VEGF-dependent and PDGF-dependent dynamic neurovascular reconstruction in the neurohypophysis of adult mice

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Abstract

Hypothalamo-neurohypophysial system (HNS) releases arginine vasopressin (AVP) and oxytocin (OXT) from axonal terminals of the neurohypophysis (NH) into blood circulation for controlling body fluid homeostasis and lactation. Chronic osmotic and suckling stimulations have been shown to cause neurovascular and neuroglial reconstruction in the NH of adult mammals and no study has been reported for vascular dynamics. The aim of this study was to elucidate the occurrence of continuous angiogenesis and growth factor-dependent neurovascular reconstruction in the NH of adult mice. Active proliferation of endothelial cells and oligodendrocyte progenitor cells (OPCs) was observed using the immunohistochemistry of bromodeoxyuridine and Ki-67. Vascular endothelial growth factor A (VEGFA) and VEGF receptor 2 (VEGFR2 (KDR)) were highly expressed at pituicytes and endothelial cells respectively. Moreover, prominent expression of platelet-derived growth factor B (PDGFB) and PDGF receptor beta was observed at OXT-containing axonal terminals and pericytes respectively. Administration of the selective tyrosine kinase inhibitor AZD2171 for VEGFRs and STI571 for PDGFRs significantly decreased proliferation of endothelial cells and OPCs. Moreover, AZD2171 treatment decreased vascular density by facilitating apoptosis of endothelial cells and the withdrawal of its treatment led to remarkable rebound proliferation of endothelial cells, so that vascular density rapidly returned to normal levels. AZD2171 decreased the density of both AVP- and OXT-containing axonal terminals, whereas STI571 selectively decreased the density of AVP-containing ones. Thus, this study demonstrates that the signaling pathways of VEGF and PDGF are crucial mediators for determining proliferation of endothelial cells and OPCs and the density of AVP- and OXT-containing axonal terminals in the HNS.

Key Words

- angiogenesis
- oxytocin
- vasopressin
- endothelial cell
- plasticity
- neurosecretion

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Introduction

Neurosecretion is an important issue to understand direct brain control of growth, maturation, reproduction, lactation, water homeostasis, and behaviors. In the neurohypophysis (NH), the hypothalamic neurosecretory cells secrete arginine vasopressin (AVP) and oxytocin (OXT) into the blood circulation from their axonal terminals (Miyata & Hatton 2002). Similarly, they release a variety of neuropeptides into the blood circulation at the median eminence (ME) to control secretion of adenohipophysial hormones (Ojeda *et al.* 1990, 2008, Müller *et al.* 1999). The vasculature of adult brains generally contains the blood–brain barrier (BBB) that consists of endothelial cell sheet and tight junction and prevents free entry of blood-derived bioactive molecules into parenchyma (Engelhardt 2003, Abbott *et al.* 2010, Daneman *et al.* 2010). The neurosecretory brain regions, the NH and ME, have fenestrated vasculature that has been defined as a characteristic of the lack of complete BBB and the direct diffusion of neuropeptides into blood circulation (Ciofi *et al.* 2009, Miyata & Morita 2011, Mullier *et al.* 2010, Morita & Miyata 2012, 2013).

The chronic physiological stimulations, such as osmotic and suckling ones, are well known to cause neurovascular and neuroglial reconstruction in the NH of adult rodents (Miyata & Hatton 2002). Under unstimulated conditions, pituicytes or neurohypophysial astrocytes generally enclose axonal terminals, while the chronic physiological stimulations increase direct contact of axonal terminals with vascular basement membrane (Miyata *et al.* 2001, Miyata & Hatton 2002). This structural reconstruction has been considered to be caused mainly by shape conversion of pituicytes via the β -adrenergic receptor (Smithson *et al.* 1990). Until now, however, evidence for the plasticity of neurohypophysial vasculature has not yet been reported.

In the ME, adenohipophysial hormone-releasing peptides, such as thyrotropin-releasing, corticotropin-releasing, growth hormone-releasing, and gonadotropin-releasing hormones (GnRH), are released from axonal terminals into blood circulation (Müller *et al.* 1999, Prevot 2002, Prevot *et al.* 2010). The axonal terminals of GnRH neurons are usually separated from the basement membrane of the capillary by cellular processes of astrocytes and tanocytes in the ME (Kozłowski & Coates 1985), while the axonal terminals of GnRH neurons and glial cells show a dynamic structural reconstruction during the estrous cycle similar to that observed in the NH (Prevot 2002, Ojeda *et al.* 2008, Prevot *et al.* 2010). These results indicate that activity-dependent structural reconstruction of

neurovascular and neuroglial interaction is a common phenomenon in two neurosecretory brain regions.

Thus far, little or no attention has been given to the point for elucidating the fundamental vascular characteristic in neurosecretory brain regions, except that they lack most of the tight junction proteins and thereby possess a fenestrated feature (Norsted *et al.* 2008, Mullier *et al.* 2010, Ciofi *et al.* 2009, 2011, Morita & Miyata 2012). However, we have recently shown the occurrence of vascular endothelial growth factor (VEGF)-dependent continuous angiogenesis and the expression of angiogenesis-associated proteins in the ME of adult mice (Morita *et al.* 2013). This study proposes that the vasculature of neurosecretory brain regions has dynamic characteristics. Angiogenesis starts at the embryonic period, persists until early the postnatal period, and then becomes quiescent along with the formation of the tight junctions (Ogunshola *et al.* 2000). Therefore, continuous angiogenesis is almost absent in adult mammalian brains except under pathological conditions such as injury or hypoxia (Brown & Thore 2011). In the angiogenic vasculature, proliferation of endothelial stalk cells and filopodia extension of endothelial tip cells are principally regulated by the signaling of VEGFA and VEGF receptor 2 (VEGFR2 (KDR); Gerhard *et al.* 2003, Somanath *et al.* 2009). The deficiency of *Vegfa* or *Vegfr2* genes appears to be lethal due to abnormal blood vessel development (Shalaby *et al.* 1995, Carmeliet *et al.* 1996). The signaling pathway of platelet-derived growth factor B (PDGFB) and PDGF receptor beta (PDGFRB) is responsible for the recruitment of pericytes to new-born endothelial cells and controls the migration and shape conversion of pericytes (Lindahl *et al.* 1997, Fukushi *et al.* 2004, Winkler *et al.* 2011). It has been shown that the embryos of *Pdgfb*- or *Pdgfrb*-deficient mice continue to develop until embryonic days 16–19, but widespread hemorrhage and edema cause embryonic lethality before birth (Lindahl *et al.* 1997).

Based on these observations, the objective of this study was to elucidate vascular dynamics by examining VEGF- and PDGF-dependent proliferation and apoptosis of endothelial cells and change in vascular density in the NH of adult mice. Moreover, we examined whether vascular reconstruction was associated with that of neurosecretory axonal terminals.

Materials and methods

Animals

This study used male mice (70–84 days old, C57BL/6J). Mice were housed in cages at a density of two per cage in a

colony room, maintained in a 12 h light:12 h darkness cycle, and allowed to access commercial chow and tap water *ad libitum*. All procedures were carried out in accordance with the National Institute of Health Guidelines and the Guideline for Proper Conduct of Animal Experiments, Science Council of Japan. The experimental protocol was approved by the Animal Ethics Experimental Committee of the Kyoto Institute of Technology.

