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Mapping the cellular expression patterns of *vascular endothelial growth factor aa* and *bb* genes and their receptors in the adult zebrafish brain during constitutive and regenerative neurogenesis

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Abstract

The complex interplay between vascular signaling and neurogenesis in the adult brain remains a subject of intense research. By exploiting the unique advantages of the zebrafish model, in particular the persistent activity of neural stem cells (NSCs) and the remarkable ability to repair brain lesions, we investigated the links between NSCs and cerebral blood vessels. In this study, we first examined the gene expression profiles of *vascular endothelial growth factors aa and bb* (*vegfaa* and *vegfbb*), under physiological and regenerative conditions. Employing fluorescence in situ hybridization combined with immunostaining and histology techniques, we demonstrated the widespread expression of *vegfaa* and *vegfbb* across the brain, and showed their presence in neurons, microglia/immune cells, endothelial cells and NSCs. At 1 day post-lesion (dpl), both *vegfaa* and *vegfbb* were up-regulated in neurons and microglia/peripheral immune cells (macrophages). Analysis of *vegf receptors* (*vegfr*) revealed high expression throughout the brain under homeostatic conditions, with *vegfr* predominantly expressed in neurons and NSCs and to a lower extent in microglia/immune cells and endothelial cells. These findings were further validated by Vegfr3 and Vegfr4 immunostainings, which showed significant expression in neurogenic radial glial cells.

Following brain lesion (1 dpl), while *vegfr* gene expression remained stable, *vegfr* transcripts were detected in proliferative cells within the injured parenchyma. Collectively, our results provide a first overview of Vegf/Vegfr signaling in the brain and suggest important roles for Vegf in neurogenesis and regenerative processes.

Keywords Brain, Vegf, Flt1, Kdr, Flt4, Kdrl, Neural stem cells, Zebrafish

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Introduction

In the sixteenth century, Andreas Vesalius described the vascularization of the brain and documented the anatomical parallels between blood vessels and nerves [63]. As an integral part of the central nervous system, the brain consumes around 20% of the body's total dioxygen and glucose, making cerebral blood vessels a key component of brain function. Among the various factors that regulate blood vessel formation, vascular endothelial growth factor (VEGF), a family of polypeptides with a highly conserved structure, are essential signaling proteins involved in the growth and maintenance of vascular and lymphatic cells [29, 63].

The VEGF family is made up of 5 main members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PGF (Placental Growth Factor) [73]. Of these proteins, VEGF-A (generally known as VEGF) is the most important. Originally described for its role in vascular permeability [65], it also acts on angiogenesis, endothelial cell growth, migration, and has anti-apoptotic properties. VEGF-B is generally associated with the maintenance of newly formed blood vessels, particularly in pathological conditions, and has neuroprotective effects. [58, 81]. In contrast, the roles of VEGF-C and VEGF-D appear to be mainly limited to lymphangiogenesis. VEGFs act on different receptors belonging to the type III receptor tyrosine kinase family: VEGFR1 (FLT1), VEGFR2 (KDR) and VEGFR3 (FLT4) [6]. VEGF signaling also plays a role in brain plasticity, in particular in synaptic plasticity, neurogenesis, and neuroprotection/neuronal regeneration [63].

Due to a third round of genomic duplication occurring in Teleosts, many genes have been duplicated in zebrafish [66], and several Vegf members have been described in the zebrafish (*vegfaa*, *vegfab*, *vegfba*, *vegfbb*, *vegfc* and *vegfd*). Zebrafish have also all the three vegf receptors *vegfr1 (flt1), vegfr2 (kdr),* and *vegfr3 (flt4)* and in addition a fourth receptor, *vegfr4* (*kdrl*), which the vertebrates lost within the eutherian lineage [6]. This last receptor seems to have a similar function to *vegfr2*. The expression and distribution of Vegf receptors along with their ligands have not been fully described in the brain of adult zebrafish. Interestingly, the zebrafish has recently emerged as an excellent model for studying brain plasticity and regeneration, given (1) the persistence of active neural stem cells into adulthood and (2) its exceptional ability to repair brain lesions [8, 16, 20, 26, 34, 47, 49, 57]. In mammals, blood vessels and neural stem/progenitor cells (radial glia and neuroblasts) are in intimate contact, but only few studies show such links between the vascular system and neural stem/progenitor cells in adult zebrafish [7, 15, 36, 38, 39, 56, 70]. Following telencephalic injury, transcriptomic studies reveal an increased expression of genes involved in angiogenesis (i.e.: *vegf, fli1, vcam, angpt1*) and neurogenesis (i.e.: *ascl1a*, *neurod4*, *gfap*, *vim*) [12]. It was thus demonstrated that both *vegfaa* and *vegfbb* were transiently upregulated after cerebral damage in zebrafish, suggesting a role of these two genes in brain regeneration [12, 22]. As well, the pharmacological modulation of Vegf signaling during zebrafish brain regeneration, was shown to modulate both regenerative angiogenesis and neurogenesis [12, 22]. In the retina, Müller glia (retinal neural stem cells) also contact blood vessels and secrete Vegf, which activates endothelial cells involved in retinal neuronal regeneration via a Notch-dependent mechanism [50]. Together, these data demonstrate the key role of Vegf signaling during regenerative neurogenesis. However, the cells expressing Vegf and their receptors in the adult zebrafish brain remains poorly documented, as do the connections between cerebral blood vessels and neural stem cells. More data are needed to clearly understand and establish the links between regenerative angiogenesis and neurogenesis.

In this work, we aimed to further describe the general distribution and cell-type expression of the main vascular endothelial growth factors, focusing on *vegfaa* and *vegfbb*, and their receptors to have a better understanding of *vegf/vegfr* signaling in the brain of adult zebrafish. We consequently performed in situ hybridization, fluorescent immunohistochemistry, and used transgenic lines to determine their potential expression in neurons, neural stem cells, microglia/immune cells and endothelial cells. Our data show that *vegfaa*/*vegfbb* and their receptors were expressed in the different brain subdivisions and in a wide variety of cells including neurons, endothelial cells, microglia/immune cells and neural stem cells. It notably suggests roles for Vegf signaling in neurogenic processes and brain regeneration.

Material & Methods

Zebrafish strains and husbandry

Adult zebrafish (*Danio rerio,* 4 to 12 month-old) wild-type and transgenic Tg(*GFAP::GFP*), Tg(*mpeg1.1:GFP*) and Tg(*fli1a:EGFP*) that allows to label neural stem cells, microglia/peripheral immune cells (macrophages) and endothelial cells respectively, were maintained in the zebrafish facility from Karlsruhe Institute of Technology (KIT—Campus North) and from DéTROI (UMR 1188, University of Reunion Island) under standard conditions of temperature (28.5°C), pH (7.4), conductivity (400 µS), and photoperiod (14 h dark/10 h light).

Ethics statement

All experiments were performed on zebrafish in accordance with the European Community Guidelines for the Use of Animals in Research and approved by the

local ethics officer and the French Minister (Ethic no: APAFIS_2023110815337464).

Telencephalic lesion: Stab Wound (SW) injury

To perform telencephalic injury, fish were anesthetized with Tricaine (0.02%) (MS-222; REF: A5040, Sigma-Aldrich). A sterile needle (BD Microlance 3; 30G×1/2") was next used to stab wound (SW) the right telencephalic hemisphere, in a medial position as previously described [18, 64]. The left hemisphere remained intact and served as an internal control.

Constructs and synthesis of antisense RNA DIG probes

Coding sequences for *vegfaa*, *vegfbb*, *vegfr1 (flt1)*, *vegfr2 (kdr)*, *vegfr3 (flt4)* and *vegfr4 (kdr-like)* were cloned into the pGEM®-T Easy Vector (Promega) by our own laboratories or by the Genecust company. These plasmids were amplified, linearized and digoxigenin (DIG)-labeled RNA probes were synthesized using DIG RNA labeling Mix (REF: 11585746910, Roche) as previously described [25, 61]. Purification of DIG-RNA labeled probes was performed on G50 Micro column kit (GE Healthcare), and resuspended in 50% DEPC water/50% hybridization buffer, before being stored at -20 °C. Table 1 lists the cloned sequences for *vegfaa*, *vegfbb*, *vegfr1 (flt1)*, *vegfr2 (kdr)*, *vegfr3 (flt4)* and *vegfr4 (kdr-like),* along with the enzymes used for linearization and antisense probe synthesis. Given that many of these genes are paralogs, we assessed the specificity of these probes by blasting their sequences against the zebrafish cDNA libraries from https://www.ensembl.org. No significant overlap was found for the *vegfaa, vegfbb, vegfr1 (flt1) and vegfr3 (flt4)* probes. The *vegfr2 (kdr)* and *vegfr4 (kdrl)* RNA probes exhibited minimal cross reactivity. Specifically, the 896 bp *vegfr2* RNA probe showed 250 to 266 bp of cross-reactivity with *vegfr1* and *vegfr3* transcripts, respectively, but these regions share only 80% identity. Similarly, the 903 bp *vegfr4* RNA probe showed minimal cross-reactivity with *vegfr2* and *vegfr3* cDNA, with overlaps of less than 300 bp and less than 80% identity in both cases. These minimal overlaps and low identities confirm the specificity of each probe for its intended target.

Specificity of in situ hybridization (ISH) staining

Since sense probes are no longer considered appropriate controls due to the increasing evidence of antisense RNA, the specificity of the ISH staining was assessed by overnight incubation in the absence of probes resulting in complete absence of staining (Suppl. Figure 1). This reveals that the chromogenic and/or fluorescent staining reaction is due to the detection of antisense probes within the tissue. Additionally, incubation with previously published probes *her4.1* and/or *id1* (genes primarily expressed in ventricular cells) produced the expected labeling (Suppl. Figure 1). Furthermore, not all cells were labeled for *vegf* and its receptors, reinforcing the specificity of the staining.

In situ hybridization and immunostaining

For chromogenic and fluorescence ISH, adult brains were processed as previously described [17, 61]. Briefly, brains were rehydrated, washed in PTw (PBS containing 0.1%Tween), incubated with proteinase K (10 µg/mL) for 30 min at room temperature, and postfixed in 4% PFA for 20 min. A prehybridization step was performed for 3 h, and then brains were incubated with the appropriate DIG-labeled probes overnight at 65 °C. Brains were subsequently incubated in blocking buffer (PTw with 2% BSA) and then embedded in 2% agarose in PBS before sectioning with a Leica VT 1000S vibratome (50 µm thickness). Incubation with anti-digoxigenin-AP, Fab fragments (1:2000, REF: AB_514497, Sigma-Aldrich) was performed overnight at 4 °C and sections were stained either with NBT/BCIP for chromogenic staining or with fast red staining solution (SIGMAFAST™ Fast Red TR/Naphthol AS-MX Tablets, REF: F4648, Sigma-Aldrich) for fluorescent staining. Alternatively, incubation with anti-digoxigenin antibodies coupled with peroxidase (1:2000, REF:11207733910, Roche) was done and staining was performed using Tyramide Cy3 solution (Akoya Biosciences, Marlborough, MA, USA) after endogenous peroxidase inhibition and according to the manufacturer's recommendations.

Following fluorescence ISH, immunostainings can be made using mouse anti-Glutamine Synthetase (REF: MAB302, 1:1000, Merck, Darmstadt, Germany), rabbit or mouse anti-HuC/D (REF: ab210554, 1:300, Abcam, Cambridge, UK and 1:100, REF: A-21271, Clone 16A11, Thermofischer), mouse anti-PCNA (REF: M0879,1:400, Agilent, Santa Clara, CA, USA) and rabbit glutamine synthetase (REF MAB302; 1:1000, Millipore), rabbit anti-Blbp (REF: ab32423, 1:300, Abcam, Paris, France) and anti-Aromatase B (AroB) (1:500; Gift from F. Brion). Sections were incubated with first antibodies overnight at 4 °C before incubation for 2 h at room temperature with secondary antibodies from Alexa series anti-rabbit Alexa 633 (1:1000; REF: A-21070; Invitrogen) and anti-mouse Alexa 488 (1:500; REF: ab150105, Abcam) with DAPI counterstaining. Finally, brain slices stained through FISH were mounted using Aqua-Poly/Mount (Polysciences, Inc, Warrington, PA, USA).

