Neurovascular Congruence during Cerebral Cortical Development

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There is evidence for interaction between the developing circulatory and nervous systems. Blood vessels provide a supporting niche in regions of adult neurogenesis. Here we present a systematic analysis of vascular development in the embryonic murine cortex and demonstrate that dividing cells, including *Tbr2*-positive intermediate progenitor cells, are closer to the vasculature than expected from a random distribution. To examine whether neurites of the newly generated embryonic neurons find blood vessels as an attractive and permissive substrate, we overlayed green fluorescent protein (GFP)-labeled dissociated cortical progenitors on embryonic organotypic cortical slice cultures with labeled vasculature. Our observations of neurites extending toward and along labeled blood vessels support the notion of vascular-neuronal interactions. The altered cortical layering had no obvious effect on the vascular patterns within the cortical plate (CP) in shaking rat Kawasaki (SRK) and the reeler mutant mouse at the ages studied (E19 and P3). It appears that similarly to other neurogenic regions in the adult, the embryonic "vascular niche" might influence neural progenitor cells during telencephalic neurogenesis, neuronal migration, and neurite extension, but the laminar phenotype of cell classes within the CP has limited influence on the developing vasculature.

Keywords: cerebral cortical blood vessels, cerebral cortical neurogenesis, intermediate progenitors, reeler mouse, shaking rat Kawasaki, subventricular zone, *Tbr2*

Introduction

Recent work has shown that numerous molecules previously associated with neuronal specification and axon guidance are also involved in the guidance and patterning of blood vessels (Carmeliet et al. 2005; Vates et al. 2005; Vasudevan et al. 2008). Axon outgrowth, guided by the amoeboid movements of the growth cone, has been described since the time of Ramon y Cajal (1928) and guidance molecules such as Robo and Semaphorin 3A have been identified (Huber et al. 2003). However, an endothelial tip cell is also capable of similar movement and is controlled by gradients of similar families of molecules (Gerhardt et al. 2003). Developmental cross talk between the developing nervous and vascular systems is thus a possibility. Indeed, recent evidence suggests such interactions. Artemin and Neurotrophin 3, when expressed on vessels, can attract axons to move alongside the "pioneer vessel" forming fascicles identical to those between different axons (Honma et al. 2002). In the adult olfactory bulb, newly generated neurons migrate radially along blood vessels (Bovetti et al. 2007). In the pathological setting, a cardinal sign of gliomas is their invasion along preferential pathways such as blood vessels and white matter tracts. Recent work using enhanced green fluorescent protein (eGFP) and Ds-Red-2-labeled C6 glioma cells shows that they can migrate along the

abluminal surface of blood vessels with increased proliferation occurring at vascular branch points (Farin et al. 2006). This study thus suggests that the vasculature not only can provide guidance cues but also exert effects, by some mechanism, on surrounding cells mitoses at these vascular branch points. Coculture experiments by Shen et al. (2004) suggest that this mechanism may be mediated via soluble growth factors released from endothelial cells maintaining neural stem cells in a proliferative state. A supportive neurovascular niche has been described in the subventricular zone (SVZ) during adult neurogenesis (Riquelme et al. 2008). It is not unreasonable to assume therefore that during development a similar "vascular niche" for neural stem cells may exist, with division in the developing nervous system influenced by the presence or absence of cortical and subcortical vasculature. Conversely, it is also possible that the neurons of the developing telencephalon orchestrate the vasculogenesis through various interactions.

In early embryogenesis, endothelial precursors called angioblasts coalesce to form a primitive plexus of the major vessels through the process of vasculogenesis. Active sprouting of new vessels, by differentiated endothelial cells, allows for the matching of vessel density to the metabolic demands of the tissues through the process of angiogenesis (Beck and D'Amore 1997; Pearce 2006). The process of angiogenic sprouting is responsible for the development of the optimal cerebral circulation (Greenberg and Jin 2005). Hypoxia-inducible factor (HIF) is a transcription factor that, in hypoxic conditions, activates genes to promote cell survival through downstream activation of numerous genes, including vascular endothelial growth factor (VEGF; Pugh and Ratcliffe 2003).

In this study, a systematic analysis of the development of vascular structure throughout corticogenesis was undertaken in the putative somatosensory cortex. Our experiments also sought to determine whether a subset of neural progenitor cells, the so-called intermediate progenitor cells (IPCs), in the cortical SVZ were closely associated to vasculature and therefore might be influenced by vascular cues. To study the possible interactions of blood vessels and neuronal progenitors and/or newly born neurons, we charted the movements and neurite extensions of individual labeled live neurons on cortical slice cultures in relation to labeled vasculature. We studied the development of vasculature in the reeler mutant mouse and shaking rat Kawasaki (SRK) to determine whether changes in the laminar pattern of cortical development had effects on the gross patterning of the developing vasculature in the inverted cortex.