Administration of tyrosine kinase inhibitors for VEGFRs and/or PDGFRs

We used the tyrosine kinase inhibitor SU11248 for both VEGFRs and PDGFRs, AZD2171 for VEGFRs, and STI571 for PDGFRs. Mice received 0.2 ml of the following solutions via oral gavage twice daily for 4 or 6 days: i) SU11248 (120 mg/kg per day; Carboxymethylcellulose, 1.8% NaCl, 0.4% Tween 80, and 0.9% benzyl alcohol), ii) AZD2171 (6 mg/kg per day, LKT Laboratories, Inc., St Paul, MN, USA) in 1% Tween 80, and iii) STI571 (200 mg/kg per day; LKT Laboratories, Inc.) in distilled deionized water.

RT-PCR

The experiment was carried out according to the method described previously (Morita *et al.* 2013). After decapitation, the pituitary glands were immediately dissected out and the anterior and intermediate pituitaries were carefully removed under the microscope and stored in RNAlater RNA stabilization reagent (Qiagen). Total RNA was extracted from the NH of adult mice using the SV Total RNA isolation system (Promega Corporation) and then subjected to cDNA synthesis with oligo(dT)₂₀ primers using the PC320 thermal controller (Astec, Fukuoka, Japan) and ReverTra Ace reverse transcriptase (Toyobo, Tokyo, Japan). The cDNA was subsequently amplified by PCR with the following set of primers: *Vegfa*, 5'-GCGGGCTGCCTCGCAGTC-3' and 5'-TCACCGCCTTGGCTTGTCAC-3'; *Vegfr2*, 5'-CTTGACAGGGGACAGCGGAC-3' and 5'-AATCGACCCTCGGCAGGGGA-3'; *Vegfr3* (*Flt4*), 5'-GCGACAGGGTTCTCATAA-3' and 5'-CGTTGCTCATTGTGATTAG-3'; *Pdgfra*, 5'-CGACTCCAGATGGGAATCCCC-3' and 5'-TGCCATCCACTTCACAGGCA-3'; and *Pdgfrb*, 5'-TGCCTCAGCCAAATGTCACC-3' and 5'-TGCTCACCACCTCGTATTCC-3'.

Western blotting

The experiments were carried out according to the method described previously (Morita *et al.* 2013). After decapitation, the pituitary glands of adult mice were immediately dissected out and the anterior and intermediate pituitaries were carefully removed under the microscope. The tissues were homogenized using a VCX T301B ultrasonic generator (Vibro Cell, Sonics & Materials, Inc., Newtown, CT, USA) in a fourfold amount of PBS containing protease inhibitors at 4 °C. The homogenate

was dissolved in an electrophoresis sample buffer at a concentration of 1 mg/ml and boiled for 90 s, and the protein sample was separated on SDS–polyacrylamide gel. Electrophoresis was performed on the membrane (GE Healthcare Bio-Science, Buckinghamshire, UK) in a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Nonspecific binding of proteins to the membrane was blocked by incubation with a 5% ECL Prime Blocking Agent (GE Healthcare Bio-Science) in 50 mM Tris-buffered saline containing 0.5% Tween 20 (TBST) overnight at 4 °C. The membrane was incubated with the rabbit antibody against VEGF-A (Santa Cruz Biotechnology; dilution 1:500) and PDGF-B (Bioss, Inc., Woburn, MA, USA; dilution 1:1000) in TBST containing 0.2% BSA for 1 h. The membrane was further incubated with a HRP-labeled anti-rabbit IgG (dilution 1:50 000) in TBST containing 5% BSA for 1 h. The approximate molecular weight for each protein was estimated using Precision Plus Protein WesternC Standards and Precision Protein Strep-Tactin HRP Conjugate (Bio-Rad Laboratories; dilution 1:50 000). The peroxidase was finally visualized using an ECL Kit (ECL Prime Western blotting detection system; GE Healthcare Bio-Sciences) and the immunoreactivity was visualized employing RX-U Medical X-ray film (Fuji Film, Tokyo, Japan).

Antibody

In this study, the following primary antibodies were used for the immunohistochemistry: rabbit IgG against AVP (Millipore–Chemicon, Temecula, CA, USA; dilution 1:1000), cleaved caspase 3 (clone 5A1E; Cell Signaling, Danvers, MA, USA; dilution 1:1000), Ki-67 (Santa Cruz Biotechnology; dilution 1:1000), PDGF-B (Bioss, Inc.; dilution 1:400), VEGF-A (Santa Cruz Biotechnology; dilution 1:200), VEGFR-2 (Sigma–Aldrich Japan; dilution 1:1000), and VEGFR-3 (e-Bioscience, San Diego, CA, USA; dilution 1:100) and rat IgG against BrdU (clone BU1/75; Abcam, Cambridge, UK; dilution 1:1000), PDGFRA (clone APA5; BD Pharmingen, Qume Drive, San Jose, CA, USA; dilution 1:500), and PDGFRB (clone APB5; e-Bioscience; dilution 1:200). The following primary antibodies were used for cellular markers: Armenian hamster IgG against CD31 (endothelial cell marker, clone 2HB; DSHB; dilution 1:20); mouse IgG against glial fibrillary acidic protein (GFAP, astrocyte marker; clone G-A-5; Sigma–Aldrich Japan; dilution 1:1000), OXT–neurophysin (clone PS38; NIH, Bethesda, MD, USA, Ben-Barak *et al.* 1985; dilution 1:20), and AVP–neurophysin (clone PS41; NIH, Ben-Barak *et al.* 1985; dilution 1:20); and guinea pig polyclonal IgG against GFAP (TS-2005, Morita *et al.* 2010; dilution 1:500) and laminin (vascular basement membrane marker, YI-2008, Imamura *et al.* 2010; dilution 1:200).

Immunohistochemistry for light microscopy

Mice were perfused transcardially with PBS (pH 7.2) containing 5 U/ml heparin followed by 4%

paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) after deep anesthesia with urethane. After perfusion of PFA, the pituitary glands, brains, and peripheral organs were dissected out and post-fixed with 4% PFA in 0.1 M PB (pH 7.4) overnight at 4 °C. Fixed tissues were cryoprotected with 30% sucrose in PBS and frozen in the Tissue-Tek OCT compound. The sections were cut coronally on a cryostat (Leica Geosystems AG, St Gallen, Switzerland) at the thickness of 30 µm. A standard immunofluorescence technique was performed on free-floating sections as described in our previous paper (Mannari *et al.* 2014). In brief, the sections were treated with 25 mM glycine in PBS for 20 min to eliminate the remaining aldehyde residues. When the mouse primary antibody was used, the sections were further treated with an unlabeled goat Fab fragment against the mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA; dilution 1:400) for 2 h to mask endogenous mouse IgG-like proteins. After blocking with 5% normal goat serum (NGS) in PBS containing 0.3% Triton X-100 (PBST) for 24 h at 4 °C, they were incubated with the primary antibody for 48 h at 4 °C. The primary rat, guinea pig, Armenian hamster, and rabbit antibodies were visualized using the Alexa 405-, 488-, or 594-conjugated secondary goat IgG antibody (Jackson ImmunoResearch; dilution 1:400). In case of the mouse primary antibody, however, Alexa 594-conjugated goat F(ab)₂ was used (Jackson ImmunoResearch; dilution 1:100) to avoid nonspecific binding of endogenous mouse Fc receptors. The coverslips were sealed with Vectashield (Vector Labs, Burlingame, CA, USA) and observation was performed using the laser-scanning confocal LSM510 (Carl Zeiss, Oberkochen, Germany) or FV10i (Olympus) microscope. Images were saved as TIF files (2048×2048) and arranged using Photoshop 7.0.

