Please cite this article in press as: Ward MC, Cunningham AM. Developmental expression of vascular endothelial growth factor receptor 3 and vascular endothelial growth factor C in forebrain. Neuroscience (2015), http://dx.doi.org/10.1016/j.neuroscience.2015.04.063

Neuroscience xxx (2015) xxx–xxx

DEVELOPMENTAL EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 3 AND VASCULAR ENDOTHELIAL GROWTH FACTOR C IN FOREBRAIN

M. C. WARD * **AND A. M. CUNNINGHAM** †

School of Women's and Children's Health, Faculty of Medicine, University of New South Wales, Sydney Children's Hospital, High St, Randwick, NSW 2031, Australia

Abstract—Increased understanding of the neurovascular niche suggests that development of the central nervous system (CNS) and its vasculature is coordinated through shared regulatory factors. These include the vascular endothelial growth factor (VEGF) family, reported to promote neuroproliferation and neuroprotection in addition to angiogenesis via its receptors VEGFR1–3. VEGFR3, a mediator of lymphangiogenesis, is expressed in murine and rodent brain from early gestation, has been associated with neural progenitors and neurons (Choi et al., 2010) and oligodendroglia (Le Bras et al., 2006) in the developing cortex and is reported to mediate adult neurogenesis in the subventricular zone (SVZ) (Calvo et al., 2011). The early expression pattern of VEGFR3 protein and its cellular associations has not as yet been comprehensively reported. We describe the temporal expression of VEGFR3 protein at cellular level and its close association with its VEGFC ligand, determined by double-labeling immunohistochemistry in the developing rat brain from embryonic day (E) 13 to postnatal day (P) 23. We found high expression of VEGFR3 in the ventricular zone and along radial glia in early gestation in association with neural stem cells and neuroblasts. Similar expression patterns were seen in the immature olfactory bulb and optic cup. In later development we found less expression by neural progenitors in proliferative regions including the SVZ and dentate gyrus of the hippocampus. In contrast, VEGFR3 expression increased with development in the cortex in neurons and astrocytes, and appeared in the emerging population of oligodendroglial progenitors. High expression in ventricular ependyma, choroid plexus and pigmented retinal epithelium was noted from E18. VEGFC ligand was found in association with VEGFR3 throughout development, with the highest expression in embryonic stages. Our findings suggest an important role for VEGFC/VEGFR3 signaling in neuronal proliferation in early forebrain development, and ongoing functions with niche neurogenesis, glial and ependymal function in the maturing postnatal brain. © 2015 Published by Elsevier Ltd. on behalf of IBRO.

Key words: VEGFR3, VEGFC, developing brain, neural progenitor, neuron, astrocyte.

BACKGROUND

The vascular endothelial growth factor (VEGF) family consists of secreted dimeric proteins known to be fundamental for angiogenesis and lymphangiogenesis, and also important in neuroproliferation during early CNS development and in later phases of tissue growth and regeneration. There are presently five known members of the mammalian VEGF family, VEGFA, VEGFB, VEGFC, VEGFD, and the placental-derived growth factor. VEGF ligands are secreted by parenchymal cells and act via binding to tyrosine kinase receptors, namely VEGFR1, VEGFR2 and VEGFR3. While VEGF ligands usually act in a paracrine manner, autocrine signaling has been reported for VEGFA in the vascular endothelium and in the pigmented retinal epithelium (RPE), and for both VEGFA and VEGFC in renal podocyte culture *in vitro* (Warner-Schmidt and Duman, 2008; Müller-Deile et al., 2009; Byeon et al., 2010; Koch et al., 2011; Tugues et al., 2011; Shibuya, 2013).