Materials and Methods

Animals Used

C57 bl6 mice were maintained on a standard diet and light cycle and were mated in the Departmental Animal Colony in the University of

Oxford, United Kingdom. The day of plug detection was defined as embryonic day 0 (E0). Time-mated pregnant females were killed by cervical dislocation and the embryos harvested by Cesarean section. The embryos were decapitated, and the heads were postfixed overnight in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 4 °C. All animal procedures used in this study were first approved by the local Ethical Review Committee and performed under license from the UK Home Office (Scientific Procedures Act, 1986). SRK brain sections were obtained from Dr Shuji Higashi (Kyoto Prefectural School of Medicine, Kyoto, Japan) (see Higashi et al. 2005). Table 1 summarizes the number of mice or rat (SRK) used for our study.

Optical Projection Tomography Scans

Sample preparation was done as follows (adapted from BioOptonics [MRC] guidelines): one E14, 3 E18, and 3 P10 C57 bl6 mice were given an IP pentobarbitone overdose (1.0 mL/kg). After complete analgesic-areflexia was established, the left ventricle was injected with 100 µL of Isolectin B4 (IB4; Griffonia Simplicifolia Lectin B4, Vector Laboratories Inc., Burlingame, CA). Then brains were removed, fixed in 4% PFA overnight. Optical Projection Tomography (OPT) scanning at MRC technologies, Edinburgh (<http://www.bioptonics.com>). Sharpe (2002) describes OPT methodology in detail. In brief: The specimen to be scanned was immersed in Murray's Clear (1:2 mixture of benzyl alcohol and benzyl benzoate) to reduce optical opacity. It was then embedded in a transparent cylinder of agarose gel (1% low-melting point). The sample was rotated within the cylinder, while being held in position for imaging by a microscope coupled to a camera imaging chip (QImaging Retiga EXi Fast 1394, British Columbia, Canada). During a complete 360° cycle, every part of the specimen was imaged in focus. The data collected were used to produce virtual sections through the specimen, using a back-projection algorithm. Data were analyzed and rendered using Volocity (Improvision, Waltham, MA).

Immunohistochemistry

Brains were removed from the skull (E12.5 brains were left in skull), fixed as described above (for a complete listing, see Table 1), and embedded in 4% agarose. In all, 40 µm sections were cut in PBS using a Leica VT1000S. Sections were washed 3 × 15 min in PBS before being blocked in 3% normal goat serum (NGS) in PBS with 0.1% Triton-X (PBST) for 1 h. Primary antibodies (Table 2) were applied in 3% NGS in

PBST overnight at 4 °C. Sections were then washed 3 × 15 min in PBS followed by a 2-h incubation in secondary antibodies (including "Griffonia Simplicifolia IB4"). Sections were then washed 3 × 15 min with PBS before mounting in *Slowfade* Antifade solutions (Invitrogen, Carlsbad, CA) on glass slides.

Microscopy

Fluorescent micrographs were obtained on a DMR upright microscope with attached DC500 camera and IM50 software (Leica Microsystems, Wetzlar, Germany). Images for the H3 immunohistochemical analysis were collected using a TCS SP1 confocal microscope (Leica). Image channels were combined using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA).

Image Analysis and H3 Statistics

All image analyses were performed using ImageJ (National Institutes of Health, Bethesda, MD). The model (see Fig. 3) assumed that a blood vessel has a "sphere of influence" within which cells are sufficiently serviced by the blood vessel (e.g., no cells are hypoxic). This was calculated by measuring distances between adjacent blood vessels in E12.5 and E14 cerebral cortex (CTX) and ganglionic eminence (GE). The "average" distance divided by 2 represents the maximum radius of the sphere of influence for a given tissue/age (the *R* term in Fig. 3). The theoretical model is effectively cumulative distance of an infinite number of points divided by an infinite number of points to yield the average distance of a point. Please see Supplementary Material for more detailed explanation on the model and quantification. Distances between adjacent blood vessels were measured in E12.5 and E14 CTX and GE. The distances between the blood vessels and H3 immunoreactive mitotic profiles were also measured in E12.5 and E14 CTX and GE. The data obtained for this analysis (Fig. 3) were pooled from 3 brains for each age studied (Table 1 in Fig. 3). Areas within the GE and CTX at both ages were optically sectioned through the entirety of a single physical section (40 µm). Because the outer limits of individual 40 µm sections may contain areas that have been cut away from vessels that were nearby in the whole brain but were not included in the actual section, only the innermost parts (at least *R* µm from surfaces, see Fig. 3*B''*) of the section were analyzed to exclude areas of sections that may have been influenced by blood vessels only apparent in previous or subsequent sections. The values of *R* depend on the age and the region of the tissue (e.g., *R* = 17.06 µm for E12.5 cortex and 19.69 µm for E14 cortex; see Fig. 3*A,B*).

