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Research Report

Cerebral blood flow and BOLD fMRI responses to hypoxia in awake and anesthetized rats

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ABSTRACT

This study investigated the functional MRI responses to graded hypoxia in awake/restrained and anesthetized animals by measuring cerebral blood flow (CBF) and blood oxygenation (BOLD) changes and estimating changes in cerebral metabolic rate of oxygen (CMRO₂). Hypoxia in isoflurane anesthetized rats reduced blood pressure but did not change heart rate and respiration rate. In contrast, hypoxia in awake animals showed compensatory responses by sustaining blood pressure, increasing heart rate and respiration rate. Basal CBF was higher under isoflurane anesthesia than awake state because isoflurane is a vasodilator. Graded hypoxia decreased BOLD signals. Surprisingly, hypoxia also decreased CBF likely because hypoxia induced hypocapnia. Hypoxia-induced CBF and BOLD decreases were smaller in awake, relative to anesthetized, rats at low pO₂, but similar at high pO₂. CBF leveled off with decreasing hypoxia-induced pCO₂ in awake rats, but monotonically decreased in anesthetized rats. CMRO₂ estimated using a biophysical BOLD model did not change under mild hypoxia but was reduced under severe hypoxia relative to baseline. These results showed that isoflurane attenuated autonomic responses to hypoxia, hypoxia-induced hypocapnia dominated CBF changes, tissues in awake conditions appeared better oxygenated, and severe hypoxia reduced oxygen metabolism. This study underscored the marked differences in BOLD and CBF MRI responses to hypoxia *in vivo* between awake and anesthetized conditions and has implications for functional MRI studies of hypoxia in anesthetized animal models.

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1. Introduction

The mammalian brain depends on a continuous and adequate supply of oxygen to maintain its structural and functional integrity. If oxygen delivery is sufficiently compromised, loss of consciousness could occur within seconds, irreversible neuronal damage and severe brain dysfunction, within minutes (Siesjo, 1978). During hypoxia, there are systematic autonomic cerebrovascular responses, such as vasodilation

and increases in respiration rate, heart rate, cerebral blood volume, and cerebral blood flow (CBF), to compensate for reduced inspired oxygen. These autonomic responses depend on the severity and duration of hypoxia, species, tissue type, and level and type of anesthetic (Siesjo, 1978).

Combined CBF and blood-oxygenation-level-dependent (BOLD; Ogawa et al., 1990) functional MRI provides a unique means to investigate the neurovascular coupling under graded hypoxia non-invasively. Stimulus-evoked CMRO₂

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changes can be estimated with combined BOLD and CBF measurements (Davis et al., 1998; Liu et al., 2004). While general anesthesia with mechanical ventilation is commonly used for immobilization in MRI studies of animal models, there has been increasing interest in imaging awake animals because of the important applications of awake models for mapping higher-order cognitive brain functions. The feasibility of performing fMRI under awake and restrained conditions has been demonstrated using the BOLD technique (Logothetis et al., 1999; Wyrwicz et al., 2000), MION (monocrystalline iron oxide nanoparticles) technique to detect stimulus-evoked changes in blood volume (Dubowitz et al., 2001; Vanduffel et al., 2001), and perfusion and perfusion-based functional MRI (Sicard et al., 2003). While fMRI studies of awake animals are generally challenging, they also offer many distinct advantages. First, the confounding effects of anesthesia on blood flow, metabolism and neuro-vascular coupling can be avoided. Second, general neural activity is not suppressed, which leads to more salient fMRI signal changes. Finally, sub-cortical and higher order cognitive functions can be studied in the awake model. Such studies would be very difficult if not impossible under anesthesia. The disadvantages of performing fMRI studies on awake animals include motion artifacts and restraint stress. Characterizing the differences and similarities in physiological and functional measures between awake and anesthetized conditions could help to better understand the fMRI signals and improve study designs.

