Feature Article

***In vivo* 3D morphology of astrocyte–vasculature interactions in the somatosensory cortex: implications for neurovascular coupling**

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Astrocytes are increasingly believed to play an important role in neurovascular coupling. Recent *in vivo* studies have shown that intracellular calcium levels in astrocytes correlate with reactivity in adjacent diving arterioles. However, the hemodynamic response to stimulation involves a complex orchestration of vessel dilations and constrictions that spread rapidly over wide distances. In this work, we study the three-dimensional cytoarchitecture of astrocytes and their interrelations with blood vessels down through layer IV of the mouse somatosensory cortex using *in vivo* two-photon microscopy. Vessels and astrocytes were visualized through intravenous dextran-conjugated fluorescein and cortically applied sulforhodamine 101 (SR101), respectively. In addition to exploring astrocyte density, vascular proximity, and microvascular density, we found that sheathing of subpial vessels by astrocyte processes was continuous along all capillaries, arterioles, and veins, comprising a highly interconnected pathway through which signals could feasibly be relayed over long distances via gap junctions. An inner SR101-positive sheath noted along pial and diving arterioles was determined to be nonastrocytic, and appears to represent selective SR101 staining of arterial endothelial cells. Our findings underscore the intimate relationship between astrocytes and all cortical blood vessels, and suggest that astrocytes could influence neurovascular regulation at a range of sites, including the capillary bed and pial arterioles.

Journal of Cerebral Blood Flow & Metabolism (2011) **31**, 795–806; doi:10.1038/jcbfm.2010.204; published online 8 December 2010

Keywords: astrocytes; immunohistochemistry; neurovascular coupling; neurovascular unit; optical imaging; two-photon microscopy

Introduction

Cerebral blood flow dynamics are functionally linked to neural activity in a process termed ‘functional hyperemia’ (Devor *et al*, 2003; Takano *et al*, 2006). Although the precise mechanisms underlying functional hyperemia remain uncertain, astrocytes have been implicated as important contributors (Takano *et al*, 2006; Winship *et al*, 2007). Astrocytes are ideally positioned to both detect neural activity and transmit

signals to nearby blood vessels. They possess stellate or spongiform morphology (Hama *et al*, 1994), with many cellular processes extending from their soma that form tripartite synapses with neurons and interact with cerebral blood vessels (Koehler *et al*, 2009). Furthermore, astrocytes control the production and release of vasodilators, such as prostaglandins and epoxyeicosatrienoic acids, and the vasoconstrictor 20-HETE (Koehler *et al*, 2009; Haydon and Carmignoto, 2006). Recent studies have also shown changes in blood vessel diameter concurrent with Ca²⁺ signaling in astrocytes (Girouard *et al*, 2010; Filosa *et al*, 2006; Takano *et al*, 2006). In addition to producing vasoactive arachidonic acid metabolites, release of Ca²⁺ within astrocytes also triggers changes in smooth muscle cell K⁺ dynamics and vascular tone through the actions of Ca²⁺-sensitive (BK) and inward-rectifying (Kir) K⁺ channels (Filosa *et al*, 2006; Girouard *et al*, 2010). Together, these characteristics of astrocytes support their potential to contribute significantly to functional hyperemia.

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Funding for this study was provided by NIH (NINDS) grants R21NS053684 and R01NS063226, NIH (NEI) grant R01EY019500, NSF CAREER 0954796, NIH (NCI) U54CA126513, the Human Frontier Science Program and The Rodriguez Family. Brenda R Chen receives support from the NSF graduate fellowship funding. Received 19 May 2010; revised and accepted 4 October 2010; published online 8 December 2010

Functional hyperemia manifests as a regional increase in blood flow that localizes to specific responding regions of the cortex. In the rodent somatosensory cortex, regions of the capillary bed exhibiting hyperemia can extend over distances exceeding 1 mm. Arterioles feeding the responding regions can dilate up to 2 mm away from the center of the responding region within less than a second after stimulation begins. After 2 to 4 seconds, or later cessation of stimulation, surface arterioles begin to constrict and blood flow reduces to baseline, sometimes with a short undershoot (Chen *et al*, 2010; Hillman *et al*, 2007). Despite evidence linking signaling in individual astrocytes to changes in adjacent blood vessels (Takano *et al*, 2006), there is as yet no model that conclusively explains how these discrete observations can account for the level of coordinated and rapid actuation required to orchestrate the complete hemodynamic response. A precise understanding of the ways in which astrocyte networks physically interact with cerebral blood vessels should provide an important foundation upon which to build models of functional hyperemia.

Previous studies examining the physical relationship between astrocytes and cerebral blood vessels have been predominantly carried out *in vitro* (Zahs and Wu, 2001; Virgintino *et al*, 1997; Kacem *et al*, 1998; Tsai *et al*, 2009). Although *in vitro* preparations allow the use of powerful visualization techniques such as immunohistochemistry (IHC) and electron microscopy, excision of tissue necessarily distorts its appearance because of the loss of pressurized blood flow and possible damage during tissue processing. Furthermore, in addition to physical tissue damage, various brain insults have been shown to trigger reactive astrogliosis, leading to the upregulated expression of the common astrocyte marker glial fibrillary acidic protein (GFAP) and changes in astrocyte morphology (Wilhelmsson *et al*, 2006). Acquiring data *in-vivo* circumvents these problems. Normal blood flow is preserved, and the intact three-dimensional (3D) structure of the cortex and pial surface may be directly examined.