Vegfr immunostainings

Transgenic zebrafish *Tg(fli1a:EGFP)*, *Tg(GFAP::GFP)*, and *Tg(mpeg1.1:GFP)* were used to label endothelial cells, neural stem cells, and microglia/immune cells,

Table 1 Cloning sequences, restriction enzyme and RNA-polymerase used for the synthesis of antisense probes

ZF Gene	Sequence	Restriction enzyme used	RNA-polymerase
vegfaa (540 bp)	ATACCCAAAGAAGGGGGAAAGAGCAAAAATGATGTGATTCCCTTCATGGATGTGTATAAAAAGAGTGCGTGCAAG ACCCGAGAGCTGCTGGTAGACATCATCCAGGAGTATCCCGATGAGATCGAGCACACGTACATCCCGTCCTGTGTG GTTCTCATGCGCTGTGCAGGATGCTGTAATGATGAGGCGCTCGAATGCGTCCCGACAGAGACACGAAACGTCACT ATGGAGGTGCTGCGGGTCAAGCAACGCGTATCGCAGCATAATTTTCAGCTGAGTTTCACAGAACACACCAAGTGT GAATGCAGGCCAAAGGCAGAAGTCAAAGCAAAGAAAGAAAACCACTGTGAGCCTTGCTCAGAGAGAAGGAAG CGCTTGTATGTGCAGGACCCCCTCACCTGTAAATGCTCCTGCAAATTCACACAAATGCAATGCAAGTCCAGACAA CTTGAGTTAAACGAAAGAACTTGCAGATGTGAAAAGCCAAGATGATGATCGAGCGAACGCCGGGCAGAACTCTGC ATGAAGGAAGGAACTTAA	NdeI	T7
vegfbb (485 bp)	TGATTGACGTAAGATGCATACGTGCTTAAGCATCCTCACCATCAAGTGGTGAAGCTCTACATGAGCACCCTTTGCAAG CCTCGTGAAACACTGGTTAAAGTTGAAGACGAGTTCTCTGAGGTGATGCTGGGTCATGTTGTGCCCTCGTGTGTC CCTTTACAGCGCTGTGGGGGATGCTGTTCAGATGAGGCTATGGTGTGTGTGAATGTGGCCAAACACACCACCATA ATTCAGTTACAGCAGTATGCCACAGATTCGGAGGGGAATATGGCCGAGAGCATTATCGAGCTGCCGTTTGTTGAA CACAGCCAGTGTCAGTGCAGGTTCAGAGACTAGTTCTGATGAAACAGCTTATCTACATGCAAGTTTTTCAGTGTTATT TAAGGAATTATTTAATATTTGTATTATGACTGTATAATTGACAGCATTATTGAGACCCCAGATTAATAAATGGAATAAGCC GAAAAGATAATGAATGAATGAAT	NdeI	T7
vegfr1 (flt1) (830 bp)	ATTTTCTCCTGTCAGCAGGTGTGTGGYAKAGCAGCGGTACGTTCCTGAATCCCGAGCAGTCAAATTCTTCAGCCCAA AATGCAGAGTATGAGAGAACTGGCCCTCCGTGAGCTCCCCATCAAGACCCGAAGCTTCATCCCAAACTGTGATTT GACTGGTTATTCTGGACCATGACAGGTCTGAATAGAGATGCCTATTGGCTATACATAGCAGACGAAGGTCACCGCCCT CTCTAGGCTCTTCCACTAAAGATGCAACGAGGCCTTCTTTGACATCTGTGACATAGAAAGGAATGTCTAGTTCATCTC TGCCTAATATGTTGGATGTGACACAGCGATAGATGCCAGAAACCAGTGCTTCGCCAACTGTCAGAACTCCAACAG TCTTATTCTTCCCTTCTAACACTTCCTGTCTGTGGCTGATGGTCAAGATGGGATTGTGGGTGGACGTGACTCCGGTCT TCTTTCTGACTGCAGTCCAGGACGAAGGAGGAGGCTTCTCACATAGGCCTTTTGGAGGGCACGGATGCCACAGCC ATTGGATTTGAGGTGGTGGGACGCCATGAGAGGTGCAGTGCAGGGCTTGTCTGCTGCCCCGCTGGACTGTCCCAG GCTGTTGGGAGGACACGCTCTTCTCTCCAATTTGGGGCTTCACATTTACCACCAGTGTGATGGTGAGGTTCTGGA AAAGTCCGTACTGCTGGATCCCAGTAAGGATAGTATAGATTCCTGCATCCTCTTCTGCAACATCCCGAATAACCAGAG AAAACCCATCCACATGATAACGGGAGCATCGCTCGGCTGCCACCATCCCATCCTTCAGCCAGATGA	SpeI	T7
vegfr2 (kdr) (896 bp)	AATTCGATTTTCTGGCCTCTCGAAAGTGTATCCATCGTGACTTGGCAGCCAGAAATATCCTACTCTCTGAAAACAGTG TGGTGAAAATCTGTGACTTTGGGCTTGCCAGAGACGTGTATAAAGACCCTGATTACGTTCGTAAAGGAGATGCTC GTCTTCCTCTCAAATGGATGGCTCCTGAGACCATCTTTGACCGCGTGTACACAACACAGAGTGACGTCTGGTCTT TCGGCGTGCTGCTCTGGGAGATCTTCTCACTTGGTGCATCTCCATATCCAGGTGTGTGCATTGATGAGTCTTTCTGCA GGCGACTTAAAGAGGGAACCAGAATGAGAGCTCCAGATTACGCCACGCCTGAAATATACCAGACCATGCTGGACT GCTGGCTTGATCGACCTTTAGACAGGCCAACTTTTACTCAGTTAGTTGAGCATCTGGGTAATCTGCTACAAGCTAGTG CTCAACAGGATGGAAAAGACTACATCCCGCTGACAAACGGAGAGATGGAGGAGGAACTGGTGGCTCCTCACCTAA ATGTGACGTCTAAAAGATCTTTCTACGCCGGCAACACAGAGGCACAGCTCCACTATGATAACGCTCCGCCTCTCG GGTTTCCTCAGCAGATGAATAGCAGTGGTGTGCCAGTGAATATGACAGGTTTTGTGGACATTCCCCTGGAGCATA CCACTGTAATGGATGGCCACGTGGACTGTGGAGTGGGTTTATCACGTGAACAGATGAAAGCTTTGGATCGTCAGG CACAAAGGCCACTCAATTTCAGCCCATTACTGCGCTGCAAGAGTAAAGAGTCTTTAGCTTCTGAATCGTCCAATC AGACCAGCGGCTATCAGTCTGGCTACCATTCTGACGATGCAGAAGCCCCGATATAATCACTA	ApaI	SP6
vegfr3 (flt4) (1219 bp)	GGCCGCCATGGCGGCCGCGGGAATTCGATTTTTCCTGAAGACTCGCCTGTGGAGTTGATGCAGGGGGAGAGTTTG GTGCTCAACTGCACTGCACTGGTCGACTTCAACACTGGTGTGGATTTCCAGTGGGACTATCCAGGCAAAAAGGAG AACCGGTTGGCGAGTTTACAGCCTGTGCGTAACGTCCTGGATGAAGCCACGGAAATCTCCAGTATCCTCTCAATC AGAAACATTCACTTGGATGATAGTGGCTACTACACCTGCTGGGCTAATACTCTGGAAATGAAACGAGAGCTCACT ACTGTAGTCATAGTTCACGAAAAGCCTTTCATCAGCTCGGACTACAGGAATGGATCAGTTATAGAGGCAAAAGAG GGACAAAAATCTGTCCGGCTATCCGTAAAAGTGTCAGCGTACCCTTCACCAGAAACACAATGGTATAAGAATGGA AAACTGATCAGCAGCAAGAATTCAAGCAGGTTTAAGGTCCAGCAGCACTCTCTACAGATCAGGGATGTGTGTAAG CAGGATGCTGGAGAATACATGCTGGTCCTGAAAAATAGCCCTGCAGCCCTGGAGAAAAGACTCAACTTTACTCTC ATAGTCAACGTTCCACCTCAGATTCATGAGAAGGAGGCAGCACCACCTACAAACCTCTATGGGAAGGGCACTCGA CAGATCCTCACTTGCACCGCAGACGGAAGCCCTCCAGCCTCCATCAGCTGGCAGTGGAGGCCCTGGAGCCCCTGC GATCTGGAGCGCACCCGCAGAGCCCTGAGACGGAGGGGTGGACGAGACCAGAGTCCTTTCTGTCACAACTGGATG GATTTGGACCCTGAACATGCTGTAAACCCTATTGAGAGTATTGACACATTGACTCAGATGGTGGACGGCAAAGAA AAGACTGTCGGCAAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTG GATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAA TTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGT GAGCTAACTCMCATTAATTGCGTTGCGCTCMCTGCCCGCTTTCCAGTCGGGAAACCTGTCGKGCCAGCTGCATTAAT GAATCGGCCAACCC	ApaI	SP6

Table 1 (continued)

ZF Gene	Sequence	Restriction enzyme used	RNA-polymerase
vegfr4 (kdrl) (903 bp)	TGGCGGCCGCGGGAATTCGATTTCTCGATGAGCAGTGTGACCGTCTACCATATGACAGCAACAAATGGGAGTTTC CTCAAGATCGCCTCAGACTCGGTAAAACTTTGGGCCATGGAGCATTTGGAAAAGTTGTAGAGGCCTCTGCATTTG GCATTGACAAGATTTCAACATGCAAAACAGTGGCTGTGAAAATGCTGAAAGTGGGAGCAACAAATAACGAATGGA GAGCCTTAATGTCTGAACTGAAGATACTGATCCATATTGGGCATCATCTCAATGTGGTTAACCTGCTAGGAGCCTGCA CAAAGCGTGGCGGCCCCCTAATGATAATCGTGGAATTCTGCAAGTATGGAAATCTTTCCAACTACCTGAGAAGTA AGAGAGGTGACTTTGTGGTTTACAAGTCTCAGGACGGTAAGGCTGTGCGTTCCAGCTCAGGCTGTGATCTGAGCG AGCTCATCAAGCGCAGGTTGGAGAGCGTGGCGAGCACCGGAAGTTCAGCCAGCTCCGGCTTCATTGAAGATAAGA GCTACTGCGACTCGGAGGAAGAGGAGGAAGAGCAAGAGGATTTGTATAAGAAAGTGCTCACATTAGAAGACTTGA TTTGCTACAGCTTTCAAGTGGCTAAAGGCATGGAGTTCTTGGCTTCGAGAAAATGTATCCACCGTGATCTGGCTG CACGTAACATCCTGCTGTCTGAAAACAATGTTGTGAAGATTTGCGATTTTGGACTTGCAAGAGATGTATACAAGGACC CAGACTATGTCCGCAAAGGAGACGCTAGACTTCCCTTAAAGTGGATGGCGCCAGAGGCCATTTTTGACAAGATCT ATACTACTCAGAGTGACGTGTGGTCTTTTGGAGTGCTTATGTGGGAGATCTTCTCTCTTGGTGCCTCCCCTT	ApaI	SP6

respectively. For brain sampling, zebrafish were euthanized and fixed in PBS-PFA as described previously in this manuscript. Briefly, for immunohistostainings, brains were washed in PTw, embedded in 2% agarose and sectioned using a Leica VT 1000S vibratome (50 µm thickness). After blocking, incubation with anti-Vegfr3 (1:125; REF: ES1002, Kerafast), anti-vegfr4 (1:125; REF: ES1003, Kerafast) and anti-HuC/D antibodies (1:100, REF: A-21271, clone 16A11, Thermofischer) were performed overnight at 4°C. The next day, sections were washed with PTw and incubated for 2 h at room temperature with the secondary antibody donkey anti-mouse Alexa Fluor 647 for HuC/D (1:500; REF: ab150107, Abcam), donkey anti-rabbit Alexa Fluor 594 (1:500; REF: ab150076, Abcam) for vegfr3 or vegfr4, and DAPI 1:500 (4′,6′-diamidino-2-phenylindole) for nuclear counterstaining. Sections were washed and mounted with Aqua-Poly/Mount (Polysciences). Note that the HuC/D staining has been artificially colored green for better visualization in the figure.

Microscopy, image analyses, and quantifications

Imaging was performed using a Nikon SMZ18 stereomicroscope for chromogenic ISH and a laser scanning confocal microscope (Leica TCS SP5 and Nikon Eclipse Ti2) for confocal imaging. The confocal brain images were analyzed with Fiji/ImageJ software as hyperstacks to manually evaluate the colocalization of PCNA, Glutamine Synthetase (GS), AroB, Blbp, Vegfr3, Vegfr4, Gfap, mpeg1.1, and HuC/D, as well as the expression of transcripts coding for Vegf and their receptors.

To quantify the number of positively colocalized cells, telencephalic brain sections (from at least three brains) were imaged at 20× magnification. Cells were manually counted on three consecutive 50-µm-thick sections, and the counts were averaged to obtain a representative value for each sample.

Neuroanatomical nomenclature

The nomenclature is according to [79]. A, anterior thalamic nucleus, APN, accessory pretectal nucleus; ATN, anterior tuberal nucleus; CCe, corpus cerebelli; Chab, habenular commissure; Chor, horizontal commissure; CM, corpus mamillare; CP, central posterior thalamic nucleus; CPN, central pretectal nucleus; Cpop, postoptic commissure; Cpost, posterior commissure; D, dorsal telencephalic area; Dc, central zone of dorsal telencephalic area; Dl, lateral zone of dorsal telencephalic area; Dm, medial zone of dorsal telencephalic area; DOT, dorsomedial optic tract; Dp, posterior zone of dorsal telencephalic area; DP, dorsal posterior thalamic nucleus; ECL, external cellular layer of olfactory bulb; EG, eminentia granularis; ENv, entopendoncular nucleus, ventral part; FR, fasciculus retroflexus; GL, glomerular layer of olfactory bulb; Had, dorsal habenular nucleus; Hav, ventral habenular nucleus; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; ICL, internal cellular layer of olfactory bulb; IL, inferior lobe; LH, lateral hypothalamic nucleus; LLF: lateral longitudinal fascicle; LR, lateral recess of diencephalic nucleus; MLF, medial longitudinal fascicle; NMLF, nucleus of medial longitudinal fascicle; PG, preglomerular nucleus; PGa, anterior preglomerular nucleus; PGl, lateral preglomerular nucleus; Pit, pituitary; PO, posterior pretectal nucleus; PP, periventricular pretectal nucleus; PPa, parvocellular preoptic nucleus, anterior part; PPp, parvocellular preoptic nucleus, posterior part; PR, posterior recess of diencephalic ventricle; PSp, parvocellular superficial pretectal nucleus; PTN, posterior tuberal nucleus; R, rostrolateral nucleus; RF, reticular formation; SC, suprachiasmatic nucleus; SD, saccus dorsalis; SO, secondary octaval population; TeO, tectum opticum; TL, torus longitudinalis; TLa, torus lateralis; TPp, periventricular nucleus of posterior tuberculum; TS, torus

semicircularis; V, ventral telencephalic area; V3, third ventricle; VCe, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalic area; Vc, central nucleus of ventral telencephalic area; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VOT, ventrolateral optic tract; Vp, postcommissural nucleus of ventral telencephalic area; Vv, ventral nucleus of dorsal telencephalic area; ZL, zona limitans.