The goal of this study was to investigate the cerebrovascular responses to graded hypoxia in awake and anesthetized (2% isoflurane) spontaneously breathing rats. CBF and BOLD fMRI signals were simultaneously measured using the continuous arterial spin-labeling technique with echo-planar-imaging acquisition. Cerebral metabolic rate of oxygen (CMRO₂) was estimated using the biophysical BOLD model. MRI data were correlated with blood pressure, heart rate, respiration rate, and blood gases in the same animals.

2. Results

2.1. Physiological parameters

MABP, heart rate, and respiration rate under basal conditions and graded hypoxia are plotted in Fig. 1. Under awake basal (21% O₂) conditions, the respiration rate, heart rate, and MABP were within normal physiological ranges (Sharp and LaRegina, 1998), consistent with previously reported values (Sicard et al., 2003). These parameters under anesthetized basal condition were clearly reduced ($P < 0.05$) but remained within normal physiological ranges (Sharp and LaRegina, 1998). During graded hypoxia in the awake state, MABP did not decrease ($P > 0.05$), and heart rate and respiration rate increased ($P < 0.05$ for 9% O₂ exposure). In contrast, in the anesthetized conditions, MABP decreased ($P < 0.05$ for 12% and 9% O₂ exposure), and heart rate and respiration rate remained unchanged ($P > 0.05$). The differences in these parameters between the awake and anesthetized conditions generally grew larger as inspired oxygen tension decreased ($P < 0.05$ for all hypoxic conditions).

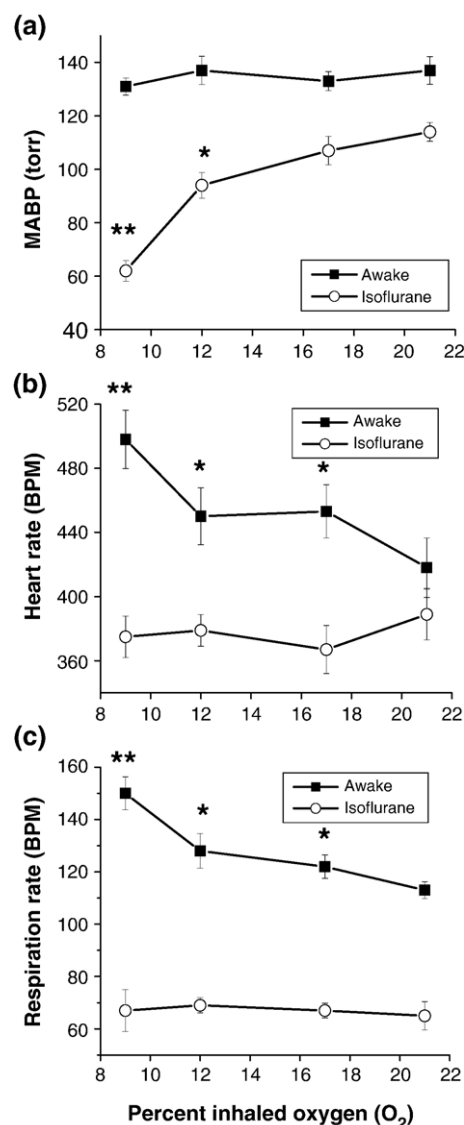


Fig. 1 – (a) Mean arterial blood pressure (MABP), (b) heart rate, (c) respiration rate under basal conditions and graded hypoxia. * $P < 0.05$, ** $P < 0.001$ relative to baseline condition (21% O₂).

Blood gases under basal conditions and graded hypoxia are shown in Fig. 2. Basal blood-gas pH, pCO₂, pO₂, and oxygenation saturation in both the awake and the anesthetized condition were within normal physiological ranges (Sharp and LaRegina, 1998) but there were systematic differences between awake and anesthetized animals. During graded hypoxia, pH increased, and pCO₂, pO₂, and O₂ saturation decreased under both conditions ($P < 0.05$ for all hypoxic conditions). The differences in O₂ saturation between the awake and anesthetized conditions grew larger as inspired oxygen tension decreased ($P < 0.05$).