Cell Culture

To investigate the interactions of blood vessels and newly born neurons, we labeled cortical cells and examined their movements and neurite extensions on cortical slice cultures in relation to labeled vasculature. Preparation of such cultures consisted of 2 distinct stages, the preparation of E14 mouse cortex sections and the dissociation of E14 cortical cells electroporated with eGFP expression vector. Briefly, pregnant mice were killed by cervical dislocation followed by harvesting of the embryos. Embryos were chilled on ice and perfused transcardially with heparin (360 U/mL in PBS) followed by intracardial injection of undiluted (5–20 µL) *Rhodamine:Griffonia Bandeira Lectin 1* (Vector Laboratories Inc.). Brains were then dissected and

Table 1

Summary of mice and rat (SRK) used for the study

Experiments	Number of animals used
OPT	1 × E14, 3 × E18, 3 × P10
Structural analysis	3 × E12.5, 3 × E14, 2 × E15, 2 × E18, 3 × adult
H3	3 × E12.5, 3 × E14
H3 + <i>Tbr2</i>	3 × E14
DCX	3 × E14
SRK, <i>Reeler</i>	3 × E19 SRK (3 × E19 WT), 3 × P9 SRK (3 × P9 WT); 3 × P8
Culture	5 × E14 (slices), 8 × E14 (dissociated)

Note: All ages refer to mice except for Shaking Rat Kawasaki (SRK). Parentheses designate controls E, embryonic day; P+, postnatal day.

Table 2

Antibodies used

Primary antibody	Manufacturer	Dilution	Host	Secondary antibody	Manufacturer	Dilution
CD31	Abcam ab7388	1:500	Rat	<i>Goat α Guinea Pig Alexa 633</i>	Molecular Probes	1:500
DCX	Chemicon ab5910	1:3000	Guinea Pig	<i>Goat α Rabbit Alexa 350</i>	Molecular Probes	1:500
GFP	Abcam ab1218	1:500	Mouse	<i>Goat α Mouse Cy2</i>	Jackson	1:500
GFAP	Sigma g9269	1:500	Mouse	<i>Goat α Rabbit Alexa 546</i>	Molecular Probes	1:500
Phospho-histone H3	Upstate 06-570	1:500	Rabbit	<i>Goat α Rat Cy3</i>	Jackson	1:500
B Galactosidase	Molecular Probes	1:500	Rabbit	<i>Goat α Rat Alexa 488</i>	Molecular Probes	1:500
MAP2	Abcam	1:500	Mouse	<i>Lectin 1 (rhodamine)</i>	Vector	1:200/Perfuse
Von Willebrand Factor	Abcam a6994	1:1000	Rabbit	<i>IB4 FITC (+IB4—Alexa 594)</i>	Vector	1:200/Perfuse

Note: Showing dilutions, host and molecular targets. Italics designate secondary antibodies/lectins. For details of use, see Materials and Methods.

embedded in 4% low molecular weight agarose in complete Hank's buffered saline solution. Brains were cut into 250 μ m thick coronal sections and plated onto poly-L-lysine/laminin-coated tissue culture membranes (Becton-Dickinson, Franklin Lakes, NJ). Separately, E14 embryonic brains were electroporated (55 V $\times$ 4 100-ms pulses with 100-ms delay) with a pCAGGS plasmid (eGFP with CMV promoter) targeting cells in the ventricular zone (VZ) and SVZ. These neurons were then dissociated immediately after electroporation and overlaid evenly onto perfused slices and incubated (37 $^{\circ}$ C, 5% CO₂; from Polleux and Ghosh 2002). Images were taken at regular intervals using a Leica DMR.

TgEomes

TgEomes:GFP (Tbr2-eGFP BAC transgenic mice were obtained from the GENSAT consortium (<http://www.gensat.org/index.html>). These mice express eGFP under the control of Tbr2 promoter elements (Kwon and Hadjantonakis 2007). Timed-pregnant dams (day of plug E0.5) were euthanized and embryos perfused with 4% PFA and then postfixed overnight at 4 $^{\circ}$ C. Brains were then removed, embedded in 4% low melt agarose, and sectioned at 50 μ m on a vibrating microtome (VT1000, Leica). Slices were incubated with isolectin GS-IB₄ conjugated to Alexa Fluor 594 (5 μ g/mL; Invitrogen), counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) or TO-PRO3 (Invitrogen), mounted on glass slides, and coverslipped with Vectashield (Vector Laboratories Inc.). Three brains were imaged on a Radiance2000 confocal microscope (Carl Zeiss, Jena, Germany) or an Axio Imager Z1 microscope with Apotome attachment (Carl Zeiss). Z-series images were processed in Axiovision (Carl Zeiss) for brightness and contrast. Distances between blood vessels and mitotic cells were measured with Axiovision's Inside 4D module. Tbr2 (Eomes) specifically identifies intermediate progenitors in the embryonic SVZ. The proximity of Tbr2-eGFP mitotic figures in the SVZ to blood vessels was determined as described above. Identical experiments were performed after Tbr2 immunostaining (Tbr2 antibody from R. Hevner, University of Washington, 1:2000).

