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Review Article

Anesthesia and the quantitative evaluation of neurovascular coupling

Kazuto Masamoto[1,2] and Iwao Kanno[2]

[1]*Center for Frontier Science and Engineering, University of Electro-Communications, Tokyo, Japan;*
[2]*Molecular Imaging Center, National Institute of Radiological Sciences, Chiba, Japan*

Anesthesia has broad actions that include changing neuronal excitability, vascular reactivity, and other baseline physiologies and eventually modifies the neurovascular coupling relationship. Here, we review the effects of anesthesia on the spatial propagation, temporal dynamics, and quantitative relationship between the neural and vascular responses to cortical stimulation. Previous studies have shown that the onset latency of evoked cerebral blood flow (CBF) changes is relatively consistent across anesthesia conditions compared with variations in the time-to-peak. This finding indicates that the mechanism of vasodilation onset is less dependent on anesthesia interference, while vasodilation dynamics are subject to this interference. The quantitative coupling relationship is largely influenced by the type and dosage of anesthesia, including the actions on neural processing, vasoactive signal transmission, and vascular reactivity. The effects of anesthesia on the spatial gap between the neural and vascular response regions are not fully understood and require further attention to elucidate the mechanism of vascular control of CBF supply to the underlying focal and surrounding neural activity. The in-depth understanding of the anesthesia actions on neurovascular elements allows for better decision-making regarding the anesthetics used in specific models for neurovascular experiments and may also help elucidate the signal source issues in hemodynamic-based neuroimaging techniques.
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Introduction

Neurovascular coupling consists of three brain cell types: neurons, supporting cells (astrocytes), and vascular cells (vascular smooth muscle, pericyte, and endothelial cells). These cells can be grouped into three conceptual components: neurons, the message senders associated with information processing; supporting cells, the potential transmission sites that mediate vasoactive signals in response to the neuronally derived messages; and vascular cells, the recipients of the signals. After evoked neural activation, vasoactive signals are transmitted directly and indirectly via supporting cells to the vascular cells, which cause redistribution of the local cerebral blood flow (CBF). The hypothetical view of the neurovascular coupling relationship is schematically shown in Figure 1. Great effort has been invested in elucidating the spatiotemporal dynamic functions of neurovascular coupling (Figure 1A), quantitative coupling relationships (Figure 1B), and the mechanisms underlying signal transmission using *in-vivo* animal models (for reviews, see Kleinfeld *et al*, 2011; Attwell *et al*, 2010; Iadecola, 2004; and Lauritzen, 2001).

The anesthesia that has been widely used for studying neurovascular coupling in *in-vivo* animal models has broad action on brain cells that include changes in neural processing, vascular reactivity, and other baseline states (e.g., spontaneous neural activity, cerebral energy metabolism, and baseline CBF). These modulatory effects eventually modify the coupling relationship between neural and vascular responses, and thus, anesthesia is a potential confounder that interferes with the neurovascular coupling relationship. In this article, we review the effects of anesthesia (e.g., anesthetic agents and

Correspondence: Dr K Masamoto, Center for Frontier Science and Engineering, University of Electro-Communications, 1-5-1 Chofugaoka, Chofu, Tokyo 182-8585, Japan.
E-mail: masamoto@mce.uec.ac.jp
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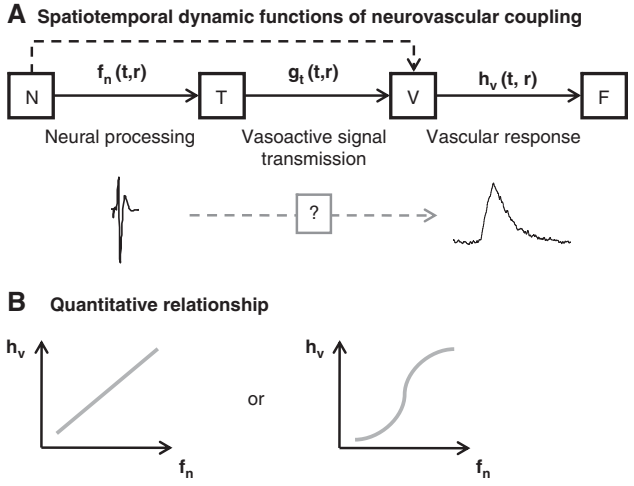


Figure 1 Compartmentalized neurovascular coupling relationship. Neurovascular cells are grouped into three compartments: neurons (N), supporting cells that are potential transmission sites of vasoactive signals (T), and vascular cells (V). The vasoactive signals that are released accompanying neural processing are transferred to the vascular cells directly or indirectly via the supporting cells and help coordinate local cerebral blood flow (CBF) (F). A sequential relationship is depicted (**A**). Each transfer function represents cortical neural processing (f_n), vasoactive signal transmission (g_t), and vascular reactivity (h_v), which are functions of time (t) and space (r). Hemodynamic response, a final output of neurovascular function, is expressed by the convolution of these transfer functions. (**B**) Linear and nonlinear relationships have been observed between neural (f_n) and vascular (h_v) signal changes. However, their relationships to transmission function (g_t) remain uncertain.

depths) on the (1) spatial coordination, (2) temporal dynamics, and (3) quantitative relationships between the neural and vascular responses. To help understand the underlying causes of these anesthesia effects, this review aims to provide comprehensive lists of the actions of anesthesia on (1) general physiology, (2) neurons, (3) supporting cells (vasoactive signal transmission), and (4) vascular cells. This may also aid decision-making regarding anesthetics for specific studies of neurovascular coupling in *in-vivo* animal models.

Why Is Anesthesia Needed?

The need for anesthesia depends on the methodology applied in neurovascular coupling studies (for reviews, see Vanzetta and Grinvald, 2008 and Villringer and Dirnagl, 1995). The electrophysiological techniques that have been used to represent neural activity as a quantitative index to compare vascular responses involves local field potentials (LFPs), and single unit and multiunit spiking activity (Lauritzen, 2001; Logothetis *et al*, 2001). Laminar electrodes have also been used to investigate neurovascular coupling with current source density analysis (Martindale *et al*, 2003). These electrophysiological techniques must fix the electrode(s) at certain locations within the brain, and thus, anesthesia has been used to minimize movement artifacts and eliminate the induced stress to the animals. For anesthesia-free neural recording, telemetry systems and linear arrays of microelectrodes have been introduced in behaving small animals (Schregardus *et al*, 2006) and humans (Keller *et al*, 2009). Electroencephalogram and magnetoencephalogram also provide less-invasive neural measurements and allow concurrent recording with cortical hemodynamics in anesthetized animals (Franceschini *et al*, 2008) and in conscious humans (Rosengarten and Kaps, 2010; Ou *et al*, 2009).

