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A close look at brain dynamics: Cells and vessels seen by *in vivo* two-photon microscopy

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ABSTRACT

The cerebral vasculature has a unique role in providing a constant supply of oxygen and nutrients to ensure normal brain functions. Blood vessels that feed the brain are far from being simply channels for passive transportation of fluids. They form complex structures made up of different cell types. These structures regulate blood supply, local concentrations of O_2 and CO_2, transport of small molecules, trafficking of plasma cells and fine cerebral functions in normal and diseased brains. Until few years ago, analysis of these functions has been typically based on *post mortem* techniques, whose interpretation is limited by the need for tissue processing at specific times. For a reliable and effective picture of the dynamic processes in the central nervous system, real-time information *in vivo* is required. There are now few *in vivo* systems, among which two-photon microscopy (2-PM) is a truly innovative tool for studying the brain. 2-PM has been used to dissect specific aspects of vascular and immune cell dynamics in the context of neurological diseases, providing exciting results that could not have been obtained with conventional methods. This review summarizes the latest findings on vascular and immune system action in the brain, with particular focus on the dynamic responses after ischemic brain injury. 2-PM has helped define the hierarchical architecture of the brain vasculature, the dynamic interaction between the vasculature and immune cells recruited to lesion sites, the effects of blood flow on neuronal and microglial activity and the ability of cells of the neurovascular unit to regulate blood flow.

Contents

Abbreviations: 2-PM, two-photon microscopy; APC, antigen presenting cell; BBB, blood–brain barrier; ChABC, bacterial enzyme chondroitinase ABC; CNS, central nervous system; EAE, experimental autoimmune encephalopathy; EATs, erythrocyte-associated transients; ECM, extracellular matrix; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; IFNγ, interferon-γ; IL, interleukin; LCMV, lymphocytic-choriomeningitis virus; LPS, lipopolysaccharide; MCAo, middle cerebral artery occlusion; MMP, matrix metalloproteinases; MRI, magnetic resonance imaging; NFAT, nuclear factor of activated T-cells; PCR, polymerase chain reaction; RBC, red blood cell; ROS, reactive oxygen species; SNR, signal to noise ratio; TGFβ, tumor growth factorβ.

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1. Introduction

The cerebral vasculature has a unique role in providing a constant supply of oxygen and nutrients to the central nervous system (CNS) to ensure normal brain functions. Blood vessels that feed the brain are not only channels for passive transportation of fluids, but are complex structures made of a number of different cell types (Abbott, 2002; Lok et al., 2007; Nedergaard et al., 2003). These structures regulate blood supply, local concentrations of O_2 and CO_2, transportation of small molecules, trafficking of plasma cells and fine cerebral functions in both normal and diseased brain (Begley and Brightman, 2003; Konsman et al., 2007). The cerebrovascular structure is generally referred to as the blood–brain barrier (BBB), and forms a physical barrier between the brain parenchyma and systemic blood circulation (Begley and Brightman, 2003; Konsman et al., 2007). The main components of the BBB are endothelial cells, astrocytes, pericytes, perivascular macrophages and microglia, all closely linked to neurons (Berezowski et al., 2004; Ramsauer et al., 2002; Schiera et al., 2003; Zenker et al., 2003). This structure as a whole is defined as the neurovascular unit.

In the case of a cerebral pathology, the neurovascular unit responds with local redistribution of the blood flow, changes in the concentrations of blood gases, or increased permeability to specific molecules (Konsman et al., 2007). Other events happen at the endothelial–brain interface and are pathological hallmarks of several CNS conditions, often involving activation of the immune response. The brain immune response can develop with the direct contribution of the neurovascular unit. Mannose-binding lectin deposition on endothelial cells has been described in brain ischemia and has deleterious effects on account of activation of the complement system cascade (Gesuete et al., 2009; Orsini et al., 2012). Moreover, in many diseased states, inflammatory cells from the periphery are recruited to the brain, and their entry into the brain parenchyma is strictly regulated by the neurovascular unit (Davoust et al., 2008; Scholz et al., 2007).