Results

In this study, we investigated the expression of *vegfaa*, *vegfbb* and their receptors (*vegfr1 (flt1)*, *vegfr2 (kdr)*, *vegfr3 (flt4)* and *vegfr4 (kdrl)*) in the adult zebrafish brain. We conducted in situ hybridization across the whole brain with a particular focus on the telencephalon, a region extensively studied due to its high plasticity and significant homology with the mammalian brain [8, 16, 26, 43, 51]. Since sense probes are no longer considered as appropriate controls due to the presence of long non coding RNA (lncRNA), we performed incubations without probes and with well-characterized probes notably specific for neural stem cells/ventricular cells (*her4.1* and *id1*). As shown in Supplementary Fig. 1, incubation without any probes resulted in a complete absence of staining, whereas incubation with *her4.1* and *id1* probes resulted in specific staining in cells from the ventricular layers as expected [17, 61, 82]. As previously described, the *her4.1* probe was also detected at lower levels in some parenchymal cells [17]. These results confirm the specificity of our in situ hybridization technique.

vegfaa and *vegfbb* gene expression in the adult zebrafish brain

As shown in Fig. 1, *vegfaa* and *vegfbb* are expressed throughout the brain, encompassing the telencephalon, diencephalon, and rhombencephalon. In the telencephalon, both genes were detected in the dorsomedial (Dm), central (Dc), lateral (Dl), and posterior parts of the pallium (Dp) (Fig. 1, A1-B2). Transcripts were also detected in the diencephalon, including the anterior and posterior nuclei of the preoptic area (PPa and PPp), as well as the anterior, mediobasal, and caudal hypothalamus (Fig. 1, A3-B6). Additionally, their expression was observed in the thalamus, optic tectum (TeO), periventricular pretectal nucleus (PP), cerebellar valvula (VCe), and corpus mammilare (CM) (Fig. 1, A4-B6). The high-power views shown in Fig. 1 (A1' to B6') highlight that the ventricular region and the periventricular layers are labeled in various brain areas, including the Dm, the PPp, the PPv and in the subgranular gray zone (SGZ) of the TeO. Interestingly, staining for *vegfaa* and *vegfbb* appeared weaker within the PPa and along the hypothalamic ventricle (Fig. 1 A2', B2', A4' and B4'). In conclusion, in all examined brain subdivisions (telencephalon, diencephalon, and rhombencephalon), *vegfaa* and *vegfbb* were detected within the brain parenchyma and along the ventricular layers where neural stem/progenitor cells are localized.

We next decided to look at their expression in neurons and neural stem/progenitor cells considering their involvement in regenerative neurogenesis. We conducted fluorescence in situ hybridization for *vegfaa* and *vegfbb* together with markers HuC/D and Aromatase B (AroB) and/or Brain lipid binding protein (Blbp) (Figs. 2 and 3, data not shown). HuC/D is a well-established pan-neuronal marker, while AroB and Blbp are known markers for neural stem cells. In zebrafish, neural stem cells are recognized for expressing a variety of well-characterized markers, including Aromatase B (AroB, encoded by the *cyp19a1b* gene), Brain lipid binding protein (Blbp, also known as fabp7), Glial fibrillary acidic protein (Gfap), S100ß and also vimentin [20, 44, 47, 57, 61, 72]. These markers are largely co-expressed in neural stem cells.

In the telencephalon, nearly all HuC/D-positive neurons expressed *vegfaa* transcripts (97.6% of neurons, see Table 2). Similar results were observed for *vegfbb*, with 96.5% of neurons showing expression (see Table 2). Likewise, HuC/D-positive neurons in the mediobasal hypothalamus were positive for both *vegfaa* and *vegfbb* (Fig. 2 I-P). These findings were corroborated by higher magnification images of the brain parenchyma within the telencephalon and hypothalamus (Fig. 2D, H, L and P). Co-expression was noted across all examined regions, including the anterior and caudal parts of the hypothalamus, the preoptic area, the pretectal periventricular nucleus, and the optic tectum (data not shown). In summary, our data demonstrate that the vast majority of HuC/D-positive neurons express both *vegfaa* and *vegfbb* (97.6% and 96.5%, respectively; see Table 2).

In addition, given the expression of *vegf* genes along the ventricular layers, we investigated their potential expression in neural stem cells. The co-labeling with the specific radial glial cell marker AroB [57], showed that most neural stem cells express *vegfaa* and *vegfbb* in the telencephalon (Fig. 3 A-H) and in the mediobasal hypothalamus (Fig. 3 I-P). Additionally, co-expression with another radial glial cell marker, Blbp, was observed (Fig. 3 Q and R). This was also observed in other periventricular regions known to exhibit AroB-positive and Blbp-positive neural stem cells (data not shown). In fact, our quantification showed that 73.2% and 78% of neural stem cells expressed *vegfaa* and *vegfbb*, respectively (Table 2).

We next decided to investigate *vegf* gene expression in both endothelial and immune cells. Endothelial cells are well-known to be characterized by elongated nuclei localized along blood vessels. This observation was confirmed by performing DAPI counterstaining on *Tg(fli1a:EGFP)*,

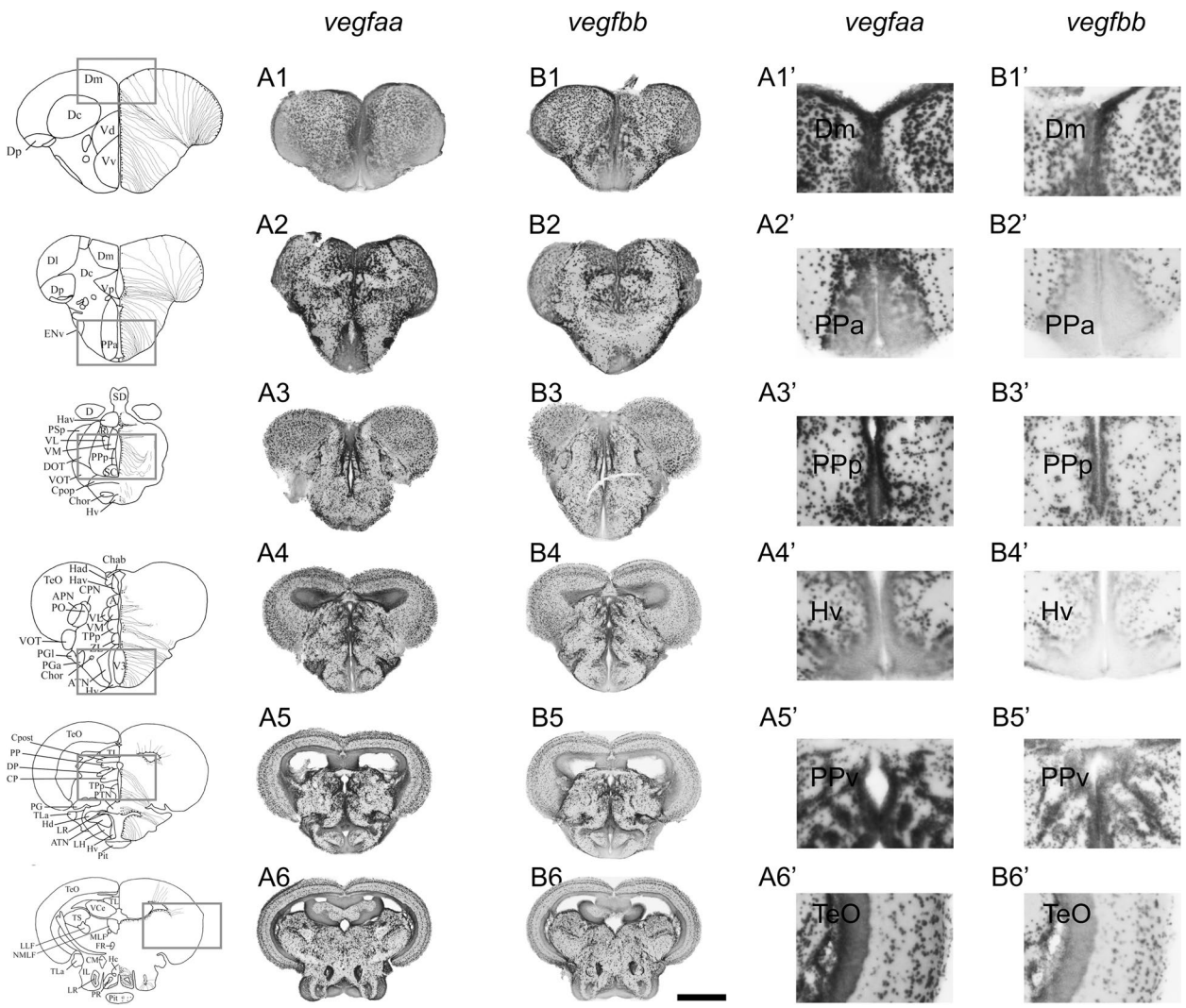

Fig. 1 Expression of *vegfaa* and *vegfbb* genes in the adult zebrafish brain. In situ hybridization was performed on transverse sections through various brain regions, including the telencephalon (**A1** and **B1**), the anterior (**A2** and **B2**) and posterior parts (**A3** and **B3**) of the preoptic area, and the anterior (**A4** and **B4**), mediobasal (**A5** and **B5**), and caudal (**A6** and **B6**) hypothalamus. Both *vegfaa* and *vegfbb* transcripts were detected in the different brain subdivisions including the telencephalon, diencephalon and mesencephalon in the parenchyma and along the ventricular/periventricular layers. A1'to B6'correspond to higher magnification images taken at the level of the square and show gene expression in the ventricular and periventricular layers of the dorsomedian telencephalon (Dm), anterior (PPa) and posterior parts (PPp) of the preoptic area, the anterior part of the hypothalamus (Hv), the periventricular pretectal nucleus (PPp) and the subgranular grey zone of the optic tectum (TeO). Bars: 200 µm (**A1** to **B3**), 300 µm (**A4** to **B6**) and 50 µm (**A1'** to **B6'**)

which expresses EGFP in endothelial cells (Fig. 4 A-C, see arrows). Since transgene expression was almost lost after the in situ hybridization protocol, even after EGFP amplification by immunostaining, we decided to analyze *vegfaa* and *vegfbb* expression in endothelial cells using only the characteristic aspect of the endothelial cell nucleus as previously done [56]. We observed *vegfaa* and *vegfbb* gene expression in some elongated DAPI-stained nuclei lining blood vessels in different parts of the brain notably in the telencephalon, the mediobasal hypothalamus and the tectum (Fig. 4 D-O, and data not shown). However, not all endothelial cells express *vegfaa* and *vegfbb* genes. Indeed, our quantification showed that approximately 51.6% of endothelial cells express *vegfaa*, while *vegfbb* is expressed by around 60.2% of endothelial cells (Table 2). Overall, our results support the expression of the *vegfaa* and *vegfbb* genes in endothelial cells, neurons, and neural stem cells as expected from mice data [11].

We also demonstrated *vegfaa* and *vegfbb* expression in microglia/immune cells in the telencephalon using

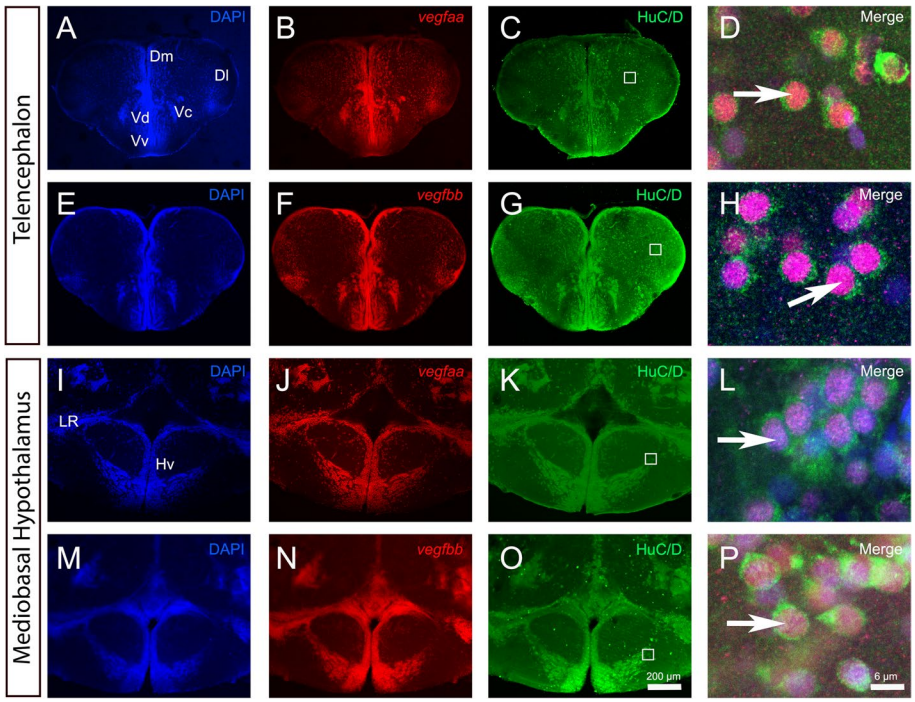

Fig. 2 *vegfaa* and *vegfbb are expressed in almost all* neurons within telencephalon and mediobasal hypothalamus. **A**-**P** *vegfaa* and *vegfbb* in situ hybridization (red) followed by HuC/D immunohistochemistry (green) in the telencephalon (**A**-**H**) and mediobasal hypothalamus (**I**-**P**) with DAPI counterstaining (blue). **D**, **H**, **L** and **P** High magnification views of the dorsomedian telencephalon (Dm) (**D** and **H**) and of the mediobasal hypothalamus (Hv) (**L** and **P**) showing that *vegfaa* and *vegfbb* are strongly expressed in HuC/D-positive neurons (see arrows). White squares (**C**, **G**, **K** and **O**) highlight the respective high-power magnifications in **D**, **H**, **L** and **P**. Arrows show examples of co-expression of *vegfaa* and *bb* with HuC/D. Bars: 200 µm (**A**-**C**, **E**–**G**, **I**-**K**, **M**–**O**) and 6 µm (**D**, **H**, **L** and **P**)

the *Tg(mpeg1.1:GFP)* fish line (Fig. 5). Our quantifications revealed that a significant proportion of microglia/immune cells expressed *vegf* genes (59% for *vegfaa* versus 49.6% for *vegfbb*). These observations were challenging to make due to the low proportion of microglia/immune cells in the telencephalon under homeostatic conditions and the reduced detection of the transgene following in situ hybridization. In posterior regions, such as in the tectum, where the proportion of microglia/immune cells is higher [53], similar observations were noted (data not shown).