To quantify vascular responses, early studies were conducted with a locally generated hydrogen clearance method with microelectrodes to measure dynamic CBF changes elicited by cortical stimulation (Leniger-Follert and Hossmann, 1979), but this technique is invasive and may disturb cortical microcirculation in the vicinity of the measurement regions. An alternative, less-invasive method, laser-Doppler flowmetry, was introduced to monitor dynamic CBF (Dirnagl *et al*, 1989). This technique has become widely used to examine the temporal and quantitative neurovascular relationship in the anesthetized rodents (Matsuura *et al*, 2000; Matsuura and Kanno, 2001; Ances *et al*, 2000; Ngai *et al*, 1999; Akgoren *et al*, 1996). Some groups have shown that laser-Doppler flowmetry can also be used to monitor longitudinal changes of CBF repeatedly in awake behaving rodents (Takuwa *et al*, 2011; Gu *et al*, 2003). Using either scanning laser-Doppler flowmetry or laser speckle flowmetry, two-dimensional maps of evoked CBF have also been reported in both anesthetized (Du and Pan, 2011; Kannurpatti and Biswal, 2006; Ayata *et al*, 2004; Weber *et al*, 2004) and unanesthetized rodents (Takuwa *et al*, 2011). Optical coherence tomography and functional magnetic resonance imaging (fMRI) techniques provide further layer-specific hemodynamic mapping in anesthetized animal cortex (Chen *et al*, 2009; Jin and Kim, 2008; Maheswari *et al*, 2003; Silva and Koretsky, 2002). These neuroimaging techniques are noninvasive, and measurements can therefore also be acquired in awake conditions with the administration of a light sedative or paralyzing agent (Sicard *et al*, 2003; Peeters *et al*, 2001; Lahti *et al*, 1998). However, caution should be exercised in interpreting the data measured in waking conditions because the obtained signals might be contaminated by restraint-related stress and discomfort or enhanced arousal due to recording noises and motion artifacts (Lahti *et al*, 1998, 1999).

Optical intrinsic signal (OIS) imaging techniques, including near infrared spectroscopy (Fuster *et al*, 2005; Obrig and Villringer, 2003) and diffuse optical imaging (Franceschini *et al*, 2008), also allow for mapping the evoked hemodynamic changes (e.g., blood oxygenation and volume) under both anesthetized (Devor *et al*, 2005; Jones *et al*, 2001) and unanesthetized conditions (Berwick *et al*, 2002;

Martin *et al*, 2002). For those techniques, waking animal experiments that require the animal to be trained to tolerate restraint in a holder and experimental set-up have been established (Berwick *et al*, 2002; Martin *et al*, 2002). Compared with other imaging methods, one major advantage that optical imaging techniques offer in neurovascular coupling studies is that they allow for imaging of neural activity on a mesoscopic scale similar to that of the hemodynamic imaging accomplished by measuring activity-induced changes in membrane potentials (Obrenovitch *et al*, 2009; Takashima *et al*, 2001; Ebner and Chen, 1995), intracellular pH (Sun *et al*, 2011), calcium ion concentrations (Homma *et al*, 2009) combined with exogenous fluorescent compounds, and endogenous fluorescent changes arising from activity-dependent cellular autofluorescence (Reinert *et al*, 2007; Shibuki *et al*, 2003). These techniques enable us to directly examine the spatial gaps between neural and vascular response regions (Weber *et al*, 2004). More directly, the *in-vivo* microscopic morphology and function of neurons, astrocytes, pericytes, and capillaries are resolved at the single-cell level with laser scanning fluorescent microscopic techniques, including confocal (Seylaz *et al*, 1999; Villringer *et al*, 1994) and two-photon excitation fluorescence microscopy (Fernández-Klett *et al*, 2010; Göbel and Helmchen, 2007; Takano *et al*, 2006; Chaigneau *et al*, 2003; Kleinfeld *et al*, 1998). In these techniques, image quality is sensitive to animal vibration, and thus, imaging is preferably conducted under anesthesia. A recent study showed that, in two-photon microscopic experiments, the image distortion was minimized to 2 to 5 μm in waking conditions by fixing animal's head to the stage and allowing the animal to move freely on a floating ball (Dombeck *et al*, 2007). This report indicates the feasible image resolution for examining neurovascular coupling in awake animals. Miniaturized two-photon microscopy has also been shown to capture images of cortical cells and capillary blood flow under unanesthetized conditions (Helmchen, 2002), which could be useful in future studies of cellular-scale neurovascular coupling in freely behaving animals.

Why Do We Need to Care About Anesthesia?

Different anesthetics have different action sites, which can potentially cause discrepancies in interpreting the mechanism of neurovascular coupling due to animal experiments conducted with different anesthetics. It was shown that intravenous infusion of cocaine in anesthetized rats provoked an increase in CBF under α-chloralose (25 mg/kg per hour), but the same experiments conducted under isoflurane (1.8% to 2.0%) showed decreases in CBF (Du *et al*, 2009). The cerebrovascular response to ethanol was shown to cause vasoconstriction under α-chloralose/urethane anesthesia (50/600 mg/kg) but vasodilation under halothane (0.5% to 1.0%) (Gordon *et al*, 1995). Furthermore, the role of the nitric oxide pathway in controlling CBF response to sensory stimulation was shown to be dominant in anesthetized rats (urethane, Gerrits *et al*, 2001; α-chloralose 40 mg/kg per hour, Nakao *et al*, 2001). However, when examined in awake rats, this nitric oxide pathway does not have a major role (Nakao *et al*, 2001). These findings indicate that particular anesthetics critically interfere with the pathway of neurovascular coupling.

