

# Expression of Vascular Endothelial Growth Factor Receptor-3 mRNA in the Developing Rat Cerebellum

Yun Hou · Jeong-Sun Choi · Yoo-Jin Shin ·  
Jung-Ho Cha · Jae-Youn Choi · Myung-Hoon Chun ·  
Mun-Yong Lee

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**Abstract** Vascular endothelial growth factor receptor (VEGFR)-3, a receptor for VEGF-C and VEGF-D, has recently been suggested to play an important role during neuronal development. To characterize its potential role in CNS ontogenesis, we investigated the spatiotemporal and cellular expression of VEGFR-3 in developing and mature rat cerebellum using in situ hybridization. VEGFR-3 expression appeared as early as E15, and was restricted to the ventricular zone of the cerebellar primordium, the germinative neuroepithelium, but was absent by E20. Instead, the expression area of VEGFR-3 in the cerebellum grew in parallel with cerebellar development. From E20 on, two populations of VEGFR-3-expressing cells can be clearly distinguished in the developing cerebellum: a population of differentiating and postmitotic neurons and the Bergmann glia. VEGFR-3 expression in neurons occurred during the period of neuronal differentiation, and increased with maturation. In particular, the expression of VEGFR-3 mRNA revealed different temporal patterns in different neuronal populations. Neurons generated early, Purkinje cells, and deep nuclear neurons expressed VEGFR-3 mRNA during late embryonic stages, whereas VEGFR-3 transcription in local interneurons appeared by P14 with weaker expression. In addition, Bergmann glia expressed VEGFR-3 throughout cerebellar maturation into adulthood. However, receptor expression was absent in the progenitors in the external granular layer and during further migration. The results of this study suggest that VEGFR-3

has even broader functions than previously thought, regulating both developmental processes and adult neuronal function in the cerebellum.

**Keywords** Purkinje cells · Bergmann glia · Deep cerebellar nuclei · Ventricular zone · Cerebellum · Development

## Introduction

The vascular endothelial growth factor (VEGF) family currently comprises seven members: VEGF-A, -B, -C, -D, viral VEGF (also known VEGF-E), snake venom VEGF (also known as VEGF-F), and placental growth factor (reviewed in Roy et al. 2006; Yamazaki and Morita 2006; Raab and Plate 2007). VEGF-C, a new VEGF member, is a main specific lymphangiogenic factor, by activating both VEGFR-2 (fetal liver kinase receptor-1; Flk-1) and VEGFR-3 (fms-like tyrosine kinase-1; Flt-4). Although much is known concerning the role of VEGF-A in the developing and adult brain (Brockington et al. 2004; Yasuhara et al. 2004), little is known about the function of VEGF-C in the brain. VEGF-C was initially identified as a specific regulator of lymphangiogenesis (Veikkola et al. 2001; Karkainen et al. 2004), a recent study demonstrated a newly discovered role for VEGF-C in the developing brain (Le Bras et al. 2006). VEGF-C and VEGFR-3 are expressed in neural progenitor cells of *Xenopus laevis* and mouse embryos, and VEGF-C deficiency leads to a severe defect in the proliferation of neuroepithelial cells expressing VEGFR-3 (Le Bras et al. 2006).

Very recently, we demonstrated the expression of VEGFR-3 in the forebrain and retina during development (Choi et al. 2010). VEGFR-3 is expressed in the ventricular

Y. Hou · J.-S. Choi · Y.-J. Shin · J.-H. Cha · J.-Y. Choi ·  
M.-H. Chun · M.-Y. Lee (✉)  
Department of Anatomy, College of Medicine,  
The Catholic University of Korea, 505 Banpo-dong,  
Socho-gu, 137-701 Seoul, Korea  
e-mail: munylee@catholic.ac.kr

(VZ) and subventricular zones (SVZ) and in postmitotic neurons in the developing cortex. Interestingly, most cells expressing VEGFR-3 in the VZ and SVZ of the lateral ventricle expressed nestin and glutamine synthetase (GS), which label radial glia in embryonic stages (Akimoto et al. 1993; Supèr et al. 2000; Mori et al. 2005). Radial glia are regarded as multipotential progenitors giving rise to both neurons and glia in the neocortex (reviewed in Barres 1999; Alvarez-Buylla et al. 2001; Tamamaki et al. 2001; Campbell and Götz 2002; Mori et al. 2005). In addition to radial glia in embryonic brain, Müller glial cells—specialized radial glial cells—exhibited weak VEGFR-3 signals in the mature retina. Thus, our previous study strongly suggests that VEGFR-3 might be involved in neuronal precursor cell proliferation and neurogenesis in association with the radial glia in the developing brain.

VEGFR-3 was shown to be abundant in the developing forebrain (Choi et al. 2010), but no data are available that describe the developmental expression of VEGFR-3 in the cerebellum, which has been extensively studied as a model system of diverse events for neuronal proliferation, migration, differentiation, and synapse formation during development (Altman and Bayer 1987; Yamada and Watanabe 2002; Bellamy 2006). Thus, to characterize its potential role in central nervous system (CNS) ontogenesis, we investigated the spatiotemporal and cellular expression of VEGFR-3 in developing and mature rat cerebellum using *in situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR) techniques. Particular attention was paid to its distribution in Bergmann glia because they are radial astrocytes developed from the radial glia in the ventricular zone and provide guidance for granule neuron migration and forming synapses with Purkinje cell dendrites (Yamada and Watanabe 2002; Bellamy 2006).