For assessing the area and diameter of microvessels and the vascular area, the sections (15 µm) were incubated with anti-laminin (YI-2008; dilution 1:1000) or anti-CD31 IgG (DSHB; dilution 1:100) in PBST for 48 h at 4 °C. They were then incubated with the biotinylated secondary guinea pig or Armenian hamster IgG (Jackson ImmunoResearch; dilution 1:200) in PBST for 2 h followed by VECTSTAIN Elite ABC Reagent (Vector Labs; dilution 1:200) for 2 h. Visualization of peroxidase was performed by treating the sections with 0.02% diaminobenzidine and 0.005% H₂O₂ in 50 mM Tris buffer (pH 7.2). After the chromogen reaction, the sections were mounted onto gelatin-coated glass slides, dehydrated, and covered with Entellan Neu (Merck) and observed under an Eclipse E600 microscope (Nikon, Tokyo, Japan).

Detection of proliferating and apoptotic cells

Mice were allowed to consume water containing BrdU (1 mg/ml) for 6 days to examine cell proliferation under normal condition and rebound proliferation after the withdrawal of AZD2171 treatment and for 4 days to investigate the effects of VEGFR- and/or PDGFR-associated tyrosine kinase inhibitors. The pituitary glands and brains

were dissected out and fixed as described above. The sections were washed with PBS, treated with 25 mM glycine in PBS for 20 min, and incubated with 10% NGS in PBST for 24 h. They were then incubated with the Armenian hamster IgG against CD31 (DSHB; dilution 1:20) or rat IgG against PDGFRA (BD Pharmingen; dilution 1:500) for 48 h at 4 °C and then with the Alexa 488-conjugated secondary Armenian hamster or rat IgG (Jackson ImmunoResearch; dilution 1:200) in PBST for 2 h. For the detection of BrdU, the sections were pretreated with 2 M HCl at 37 °C for 30 min and then neutralized with 0.1 M borate buffer (pH 8.4) for 20 min. They were incubated with the rat anti-BrdU IgG (Abcam; dilution 1:1000) in PBST containing 5% NGS for 48 h at 4 °C. The sections were then incubated with the Alexa 594-conjugated anti-rat IgG (Jackson ImmunoResearch; dilution 1:400) in PBST for 2 h. For visualization of nuclei, the sections were incubated with propidium iodide (Sigma-Aldrich Japan; 40 µg/ml).

For the immunohistochemistry of Ki-67 and caspase 3, the sections were incubated with the Armenian hamster IgG against CD31 (DSHB; dilution 1:20) for 48 h at 4 °C followed by the Alexa 488-conjugated secondary Armenian hamster IgG (Jackson ImmunoResearch; dilution 1:200) in PBST for 2 h. They were then treated with 0.05% citraconic anhydride solution (Immunosaver; Nissin EM Co. Ltd, Tokyo, Japan) for 30 min at 95 °C according to the previous study (Namimatsu *et al.* 2005). In the case for Ki-67, the sections were further treated with 2 N HCl for 20 min at 37 °C. After blocking with 5% NGS for 24 h at 4 °C, the sections were incubated with the rabbit IgG against Ki-67 (Santa Cruz Biotechnology; dilution 1:1000) or cleaved caspase 3 (Cell Signaling; dilution 1:1000) for 48 h at 4 °C. The sections were then incubated with the Alexa 594-conjugated anti-rat IgG or Alexa 488-conjugated anti-rabbit IgG (Jackson ImmunoResearch; dilution 1:400) in PBST for 2 h.

Quantitative and statistical analyses

For the quantitative analysis, confocal images were obtained under the same pinhole size, brightness, and contrast setting. The images (1024×1024 pixels) were saved as TIF files by employing LSM510 Image Browser (Carl Zeiss) or FV10-ASW Ver 1.7 Viewer (Olympus) for Windows, and arranged using Photoshop 7.0. For the quantitative analysis, BrdU- and PI-labeled nuclei were carefully identified by three-dimensional (3D) images (interval of optical slice at 1 µm). The number of BrdU-labeled endothelial cells, oligodendrocyte progenitor cells (OPCs), and CD31-immunoreactive endothelial cells, and the area of the laminin-positive vasculature and AVP- and OXT-immunoreactive axonal terminals were counted using WinRoof (Mitani Corporation, Fukui, Japan) for which the threshold intensity was set to be included for measurement profile by visual inspection and kept constant.

For assessing the area and diameter of microvessels, images of CD31 and laminin immunoreactivity visualized

with chromogen were obtained using the Eclipse E600 microscope (Nikon), saved as TIF files (1024×1024 pixels), and arranged using Photoshop 7.0. The area of the CD31-positive endothelial cells and the laminin-positive vasculature and the total area were measured using WinRoof, for which the threshold intensity was set to be included for measurement profile (only immunoreactive) by visual inspection and kept constant. The diameter of microvessels was manually measured at 10 µm interval and their mean diameter was obtained. Analysis of all images was performed such that the experimenter was blind to the treatment group. Statistical difference was assessed using a significance level of $P < 0.05$ by unpaired Student's *t*-test or ANOVA with Tukey's *post hoc* test.

Results

Proliferation of endothelial cells

We performed BrdU and Ki-67 immunohistochemistry to detect proliferating cells in the NH of adult mice. Double-labeling immunohistochemistry of BrdU and a vascular basement membrane marker laminin revealed that many BrdU-positive nuclei were observed at the neurohypophyseal vasculature after administration of BrdU for 6 days (Fig. 1A). Analysis of the 3D image revealed the presence of a BrdU-labeled nucleus at the neurohypophyseal vasculature (Fig. 1B). Double-labeling immunohistochemistry of BrdU and an endothelial cell marker CD31 revealed that BrdU-labeled nuclei were often seen at endothelial cells and parenchyma cells (Fig. 1C). The number of BrdU-labeled endothelial cells (mean number of BrdU-labeled cells $\pm$ S.E.M./mm²) was 131.66 ± 19.84 in the NH after administration of BrdU for 6 days. It has been shown that proliferating parenchyma cells are OPCs that differentiate into pituicytes in the NH of adult rats (Virard *et al.* 2008). Analysis of the 3D image revealed the presence of a BrdU-labeled nucleus within an endothelial cell (Fig. 1D). Double-labeling immunohistochemistry showed the presence of Ki-67-positive nuclei in CD31-positive endothelial cells (Fig. 1E). The expression of Ki-67 (MKI67) is strictly associated with all active phases of the cell cycle (G₁, S, G₂, and mitosis), but absent from resting cells (G₀). Analysis of the 3D image showed the presence of a Ki-67-positive nucleus within a CD31-positive endothelial cell (Fig. 1F).

