



A guide to delineate the logic of neurovascular signaling in the brain

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The neurovascular system may be viewed as a distributed nervous system within the brain. It transforms local neuronal activity into a change in the tone of smooth muscle that lines the walls of arterioles and microvessels. We review the current state of neurovascular coupling, with an emphasis on signaling molecules that convey information from neurons to neighboring vessels. At the level of neocortex, this coupling is mediated by: (i) a likely direct interaction with inhibitory neurons, (ii) indirect interaction, via astrocytes, with excitatory neurons, and (iii) fiber tracts from subcortical layers. Substantial evidence shows that control involves competition between signals that promote vasoconstriction versus vasodilation. Consistent with this picture is evidence that, under certain circumstances, increased neuronal activity can lead to vasoconstriction rather than vasodilation. This confounds naïve interpretations of functional brain images. We discuss experimental approaches to detect signaling molecules *in vivo* with the goal of formulating an empirical basis for the observed logic of neurovascular control.

Keywords: astrocytes, blood flow, channelrhodopsin, interneurons, neurotransmitter, two-photon

Blood is a vital and limited resource in the brain. All aspects of neuronal and non-neuronal activity require a supply of oxygen and glucose – a need that constantly evolves with changes in brain activity (Fox and Raichle, 1986; Leybaert, 2005). How is the distribution of blood controlled relative to these changing needs? More basically, what are the signals that different classes of cells use to communicate changes in their metabolic load? How are these signals integrated to change the flow in the blood vessels that intercalate cortical neurons and glia (Figure 1)?

An understanding of neurovascular signaling bears directly on the limit to cortical function and, more generally, on resource management by the central nervous system. From a clinical perspective, understanding the relation of neuronal activity to changes in blood oxygen and flow is an essential step toward addressing the role of vascular dysfunction and disease in dementia (Paulson et al., 1990; Kövari et al., 2007). From the perspective of cognitive science, a quantitative understanding of neurovascular signaling is crucial for the interpretation of functional brain images (LeDoux et al., 1983) – especially those obtained with blood oxygenation level dependent functional magnetic resonance imaging (BOLD fMRI). Magnetic resonance imaging and other macroscopic diagnostic techniques have provided a “window” into the brain from which modulation of macroscopic blood by neuronal activity may be assessed (Belliveau et al., 1991; Ogawa et al., 1992). These techniques are likely to evolve into essential tools for judging cognitive capabilities in a clinical setting and may be critical for the early identification and hopefully containment of dementia.

We view neurovascular control in terms of the net action of a tractable number of signaling molecules that arrive via one global and two local pathways (Figure 2). All of these function in a push–pull

manner, i.e., one set of signals induces vasodilation through relaxation of arteriole smooth muscle, while a second set induces vasoconstriction (Cauli et al., 2004). The first local pathway involves local inhibitory interneurons that act directly on smooth muscle. Different subclasses of interneurons co-release different transmitters that act to either dilate vessels, including nitric oxide (NO) and vasoactive intestinal peptide (VIP), or to constrict vessels, including the neuropeptides somatostatin (SOM) and neuropeptide Y (NPY). In some cases the same interneuron can cause both effects; e.g., some SOM-expressing cells further express nitric oxide synthetase (Kawaguchi, 2001). Additional evidence for a role of interneurons in the modulation of blood flow comes from an increase in flow concurrent with γ -band oscillations in inhibitory networks (Niessing et al., 2005). Increases in flow correlate with such global rhythms. It is thus natural to hypothesize that the competition between dilation and constriction depends on the relative activation of different inhibitory networks (Gibson et al., 1999); this hypothesis may be tested with cell-specific markers of neuronal activity.

The second local pathway for the control of blood flow originates with excitatory neurons and acts via astrocytes (Filosa et al., 2004; Iadecola and Nedergaard, 2007). Volume conduction of the excitatory transmitter glutamate can cause an increase in intracellular Ca^{2+} in astrocytes, which in turn initiates the conversion of arachidonic acid to the dilators prostaglandin E (PGE_2) and epoxyeicosatrienoic acid (EET) or the constrictor 20-hydroxyeicosatetraenoic acid (20-HETE). Recent work shows that the competition between dilation and constriction depends on the partial pressure of oxygen (pO_2) (Gordon et al., 2008); dilation dominates at low pO_2 .

