follow-up period ranged from 1 to 9 years.

Normal-appearing control cortex/white matter from temporal region was obtained at autopsy from five adult control patients (male/female: 2/3; mean age 42, range 17–55) without history of neurological diseases. All autopsies were performed within 12 h after death (post mortem delay: 11, 11.5, 9, 8.5, 6). The cause of death was represented by acute myocardial infarction. In addition, four of the nine FCD cases contained sufficient amount of perilesional zone (normal-appearing cortex/white matter adjacent to the lesion), for comparison with the autopsy specimens. This

Table 1 Summary of clinical findings of patients with focal cortical dysplasia

Patient/sex/age (years)	Diagnosis	Duration of epilepsy (years)	Seizure type	Engel class
1/M/11	FCD IIB	11	CPS	I
2/M/31	FCD IIB	20	CPS	I
3/F/25	FCD IIB	9	CPS	I
4/F/22	FCD IIB	21	CPS/SGS	I
5/M/18	FCD IIB	14	CPS	I
6/M/17	FCD IIB	10	CPS	I
7/F/16	FCD IIB	11	CPS	I
8/M/29	FCD IIB	21	CPS	I
9/M/28	FCD IIB	21	CPS	I

FCD focal cortical dysplasia, *CPS* complex partial seizures, *SGS* secondary generalized seizures

material represents good disease control tissue, since it is exposed to the same seizure activity, drugs, fixation time, and the age and gender are also the same.

Tissue preparation

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Two representative paraffin blocks per case (containing the complete lesion or the largest part of the lesion resected at surgery) were sectioned, stained, and assessed. Paraffin-embedded tissue was sectioned at 6 µm, mounted on organosilane-coated slides (Sigma, St Louis, MO) and used for histological and immunocytochemical reactions as described below. Frozen tissue from control cortex and FCD tissue, stored at −80°C, was used for western blot analysis.

Antibody characterization

To document the presence of a heterogeneous population of cells, we used the following antibodies: glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4,000; monoclonal mouse, DAKO; 1:50), vimentin (mouse clone V9, DAKO; 1:1,000), MAP2 (polyclonal rabbit; Chemicon; 1:500), neuronal nuclear protein (NeuN; mouse clone MAB377, Chemicon, Temecula, CA, USA; 1:2,000), non-phosphorylated neurofilament (SMI311; Sternberger monoclonals, Lutherville, MD; 1:1,000), human leukocyte antigen (HLA)-DP, -DQ, -DR (CR3/43; monoclonal mouse, DAKO; 1:400), CD68 (mouse clone PG-M1, DAKO; 1:200) and CD31 (mouse clone JC70A, DAKO; 1:100).

For the detection of VEGFA, VEGFB, and their receptors, the following antibodies (Abs) were used: VEGFA (G153-694, monoclonal mouse; recognizing VEGF 165 and 189 [51], Pharmingen, CA, USA; 1:100), VEGFA (A-20, SC-152, polyclonal rabbit; raised against the N-terminus of VEGFA, recognizing VEGF 121, 165 and 189, Santa Cruz Bio., CA, USA; 1:100), VEGFB (H-70, SC-13083, polyclonal rabbit; raised against amino acids 1–70 of human VEGFB, Santa Cruz Bio.; 1:20), Flt-1 (VEGFR-1; C-17, SC-316, polyclonal rabbit, Santa Cruz Bio.; 1:100), Flk-1 (VEGFR-2; A-3, SC-6251, monoclonal mouse; Santa Cruz Bio.; 1:100). To allow comparative analysis, we used on frozen specimens of normal ($n = 3$) and FCD tissue ($n = 2$), two additional antibodies (which are not suitable for staining paraffin-embedded, formalin-fixed tissue): VEGFR-1 (clone Flt-19, 1:400; developed against the recombinant human extracellular domain of VEGFR-1) and VEGFR-2 (clone KDR-1, 1:400; developed against the recombinant human extracellular domain of VEGFR-2), kindly provided by Dr. H. A. Weich (National Research Center for Biotechnology, Braunschweig, Germany), and previously characterized on human tissues [49, 64, 80]. Similar immunoreactivity patterns were observed on paraffin-embedded and frozen tissue.

The specificity of the antibodies used for immunocytochemistry on paraffin-embedded, formalin-fixed tissue (VEGFA, VEGFB, VEGFR-1, VEGFR-2; Santa Cruz Bio.), was further tested by performing western blot analysis of total homogenates of human control cortex. We also include one FCD case of which sufficient frozen material for blot analysis was available (Fig. 1). VEGFR1 and VEGFR-2 receptor proteins were detectable as a band of approximately 180 and 200 kDa, respectively; VEGFB was detectable as a band of approximately 40 kDa; VEGFA labeled a prominent band at approximately 48 kDa and a

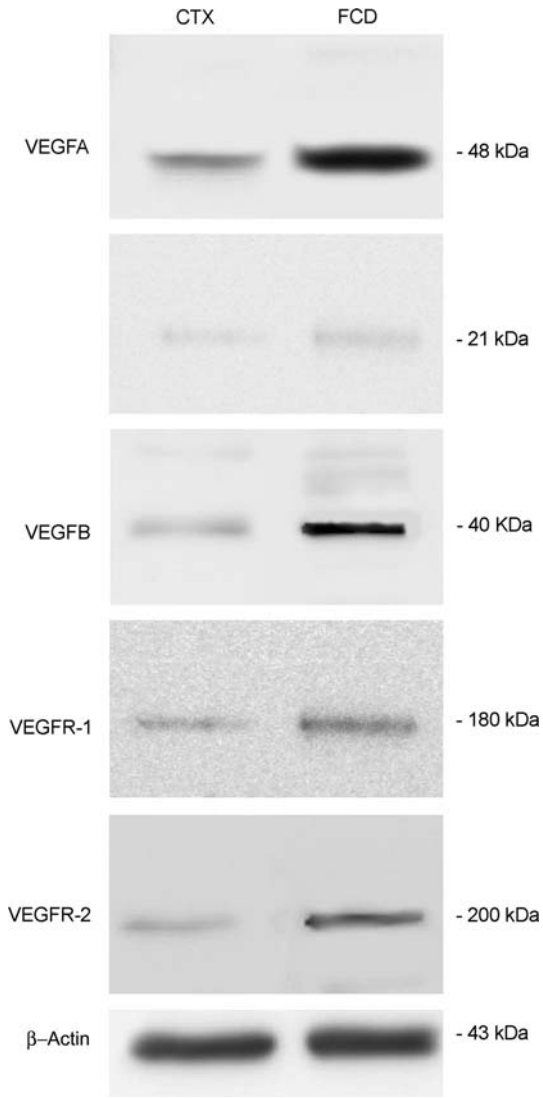

Fig. 1 Representative immunoblot of VEGFA, VEGFB, VEGFR-1, and VEGFR-2 in total homogenates from control cortex and FCD tissue. Expression of β-actin (as reference protein) is shown in the same protein extracts

light band at 21 kDa (reducing conditions), as recently reported in human brain tissue (control hippocampus and FCD; [56]). All immunoreactive bands disappeared after preadsorption with the corresponding peptide.