Results

Gross Pattern of Vasculature

We used OPT to monitor the gross pattern of vascular development on the convexity and base of the brain perfused with IB₄ (Fig. 1 A,B in Panel 1). The circle of Willis and related major landmarks (anterior and middle cerebral arteries) were present from E14. Between E14 and 18, the blood vessels became progressively more apparent on the cortical convexity. The 3D reconstructions revealed the developing vasculature with great clarity and allowed us to generate 2D optical slices from the same data sets (Fig. 1C in Panel 1). The 2D optical slices obtained from OPT (Fig. 1C in Panel 1) were similar to the results obtained in histological sections (Fig. 1D in Panel 1).

Changing Pattern of Cortical Vascular Plexi at Different Ages

We studied the changes of vasculature patterning throughout corticogenesis and later in postnatal life. We focused on the putative somatosensory area, but other cortical areas had a similar pattern. The earliest cortical blood vessels originate from pial arteries as penetrating branches forming loops heading toward the ventricle. These loops start to form a distinct dense plexus surrounding the ventricle from E12.5 (Fig. 1A,G in Panel 2) with occasional radial blood vessels connecting them to the pial surface. These penetrate the cortical plate (CP) perpendicular to the pial surface. By E14, the thickness of the cortex increases, and a second plexus can be seen just beneath the forming CP with a similar structure of loops. The 2 plexi are interconnected by a few penetrating branches bridging the intermediate zone (Fig. 1B,H in Panel 2).

By E15, a few blood vessels tangential to the pial surface start to appear in the intermediate zone (see arrow in Fig. 1C in Panel 2), but this region remains relatively less vascularized compared with the plexi in the VZ/SVZ and the CP. At E18, toward the end of the neurogenesis, the ventricular plexus has lost much of its definition; and by P8 and adulthood, a more homogeneous structure with many small parenchymal arteries is present (Fig. 1E,F,K in Panel 2).

Measuring Distance between Blood Vessels and Dividing Cells in the SVZ

In the embryonic CTX, most of the divisions occur in 2 zones, the VZ and the SVZ (Noctor et al. 2008). The appearance of the vascular plexi in the SVZ coincides with the formation of the SVZ as a distinct histological and neurogenic zone (Carney et al. 2007; Cheung et al. 2007). To study the possible relationship between blood vessels and cell divisions, we revealed dividing (M phase) cells with phospho-histone H3 immunohistochemistry and labeled vasculature through the affinity of IB₄ to vascular endothelial cells (Fig. 1 in Panel 3). We noted the apparent proximity of mitotic profiles and the blood vessels in both the cerebral cortical SVZ and the intermediate zone (Fig. 1A,B in Panel 3) and in the GE (Fig. 1C in Panel 3).

Testing the Idea That Mitotic SVZ Cells Are Closer to Blood Vessels than Predicted

To test whether divisions occur closer to blood vessels than chance, the mathematical model shown in Figure 2A was used to calculate an estimate of the average distance a cell was from a blood vessel based on intervessel measurements. This was done by measuring distances between adjacent blood vessels and then obtaining average values for E12.5 and E14 cortex (CTX) and GE. The average distance was then divided by 2 to give the R term (Fig. 2A). Results show that dividing (H3) cells are, regardless of age and location, closer to a vessel than predicted by theory for randomly distributed cells ($P < 0.005$) (Fig. 2, Table 1). In both GE and cortex, many H3-positive cells were in contact with the blood vessels (between 10% and 30%) ($n = 349$). Furthermore, dividing cells at E12.5 were closer to vessels than in the corresponding location at E14 ($P < 0.005$), although this is potentially due to the limited possible distance in smaller brain.

Tbr2 Positive Cells Are Found Adjacent to Blood Vessels

IPCs in the cortical VZ and SVZ express the transcription factor Tbr2 (Englund et al. 2005). The Tbr2-eGFP transgenic mouse expresses eGFP under control of the Tbr2 promoter and serves as an excellent correlate for Tbr2 expression (Kwon and Hadjantonakis 2007; compare panels Fig. 3A-E' obtained using Tbr2-eGFP transgenic mouse with Fig. 3 panels F-F' obtained after Tbr2 immunohistochemistry). To further examine the proximity of dividing cells in the SVZ to blood vessels by additional methods, we investigated the proximity of GFP positive dividing cortical progenitors to vascular profiles in embryonic Tbr2-GFP transgenic mice. Tbr2-eGFP-positive cells in M-phase were found adjacent to vasculature in the VZ and SVZ throughout neurogenesis (see Fig. 3A-E'). The blood vessels change their orientation from radial to tangential as they ascend from VZ to SVZ to form the loops in the germinal zone (Fig. 1). The eGFP-positive (Tbr2-positive intermediate progenitor) cells have a close association to blood vessels in the