Lastly, global pathways involve the vasodilator acetylcholine (ACh) and the constrictor serotonin (5HT), released from their respective subcortical nuclei, i.e., nucleus basalis magnocellularis

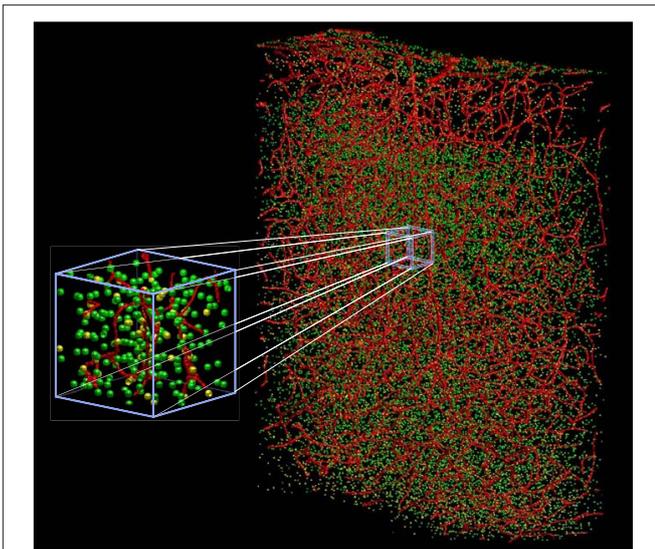


FIGURE 1 | Three dimensional vectorized reconstruction of all of the cell soma and blood vessels in a slab of mouse cerebral cortex. Features in the raw data are analyzed and transformed into a digital map that represents them as cylinders and spheres with vector coordinates and associated radii. The vascular network is in red, the neuronal nuclei are in green, and non-neuronal nuclei are in yellow. The total volume is a 600- μm \times 900- μm \times 250- μm . Derived from Tsai et al. (2003, 2009).

and the dorsal raphe nucleus (Hamel, 2004, 2006). An additional neural pathway for the modulation of cortical blood flow occurs via the rostral ventrolateral medulla (RVLM; Golanov and Reis, 1996), whose projections to cortex are relayed by the interlaminar thalamic nucleus. Neurons in the RVLM are sensitive to oxygen levels. Their activation causes bilateral increase in cortical blood flow via thalamic intermediates. This raises the possibility that changes in cortical blood flow and activity are slaved to fluctuations in breathing and blood oxygenation (Wise et al., 2004; Shmueli et al., 2007; Drew et al., 2008). Blood flow is further driven by cortical state (Jones et al., 2008); this mode of control may well lie in changes in the patterns of activation of excitatory cells and subpopulations of inhibitory cells (Figure 2) that may involve modulatory control via Ach and 5HT pathways, as well as noradrenergic inputs (NA) from locus coeruleus (Cohen et al., 1997).

POTENTIAL FOCUS OF AN EXPERIMENTAL PROGRAM

We consider competition among vasoactive pathway that predominantly effect parenchymal microvessels. We hypothesize that microvascular tone may be defined as a function of the local concentration of specific signaling molecules, such as EET, 20-HETE, NO, NPY, PG_E , SOM, and VIP. The driving term is neuronal activity, which may be a one-to-one function of the neuronal stimulus for primary sensory areas. Formally, we seek a relation of the form

Smooth muscle tension

$$\equiv f \left(\begin{array}{l} \text{SOM}^+ \text{ inhibitory interneuron activation} \\ \text{VIP}^+ \text{ inhibitory interneuron activation} \\ \vdots \\ \text{Astrocyte activation} = f(\text{excitatory cell activation}) \\ \text{Extracortical and endothelial contributions} \\ [\text{O}_2]_{\text{tissue}} \end{array} \right) \quad (1)$$

and

$$\text{Vascular output} \equiv f \left(\begin{array}{l} \text{Smooth muscle tension} \\ \vdots \\ \text{Vascular architecture} \end{array} \right) \quad (2)$$

where the functionalities, denoted f , are yet to be determined. Nonetheless, this general formalism emphasizes the need to study muscle tension on a cell type-by-type or transmitter-by-transmitter basis. It further reinforces the need to map the geometry of the vasculature (Figure 1) and the location of neuronal control regions (Figure 2).

The greatest uncertainty in the hypothesis that the control of cortical blood flow depends on the balance of neurotransmitters that lead to vasoconstriction versus dilation is that much of our understanding of signaling comes from experiments with brain slice preparations. Not only is vascular pressurization absent, but responses take place on tens of seconds (Cauli et al., 2004; Gordon et al., 2008), while the vasculature responds to changes in neuronal activity on the 0.5-s time-scale *in vivo*. It remains an open issue if signaling is faster *in vivo*, although such long times are observed when astrocytic Ca^{2+} levels are directly excited *in vivo* (Wang et al., 2006; Figure 3).