## Methods

### Experimental Animals and Tissue Processing

All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea and were in accordance with the US National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Pregnant Sprague–Dawley rats with the exact date of conception known were purchased from Orient Bio Inc. (Seongnam-Si, Korea). The day of vaginal plug discovery was considered embryonic day (E) 0. For *in situ* hybridization histochemistry, 54 rats of various ages (embryonic days E15, E17, E20, postnatal days (P) 1, P3, P7, P14, P28,

P56;  $n = 6$  each) were used. Pregnant animals were anesthetized with 4% (w/v) chloral hydrate (1 ml/100 g body weight, intraperitoneally). Embryonic pups were removed from the mothers, killed by decapitation and immediately fixed in 4% (v/v) paraformaldehyde buffered with 0.1-M phosphate buffer (PB, pH 7.2) overnight at 4°C. Postnatal rat pups were anesthetized with ether or with 4% chloral hydrate. The rats were perfused through the left ventricle with 4% paraformaldehyde buffered with 0.1-M PB. Brains were dissected and postfixed in the same fixative for 5 h at 4°C, and equilibrated with 30% sucrose in 0.1-M PB, and frozen until used.

### Semi-Quantitative RT-PCR

Semi-quantitative RT-PCR analysis was carried out as described previously (Shin et al. 2008). In brief, total RNA was extracted from whole cerebellum homogenates of rats at several stages of development (E20, P1, P7, P56;  $n = 3$  each) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using Reverse Transcriptase M-MLV (Takara Korea Biomedical Inc., Korea) according to the manufacturer's instructions. Equal amounts (1  $\mu$ l) of the reverse transcription products were then PCR-amplified using Perfect Premix Version 2.1 (Ex Taq version; Takara Korea Biomedical Inc.). One picomole of primer, which was specific for rat VEGFR-3 (sense, 5'-ctgaggcagaatcatcagtctggag-3', antisense, 5'-agatgctca-tactgttagttgcc-3'; GenBank accession no. NM\_053652.1; nucleotides 1222–1763), was used in the amplification reaction. Amplification commenced with denaturation at 94°C for 4 min followed by 25–30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The final extension was made at 72°C for 10 min. Ten microliters of each PCR reaction product were electrophoresed on 1.5% (w/v) agarose gels containing ethidium bromide (1  $\mu$ g/ $\mu$ l). For semiquantitative measurements, we amplified the VEGFR-3 mRNAs with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and optimized the number of PCR cycles to maintain amplification within a linear range. RT-PCR products were quantified by photographic densitometry of the ethidium bromide-stained agarose gel, and VEGFR-3/GAPDH product ratios were calculated as indices of VEGFR-3 mRNA expression. Three animals were used for PCR at each time point and each sample per animal was tested in triplicate. The average value for the optical density was calculated separately for every animal before the means and standard errors were determined for the total number of animals ( $n = 3$ ) included per group. Results were expressed as a percentage of expression in E20 cerebellum. Statistical significance was analyzed by analysis of variance (ANOVA) followed by Dunnett's *t* test;  $P < 0.05$  was regarded as significant.

### In Situ Hybridization Histochemistry

The sequences for VEGFR-3 from RT-PCR product corresponding to nucleotides 1222–1763 (see above) were cloned into the TA site of pGEM-T Easy vector (Promega Co., Madison, WI, USA), and sequenced. The antisense and sense riboprobes were labeled with digoxigenin (DIG) using *in vitro* transcription, as described in detail previously (Shin et al. 2008).

Coronal or sagittal cryostat sections through the cerebellum (30- $\mu$ m thick) were hybridized with antisense or sense probes diluted in hybridization solution (150 ng/ml) at 52°C for 18 h. Hybridization was visualized using an alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche, Germany; dilution 1:2000) with 4-nitroblue tetrazolium chloride (0.35 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.18 mg/ml) as substrates. Tissue sections were viewed with a microscope and photographed with a digital camera (Jenoptik, Germany). Images were converted to TIFF format and contrast levels adjusted using Adobe Photoshop v. 7.0 (Adobe Systems, San Jose, CA, USA). Images were acquired with the same intensity of light for the microscopy and the same parameters for the digital photography, and so only minor adjustments were made to establish uniform contrast for all figures.

### Double- or Triple-Labeling

After hybridization, as described above, some sections were incubated with biotin-conjugated mouse monoclonal anti-DIG antibody (Jackson ImmunoResearch, West Grove, PA, USA; dilution 1:200) overnight at 4°C. For double- or triple-immunofluorescence histochemistry, sections were incubated at 4°C overnight with following antibodies; monoclonal mouse anti-*nestin* (Biogenesis, Poole, UK; dilution 1:100), polyclonal rabbit anti-*glutamine synthetase* (GS; Abcam Inc. Cambridge, MA, USA; dilution 1:100), monoclonal mouse anti-*microtubule-associated protein-2* (MAP-2; Sigma, St Louis, MO, USA; dilution 1:500), monoclonal mouse anti-*polysialic acid-neural cell adhesion molecule* (PSA-NCAM; Chemicon International; dilution 1:200), monoclonal mouse anti-*calbindin* (Sigma; dilution 1:1000), monoclonal mouse anti-*neuronal nuclear antigen* (NeuN; Chemicon International Inc.; dilution 1:100), and monoclonal mouse anti-*glial fibrillary acidic protein* (GFAP; Chemicon International; dilution 1:400). Antibody staining was visualized with the following secondary antibodies; Cy3-conjugated streptavidin (Jackson ImmunoResearch; dilution 1:1500), Alexa488-conjugated anti-mouse antibody (Invitrogen; dilution 1:300) and Cy5-conjugated anti-rabbit antibody (Jackson ImmunoResearch; dilution 1:500). In controls, the primary antibody was omitted from the incubation solution.