For immunoblot analysis, human normal cortex ($n = 3$) and FCD ($n = 1$) samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, Na-orthevanadate (10.4 mg/ml), 5 mM EDTA (pH 8.0), 5 mM NaF, and protease inhibitor cocktail (Boehringer Mannheim, Germany). Protein content was determined using the bicinchoninic acid method [65]. Non-reducing conditions were used to improve the detection of the VEGFA antibody, as previously reported [8]. For electrophoresis, equal amounts of proteins (30 µg/lane) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis in 7.5–12.5% gels. Separated proteins were transferred to nitrocellulose paper for 1 h and 30 min, using a semi-dry electroblotting system (BioRad, Transblot SD, Hercules, CA, USA). Blots were incubated overnight in TTBS (20 mM Tris , 150 mM NaCl, 0.1% Tween, pH 7.5)/5% non-fat dry milk, containing the primary antibody (VEGFA, VEGFR-1, and VEGFR-2, 1:1,000; VEGFB, 1:200). After several washes in TTBS, the membranes were incubated in TTBS/5% non-fat dry milk/1% BSA, containing the goat anti-rabbit coupled to horseradish peroxidase (1:2,500; Dako, Denmark) for 1 h. After washes in TTBS, immunoreactivity was visualized using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Expression of β-actin (monoclonal mouse, Sigma, St Louis, MO; 1:50,000) was used as reference protein. Because of the limited availability of frozen material from FCD cases, a complete analysis with statistical comparison between control and FCD by immunoblot could not be performed.

Immunocytochemistry

Sections were deparaffinized, re-hydrated, and incubated for 20 min in 0.3% H_2O_2 diluted in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed by incubation for 10 min at 121°C in citrate buffer (0.01 M, pH 6.0); sections were washed with phosphate-buffered saline (PBS), and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK). We incubated the sections with the primary antibodies overnight at 4°C. Hereafter, sections were washed in PBS and we used the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3′-diaminobenzidine as chromogen to develop the staining. Sections were counterstained with hematoxylin, dehydrated, and coverslipped. Sections incubated without the primary antibody and excess of the antigenic peptide were essentially blank.

For double-labeling studies, after incubation overnight at 4°C with the primary antibodies, sections were incubated for 2 h at RT with Alexa Fluor® 568-conjugated anti-rabbit IgG and Alexa Fluor® 488 anti-mouse IgG (1:1,000; Molecular Probes, The Netherlands). The VEGFR-2 antibody (monoclonal mouse) could only be combined with MAP2 and GFAP (polyclonal rabbit). Sections were analyzed by means of a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA; MRC1024) equipped with an argon-ion laser.

Evaluation of immunostaining

Semi-quantitative evaluation of immunoreactivity

As previously reported [3, 54], a semi-quantitative analysis was done using an Olympus microscope and examining in each section, high-power non overlapping fields (of 0.0655 × 0.0655 mm width, each corresponding to 4.290 µm^2), defined in the center of the lesion using a square grid inserted into the eyepiece. A total microscopical area of 858.050 µm^2 was assessed per case. Neuronal cell bodies were differentiated from glia and glia-neuronal balloon cells on the basis of morphology. Balloon cells have eccentric nuclei and ballooned opalescent eosinophilic cytoplasm. The staining intensity was evaluated using a semi-quantitative three-point scale where immunoreactivity was defined as: − absent (0); + moderate (1); ++ strong staining (2); intensity score (Table 2). This score represents the predominant cell staining intensity found in each section for the different cell types (neurons, astrocytes, microglial cells, and balloon cells) as averaged from the selected fields (as previously described [3, 54]).

Frequency of cell staining

In each slice, we assessed the number of neurons and astrocytes labeled by a specific Ab on the total number of each

Table 2 VEGFA, VEGFB, VEGFR-1, and VEGFR-2 distribution in different cellular types in cases of FCD (% of cases with immunoreactive cells)

	Focal cortical dysplasia ($n = 9$)								
	Neurons			Astrocytes			Balloon cells		
	−	+	++	−	+	++	−	+	++
VEGFA	0	22%	78%	0	11%	89%	0	11%	89%
VEGFB	0	33%	67%	78%	22%	0	78%	22%	0
VEGFR-1	0	55%	45%	0	22%	78%	0	33%	67%
VEGFR-2	0	11%	89%	55%	45%	0	22%	45%	33%

FCD focal cortical dysplasia; immunoreactivity: − not present, + moderate, ++ strong

cell type within the lesion using an ocular grid [4]. This frequency score was assigned using three distinct categories: (1) <10%, rare; (2) 11–30%, sparse; (3) >30%, high. The product of the intensity and the frequency scores was taken to give the total immunoreactivity score, as previously reported [23, 54].

For statistical analysis of data, SPSS for Windows was used. Data were compared using a non-parametric Kruskal–Wallis test followed by a Mann–Whitney test to assess the difference between groups. $P < 0.05$ was taken as the level of significance.

Results

Human material and histological features

All nine patients had chronic pharmacoresistant epilepsy and were all seizure-free postoperatively (Engel's class I; Table 1). The FCD cases included in this study have all the histopathological features of severe (type IIB) FCD, according to the classification of Palmini et al. [48]. The resected specimens consisted of disorganized neocortex containing immature neurons, giant neurons, dysmorphic neurons, and balloon cells. Neurons and balloon cells were also observed in the subcortical white matter and there was a prominent population of reactive astrocytes. Cells of the microglia/macrophage lineage were also observed within the dysplastic cortex, suggesting activation of inflammatory processes in FCD [6].