Expression of VEGFA and VEGFRs

VEGFA and VEGFRs are principal signaling pathways for proliferation of endothelial cells in the developing vasculature (Gerhardt *et al.* 2003, Mackenzie & Ruhrberg 2012).

The immunohistochemistry showed that the expression of VEGFA was higher in the NH of adult mice when compared with that of the anterior and intermediate pituitaries (Fig. 2A). Double-labeling immunohistochemistry of VEGFA and a pituicyte marker GFAP revealed that many VEGFA-immunoreactive puncta were seen at pituicytes (Fig. 2B). RT-PCR analysis revealed the expression of *Vegfa*₁₂₀ but not *Vegfa*₁₆₄ and *Vegfa*₁₈₈ mRNA in the NH (Fig. 2C). Western blotting further confirmed the predominant expression of the mature form of VEGFA₁₂₀ by judging from their molecular mass (Fig. 2C). Triple-labeling immunohistochemistry of VEGFR2, CD31, and laminin revealed that VEGFR2 was expressed specifically in endothelial cells in the neurohypophyseal vasculature (Fig. 2D). RT-PCR analysis further showed the expression of *Vegfr2* mRNA in the NH of adult mice (Fig. 2E). Double-labeling immunohistochemistry showed that VEGFR3 was expressed in AVP- (Fig. 2H) and OXT-containing (data not shown) axonal terminals and pituicytes (Fig. 2I) but not in endothelial cells (Fig. 2F). RT-PCR analysis showed the expression of *Vegfr3* mRNA in the NH of adult mice (Fig. 2G).

Expression of PDGFB and PDGFRs

PDGFB and PDGFRB are generally highly expressed in endothelial cells and pericytes in the developing vasculature and mediate the interaction between endothelial cells and pericytes and the migration and shape conversion of pericytes (Lindahl *et al.* 1997, Fukushi *et al.* 2004, Daneman *et al.* 2010). Triple-labeling immunohistochemistry revealed that PDGFB was localized to OXT-containing axonal terminals but not to CD31-positive endothelial cells (Fig. 3A). The immunoreactivity of PDGFB was observed as numerous puncta within OXT-containing axonal terminals (Fig. 3C) but not in the AVP-containing ones (Fig. 3B). High-magnification views showed the localization of PDGFB to neurosecretory granules of OXT-containing axonal terminals (Fig. 3D). Western blotting confirmed the expression of PDGFB in the NH of adult mice (Fig. 3E). It has been demonstrated that PDGFRA-positive OPCs differentiate into pituicytes in the NH (Virard *et al.* 2008). Double-labeling immunohistochemistry of PDGFRA and a pituicyte marker GFAP revealed that the expression of PDGFRA in pituicytes was at an undetectable level (Fig. 3F). RT-PCR analysis further indicated the expression of *Pdgfra* mRNA from neurohypophyseal cDNA of adult mice (Fig. 3G). Double immunolabeling of PDGFRB and a pericyte marker desmin revealed that the expression of PDGFRB was

Figure 1

Proliferation of endothelial cells in the NH of adult mice. Proliferating cells were detected by the immunohistochemistry of BrdU and Ki-67. For BrdU-labeling, mice were allowed to consume water containing BrdU (1 mg/ml) for 6 days. (A and B) Confocal images revealing that BrdU-labeled nuclei were frequently seen in the laminin-positive vasculature (arrowheads in (A)). Analysis of the 3D image revealed the presence of the BrdU-labeled nucleus within the laminin-positive vasculature (arrowhead in (B)). (C and D) Double-labeling immunohistochemistry with an endothelial cell marker CD31 showed that BrdU-labeled nuclei were frequently seen

in endothelial cells (arrowheads in (C)) and CD31-negative parenchyma cells (arrows in (C)). Analysis of the 3D image revealed the presence of the BrdU-labeled nucleus within a CD31-positive endothelial cell (D). (E and F) Ki-67-immunoreactive nuclei were detected in CD31-positive endothelial cells (arrowheads in (E)) and CD31-negative parenchyma cells (arrows in (E)). Analysis of the 3D image confirmed the presence of the Ki-67-positive nucleus within a CD31-positive endothelial cell (F). Scale bars represent 10 μ m (B, D and F) and 50 μ m (A, C and E).

specific at pericytes (Fig. 3H). RT-PCR analysis further indicated the expression of *Pdgfrb* mRNA in the NH of adult mice (Fig. 3I).

Effects of tyrosine kinase inhibitor for VEGFRs and/or PDGFRs on proliferation of endothelial cells and OPCs

Administration of the tyrosine kinase inhibitor SU11248 for both VEGFRs and PDGFRs, AZD2171 for VEGFRs, and STI571 for PDGFRs apparently decreased the number of

BrdU-labeled endothelial cells when compared with the control (Fig. 4A). Similarly, administration of AZD2171 decreased the number of BrdU-labeled OPCs when compared with the control (Fig. 4B). The quantitative analysis revealed that treatment with SU11248 significantly decreased the number of BrdU-labeled endothelial cells (% of control: 5.62 ± 1.35 , $P=0.00037$ by ANOVA with Turkey's *post hoc* tests, $n=4$) and OPCs (12.91 ± 1.54 , $P=0.00049$) when compared with that of the control (Fig. 4C). Administration of AZD2171 significantly decreased the number of

Figure 2

The localization of VEGFA and VEGFRs in the NH of adult mice. (A, B and C) The immunoreactivity of VEGFA was prominently higher in the NH when compared with that of the anterior and intermediate pituitary (A). There were many VEGFA-immunoreactive puncta in GFAP-positive pituicytes (arrowheads at inset of (B)). RT-PCR analysis showed a prominent signal at a band corresponding to *Vegfa*₁₂₀ mRNA (512 bp) but not *Vegf-f-A*₁₈₈ (716 bp) and *Vegfa*₁₆₄ (644 bp) mRNAs from neurohypophysial cDNA (C). A mature form of VEGFA₁₂₀ was detected with a molecular weight of 18 kDa by western blotting (C). (D and E) The immunoreactivity of VEGFR2

was observed specifically in CD31-positive endothelial cells of the vasculature (arrowheads in (D)). RT-PCR analysis revealed a specific signal corresponding to the predicted size (314 bp) of *Vegfr2* mRNA (E). (F, G, H and I) The immunoreactivity of VEGFR3 was observed in axonal terminals (arrowheads in (F) and (H)) and GFAP-positive pituicyte (arrows in (F) and (I)). RT-PCR analysis revealed a specific signal corresponding to the predicted size (290 bp) of *Vegfr3* mRNA (G). AP, anterior pituitary; IP, intermediate pituitary; Lam, laminin. Scale bars represent 10 μ m (insert in (B)) and 50 μ m (A, B, D, F, H and I).