This study quantitatively examines the interrelations between astrocytes and blood vessels in living, intact rodent brains using *in vivo* two-photon microscopy to depths exceeding 500 μ m. The cortical surface and pial blood vessels are also examined in detail. Astrocyte density, astrocyte proximity to capillaries, and capillary density are quantified as a function of cortical depth, followed by an analysis of the frequency with which astrocyte processes contact blood vessels, and a detailed characterization of the astrocytic sheath encircling the vasculature. Analysis was conducted separately for arteries, capillaries, and veins. *In vivo* contrast was provided by intravascular dextran-conjugated fluorescein, cortically applied sulforhodamine 101 (SR101), a dye shown to preferentially label astrocytes *in vivo* (Nimmerjahn *et al*, 2004), and in some cases, green fluorescent protein (GFP). After *in vivo* imaging, animals were euthanized, their brains

were sectioned, and IHC was used for validation studies with antibodies for GFAP and aquaporin-4 to target astrocyte cell bodies and processes, claudin-5 to label tight junctions, and smooth muscle actin (SMA) to visualize arterial smooth muscle.

Materials and methods

Animal Preparation

All procedures were approved by the Columbia University Institutional Animal Care and Use Committee. Five male C57BL/6J mice (JAX, Bar Harbor, ME, USA), two GFAP-GFP mice (from local colonies), and one endothelial-specific receptor tyrosine kinase (TIE2)-GFP mouse (JAX) weighing between 20 and 30 g were anesthetized with either 2% $\pm$ 0.5% isoflurane in a 3:1 air to oxygen mix or 100 mg/kg ketamine and 10 mg/kg xylazine and secured in a custom stereotaxic apparatus. A section of the skull over the somatosensory cortex measuring $\sim 3 \times 4$ mm² was then removed, taking care to preserve the underlying dura mater. The meninges covering the IVth ventricle were punctured to relieve cerebrospinal fluid pressure. Sulforhodamine 101 (SR101; S-359, Invitrogen, Carlsbad, CA, USA) was prepared at a 0.2 mmol/L concentration in artificial cerebrospinal fluid (ACSF), and its fixable analog Texas red hydrazide (T-6256; Invitrogen) (Nimmerjahn *et al*, 2004) was prepared by first adding 80 μ L/mg methanol to the dry hydrazide stock and then diluting to 0.2 mmol/L in ACSF. A reduced concentration of 0.04 mmol/L SR101 was used for GFP mice. These solutions were applied to the surface of the cortex just below the dura using a glass micropipette connected to a Picospritzer III microinjection system (Parker Instrumentation, Cleveland, OH, USA). Approximately 10 μ L of the dye solution was also placed onto the dura and allowed to incubate for 30 minutes before washing with ACSF. Bulk loading of SR101 into the cortex was unnecessary because it distributes readily throughout astrocytes within the cortex through their gap junctions (Nimmerjahn *et al*, 2004). A glass coverslip with a drop of 0.5% agarose in artificial cerebrospinal fluid was then sealed in place over the craniotomy using dental acrylic (Henry Schein, Melville, NY, USA), thereby reducing brain motion and contamination. In wild-type mice, dextran-conjugated fluorescein (FD2000S, Sigma-Aldrich, St Louis, MO, USA, 5 mg in 0.1 mL saline) was administered by a tail vein injection to provide blood vessel contrast.

In Vivo Imaging

After craniotomy and dye application, mice were maintained on intraperitoneal ketamine anesthesia at a dose of 30 to 50 mg/kg per hour before imaging. A custom-built two-photon laser-scanning microscope (Radosevich *et al*, 2008) equipped with a Ti:Sapphire laser (Mai Tai XF, Spectra Physics, Mountain View, CA, USA), three emission channels (350 to 505 nm, 505 to 560 nm, and 560 to 650 nm), and a $\times 20$ objective lens (XLUMPLanFl 20X 0.95W; Olympus, Tokyo, Japan) was then used to image the

somatosensory cortex with excitation wavelengths between 800 and 850 nm at depths up to and exceeding 500 μm below the cortical surface.

Vessels were identified as arteries, veins, or capillaries based on morphology, flow speed, and the direction of blood flow. Arteries typically have fewer branches, more linear morphology, and higher flow speeds relative to veins. Serial stacks of $250 \times 250 \mu\text{m}^2$ images (400×400 pixels at 400,000 Hz pixel rate) separated by 1 to 2 μm increments and running from the cortical surface to depths of $\sim 500 \mu\text{m}$ were acquired by vertically translating the system's objective (using a M-112.1DG stage; Physik Instrumente, Karlsruhe, Germany). These image stacks recorded the interactions between astrocytes and cerebral blood vessels at multiple cortical depths through layer IV, which terminates at $\sim 450 \mu\text{m}$ below the surface of the mouse somatosensory cortex (Altamura *et al*, 2007).