Expression of VEGF and VEGFR in normal temporal cortex and FCD

Cellular distribution of VEGFA

VEGFA staining was observed within the histologically normal cortex (Fig. 2a, b). The staining was strongest in pyramidal neurons, which displayed somatic staining and staining of the apical dendrites (Fig. 2b). Neuropil staining was weak and resting glial cells did not show VEGFA immunoreactivity (IR). Weak staining was observed in endothelial cells. Autopsy material and the perilesional cortex showed similar IR.

In the majority of FCD cases, strong VEGFA immunoreactivity (IR) was observed in dysplastic neurons located throughout the dysplastic cortex (Fig. 2c–e; Table 2; Fig. 6). Strong staining was also detected in balloon cells, reactive astrocytes, and in perivascular astrocytic end-feet (Fig. 2f–i; Table 2; Fig. 6). Endothelial IR was weak. Double-labeling experiments confirmed expression in reactive astrocytes, neurons, and in CD68+ macrophages (Fig. 2p). Immunocytochemistry using two different antibodies to VEGF (Pharmingen and Santa Cruz Bio.) demonstrated similar patterns.

Cellular distribution of VEGFB

Histologically normal cortex displayed only weak VEGFB IR (Fig. 3a). Both autopsy and surgical specimens showed light staining in pyramidal neurons and in endothelial cells. Glial cells did not show VEGFB IR.

In FCD specimens, moderate to strong VEGFB IR was observed within the dysplastic cortex (Fig. 3b; Table 2; Fig. 6) with strong VEGFB IR in dysplastic neurons (Fig. 3c). In the majority of cases (seven out of nine), balloon cells and reactive astrocytes did not express VEGFB (Fig. 3d; Table 2; Fig. 6). Double-labeling experiments confirmed the absence of VEGFB IR in GFAP-positive cells (astrocytes), whereas co-localization was found with neurofilament in dysplastic neurons (Fig. 3h–j). VEGFB IR was also observed in CD68+ macrophages (Fig. 3k).

Cellular distribution of VEGFR-1

Histologically normal cortex (autopsy and surgical specimens) displayed only weak VEGFR-1 IR, which was restricted to pyramidal neurons (Fig. 4a). IR in blood vessels was weak (Fig. 4b). Glial cells did not show VEGFR-1 IR.

In FCD specimens, moderate to strong VEGFR-1 staining was observed within the dysplastic cortex (Fig. 4c–g; Table 2; Fig. 6). VEGFR-1 IR was observed in different cell types, including dysplastic neurons, astrocytes, and endothelial cells. Double-labeling experiments confirmed the co-localization of VEGFR-1 IR with neuronal (inset in Fig. 4c), endothelial (inset in Fig. 4d), and glial (Fig. 4h–j) markers. Additionally, expression of VEGFR-1 was observed in CD68+ macrophages (Fig. 4k–m).

Cellular distribution of VEGFR-2

VEGFR-2 staining was observed within the histologically normal cortex (autopsy and perilesional zone) in pyramidal neurons (Fig. 5a). IR in blood vessels was weak (Fig. 5b). Glial cells did not show VEGFB IR.

In the large majority of FCD cases (eight out of nine), VEGFR-2 was strongly expressed in dysplastic neurons (Fig. 5c, e; Table 2; Fig. 6). VEGFR-2 IR was also detected in balloon cells, but only three out of nine cases displayed strong staining for VEGFR-2 (Fig. 5f; Table 2). Endothelial expression was weak. In many FCD cases (five out of nine), expression of VEGFR-2 was undetectable in reactive astrocytes (Table 2). Double-labeling experiments confirmed the co-localization of VEGFR-2 IR with neuronal markers