Figure 1. Panel 1: Gross pattern of vascular development revealed by OPT (A–C) and on histological section (D). (A, B) Reconstructions of the E18 brain perfused with IB4 and scanned with OPT. The same brain is shown from the dorsal (A) and ventral (B) surface. The developing vasculature is revealed to an impressive degree of clarity. (C) An example for a parasagittal 2D slice generated from the same E18 data set shown in panels (A, B) using Volocity. (D) Coronal section (40 μ m thick) of an E14.5 brain perfused with IB4. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; and MZ, marginal zone. Scale bar for (A–C): 1 mm; 50 μ m for (D). Panel 2: Developmental stages of blood vessel formation in putative somatosensory cortex in the mouse. Panels (A–F; left column) show fluorescent images of blood vessels labeled with IB4 on coronal sections (40 μ m thick) at E12.5, 14, 15, 18 (2 examples from 2 different brains, A, A'–D, D'); one for P8 (E) and adult (F). (G–K) Show generalized schematic drawings of the layout of cortical blood vessel plexi at each stage. At E14 and 15, there are 2 dense plexi in the germinal zone (VZ and SVZ) and CP, connected by tangential blood vessels (arrows in B, C). The region of SP and white matter might correspond to region of poorer perfusion. MZ, marginal zone; I–VI represent cortical layers in adult. Scale: 100 μ m. Panel 3: Association of SVZ blood vessels to mitotic profiles in CTX and GE in an E14 brain. (A) Demonstrates the overall pattern of blood vessels (revealed with IB4–green) and the mitotic profiles (revealed with H3–red immunoreactivity) on a low power image of the cortex. (A) Demonstrates the cortical plexi and the mitotic profiles in the SVZ and VZ. The section was counterstained with DAPI, appears blue. Panels (B, C) show examples for confocal microscopic reconstructions of mitotic cells in contact with blood vessels in the cortical SVZ (B) and in the GE (C). Scale bar for (A): 100 μ m.

radially oriented VZ (Fig. 3D) and continue in the tangentially oriented SVZ (Fig. 3E,E'). E' is a high-power image from E to provide example of the close association. Some of the GFP-positive cells were clustered on blood vessels. We measured

the distance between blood vessels and the average distance of eGFP expressing mitotic cells in the cortical intermediate zone of 3 E14.5 Tbr2-eGFP mice and compared with theoretical average cell distance ($\bar{r}$) from a blood vessel as calculated using

A $\frac{4\pi R^3}{3}$ represents total number of cells within the “sphere of influence”

$4\pi R^2$ represents total number of cells along the surface of a sphere with radius r

$$\bar{r} \times \frac{4\pi R^3}{3} = \int_0^R 4\pi r^2 \times dr$$

$$\bar{r} \times \frac{4\pi R^3}{3} = \left[\frac{4\pi r^3}{3} \right]_0^R$$

$$\bar{r} = \frac{3\pi R^4}{4\pi R^3}$$

$$\bar{r} = \frac{3R}{4}$$

Table 1: H3 Measurements

Age/Region	Theoretical Distance (μm)	H3 Positive Distance (μm)	s	n
E12.5 Ctx	12.8	5.97	5.83	29
E12.5 GE	13.29	4.15	6.06	119
E14 Ctx	14.77	10.64	6.61	91
E14 GE	11.73	8.44	6.22	258

Table 2: Tbr2eGFP Mitotic Profile to BV distance

Theoretical Distance (μm)	14.77
Actual Distance (μm)	8.76
SD	5.62
n	109

Table 3: Tbr2+ cell - BV distance

Theoretical Distance (μm)	17.46
Actual Distance (μm)	8.91
SD	0.46
n	204

Figure 2. Model and formula derivation for calculating the expected distance between a cell and blood vessel (A) and actual measurement performed (B–B’’) together with the data obtained with the different methods (Tables 1–3). (A) Derivation of the model. The model states that the average distance of a cell ($\bar{r}$) equals the total number of cells divided by their cumulative total distance. (B–B’’) Schematic diagram explaining the process of quantification. (B) Whole brains were perfused, embedded, and cut into 40 μm coronal sections. (B’) The outer limits of individual 40 μm sections may contain areas that have been cut away from vessels that were nearby in the whole brain but were not included in the actual section. (B’’) Only the tissue at least R μm from surfaces of the section (the innermost parts) was analyzed to exclude areas of sections that may have been influenced by undocumented blood vessels, only apparent in previous or subsequent sections. For example, $R = 17.06$ μm for E12.5 cortex and 19.69 μm for E14 cortex. $\bar{r}$ Values were calculated from these values (Figs 4 and 5). Tables 1–3: Table 1—The distances between the blood vessels and H3 immunoreactive mitotic profiles were measured in E12.5 and E14 CTX and GE. The data obtained for this analysis were pooled from 3 brains for each age studied. Theoretical distance and the actual distance have been compared. Table 2 similar as Table 2 but for Tbr2-eGFP mitotic profiles. Table 3 similar to Tables 1 and 2 but for Tbr2 immunoreactive mitotic profiles. Significance between model and obtained data was determined using a 1-sample t -test. All were significant ($P < 0.001$). No standard deviation can be calculated for the model distance. These results show that H3 immunoreactive, Tbr2-eGFP mitotic profiles, or Tbr2 immunoreactive mitotic profiles are closer to the vasculature than a quiescent cell is expected to be.