Immunohistochemistry

After *in vivo* imaging, mice were euthanized and intracardially perfused with phosphate-buffered saline (PBS), pH 7.4. Their brains were then removed, bathed for 5 minutes in SR101/hydrizide solution to increase staining, and then fixed overnight in 4% paraformaldehyde. Oblique 100- μm -thick sections of tissue running parallel to the surface of the somatosensory cortex were obtained using a vibratome (Vibratome, Richmond, IL, USA) and washed in PBS. Slices then underwent antigen retrieval using protease digestion. This involved incubating slices in a 0.05% solution of protease from *Streptomyces griseus* (Sigma-Aldrich) at $\sim 37^\circ\text{C}$ in a humidified chamber for 1 minute before returning them to PBS. The slices were then transferred to a blocking solution consisting of 10% normal goat serum (Invitrogen) and either 0.3% or 2% Triton X-100 (Sigma-Aldrich) for 30 minutes. The blocking solution was replaced with a staining solution consisting of 0.3% or 2% Triton X-100 in PBS to which primary antibodies were added. Polyclonal rabbit anti-aquaporin-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added at a 1:50 dilution, polyclonal rabbit anti-GFAP was added to separate wells at a 1:50 dilution, and polyclonal rabbit anti-claudin-5 (Abcam, Cambridge, MA, USA) was used at a 1:100 dilution, and monoclonal rabbit anti-smooth muscle actin was applied at a 1:50 dilution. Primary antibodies were incubated overnight at room temperature. Sections were then washed four times in PBS for 5 minutes each wash. Goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 405 (Invitrogen) were then added at a 1:300 dilution. Sections were washed again three times in PBS for 5 minutes each wash and then mounted on glass slides using the Vectashield mounting medium (Vector Labs, Burlingame, CA, USA). Slides were then imaged using our two-photon microscope.

Data Analysis

Custom analysis software was developed in MATLAB (Mathworks, Sherborn, MA, USA) to allow us to precisely determine each astrocyte's 3D position, dimensions, depth, and vessel proximity, as well as blood vessel density as described below.

To calculate the locations of astrocytes within the cortex, a single point that lay midway between the upper and lower boundaries of the soma was chosen for each cell. The depth dependence of astrocyte density was determined by mapping the total number of these points representing astrocyte somata contained within consecutive, nonoverlapping 20- μm depth increments. Although astrocyte domains may occupy areas much larger than 20 μm , across the use of a single representative voxel for each soma allowed clean grouping of astrocyte cell bodies into nonoverlapping 20- μm segments without the danger of double counting any cells. The glia limitans superficialis, which spans from the surface of the brain to a depth of ~ 10 to 15 μm , was excluded from our analyses because of high cell volume and poor contrast within this region.

Masks of blood vessels were generated using a thresholding system and growing algorithm to mark pixels of sufficient brightness along a manually selected seed vessel in the 3D image stack. Capillary density was then calculated by excluding large diving arterioles and venules (identified by their connectivity, diameter, and tortuosity), and then by calculating the total number of capillary mask pixels at 2- μm depth increments below the surface of the cortex. Every voxel in the 3D image stack was considered, and the depth of each vessel pixel was taken as the z-separation between that image slice and the brain surface.

The separation between astrocytes and their nearest capillary was calculated by sampling the pixels in expanding spheres around each cell. When a capillary pixel was detected, the vector linking this pixel to the astrocyte soma marker was obtained, and all pixels between these two points were examined. The distance between the capillary pixel and the most peripheral point of the astrocyte cell body closest to the capillary was used as our measure of the separation between the two.

The diameter of blood vessels and their perivascular astrocyte sheaths were manually measured for arteries, capillaries, and veins using the average of numerous sample points along the length of each vessel. Locations at which astrocyte processes connected with vessels and locations of perivascular astrocyte somata in direct contact with vessels were also manually recorded for each type of vessel. These data were then used to calculate the density of astrocyte process contacts along each type of vessel and the spacing of perivascular astrocytes. The density of contacts between astrocyte processes and blood vessels was calculated per unit of vessel length (total visible processes per 100 μm of vessel length) and as a function of vessel surface area (processes per 100 μm^2 of blood vessel).

Trends in the data were quantified using a combination of Pearson's correlation and one-way ANOVA (analysis of variance) implemented in PASW (Predictive Analytics Software, Somers, NY, USA). Error in all data presented was calculated as s.e.m.

Spectral Unmixing

For analysis of images obtained from transgenic GFP mice, it was necessary to exclude any potential overlap between

the fluorescence emission of SR101 and GFP. Therefore, to generate the images in Figure 3, we used a spectral unmixing strategy as previously used in the studies by Radosevich *et al* (2008) and Tsai *et al* (2009). In brief, the pure red–green–blue emission spectral signatures of SR101 and GFP were identified by sampling regions of images that contained only one of these two contrasts (e.g., an astrocyte in a GFAP-GFP mouse before SR101 staining and an astrocyte in a wild-type mouse with only SR101 staining). This yielded a unique set of three emission values for each contrast species (SR101 and GFP) that could be used in a nonnegative least squares fit to all of the tricolor pixels in images acquired in mice in whom both forms of contrast were present. The results of this fit are two images that represent the ‘concentration’ of either SR101 or GFP within each pixel, based on the detected red–green–blue spectrum of that pixel. This approach allows quantitative analysis of any true spatial overlap between these two contrasts without the possible confound of spectral overlap.