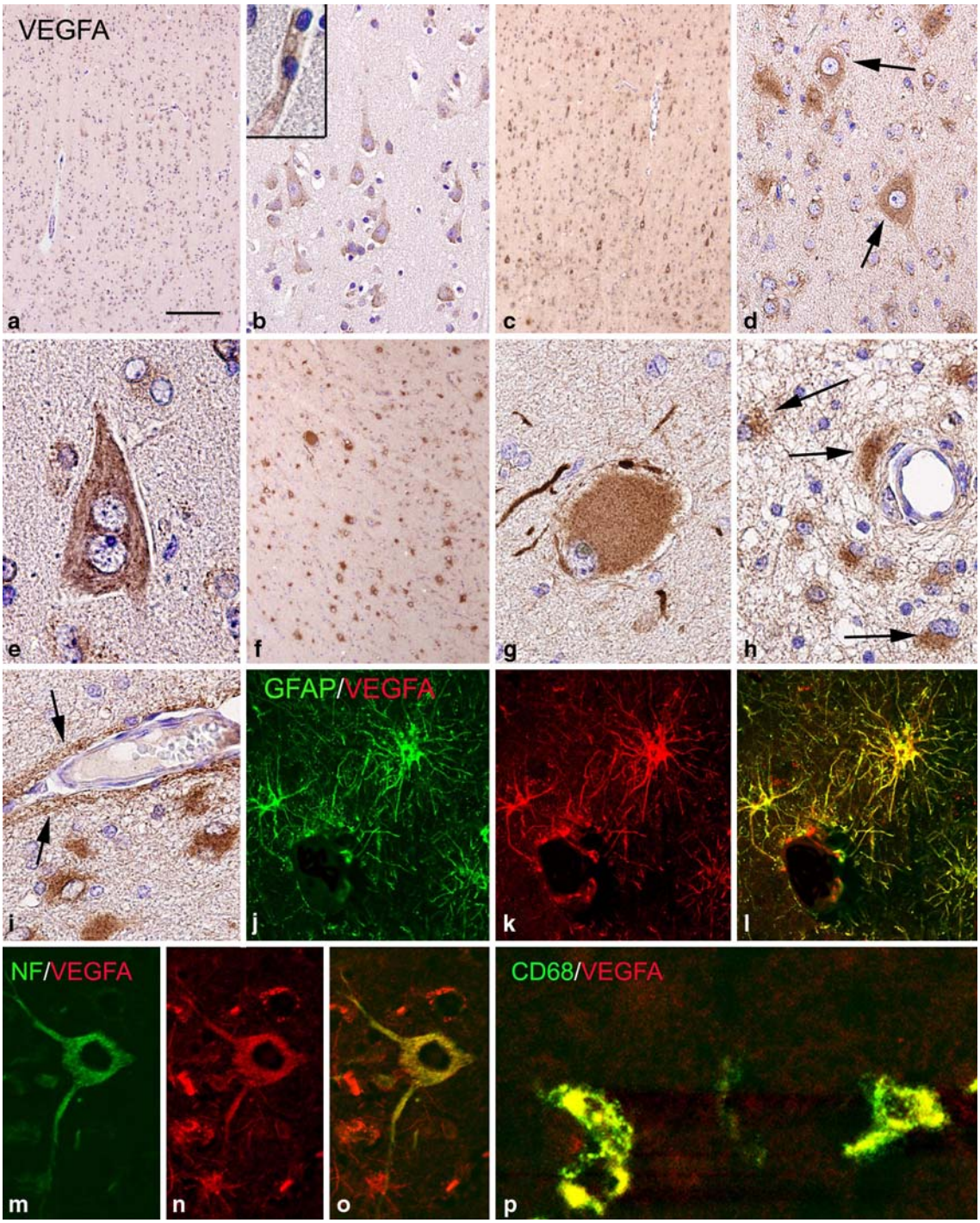

Fig. 2 VEGFA immunoreactivity in focal cortical dysplasia type IIB. **a** VEGFA immunoreactivity (IR) within the histologically normal adult cortex. **b** High magnification showing somatic staining in pyramidal neurons (*inset*: vascular staining). **c** VEGFA in focal cortical dysplasia (FCD; type IIB) showing strong IR within the dysplastic cortex. **d** High magnification showing VEGFA IR in dysplastic neurons (*arrows*). **e** A binucleated VEGFA positive dysplastic neuron. **f** VEGFA IR within the subcortical dysplastic region. **g** VEGFA expression in a balloon cell. **h**, **i** VEGFA expression in reactive astrocytes (*arrows* in **i** indicate perivascular astrocytic end-feet) **j–l** double-labeling of GFAP (*green*, **j**) with VEGFA (*red*, **k**) shows co-localization (*yellow*, **l**) in astrocytes. **m–o** Double-labeling of non-phosphorylated neurofilament (SMI311; NF, *green*, **m**) with VEGFA (*red*, **n**) shows co-localization (*yellow*, **o**) in dysplastic neurons. **p** Merged image showing co-localization of CD68 (*green*) with VEGFA (*red*) in macrophages. *Scale bar* in **a** a, c, f 400 μm; b, d 120 μm; e, g–i 35 μm; j–o 40 μm; p 18 μm