Another issue concerning the use of anesthesia in neurovascular coupling experiments is that anesthesia profoundly affects the stability and reproducibility of neurovascular imaging. Austin *et al* (2005) showed that fMRI blood oxygen level-dependent responses to sensory stimulation varied over 6 hours of measurements in rats under α-chloralose (10 to 30 mg/kg per hour), accompanied with the varying electroencephalogram. However, in rats under urethane anesthesia (1.1 g/kg), electroencephalogram activity was shown to be stable for a prolonged time (8 to 12 hours) (Lincoln, 1969). To maintain steady electroencephalogram and fMRI responses with rats receiving medetomidine infusion (0.1 to 0.3 mg/kg per hour), the infusion rate needs to be adjusted over time due to potential pharmacokinetic changes in long-term experiments (>3 hours) (Pawela *et al*, 2009). Moreover, functional connectivity examined with blood oxygen level-dependent fMRI was well localized in rats anesthetized with either α-chloralose (27 mg/kg per hour) or medetomidine (0.1 mg/kg per hour) but less so under isoflurane (2%) (Williams *et al*, 2010). These reports indicate that choosing the appropriate anesthesia and adjusting its dosage are critical for achieving stable and reproducible experimental conditions. In the following sections, we will discuss the effects of anesthesia on the major properties of neurovascular coupling: (1) the spatial coordination of the vascular response with respect to the neurally derived map; (2) the temporal dynamics; and (3) the quantitative relationships examined under a variety of anesthesia conditions compared with those under unanesthetized conditions.

Anesthesia interference with neurovascular coupling

Spatial Coordination

Anesthesia-dependent variations in cortical mapping with OIS (630 nm) based on fingerpad stimulation were reported in the monkey somatosensory cortex (pentothal 1 to 2 mg/kg per hour versus isoflurane 0.8% to 1.5%) (Chen *et al*, 2001). In this study, mapping under pentothal produced focal localization, whereas maps obtained under isoflurane were less uniform and more broad. This observation was thought to occur because pentothal has suppressive actions on cortical activity due to both the

potentiation of GABAergic interneurons and the suppression of the excitability of glutamatergic neurons, whereas isoflurane enhances surround inhibition (Chen *et al*, 2001). Although the effects of focal excitation and surround inhibition on hemodynamic regulation are not fully understood (Boorman *et al*, 2010; Devor *et al*, 2007), the observed variation of the cortical mapping could depend on the actions of the anesthesia on both the neural and vascular components. Enhanced stimulus-specific localization of the cerebral blood volume (CBV)-weighted OIS (570 nm) under waking conditions relative to anesthetized conditions was observed for orientation column mapping in the cat visual cortex (Fukuda *et al*, 2005). Shtoyerman *et al* (2000) suggested that the increased specificity for the CBV response is due to an increased vascular response and signal transmission from neurons to vessels at the active columns because of the relatively unchanged spatial properties of neural responses. With increased focal activity, one may expect that the CBF/CBV response region would expand due to the activity-dependent spread of the vasodilation region (Kannurpatti and Biswal, 2011; Masamoto *et al*, 2010*a*; Durduran *et al*, 2004). However, enhanced surround inhibition may also cause vasoconstriction in that region (Devor *et al*, 2007), leading to a sharp tuning of the CBF/CBV supply to the focal active region.

To further explore the spatial gap between vascular response regions and activated neural sites at the cellular scale of the spatial resolution, Chaigneau *et al* (2003, 2007) applied two-photon imaging techniques to a rodent olfactory bulb model. A series of experiments showed that no tight coupling exists between activated glomeruli, and nearby capillary flow changes depending on inducing odor stimuli in rats or mice (either urethane 1.5 g/kg or ketamine/xylazine 90/10 to 100/16 mg/kg, Jukovskaya *et al*, 2011; Chaigneau *et al*, 2007). Based on these findings, it was suggested that the spatial mismatch originates from a vascular mechanism, such as the nonspecific orientation of capillaries, which pass through several neural modules. Furthermore, this spatial mismatch may also be related to the regulatory mechanism of capillary blood flow in which control may not be localized within a single capillary. The findings further suggest a role for precapillary arterioles in controlling capillary blood flow during cortical activation (Fernández-Klett *et al*, 2010). Overall, anesthesia interference with spatial CBF coordination could be caused by both anesthesia-dependent modulation of neural processing, such as local balance between excitatory and inhibitory activity, and changes in vascular response sensitivity to underlying neural activity. However, the mechanisms that (1) detect focal excitatory and surrounding inhibitory activity in neighboring capillaries, (2) transport the vasoactive signals to upstream parent arteries (arterioles), and (3) control CBF balances between the active and inactive regions remain unknown.

Temporal Dynamics

The factors affecting the time delay in the vascular response relative to the onset of a neural response are the following: (1) the release of vasoactive signals from neurons, (2) the transmission of the signals, (3) the uptake of the signals by vascular cells, and (4) the action of the vascular cells. Hayton *et al* (1999) reported that the onset latency of somatosensory-evoked potential after electrical stimulation to rat paws was slightly changed by anesthesia: 8.2 $\pm$ 5.0 ms for ketamine/xylazine (90/10 mg/kg), 7.5 $\pm$ 3.5 ms for medetomidine (0.3 mg/kg), 5.4 $\pm$ 2.6 ms for isoflurane (2%), and 6.3 $\pm$ 2.6 ms for fentanyl/fluanisone-midazolam (0.85/27 and 13.5 mg/kg, respectively), after the onset of forelimb stimulation. Dose-dependent increases in the latency of somatosensory-evoked potential (1.8 $\pm$ 0.8 ms per % isoflurane concentration) have also been observed in human subjects with inhaled anesthetics (0% to 1.65% isoflurane) (Sebel *et al*, 1986). These reports showed that the effect of anesthesia on the temporal dynamics of synaptic transmission is relatively small, indicating minimum interference with the latency of vasoactive signal release (i.e., within a couple of milliseconds).