2.2. CBF and BOLD signal responses to hypoxia

CBF and BOLD responses to hypoxia were initially analyzed for five different brain regions (cortex, hippocampus, thalamus,

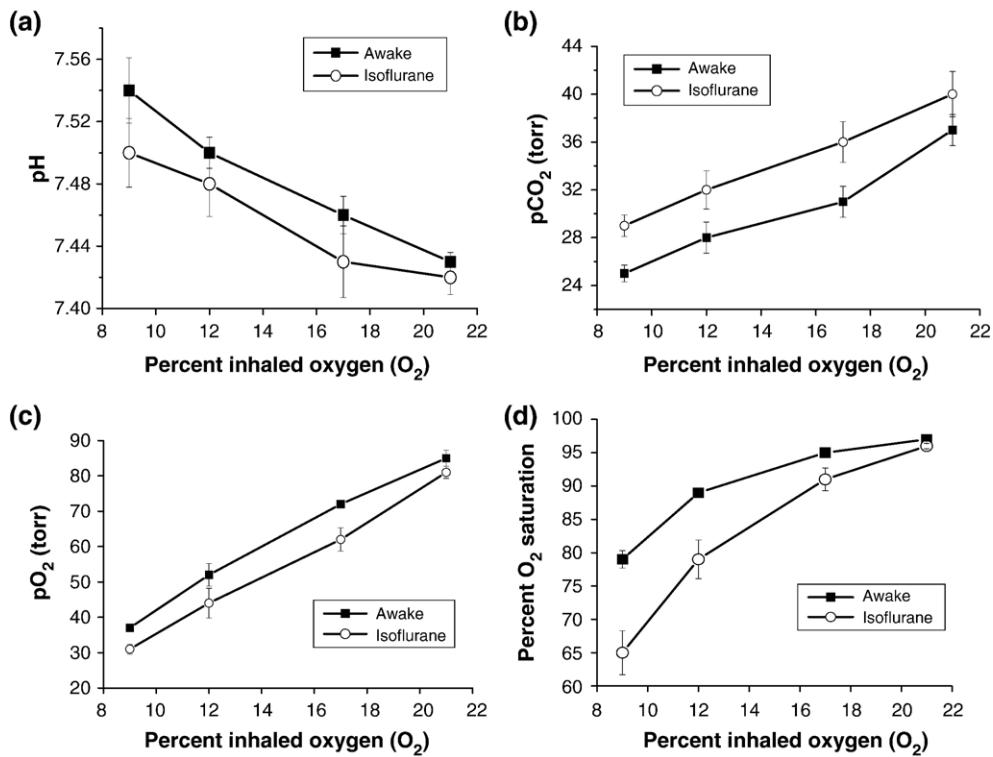


Fig. 2 – Blood gases (pH, pCO₂, pO₂, O₂ saturation) under basal conditions and graded hypoxia. Basal blood gases were not statistically different between awake and anesthetized rats ($P > 0.05$). During graded hypoxia, pH increased, and pCO₂, pO₂, and O₂ saturation decreased under both awake and anesthetized conditions ($P < 0.05$).

corpus callosum, and caudoputamen). The data trends were similar and thus we chose to perform whole-brain ROI analysis for high signal-to-noise ratios.