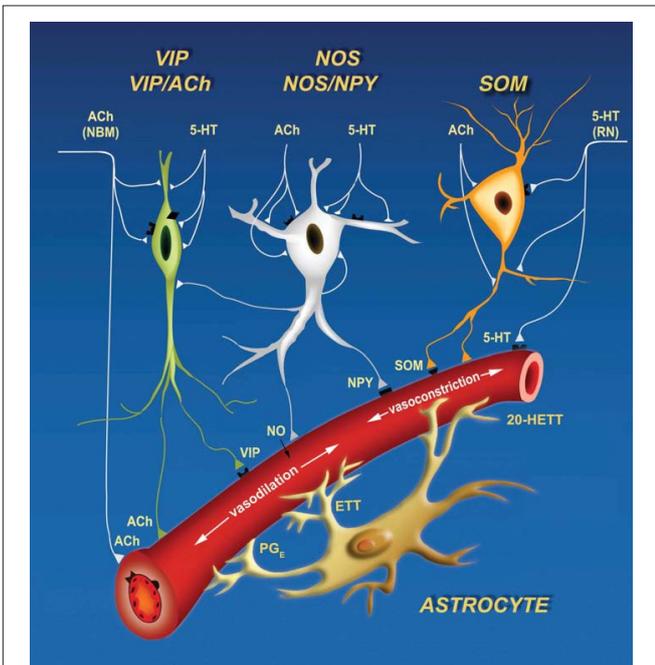


FIGURE 2 | Cartoon of three signaling pathways, two local and one global, that can both constrict and dilate the arteriole vasculature. Inhibitory interneurons can drive dilation via nitric oxide (NO), catalyzed by nitric oxide synthetase (NOS), and vasoactive intestinal peptide (VIP), and drive constriction via somatostatin (SOM) and neuropeptide Y (NPY). Astrocytes can dilate via protoglandin E (PG_E) and epoxyeicosatrienoic acid (EET) and constrict via 20-Hydroxyeicosatetraenoic acid (20-HETE). Lastly, extrinsic input of acetylcholine (ACh) will dilate while serotonin (5HT) will constrict. Derived from Cauli et al. (2004).

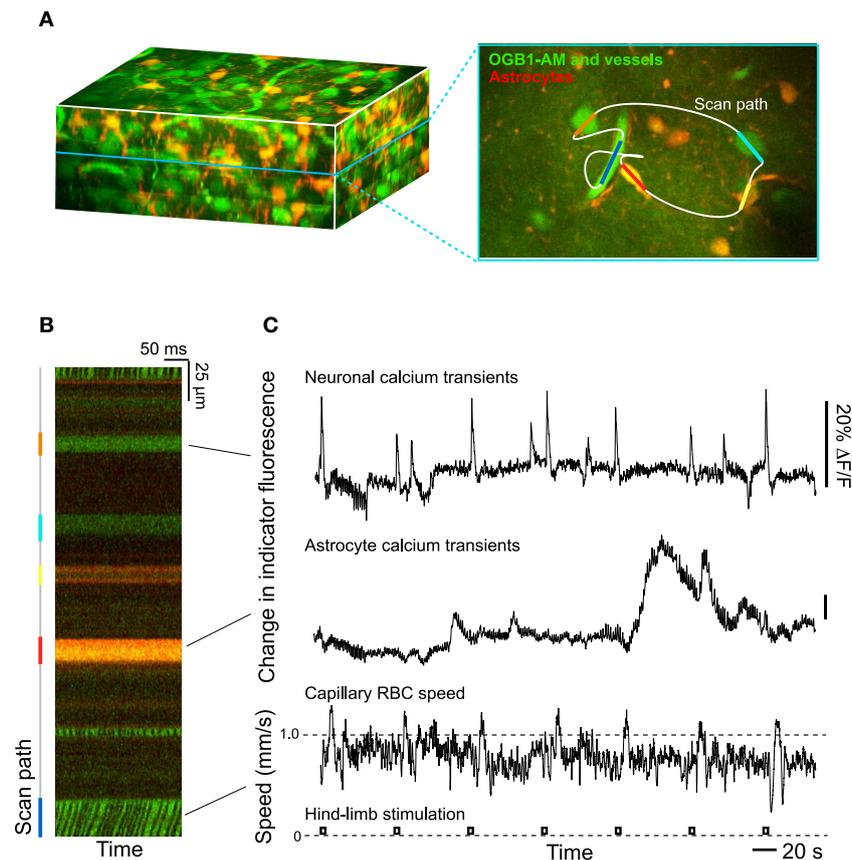


FIGURE 3 | Two-photon laser scanning microscopy with arbitrary scan pathways to record from functionally labeled cells and vessels.

(A) Neurons and glia are labeled with the Ca^{2+} reporter Oregon Green BAPTA-1 AM and glia have sulforhodamine 101 as an additional label. Blood plasma is stained with fluorescein so that blood cell

motion is measured. (B) Intensity along the scan path records Ca^{2+} signals and blood cell movement. (C) Traces of functional changes in intracellular Ca^{2+} and blood flow; note flow changes concurrent with stimulation. Algorithms from Helmchen and Kleinfeld (2008), Drew et al. (2010), Driscoll et al. (2011).