Counterstaining of cell nuclei was carried out by incubating the sections with DAPI (4',6-diamidino-2'-phenylindole; Roche; 0.5–1  $\mu$ g/ml) for 10 min.

Slides were viewed with a confocal microscope (LSM 510 Meta; Carl Zeiss Co., Ltd., Germany) equipped with four lasers (Diode 405, Argon 488, HeNe 543, HeNe 633). Images were converted to TIFF format, and contrast levels were adjusted by using Adobe Photoshop v. 7.0.

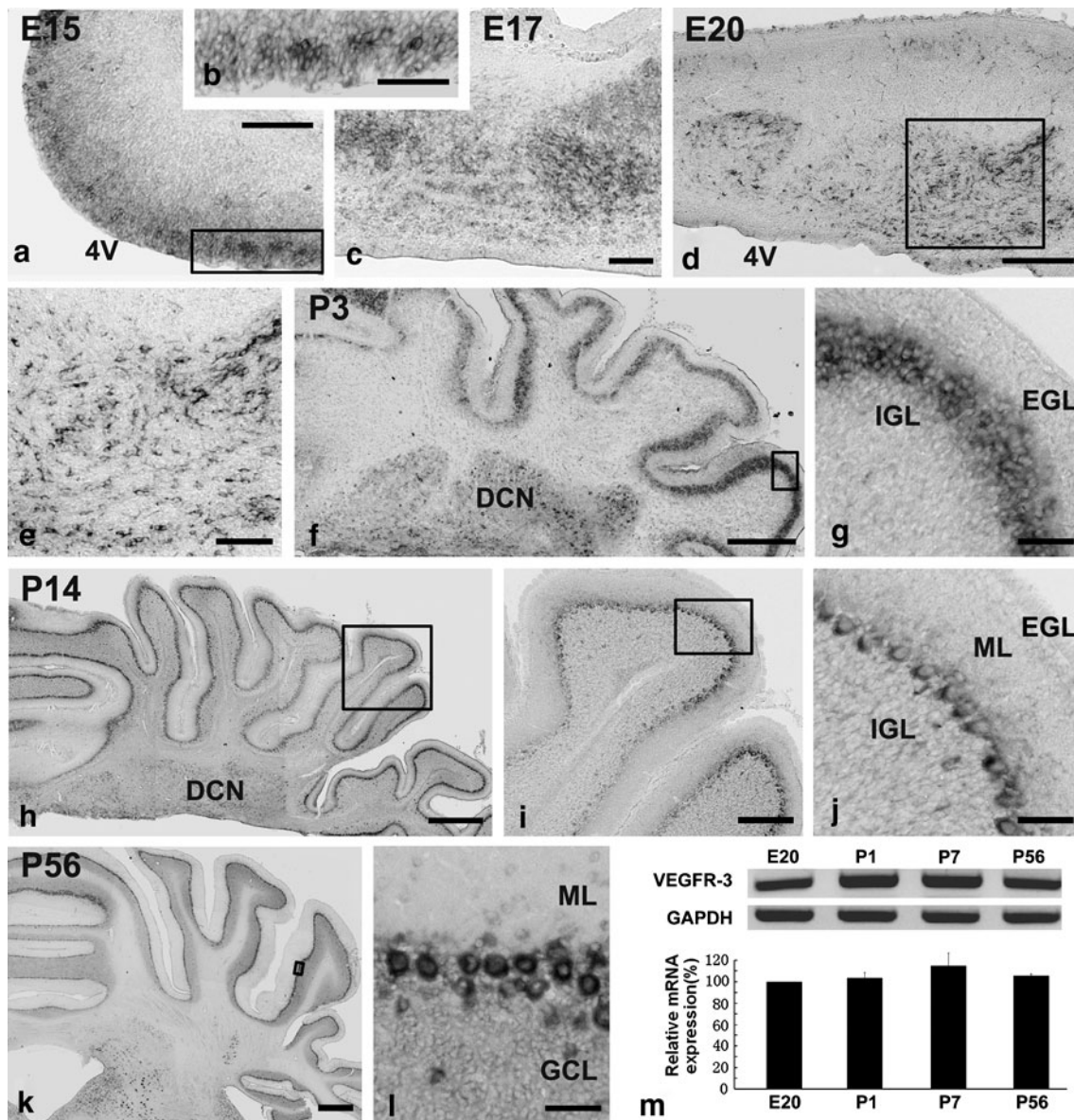
### Results

#### Expression of VEGFR-3 mRNA in the Developing and Adult Cerebellum

The spatial expression pattern of VEGFR-3 mRNA in the developing rat cerebellum was examined using *in situ* hybridization histochemistry. Hybridization localized specific VEGFR-3 signals in distinct regions in the cerebellum at different developmental stages from E15 to adulthood. However, *in situ* hybridization with a sense-stranded probe did not detect any cellular labeling, even when used at a concentration three times that of the antisense probe (data not shown).