Figure 3. GFP expressing cells in the Tbr2-eGFP mouse (A–E’) or Tbr2 immunoreactive cells in SVZ (F–F’) undergo mitosis near blood vessels in the neocortical germinal zones. (A) Endogenous GFP expression in an E14.5 Tbr2-eGFP mouse neocortex shows a GFP+ cell undergoing mitosis (arrow) adjacent to a profile of a blood vessel (arrowheads). (B) IB4-Alexa594 (red) staining reveals a blood vessel (arrowheads) adjacent to Tbr2-eGFP+ mitotic figure (arrow). (C) The 3D reconstruction of a Z-series reveals a Tbr2-eGFP-positive cell undergoing mitosis (arrow) in the SVZ on E14.5 in close proximity to a large blood vessel (red). Cross section delineated by white lines. Blue counterstain TO-PRO (A) and DAPI (B, C). Scale bar: 50 μm. (D–E) Low-power confocal microscopic images taken from horizontal sections through the SVZ of E14.5 Tbr2-eGFP brain. The figure demonstrates that the orientation of blood vessels changes from radial to tangential as they ascend from VZ to SVZ. The panels in (D–E) demonstrate that the eGFP-positive cells have a close association to blood vessels in the radially oriented VZ (D) and continue in the tangentially oriented SVZ (E). (E’) Is a high-power image from (E) to provide example of the close association. Scale: 100 μm for (D, E) and 25 μm for (E’). (F) E14.5 sections were processed Tbr2 immunohistochemistry to further validate the results that eGFP expressing dividing cells (presumed intermediate progenitors) in the Tbr2-eGFP mouse are closer to the vasculature than a quiescent cell is expected to be. Tbr2 immunoreactivity has been the most intensive along a band through cortical SVZ (appear green). (F’) Confocal microscopic reconstruction of the region in SVZ labeled with a box in (F) to demonstrate the Tbr2 and H3 immunoreactivity and the IB4-positive blood vessel. Scale: 100 μm in (F) and 10 μm in (F’).

the model in Figure 2 and used previously in Table 1 for H3 immunoreactive profiles. The results in Table 2 in Figure 2 show that eGFP expressing dividing cells (presumed intermediate progenitors) in the Tbr2-eGFP mouse are closer to the vasculature than a quiescent cell is expected to be. We performed H3 immunostaining and combined this with Tbr2 immunoreactivity to specifically study the Tbr2-positive intermediate progenitor population and their proximity to blood vessels (Fig. 3*F–F'*). The quantification of these results (Fig. 2, table 3) also demonstrated the closer position of these dividing populations to blood vessels than chance would indicate.

Early Neurite Outgrowth Associated to VZ Blood Vessels In Vivo

The intersection of newly generated neurons and their occasional association with blood vessels was investigated further using the neuronal marker doublecortin to visualize newly differentiated neurons in brains perfused with IB4 (Fig. 4*A–C* in Panel 1). The results showed some association between the doublecortin positive neurons and their processes and blood vessels at the junction of the germinal zone and intermediate zone at E14 (Fig. 4*A–C* in Panel 1). Some, but not all, doublecortin immunoreactive neurites extend along labeled blood vessels into the SVZ and VZ, suggesting that some of the initial neurite outgrowth is related to blood vessels and a “vascular scaffold” might be present for developing cortical neurons in vivo.

Blood Vessels Appear to Attract Dissociated Neuronal Progenitors/Newly Generated Neurons in Overlay Cultures

To determine whether the association between vasculature and cell divisions and newly generated neurons was an active or passive process, we cultured dissociated VZ progenitors to see if blood vessels could guide embryonic cortical cells in a manner similar to that suggested by the images in Figure 4, Panel 1. We charted the movements and neurite extensions of individual labeled live dissociated cortical VZ cells seeded onto organotypic cortical slice cultures in relation to labeled vasculature for prolonged periods (up to 42 h). In our overlay experiments, GFP positive newly born cortical cells could be seen in contact with the Rhodamine positive blood vessels from as little as 9 h after dissociation and coculturing (Fig. 4*A* in Panel 1). Our culture experiment shows examples for GFP-positive neuronal processes extending from the overlaid cortical neurons toward a presumptive vascular structure and labeled cells approach vasculature after continued culturing (compare Fig. 4*A,B* in Panel 2). After prolonged culturing, the labeled vascular structures and some of the labeled overlaid cells appeared in register (Fig. 2*C,D* in Panel 2).

Reeler and SRK Mutations Have No Effect on Establishment of Cortical Vascular Plexi

Our characterization of early cortical vascular development revealed changing laminar pattern of developing vasculature according to the lamination of the germinal zone and developing CP. We decided to investigate whether the altered placement of cortical cell types has an effect on the gross patterning of the CP vasculature. We stained blood vessels in 2 mutants, the SRK (Aikawa et al. 1988) and the reeler mouse mutant (Caviness and Rakic 1978) where the cortical layers form in a reversed outside

first, inside last pattern due to well-characterized cortical migration defect. The SRK showed no major effect on the formation of the penetrating CP blood vessels. Panel 3 in Figure 4 shows images of E19 WT rat (*A*) and SRK mutant (*B*). The pial origin of the vessels is preserved with both plexi, and the plexi in the germinal zone and the CP show similar patterns between SRK and WT. Although plexi in the CP and germinal zone form with similar interconnections, the intermediate zone in SRK appears to contain fewer blood vessels oriented tangentially with the lower edge of the CP. The CP vasculature in the postnatal reeler mouse is virtually indistinguishable from wild type (Fig. 4*C,D* in Panel 3) suggesting that the altered cortical cell migrations have little influence on the vascular development in the cortex.