Results

Astrocyte Distribution

On the basis of an examination of 4,167 cortical astrocytes in 8 image stacks ($250 \times 250 \times 500 \mu\text{m}^3$ field of view) from 5 C57BL/6J mice, astrocyte density was found to vary in a consistent pattern as cortical depth increases (Figure 1A). Although the glia limitans (which was excluded from our automated analysis) has high astrocyte density, the region immediately below it from ~ 10 to $40 \mu\text{m}$ deep contains very few astrocyte cell bodies. However, a dense network of astrocyte processes exists in this region. Astrocyte density (the number of astrocyte soma per unit volume) spikes sharply approaching 40 to $60 \mu\text{m}$ in depth before falling away by $180 \mu\text{m}$. Below $180 \mu\text{m}$, astrocyte density increases quite steadily as depth increases (Pearson’s correlation between depth and astrocyte density below $180 \mu\text{m}$ is positive and significant: $r(113) = 0.581$; $P < 0.0005$) (Figure 1A). One-way ANOVA of $20 \mu\text{m}$ groups with *post hoc* least significant difference testing shows that beyond $180 \mu\text{m}$, astrocyte density never varies significantly from the density of neighboring groups, suggesting that the small fluctuations in this distribution are not significant. A similar methodology shows that the peak in astrocyte density at 40 to $60 \mu\text{m}$ is highly significant relative to nearly every other group, including 0 to $20 \mu\text{m}$ ($P < 0.0005$), 20 to $40 \mu\text{m}$ ($P < 0.0005$), and all groups from 80 to $400 \mu\text{m}$ ($P < 0.0005$ for 9/16 groups). The peak astrocyte density of $\sim 30,000$ astrocytes/ mm^3 that occurs at 40 to $60 \mu\text{m}$ and then again between depths of 440 and $500 \mu\text{m}$ agrees well with two-photon microscopy studies of intact *ex vivo* brain sections performed by Tsai *et al* (2009), who showed that the combined density of astrocytes and all other nonneuronal cells, including endothelial cells and other glia, reaches $\sim 43,000$ cells/ mm^3 , with a spike

in density around $50 \mu\text{m}$ below the surface of the cortex. It should be noted that we did not attempt to identify cortical layer boundaries from our data, and therefore we cannot say whether this peak in astrocyte density coincides with layer I, layer II, or the boundary between them. Layer boundaries shown in Figures 1A–C are based on literature values. Representative images from key cortical depths are shown in Figures 1D to 1G.

Capillary Density

Relatively few capillaries were found to occupy the region immediately below the glia limitans, but capillary density increases sharply as depth approaches 30 to $40 \mu\text{m}$. Capillary density continues to increase at a reduced rate beyond $40 \mu\text{m}$, eventually reaching an apparent maximum near $500 \mu\text{m}$ below the surface of the somatosensory cortex (Figure 1B) (Pearson’s correlation between depth and capillary density is positive and significant: $r(1,448) = 0.505$; $P < 0.0005$). One-way ANOVA showed that the smaller spikes in this trend beyond $40 \mu\text{m}$ are not significant.

Astrocyte–Capillary Separation

The separation between astrocytes and capillaries was found to slightly decrease, on average, as cortical depth increases (Pearson’s correlation with depth is negative and significant: $r(142) = -0.339$; $P < 0.0005$) (Figure 1C). This relationship follows a roughly linear pattern, decreasing quite steadily with only minor spikes in separation as depth increases, despite the fact that astrocyte density and capillary density follow more complex patterns. One-way ANOVA shows that the small spikes in separation are not significant. The average distance between the edge of astrocyte somata and the nearest capillary at any given cortical depth ranges between ~ 6 and $10 \mu\text{m}$, although many astrocytes may be found within a single micron of a microvessel, and others may be found $> 20 \mu\text{m}$ away. Tsai *et al* (2009) found that neuronal nuclei were located, on average, $\sim 15 \mu\text{m}$ from a microvessel, whereas Zhang *et al* (2005) showed that dendritic spines of somatosensory neurons were typically $13 \mu\text{m}$ from a capillary. Our results show that astrocyte somata tend to be much closer to the microvasculature than either neuronal somata or dendrites, supporting a close affinity between astrocytes and microvessels.

Perivascular Sheathing Morphology

All blood vessels below the glia limitans were found to be surrounded by a perivascular sheath, brightly staining with SR101. Arteries on the pial surface, but never surface veins, were also found to exhibit robust perivascular SR101 staining (Figure 2A). For subpial veins, the strong SR101 sheath appears to merge with

Figure 1 Astrocyte density, capillary density, and astrocyte–vessel separation. **(A)** Astrocyte density as a function of cortical depth (excluding the glia limitans superficialis between 0 and 20 μm). Cell density was sampled in 20- μm increments. **(B)** Capillary density as a function of cortical depth, sampled in 2- μm increments. **(C)** The average separation between astrocytes and capillaries as a function of cortical depth sampled in 20- μm increments. All error bars show s.e.m. Layer I to IV positions are indicated in accordance with Altamura *et al* (2007), although it should be noted that these boundaries are simply guidelines, and that Tsai *et al* (2009) found that layer I may start at depths of up to 100 μm in unprocessed tissue. **(D to G)** Representative images from specific cortical depths. Red = SR101 labeling, green = intravascular dextran-conjugated fluorescein. (Panel **D**) Image of the glia limitans at the cortical surface. Pial veins are visible with sub-pial perivascular SR101 sheathing merging into the numerous cell bodies of the glia limitans. (Panel **E**) Dense astrocyte cell bodies 50 μm below the surface of the brain. (Panel **F**) Sparse astrocyte cell bodies at a depth of 150 μm . Neuron cell bodies are visible as dark, unstained areas. (Panel **G**) Astrocyte density recovers approaching 450 μm . Capillary density is also elevated. This full stack is provided as a movie and 3D rendering in Supplementary data. SR101, sulforhodamine 101.

the glia limitans and disappear as the vein ascends to the pial surface (Figure 2B). A sheath can also be seen around capillaries, forming from an accumulation of processes from the adjacent astrocyte somata (Figure 2C).

A closer inspection of cortical arterioles and arteries revealed two distinct layers of SR101 sheathing (Figure 2D). Similar to ascending veins, diving arterioles possess an outer perivascular sheath that joins with the cells of the glia limitans and is no longer present above the pial surface (Figure 2E).