At the earliest age studied (E15), hybridization of the VEGFR-3 probe was restricted to the VZ of the cerebellar primordium where clusters of intensely labeled cells were observed (Fig. 1a, b). However, no significant labeling was observed in the overlying intermediate zone (Fig. 1a) and rhombic lip located at the dorsal-most VZ (data not shown). At E17, the intensity of VEGFR-3 expression had been reduced in the VZ of the fourth ventricle and very faint labeling appeared within the VZ (Fig. 1c). Instead, VEGFR-3 mRNA was detected in many cells distributed throughout the cerebellum; the differentiating fields of cerebellar hemisphere and vermis. At E20, the labeling patterns remained similar to those observed in the E17 cerebellum with strong expression in the primitive deep cerebellar nuclei (Fig. 1d, e). In addition, labeled cells were aligned in the cortical area, forming multiple cell layers, the incipient Purkinje cell layer, even though they are weaker than deep cerebellar nuclei, but not observed in forming the external granular layer.

At P3, an intense hybridization signal was observed in deep cerebellar nuclei and in the cerebellar cortex. In the cerebellar cortex, expression was observed in the Purkinje cell layer, which comprises several cell layers at this stage (Fig. 1f, g). The external granular layer containing proliferating cells, and immature, migrating granule cells, and the forming molecular layer were devoid of labeled cells. In addition, no significant expression was detectable in the nascent internal granule cell layer. In the P14 cerebellum, VEGFR-3 expression persisted in the deep cerebellar



**Fig. 1** Developmental changes in expression of VEGFR-3 mRNA in the rat cerebellum. **a, b** Parasagittal section of E15 cerebellum. Hybridization signals were restricted to the cerebellar ventricular zone (VZ). 4 V, the fourth ventricle. **b** Higher magnification view of the boxed area in (**a**). Note that clusters of intensely labeled cells resided in the VZ. **c** Coronal section of E17 cerebellum. VEGFR-3 expression was localized in many cells distributed throughout the cerebellum, but was very weak in the cerebellar VZ. **d** Coronal section of E20 cerebellum. The signals were localized in the developing deep cerebellar nuclei and in the incipient Purkinje cell layer. **e** Higher magnification view of the boxed area in (**d**). Note the strong VEGFR-3 expression in the deep nuclei. **f, g** Coronal section of P3 cerebellum. Hybridization signals were localized in the deep cerebellar nuclei (DCN) and in the cerebellar cortex. **g** Higher magnification view of the boxed area in (**f**). VEGFR-3 expression was localized in the Purkinje cell layer, but was negative in the external (EGL) and internal granular layers (IGL). **h–j** Coronal section of P14 cerebellum. The signals were localized in the deep nuclei and in the

cortex. (**i, j**). Higher magnification views of the boxed areas in (**i**) and (**j**), respectively. Note the intense signal in cells located in the Purkinje cell layer and the weak signal in some scattered cells in the molecular (ML) and internal granule cell layers. **k, l** Coronal section of P56 cerebellum. **l** Higher magnification view of the boxed area in **k**. VEGFR-3 was intensely expressed in the deep nuclei and in the Purkinje cell layer with weaker labeling in a subset of cells in the molecular and granular layers (GCL). **m** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the VEGFR-3 gene in the cerebellum at different ages (E20, P1, P7, and P56). As an internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used. Results were expressed as a percentage of expression in E20 cerebellum. PCR yielded the expected product size (542 bp) and showed that the difference between developmental ages examined did not reach any significance. Scale bars = 1000  $\mu$ m for (**h, k**); 500  $\mu$ m for (**f**); 200  $\mu$ m for (**c, i**); 100  $\mu$ m for (**a, d, e**); and 50  $\mu$ m for (**b, g, j, l**)

nuclei and the Purkinje cell layer, which, as typical for this age, establishes monolayer alignment (Fig. 1h–j). In addition, a somewhat weaker signal appeared in scattered cells in the molecular and internal granule cell layers. In the adult cerebellum (P56), the labeling pattern was maintained, with increasing expression levels in the Purkinje cell layer, and also in the granular and molecular layers (Fig. 1k–l). Labeling in the cerebellar cortex of P56, especially in the Purkinje cell layer, showed no differential expression along the mediolateral axis. This was also true for the postnatal cerebellum (see Fig. 1f, h).

The developmental profile of VEGFR-3 mRNA was analyzed by semiquantitative RT-PCR on whole cerebellum homogenates from embryonic (E20) and postnatal (P1, P7, P56) animals (Fig. 1m). The signal was observed consistently, with similar expression levels at all developmental ages examined, and the difference between developmental ages examined did not reach any significance.

#### Characterization of VEGFR-3-Expressing Cells in the Developing and Adult Cerebellum

To define the phenotype of cells expressing VEGFR-3 in the developing cerebellum, we performed double- or triple-labeling using markers of neural progenitor cell development. In the cerebellum of E20 (Fig. 2a–d), VEGFR-3 expression was observed in the developing cortical mantle, whereas most cells expressing VEGFR-3 were immunoreactive for nestin, a neuronal stem cell marker (Hockfield and McKay 1985; Matsuda et al. 1996), and GS, a marker for radial glial cells and Bergmann glia (Akimoto et al. 1993; Yamada and Watanabe 2002; Mori et al. 2005), suggesting that these cells are prospective Bergmann glia. These cells were dispersed rather evenly throughout the cortical mantle zone. In addition, double labeling of VEGFR-3 and calbindin, which starts being expressed in postmitotic Purkinje cells (Yuasa et al. 1991) showed that weak expression of VEGFR-3 was localized to the Purkinje cells that were concentrated in a broad cellular zone in the cortical mantle zone (Fig. 2e–g). We further characterized the cells expressing VEGFR-3 in the area of the deep cerebellar nuclei and the white matter by double labeling with PSA-NCAM or MAP-2, which are markers of neural differentiation. We found a clear separation of the fluorescence signals for VEGFR-3 and PSA-NCAM (Fig. 2h–j), whereas the majority of VEGFR-3-expressing cells were immunoreactive for MAP-2 (Fig. 2k–m).