BrdU-labeled endothelial cells (1.5 mg , 30.05 ± 10.54 , $P=0.0072$; 3 mg , 38.42 ± 5.66 , $P=0.0066$; and 6 mg , 4.33 ± 1.11 , $P=0.00024$) when compared with that of the control. Similarly, treatment with AZD2171 significantly

decreased the number of BrdU-labeled OPCs (1.5 mg , 44.07 ± 12.17 , $P=0.034$; 3 mg , 46.92 ± 3.82 , $P=0.018$; and 6 mg , 28.21 ± 2.59 , $P=0.011$) when compared with that of the control. Administration of STI571 significantly

Figure 3

The localization of PDGFB and PDGFRs in the NH of adult mice. (A, B, C, D and E) The immunoreactivity of PDGFB was observed in OXT-containing axonal terminals (arrowheads in (A)) but not AVP-containing axonal terminals (B) and CD31-positive endothelial cells (arrows in (A)). A high-magnification view showed that the immunoreactivity of PDGFB was observed within neurosecretory granules of OXT-containing axonal terminals (C and D). Western blotting showing a prominent signal of PDGFB with a molecular weight of 24 kDa and faint signal of PDGFB with a molecular weight of

48 kDa (E). (F and G) The immunoreactivity of PDGFRA (arrowheads in (F)) was not observed in GFAP-positive pituitary cells. RT-PCR analysis revealed a specific signal corresponding to the predicted size (823 bp) of *Pdgfra* mRNA (G). (F and G) Double-labeling immunohistochemistry using a pericytes marker desmin revealed that the immunoreactivity of PDGFRB was specifically observed in pericytes (arrows in (H)). RT-PCR analysis revealed a specific signal corresponding to the predicted size (162 bp) of *Pdgfrb* mRNA (I). Scale bars represent 500 nm (D), 10 μm (C), and 50 μm (A, B, F and H).

decreased the number of BrdU-labeled endothelial cells (34.77 ± 6.21 , $P=0.0049$) and OPCs (20.78 ± 2.70 , $P=0.00096$) when compared with that of the control.

Rebound proliferation of endothelial cells after the withdrawal of VEGFR signaling inhibition

To examine the reversibility of cellular proliferation decreased by VEGFR signaling inhibition, mice received

oral administration of AZD2171 (6 mg/kg per day) for 6 days and then kept without AZD2171 treatment for 6 days. Double-labeling immunohistochemistry revealed that BrdU-labeled endothelial cells were conspicuously increased after the withdrawal of AZD2171 treatment when compared with the control (Fig. 5A and B). BrdU-labeled endothelial cells often lay close to the same line of the microvessel (Fig. 5A and B). The quantitative analysis showed that the number of BrdU-labeled

Figure 4

The effects of tyrosine kinase inhibitors for VEGFRs and/or PDGFRs on proliferation of endothelial cells and OPCs in the NH of adult mice. Animals received oral administration of the tyrosine kinase inhibitor SU11248 for both VEGFRs and PDGFRs, AZD2171 for VEGFRs, and STI571 for PDGFRs together with drinking of BrdU-containing water (1 mg/ml) for 4 days. (A and B) There were many BrdU-labeled endothelial cells (arrowheads in (A)) and OPCs (arrowheads in (B)) in the NH of control mice, but treatment with SU11248 (120 mg/kg per day), AZD2171 (6 mg/kg per day), and STI571

(200 mg/kg per day) largely diminished BrdU-labeled endothelial cells and OPCs. Scale bars represent 50 μ m. (C) The quantitative analysis showed that the number of BrdU-labeled endothelial cells and OPCs was significantly decreased by treatment with SU11248 (120 mg/kg per day), AZD2171 (1.5, 3, and 6 mg/kg per day), and STI571 (200 mg/kg per day) when compared with that of the vehicle control. Data are expressed as mean $\pm$ s.e.m., $n=4$, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs the control by ANOVA using Turkey's *post hoc* tests.

endothelial cells was 10.59 times higher in AZD2171-treated mice ($P=0.00000015$ by unpaired Student's *t*-test, $n=5$) when compared with the control (Fig. 5C). By contrast, administration of AZD2171 significantly ($P=0.017$) decreased the number of BrdU-labeled OPCs by 33.0% when compared with the control. The basal proliferation of endothelial cells was significantly lower

in the somatosensory cortex (number of BrdU-labeled cells/mm²; 0.71 ± 0.14 , $P=0.00035$ by Student's *t*-test) when compared with the NH (131.66 ± 19.84). Proliferation of endothelial cells was not significantly changed in the somatosensory cortex (0.47 ± 0.13 , $P>0.05$) after the withdrawal of AZD2171 treatment when compared with the control.

Figure 5

Dramatic rebound proliferation of endothelial cells but not OPCs after the withdrawal of the VEGFR-associated tyrosine kinase inhibitor AZD2171 treatment in the NH of adult mice. Mice received oral administration of the tyrosine kinase inhibitor AZD2171 (6 mg/kg per day) or vehicle for 6 days and then were kept without AZD2171 treatment for 6 days. After the withdrawal of AZD2171 treatment, animals were allowed to consume BrdU-containing water (1 mg/ml) for 6 days. (A and B) A large number of BrdU-labeled endothelial cells were observed after the withdrawal of 6-day administration of AZD2171 when compared with the control. Arrowheads

indicate BrdU-labeled endothelial cells. Scale bars represent 50 μm. (C) The quantitative analysis showed that the number of BrdU-labeled endothelial cells was significantly increased after the withdrawal of 6-day administration of AZD2171 when compared with the control. By contrast, the number of BrdU-labeled OPCs was significantly lower after the withdrawal of 6-day administration of AZD2171 than the control. AZD2171 + R, 6-day recovery after the withdrawal of AZD2171 treatment. Data are expressed as mean $\pm$ S.E.M., $n=4$, * $P<0.05$ and *** $P<0.001$ vs the control by unpaired Student's t -test.

Effects of VEGFR and PDGFR signaling inhibition on vascular structure

The vascular structure of the NH is unique, as endothelial cells are surrounded by a large perivascular space consisting of two layers of the basement membrane (Morita & Miyata 2012). Double-labeling immunohistochemistry showed that CD31-positive endothelial cells were covered by a large laminin-positive perivascular space (Fig. 6A). High-magnification views showed that the inner basement membrane covered endothelial cells and the outer basement membrane enveloped the large perivascular space (Fig. 6B). Administration of the VEGF-associated tyrosine kinase inhibitor AZD2171 significantly decreased the area of CD31-positive endothelial cells (Fig. 6C) and the laminin-positive vasculature (Fig. 6D) when compared with the control. AZD2171 also decreased the number of CD31-positive endothelial cells when compared with the control (Fig. 6E). The quantitative analysis revealed that AZD2171 significantly decreased the area of CD31-positive endothelial cells (% of CD31-immunoreactive area/total area: control, 23.39 ± 1.60 and AZD2171, 15.44 ± 1.06 ; $P=0.0059$ by ANOVA with Turkey's *post hoc* tests, $n=5$; Fig. 6F). Administration of AZD2171 significantly reduced the density of CD31-positive endothelial cells (% of control: control, 100.00 ± 1.48 and AZD2171, 79.92 ± 0.69 ; $P=0.00000083$; Fig. 6F). Treatment with AZD2171 did not change ($P>0.05$) the diameter of CD31-positive microvessels (Fig. 6F). Treatment with AZD2171 significantly decreased the area of the laminin-positive vasculature (% of laminin-immunoreactive area/total area: control, 54.06 ± 1.74 and AZD2171, 46.83 ± 0.60 ; $P=0.0000062$) when compared with that of the control (Fig. 6F). The area and number of CD31-positive endothelial cells and the area of the laminin-positive vasculature returned to control levels 6 days after the withdrawal of AZD2171 treatment. Administration of AZD2171 did not significantly ($P>0.05$) change the area of CD31-positive endothelial cells in the anterior pituitary, the somatosensory cortex, the kidney, the liver, the adrenal cortex, and the adrenal medulla (Fig. 6G).