However, the inner SR101-stained arteriolar sheath extends up through the glia limitans and along all pial arteries and arterioles. This inner arteriolar SR101 sheath exhibits consistent substructure appearing to be composed of elongated striations running parallel to the long axis of the vessel (Figure 2F). These inner structures were not found to be labeled in cortical veins either above or below the glia limitans.

Data acquired *in vivo* in TIE2-GFP transgenic mice, whose endothelial cells express GFP, showed

Figure 2 Perivascular sheathing morphology. (A) Pial arteries exhibit bright perivascular SR101 staining, whereas pial veins show no evidence of such staining. (B) The perivascular astrocyte sheath of diving veins merges with the cells of the glia limitans. (C) Capillaries exhibit astrocyte sheathing composed of processes from perivascular and intervascular astrocytes (arrow). Cell soma contacting an ascending vein are also visible (arrowheads). (D) Diving arteries possess two distinct layers of SR101 sheathing, thereby defining four domains of the vessel: (1) the arterial lumen; (2) the nonastrocytic inner SR101 sheath immediately apposing the vessel lumen; (3) a negative staining space between the inner and outer SR101 sheaths, presumably occupied by smooth muscle; and (4) the astrocytic outer SR101 sheath. (E) The outer SR101 sheath of diving arterioles merges with astrocytes of the glia limitans, whereas the inner sheath continues along the pial arteriole. (F) SR101 sheathing of pial arteries exhibits consistent substructure, manifested as parallel-staining lines running along the long axis of the vessel. SR101, sulforhodamine 101.

that the strongly staining SR101 sheath visible on pial arteries as well as the inner SR101 sheath of diving arterioles appear to colocalize with arteriolar endothelial cells (Figures 3A and 3B). In contrast, GFP-positive endothelial cells of pial veins show no colocalized SR101 staining. Similarly, SR101 labeling in transgenic GFAP-GFP mice showed that the outer SR101 sheath of diving arterioles expresses GFAP (indicating the presence of astrocytes), whereas the inner arteriolar SR101 sheath does not (Figure 3C).

Perivascular Sheath Thickness

Arteries were found to have the thickest astrocytic perivascular sheaths with an average of $2.6 \pm 0.30 \mu\text{m}$. This number represents the thickness of the outer SR101 sheath around diving arterioles. Veins were found to have the second thickest sheath ($2.1 \pm 0.05 \mu\text{m}$), and capillaries had the thinnest astrocytic perivascular SR101 sheaths ($1.2 \pm 0.04 \mu\text{m}$). These differences are all significant ($F(2, 24) = 40.859$; $P < 0.0005$) (Figure 4A). Despite having the thinnest perivascular sheaths however, capillaries have the highest ratio of sheath thickness to vessel thickness ($F(2, 24) = 13.907$; $P < 0.0005$) (Figures 4B and 4C). On average, capillary sheath cross-sectional thickness is $36.60\% \pm 1.31\%$ of capillary vessel thickness. Artery sheaths measure $25.63\% \pm 2.94\%$ of artery thickness, and vein sheaths measure $21.76\% \pm 3.55\%$ of vein thickness.

There was no significant difference between artery and vein sheath thickness relative to vessel diameter.

Perivascular astrocytes (astrocytes with somata physically contacting and arcing around a vessel wall) were more commonly found on arteries and veins compared with capillaries ($F(2, 24) = 20.091$; $P < 0.0005$) (Figure 4C). On average, 1.79 ± 0.43 perivascular astrocytes were found per $100 \mu\text{m}$ of arteriole, 0.19 ± 0.06 were found per $100 \mu\text{m}$ capillary, and 1.08 ± 0.20 were found per $100 \mu\text{m}$ of vein. Care was taken when calculating each vessel's perivascular sheath thickness to measure areas unaffected by the presence of perivascular astrocytes. Perivascular astrocytes were almost always found at branch points along diving veins, but typically not at branch points along arteries or capillaries.

Astrocyte Process Density

On average, 17.07 ± 1.05 astrocyte processes were found to contact a $100 \mu\text{m}$ segment of arteriole, 16.80 ± 0.77 processes contact $100 \mu\text{m}$ of capillary, and 18.59 ± 1.28 processes contact an equal length of venule. These differences are not statistically significant ($F(2, 24) = 0.848$; $P = \text{NS}$) (Figure 4E). However, when the number of visible astrocyte processes contacting each type of vessel was compared with estimates of vessel surface area, the highest density of contacting processes was found for capillaries

Figure 3 SR101 staining in transgenic TIE2-GFP and GFAP-GFP mice. **(A)** The inner arteriolar SR101 sheath of the pial arteriole colocalizes with GFP expressed in arterial endothelial cells (top arrow). Endothelial cells composing the wall of the pial vein do not colocalize with SR101 staining (bottom arrow). **(B)** The inner sheath of a diving arteriole exhibits both SR101 staining and endothelial GFP expression (arrow), whereas the outer sheath is only stained with SR101 (inset shows magnified diving arteriole). **(C)** A diving arteriole in a GFAP-GFP mouse has SR101 staining of both its inner and outer sheaths, but GFAP-GFP expression is only seen in astrocytes of the outer sheath. Images were processed using spectral unmixing to avoid confounding spectral overlap between GFP and SR101. Left column shows unmixed SR101, middle column shows unmixed GFP, and right column shows merge of the two. GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; SR101, sulforhodamine 101.