Discussion

Our characterization of embryonic cortical vascular development shows the establishment of 2 plexi; one originating from the VZ and one from the pial surface with relatively few connections between them. This could explain the selective vulnerability of the intermediate zone to ischemic injury at embryonic cortex and how the ischemic necrosis of periventricular leukomalacia can cause such extreme morbidity (Volpe 2005). Interestingly, our data demonstrate close association between dividing cells in the cortical SVZ and suggest that vascular disruption could have consequences beyond simple ischemic necrosis with far-reaching consequences on cortical development.

Model Critique and Statistical Analysis

Our data show that mitotic cells are closer to blood vessels than predicted by chance. This, in light of in vitro and in vivo work is suggestive of a vascular niche for precursors (Palmer et al. 2000; Shen et al. 2004; Ernst and Christie 2006; Ohab et al. 2006). Our model essentially predicts the average distance from the center of this sphere to an “average cell” multiplied by the total number of cells (i.e., the area) is equal to the integral of all the potential distances (i.e., the distance to an infinite number of mini spheres sitting within this sphere) multiplied by the number of cells at this point. In essence, it is a “weighted average” of all the potential distances.

This model can only be considered as a framework; yet, it does fit existing in vitro data despite its assumptions. For example, the model abstracts cells and blood vessels as points. It would be possible to incorporate an average cellular width but the varied diameters of neuronal and glial subtypes means that this would not necessarily ensure a more representative result. Further refinements to the model are undoubtedly possible, but we felt that modeling as points most represented the dynamic and convoluted structures depicted in our structural analysis. Perhaps, the most important consideration is that we are only testing the subpopulation of mitotic cells within the sphere given by the *R* value from Figure 2*A*. This was based on the premise that a certain volume of cells are responsible for the ingrowth of the vessel via the HIF-VEGF angiogenesis pathway; thus, it is reasonable to assume that any factors influencing cellular activity will act within a similar range. It was important to obtain a comparative value for testing and the model permitted us to do this while removing influences from blood vessels outside of the block of sectioned

Figure 4. Panel 1: The close relationship between the IB4-labeled blood vessels (green) and doublecortin (DCX) immunoreactive cortical neurons and their neurites (red) suggests early cortical vascular scaffold. (A) Single optical slice through confocal stack (B), close association between DCX immunoreactive processes and IB4 immunoreactive blood vessels. (C) Enlargement from the area indicated with a box in (B) showing association between blood vessels and some DCX neurons/neurites in the germinal zone. Panel 2: Association of GFP labeled cortical germinal zone neurites and close apposition of labeled cells with labeled cortical vasculature in vitro suggests interaction with vascular scaffold. (A, B) Results from an experiment showing eGFP-positive newly born cells (labeled with VZ electroporation shortly before dissociation) and their processes extending toward and in contact with vasculature (prelabeled with IB4 perfusion before culturing) and examined after $t = 26$ h (A) and $t = 42$ h (B), respectively. Note that after 26 h, the cell in (A) extends its process toward the blood vessel (insert in A) and approaches the blood vessel by 42 h (B). (B) Has been taken from the same region as (A). (C, D) Results from an additional series of overlay culture experiment showing GFP-positive signal in contact with vascular plexus (C, 9 h and D, 25 h). The red and green profiles align in close register in the 25-h culture. The greater number of cells in later picture is presumably due to increased GFP

tissue. This was achieved by excluding optical sections lying within R μm of top and bottom. When all H3 distances are pooled, we can see that the majority of cells reside in a distance <30 μm (63–84% depending on age and location—data not shown); thus, it is possible that we underestimated the radius of our sphere of influence. New methods are needed to more accurately calculate this range for statistical comparison. One hypothetical way in which this could be done would be to measure oxygen tensions at different distances from an isolated vessel. The position where no oxygen was recorded would mark the theoretical limit from a blood vessel where any cell could survive and divide. However, we feel that this rather complicated set of experiments would merely be an in vitro estimate of the thought process behind our image analysis method. The fact that the HIF-VEGF angiogenesis pathway carefully regulates oxygen tension in tissues means that vascular supply is inextricably matched to the metabolic needs of a tissue. Thus, our method of measuring intervessel distances and halving them is actually reflecting this Fickian diffusion of oxygen and as such provides a reasonable estimate of the cells upon which local, autocrine, and paracrine factors could have an effect.