A first approach to define the inputs and output to a model of neurovascular coupling is to measure the activity of identified cell types concurrent with blood flow in a neighboring microvessel. The activity of neurons and glia in cortex may be established by measuring their internal calcium levels with optical contrast agents (Garaschuk et al., 2006; **Figure 3**) and *in vivo* two-photon laser scanning microscopy (TPLSM; Svoboda et al., 1997; **Figure 3**). Similarly, both the speed of blood cells and vascular tone may be concurrently established with two-photon microscopy (Helmchen and Kleinfeld, 2008; **Figure 3**). The clarity of these measurements depends on the ability to record from specific cell types and the specificity of co-release of different neuropeptides by interneurons. This implies the need to use transgenic animals that either express a functional indicator in specific cell types or, as a more general approach, to use mice that express Cre recombinase in specific cell types (Luo et al., 2008; Bernard et al., 2009; **Table 1**). Cre recombinase drives the labeling of these cell types by crossing these mouse lines with fluorescent reporter mice (Madisen et al., 2010), in which a member of the fluorescent protein (FP) family (Tsien, 1998; Shaner et al., 2004) and/or a genetically encoded functional indicator is coded between lox-sites (Wallace et al., 2008; **Table 2**); Cre recombinase activates sequences between these sites (Mallo,

2006). A complementary and common method is to infect cells that express Cre recombinase with a virus whose genetic material is modified to code a fluorescent indicator between lox-sites (**Table 2**). As a technical issue, a construct with double lox-sites improves specificity of Cre recombinase targeting strategies (Atasoy et al., 2008).

A second, albeit related approach is to manipulate the output of the neurons and astrocytes that release signaling factors and measure the volume concentration of signaling molecules, particularly under conditions of changing vascular dilation versus constriction. The activity of neurons and glia may be manipulated with photoexcitation of caged molecules, with ectopic expression of chemical receptors modified to have unnatural affinities to a specific drug (Alexander et al., 2009), with ectopic expression of receptors with native binding sites (Arenkiel et al., 2008), and with optogenetic agents that are targeted to specific cell types (Cardin et al., 2009, 2010; Sohal et al., 2009). The latter strategy includes the use of channelrhodopsin (ChR2) to depolarize cells (Boyden et al., 2005; Arenkiel et al., 2007; Gradinaru et al., 2009) and halorhodopsin (NpHR; Zhang et al., 2007) or archaerhodopsin-3 (Arch; Chow et al., 2010) to inactivate cells. Other agents act directly on specific G-protein coupled pathways (Airan et al., 2009). All of these agents are typically delivered

by viral injection in combination with Cre recombinase labeled animals, as described above (**Tables 1 and 2**). A complementary strategy is the use of short hairpin RNA (shRNAs) to silence specific cell signaling pathways (**Table 3**).

The presence of multiple sources for the different signaling molecules suggests the additional need to measure receptor activation by these molecules directly. Receptor activation via the volume

conduction of vasoactive signaling molecules may be observed with new cell-based indicators, CNiFERS, that can be made sensitive to any molecule that has a G-coupled protein receptor (**Figures 4A,B**). In a first realization with ACh sensing as the task, HEK cells were transfected with the muscarinic (M1) G_q -protein receptor along with the $[Ca^{2+}]_{int}$ reporter TN-XXL (Heim and Griesbeck, 2004). These show a strong response to the release of endogenous ACh

Table 1 | Mice that express fluorescent proteins or Cre recombinase in cell-types relevant to neurovascular regulation.