Representative CBF and BOLD responses to graded hypoxia from a single animal in the awake and anesthetized conditions are shown in Fig. 3. BOLD and CBF image time courses in

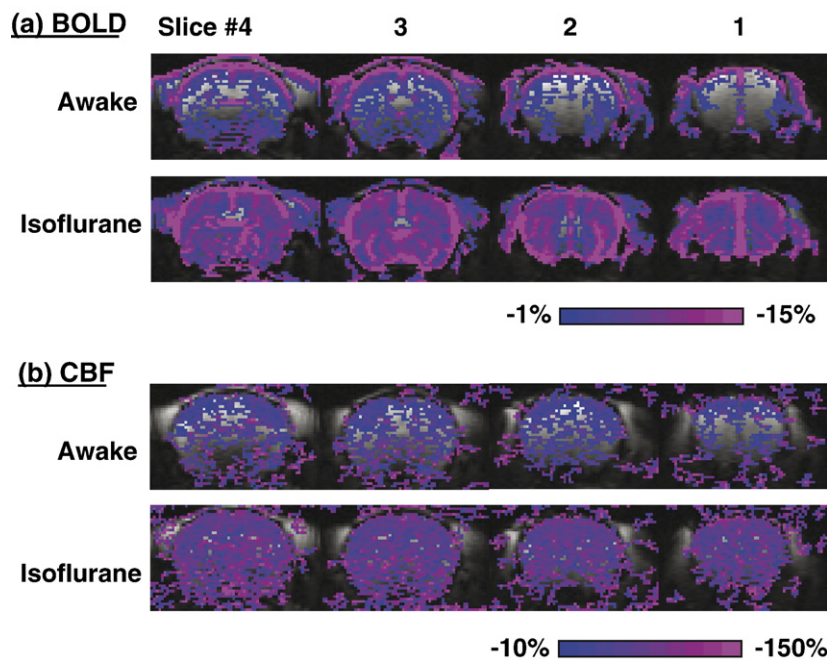


Fig. 3 – Representative BOLD and CBF responses to hypoxia (9% O₂) under anesthetized and awake condition from one animal. BOLD and CBF percent-change maps were calculated and color-coded. The threshold was set the same to allow comparison between awake and anesthetized conditions. Percent changes in text employed the same whole-brain ROI without statistical threshold.

anesthetized and awake rats generally showed no ostensible movement artifacts. Surprisingly, no hypoxia-induced CBF increase was observed. Both CBF and BOLD decreased with graded hypoxia. However, hypoxia-induced CBF and BOLD percent decreases in the awake state were smaller than those in the anesthetized state.

The group-averaged quantitative CBF values were evaluated as a function of percent inhaled oxygen under awake and anesthetized conditions (Fig. 4a). Basal (21% O₂) CBF in the anesthetized condition (1.13±0.12 ml/g/min) was greater than that in the awake condition (0.93±0.10 ml/g/min). However, this was reversed under 9% O₂ hypoxia—that is basal CBF in the awake condition was greater than that in the anesthetized condition. Fig. 4b re-plots the quantitative CBF as a function of arterial blood pO₂. For each hypoxic gas condition, pO₂ was slightly higher in the awake relative to the anesthetized state,