In the P3 cerebellar cortex, triple labeling with VEGFR-3, GS, and nestin revealed that GS and nestin double-labeled cells were condensed beneath the multicellular layer of Purkinje cells, most of which expressed VEGFR-3 (Fig. 3a–e). Based on their location, association with Purkinje cells, and co-labeling with GS and nestin, it is

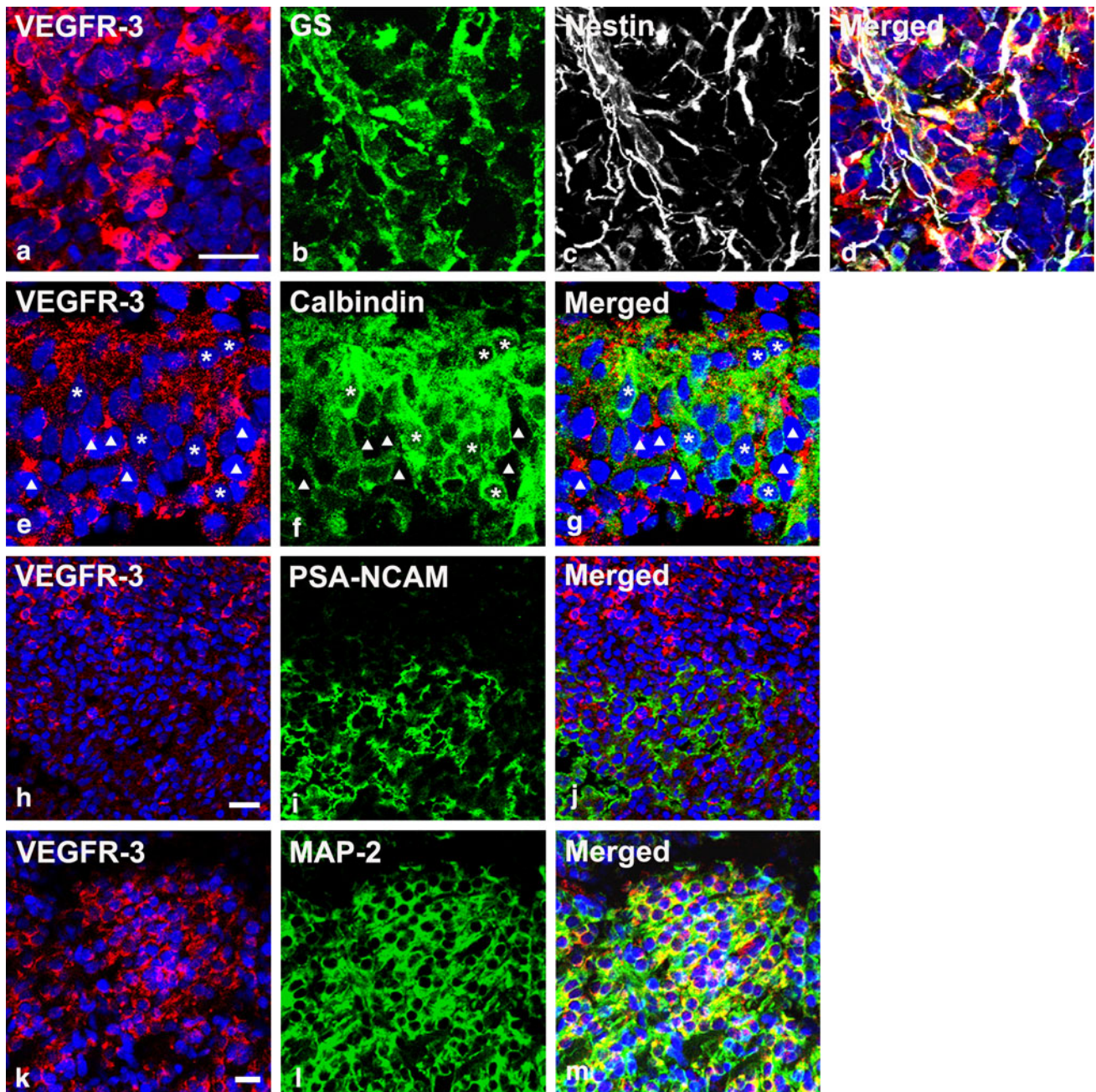
apparent that they are Bergmann glia. In addition, intense VEGFR-3 expression was observed in the Purkinje cells showing calbindin immunoreactivity (Fig. 3f–i). As shown in Fig. 3j–m, intense VEGFR-3 expression in the Purkinje cell layer of P14 cerebellum overlapped with that of GS and GFAP, and these cells, Bergmann glia, were in the close vicinity of the Purkinje cells that themselves were also labeled with VEGFR-3.

In the adult cerebellum (P56), intense VEGFR-3 expression was observed in almost all Purkinje cells and the scattered cells in the molecular layer (Fig. 4a–d). In addition, triple labeling with VEGFR-3, GS, and NeuN, which preferentially labels the nucleus of granule cells, identified that strong VEGFR-3 expression was localized in Bergmann glia that were located around Purkinje cell somata, while granule cells exhibited weak VEGFR-3 signals (Fig. 4e–h).