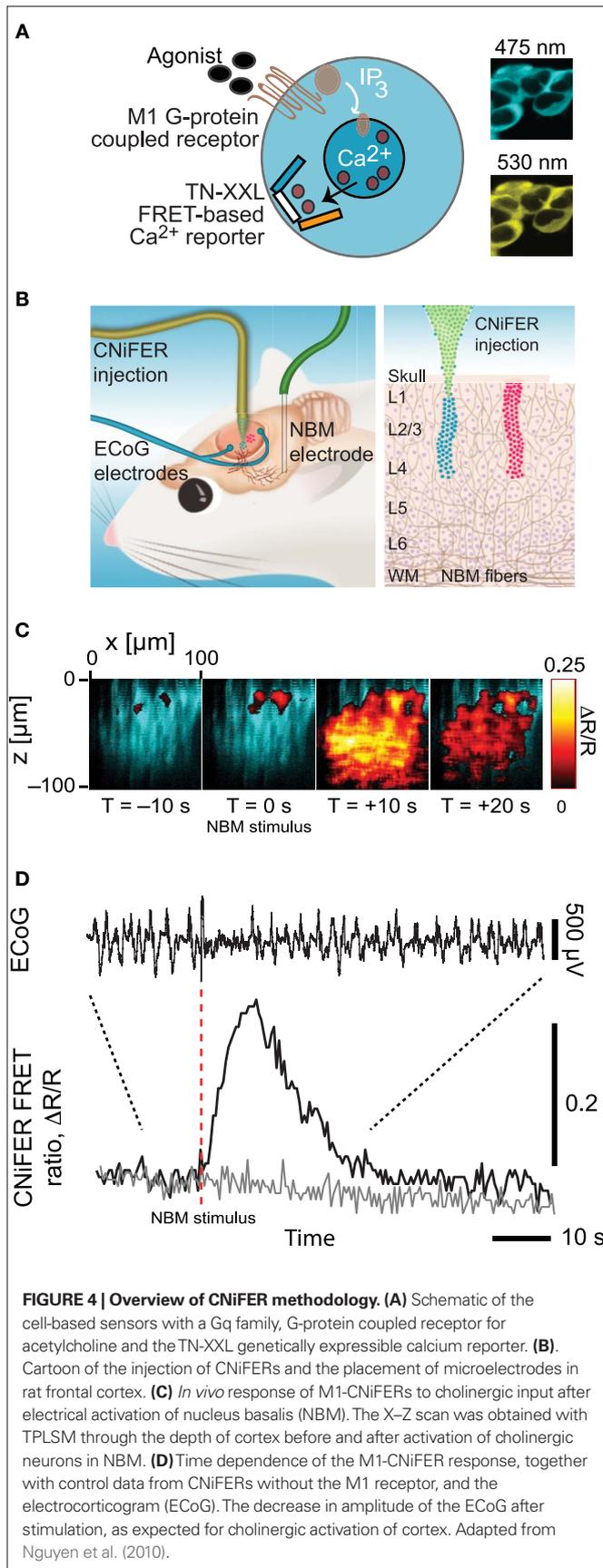
Promotor	Expression	Goal	Jax number	References
hGFAP	eGFP	Visualization of cortical astrocytes		Nolte et al. (2001)
GAD67	GFP	Visualization of parvalbumin, calretinin, or somatostatin-positive interneurons	003718, 007677, 006334	Oliva Jr. et al. (2000), Tamamaki et al. (2003), Chattopadhyaya et al. (2004), Ma et al. (2006)
Camk2a	tdTomato (a red FP)	Visualization of cortical excitatory neurons		Madisen et al. (2010)
Camk2a	Cre recombinase	Gene targeting to cortical excitatory neurons	005359	Tsien et al. (1996)
hGFAP	Cre recombinase	Gene targeting to cortical astrocytes	004600	Brenner et al. (1994), Zhuo et al. (2001)
Parvalbumin	Cre recombinase	Gene targeting of parvalbumin-positive cortical interneurons	008069	Wulff et al. (2008)
Smooth muscle myosin heavy chain	Cre recombinase and GFP	Gene targeting and visualization of pial vascular smooth muscle	007742	Xin et al. (2002a,b)

Table 2 | Viral expression cassettes for *in vivo* manipulation and detection of astrocyte and interneuronal signaling.

Protein expressed	Goal	Targeting strategies	References
Channelrhodopsin 2	Light-mediated cell depolarization		Arenkiel et al. (2007), Gradinaru et al. (2009)
Halorhodopsin	Light-mediated cell hyperpolarization	(i) Cre-lox	Zhang et al. (2007), Chow et al. (2010)
Archaerhodopsin-3		(ii) Cell type – specific promoters	
hM3Dq (G-coupled protein receptor for cell activation)	Drug-based (clozapine-N-oxide) regulation of activity	(iii) Cell type – trophic viruses	Alexander et al. (2009)
TRPV1	Ligand-based (capsaicin) regulation of activity		Arenkiel et al. (2008)
Tn-XXL or GCaMP3 (Fluorescent protein based Ca^{2+} sensors)	Cell-specific detection of intracellular calcium increases		Mank et al. (2008), Tian et al. (2009)