An early study using an impedance technique suggested that the earliest onset of evoked vasodilation in response to auditory stimulation is $\sim$0.15 to 0.25 seconds from the onset of neural activation in conscious human brains (Sandman *et al*, 1984). In accordance with this observation, Nielsen and Lauritzen (2001) reported that the earliest onset latency of laser-Doppler flowmetry response measured in upper cortical layers was 0.2 $\pm$ 0.2 seconds after infraorbital nerve stimulation in anesthetized rats (α-chloralose 45 to 60 mg/kg per hour). Short onset latencies (0.2 to 0.4 seconds) of the red blood cell speed change after sensory stimulation were also observed in the parenchymal capillaries of awake mice with two-photon microscopy (Drew *et al*, 2011). These observations are also in good agreement with the reported onset time of the earliest plasma volume increases ($<$0.5 second latency) that originate from the arterioles of the middle cortical layers (α-chloralose 40 mg/kg per hour) (Tian *et al*, 2010), and the CBV onset (0.35 second) that starts from middle cortical layers measured with fMRI in rats (α-chloralose 26.7 mg/kg per hour) (Hirano *et al*, 2011). Considering these small variations in the reported onset latency of evoked vasodilation and capillary red blood cell speed changes, it can be expected that the transmission of vasodilatory signals to vascular cells is minimally influenced by anesthesia (e.g., α-chloralose) or has minimal variations that are not detectable with current methodologies. Because the frame rates of the MRI and two-photon imaging conducted in those studies were reported to be 4 and 5 to 30 frames per second, respectively, these resolutions may be not sufficient to stably track the fast responses of the vascular reaction. Furthermore,

Table 1 Onset delay of hemodynamic response relative to neural response in rats

Onset time (seconds)	*Criteria*	*Anesthetic (dosage)*	*Stimulation (pulse width, current, frequency)*	*Measurement*	*Reference*
1.8 ± 0.2	> 2 s.d.	Ure (1.5 g/kg)	Od (2 seconds)	Two-photon MS (capillary RBC speed)	Chaigneau *et al* (2003)
0.50 ± 0.10 0.63 ± 0.25 0.56 ± 0.19	Intersection of initial slope	Ure (1.25 g/kg)	WP (0.3 ms, 1.2 mA, 5 Hz)	OIS (parenchyma) OIS (artery) OIS (vein)	Berwick *et al* (2005)
0.7 ± 0.1	Not described	Ket (6 mg/kg)	WD (2 Hz, 10 seconds)	H_2 clearance	Khananashvili and Demidova (2002)
0.2 ± 0.2	> 2 s.d.	AC (45–60 mg/kg per hour)	IO (1 ms, 1.5 mA, 2 Hz)	LDF	Nielsen and Lauritzen (2001)
0.34 ± 0.06	Intersection of initial slope	AC (35 mg/kg per hour)	Cor (1 ms, 10–15 μA, 5–50 Hz)	LDF	Matsuura *et al* (1999)
0.4 ± 0.1	> 2 s.d.	AC (30 mg/kg per hour)	HP (1 ms, 1.2 mA, 2–20 Hz)	LDF	Sheth *et al* (2005)
0.52 ± 0.06	Intersection of initial slope	AC (45 mg/kg per hour)	HP (0.1 ms, 1.5 mA, 5 Hz)	LDF	Matsuura *et al* (2000)
0.54 ± 0.07 0.75 ± 0.13	Statistical analysis	AC (40 mg/kg per hour)	FP or HP (3 ms, 1 mA, 3 Hz)	OIS (parenchyma) OIS (artery)	Chen *et al* (2011)
< 0.5	Intersection of initial slope	AC (40 mg/kg per hour)	FP (0.3 ms, 1 mA, 3 Hz)	Two-photon MS (plasma volume)	Tian *et al* (2010)
0.7 ± 0.4	> 1 s.d.	AC (27 mg/kg per hour)	FP (0.3 ms, 1.5 mA, 3 Hz)	fMRI (CBF)	Silva *et al* (2000)
0.40 ± 0.22 0.43 ± 0.18 0.61 ± 0.34	> 1 s.d.	AC (27 mg/kg per hour)	BiFP (0.3 ms, 2 mA, 3 Hz)	fMRI (CBF, layers I–II) fMRI (CBF, layers III–V) fMRI (CBF, layer VI)	Hirano *et al* (2011)
0.34 ± 0.19 0.35 ± 0.16 0.58 ± 0.25	> 1 s.d.	AC (27 mg/kg per hour)	BiFP (0.3 ms, 2 mA, 3 Hz)	fMRI (CBV, layers I–II) fMRI (CBV, layers III–V) fMRI (CBV, layer VI)	Hirano *et al* (2011)
1.3	30% peak	AC (30–40 mg/kg per hour)	FP (3 ms, 1.6 mA, 3 Hz)	OIS (total Hb)	Hillman *et al* (2007)
0.4–0.6	Intersection of initial slope	Iso (1.4%)	FP (1.0 ms, 1.0 mA, 2–20 Hz)	LDF	Masamoto *et al* (2007)

AC, α-chloralose; BiFP, bilateral forepaw; CBF, cerebral blood flow; CBV, cerebral blood volume; Cor, cortex; fMRI, functional magnetic resonance imaging; FP, forepaw; HP, hindpaw; IO, infraorbital nerve; Iso, isoflurane; Ket, ketamine; LDF, laser-Doppler flowmetry; MS, microscope; Od, odor; OIS, optical intrinsic signal; RBC, red blood cell; s.d., standard deviation; Ure, urethane; WD, whisker deflection; WP, whisker pad.

additional tests of other cortical regions and with different anesthetics are also needed.

Moreover, the definition of the onset timing of the evoked vascular response may be biased depending on the criteria by which threshold levels for activity-induced changes are determined relative to baseline fluctuations (e.g., a threshold at twofold the standard deviation of baseline or an intersection between baseline and initial slope). Nevertheless, we found that the literature showed narrow ranges for the onset time (0.2 to 0.7 seconds) of evoked CBF in the rat somatosensory cortex regardless of the criteria used to define onset time under α-chloralose (Hirano *et al*, 2011; Sheth *et al*, 2005; Nielsen and Lauritzen, 2001; Silva *et al*, 2000; Matsuura *et al*, 1999, 2000). These findings are also in good agreement with the results measured under isoflurane anesthesia (0.4 to 0.6 seconds; Masamoto *et al*, 2007; Table 1). In contrast, relatively variable reports were found for the onset time of CBV changes (0.5 to 1.3 seconds) with OIS in a rat somatosensory model (α-chloralose, Chen *et al*, 2011; Hillman *et al*, 2007, versus urethane, Berwick *et al*, 2005). The large variations observed in the OIS results could be related to technical issues concerning spectral decomposition to calculate total hemoglobin content and the different signal-to-noise ratios of the measurements. Differing regions of interest also potentially contributed to the observed discrepancies, as it is well known that CBV changes spread along the vasculature from the activated hot spots (Chen *et al*, 2011; Sheth *et al*, 2005).