#### Discussion

In this study, we first showed the developmental regulation and distribution of VEGFR-3 mRNA in the embryonic and postnatal rat cerebellum at the cellular level. Prominent VEGFR-3 expression was detected in the cerebellar VZ at the earliest stage examined (E15), but disappeared in the VZ by E20. Instead, VEGFR-3 expression emerged and increased throughout the cerebellum; especially, in the incipient Purkinje cell layer and deep cerebellar nuclei during late embryonic and postnatal development. In the P14 rats or older rats (P56), VEGFR-3 was also expressed in a subset of cells in the molecular and granular layers, though the cells were weaker than in the Purkinje cell layer and deep cerebellar nuclei.

VEGFR-3 expression in the cerebellar VZ progenitors giving rise to Purkinje cells and interneurons including Golgi cells, basket cells, and stellate cells (reviewed in Carletti and Rossi 2008) indicates that VEGFR-3 might be involved in neurogenesis during early cerebellar development. Supporting our hypothesis in their elegant study, Le Bras et al. (2006) showed that VEGF-C and VEGFR-3 are expressed by murine neural progenitor cells and that VEGF-C deficiency leads to a severe defect in the proliferation of neuroepithelial cells expressing VEGFR-3, suggesting that VEGF-C–VEGFR-3 signaling provides a direct trophic support to neural progenitor cells in the embryonic brain. In addition, we have previously shown that VEGFR-3 is expressed in the proliferating stem cells of the VZ and SVZ in the developing forebrain, suggesting that VEGFR-3 might mediate the regulation of neuronal precursor cell proliferation and neurogenesis in the embryonic forebrain (Choi et al. 2010). Receptor expression, however, is not generally associated with the



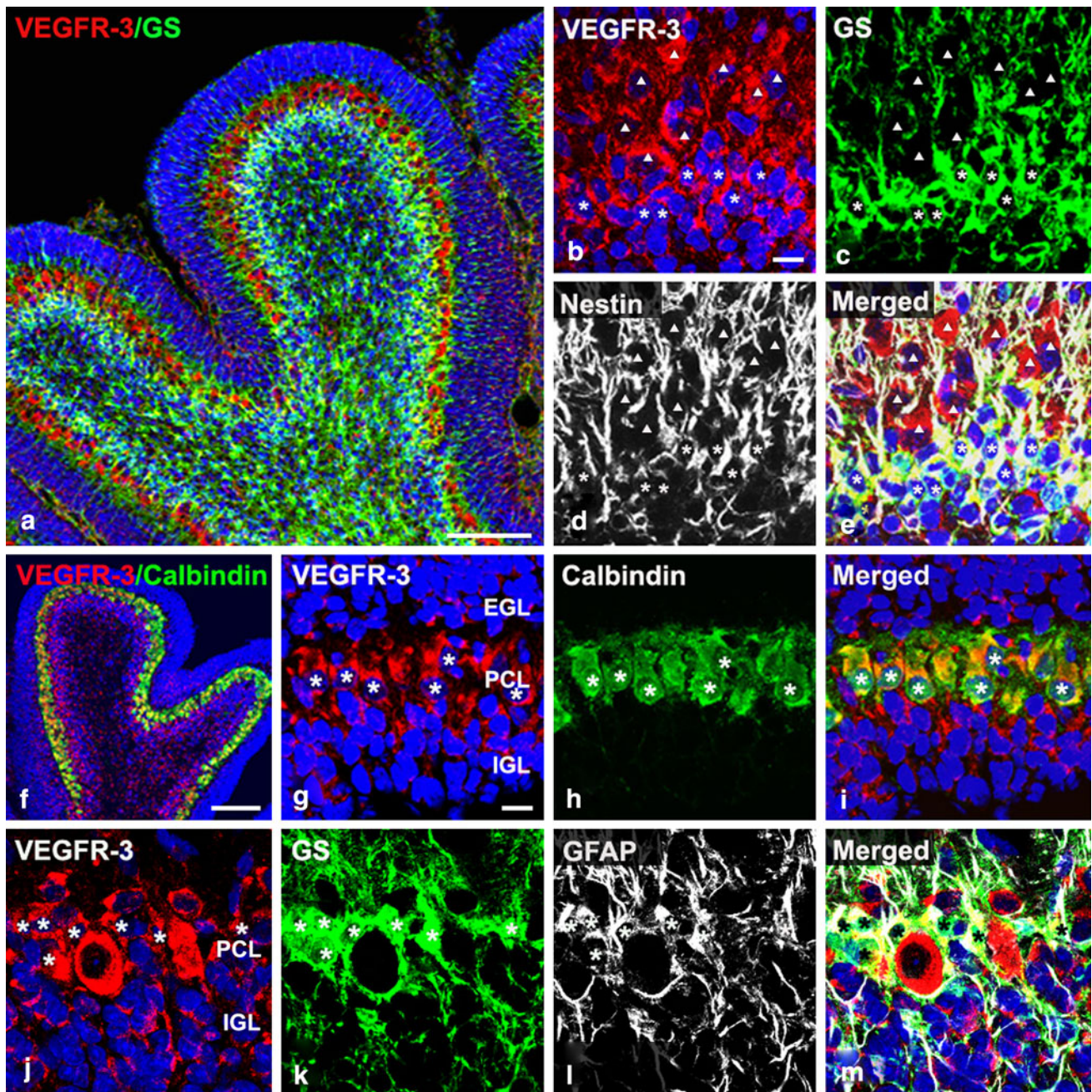
**Fig. 2** Characterization of VEGFR-3-expressing cells in E20 cerebellum. **a–g** The developing cortical mantle. Note that the pattern of intense VEGFR-3 expression overlapped with that of GS and nestin. Also note that VEGFR-3 was weakly expressed in Purkinje cells showing calbindin immunoreactivity (*asterisks in e–g*). *Triangles in (e–g)* indicate VEGFR-3-expressing cells, which were not positive for