vegfaa and *vegfbb* gene expression after telencephalic injury in adult zebrafish

Recent data have shown that *vegfaa* and *vegfbb* are upregulated following telencephalic injury at 1 dpl and returned to basal levels by 3 dpl [12, 22]. We therefore decided to investigate the cellular expression pattern of *vegfaa* and *vegfbb* in the adult telencephalon and the nature of cell types expressing these genes at the lesion site. It is known that at 1 dpl, microglia and macrophages are rapidly recruited to the site of injury and represent an important modulator of regenerative neurogenesis [24, 35, 42]. To identify the cells expressing *vegfaa* and *vegfbb* after stab wound injury, we used Tg*(mpeg1.1:GFP)*, which marks microglia and infiltrating peripheral immune cells (macrophages) when the blood–brain barrier is dysfunctional, and performed HuC/D immunohistochemistry to label neurons. At 1 dpl, *vegfaa* and *vegfbb* genes were increased in the ipsilateral hemisphere compared with the contralateral hemisphere (Fig. 5A vs. 5F and 5 K vs. 5P). The *vegfaa* expression was mainly observed in HuC/D-positive neurons (Fig. 5D and U), and also in mpeg1.1^{+} cells (Fig. 5E and J). Regarding *vegfbb* overexpression, it seems to occur mainly in microglia. Together, these data suggest that *vegfaa* and *vegfbb* are upregulated in neurons and in microglia/immune cells following an injury (Fig. 5).

vegf receptor gene expression in the adult zebrafish brain

In the second part of this work, we investigated the expression levels of *vegf* receptors by extracting RNA seq data from whole brain samples [78] and from telencephalic samples [27, 61]. As shown in Fig. 6, in the whole brain, *vegfr4* has the highest level of gene expression, followed by *vegfr1* and *vegfr2*, and finally, the lowest expression was observed for *vegfr3*. Accordingly, bulk RNA sequencing data from zebrafish telencephalon [27, 61],

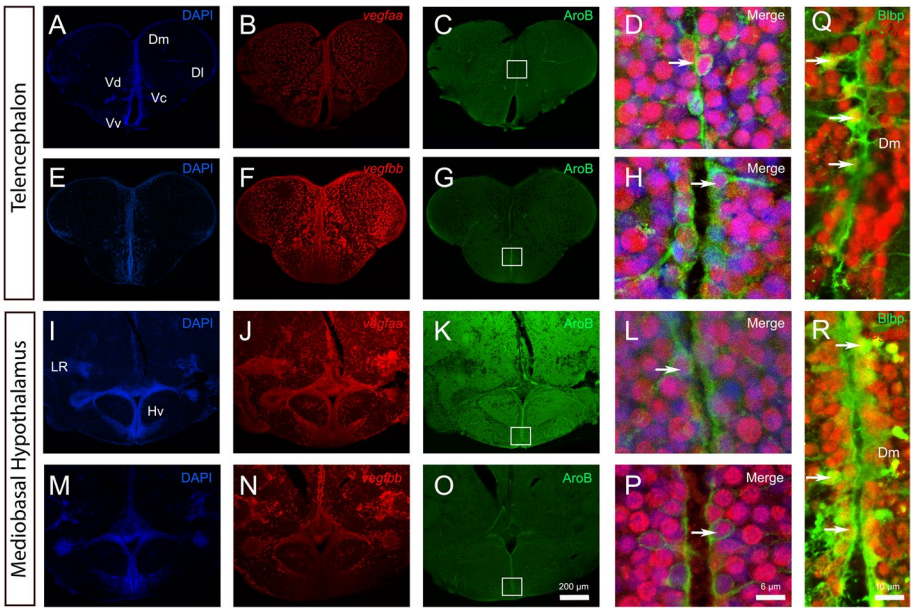

Fig. 3 *vegfaa* and *vegfbb* are expressed in almost all neural stem cells. **A**-**P** *vegfaa* and *vegfbb* in situ hybridization (red) followed by AroB immunohistochemistry (green) in the telencephalon (**A**-**H**) and mediobasal hypothalamus (**I**-**P**) with DAPI counterstaining (blue). High magnification views of the dorsomedian telencephalon (Dm) (**D** and **H**) and of the mediobasal hypothalamus (Hv) (**L** and **P**) showing that *vegfaa* and *vegfbb* (in red) are detected in some AroB-positive neural stem cells (see arrows). White squares (**C**, **G**, **K** and **O**) highlight the respective high-power magnifications in **D**, **H**, **L** and **P**. **Q** and **R** *vegfaa* and *vegfbb* in situ hybridization (red) followed by Blbp immunohistochemistry (green) in the dorsomedian telencephalon. Arrows show examples of co-expression of *vegfaa* and *bb* with AroB and Blbp. Bars: 200 µm (**A**-**C**, **E**–**G**, **I**-**K**, **M**–**O**), 10 µm (**Q** and **R**) and 6 µm (**D**, **H**, **L** and **P**)

Table 2 Quantification of neurons, neural stem cells, endothelial cells, and immune cells expressing *vegfaa* and *vegfbb* genes in the adult zebrafish telencephalon

	Neurons	Neural stem cells	Endothelial cells	Microglia/Immune cells
vegfaa	97.6% (+/- 1.8) Counted cells: 203	73.2 % (+/- 37.2) Counted cells: 52	51.6 % (+/- 34) Counted cells: 52	59 % (+/- 25.1) Counted cells:86
vegfbb	96.5 % (+/- 2.2) Counted cells: 199	78 % (+/- 39) Counted cells: 59	60.2 % (+/- 34) Counted cells: 47	49.6% (+/- 25.9) Counted cells: 54

The table presents the percentage of various cell types expressing *vegfaa* and *vegfbb* genes, along with standard deviations. The lower counts of immune and endothelial cells are attributed to the low number of immune cells in the telencephalon under homeostatic conditions and the potential autofluorescence of blood vessels, which can limit accurate quantification of endothelial cells. The data are based on the examination of telencephalic sections from different brains. Note that for neural stem cell counting, quantifications have been made in 3 sections from the same brain

showed the same profile of gene expression than in the whole brain (Fig. 6). For reference, we also provided gene expression data for neural progenitor/stem cell (*her4.1*), microglia/immune cell (*mpeg1.1*), and endothelial (*fli1a*) markers (Fig. 6, right panels), showing that *vegfr* expression remained relatively significant compared to these markers.

We, therefore, decided to investigate *vegfr1*, *vegfr2*, *vegfr3* and *vegfr4* gene expression by ISH. The distribution of *vegfr* largely overlapped and was observed in all major brain subdivisions from telencephalon to rhombencephalon. The expression of the four receptors was detected in the dorsomedial (Dm), central (Dc), lateral (Dl), and posterior (Dp) parts of the pallium, in the brain parenchyma, and also in the ventricular layers where neural stem cells are located (Figs. 7 and 8). They were detected in the diencephalon, in the anterior and posterior parts of the preoptic area (PPa and PPp), and in the various subdivisions of the hypothalamus, namely in the anterior, medio-basal and caudal regions (Figs. 7 and 8). Expression was also found in the optic tectum (TeO), pretectal periventricular nucleus (PP), valvula cerebelli (VCe) and corpus mammilare (CM). Consequently, in all brain subdivisions examined, *vegfr* were detected in the

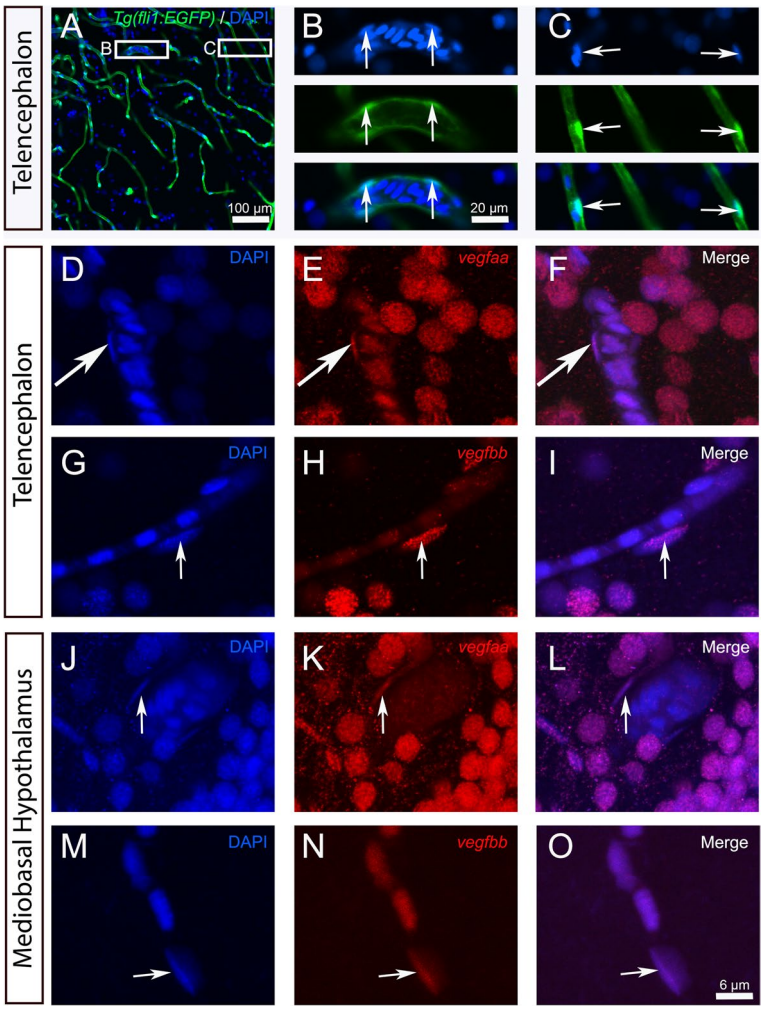

Fig. 4 *vegfaa* and *vegfbb* are expressed in endothelial cells. **A**-**C** Representative image of *Tg(fli1a:EGFP)* zebrafish brain section through the telencephalon with DAPI counterstaining. **B** and **C** show high-power views of the white squares in A, with split channels showing DAPI in blue, the *fli1a:EGFP* transgene in green, and the merged images. Arrows indicate that elongated nuclei lining the blood vessels correspond to *fli1a:EGFP*-positive endothelial cells. (**D**-**O**) *vegfaa* and *vegfbb* in situ hybridization (red) with DAPI staining in the telencephalon (**D**-**I**) and mediobasal hypothalamus (**J**-**O**). *vegfaa* and *vegfbb* (in red) are expressed in some elongated nuclei localized along blood vessels and corresponding to endothelial cells (see arrows). Bar: 100 µm (**A**), 20 µm (**B**-**C**).6 µm (**D**-**O**)

brain parenchyma and along the ventricular layers (e.g., telencephalic, diencephalic, tectal ventricles).

The nature of the cells expressing the different *vegfr* genes was investigated. By performing fluorescence-based ISH of *vegfr* and DAPI staining, we found that *vegfr1*, *vegfr2* and *vegfr4* were expressed in endothelial cells, while *vegfr3* was not or only weakly detected in these cells (Fig. 9). These expressions were observed in different regions of the brain, such as the telencephalon, diencephalon, and rhombencephalon, in both small and large blood vessels. By focusing on the telencephalon, our quantifications showed that in the subset of endothelial cells studied, the different *vegf receptors* were expressed in more than 35% to 53% of the endothelial cells (Table 3); the *vegfr4* mRNA being expressed in a relatively greater proportion of endothelial cells.