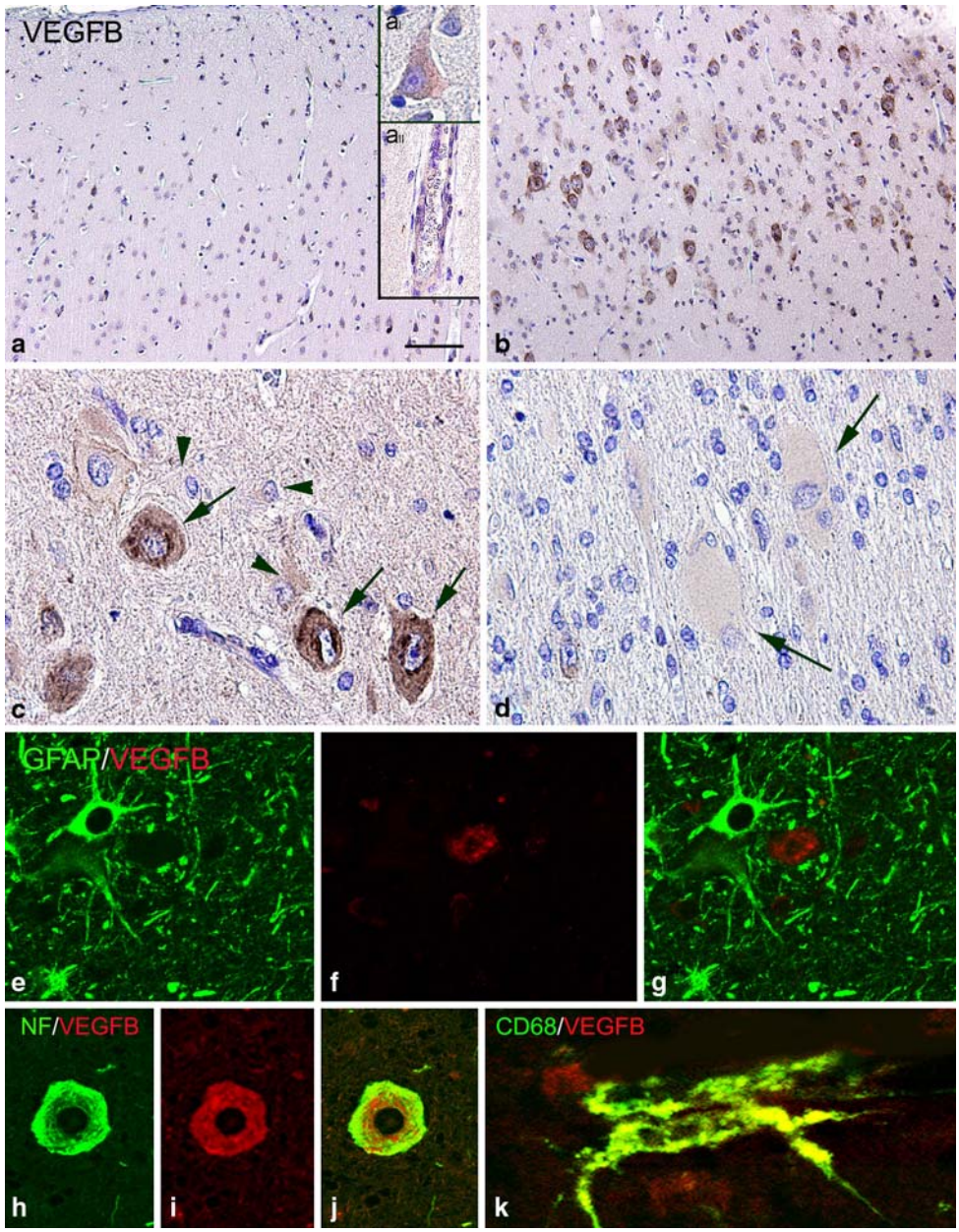

Fig. 3 VEGFB immunoreactivity in focal cortical dysplasia type IIB. **a** Histologically normal adult cortex, showing neuronal distribution of VEGFB with weak immunoreactivity (IR) in pyramidal cell neurons (high magnification of a pyramidal neuron is shown in the *inset aI*); weak staining was also observed in blood vessels (*inset aII*). **b** VEGFB in focal cortical dysplasia (FCD; type IIB) showing strong IR within the dysplastic cortex. **c** Strong VEGFB IR in dysplastic neurons of different size and shape (*arrows*). **d** Undetectable VEGFB IR in balloon cells (*arrows*). **e–g** Absence of co-localization between GFAP (*green*, **e**) with VEGFB (*red*, **f**) in astrocytes (**g**, merged image). **h–j** Double-labeling of non-phosphorylated neurofilament (SMI311; NF, *green*, **h**) with VEGFB (*red*, **i**) shows co-localization (*yellow*, **j**) in dysplastic neurons. **k** Merged image showing co-localization of CD68 (CD68; *green*) with VEGFB (*red*) in macrophages. *Scale bar* in **a** a, b 200 μm; c–j 40 μm; k 18 μm

(neurofilament or MAP2, Fig. 5e, g–i) within the dysplastic cortex.

Discussion

In addition to their role in angiogenesis, VEGF proteins and their receptors have been implicated in several neurological disorders, including epilepsy [13, 44, 56]. In the present study, we demonstrate a prominent expression of VEGFA, VEGFB, and their signaling receptors in FCD type IIB, a malformation of cortical development associated with intractable epilepsy. This is particularly interesting in view of the recently proposed role of VEGFs and their signaling pathways during development and in epilepsy-associated pathologies [13, 26, 33, 69, 76].

Expression of VEGFA and VEGFB in normal temporal cortex

In histologically normal temporal cortex (autopsy and perilesional zone), we have shown weak expression of both VEGFA and VEGFB in cortical neurons. Expression of VEGFA and VEGFB, including both mRNA and protein, has been demonstrated in neurons in adult rodent brain [39, 41, 42, 72]. In human adult brain, only few studies have described neuronal expression of VEGFA in control tissue [8, 78], which was similar to

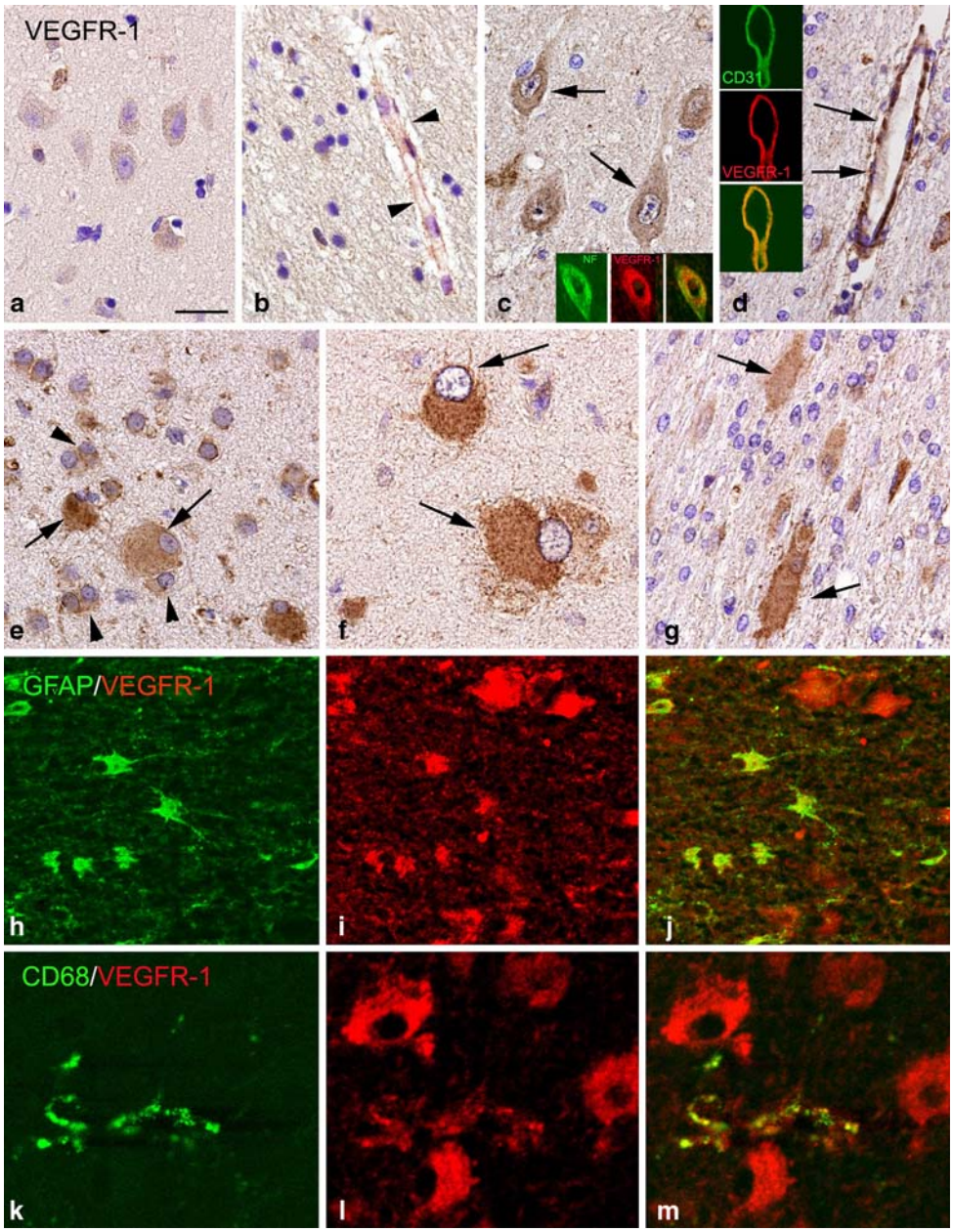

Fig. 4 VEGFR-1 immunoreactivity in focal cortical dysplasia type IIB. **a**, **b** Histologically normal adult cortex (**a**) and white matter (**b**) showing weak immunoreactivity (IR) in neurons (**a**) and blood vessels (**b**; *arrowheads*). **c**, **d** VEGFR-1 in focal cortical dysplasia (FCD; type IIB) showing strong IR in dysplastic neurons (**c**; *arrows*) and in blood vessels (**d**; *arrows*). *Inset* in **c** Co-localization between non-phosphorylated neurofilament (SMI311; NF, *green*) with VEGFR-1 (*red*) in dysplastic neurons. *Inset* in **d** Co-localization between CD31 (endothelial marker; *green*) with VEGFR-1 (*red*) in blood vessels. **e**–**g** Strong IR in balloon cells of different size (*arrows*) and glial cells (*arrowheads* in **e**). **h**–**j** Co-localization between GFAP (*green*, **h**) with VEGFR-1 (*red*, **i**) in astrocytes (**j**, merged image). **k**–**m** Co-localization of CD68 (CD68; *green*, **k**) with VEGFR-1 (*red*, **l**) in macrophages (**m**, merged image). *Scale bar* in **a** a–d and h–m 40 μm; e–g 35 μm