calbindin. **h–m** Double labeling of VEGFR-3 and either PSA-NCAM or MAP-2 in the area of the deep cerebellar nuclei. Note that cells expressing VEGFR-3 were negative or weakly positive for PSA-NCAM (**h–j**), but strongly positive for MAP2 (**k–m**). *Scale bars = 20  $\mu$ m for (a–m)*

proliferation of neuronal progenitors in the cerebellum, because no signals are detected in the rhombic lip and external granular layer, the second cerebellar germinal center, where granule cells are produced (reviewed in Carletti and Rossi 2008; Millen and Gleeson 2008). Why these germinal centers were devoid of VEGFR-3

expression and why VEGFR-3 expression was observed exclusively in the cerebellar VZ remains to be examined.

After the transient expression in the VZ, VEGFR-3 was upregulated in two distinct cell types in the late embryonic and early postnatal cerebellum: first in the postmitotic neurons, Purkinje cells and deep nuclear neurons, and

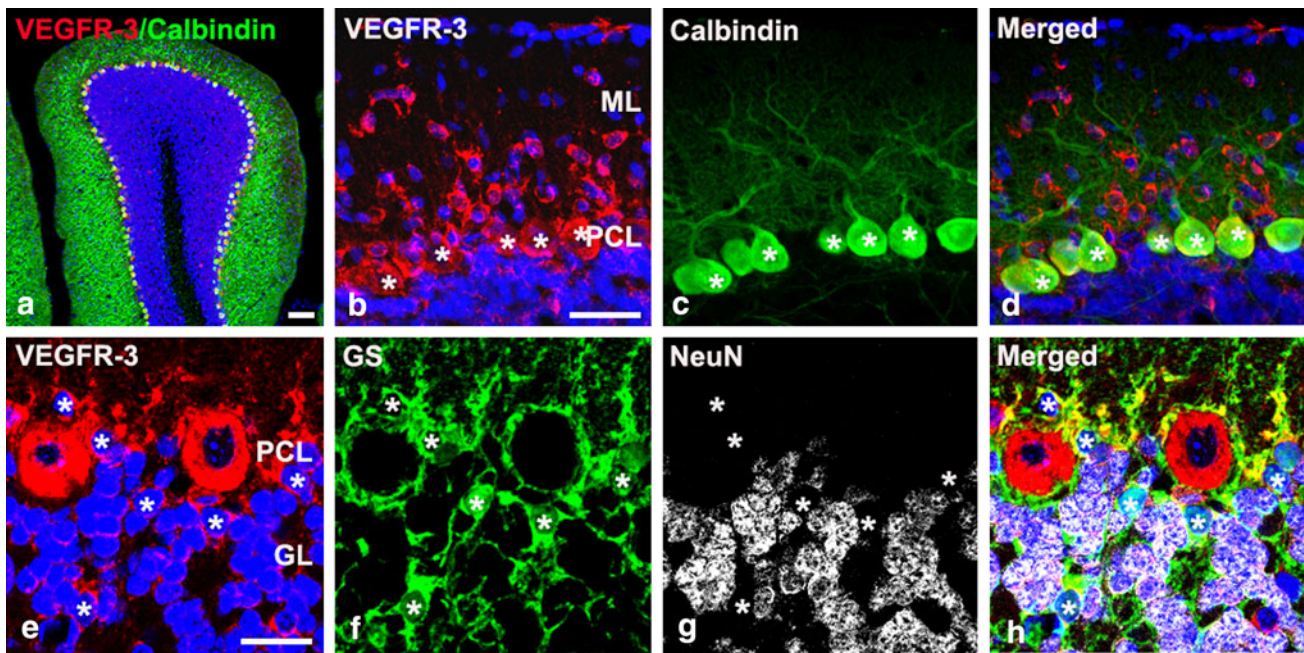


**Fig. 3** Characterization of VEGFR-3-expressing cells in P3 (**a–i**) and P14 (**j–m**) cerebellum. **a–e** Triple labeling for VEGFR-3, GS, and nestin in the P3 cerebellar cortex showing that VEGFR-3-expressing profiles beneath the somata of Purkinje cells were positive for GS and nestin (*asterisks* in **b–e**). *Triangles* in (**b–e**) indicate Purkinje cell somata, which were positive for VEGFR-3, but not for GS and nestin. **b–e** Higher magnification views of the section in (**a**). **f–i** Double labeling of VEGFR-3 and calbindin showing that VEGFR-3 was

strongly expressed in the somata of Purkinje cells (*asterisks* in **g–i**), but weak or undetectable in the external (EGL) and internal granular layers (IGL). **g–i** Higher magnification views of the section in (**f**). **j–m** Triple labeling for VEGFR-3, GS, and GFAP in the P14 cerebellar cortex showing that VEGFR-3-expressing cells surrounding the Purkinje cell somata were immunoreactive for GS and GFAP (*asterisks* in **j–m**). *Scale bars* = 100  $\mu$ m for (**a**, **f**); and 20  $\mu$ m for (**b–e**, **g–m**)

second in the Bergmann glial cells. VEGFR-3 expression in Purkinje cells and deep nuclear neurons was faint from E20 and increased during early postnatal periods, coinciding with the ongoing maturation of these neurons, suggesting that the VEGFR-3 might be involved in the

differentiation and maturation of these cerebellar neurons. In support of this, different cell populations began to express VEGFR-3 according to a characteristic time schedule in the developing cerebellum. Considering that projection neurons, Purkinje cells, and deep nuclear