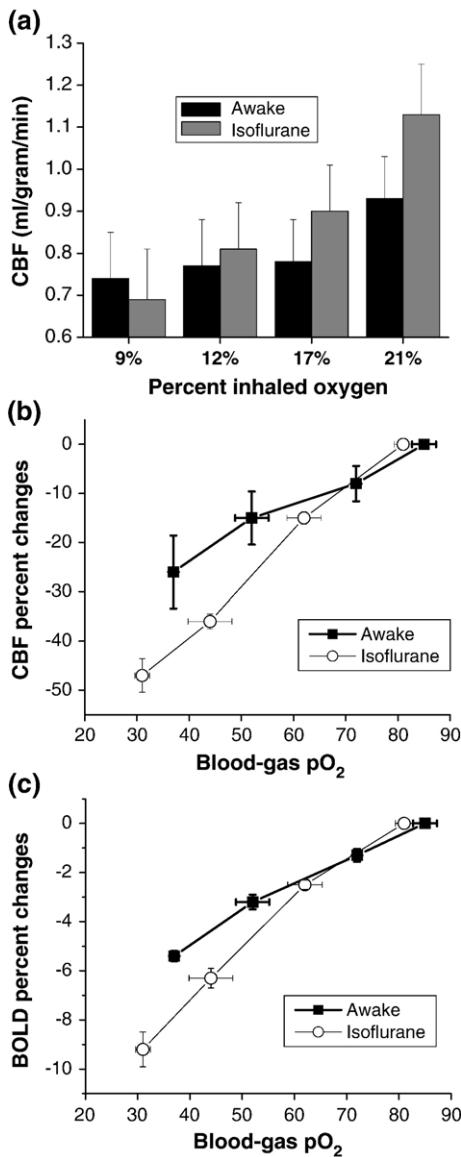


Fig. 4 – (a) Quantitative CBF values as a function of percent inhaled oxygen under awake and anesthetized conditions. (b) CBF and (c) BOLD percent changes as a function of arterial blood pO₂.

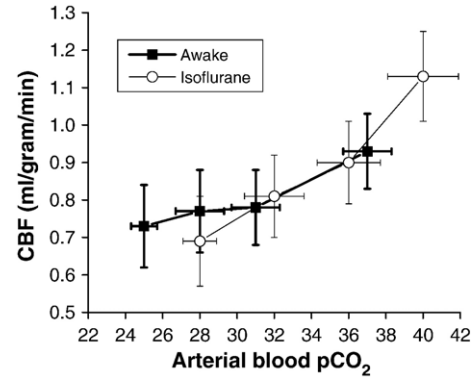


Fig. 5 – Cerebral blood flow (CBF) as a function of arterial blood pCO₂ during graded hypoxia under awake and anesthetized conditions. CBF leveled off with decreasing arterial pCO₂ under awake condition, whereas CBF decreased monotonically with decreasing arterial pCO₂ under anesthetized condition.

resulting in a shift between the two curves. CBF under isoflurane decreased markedly with decreasing arterial blood pO₂. In contrast, CBF in the awake condition decreased more gradually and appeared to level off.

BOLD percent changes were re-plotted as a function of blood pO₂ (Figs. 4c). CBF and BOLD percent decreases were more negative in the anesthetized condition than in the awake state at low arterial pO₂ (<50 mm Hg), whereas there were no statistical differences at high arterial pO₂ (>50 mm Hg).

The correlation between CBF and arterial pCO₂ is shown in Fig. 5. At high pCO₂ (non-hypoxic condition), the difference in CBF under graded hypoxia between awake and anesthetized conditions could be explained by the differences in pCO₂. At low pCO₂ (hypoxic condition), CBF leveled off slightly with decreasing arterial pCO₂ in the awake condition, whereas CBF decreased monotonically with decreasing pCO₂ in the anesthetized condition.

Fig. 6 shows the CBF and BOLD hypoxia data superimposed on iso-CMRO₂ contour plots. The CBF and BOLD hypoxia data herein (▲ and ▼ symbols) mostly fell on or near the 0% iso-CMRO₂ line as expected because mild hypoxia does not alter CMRO₂. Similarly, previously published data (Sicard et al., 2003; Liu et al., 2004; Sicard and Duong, 2005; Schmidt et al., 2006) obtained under mild hypoxia and hypercapnia also fell on or near 0% iso-CMRO₂ line. Three data points from physiological perturbation (encircled in Fig. 6), however, deviated significantly from the 0% iso-CMRO₂ line and they are those under severe hypoxia (9% O₂) and severe hypercapnia (10% CO₂) for anesthetized conditions. Forepaw and pharmacological stimulation data are also superimposed on the iso-CMRO₂ contour plots and these data showed positive increase in CMRO₂ as expected.