($F(2, 24) = 44.945$; $P < 0.0005$) (Figure 4F). On average, 0.94 ± 0.05 processes were found within $100 \mu\text{m}^2$ of capillary surface area compared with 0.36 ± 0.03 processes per $100 \mu\text{m}^2$ of arteriole and 0.41 ± 0.04 processes per $100 \mu\text{m}^2$ of venules.

Immunohistochemistry Validation

In addition to using transgenic mice as controls, we also used IHC to verify the identity of structures stained by SR101. We found that although IHC GFAP labeling revealed astrocyte processes interacting with diving arterioles, staining did not persist above the glia limitans (Figure 5A), supporting findings from GFAP-GFP mice that the inner SR101 sheath of pial arteries is likely not due to the presence of astrocytes or their processes. Similarly, aquaporin-4 expression was observed around every vessel below the glia limitans, and robust staining was observed within the glia limitans itself (Figure 5B).

However, perivascular aquaporin-4 staining was not observed on pial vessels and did not colocalize with the inner SR101 sheath of pial or diving arterioles.

Conversely, IHC for the endothelial cell tight junction marker claudin-5 strongly costained with SR101 in the inner sheath of pial arteries. Furthermore, claudin-5 staining mimicked the fine structure of this SR101 sheath by staining elongated, parallel structures running along the long axis of the vessel (Figures 5D to 5I). Claudin-5 staining was also observed along pial veins, although the morphology of the stained structures was quite different from that seen on pial arterioles, being much less unidirectional (Figures 5D to 5F). No corresponding SR101 staining was seen on these surface veins. Immunohistochemistry for smooth muscle actin in TIE2-GFP mice further confirmed the location of the inner arteriolar SR101 sheath, showing that the arterial smooth muscle is present outside the colocalized inner SR101 sheath and endothelial GFP in pial arterioles (Figure 5C).

Figure 4 Measured astrocyte–vascular parameters. **(A)** Perivascular sheathing is thickest around arteries, followed by veins, with capillaries having the thinnest sheaths. **(B)** Diving arteries and ascending veins have diameters averaging 10 to 12 μm . Capillary diameters average $\sim 3.5 \mu\text{m}$. **(C)** Capillaries have the thickest perivascular astrocyte sheaths relative to vessel diameter. There is no significant difference between the thickness of artery and vein sheaths relative to vessel diameter. **(D)** Significantly more perivascular astrocytes are found along diving arterioles than around either capillaries or ascending venules. Ascending venules interact with significantly more perivascular astrocytes than capillaries. **(E)** There is no difference in the number of visible astrocyte processes contacting equal length segments of diving arterioles, capillaries, and diving venules. **(F)** The surface density of astrocyte processes is significantly higher on capillaries than on diving arterioles or ascending venules. All error bars are s.e.m.

Discussion

The purpose of this study was to document the *in vivo* 3D cytoarchitecture of astrocytes and their interactions with blood vessels to explore structural evidence for the potential role of astrocytes in neurovascular coupling. We investigated the depth dependence of both astrocyte and microvascular density, finding that the two have similar but not identical trends. We also observed that all vessels below the pial surface are covered with extensive sheathing composed of astrocyte processes. Capillaries were found to have substantially more processes contacting them per unit surface area than arterioles and veins. We concluded that SR101 staining of pial arterioles above the glia limitans is not attributable to the presence of astrocyte processes. We discuss the implications of these findings below.

Neurovascular Signaling: Beyond the Diving Arteriole

Our Results Suggest that Astrocytic Neurovascular Control May Not be Restricted to Diving Arterioles: It has been shown that astrocyte processes contacting diving arterioles exhibit increases in intracellular calcium $[\text{Ca}^{2+}]$ in response to glutamate and/or inositol triphosphate and may release vasoactive metabolites or alter perivascular K^+ dynamics to induce changes in vascular tone (Girouard *et al*,

2010; Koehler *et al*, 2009; Iadecola and Nedergaard, 2007; Jakovcevic and Harder, 2007; Filosa *et al*, 2006). Neurovascular coupling literature to date has targeted diving arterioles as the likely point of control of functional hyperemia, assuming that changes in their diameter will have the highest impact on changing vascular resistance and therefore blood flow (Iadecola and Nedergaard, 2007; Nishimura *et al*, 2007; Takano *et al*, 2006). This, combined with the presence of smooth muscle, makes arterioles an obvious place to look when examining regulation of blood flow by astrocytes.

However, our findings show that every capillary and vein below the pia exhibits the same general morphology of interaction with astrocyte processes as diving arterioles. Given this lack of selectivity, we posit that neurovascular interactions involving astrocytes might equally occur at other locations, beyond diving arterioles. For example, if astrocytes are assumed to only sense and act discretely within their immediate vicinity, then these interactions would most likely occur in the capillary beds, since this is where most nutrient exchange occurs, and is therefore where increased neuronal activity or energy demands could be most rapidly sensed (Borowsky and Collins, 1989).