**Fig. 4** Characterization of VEGFR-3-expressing cells in P56 cerebellum. **a–d** Double labeling for VEGFR-3 and calbindin in the cerebellar cortex showing that intense VEGFR-3 expression was observed in the somata of almost all Purkinje cells (*asterisks* in **b–d**). Note that VEGFR-3-expressing profiles were also localized in the molecular layer (ML). **b–d** Higher magnification views of the section

in **(a)**. PCL, the Purkinje cell layer. **e–h** Triple labeling with VEGFR-3, GS, and NeuN showing that cells expressing VEGFR-3 located around Purkinje cell somata were positive for GS (*asterisks* in **e–h**). Note that weak signals were also localized in some granule cells, which were immunoreactive for NeuN. Scale bars = 100  $\mu$ m for **(a)**; and 20  $\mu$ m for **(b–h)**

neurons, are generated first, whereas local interneurons appear during late embryonic and early postnatal life (Altman and Bayer 1987; Sotelo 2004; Carletti and Rossi 2008), there seems to be close correlation between the appearance of VEGFR-3-expressing neurons and the time at which they first express VEGFR-3. In addition, VEGFR-3 is constitutively expressed in these differentiated neurons in the mature rat cerebellum. Taken together, VEGFR-3 may contribute to developmental processes and adult neuronal function in the cerebellum.

However, hardly any signal emerged in the superficial region of the mantle zone at E17 when a considerable number of Purkinje cells are already migrating radially toward the pial surface of the cerebellar anlage (Altman and Bayer 1985; Takahashi-Iwanaga et al. 1986). Further, our study revealed nonoverlapping expression of VEGFR-3 and PSA-NCAM, a marker for immature migratory neurons (Seki and Arai 1991), in the area of the deep cerebellar nuclei and the white matter, indicating that the majority of migratory cells do not express VEGFR-3. Instead, VEGFR-3-expressing neurons were labeled by MAP-2, a marker for differentiating and postmitotic neurons during neurogenesis (Johnson and Jope 1992). Thus, expression is absent during migration, and then induced in postmitotic and maturing cerebellar neurons during the period of neuronal

differentiation. In support of this, cells in the molecular and granular layers also began to show detectable signals by P14 and the later, although much later and weaker than in projection neurons. Thus, only differentiated granule cells in the internal granular layer expressed VEGFR-3, while granule cells in the external granular layer, i.e., proliferating and migrating granule cells, did not.

The most significant finding of this study is the expression of VEGFR-3 in the Bergmann glia of the embryonic and postnatal cerebellum. VEGFR-3 expression was detected in Bergmann glial precursors as early as E20, when glial precursors migrating from the VZ settle down in the nascent Purkinje cell layer (Yamada and Watanabe 2002; Bellamy 2006), and this expression is then maintained in adults. Thus, Bergmann glia express VEGFR-3 from an early stage, especially prior to the expression of GFAP and its expression, coincident with the time during which Bergmann glia undergo differentiation during cerebellar development. Recently, Kranich et al. (2009) reported that VEGFR-3 mediates proliferation and chemotaxis in glial precursor cells in the developing brain. Thus, our data suggest a role for VEGFR-3 in the development and maturation of Bergmann glia. Further, considering that Bergmann glia have a scaffolding role for both the guidance of Purkinje cell dendrites and the



migration of granule cell precursors in the developing cerebellum (Yamada and Watanabe 2002; Lordkipanidze and Dunaevsky 2005), VEGFR-3 may be involved in development-specific functions of Bergmann glia. However, future investigations to elucidate how VEGFR-3 in the Bergmann glia actually functions during cerebellar ontogenesis are needed.

It is now well established that radial glia, in addition to their role as a transient scaffold for neuronal migration, represent multipotential progenitors giving rise to both neurons and glia in the developing and adult forebrain (reviewed in Barres 1999; Alvarez-Buylla et al. 2001; Tamamaki et al. 2001; Campbell and Götz 2002; Mori et al. 2005; Gubert et al. 2009). Interestingly, our previous data showed that radial glia in embryonic forebrain and Müller glial cells—specialized radial glial cells—expressed VEGFR-3 (Choi et al. 2010). In addition, VEGFR-3 was upregulated in SVZ astrocytes, type B cells in the SVZ of stroke-lesioned rats, which express GFAP and nestin and share some characteristics with radial glia-like cells (Shin et al. 2010). Considering that Bergmann glia are thought to be a remnant of radial glia in the adult cerebellum, expression of VEGFR-3 can be applied as a marker of radial glia in the developing and adult brain.

In summary, we have characterized, to our knowledge for the first time, the expression of VEGFR-3 in the cerebellum during development. Two different populations of cells, distinct neuronal subpopulations and Bergmann glia express VEGFR-3, in addition to being transiently expressed within the VZ of the cerebellar primordium. The receptor expression in distinct neuronal populations occurs during the period of neuronal differentiation, and increased during early postnatal periods, coinciding with the ongoing maturation of these neurons. In addition, Bergmann glia express VEGFR-3 throughout cerebellar maturation into adulthood. However, receptor expression was absent in the progenitors in the external granular layer and during further migration. Taken together, these results suggest a possible involvement of VEGFR-3 in developmental processes and adult neuronal function in the cerebellum.

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