To study the potential expression of *vegfr* in neurons and neural stem cells, we performed ISH followed by HuC/D and AroB/Blbp immunostainings, respectively. As shown in Fig. 10, *vegfr* were expressed by almost all neurons in the telencephalon at the level of the dorso-median region (Dm), the dorsolateral (Dl), dorsoposterior (Dp) domains as well as in the subpallium in the ventral (Vv), dorsal (Vd) and central nuclei (Vc). Such a co-expression was observed in the whole brain within the telencephalon, diencephalon and rhombencephalon (i.e., hypothalamus, optic tectum). Altogether, our quantification demonstrated that *vegfr1-4* were expressed in more

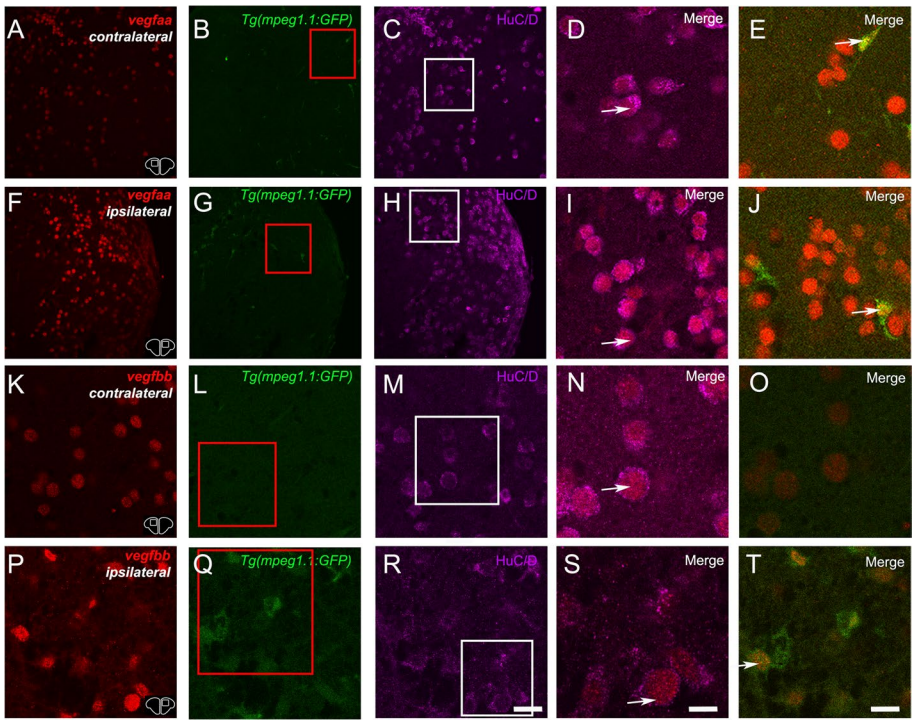

Fig. 5 *vegfaa* and *vegfbb* are up-regulated after brain lesion notably in neurons and microglia. Fluorescent ISH (red) followed by HuC/D immunostaining (purple) on Tg(*mpeg1.1*:GFP) (green) showing *vegfaa* and *vegfbb* at 1 day post-lesion in contralateral and ipsilateral telencephalic hemispheres. (**D**, **I** and **E**, **J**) High magnification views at the levels of the white and red squares showing *vegfaa* expression in neurons (**D** and **I**) and in microglia (**E** and **J**). (**N**, **S** and **O**, **T**) High magnification views at the levels of the white and red squares showing *vegfbb* expression in neurons (**N** and **S**) and in microglia/immune cells (**O** and **T**). Bar: 50 µm (**A**-**C** and **F**–**H**), 14 µm (**D**-**E**, **I**-**J**, **K**-**M** and **P**-**R**), 7 µm (**N**–**O** and **S**-**T**)

than 98–99% of HuC/D-positive neurons (Table 3). Neural stem cell labelling using AroB and/or Blbp antibodies showed that *vegfr* genes were detected in radial glial cells (neural stem cells) studied from various locations, such as at the level of the dorsomedian telencephalon or to the ventral/dorsal nuclei of the ventral telencephalon (Fig. 11). This has also been shown in posterior parts of the brain such as the hypothalamus (data not shown). The quantification showed that more than 96% of neural stem cells express the *vegfr* (Table 3).

Such an expression of *vegfr* in neurons and in neural stem cells was confirmed with the use of other neural stem cell markers such as Blbp and Glutamine Synthetase (GS), and by other ISH techniques based on peroxidase staining (Suppl. Figure 2 and not shown). Weak discrepancies between the two techniques can be observed and could be attributed to the staining methods and/or the use of different neural stem cells markers given that radial glia cells have been shown to form a heterogeneous population [19, 20, 48]. Furthermore, the length of the staining period may result in an increased number of positive cells being identified.

Using the transgenic Tg(*mpeg1.1:GFP*) fish, we observed that a small proportion of microglia/immune cells (21 to 47%) in the telencephalon express *vegfr1*, *vegfr2*, *vegfr3* and *vegfr4* (Fig. 12 and Table 3), with similar observations in more posterior regions (data not shown). Quantifying the latter was challenging due to the low number of mpeg1.1-positive cells in the telencephalon, the decreased detection of the mpeg1.1 transgene after in situ hybridization.

Overall, our results demonstrate that a high proportion of neurons and neural stem cells express *vegfr*, while the proportion of endothelial and immune cells expressing *vegfr* was significantly lower.

Finally, we performed immunohistostainings for Vegfr3 and Vegfr4 using specific antibodies raised against these zebrafish proteins. Using the *Tg(fli1a:GFP)* fish line, which allows labeling of endothelial cells from blood vessels, both Vegf receptors were detected in these cells (Fig. 13 A-H). We also showed that Vegfr3 and Vegfr4 were highly expressed in neurons labeled with the anti-HuC/D antibody in the telencephalon (Fig. 13 I-P) and also in all the other brain regions including the diencephalon (preoptic area and hypothalamus), the optic tectum and other tectal regions (data not shown). Both immunostainings also confirmed expression in Gfap-positive radial

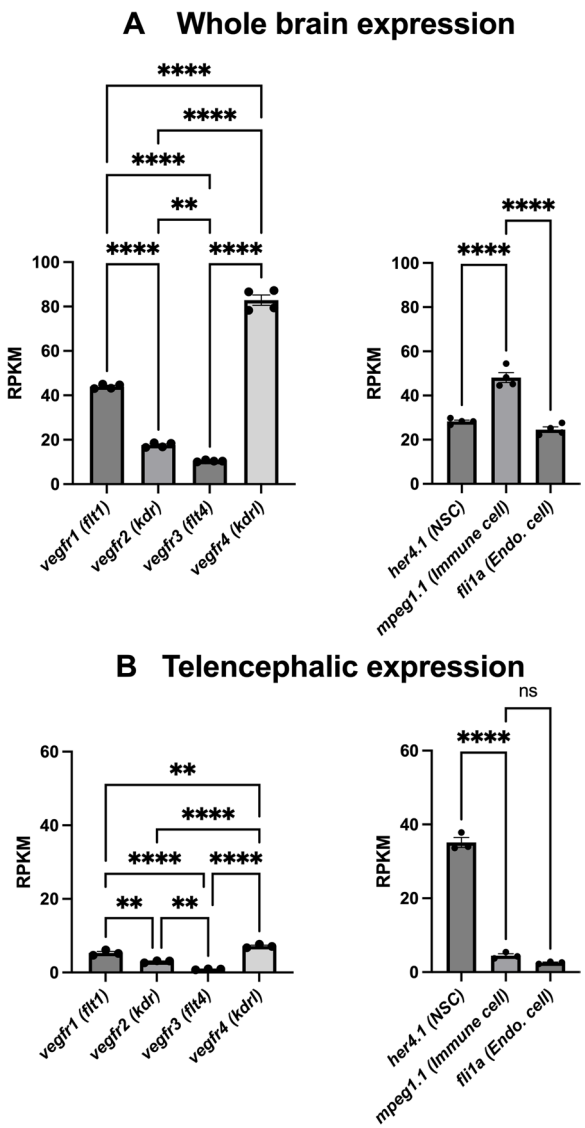

Fig. 6 Gene expression levels of the different *vegf receptors* in the whole brain and the telencephalon. Extraction of RNA sequencing data sets from [27, 61, 78] showing relative expression levels of genes for different *vegf receptors* in the whole brain (**A**) or telencephalon (**B**). Note that specific neural stem cell (*her4.1*), microglia/immune cell (*mpeg1.1*), and endothelial gene (*fli1a*) markers were provided for comparison of gene expression levels. These data show that *vegfr* are expressed at significant levels in a manner similar to most of the reference markers

glial cells (Fig. 13 S-T). Our quantification showed that 57.4% (±6.8) and 70% (±5 0.2) of Gfap-positive cells from the dorsomedian telencephalon expressed Vegfr3 and Vegfr4 proteins, respectively (total number of cells counted: 383 and 439 cells, respectively from 3 brains). However, Vegfr3 and Vegfr4 were mainly not detected in microglia as shown in Tg(*mpeg1.1:GFP*), in contrast to their transcripts (Fig. 13 Q-R).

In conclusion, *vegf receptors* were detected in all cell types studied, with the neuronal and neural stem cell populations expressing these genes predominantly. In contrast, the proportion of microglial/immune and endothelial cells expressing these transcripts was lower. Immunostainings for Vegfr3 and Vegfr4 confirm their strong expression in neurons and neural stem cells. Expression of Vegf receptors in radial glial cells suggest that Vegf signaling may modulate the neural stem cell activity. However, some discrepancies were observed between RNA and protein expression levels in microglia/immune cells and endothelial cells.

Gene expression of vegf receptors after telencephalic injury in adult zebrafish

We proceeded to investigate whether the *vegfr* gene expression was modulated following a stab wound injury in the adult zebrafish telencephalon, with a view to determining whether they exert a potential influence on regenerative neurogenesis. To this aim zebrafish were stab-wounded in the telencephalon and the expression of the *vegfr* was observed at 1, 3 and 5 dpl. No striking difference in ISH staining was observed between the ipsilateral and contralateral hemispheres (Data not shown), these results being comforted by reanalysis of RNA data seq [12, 27, 61] (data no shown). However, in the brain parenchyma, numerous proliferative cells were shown to be *vegfr*-positive (Fig. 14, first and second columns), while the proliferative cells from the ventricular zone (neurogenic niche) were not particularly positive for *vegfr*, except for *vegfr2* (Fig. 14, last column). Unfortunately, we could not confirm these results at the protein level for Vegfr3 and Vegfr4 because PCNA staining required antigen retrieval by MeOH incubation overnight at -20 °C, which suppresses Vegfr3/4 immunostaining.

Discussion

The vascular system plays a critical role in the normal functioning of the brain by supplying dioxygen and nutrients. Vascular Endothelial Growth Factor (VEGF), an important factor in angiogenesis and maintaining blood vessel integrity, is also involved in various brain processes such as neurogenesis. This study presents, for the first time in the brain of adult zebrafish, the distribution and expression sites of *vegfaa* and *vegfbb* transcripts, along with their four receptors: *vegfr1* (*flt1*), *vegfr2* (*kdr*), *vegfr3* (*flt4*), and *vegfr4* (*kdrl*). While their expression and the nature of the cells expressing them is relatively well-known in mammals, there are only a few data in the brain of adult fish, which has become a model for studying brain plasticity and regeneration. Both *vegfaa* and *vegfbb* genes appeared to be expressed in neurons, microglia/immune cells, endothelial cells

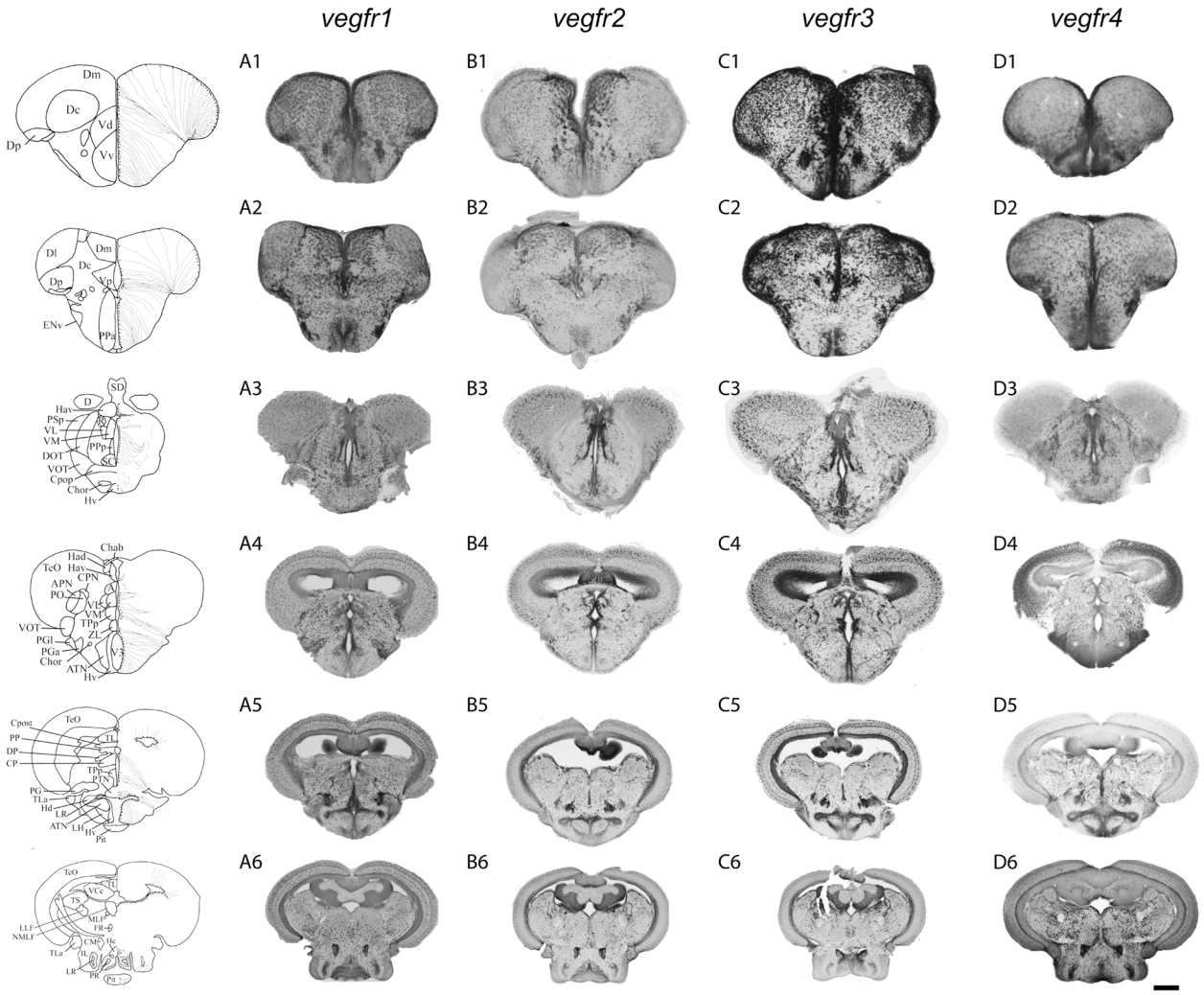

Fig. 7 Overview of *vegfr1*, *vegfr2*, *vegfr3,* and *vegfr4* expression in the adult zebrafish brain. *vegfr1*, *vegfr2*, *vegfr3,* and *vegfr4* in situ hybridization on transverse sections through the telencephalon (**A1**, **B1**, **C1** and **D1**), the anterior (**A2**, **B2**, **C2** and **D2**), and posterior part (**A3**, **B3**, **C3** and **D3**) of the preoptic area, and the anterior (**A4**, **B4**, **C4** and **D4**), the mediobasal (**A5**, **B5**, **C5** and **D5**), and the caudal (**A6**, **B6**, **C6** and **D6**), hypothalamus. The expression of the different *vegfr* genes is widely detected throughout the whole brain, in the parenchyma and along the periventricular/ventricular layers corresponding to neurogenic regions. Expression is somehow ubiquitous. Bars: 200 µm (**A1** to **D3**); 300 µm (**A4** to **D6**)