Whether certain anesthetics directly interfere with the dynamics of vascular responses (e.g., the propagation speeds of the vasodilatory signals) remains incompletely understood. In rats anesthetized with urethane (1.25 g/kg), the times-to-peak of CBF responses were observed to be longer (0.6 to 1.2 seconds) than those observed under waking conditions (Martin *et al*, 2006). In contrast, a similar time course of evoked change was observed for arterial vascular responses measured under either awake or urethane anesthesia (1 g/kg) conditions in the mouse somatosensory cortex (Drew *et al*, 2011). Although the cause of the controversial results (i.e., urethane effects) remains unclear in those studies, anesthesia may affect the temporal dynamics of vascular responses in varying degrees via its actions on the intracellular

calcium dynamics of smooth muscle cells and the reactivity to the vasoactive substances (Altura *et al*, 1980), and secondary effects via anesthesia-induced changes in systemic conditions, such as hypercapnia and hypotension.

Quantitative Relationships

The quantitative coupling relationships between evoked neural and vascular responses are largely influenced by the anesthesia. The route of this action involves anesthesia-dependent modulation of the following: (1) neural processing, (2) the vasoactive signal pathway, and (3) vascular cell reactivity. In awake conditions, larger evoked CBF/CBV changes have generally been observed than those observed in anesthetized conditions (Fukuda *et al*, 2005; Martin *et al*, 2006). Simultaneous recordings of evoked neural and vascular responses in rats have shown that the reduced hemodynamic responses under anesthesia (urethane 1.25 g/kg) were mainly due to the suppression of cortical excitability (Martin *et al*, 2006). The suppression of cortical activity is thought to be due to the suppression of thalamocortical inputs and suppression of cortical processing. Franceschini *et al* (2010) showed strong reduction of thalamocortical input under either ketamine/xylazine (20/2 mg/kg per hour) or fentanyl/droperidol (0.09/4.5 mg/kg per hour) anesthesia measured with somatosensory-evoked potential (i.e., a first positive peak, P1) in rats, whereas relatively preserved thalamocortical input was observed with α-chloralose (40 mg/kg per hour). Either pentobarbital (25 mg/kg) or propofol (50 mg/kg per hour) preserved thalamocortical inputs but reduced cortical activity, leading to the lowest hemodynamic responses among the anesthesia conditions tested (Franceschini *et al*, 2010). These findings strongly indicate that the reduced magnitude of cortical vascular response under general anesthesia originates from the anesthesia-dependent modulation of cortical processing.

In the rat primary somatosensory cortex, it was shown that anesthesia profoundly affects neural refractory periods with different degrees of potentiation depending on the anesthesia type and dosage (Masamoto *et al*, 2007, 2009). A prolonged refractory period was observed under α-chloralose (Masamoto *et al*, 2007; Ogawa *et al*, 2000), whereas short refractory periods were maintained under isoflurane and enflurane anesthesia. The latter anesthetics maintained robust hemodynamic responses to higher frequency stimulation (6 to 10 Hz; Kim *et al*, 2010; Masamoto *et al*, 2007; Sheth *et al*, 2003), which is in contrast to the well-known frequency dependencies in the rat somatosensory models using α-chloralose (a peak at a frequency of 1.5 to 5 Hz; Table 2). In addition, under different levels of isoflurane (0.8% to 2.2%), cortical adaptation was enhanced in a dose-dependent manner (Masamoto *et al*, 2009). In this condition, the evoked CBF induced by single-pulse local field potential (i.e., hemodynamic impulse response) was, however, found to increase in a dose-dependent manner (Masamoto *et al*, 2009). As a result, the optimum stimulus frequency that evoked the highest CBF response per given stimulus duration was shifted from high to low frequency stimulation with increases in anesthesia depths (Masamoto *et al*, 2009). Strong cortical adaptation under isoflurane (1.2%) was also consistently found in the rat somatosensory model with diffuse optical imaging (Franceschini *et al*, 2010). In this study, the highest hemodynamic response per given neural activity (P2 or N1 of somatosensory-evoked potential) was observed for either α-chloralose (40 mg/kg per hour) or isoflurane (1.2%), while a 10-fold higher CBF response to CO_2 challenges (5% CO_2 gas) was observed for isoflurane compared with ketamine/xylazine, propofol, or α-chloralose anesthesia (Franceschini *et al*, 2010). These findings suggest that separate mechanisms are involved in the vasodilatory response to neural stimulation and CO_2 challenge. In conclusion, the effects of anesthesia on quantitative neurovascular coupling primarily consist of the modulation of cortical processing and, thus, vasoactive pathways, while the effects of vascular reactivity differences on evoked vascular responses are weaker and the degree of these effects depends on anesthesia type and depth. Because different stimulus frequencies (4 to 30 Hz) were shown to evoke different populations of excitatory pyramidal cells and vasoactive inhibitory interneurons (Enager *et al*, 2009), it is likely that this anesthesia interference with cortical processing involves actions on the variable populations of vasoactive local interneurons (Lecrux *et al*, 2011; Harris *et al*, 2010). The cellular mechanism of the anesthesia action and its involvement in neurovascular transmission must be better identified in future studies.

Sites of action of anesthesia and neurovascular coupling

The potential target sites of anesthesia actions involved in neurovascular coupling are summarized in this section with emphasis on the anesthetics frequently used in animal experiments of neurovascular coupling and imaging, such as α-chloralose, isoflurane, pentobarbital, urethane, and medetomidine. The appropriate dosages and type of anesthetic for acute and chronic experiments in both rats and mice can be referenced in the literature (Lukasik and Gillies, 2003).