In contrast to AZD2171, administration of the PDGF-associated tyrosine kinase inhibitor STI571 did not significantly ($P>0.05$) change the area of CD31-positive endothelial cells (Fig. 7A and B) and the laminin-positive vasculature (Fig. 7C and D) when compared with the control. The withdrawal of STI571 treatment slightly increased the number of BrdU-labeled OPCs (control, 100 ± 15.07 and STI571, 152.28 ± 5.31 ; $P=0.018$ by unpaired Student's *t*-test, $n=4$), but did not significantly

($P=0.05$) change the number of BrdU-labeled endothelial cells (Fig. 7E, F and G).

Effects of VEGF signaling inhibition on apoptosis of endothelial cells

We examined the effects of AZD2171 on the expression of an apoptosis marker caspase 3 in endothelial cells to demonstrate that the decrease in vascular density caused by VEGFR signaling inhibition is due to the apoptosis of endothelial cells. Mice received oral administration of AZD2171 (6 mg/kg per day) for 1, 2, and 3 days and were then fixed for the immunohistochemistry of cleaved caspase 3. Double-labeling immunohistochemistry revealed that caspase 3-positive endothelial cells were more often seen after administration of AZD2171 when compared with the control (Fig. 8A, B, C and D). High-magnification views revealed continuous distribution of caspase 3-positive endothelial cells in the same microvessel (Fig. 8E, F, G and H). The quantitative analysis showed that the number of caspase 3-positive endothelial cells was 5.26 times on day 1 ($P=0.01309$ by ANOVA with Turkey's *post hoc* tests, $n=4$), 4.97 times on day 2 ($P=0.02121$), and 6.70 times on day 3 ($P=0.001314$) in AZD2171-treated mice when compared with the control (Fig. 8I).

Effects of VEGFR and PDGFR signaling inhibition on the density of AVP- and OXT-containing axonal terminals

We examined the effects of AZD2171 and STI571 on the density of AVP- and OXT-immunoreactive axonal terminals to demonstrate that inhibition of VEGFR and PDGFR signaling causes reconstruction of AVP- and OXT-containing axonal terminals. Mice received oral administration of AZD2171 (6 mg/kg per day) or STI571 (200 mg/kg per day) for 6 days and were then fixed for the immunohistochemistry. Double-labeling immunohistochemistry revealed that OXT-immunoreactive axonal terminals were more likely to localize in close proximity to the vasculature than the AVP-containing ones in control animals (Fig. 9A and D). The number of AVP-immunoreactive axonal terminals was decreased after treatment with AZD2171 (Fig. 9B) and STI571 (Fig. 9C) when compared with the control (Fig. 9A). The number of OXT-immunoreactive axonal terminals was decreased by treatment with AZD2171 (Fig. 9E), but not STI571 (Fig. 9F), when compared with the control (Fig. 9D).

The quantitative analysis revealed that treatment with AZD2171 significantly decreased the density of both

Figure 6

The effects of the VEGFR-associated tyrosine kinase inhibitor AZD2171 on vascular structure in the NH of adult mice. Animals received oral administration of AZD2171 (6 mg/kg per day) for 6 days and were fixed for the immunohistochemistry of CD31 and laminin. (A and B) Confocal images showing the presence of a large perivascular space around CD31-positive endothelial cells (A) and two layers of the laminin-positive basement membrane (arrows and arrowheads in (B)). (C, D and E) Light microscopes, using a conventional chromogen method, revealing the reduction in the area of CD31-positive endothelial cells (C) and vascular area surrounded by a laminin-positive outer basement membrane (D) by administration of AZD2171. Confocal images showing that the number of CD31-positive endothelial cells (arrowheads) was decreased by administration of

AZD2171 (E). Scale bars represent 50 μm. (F and G) Administration of AZD2171 significantly decreased the area of CD31-immunoreactive endothelial cells and laminin-positive vascular area, and the number of CD31-positive endothelial cells, but did not change the diameter of CD31-immunoreactive endothelial microvessels (F). Administration of AZD2171 did not change the area of CD31-immunoreactive endothelial cells in the anterior pituitary, the somatosensory cortex layers II-III and V, and the peripheral organs (G). 6d, 6-day administration of AZD2171; 6d+R, 6-day recovery after the withdrawal of AZD2171 treatment; ECs, endothelial cells; PI, propidium iodide. Data are expressed as mean $\pm$ s.e.m., $n=5$, $**P<0.01$ and $***P<0.001$ vs the control by ANOVA using Turkey's *post hoc* tests (F) and unpaired Student's *t*-test (G).

Figure 7

The effects of the PDGFR-associated tyrosine kinase inhibitor STI571 on vascular structure and proliferation of endothelial cells and OPCs after the withdrawal of STI571 treatment in the NH of adult mice. (A, B, C and D) Animals received oral administration of STI571 (200 mg/kg per day) for 6 days and were fixed for the immunohistochemistry of CD31 and laminin. Light microscopes (A and C) and quantitative analysis (B and D) revealing no change in the area of CD31-positive endothelial cells and the laminin-positive vasculature by administration of STI571. (E, F and G) Animals received oral administration of the tyrosine kinase inhibitor STI571 (200 mg/kg per day) or vehicle for 6 days and then were kept without

STI571 treatment for 6 days. After the withdrawal of STI571 treatment, animals were allowed to consume BrdU-containing water (1 mg/ml) for 6 days. The withdrawal of administration of STI571 slightly increased the number of BrdU-labeled OPCs (arrowheads) but not endothelial cells (arrows). (E and F) The quantitative analysis showed that the number of BrdU-labeled OPCs but not endothelial cells was significantly increased after the cessation of 6-day administration of STI571 when compared with the control (G). Scale bars represent 50 μ m. Data are expressed as mean $\pm$ S.E.M., $n=5$, * $P<0.05$ vs the control by unpaired Student's t -test.