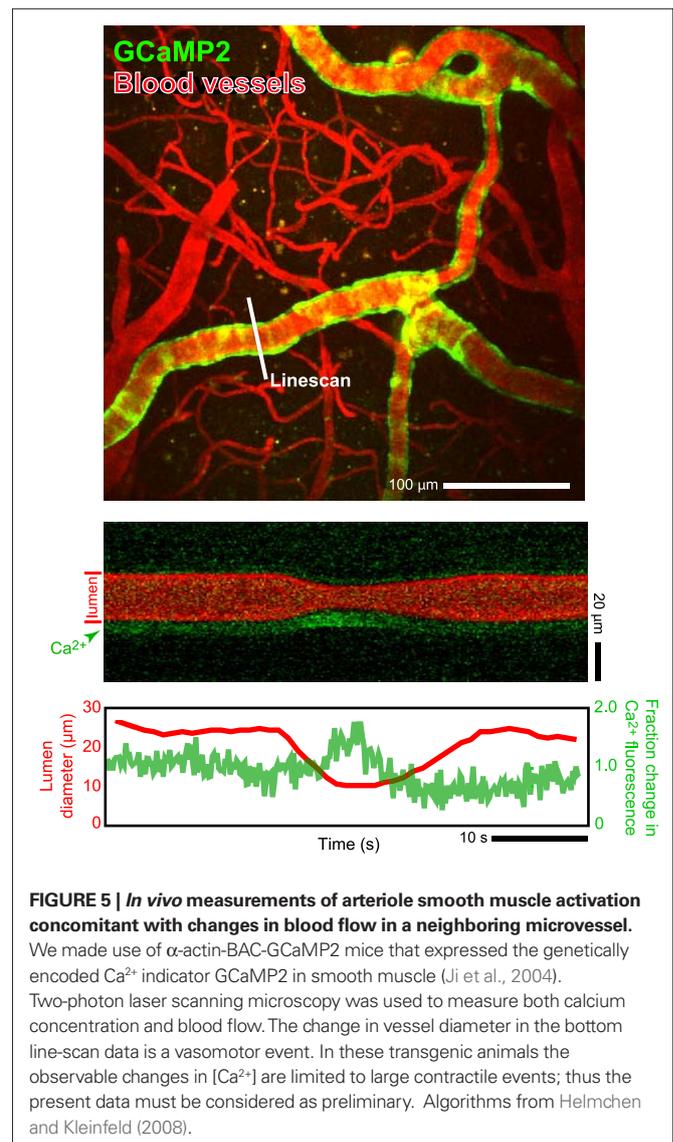
Table 3 | Viral expression cassettes for *in vivo* genetic manipulation of astrocyte and interneuronal signaling.

shRNA target	Pathway	Targeting strategies	References
mGluR	Astrocyte glutamate signaling pathway		Zonta et al. (2003)
phospholipase A_2 , cyclooxygenase-1, or cytochrome p450	Astrocyte arachidonic acid metabolism		Zonta et al. (2003), Mulligan and MacVicar (2004)
Ca^{2+} sensitive large conductance BK channels	Astrocytic potassium release		Filosa et al. (2006), Weaver et al. (2006), Girouard et al. (2010)
VIP or vascular receptor VPAC1	Interneuron VIP release	Cre-lox	Fahrenkrug et al. (2000), Cauli et al. (2004)
NOS	Interneuron NO release		Cauli et al. (2004), Enager et al. (2008)
SOM or vascular receptor SSTR	Interneuron SOM release		Cauli et al. (2004), Enager et al. (2008), Kocharyan et al. (2008)
NPY or vascular receptor NPY-Y1	Interneuron NPY release		Cauli et al. (2004)



(Figures 4C,D). CNiFER technology may be extended, by the use of $G_{i/o}$ - and G_s -protein receptors and chimeric $G\alpha$ -q proteins (Coward et al., 1999), to sense neuropeptides such as VIP and SOM.

In toto, the combination of many *in vivo* tools, including electrophysiology, *in vivo* two-photon imaging, intracellular ion measurements (Figure 3), neurotransmitter receptor activation measurements (Figure 4), microdialysis measurements, single vessel blood flow measurements (Figure 3) as well as direct measurement of the activation of smooth muscle (Figure 5), and measurement of tissue oxygen levels (Sakadzic et al., 2010), can allow one to assess the influence of single neurons and networks of neurons on vascular control. These can be further supported by optical and chemical activation of specific cell types, as illustrated by the optical activation of astrocytes to increase blood flow in neighboring capillaries (Figure 6) in a manner similar to that seen with caged compounds (Takano et al., 2006). Lastly, automated reconstruction techniques allow one to map local architectonics (Figure 1). In all such endeavors, it important to realize that one may conceive new



tools as they are required. One anticipated need is a calcium-clamp to control smooth muscle tension, possibly realized with caged molecules and optical indicators, as a means to fix the flow in a region and observe changes in signaling molecule concentration.