General Physiology

Alpha-chloralose is thus far the most commonly used anesthesia in neurovascular coupling imaging and physiology experiments in rodents because the

Table 2 Stimulation frequency dependences of hemodynamic responses in rat somatosensory cortex

Optimum frequency (Hz)	*Anesthetic (dosage)*	*Stimulation (pulse width, current)*	*Range of frequency (Hz)*	*Duration (seconds)*	*Techniques*	*Reference*
40	Waking	WP (0.3 ms, 0.3 mA)	1–40	2	LDF	Martin *et al* (2006)
5	Ure (1.25 g/kg)	WP (0.3 ms, 1.2 mA)	1–40	2		
3	Ure (1 g/kg)	WD (1 mm)	1–20	60	H_2 clearance	Moskalenko *et al* (1996)
5	Eto (1.5–2.1 mg/kg per hour)	WD (maximum)	1–7	120	ARG (^{14}C-IAP)	Vogel and Kuschinsky (1996)
10.5	Ure (1.2 g/kg)	WD (5 mm)	1.5–10.5	15	LDF	Gerrits *et al* (1998)
10	Ure (1.2 g/kg)	WAP (15 ms)	1–10	44	2D-LDFI	Kannurpatti and Biswal (2011)
12	AC (40 mg/kg per hour)	WAP (16.7–125 ms)	4–30	30	fMRI (BOLD)	Sanganahalli *et al* (2008)
1.5		FP (0.3 ms, 2 mA)	0.5–30	30		
9–15	Ure (1.25 g/kg)	FP (0.3 ms, 1–1.2 mA)	1–15	30	fMRI (BOLD)	Huttunen *et al* (2008)
10	Enf (1–2%)	HP (1.0 ms, 1.0 mA)	2–20	2	OIS (570 nm)	Sheth *et al* (2003)
12	Iso (1.4%)	FP (1.0 ms, 1.0 mA)	2–20	0.5–5	LDF and fMRI	Masamoto *et al* (2007)
6–8	Iso (1.3–1.5%)	FP (1.0 ms, 1.5 mA)	1–24	30	LDF and fMRI	Kim *et al* (2010)
9	Me (0.1 mg/kg per hour)	FP (0.3 ms, 2 mA)	1–18	20	fMRI (BOLD)	Zhao *et al* (2008)
8	K/X (75/5 mg/kg)+ AC (60 mg/kg)	BiFP (10 ms, 1 mA)	1–12	40	fMRI (BOLD)	van Camp *et al* (2006)
3	AC (27 mg/kg per hour)	BiFP (0.3 ms, 2 mA)	1–8	45	fMRI (BOLD)	Keilholz *et al* (2004)
2	AC (30 mg/kg per hour)	HP (1.0 ms, 0.8 mA)	2–20	2	LDF and OIS	Sheth *et al* (2004)
5	AC (45 mg/kg per hour)	HP (0.1 ms, 1.5 mA)	0.2–10	5	LDF	Matsuura and Kanno (2001)
3	AC (27 mg/kg per hour)	FP (0.3 ms, 1.5 mA)	1–5	40	LDF	Silva *et al* (1999)
1.5	AC (27 mg/kg per hour)	FP (0.3 ms, 0.5 mA)	1.5–6	50	fMRI (BOLD)	Brinker *et al* (1999)
1.5	AC (27 mg/kg per hour)	FP (0.3 ms, 0.5 mA)	1.5–9	40	fMRI (BOLD)	Gyngell *et al* (1996)

AC, α-chloralose; ARG, autoradiography; BiFP, bilateral forepaw; BOLD, blood oxygen level-dependent; ^{14}C-IAP, [^{14}C]iodoantipyrine; 2D-LDFI, two-dimensional laser-Doppler flowmetry imaging; Enf, enflurane; Eto, etomidate; fMRI, functional magnetic resonance imaging; FP, forepaw; HP, hindpaw; Iso, isoflurane; K/X, ketamine/xylazine; LDF, laser-Doppler flowmetry; Me, medotomidine; OIS, optical intrinsic signal; Ure, urethane; WAP; whisker air-puff; WD, whisker deflection; WP, whisker pad.

early studies have shown that this agent preserves robust and stable hemodynamic and metabolic coupling to sensory stimulation (Lindauer *et al*, 1993; Ueki *et al*, 1992). The effects of α-chloralose on general physiology include respiratory depression, metabolic acidosis, and hyperreactivity (Arfors *et al*, 1971). The analgesic properties of this agent are questionable (Silverman and Muir, 1993), and thus, any surgical preparation must be performed with administration of another anesthetic. This procedural complication before the experiment contributes to the variable results regarding neurovascular coupling relationships in α-chloralose-anesthetized rats (Bonvento *et al*, 1994). In addition, the use of α-chloralose is limited to experiments with nonsurvival protocols (Silverman and Muir, 1993), which hampers the use of this animal model for wider applications, such as repeated longitudinal experiments.

Alternatively, several groups have introduced inhaled anesthetics (e.g., halothane, enflurane, and isoflurane) for rodent neurovascular coupling studies (Kim *et al*, 2010; Masamoto *et al*, 2007; Schulte and Hudetz, 2006; Sheth *et al*, 2003). A major advantage of inhaled anesthetics is that the fast induction and rapid recovery achieved with these anesthetics make them usable for repeated longitudinal experiments. However, a known disadvantage of these agents is that volatile anesthetics themselves are potent vasodilators and, thus, cause cerebral vasodilation, which leads to increased baseline CBF that is uncoupled from the cerebral energy metabolism (van Aken and van Hemelrijck, 1991). The effects might be more severe in mice compared with rats. Moreover, it was shown that high concentrations of isoflurane (>3%) break down the cortical blood–brain barrier (Tétrault *et al*, 2008), and isoflurane impairs glucose-stimulated insulin release (Tanaka *et al*, 2011).

Barbiturate anesthetics, including pentobarbital and thiopental, suppresses cardiac output and often cause hypotension. Respiratory depressant effects of pentobarbital have also been reported (Field *et al*, 1993). In contrast, urethane provides moderate depression of blood pressure and heart rate, and may cause hyperventilation (Field *et al*, 1993). Intraperitoneal administration of urethane (1.2 g/kg) was shown to cause hyperglycemia associated with hypothalamic activation (Reinert, 1964; Maggi and Meli, 1986). Urethane is also known as potential mutagen and carcinogen (Field and Lang, 1988). Medetomidine and xylazine are specific α2-adrenoreceptor agonists that block norepinephrine release. Due to the limited analgesic properties of these agents, surgical procedures should be performed in combination with other anesthetic agents. The α2-adrenoreceptor was also known to significantly affect cardiovascular function and depress respiratory function (Sinclair, 2003). Animals can recover after the administration of a reversible α2-antagonist, making this agent also suitable for use in repeated longitudinal imaging experiments (Pawela *et al*, 2009; Weber *et al*, 2006).