There is increasing interest in the role of the VEGF family and its receptors in the CNS. While in normal adult brain low levels of VEGF receptor has been described, upregulation is seen in the CNS in a variety of pathological insults, including ischemia and spinal cord injuries (Krum et al., 2008). Both endogenous and exogenous VEGF have been shown to promote adult neurogenesis in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus of the hippocampus (DG) (Jin et al., 2002; Sun et al., 2003; Warner-Schmidt and Duman, 2008). In the adult rodent hippocampus, Lee and Agoston (2009) found that inhibition of VEGFR2 increased cell death in hilar neurons in the DG in a traumatic brain injury model, and VEGF signaling has recently been implicated in the action of antidepressant medication in promoting hippocampal neurogenesis (Fournier and Duman, 2012; Sun et al., 2012). In addition to a role in

*Corresponding author. Tel: +61-2-92836190; fax: +61-2-93826191.
E-mail addresses: Meredith.Ward@sesiahs.health.nsw.gov.au (M. C. Ward), a.cunningham@unsw.edu.au (A. M. Cunningham).
† Tel: +61-2-93820613; fax: +61-2-93821401.

Abbreviations: 3V, ventral third ventricle; BTIII, Tubulin beta III isoform; CNS, central nervous system; DG, dentate gyrus of the hippocampus; E, embryonic day; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; LV, lateral ventricle; NeuN, Neuronal Nuclei clone A60; NG2, NG2 Chondroitin sulfate proteoglycan; nNOS, neuronal nitric oxide synthase; NSCs, neural stem cells; P, postnatal day; PFA, paraformaldehyde; RMS, rostral migratory stream; RPE, pigmented retinal epithelium; SVZ, subventricular zone; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VZ, ventricular zone.

http://dx.doi.org/10.1016/j.neuroscience.2015.04.063
0306-4522/

neurogenesis, VEGF may also regulate oligodendroglial behavior, based on reports that VEGFA promotes the migration of oligodendrocyte precursors *in vitro* (Hayakawa et al., 2012).

VEGFR3, initially identified in lymphatic endothelial cells (Kukk et al., 1996; Karkkainen et al., 2004) was subsequently reported in normal adult brain and glioblastomas (Jenny et al., 2006). A more recent study reported widespread expression of VEGFR3 mRNA in the adult rat CNS in neurons, astrocytes and ependyma in the forebrain, brainstem and spinal cord (Hou et al., 2011). While the precise functions of VEGFR3 in the mature CNS remain uncertain, there is growing evidence for a role in neurogenesis. *In vitro* studies of adult hippocampal-derived neural stem cells (NSCs) found induction of VEGFR3 expression after severe anoxia (Maurer et al., 2003), and *in vivo*. VEGFR3 and VEGFC, its high-affinity ligand, were upregulated in activated glial cells after transient ischemia in the adult rat hippocampus (Shin Yoo-Jin et al., 2008). VEGFR3 upregulation has also been shown in the SVZ of adult rats after cerebral ischemia (Shin et al., 2010a) and demonstrated in the peri-infarct penumbra zone of the ischemic cortex in association with glial fibrillary acidic protein (GFAP) and nestin-expressing reactive astrocytes (Shin et al., 2010b). Most recently, VEGFR3 expression has been reported by adult-murine NSCs with direct stimulation of neurogenesis shown *in vivo* via the VEGFC–VEGFR3 signaling pathway (Calvo et al., 2011).

In earlier development, VEGFR3 has been reported in embryonic mouse brain in association with neural and oligodendroglial precursor cells (Le Bras et al., 2006; Kranich et al., 2009) and in a VEGFC knockout mouse there was marked oligodendroglial depletion and partial neural progenitor depletion (Le Bras et al., 2006).

Choi et al. extended knowledge of the expression of VEGFR3 in the developing rat brain using *in situ* hybridization histochemistry for VEGFR3 mRNA in combination with phenotyping immunohistochemistry (Choi et al., 2010). They reported early expression in the ventricular zone (VZ), SVZ and rostral migratory stream (RMS) commencing from E13 in association with NSCs, and early neuronal and astrocytic markers but with down regulation in the VZ and cortical subplate in the early postnatal period. Expression of VEGFR3 persisted until adulthood in the SVZ, RMS and cortical plate.