AVP-immunoreactive axonal terminals ($P=0.0029$ by unpaired Student's t -test, $n=4$) and OXT-immunoreactive axonal terminals ($P=0.0020$) when compared with the control (Fig. 9G). The density of AVP- and OXT-immunoreactive axonal terminals localized in close proximity to the vasculature was significantly decreased (AVP, $P=0.0060$ and

OXT, $P=0.00077$) by treatment with AZD2171, while the density of AVP- and OXT-immunoreactive axonal terminals that were away from the vasculature was significantly increased (AVP, $P=0.0025$ and OXT, $P=0.00030$). Treatment with AZD2171 significantly increased the ratio of OXT-immunoreactive axonal terminal area, but not

Figure 8

The effects of the VEGFR-associated tyrosine kinase inhibitor AZD2171 on apoptosis of endothelial cells in the NH of adult mice. Animals received oral administration of the tyrosine kinase inhibitor AZD2171 for VEGFRs (6 mg/kg per day) twice daily for 1, 2, and 3 days. The rabbit IgG against caspase 3 detects endogenous levels of the activated caspase 3 resulting from a cleavage adjacent to Asp175 but does not recognize full-length caspase 3 or other cleaved caspases. (A, B, C, D, E, F, G and H) Confocal images showing that caspase 3-positive endothelial cells (arrowheads) were more frequently

observed in AZD2171-treated mice when compared with the control (A, B, C and D). High-magnification views revealed that caspase3-positive endothelial cells (arrows) were continuously distributed (E, F, G and H). Scale bars represent 50 μ m. (I) The quantitative analysis showed that the number of caspase 3-positive endothelial cells was significantly higher at days 1, 2, and 3 of administration of AZD2171 when compared with the control. Data are expressed as mean $\pm$ s.e.m., $n=4$, * $P<0.05$ and ** $P<0.01$ vs the control by ANOVA using Turkey's *post hoc* tests.

AVP-immunoreactive axonal terminal area, to laminin-positive vascular area (AVP, $P>0.05$ and OXT, $P=0.0031$).

The quantitative analysis revealed that STI571 significantly decreased the density of total axonal terminals ($P=0.00029$ by unpaired Student's *t*-test, $n=4$), axonal terminals localized in close proximity to the vasculature ($P=0.0013$) and away from the vasculature ($P=0.00063$) of AVP-immunoreactive neurons, and the area ratio of axonal terminals to the vasculature ($P=0.0019$) when compared with the control (Fig. 9H). By contrast, all examined parameters of OXT-immunoreactive neurons were not changed by treatment with STI571 ($P>0.05$).

Discussion

The present main finding is the demonstration of vascular dynamics in the NH of adult mice.

The inhibition of VEGF signaling decreased proliferation of endothelial cells and OPCs but promoted apoptosis of endothelial cells, which resulted in a low vascular density. Moreover, the withdrawal of VEGFR signaling inhibition caused prominent rebound proliferation of endothelial cells but not OPCs. The inhibition of PDGF signaling did not change the vascular structure and cause rebound proliferation of endothelial cells, although it decreased proliferation of endothelial cells and OPCs. The inhibition of VEGF signaling decreased the density of both AVP- and OXT-containing axonal terminals and that of PDGF signaling decreased the density of only AVP-containing axonal terminals. These results offer the key to an understanding of the fundamental property of fenestrated vasculature in neurosecretory brain regions and, moreover, shed light on a new aspect to understand neurosecretion.

Figure 9

The effects of VEGFR- and PDGFR-associated tyrosine kinase inhibitors on the density of AVP- and OXT-containing axonal terminals in the NH of adult mice. Animals received oral administration of AZD2171 (6 mg/kg per day) or STI571 (200 mg/kg per day) for 6 days and were fixed for the immunohistochemistry. (A, B, C, D, E and F) Confocal images showed that the density of AVP-immunoreactive axonal terminals was reduced by administration of AZD2171 and STI571 (A, B and C). The density of OXT-immunoreactive axonal terminals was reduced by administration of AZD2171 but not STI571 (D, E and F). Arrowheads and arrows indicate axonal terminals in contact and without contact with the vasculature respectively. Scale bar represents 50 μm. (G and H) The quantitative analysis showed that treatment with AZD2171 decreased the area of total

AVP-immunoreactive axonal terminals and AVP-immunoreactive axonal terminals in contact with the vasculature but increased the area of AVP-immunoreactive axonal terminals not in contact with the vasculature (G). Same changes were observed in OXT-immunoreactive axonal terminals of AZD2171-treated animals, except that the area ratio of axonal terminal to the vasculature was increased. Treatment with STI571 decreased the area of total axonal terminals, axonal terminals in contact and without contact with the vasculature, and the ratio of axonal terminals to the vasculature in AVP-immunoreactive neurons but not the OXT-immunoreactive ones (H). Data are expressed as mean $\pm$ s.e.m., $n=4$, ** $P<0.01$ and *** $P<0.001$ vs the control by unpaired Student's t -test.

Continuous angiogenesis

In this study, we found many BrdU-labeled and Ki-67-positive nuclei in the endothelial cells of adult mice. Moreover, the expression of principal angiogenesis-inducing mediators, VEGFA and VEGFR2, was found in pituicytes and endothelial cells, respectively, in the

NH of adult mice. A previous *in situ* hybridization study has reported higher expression of *Vegfa* mRNA in the NH when compared with the anterior and intermediate pituitary (Ochoa *et al.* 2000). It has been shown that astrocyte-derived VEGFA plays a crucial role in the formation and growth of new blood vessels in the

developing brain (Bozoyan *et al.* 2012) and retina (Gerhardt *et al.* 2003, Scott *et al.* 2010). Proliferation of endothelial cells is mainly regulated by the signaling of VEGFA and VEGFR2 in the developing vasculature (Gerhardt *et al.* 2003, Somanath *et al.* 2009). The expression level of total *Vegfa* mRNA is high during early embryonic period but it gradually decreases along with brain maturation (Ng *et al.* 2001, Mackenzie & Ruhrberg 2012). It is also known that the expression pattern of the VEGFA isoform is different depending on developmental stages and tissues. Although the expression level of *Vegfa*₁₂₀ mRNA is remarkably high at the early embryonic period and gradually decreases thereafter, the level of *Vegfa*₁₆₄ mRNA increases along with the brain development and the level of *Vegfa*₁₈₈ is relatively low and constant throughout life (Ng *et al.* 2001). Transgenic mice that express only *Vegfa*₁₂₀ result in lethality just before or after the birth (Karkkainen *et al.* 2004), while animals overexpressing *Vegfa*₁₂₀ in the forebrain augment angiogenesis in the hippocampus (Udo *et al.* 2008). The present pharmacological experiment using the selective VEGFR inhibitor AZD2171 largely attenuated proliferation of endothelial cells in the NH. Moreover, the withdrawal of administration of the VEGFR signaling inhibitor induced drastic rebound proliferation of endothelial cells. On the other hand, the expression of VEGFR3 was observed at AVP- and OXT-containing axonal terminals and pituicytes. VEGFR3 is reported to bind with VEGFC and VEGFD but not with VEGFA (Karkkainen *et al.* 2004, Roberts *et al.* 2006). VEGFR1 has a binding affinity for VEGFA and VEGFB and an inhibitory effect on proliferation of endothelial cells (Keyt *et al.* 1996, Fong *et al.* 1999). Our previous study has demonstrated continuous proliferation of endothelial cells and the expression of angiogenesis-associated proteins such as NG2 (CSPG4), PDGFRB, and Delta-like ligand 4 in the ME of adult mice (Morita *et al.* 2013). It is concluded that VEGFA and VEGFR2 together play a crucial role in continuous angiogenesis in the NH of adult mice, which is a common feature in two neurosecretory brain regions.