our observed staining pattern in the control temporal specimens.

To our knowledge, previous studies of VEGFB protein expression in human control cortex have not been described. However, VEGFB mRNA was detected in human hippocampal cortex [24], and VEGFB mRNA and protein expression has been described in adult rodent brain [36, 42]. In control rat brain, VEGFB was constitutively expressed in endothelial cells [42]. In our study, we observed only weak endothelial VEGFB IR in blood vessels. In agreement with previous studies [8, 36, 78], immunoreactivity (IR) for both VEGFs was not observed in glial cells within control specimens.

Differential cellular distribution of VEGFA and VEGFB in FCD

In the present study, we provide evidence for a consistent expression of both VEGFA and VEGFB within the dysplastic cortex of patients with FCD. Both VEGFs are highly expressed in dysplastic neurons; however, only the VEGFA protein is prominently expressed in reactive astrocytes. Expression of VEGFA in astrocytes has been shown in several other pathologies associated with reactive gliosis, such as ischemic stroke, traumatic brain injury, neurodegenerative disorders, and the hippocampus following entorhinal deafferentation [8, 27, 59, 78, 79]. In addition, we previously

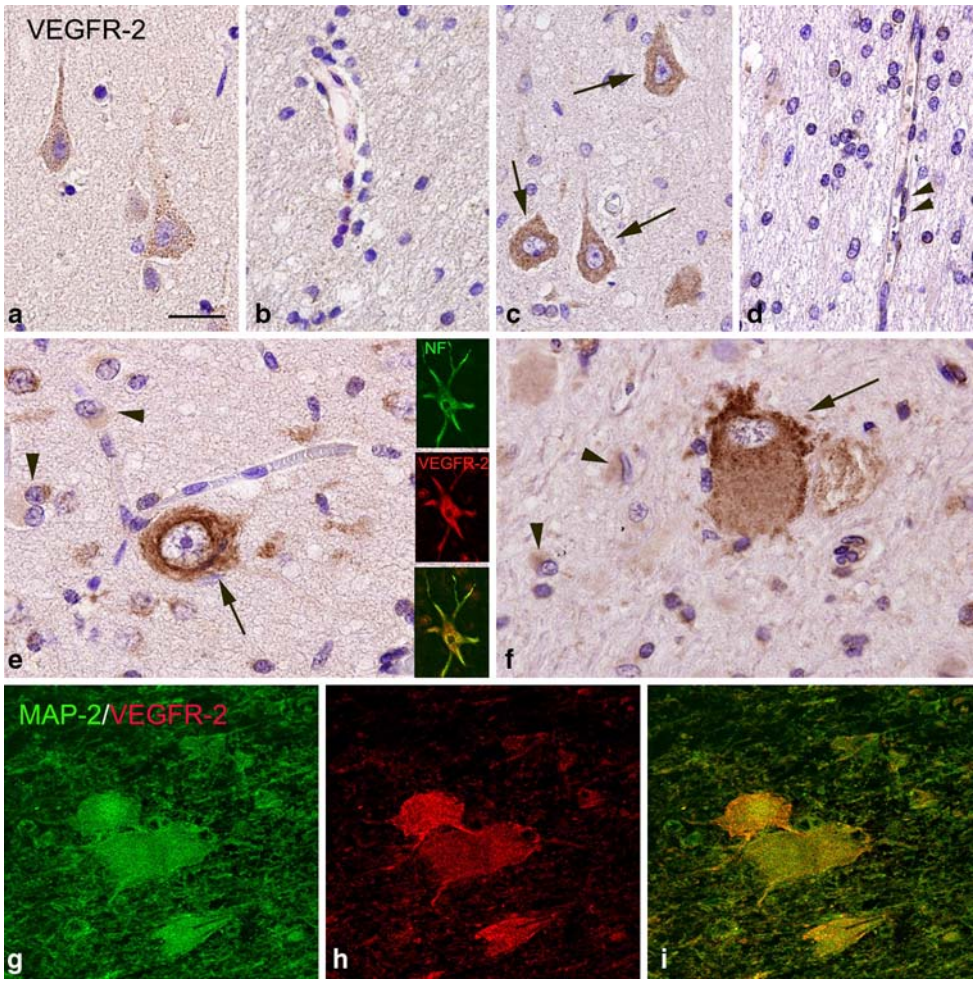

Fig. 5 VEGFR-2 immunoreactivity in focal cortical dysplasia type IIB. **a**, **b** Histologically normal adult cortex (**a**) and white matter (**b**) showing moderate immunoreactivity (IR) in pyramidal cells (**a**); in the white matter, endothelial IR was weak and glial IR was under detection level (**b**). **c**, **d** VEGFR-2 in focal cortical dysplasia (FCD; type IIB) showing strong IR in dysplastic neurons (*arrows* in **c**), but weak IR in blood vessels (*arrowheads* in **d**). **e**, **f** Strong IR in a dysplastic neuron (*arrow* in **e**) and in a balloon cell (*arrow* in **f**), but weak IR in glial cells (*arrowheads* in **e**, **f**). *Inset* in **e** Co-localization between non-phosphorylated neurofilament (SMI311; NF, *green*) with VEGFR-2 (*red*) in a dysplastic neuron. **g–i** Co-localization between MAP-2 (*green*, **g**) with VEGFR-2 (*red*, **h**) in balloon cells (**i**, merged image). *Scale bar* in **a** a–d and g–i 40 µm; e, f 35 µm

reported upregulation of both neuronal and glial VEGFA expression in patients with hemimegalenchephaly, an epilepsy-associated malformation of cortical development [5, 7]. In the present study, we also observed expression of VEGFA in balloon cells, which are characteristic cell types of severe FCD [48]. Whether these cells are glial or neuronal in nature is still controversial [12].