and neural stem cells. Upon injury, they are transiently up-regulated at the vicinity of the lesion in cells corresponding to neurons and microglia/immune cells. As for their receptors, they are predominantly found in neurons, and neural stem cells with inconsistent expression in microglia/immune cells and in endothelial cells. The presence of *vegfr* in radial glial cells suggests a potential role for Vegf signaling in neural stem cell activity. Overall, this study provides further evidence for the involvement of Vegf in brain functions under both normal and pathological conditions, beyond its vasculogenic role.

Focus on the expression of vegf and vegf receptors: specificity and nuclear localization

In this study, many of the genes examined are paralogs, which raises the potential for significant cross-reactivity between the probes and their mRNA targets. After blasting the sequences of our probes against the zebrafish cDNA library from Ensembl.org, we found that the probes for *vegfaa*, *vegfbb*, *vegfr1* (*flt1*), and *vegfr3* (*flt4*) did not show significant overlap with other transcripts. The *vegfr2* (*kdr*) and *vegfr4* (*kdrl*) RNA probes displayed minimal cross-reactivity with other vegfr probes, with overlaps limited to 200–300 bp regions of probes that are

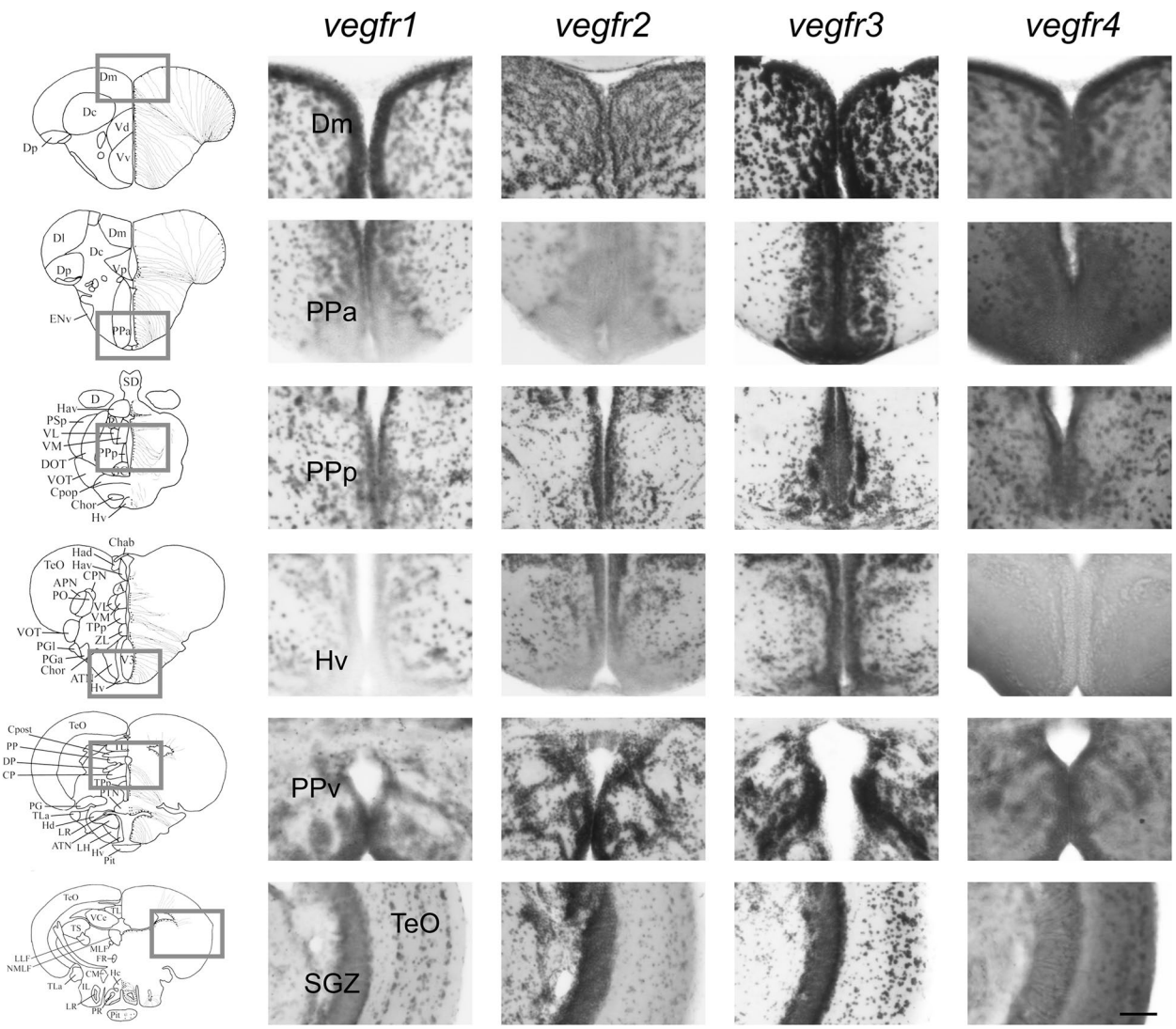

Fig. 8 *vegfr1*, *vegfr2*, *vegfr3*, and *vegfr4* expression in the adult zebrafish brain. In situ hybridization for *vegfr1*, *vegfr2*, *vegfr3*, and *vegfr4* performed on transverse sections through the dorsomedian telencephalon (Dm), the anterior (PPa) and posterior part (PPp) of the preoptic area, the anterior hypothalamus (Hv), the periventricular pretectal nucleus (PP) and the optic tectum (TeO), with a focus on the subgranular grey zone (SGZ). Expression of *vegfr* genes is detected widely throughout the brain, in both the parenchyma and along the periventricular/ventricular layers which are associated with neurogenic regions. The expression is relatively ubiquitous. Bars: 100 µm

over 800 bp long, and showing only 80% or less relative identity. These minimal overlaps are consistent with the specificity of each probe for its target.

We observed broad expression of all studied transcripts throughout the brain, though not ubiquitously; some regions, such as the subpallium (ventral and dorsal nuclei of the ventral telencephalon) and the anterior part of the preoptic area, showed less staining. Additionally, not all cells were labeled, supporting the specificity of our staining (Tables 2 and 3).

Interestingly, the cellular localization of the targeted mRNAs was predominantly nuclear, which has been previously observed in our in situ hybridizations of transcriptional regulators and with other probes [17, 60]. While mRNA is generally considered to localize in the cytoplasm, there has been increasing documentation of nuclear mRNA detection, with some evidence suggesting nuclear translation [10]. It is proposed that nuclear retention may buffer gene expression noise and attenuate fluctuations in cytoplasmic mRNA concentrations, potentially leading to reduced variability in cytoplasmic mRNA [4]. A key idea is that nuclear mRNA is involved in regulatory networks that control

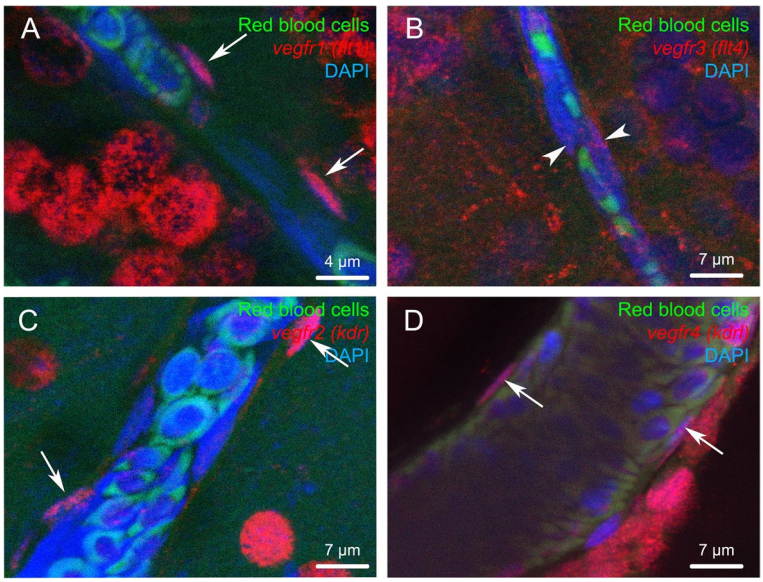

Fig. 9 *Vegf receptor* gene expression in endothelial cells. Fluorescent in situ hybridization of *vegfr* (red) showing expression in endothelial cells for *vegfr1*, *2* and *4*. Note that endothelial cells correspond to cells with an elongated nucleus (arrows) localized along the blood vessels. Arrowheads show a very weak *vegfr3* labeling in endothelial cells. The red blood cells exhibit green autofluorescence. DAPI counterstaining allows to visualize cell nuclei (blue). Bars: 4 µm (**A**), 7 µm (**B**, **C** and **D**)

Table 3 Quantification of neurons, neural stem cells, endothelial cells, and microglia/immune cells expressing *vegfr* genes in the adult zebrafish telencephalon

	Neurons	Neural stem cells	Endothelial cells	Microglia/Immune cells
Vegfr1	98.7 % (+/- 1.8) Counted cells: 230	98.29 % (+/- 0.2) Counted cells: 411	35.9 % (+/- 19.2) Counted cells: 53	45.2 % (+/- 14.8) Counted cells: 20
Vegfr2	99.1 % (+/- 7.4) Counted cells: 294	97.82 % (+/- 1.5) Counted cells: 395	48.4 % (+/- 12.2) Counted cells: 69	44.6% (+/- 8.8) Counted cells: 59
Vegfr3	99.5 % (+/- 4.6) Counted cells: 273	97.0 % (+/- 1.6) Counted cells: 373	40.2% (+/- 27) Counted cells: 58	21 % (+/-3.5) Counted cells: 28
Vegfr4	99.5 % (+/- 2.7) Counted cells: 251	96.66 % (+/- 0.9) Counted cells: 479	53.8 % (+/- 25.8) Counted cells: 67	47.4 % (+/- 29.6) Counted cells: 52

The table presents the percentage of various cell types expressing *vegfr1-r4*, along with standard deviations. The lower counts of microglia/immune and endothelial cells are attributed to the low number of microglia/immune cells in the telencephalon under homeostatic conditions and the potential autofluorescence of blood vessels, which can limit accurate quantification of endothelial cells. The data are based on the examination of telencephalic sections from different brains

gene expression [59]. However, the significance of nuclear translation remains debated [21].

In our study, the specific detection of mRNA in the nucleus raises questions about their role and translation. Nonetheless, the immunostaining for Vegfr3 and Vegfr4, which shows clear overlap between mRNA and protein distributions, supports the specificity of our in situ hybridizations and suggests effective translation of these transcripts.

vegfaa and *vegfbb* genes and their receptors are widely expressed in the brain of adult zebrafish

Our data show that *vegfaa* and *vegfbb* are widely expressed in the adult zebrafish brain, not only throughout the telencephalon, but also in the diencephalon (preoptic area, thalamus, hypothalamus) and mesencephalon, which is consistent with the expression found in mammals including mice and humans [54](Allen brain atlas). In the whole brain, the *vegfaa* and *vegfbb* transcripts

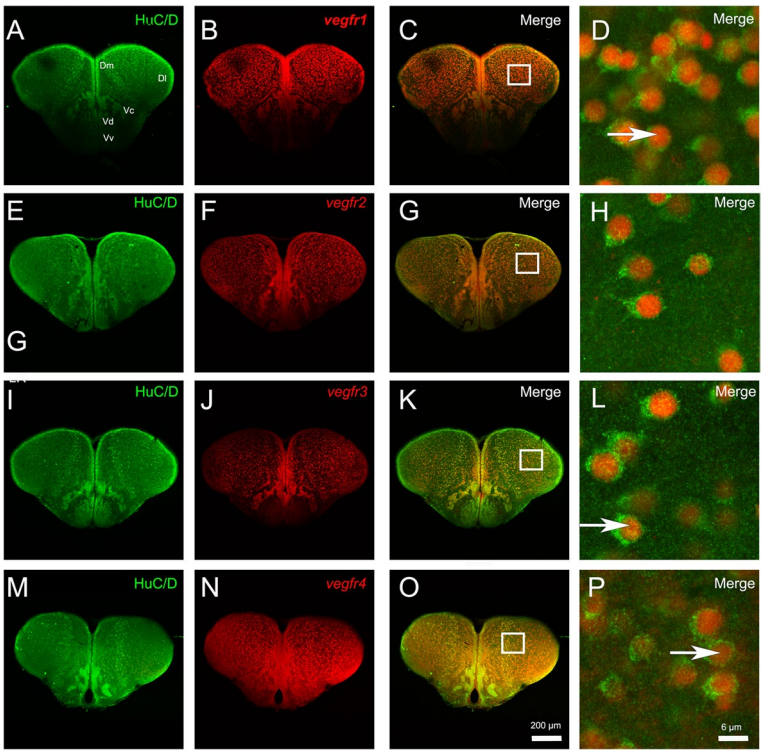

Fig. 10 *vegf receptor* expression is observed in most neurons within the telencephalon. **A**-**P** *vegfr1, vegfr2, vegfr3,* and *vegfr4* in situ hybridization (red) followed by HuC/D immunohistochemistry (green) in the telencephalon. **D**, **H**, **L** and **P** High magnification views of the respective white squares in the telencephalon showing that most *vegfr*-positive cells correspond to HuC/D-positive neurons. Bars: 200 µm (**A**-**C**, **E**–**G**, **I**-**K**, **M**–**O**) and 6 µm (**D**, **H**, **L** and **P**)

were detected along the neurogenic niches (ventricular zone) and in the parenchyma. In mice and humans, their respective orthologues VEGF-A and VEGF-B have also been detected in many brain regions, including the cerebral cortex, hypothalamus, thalamus, and some neurogenic regions such as the hippocampus and the subventricular zone of the lateral ventricle [54] (Allen brain atlas).