Intermediate Progenitors

A subset of neuronal precursors called IPCs is present in the SVZ and VZ at nonsurface locations as well as at scattered locations in the CP and the IZ. The IPCs contrast with radial glial precursors, which divide at the ventricular/apical surface. Recent work has shown that a potential marker of these cells is the transcription factor *Tbr2* (Englund et al. 2005). We demonstrate using immunohistochemistry and a *Tbr2*/GFP transgenic mouse that *Tbr2*-positive cells frequently undergo mitosis in close proximity to blood vessels. Our work shows that the start of subventricular divisions is synchronized with the establishment of ventricular vascular plexi, and these dividing SVZ cells are located statistically closer to blood vessels than predicted by theory. Moreover, the IPCs appeared to have stronger interactions with blood vessels (for *Tbr2*-eGFP cells, actual distance = 8.76 μm and theoretical distance = 14.77 μm ; for *Tbr2* immunoreactive cells, actual distance = 8.91 μm and theoretical distance = 17.46 μm) than did the total population of phosphohistone H3-positive progenitors (actual distance = 10.64 μm in E14 cortex and theoretical distance = 14.77 μm). Further work to determine the significance, mechanisms, and efficacy of this vascular influence on different progenitor types is crucial.

expression; the less clear vascular pattern is due to removal of some of the endothelial cells after longer culturing period. Scale: 100 μm . Panel 3: Normal CP vasculature in the SRK and reeler mouse. (A, B) Inversion of cortical lamination does not alter the general pattern of the cortical vascular plexi in E19 SRK cortex. Blood vessels have been stained with IB4 (appear green) on coronal sections through WT (A) rat and SRK (B) putative somatosensory cortices. There is little difference between mutant and wild type with similar penetrating vessels through CP and dense plexi in the germinal zones. At the end of neurogenesis, the plexi around the VZ is still the densest in both. The CP is penetrated with blood vessels according to very similar pattern in the WT and SRK, but the intermediate zone contains less tangential blood vessels in the SRK cortex. Nevertheless, the plexi present in CP and germinal zone are still interconnected in both. Panels (C, D) show indistinguishable cortical vascular pattern in a P8 WT (C) and reeler mutant mouse (D) somatosensory cortex. Scale in (A) 100 μm and applies for (B); in (C) 100 μm and applies for (D).

Migration

We investigated whether postmitotic doublecortin-labeled neurons were associated with cortical vessels. Glioma cells are known to use vascular structures as permissive pathways, explaining the massive infiltration demonstrated by these tumors (Farin et al. 2006), and new neurons in the adult olfactory bulb utilize blood vessels for radial migration (Bovetti et al. 2007). Our results showing that doublecortin and glial fibrillary acidic protein (GFAP)-positive cells associate with blood vessels suggest that the vascular plexus in the VZ/SVZ could play a similar role in the migration of newly born cells into the cortical layers. Electroporation of the progenitors lining the VZ surface with GFP could be used as an accurate method to assess this process in vitro. Elucidation of vascular molecular factors that influence IPCs and other dividing cells is crucial to fully understanding this phenomenon.

Lack of Interactions from Cortical to Vascular Components

Our cell culture and immunohistochemistry work suggests that embryonic vasculature has an influence on cell divisions and on neuronal position and neurite extension. However, the laminar position of subsets of cortical neurons has very little effect on the overall vascular pattern in cortex. Our results in *SRK* and *reeler* mutants showed no alteration of the vascular plexi within the CP despite the inversion of the cortical layers. However, the stages we studied (E19 and P3) were relatively late to observe possible differences in preplate stages. However, such early effects are also unlikely, since Vasudevan et al. (2008) recently investigated the early stages (E10–11) of periventricular vessel development in the *reeler* mouse telencephalon and found it to be unaffected.

Interactions between developing vasculature and neuronal migration and axonal pathfinding should be considered in the pathomechanisms of clinical syndromes. Neurovascular interactions could be important in CRASH syndrome, caused by aberrant axon guidance due to mutations in L1CAM guidance molecule (Fransen et al. 1998), or in epileptogenic diseases, caused by failures in radial migration such as Schizencephaly (Guerrini and Carrozzo 2001), which have been hypothesized to be due to a failure in migration from the VZ. Focal necrosis, ischemia, and inherited genetic defects have all been proposed as causes but altered vascular plexi could conceivably contribute.

Therefore, we conclude that the development of the vascular plexi may have crucial roles in the developmental processes of neurogenesis and early migration. In particular, the *Tbr2*-positive IPCs are one cell type that appears to be most influenced by these vascular factors. If this is the case, then it could have profound consequences on our understanding of cortical development and malformation. The analysis of mitotic cells presented herein, when considered in light of previous in vitro work, could allow a new understanding of the progenitor cell niche.

Supplementary Material

Supplementary Material can be found at: <http://www.cercor.oxfordjournals.org/>.

Funding

Medical Research Council (G0700377 and G0300200) and Biotechnology and Biological Sciences Research Council (BB/F003285/1) to Z.M. and NIH R01 N5050248 to R.F.H.

Notes

Our thanks go to Michael Wilson for his thoughtful comments on an earlier version of this manuscript. *Conflict of Interest*: None declared.

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