Neural Activity

The action sites at which anesthesia influences neural pathways have been shown to involve common targets, such as ligand-gated ion channels (potentiation of GABA type A and glycine receptors, and suppression of NMDA (*N*-methyl-D-aspartate) receptor) and presynaptic actions on calcium, potassium, and sodium channels (for reviews, see Chau, 2010; Hemmings 2009; Franks, 2008). Isoflurane was shown to predominantly reduce presynaptic excitability via sodium channel blockade (Hemmings 2009) and, thus, glutamate release (Sandstrom, 2004; Wu *et al*, 2004). Moreover, the inhibition of the NMDA receptor, potentiation of GABA type A receptors, and suppression of acetylcholine transmission and receptors have also been reported (Dickinson *et al*, 2007; Hentschke *et al*, 2005; Violet *et al*, 1997).

The action site of α-chloralose has been shown to involve the potentiation of GABA-induced currents by increasing affinity for GABA (Garrett and Gan, 1998), whereas preserved synaptic transmission and glutamate-, glycine-, and acetylcholoine-induced current were observed at low concentration of α-chloralose (Wang *et al*, 2008). Pentobarbital binds to GABA type A receptors and enhances GABA-mediated inhibitory neurotransmission (Curtis and Lodge, 1977). Pentobarbital and thiopental have also been shown to inhibit the release of acetylcholine, norepinephrine, and glutamate (Nicoll, 1978; Curtis and Lodge, 1977). Significant inhibition of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor by pentobarbital has also been found, but pentobarbital has small effects on NMDA, glycine, and GABA receptors (Hara and Harris, 2002). Medetomidine selectively inhibits noradrenergic neurons in the locus coeruleus and has been shown to disrupt thalamocortical transmission (Sinclair, 2003). Urethane is shown to have modest effects on multiple ligand-gated ion channels, including potentiation of GABA type A and glycine receptors, and mild inhibition of NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Hara and Harris, 2002; Maggi and Meli, 1986; Minchin, 1981), which shows that this agent is suitable for pharmacological studies of neurotransmitter release and uptake.

Vasoactive Signal Transmission

Determining the action site on neural processing and the resultant effects on the vascular responses would provide an insight into the mechanism of the neurovascular transmission pathway. However, it is difficult to directly identify and purify the action site of anesthesia because most neurovascular transmissions share a common mechanism of neural processing. Volatile anesthetics, but not ketamine or pentobarbital, have been shown to enhance glutamate uptake by astrocytes (Miyazaki *et al*, 1997), and dose-dependent closure of astrocytic gap junctions has been observed with volatile anesthetics in cultured astrocytes (Mantz *et al*, 1993). Whether these effects of isoflurane on astrocyte function are related to the observed dose-dependent increase in hemodynamic impulse responses (Masamoto *et al*, 2009) remains unclear. Future studies should determine the exact contribution of the actions of anesthetics on astrocyte mechanisms using pharmacological approaches. Furthermore, experimental models with well-defined neural circuits would be useful to further determine the action site in *in-vivo* conditions and its contribution to the generation of the blood oxygen level-dependent fMRI signal (Krautwald and Angenstein, 2012).

Vascular Responses

Some anesthetics have been shown to directly affect vascular physiology. Isoflurane dose dependently induced the relaxation of cerebral arteries (i.e., vasodilation) via its actions on ATP-sensitive potassium channels and reduced calcium current in smooth muscle cells (Iida *et al*, 1998; Flynn *et al*, 1991, 1992). In contrast, endothelium-dependent vasodilation induced by acetylcholine was inhibited by isoflurane due to its inhibiting effects on formation of nitric oxide in endothelium (Nakamura *et al*, 1994; Toda *et al*, 1992). Autoregulatory responses, and the CBF response to CO_2 inhalation, were shown to be preserved under isoflurane anesthesia (Lee *et al*, 1994, 1995). However, a depression of the vascular response to CO_2 inhalation has been found in rats anesthetized with 2% isoflurane compared with the waking condition (Sicard *et al*, 2003). It is well known that pentobarbital causes a reduction of CBF relative to awake conditions (Wei *et al*, 1993). However, the effects of barbiturate anesthetics on cerebral vessels have been shown to be controversial; they can act as potent vasoconstrictors (Tsuji and Chiba, 1987) or vasodilators (Ogura *et al*, 1991). Medetomidine also causes α2-adenoreceptor-mediated vasoconstriction of cerebral arteries and results in reduced CBF (Sinclair, 2003; Ganjoo *et al*, 1998). Decreased sensitivity of the cerebrovascular response to arterial CO_2 has been reported for α-chloralose (100 mg/kg; Sándor *et al*, 1977). Furthermore, we observed that the capillary diameter in the resting state was slightly larger under 45 mg/kg per hour α-chloralose ($5.1 \pm 1.2\,\mu$m) than 1.4% isoflurane ($4.8 \pm 1.1\,\mu$m) (Masamoto *et al*, 2010*b*), which may further contribute to the anesthesia-dependent variations of microvascular responses to physiological perturbations, such as the contribution of capillary diameter changes measured under different anesthesia conditions.

Other Considerations

As discussed above, one should consider the effect of anesthesia on baseline (prestimulus resting) conditions. It has been reported that activation-induced

changes of brain activity were largely dependent on the baseline states, such as the anesthesia-dependent reduction of oxygen and glucose metabolism and unit neural activity (Hyder *et al*, 2002; Shulman *et al*, 1999). In these studies, lower baseline states induced by anesthesia have been shown to cause larger activation changes. Because the resting-state energy metabolism is known to be coupled to baseline CBF, it can therefore be expected that baseline CBF also differs depending on the anesthesia. Some previous works regarding the baseline CBF measured under a variety of anesthesia conditions in the rat cerebral cortex are summarized in Table 3. Overall, injectable anesthetics (α-chloralose and pentobarbital) reduced baseline CBF, whereas low concentration of isoflurane (1.3% to 1.5%) maintained CBF values close to those of the awake condition (Table 3). Whether these modulatory effects of anesthesia on baseline CBF affect activation-induced vascular responses remains relatively unknown (Franceschini *et al*, 2010). However, caution should be exercised in comparing the quantitative data examined under different baseline states with different anesthesia.

Finally, repeated longitudinal experiments are becoming increasingly more important for further understanding the biological implications and plasticity of neurovascular coupling (Brown *et al*, 2010; Colonnese *et al*, 2008). For longitudinal experiments, the same anesthetics have been repeatedly used in single animals; i.e., isoflurane (Colonnese *et al*, 2008; Tomita *et al*, 2005) and ketamine/xylazine (Brown *et al*, 2010). For those experiments, good recovery from anesthesia discontinuation is important in the choice of anesthetics for performing controlled experiments. Hayton *et al* (1999) reported that ketamine/xylazine, medetomidine, and fentanyl/fluanisone-midazolam cause losses in body weight; however, isoflurane does not have this effect. No effects on cell proliferation were found for isoflurane, propofol, medetomidine, or ketamine in young rats (Tung *et al*, 2008); these results are particularly important for developmental and regeneration studies with long-term imaging experiments.