The neurovascular unit is thus involved in metabolic and inflammatory pathways, being important in generating and sustaining many pathophysiological cascades that precede or follow a diseased condition of the brain. The inflammatory cascade, in particular, offers a potential, promising therapeutic target, with its role in injury progression in most – virtually all – CNS diseases and arises early in the pathology, with long-lasting effects (Magnus et al., 2012; Dirnagl et al., 1999).

The interplay between the immune system and blood vasculature seems fundamental in the pathobiology of CNS diseases, and has stimulated increasing interest in recent years. The dynamic nature of cerebrovascular events and immune cell trafficking, as they evolve over time, requires adequate analysis methods. To date, our knowledge of vascular and immune responses in the diseased brain has been typically based on *post mortem* techniques, *i.e.* immunohistochemistry and flow cytometry (Gelderblom et al., 2009). The interpretation of these data is limited by the need for tissue processing at specific time points, but, to get a reliable and effective picture of the dynamic processes involved in vascular and immune events, we need real-time information *in vivo*. Various approaches are available for *in vivo* imaging, and two-photon microscopy (2-PM) offers a major qualitative advance. This technique allows high-resolution visualization of individual blood vessels and cells and maps of cellular connectivity within a three-dimensional space (Oh et al., 2014; Ortolano et al., 2010; Svoboda and Yasuda, 2006), overcoming the main limitations in spatial resolution and three-dimensional acquisition associated with other *in vivo* imaging approaches (*e.g.* MRI, intravital microscopy). Furthermore, 2-PM can provide time-lapse recordings of dynamic events, with timescales range from tens of millimeters per second (blood flow) to days (modification of vascular geometry during development, Fig. 1). The frame acquisition rate must therefore be set appropriately (Table 1).

2-PM has been successfully used to dissect specific aspects of vascular and immune cell dynamics in neurological diseases (Cordiglieri et al., 2010; Fumagalli et al., 2011; Schaffer et al., 2006; Shih et al., 2012) yielding results that could not be obtained with standard methods. This review gathers the latest 2-PM findings on vascular and immune system action in the brain, contributing to the picture of their dynamic interactions.

2. Blood vessel dynamics by 2-PM

Blood flow in the brain has been typically studied and measured *in vivo* using approaches such as laser Doppler flowmetry or MRI (Nakase et al., 1997). These instruments focus on an area measuring hundreds of microns, yielding a mean value obtained from the blood vessels pertinent to that area.

MRI allows the acquisition of three-dimensional images which can be processed to yield structural information, including gross vascular architecture, tissue perfusion, BBB integrity, onset of hemorrhage and immune cell infiltration (Klohs et al., 2014). To image vascular architecture and measure perfusion, MRI exploits the different relaxation times between tissue and blood in response to the application of a magnetic field pulse. This is briefly turned on so to align proton spins. As the pulse is turned off, the tissue recovers its initial proton alignment, while the intravascular bed is replenished with fresh proton spins due to blood flowing, allowing to distinguish tissue *vs.* blood signals. Poor perfusion results in delayed fresh spin replenishment and decrease in oxygen content, allowing to detect disease-related perfusion impairments (Santosh et al., 2008). Blood-associated signal can be enhanced using contrast agents that can be exploited to study the occurrence of hemorrhage (Strbian et al., 2008). Due to their non-invasive nature, imaging solutions based on MRI are readily translatable to clinical use. However, MRI *in vivo* cannot provide sufficient spatial resolution to proper study small vessels, individual vascular events or interactions between cell and vessels. Moreover, MRI *in vivo* is limited by the long delays between sequential images, hampering the acquisition of very fast dynamic events (Cho et al., 2011). With the introduction of 2-PM, the possibility of imaging at high spatial resolution and acquisition rate meant that single vessels could be visualized to get parameters associated with vessel architecture (diameter, length, number of