Interestingly, *vegfaa* and *vegfbb* transcripts were detected in neurons, microglia/immune cells (macrophages), endothelial cells, and neural stem cells. Obviously, a high proportion of neurons appears to express *vegfaa* and *vegfbb* (≈97% and 96%, respectively). This observation lends support to the hypothesis that neurons may be a primary source of *vegfaa* and *vegfbb*, given their high abundance in the brain. In mammals, VEGF-A and VEGF-B are mostly believed to be secreted by the neurons, with VEGF-A contributing to chemoattractant activity along with promoting self-renewal of neural stem cells, and VEGF-B playing a neuroprotective activity [45, 58, 62]. VEGF-A and/or VEGF-B are also secreted under inflammatory processes by microglia [2, 14, 75] and astrocytes [5, 13]. RNA sequencing data from mouse cerebral cortex showed strong expression of VEGF-A in astrocytes, lower expression in neurons and oligodendrocytes, and almost no expression in endothelial cells and microglia [30, 84]. In contrast, VEGF-B is more expressed in microglia and shows similar expression levels in astrocytes, neurons and oligodendrocytes [30, 84]. In the human brain, both isoforms have also been detected in these different cell types, but in proportions that may differ compared to mice [30, 85]. For example, astrocytes are the major cells expressing VEGF-B in human brain compared to microglia in the mouse [30, 84, 85]. These RNA seq data establish some discrepancies between the expression of VEGF-A and VEGF-B in mice and humans. Surprisingly, neural stem cells from the adult hippocampus have also been shown to synthesize and secrete large quantities of VEGF, establishing the fact that neural stem cell can shape their own neurogenic niche via secreted proteins [37]. Taken together, these data highlight the evolutionarily conserved expression of *vegf* in the brain across species. It also reveals differences between species in the nature of the cells that are the major sources of VEGF-A *and* VEGF-B.

Similarly, the expression of *vegfr1 (flt1), vegfr2 (kdr),* and *vegfr3 (flt4)* and *vegfr4* (*kdrl*) genes is broad and overlapping in the telencephalon, diencephalon, and

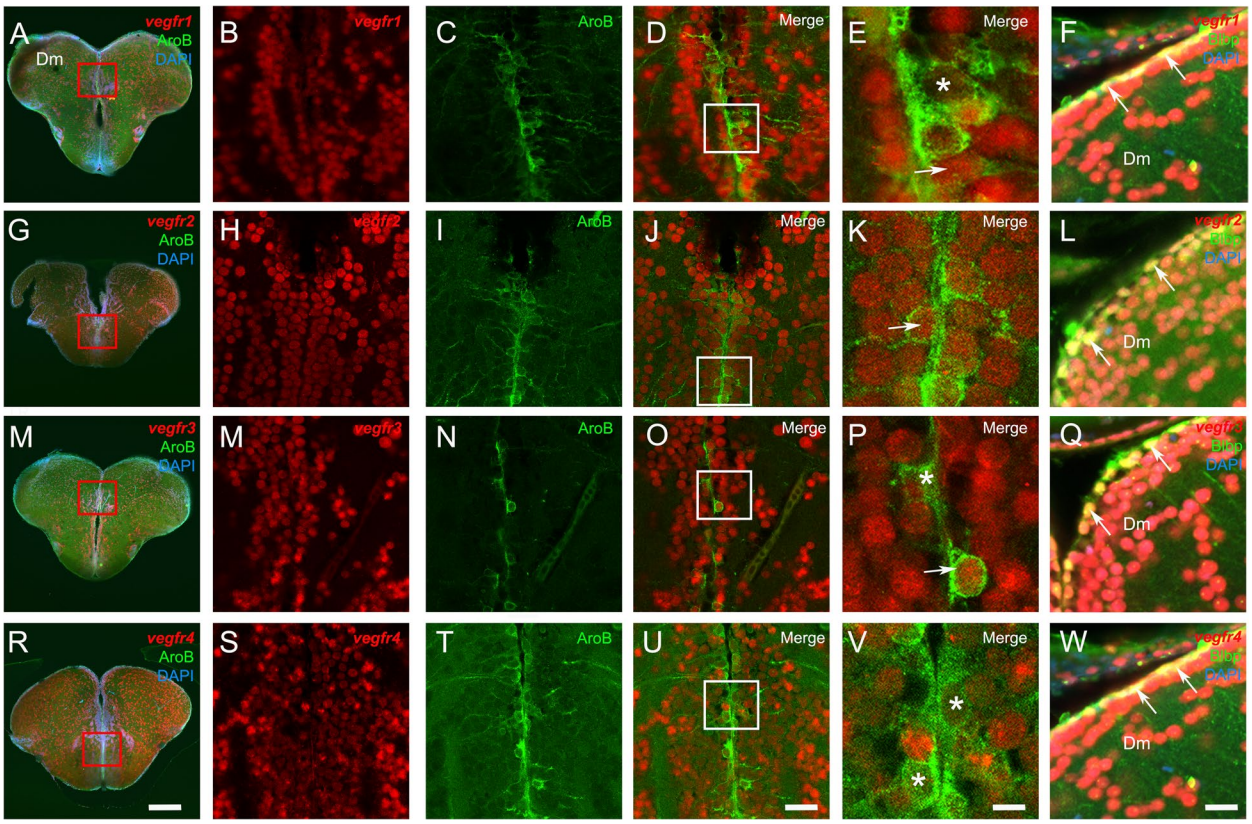

Fig. 11 *vegfr* genes are expressed in neural stem cells. **A**-**R** *vegfr1*, *vegfr2*, *vegfr3*, and *vegfr4* in situ hybridization (red) followed by AroB immunohistochemistry (green) in the telencephalon with DAPI counterstaining (blue). **B**, **H**, **M**, and **S** High magnification views of the ventricular layer at the levels of the red squares in the telencephalon showing *vegfr* gene expression along the ventricular layer. **C**, **I**, **N** and **T** High magnification views of the respective red squares showing AroB-positive cells. **D**, **J**, **O** and **U** Merged pictures showing the colocalization between the *vegfr* and the AroB-positive cells. The asterisks (*) and arrows (last columns) represent the absence or presence of *vegfr* expression in neural stem cells, respectively. **F**, **L**, **Q** and **W** *vegfr1*, *vegfr2*, *vegfr3*, and *vegfr4* in situ hybridization (red) followed by Blbp immunohistochemistry (green) in the dorsomedian telencephalon (Dm) with DAPI counterstaining (blue) showing that almost all Blbp-positive neural stem cells are *vegfr*-positive (see arrows). Bar: 200 µm (**A**, **G**, **M**, and **R**), 30 µm (**B**-**D**, **H**-**J**, **M**–**O** and **S**-**U**), 15 µm (**F**, **L**, **Q**, and **W**), 7 µm (**E**, **K**, **P**, and **V**)

mesencephalon. Our experiments demonstrate the expression of *vegf* receptors in different cell types in the adult zebrafish brain, including neurons, neural stem cells and to a lower extent microglia/immune cells and endothelial cells. Importantly, in homeostatic state, *vegfr* gene expression was observed in most of the neural stem cells examined (>96%). This was partially confirmed by Vegfr3 and Vegfr4 immunostaining, with 57.4% and 70% of Gfap-positive cells expressing these proteins, respectively. Although a notable discrepancy was observed between transcript and protein detection, the findings nonetheless substantiate the expression of Vegf receptors by neural stem cells and imply that their neurogenic activity can be influenced by Vegf signaling. The analysis of transgenic reporter lines in zebrafish (*flt1* and *kdrl*:GFP for instance) showed that these transgenes are mainly expressed in endothelial cells [3, 32, 40, 52, 76]. However, the study of the *Tg(flt1:YFP)hu4624* at the level of the spinal cord demonstrated expression in some neurons [76]. It consequently seems that the endothelial cells are not the only site of expression of Vegf receptors. This was further supported by our Vegfr3 and Vegfr4 immunostaining showing expression in endothelial cells, neurons, and neural stem cells. Further investigation is consequently required to ascertain the significance of the observed differences in expression pattern in zebrafish.

Of interest, vegfr*1 (flt1)*, *vegfr2 (kdr)*, and *vegfr3 (flt4)* appear to be mainly expressed in endothelial cells in the mouse cerebral cortex [84]. In the human brain, although *vegfr* expression is mostly detected in endothelial cells, it is also found at significant levels in microglia, neurons and oligodendrocytes for *vegfr1 (flt1)*, oligodendrocytes for *vegfr2 (kdr)*, and astrocytes, microglia, neurons and oligodendrocytes for *vegfr3 (flt4)* [85]. In addition, these receptors have also been described in the mammalian literature in neural stem/progenitor cells [33, 45, 46, 74, 77].

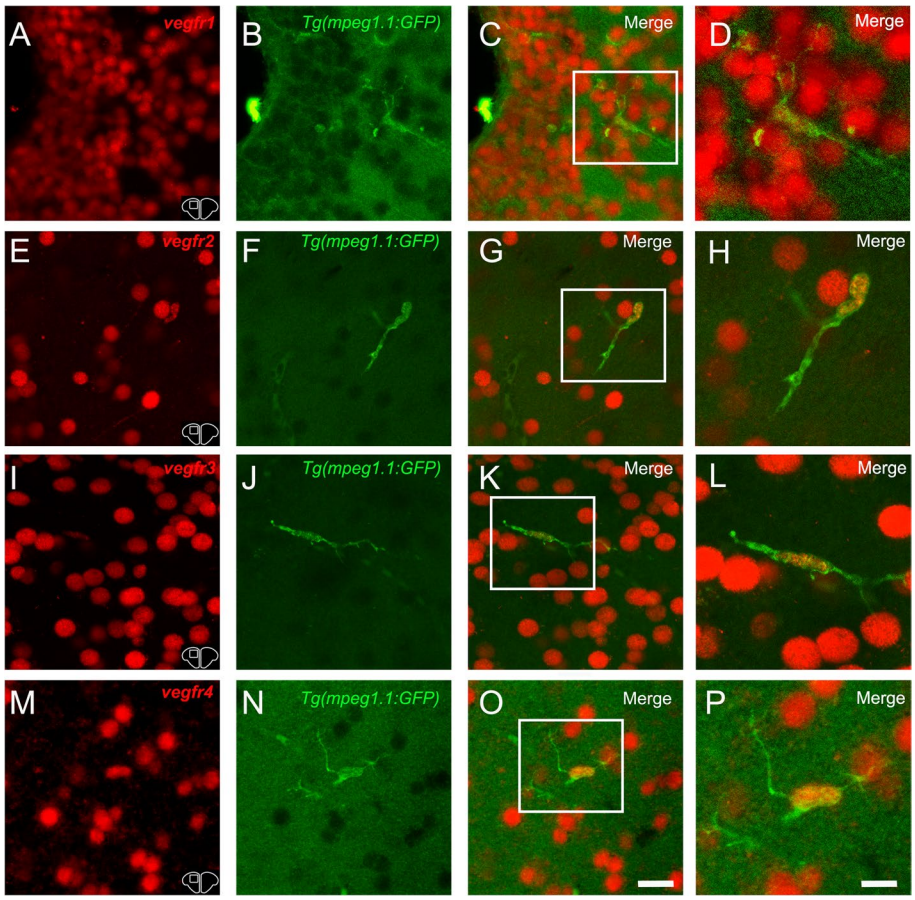

Fig. 12 *vegfr1, vegfr2, vegfr3,* and *vegfr4* expression in microglia/immune cells. **A**-**C**, **E**–**G**, **I**-**K**, and **M**–**O** *vegfr1, vegfr2, vegfr3,* and *vegfr4* in situ hybridization (red) in Tg(*mpeg1.1:GFP*) zebrafish (green) at the level of the telencephalon. **C**, **G**, **K** and **O** Merged images showing colocalization between *vegfr* and microglial cells. Although expression is mostly present in other cell types, a colocalization is found between *vegfr* and microglia/immune cells. **D**, **H**, **L** and **P** Higher magnification view of the square in the preceded picture further presenting the *vegfr* expression in microglia/immune cells. Bar: 6 µm (**D**, **H**, **L** and **P**)

Taken together, these data show that Vegf signaling is likely important for maintaining brain homeostasis in adult zebrafish. They shed light on evolutionarily conserved expressions with mice and humans and also reveal discrepancies in the nature of the cells expressing these different genes, particularly *vegfr*, between mice, humans and zebrafish. The respective roles of Vegf signaling in the different cell types, other than endothelial cells, should be further investigated.