Induction of both neuronal and astroglial VEGFA expression has been shown in different experimental models of seizures and human temporal lobe epilepsy (TLE) [13, 44, 56]. Rigau et al. [56] showed increased levels of VEGFA in the hippocampus of several cases of TLE, including two cases of focal dysplasia. All TLE cases showed VEGFA expression in pyramidal neurons and granule cells of the hippocampus [56]. However, immunocytochemical analysis of the temporal cortex and the FCD cases was not performed.

The molecular mechanism underlying the induction of VEGFA expression after seizures remains unclear. One possible mechanism, which has been proposed to explain the association between seizure activity and the induction of VEGFA expression, is represented by the stabilization of the hypoxia inducible factor-1α (HIF-1α). HIF-1α is a transcription factor which upregulates VEGFA transcription under hypoxic conditions [21, 38, 61]. Hypoxia may occur during seizures, representing an important trigger in the induction of VEGFA expression, particularly in case of long lasting seizures, such as in status epilepticus models. However, VEGFA expression is already induced after acute seizures [44] and the mechanisms that regulate VEGFA expression are complex. Several transcription factors, including AP-1, HIF-1α, and NF-κB, have been identified to regulate VEGFA expression [31], and recently it has also been shown that inflammatory cytokines, such as interleukin-1β (IL-1β), activate *HIF1α* and *VEGFA* gene expression in primary human astrocytes [2]. Interestingly, increased expression of proinflammatory cytokines and related molecules has been reported in both animal models and human epilepsy-associated pathologies, including FCD [3, 15, 54, 77]. In addition, VEGFA has been demonstrated to be a key mediator of the inflammatory process [14, 35]. Thus, we might speculate that the prominent expression of VEGFA within the dysplastic cortex could be a critical component of the complex cascade of events leading to a chronic inflammatory state and the sustained seizure activity

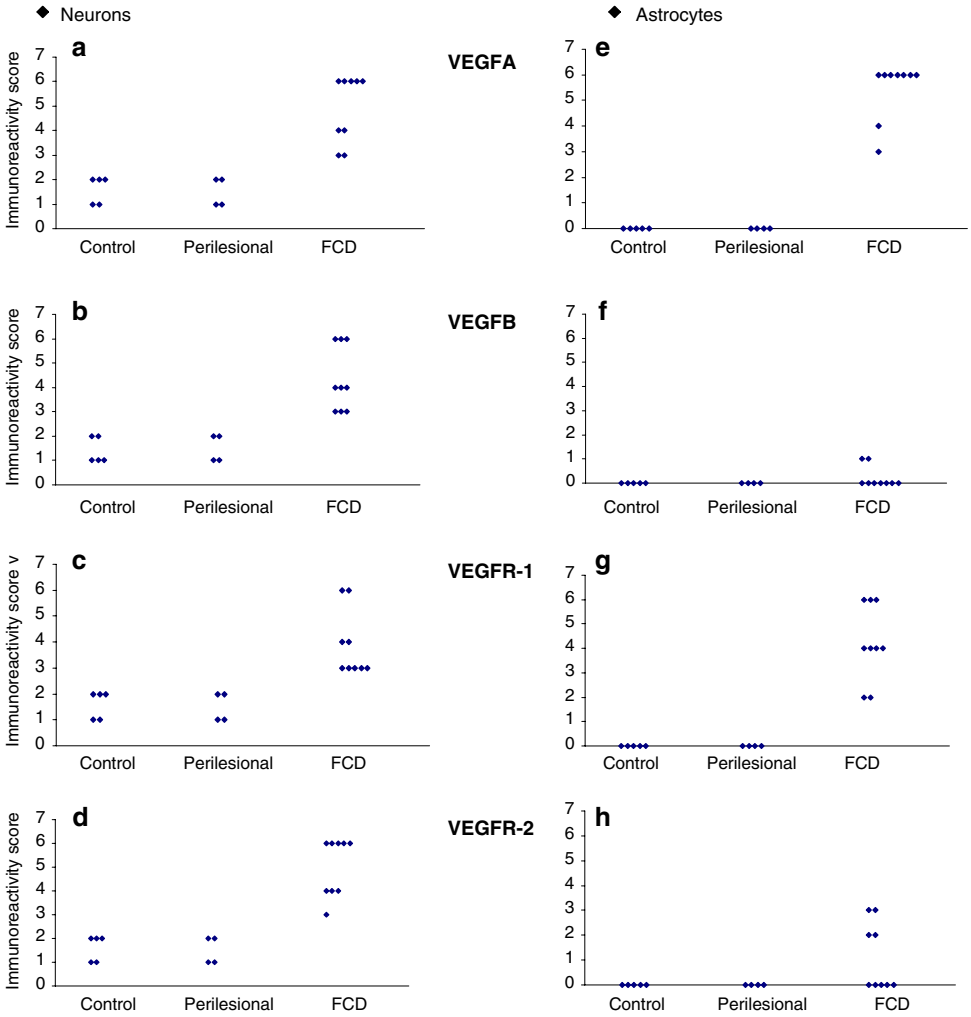

Fig. 6 Distribution of immunoreactivity scores (total score; see details in "Materials and methods" section) in neurons and astrocytes of control, perilesional, and FCD specimens. **a**, **e** VEGFA; **b**, **f** VEGFB; **c**, **g** VEGFR-1; **d**, **h** VEGFR-2. **a**–**d** Neurons; **e**–**h** astrocytes. IR scores of VEGFs and VEGFRs in neurons of FCD were greater than IR scores of VEGFs and VEGFRs of control and perilesional cortex; scores of VEGFA and VEGFRs in astrocytes of FCD were greater than IR scores of VEGFA and VEGFRs of control and perilesional cortex ($P < 0.05$). There were no significant differences in IR scores of VEGFs and VEGFRs between control and perilesional cortex

[76, 77]. With respect to inflammation, inflammatory cells, such as macrophages, can also release various angiogenic cytokines including VEGFs [71]. Accordingly, we observed expression of both VEGFA and VEGFB in macrophages (CD68-positive cells), as previously shown in animal models of brain ischemia [11, 42, 52].