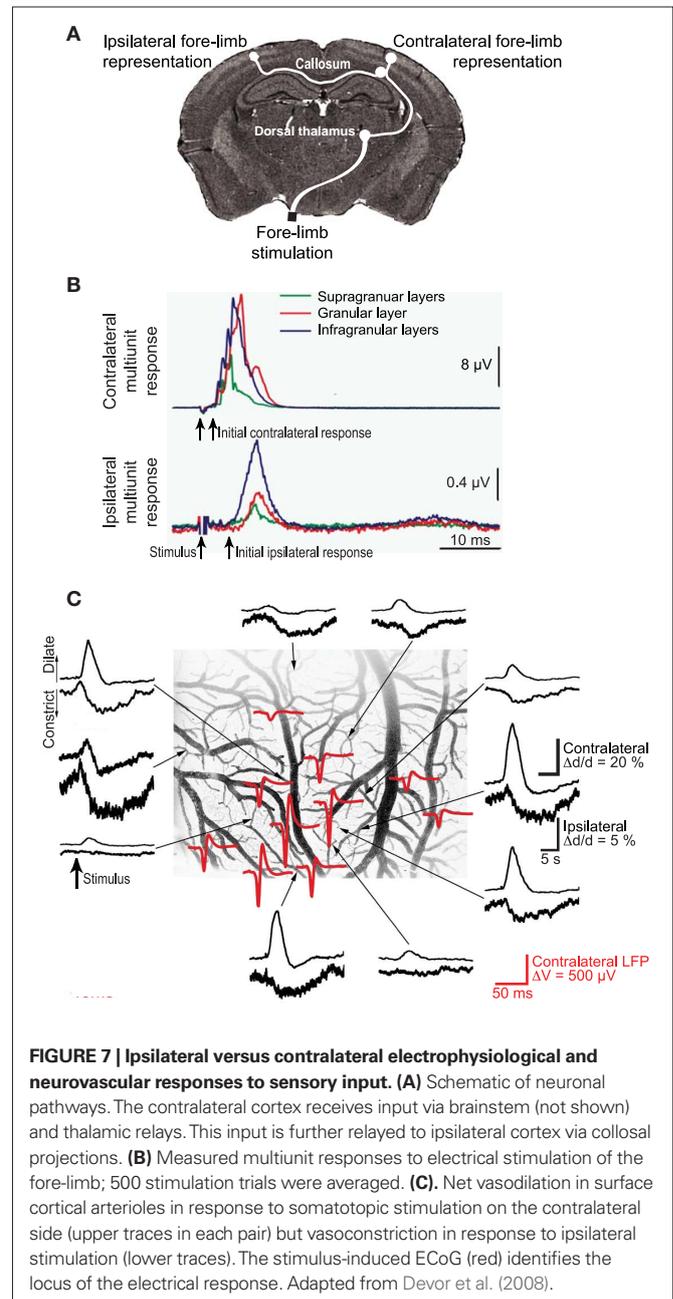
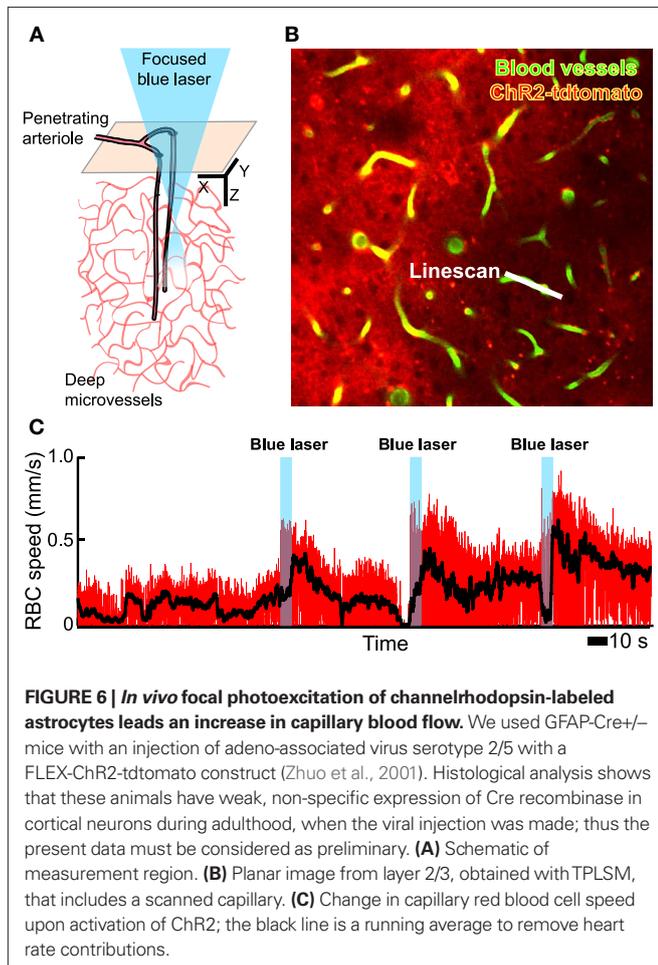
EXAMPLE PROPOSED EXPERIMENT