Significance of VEGF signaling

This study revealed that administration of the VEGFR signaling inhibitor AZD2171 for 6 days largely reduced the area of CD31-positive endothelial cells and the laminin-positive vasculature in the NH, but not in the anterior pituitary, the cerebral cortex, and the peripheral organs. It is reported that administration of VEGF signaling inhibitors for 14 and 21 days reduces the area of endothelial cells in the

islets of the pancreas, the thyroid, the adrenal cortex, the anterior pituitary, and the NH, but not in the acini of pancreas, the adrenal medulla, and the cerebral cortex of brains (Kamba *et al.* 2006). This difference is probably due to the time period of VEGF inhibitor treatment, indicating that the control of neurohypophyseal vascular density is much more dependent on the VEGF signaling when compared with other tissues. In this study, moreover, we found that the decrease in the number of endothelial cells resulted from the promotion of endothelial cell apoptosis rather than simply the attenuation of their proliferation. Moreover, the withdrawal of AZD2171 treatment increased the number of BrdU-labeled endothelial cells and rapidly returned to the control level, while such dramatic rebound proliferation was not observed after the withdrawal of the PDGFR inhibitor STI571. There is no doubt that the surface of the vascular basement membrane is important for neurosecretion to interact with axonal terminals of AVP- and OXT-containing neurons (Tweedle & Hatton 1987, Miyata *et al.* 2001, Miyata & Hatton 2002). Thus, these experiments demonstrate that VEGF signaling is a crucial mediator for proliferation and apoptosis of endothelial cells, indicating the presence of VEGF-dependent regulatory mechanism to control the vascular density in the NH of adult mice.

In this study, the inhibition of VEGF signaling largely decreased the density of AVP- and OXT-containing axonal terminals. The density of axonal terminals in contact with the vasculature was reduced, while that of axonal terminals not in contact with the vasculature was increased. In the adult songbird brain, new neurons arise from the ventricular zone and are recruited to the higher vocal center. VEGF-dependent endothelial cell division and angiogenesis provide microvascular expansion in the higher vocal center, which in turn secretes the brain-derived neurotrophic factor to support the recruitment of newly generated neurons (Louissaint *et al.* 2002, Chen *et al.* 2013a,b). The perivascular niche provides a source of both trophic support and migratory cues for the newly generated neural stem/progenitor cells. The tenascin, laminin, heparan sulfates, and chondroitin sulfate proteoglycans have all been identified as perivascular substrates for neuronal migration in the subventricular zone and rostral migratory stream (Goldman & Chen 2011). The expression of tenascin C (Theodosios *et al.* 1997), laminin (Imamura *et al.* 2010), and chondroitin sulfate proteoglycans (Morita *et al.* 2010) has been reported in the NH. These results imply the presence of perivascular niche for VEGF-dependent coordinate regulation of axonal terminals and endothelial cells in the NH of adult mice.

VEGF has been shown to increase microvascular permeability and endothelial fenestration (Roberts & Palade 1995). The activation of VEGFR2 also alters extracellular matrix, as well as adherens junctions and tight junctions (Zhang & Chopp 2002, Sandoval & Witt 2008). A role of the *Src* pathway in the downregulation of adherens and tight junction proteins has already been demonstrated (Gavard & Gutkind 2006, Takenaga *et al.* 2009). In fetal brains, vascular permeability of high-molecular-mass molecules is very low as in the adult brains (Armulik *et al.* 2010, Daneman *et al.* 2010), whereas the permeability of low-molecular-mass molecules (<1000) is higher in fetal brains than in adult brains (Tuor *et al.* 1992, Keep *et al.* 1995). Thus, VEGFA may be a possible mediator to determine the fenestrated feature of the neurohypophyseal vasculature.

Significance of PDGF signaling

PDGFB and PDGFRB are highly expressed in endothelial cells and pericytes, respectively, in the developing vasculature (Lindahl *et al.* 1997). PDGFRB has a binding affinity for PDGFB but not PDGFA, whereas PDGFRA is able to bind to both PDGFA and PDGFB (Chen *et al.* 2013a,b). The present immunohistochemistry revealed that PDGFB and PDGFRB were highly expressed in OXT-containing axonal terminals and pericytes respectively. It has been demonstrated that PDGFRA is expressed specifically in OPCs in the NH of adult rats (Virard *et al.* 2008). The present pharmacological experiment using the selective PDGFR inhibitor STI571 largely diminished proliferation of endothelial cells in the NH of adult mice. The signaling of PDGFB and PDGFRB plays a crucial role in the interaction of pericytes with endothelial cells by controlling the migration and shape conversion of pericytes (Lindahl *et al.* 1997, Fukushi *et al.* 2004, Ribatti *et al.* 2011, Winkler *et al.* 2011). The PDGFR inhibitor STI571 has been shown to inhibit angiogenesis by preventing PDGFRB of pericytes (Bauman *et al.* 2007, Rocha *et al.* 2007). In this study, PDGFB is stored within OXT-containing neurosecretory granules, suggesting that PDGFB is co-released with OXT from axonal terminals. It has been suggested that localized secretion of OXT is important for the integrity of neurovascular contacts in the NH of developing zebrafish (Gutnick *et al.* 2011). Axonal fibers of the peripheral sensory neurons regulate the pattern of arterial branching via local secretion of VEGFA_{160/120} (Mukouyama *et al.* 2002, 2005). Therefore, it is possible that PDGF signaling controls proliferation of endothelial cells possibly through an indirect interaction between pericytes and endothelial cells.

This study demonstrated that the inhibition of PDGF signaling reduced the density of AVP-containing axonal terminals without changing the vascular density. Recently, we have found that Notch3 and a Notch ligand Delta-like ligand 4 are specifically expressed in OXT-containing axonal terminals and pituicytes respectively (Mannari & Miyata 2014). This observation indicates the specific interaction between OXT-containing axonal terminals and pituicytes and/or OPCs to discriminate OXT-containing axonal terminals from the AVP-containing ones. This study demonstrated that PDGFB was highly expressed in OXT-containing axonal terminals but not in the AVP-containing ones. Thus, it is possible that PDGFB derived from OXT-containing axonal terminals may be necessary for the maintenance of AVP-containing axonal terminals.

In this study, the inhibition of PDGFR signaling prominently inhibited proliferation of OPCs in the NH. It has been demonstrated that PDGFRA-expressing OPCs are shown to proliferate and differentiate into pituicytes and the chronic salt loading significantly increased proliferation of OPCs in the NH of adult rats (Virard *et al.* 2008). It has been reported that proliferation of OPCs is mediated through PDGF and neurotrophin 3 (Robinson & Miller 1996). Fibroblast growth factor 2 and PDGF promote the survival, migration, and differentiation of OPCs in the subventricular zone (Lachapelle *et al.* 2002). While physiological concentrations of PDGF were not in themselves sufficient to promote OPC proliferation, PDGF-mediated integrin activation is the critical integrative step in proliferation signaling of OPCs (Baron *et al.* 2002). These results suggest that proliferation of OPCs may be controlled partly through the signaling of PDGF in the NH.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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