Studies focusing on diving arterioles also do not address the fact that the hemodynamic response manifests as a sequence of coordinated, rapidly propagated vasodilations and vasoconstrictions across

Figure 5 GFAP, AQP-4, SMA, and claudin-5 *ex vivo* immunohistochemistry. (A) GFAP staining reveals astrocytes entwining a diving arteriole, but no GFAP is visible along the larger pial surface artery. (B) AQP-4 staining reveals high levels of AQP-4 expression around capillaries and within the glia limitans. Although AQP-4 does not costain the inner SR101 sheath of the diving arteriole, it appears to be localized in the space occupied by the outer SR101 sheath (arrows). (C) SMA staining is visible outside SR101 staining that is colocalized with TIE2-GFP on this pial arteriole. (D to F) Pial surface artery showing fine structure of claudin-5 and SR101/Texas red hydrazide staining. A surface vein, with disordered claudin-5 staining, but no staining with SR101, is also visible. Arterial tight junctions outline elongated parallel structures running along the arteriole, whereas venous tight junctions have a less uniform arrangement. (G to I) Magnified region showing close apposition of claudin-5 expression and SR101 staining, suggesting that SR101 is localized within structures intimately associated with arteriolar endothelial tight junctions. AQP-4, aquaporin-4; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; SMA, smooth muscle actin; SR101, sulforhodamine 101.

several millimeters of the cortex (Hillman *et al*, 2007, Chen *et al*, 2010). If astrocytes do not simply act locally, but are exclusively responsible for modulating vascular tone, then they would need to be able to communicate and act over long distances in a coordinated manner. We discuss these two possibilities with regard to our findings below.

An Astrocyte Network for Neurovascular Signaling?

The Ubiquitous Presence of an Astrocytic Sheath Around All Subpial Vessels May Indicate that Astrocytes Can Indeed Signal Over Long Distances: Previous experiments using dye filling and transgenic mice have shown that processes from astrocyte cell bodies occupy discrete regions in space that only minimally overlap with adjacent astrocytes (Halassa *et al*, 2007; Wilhelmsson *et al*, 2006; Bushong *et al*, 2002). This discretization of astrocyte domains implies that astrocytes are unlikely to individually

exert influences over distances of >100 to 200 μm. However, a recent study showed that gap junctions are highly expressed within perivascular astrocyte sheaths (Giaume *et al*, 2010), connecting the cytosols of adjacent astrocyte processes. Furthermore, astrocytes have been shown to be capable of communicating through Ca^{2+} signaling, and increases in astrocytic Ca^{2+} levels have in turn been linked to modulations in vascular tone (Takano *et al*, 2006; Zonta *et al*, 2003). Therefore, the comprehensive sheathing that we have observed along all subpial vessels could feasibly form a system of interconnected conduits capable of transmitting signals along the vasculature over distances that far exceed the discrete range of a single astrocyte.

We also observed that SR101 consistently labels the walls of pial surface arteries but not pial surface veins (Figures 2A and 3A), and that this staining did not correspond to the presence of astrocytes (Figures 3C, 5A, and 5B). Instead, our results suggest

that SR101 is staining structures within the arterial endothelium (Figures 3A, 3B, and 5D to 5I). No corresponding SR101 labeling was seen on pial veins, suggesting that it is not simply the presence of endothelial cells or tight junctions that is causing the SR101 staining, but rather some difference between arterial and venous endothelial cells that causes the uptake of SR101 in arteries but not veins.

Nimmerjahn *et al* (2004) showed that SR101 is efficiently distributed to astrocytes deep within the cortex through their gap junctions (because this distribution was inhibited by gap junction/hemichannel blocker carbenoxolone, Cbx). Therefore, one explanation for the specific staining of the inner arteriolar SR101 sheath may be that astrocytes share gap junctions with the arteriolar endothelium. The possibility of a connection between the subpial astrocyte network and the endothelium of far-reaching pial arterioles is particularly compelling because it might suggest that vasomodulatory signals of astrocyte origin could be directly relayed over the long distances necessary for orchestration of the hemodynamic response to functional stimulus (consistent with the findings of Xu *et al* (2008)). If SR101 staining of the inner arteriolar SR101 sheath is not an indication of a functional connection between astrocytes and the arteriolar endothelium, then it is still evidence that the arteriolar endothelium possesses unique properties compared with capillary or venous endothelium, allowing it to uptake a substance that otherwise appears to be highly specific only to astrocytes.

Previously proposed mechanisms for the propagation of vasodilation have included possible contributions of endothelial cells (Andresen *et al*, 2006; Girouard and Iadecola, 2006), vasoactive interneurons (Cauli *et al*, 2004; Fahrenkrug *et al*, 2000), and smooth muscle cells (Girouard and Iadecola, 2006). If the network of astrocyte connectivity that we have observed has a role in this propagation, the next question that must be addressed is whether the speed of Ca^{2+} propagation between interconnected astrocytes could be sufficient to account for the rapid evolution of the hemodynamic response observed *in vivo*.

Do Astrocytes Interact Preferentially with Capillaries?

Capillary-Astrocyte Proximity Suggests a Preferential Relationship Between Them that Overrides Spatial Variations in Microvascular and Astrocyte Density: Whether astrocytes propagate vasomodulatory signals directly or act via other intermediate cell types, the potential role of astrocytes as local sensors and even actuators remains. However, particularly if astrocytes are only able to act locally, their proximity to vessels and their relative densities might be important indicators of their role in neurovascular coupling.

Our *in vivo* imaging results revealed a particularly strong affinity between astrocytes and capillaries, rather than diving arterioles. Our findings supporting

this assertion are first that capillaries appear to have more astrocyte processes connecting to them per unit vessel area than either arterioles or venules (Figure 4F). Second, we note that Figure 1C reveals fairly uniform spacing between astrocytes and vessels as a function of depth, in spite of significant depth-dependent variations in both astrocyte and microvascular density (Figures 1A and 1B). If the relative locations of astrocytes and microvessels were random, we would expect the average separations between them to have a more variable depth-dependent structure. For example, the peak in astrocyte density at a depth of $\sim 60 \mu\text{m}$ (Figure 1A) is not mirrored by a change in microvascular density (Figure 1B), and so should correspond to a marked decrease in astrocyte-vessel separation rather than the slight increase that is observed. We conclude then, that astrocytes are preferentially located ~ 6 to $8 \mu\text{m}$ from vessels, and that their proximity is not simply a function of their relative densities. This close interrelation suggests that astrocytes and capillaries are functionally interdependent.