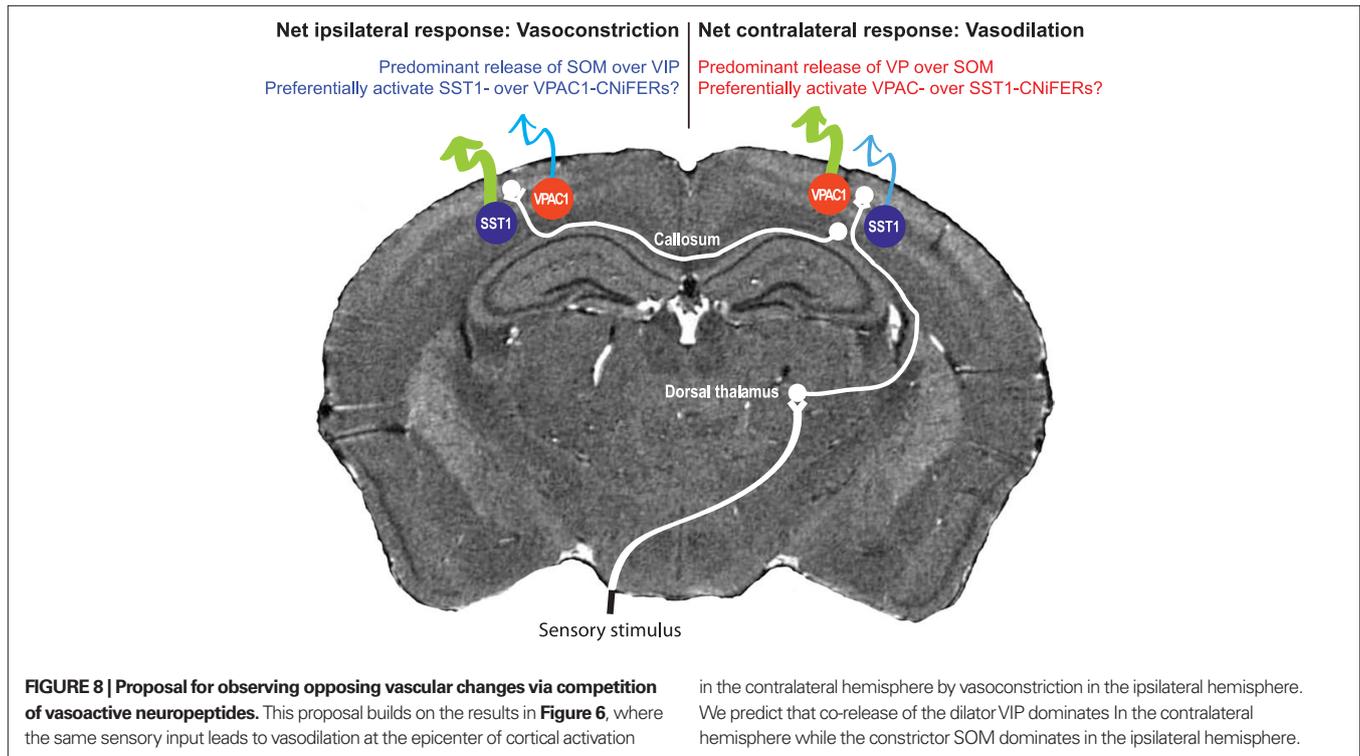
Somatosensory input leads to neuronal activation in ipsilateral parietal cortex as well as contralateral cortex, albeit with a reduced amplitude (Figures 7A,B). Unexpectedly, contralateral sensory input leads to net dilation of vessels, while ipsilateral input has the paradoxical effect of net constriction (Devor et al., 2008; Figure 7). One possible explanation is that contralateral input leads to rapid release of VIP while ipsilateral input preferentially excites SOM+ interneurons more slowly. One can make use of microdialysis, or for greater spatial localization an extension of CNiFER technology to form VPAC1-CNiFERs to detect vasoactive intestinal peptide and SST1-CNiFERs to detect somatostatin. We predict that the ratio of VPAC1-CNiFER to SST1-CNiFER signals will be greater for contralateral versus ipsilateral stimulation (Figure 8).

PARADIGM-SHIFT TO CONSIDERING NEUROVASCULAR COUPLING IN TERMS OF SIGNALING MOLECULES

The conventional view is that neuronal spiking leads to an increase in local metabolism, and that metabolism leads to increased oxygen extraction from the blood as well as an increase in blood speed (Fox and Raichle, 1986; Leybaert, 2005). Thus neuronal activity is

mirrored by changes in blood oxygenation. This paradigm is the accepted view for the interpretation of BOLD fMRI (Logothetis et al., 2001). But this causal relation breaks down, as shown by a multiplicity of recent experiments (Metea and Newman, 2006; Devor et al., 2008; Gordon et al., 2008; Sirotin and Das, 2008; Girouard et al., 2010; Lindauer et al., 2010; Figure 7C). We envision a model of neurovascular control that maps the activity of different neuronal subtypes to changes in vascular tone (Eqs 1 and 2). In our hypothesized paradigm, neuronal activity forms the inputs, the dynamics of the underlying signaling molecules form the internal state variables, and the vascular tone is the output state variable. This is not unlike the case of neuronal dynamics, where the input is derived from synaptic activity, the opening probabilities of channels form the internal state variables, and voltage is the output.





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