3. Discussion

Relative to normoxia, hypoxia (i.e., 9% O₂) in 2%-isoflurane anesthetized rats reduces in blood pressure and does not

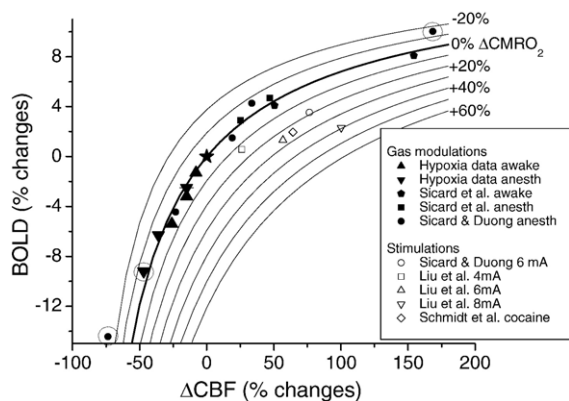


Fig. 6 – Iso-CMRO₂ contour lines from –20% to 60% in steps of 10% are overlaid on the plot of BOLD versus CBF percent changes under different physiological modulations and functional stimulations. CMRO₂ contour lines were calculated using $M=0.16$, α of 0.4, and β of 1.2. The open data points were functional (forepaw or cocaine stimuli) stimulations and the solid data points were physiological modulations (normal conditions, hypercapnia, hyperoxia or hypoxia). ▲ and ▼ are awake and anesthetized hypoxia data obtained from this study; ● and ■ are CO₂ challenges data under awake and anesthetized conditions, respectively, obtained from (Sicard et al., 2003); ● and ○ are CO₂ challenges and 6 mA forepaw stimulation data, respectively, obtained from (Sicard and Duong, 2005); □, △, and ▽ are 4, 6, and 8 mA forepaw stimulation data, respectively, obtained from Liu et al. (2004); ◇ is cocaine data (1 mg/kg) from Schmidt et al. (2006). Three data points that are encircled show deviation from the 0% CMRO₂ iso-contour line: ▼ at ~50% ΔCBF and ● at ~70% ΔCBF were 9% O₂ hypoxia under anesthesia, and ● at 170% ΔCBF was 10% CO₂ hypercapnia under anesthesia. ★ indicates the point of origin.

change heart and respiration rate. In contrast, hypoxia in awake animals shows compensatory responses by sustaining blood pressure, and increasing heart rate and respiration rate. Hypoxia induces hypocapnia. Basal CBF under anesthesia is higher than under the awake state because isoflurane is a potent vasodilator. Graded hypoxia decreases BOLD signals. Surprisingly, hypoxia decreases CBF because hypoxia induces hypocapnia. However, hypoxia-induced CBF and BOLD reductions are smaller in awake relative to anesthetized rats at low pO_2 , but similar at high pO_2 . CBF levels off with decreasing hypoxia-induced pCO_2 in awake rats, but monotonically decreases in anesthetized rats. CMRO₂ does not change under mild hypoxia but appears to decrease under severe hypoxia and severe hypercapnia. These results further support the feasibility of awake fMRI studies, show that isoflurane attenuates autonomic responses to hypoxia, hypoxia-induced hypocapnia dominates CBF changes, tissues in awake conditions appear better oxygenated under identical hypoxic conditions, and severe hypoxia appears to reduce cerebral oxygen metabolism. These findings have strong implications for functional MRI studies of awake animals and hypoxia studies in anesthetized animals.

3.1. Potential issues

MRI offers a non-invasive tool to obtain quantitative CBF images at reasonably high spatial and temporal resolution at the level of the capillary (Duong et al., 2001b). Although consistent with many established invasive CBF techniques, the continuous arterial spin-labeling CBF technique could yield CBF data that are subject to errors. These errors could arise from magnetization transfer, transit time, and water exchange effects. With the actively decoupled two-coil system for CBF measurements used herein, there was no error arising from magnetization transfer effect on the quantitative CBF values (Silva et al., 1999; Duong et al., 2000). Transit time (Zhou and van Zijl, 1999) and water-exchange effect (Zhou et al., 2001) are likely to be small (Zhou et al., 2001) and are not expected to alter the overall conclusions of this study.