We have further characterized VEGFR3 in the developing rat brain, describing its expression at protein level and its association with various cell lineages using double-labeling immunohistochemistry from embryonic to adolescent time points. We have characterized its expression in choroid plexus and RPE, both critical CNS–CSF interfaces. We also have directly compared the spatiotemporal expression of VEGFR3 and that of its ligand VEGFC during early brain development.

EXPERIMENTAL PROCEDURES

Experimental animals and animal tissue preparation

All animal experimental work was undertaken with the approval of the University of New South Wales Animal Ethics Committee, in accordance with their guidelines, the National Health and Medical Research Council animal experimentation guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990).

Wistar rats were supplied by the Animal Resources Centre (Perth, Australia) with exact ages dated from conception E13, E16, E18 and postnatal day (P) 2, P7, P15, and P23 ($n = 3$ at each time point).

For embryonic brain tissue, pregnant dams at E13, E16, and E18 were sacrificed by intra-peritoneal injection of pentobarbital sodium solution Lethabarb™ (Virbac, Peakhurst, Australia) (6 mg/100 g body weight). Uteri were dissected and placed on ice. Whole embryos were dissected from uteri and immersion-fixed in 4% paraformaldehyde (PFA) for 20–60 min, prior to dissection of the whole embryonic brain which was then halved in a sagittal or coronal plane. Tissue underwent overnight immersion fixation in ice-cold 4% PFA before transfer into 70% ethanol (v/v) and subsequent paraffin embedding.

For neonatal brain tissue, P2–P7 rat pups were sacrificed by decapitation. P15 and P23 rat pups were anaesthetized with intra-peritoneal Lethabarb injection as above and were sacrificed with intra-cardiac perfusion with phosphate-buffered saline (PBS) followed by ice-cold 4% PFA. Whole brains were dissected and immersion fixed overnight in ice-cold 4% PFA before transfer to 70% ethanol and paraffin embedding.

Brain areas of interest were sectioned at 4 μm using a Leica RM microtome (Leica, Germany), with sections taken sequentially from the ventral surface in a coronal orientation. A minimum of eight sequential coronal sections from two animals at each time point were used to define VEGFR3 expression. Double-labeling immunohistochemistry using cell-type-specific markers was performed at each time point in a minimum of duplicate on tissue from a minimum of two different animals.

Immunohistochemistry

Paraffin tissue sections were dewaxed followed by heating in citrate buffer for antigen retrieval, and blocking with 10% normal donkey serum (NDS) prior to application of primary antibodies. Primary antibodies were applied for approximately 1 h in a humidified chamber at room temperature, and secondary antibodies for 30 min. Primary antibodies used were nestin (mouse monoclonal, Chemicon, MAB353, 1:100 dilution); GFAP (mouse monoclonal, Chemicon, MAB360, 1:400 dilution); NG2 Chondroitin sulfate proteoglycan (NG2) (mouse monoclonal, Chemicon, MAB5384, 1:50 dilution); Neuronal Nuclei clone A60 (NeuN) (mouse monoclonal, Chemicon, MAB377, 1:100 dilution); Tubulin beta III isoform (BTIII) (mouse monoclonal, Chemicon, MAB1637, 1:200 dilution); VEGFR3 (rabbit polyclonal, Santa Cruz, SC-321, 1:25 dilution); VEGFC (mouse monoclonal, Santa Cruz, SC-74584, 1:100 dilution); Iba1 (goat polyclonal, Abcam, AB5076, 1:100 dilution) and neuronal nitric oxide synthase (nNOS) (mouse monoclonal, BD Biosciences, 610308, 1:100 dilution),

Please cite this article in press as: Ward MC, Cunningham AM. Developmental expression of vascular endothelial growth factor receptor 3 and vascular endothelial growth factor C in forebrain. Neuroscience (2015), http://dx.doi.org/10.1016/j.neuroscience.2015.04.063