If astrocytes are predominantly acting locally, this also means that spatial variations in their density are noteworthy. For example, the peak in astrocyte density at $\sim 60 \mu\text{m}$ (Figure 1A) may indicate that this region has particular significance for neurovascular control. Several studies have attempted to explore the depth dependence of blood flow changes in the cortex (Nielsen and Lauritzen, 2001; Hillman *et al*, 2007); however, none to date have had sufficient resolution to determine whether this astrocyte-dense layer might be of particular functional importance.

What Might the Role of Astrocytes at the Capillary Level Be?

Are Astrocytes Involved in Hemodynamic Modulation of Capillaries, or is Their Presence Primarily for Nutrient Exchange and/or Neurotransmitter Scavenger?: Astrocytes have been hypothesized to serve many different roles in the brain. Therefore, the physical linkage between astrocytes and capillaries can be interpreted in a number of ways. For example, the majority of oxygen, nutrient, and waste exchange occurs at the capillary level, and therefore astrocytes might simply be positioned to ensure that they have adequate access to this supply (Borowsky and Collins, 1989). As neurons do not appear to exhibit similar perivascular morphology to astrocytes (Cauli *et al*, 2004), close connectivity between astrocytes and capillaries may support hypotheses that astrocytes have vital roles as intermediaries in providing nutrients, such as lactate to active neurons (Giaume *et al*, 2010; Rouach *et al*, 2008; Pellerin *et al*, 1998).

However, if astrocytes' roles extend beyond nutrient and waste exchange, their physical interrelation with capillaries may suggest that they sense

local increases in activity and metabolic demand (Pellerin *et al*, 1998) to mediate changes in vascular tone (either by propagation through the perivascular astrocyte sheath network described above or as intermediaries with other cell types). A further possibility is that the physical connectivity between astrocytes and capillaries may actually form part of a mechanism capable of actively mediating changes in capillary tone or even permeability.

Recent studies have suggested that initiation of the hemodynamic response manifests as a rapid increase in tissue hemoglobin concentration in the capillary bed, with subsequent upstream propagation of dilation along the arteriolar tree (Chen *et al*, 2010; Sirotnin *et al*, 2009). To account for these observations, an active mechanism around the level of the capillary bed (including precapillary arterioles or postcapillary venules) must be capable of inducing a rapid increase in the number of red blood cells in the capillary bed before upstream arteriolar dilation. Mechanisms by which astrocytes might modulate capillary hemoglobin concentrations in the absence of smooth muscle cells are uncertain (Hutchinson *et al*, 2006). One possibility is through interactions with pericytes, which are contractile cells located along central nervous system capillaries (Peppiatt *et al*, 2006). An alternative possibility might be that Ca^{2+} -sensitive K^+ (BK) channels on astrocyte endfeet (Filosa *et al*, 2006; Girouard *et al*, 2010) could vary intravascular hematocrit levels by altering the osmotic properties of the capillary walls.

Methodological Conclusions

We have shown that two-photon microscopy can be used to perform ‘*in vivo* 3D histology’ of the cellular and vascular morphology of the brain. Although our penetration depth was limited to $\sim 500\ \mu\text{m}$, this was sufficient to see beyond layer IV in mouse cortex, and with the use of longer wavelengths, could feasibly be performed to deeper layers (Kobat *et al*, 2009). We used SR101 as an *in vivo* marker for astrocytes, which we found to provide a much more comprehensive and stronger labeling of astrocytes than GFAP-GFP transgenic mice. However, it should be noted that although Nimmerjahn *et al* (2004) performed a very detailed characterization of the specificity of SR101 for astrocytes in the cortex that, as with all labeling techniques, there is some possibility that our findings were influenced by the labeling specificity of SR101. Representative of this, our analysis showing that the SR101-labeled inner sheath of pial and diving arterioles is not of astrocytic origin is important for those using SR101 for *in vivo* studies of astrocytes.

Summary

In summary, although control of functional hyperemia most likely involves the interactions of

many different cell and vessel types, our findings have provided greater insights into the potential role of astrocytes in neurovascular coupling. Where astrocyte domains were previously believed to be small and discrete, we have found compelling evidence that astrocyte processes form conduits of connectivity that trace along all vessels within the cortex, potentially forming an ‘astrocytic superhighway’ capable of rapidly distributing vasomodulatory signals. We did not find strong evidence that astrocytes have preferential associations with diving arterioles. In fact, our results suggest that the capillary beds may be a more important site for astrocyte–vascular interactions. Our studies also present many questions, including whether the presence of a sheath on ascending veins obviates the significance of sheathing on arterioles and capillaries, and whether astrocyte signal propagation could be fast enough to account for observations of the hemodynamic response.

Acknowledgements

The authors thank James Goldman, Peter Canoll, and Edith Hamel for guidance and useful discussions, and Fiona Doetsch for generous donation of GFAP-GFP mice.

Disclosure/Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Journal of Cerebral Blood Flow & Metabolism website (<http://www.nature.com/jcbfm>)