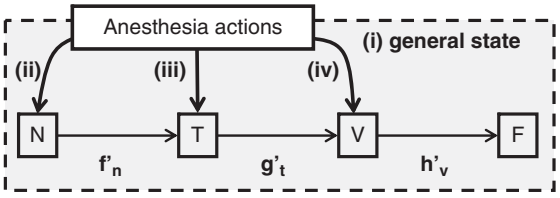
Summary

The effects of anesthesia on neurovascular coupling involve the following: (1) changes in general physiology, (2) direct interference with cortical neural processing, (3) modulation of vasoactive signal transmission, and (4) suppression of vascular cell activities (Figure 2). It is well known that anesthesia modifies the balance between focal excitation and surround inhibition in a manner dependent on the type and dosage of anesthesia. However, the resultant effects on spatial coordination of vascular responses, such as activity-dependent vasodilation and vasoconstriction, are not well understood. Propagation time, in particular that for the onset latency of vasodilation evoked by neural stimulation, is relatively preserved across anesthesia states (Table 1), but the temporal dynamics of vasodilation may vary between anesthetics. Finally, the quantitative coupling relationship of neurovascular responses is strongly influenced by anesthesia types and dosages. The fact that different anesthetics differentially modify the hemodynamic impulse response functions indicates that different anesthetics act specifically on

Table 3 Baseline CBF in rat cortex

CBF (ml/100 g per minute)	*Anesthetic (dosage)*	*Region*	*Technique*	*Reference*
167 ± 45	Waking	Sensorimotor	ARG (^{14}C-IAP)	Kuschinsky *et al* (1985)
155 ± 30	Waking	Sensorimotor	ARG (^{14}C-IAP)	Maekawa *et al* (1986)
102 ± 35	Iso (0.7%)			
147 ± 40	Iso (1.4%)			
183 ± 63	Iso (2.1%)			
247 ± 67	Iso (2.8%)			
134 ± 8	Waking	Sensorimotor	ARG (^{14}C-IAP)	Lenz *et al* (1998)
132 ± 26	Iso (1.4%)			
153 ± 14	Iso (2.8%)			
151 ± 23	Iso (1.3–1.5%)	Somatosensory	MRI (ASL)	Kim *et al* (2007)
146 ± 13	Ure (1.2 g/kg)	Somatosensory	ARG (^{14}C-IAP)	Gerrits *et al* (2000)
168 ± 12	Waking	Sensorimotor	ARG (^{14}C-IAP)	Otsuka *et al* (1991)
55 ± 5	Pen (50 mg/kg)			
180 ± 15	Waking	Somatosensory	ARG (^{14}C-IAP)	Nakao *et al* (2001)
65 ± 5	AC (40 mg/kg per hour)			
58 ± 3	AC (26.7 mg/kg per hour)/50% N_2O	Cortex	MRI (CASL)	Lee *et al* (2001)
75 ± 9	Morphine (60 mg/kg per hour)/70% N_2O	Somatosensory	MRI	Hyder *et al* (2000)
40 ± 9	AC (40 mg/kg per hour)/70% N_2O			
90 ± 20	AC (36 mg/kg per hour)/70% N_2O	Somatosensory	MRI	Smith *et al* (2002)
60 ± 20	AC (46 mg/kg per hour)/70% N_2O			

AC, α-chloralose; ARG, autoradiography; ASL, arterial spin labeling; CASL, continuous arterial spin labeling; CBF, cerebral blood flow; ^{14}C-IAP, [^{14}C]iodoantipyrine; Iso, isoflurane; MRI, magnetic resonance imaging; Pen, pentobarbital.



(i) Systemic physiology
- blood gas conditions
- cardiac output
- arterial blood pressure

(iii) Vasoactive signal Transmission
- release and uptake of transmitters
- efficiency of transmission
- turnover

(ii) Neural activity
- synaptic transmission
- pre-synaptic release
- post-synaptic receptors
- removal of transmitters
- membrane potentials

(iv) Vascular reactivity
- baseline CBF
- vascular tone
- vasomotion
- receptor functions
- calcium ion mobility

Figure 2 Summary of the effects of anesthesia on neurovascular coupling. The effects of anesthetics involve systemic physiology (i), neural processing (ii), vasoactive signal transmission (iii), and vascular responses (iv). Depending on the type and dose of anesthetic, the anesthesia differentially modifies the individual transfer functions of neural processing (f'_n), vasoactive signal transmission (g'_t), and vascular reactivity (h'_v). CBF, cerebral blood flow.

the different cell populations that participate in the vasoactive pathways. In addition, this anesthesia interference may involve variations in baseline states, such as spontaneous neural activity, energy metabolism, and baseline CBF.

For *in-vivo* rodent somatosensory models, a large amount of neurovascular physiology and imaging data have been accumulated under α-chloralose anesthesia. Some findings might be specific to this anesthetic, such as the optimum stimulus frequency (Table 2) and low baseline CBF (Table 3). For repeated longitudinal experiments, isoflurane is recommended as an alternative agent because it provides easy control, good anesthesia recovery, and robust activity-induced vascular response that is comparable to that of α-chloralose in rat somatosensory models (Masamoto *et al*, 2007; Franceschini *et al*, 2010). Intravenous injection anesthesia (e.g., α-chloralose and medetomidine) carries concerns about the stability and reproducibility for long-term experiments (>3 hours), whereas urethane provides relatively long-term stability and balanced actions on multiple neurotransmitter receptors. Although some anesthetics, such as isoflurane, pentobarbital, and medetomidine, directly affect vascular physiology (i.e., dose-dependent vasodilation or vasoconstriction) independent of neural processing, the practical effects of these actions on neurovascular signal transmission remain relatively unknown. Because specific anesthetics may influence the specific cellular mechanisms of neurovascular elements, conducting multiple tests under different anesthesia conditions is recommended to ensure the exclusion of anesthesia confounds. This approach also helps determine the mechanisms of the generation of the signal in hemodynamic-based neuroimaging techniques.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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