VEGFA effects can also compromise the integrity of the blood–brain barrier (BBB; [60]). Interestingly, alterations of the BBB permeability have been recently observed in both human and experimental temporal lobe epilepsy with positive correlation between the increased vascular permeability and the occurrence of spontaneous seizures in chronic epileptic rats [55, 56, 75].

In contrast, several studies highlight a dichotomous function of VEGFA, also demonstrating a neuroprotective role [13, 47]. Administration of VEGFA and neuronal expression of VEGFA have been shown to stimulate neurogenesis in vitro and in vivo [9, 28]. In addition, it has been suggested that the neuroprotective effects of VEGFA are mediated by the neuronal VEGFR-2 and the subsequent activation of the PI3K/Akt survival pathway [32, 67].

VEGFB expression is not induced by hypoxia or several transcription factors known to regulate VEGFA expression [18], as the promoter region of VEGFB lacks HIF-1 and AP-1 sites [43, 62]. The regulation of the expression of VEGFB remains unknown. Since all cases examined were associated with epilepsy, we cannot exclude that chronic seizure activity could also contribute to the VEGFB expression in FCD specimens. Alternatively, since VEGFB expression has been shown to be prominent during early brain development [36], the strong neuronal expression of VEGFB could represent an intrinsic and immature feature of the dysplastic neuronal cells that could contribute to their survival. Recent studies using VEGFB knock-out mice demonstrate a neurotrophic and neuroprotective activity of VEGFB, exerting a direct action on neurons, and promoting neurogenesis [68, 69].

This is an observational study and we were, therefore, not able to investigate the spatio-temporal regulation of the VEGF system. Further research in animal models of cortical dysplasia is clearly needed to elucidate the role of VEGFs and their signaling pathways in the histogenesis or epileptogenesis of developmental disorders.

Expression of VEGF receptors in normal temporal cortex

In histologically normal temporal cortex (autopsy and perilesional zone), VEGFR-1 and VEGFR-2 showed a similar pattern of expression, with weak to moderate immunostaining in pyramidal neurons. Neuronal expression of VEGFRs' mRNA and protein has been reported in adult human and rodent brain, with strong expression in the hippocampus [8, 11, 78, 79]. In agreement with these studies, we did not detect glial VEGFR expression in histologically normal cortex and only weak VEGFR expression was observed in endothelial cells.

Differential cellular distribution of VEGFR-1 and VEGFR-2 in FCD

Consistent expression of both VEGFR-1 and VEGFR-2 was detected within the dysplastic cortex of patients with FCD. Both receptors were upregulated in dysplastic neurons. Increased expression of the VEGFRs and, in particular VEGFR-1, was observed in reactive astrocytes. Upregulation of VEGFRs in neurons and reactive astrocytes has been shown in several other pathological conditions including ischemia, neurodegenerative diseases, and trauma [8, 11, 37, 39, 66, 79]. Recently, increased expression of VEGFR-2 has been shown in several cases of TLE, including two cases of cortical dysplasia [56]. Immunocytochemical analysis demonstrated expression only in endothelial cells, whereas neuronal VEGFR-2 IR was not detected in either control hippocampus or TLE specimens [56]. Differences in the phenotypes of cells expressing VEGFRs have been observed in several other studies [8, 30, 52]. These discrepancies may be caused by differences in experimental methods, tissue processing, or the use of different antibodies that recognize different epitopes.

Our results support the neuronal expression of VEGFR-2 recently reported in human brain [8]. The similar expression pattern of VEGFA and VEGFR-2, with prominent neuronal IR, suggests autocrine/paracrine effects on dysplastic neurons, supporting the hypothesis of a mechanism to protect abnormal neurons from cell death associated with seizures. Autocrine and/or paracrine effects of VEGFA are supported by the observation that administration of VEGFA has been shown to induce mRNA and protein expression of both receptors in adult rat brain [34, 57]. A protective mechanism of VEGFA has been suggested in epileptic rats, showing that VEGFA may reduce spontaneous discharges in epileptic rats [40]. Therefore, upregulation of VEGFA could represent an endogenous compensatory mechanism to reduce excitability and to prevent cell loss after severe seizures. Accordingly, infusion of VEGFA into the hippocampus has been shown to protect against neuronal cell loss after pilocarpine-induced status epilepticus [45].

In the present study, we also provide evidence for the expression of VEGFR-1 in activated cells of the microglia/macrophage lineage, which have been shown to be present in FCD specimens [6]. This is in agreement with previous in vitro and in vivo studies [11, 20] showing VEGFR-1 expression in activated microglial cells. These observations suggest that the microglia/macrophage lineage is also a target for VEGF, which may affect chemotaxis and proliferation of these cells, contributing to the inflammatory state in the epileptic brain.

There is substantial information about the function and the signaling through VEGFR-2; in contrast, signaling through VEGFR-1 remains poorly understood and has been a matter of discussion. A decoy role has been proposed for VEGFR-1, but more recently, functional signaling via VEGFR-1 has been reported (for reviews see [43, 67]). These observations may give rise to new therapeutic strategies focusing on VEGFR-1 specific ligands, such as VEGFB [43].

Although a rapid induction of VEGF and its receptors has been shown in different experimental models of seizures [13, 44, 56], seizures alone cannot account for changes in neuronal and glial expression in FCD since perilesional tissue was exposed to seizures but did not show significant upregulation of VEGFs and/or VEGFRs. Therefore, the lesion per se or the concomitant presence of the lesion and the epileptic activity, are likely to play a role in modulating the VEGF system in these developmental disorders.

Conclusions

Our observed cellular distribution of VEGFA, VEGFB, and their signaling receptors indicate that different cellular components of FCD are involved in VEGF-signaling. In this context, future studies, using both in vivo and in vitro models, will be important to achieve a better understanding of the role of the VEGF-mediated pathways in the histogenesis and epileptogenesis of developmental lesions associated with intractable chronic epilepsy. Presently, signaling via VEGF receptors is not targeted by existing therapies in epileptic patients, but it can be potentially useful in view of its involvement in the regulation of neurogenesis, inflammation, and BBB integrity. However, an effective therapeutic intervention based on modulation of the VEGF system has to take into consideration the specific role of VEGFA and VEGFB and the multiple effects (protective and/or detrimental) reported for VEGFA.

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