Vegf signaling: involvement in neurogenesis?

After brain injury, whether a stroke or traumatic injury, there is an interplay between neurogenesis and angiogenesis to replenish the injured area with new blood vessels, dioxygen and nutrients, and to replace the dead and damaged neurons [9, 22, 23, 28, 55, 80, 83]. A key regulator of both processes is the Vegf signaling pathway, which activates both endothelial cell proliferation and neural stem/progenitor cells after traumatic brain injury or ischemic stroke in mammalian and zebrafish models [1, 22, 41, 71].

The constitutive expression of VEGF by neural stem cells in the adult mouse highlights the fact that neural stem cells contribute to VEGF secretion and establishes that neural stem cells can shape their own neurogenic niche via secreted proteins [37]. In this line, neural stem cell-derived VEGF is specifically involved in the control of the neural stem cell pool in the adult hippocampus [37]. This is particularly interesting given that in zebrafish, Müller glia have been shown to secrete Vegfaa, which acts on endothelial cells to down-regulate Notch signaling between the endothelial cell and neural stem cells, ultimately leading to increased Müller glial proliferation [50]. In the same line of evidence, we have recently shown that activation of Vegf signaling during brain injury promotes regenerative processes (angiogenesis and neurogenesis) through a microglia-dependent process,

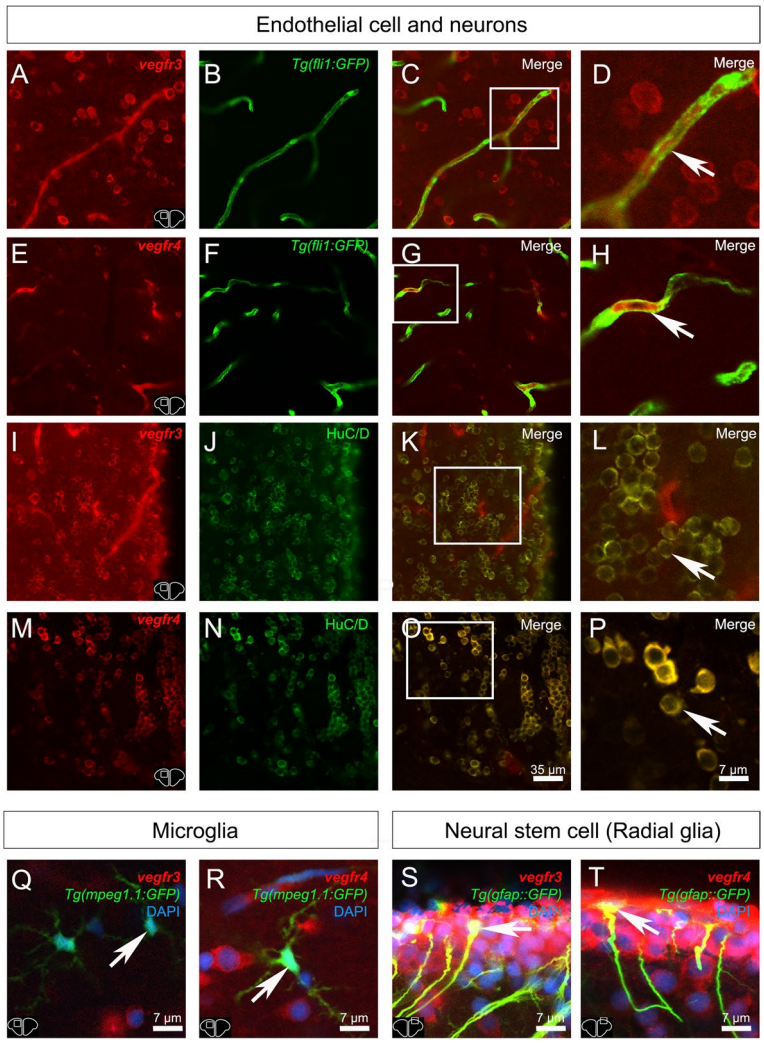

Fig. 13 Vegfr3 and Vegfr4 are expressed in neurons, endothelial cells and neural stem cells. **A**-**T** Vegfr3 and Vegfr4 immunostainings (red) in adult zebrafish telencephalon. **A**-**D** and **E**–**H** Vegfr3 (**A**-**D**) and Vegfr4 (**E**–**H**) expression (red) in endothelial cells (green) from *Tg(fli1a:EGFP)* zebrafish (see arrows). **I**-**L** and **M**-**P** Vegfr3 (**I**-**L**) and Vegfr4 (**M**-**P**) expression (red) in HuC/D -positive cells (neurons) cells in adult zebrafish (see arrows). **D**, **H**, **L** and **P** High magnifications views of the respective white square. **Q**, **R** Merged images showing no colocalization between Vegfr3 (**Q**) and Vegfr4 (**R**) in microglial cells/immune cells (green) from Tg(mpeg1.1:GFP) (see arrows). (**S**-**T**) Merged images showing colocalization between Vegfr3 (**Q**) and Vegfr4 (**R**) in neural stem cells (green) from *Tg(GFAP::GFP)* (see arrows). Bars: 35 µm (**A**-**C**, **E**–**G**, **I**-**K**) Bar: 7 µm (**D**, **H**, **L**, **P**, **Q**, **R**, **S** and **T**)

while its inhibition reduces them [22]. We can speculate that in the adult zebrafish telencephalon, *Vegf* expression and secretion by neural stem cells could be involved in the control of their proliferation through a multi-component cell interaction as in the retina and mammals.

Upon telencephalic injury, the expression of the genes coding for the four *vegfr* remained unchanged in contrast to *vegfaa* and *vegfbb* genes that were transiently increased. In mammals, Vegf increases neural stem cell proliferation and subsequent neurogenesis [31, 67–69]. In addition, VEGF secreted by neural stem cells has been shown to support the maintenance of gene expression associated with cell migration and adhesion in neural stem cells. These functions were confirmed in vitro by blockade of VEGF receptor 2, which impaired neural stem cell motility and adhesion in vitro [11].

In conclusion, we have suggested that neurons and microglia/immune cells are likely to be the major sources of Vegf after zebrafish brain injury. Our recent pharmacological modulation of the Vegf signaling during brain injury shows that Vegf signaling promotes microglia/immune cell recruitment and regenerative processes (angiogenesis and neurogenesis) [22]. However, the specific role of Vegf signaling on constitutive neurogenesis remains to be elucidated as does its impact on neuronal survival, migration, and differentiation.

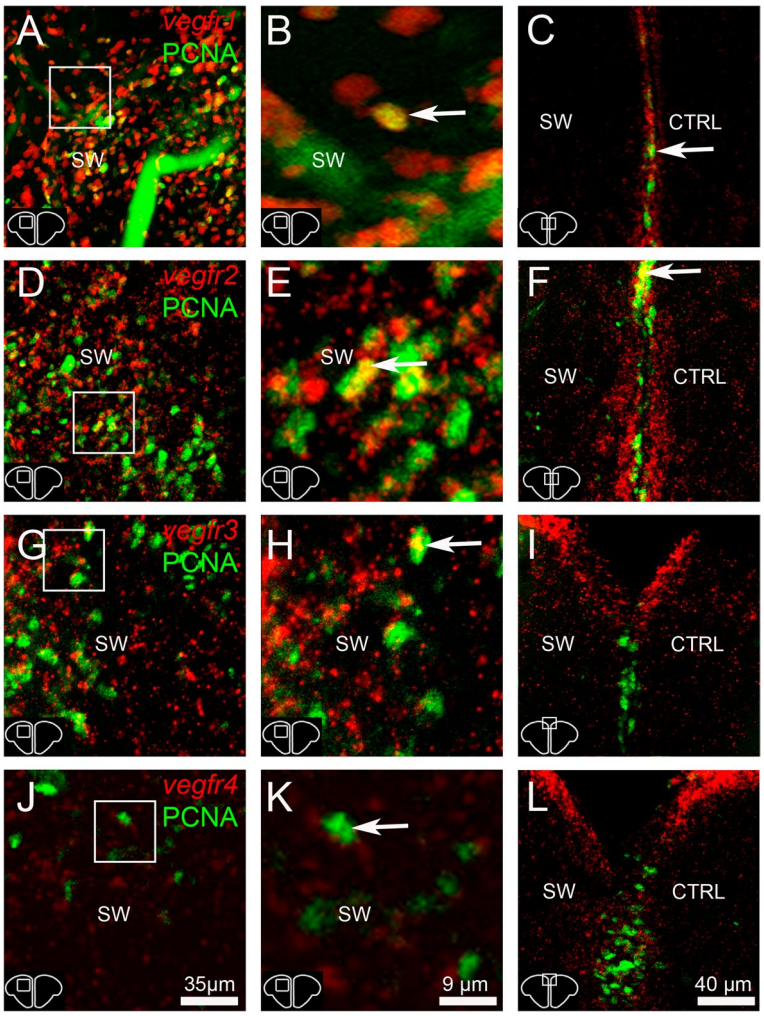

Fig. 14 Numerous proliferative cells in the parenchyma are *vegfr*-positive. **A**, **B**, **D**, **E**, **G**, **H**, **J** and **K** *vegfr1*, *vegfr2*, *vegfr3*, and *vegfr4* (red) in situ hybridization followed by PCNA immunohistochemistry (green) at 3 dpl in the stabwounded (SW) hemisphere. Arrows show examples of proliferative cells expressing *vegfr*. **C**, **F**, **I** and **L** *vegfr1*, *vegfr2*, *vegfr3*, and *vegfr4* (red) in situ hybridization followed by PCNA immunohistochemistry (green) at 5 dpl in the ventricular zone containing neural stem cells that actively proliferate following injury at this time. Most of proliferative cells did not express vegfr (or barely), except for some of them (see arrows). Scale bar = 35 µm (**A**, **D**, **G** and **J**), 9 µm (**B**, **E**, **H** and **K**), 40 µm (**C**, **F**, **I** and **L**)

Conclusion

In this work, we have mapped the expression of *vegfaa*, *vegfbb* and their major receptors *vegfr1 (flt1)*, *vegfr2 (kdr)*, *vegfr3 (flt4)* and *vegfr4* (*kdrl*) across various brain regions and cell types. The broad and overlapping distribution of these genes indicates a significant role for Vegf signaling in both normal and injury-induced neurogenesis. Our findings strongly suggest that modulation of Vegf signaling could enhance brain plasticity and support regenerative processes considering Vegfr expression in neural stem cells. Future research should focus on the expression of each receptor in specific cell types to elucidate their distinct roles in different brain functions.

Abbreviations

AroB	Aromatase B
CM	Corpus mammilare
Dm, Dc, Dl and Dp	Dorsomedial, central, lateral, and posterior parts of the pallium.
Dpl	Days post-lesion
GS	Glutamine synthetase
ISH	*In situ* Hybridization
NSC	Neural Stem Cells
PCNA	Proliferating cell nuclear antigen
PP	Pretectal periventricular nucleus
PPa and PPp	Anterior and posterior parts of the preoptic area
SW	Stab Wound
TeO	Optic tectum
VCe	Cerebellar valve
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13064-024-00195-1.

Additional file 1: Suppl Fig. 1: Specificity of the in situ hybridization technique. Incubation with *id1* (A) and *her4.1* (B) probes strongly labels the ventricular/periventricular zone (arrows), where neural stem/progenitor cells are localized. Both *id1* and *her4.1* in situ hybridizations were consistent with previously published data [17, 61]. In contrast, the in situ protocol realized without incubation with RNA probes resulted in the total absence of labeling (C).

Additional file 2: Suppl. Figure 2: The four *vegfr* genes are expressed more in neurons marked by HuC/D than in radial glia cells marked with GS in the adult zebrafish telencephalon. Expression of *flt1*, *kdr*, *flt4* and *kdr-like* mRNA revealed by fluorescent FISH (red) on cross sections of WT telencephala, together with either immunofluorescent staining for post-mitotic neurons (HuC/D, cyan) or neural stem cells (glutamine synthetase (GS), blue). The *flt1*, *kdr*, *flt4* and *kdr-like* mRNA is co-expressed with HuC/D in neurons and less expressed in GS + neural stem cells. White rectangles (A-H) represent the region magnified in A´-A´´´; B´-B´´´; C-´C´´´; D´-D´´´; E´-E´´´; F´-F´´´; G´-G´´´; H´-H´´´. Scale bars = 300 µm (A-H), 50 µm (A´-A´´´; B´-B´´´; C-´C´´´; D´-D´´´; E´-E´´´; F´-F´´´; G´-G´´´; H´-H´´´).

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Authors' contributions
Conceptualization (ND, DF and SR), Formal analysis (all authors), Funding acquisition (ND and SR), Investigation (DF, ND and SP), Methodology (all authors), Project administration (ND and SR), Supervision (ND and SR), Validation (ND, SR, DF), Visualization (all authors), Writing – original draft (DF, ND, SP and SR), Writing – review & editing (all authors). DF and ND prepared Figs. 1–13; SP and SR prepared Fig. 14 and suppl. Figure 2. Vegfr plasmids were designed and produced by SP and SR.

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Availability of data and materials
The data that support the findings of this study are available on request from the corresponding author (N.D. and S.R.).

Data Availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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