Perfusion imaging is particularly sensitive to movement artifacts because it has relatively low signal-to-noise ratio and it involves paired images acquired at two different time points between which movement could occur. The use of a proper restrainer provided BOLD and quantitative perfusion images free of movement artifacts. Echo-planar-imaging technique was also helpful because EPI samples the entire k -space in a single shot acquisition within ~20 ms. Therefore, the approach taken herein offers a means to quantitatively evaluate regional CBF in fully conscious rats, allowing direct comparison of quantitative CBF in graded hypoxia under awake and anesthetized conditions in the same animals.

Restraint could have a substantial effect on animal physiology which could complicate the comparison between awake and anesthetized conditions. A detailed investigation of acclimation and restraint effects has been described elsewhere (King et al., 2005). In this study, blood gases, heart rate, respiration rate, and mean arterial blood pressure in the awake animals were within normal physiological ranges, and the number of struggling movements markedly decreased after the first hour of being awake in the restrainer. Taken together, these observations suggest that the animals had at least partially acclimated to the restrainer during the imaging session. Nonetheless, regional differences in CBF as well as the CBF and BOLD responses to hypoxia could be influenced in part by various stress factors which include restraint stress. Similarly, hypoxia could also activate brain regions related to hypoxia exposure differently between awake and anesthetized conditions. These changes, however, are unlikely to alter the conclusions where the whole-brain signals were analyzed. Furthermore, only isoflurane was used for comparison in this study. Other anesthetics could have different effects on autonomic responses under hypoxia and may show different BOLD and CBF responses to hypoxia. The advantages and disadvantages of isoflurane anesthesia under physiological and stimulation studies have been extensively discussed (Sicard et al., 2003; Liu et al., 2004).

3.2. CBF in anesthetized conditions

Oxygen delivery to the brain during hypoxia involves many complicated physiological factors. Hypoxia induced respiration and heart rate increases were observed in awake but not in isoflurane-anesthetized animals (Fig. 1). MABP was main-

tained in awake rats under graded hypoxia but was markedly reduced in anesthetized rats. These observations suggest that hypoxia-induced compensatory responses were attenuated under 2% isoflurane anesthesia. Increased respiration in awake rats is expected to be helpful in compensating for reduced inspired oxygen tension. Ironically, hyperventilation greatly reduces CBF and thus tissue oxygen delivery because hyperventilation-induced hypocapnia leads to vascular constriction (Fig. 2), although reduced CBF increases oxygen extraction to some extent. Furthermore, the hypoxia-related changes in $p\text{CO}_2$ and pH also affect the oxygen unloading from oxyhemoglobin via the Bohr effect; this also has an effect on oxygen delivery.

In the anesthetized and spontaneously breathing animals, no hypoxia-induced CBF increase was observed (Figs. 3 and 4). Instead, CBF decreased from 1.13 ± 0.12 ml/g/min during 21% O_2 (arterial $p\text{O}_2 = 81 \pm 4$ mm Hg, $p\text{CO}_2 = 40 \pm 3$ mm Hg) to 0.69 ± 0.12 ml/g/min during 9% O_2 exposure (arterial $p\text{O}_2 = 31 \pm 3$ mm Hg, $p\text{CO}_2 = 29 \pm 2$ mm Hg). In a previous study in which the animals were anesthetized with 2% isoflurane but mechanically ventilated (Duong et al., 2001a), CBF decreased from 1.3 ml/g/min under 21% O_2 (arterial $p\text{O}_2 \sim 100$ mm Hg, $p\text{CO}_2 = 30\text{--}40$ mm Hg) to 0.5 ml/g/min under 10–12% O_2 (arterial $p\text{O}_2 \sim 30$ mm Hg, $p\text{CO}_2 = 30\text{--}40$ mm Hg). These two studies are qualitatively similar. Both studies reported hypoxia-induced CBF decreases, likely due to hypoxia-induced hypocapnia and the suppression of the autonomic responses to hypoxia by isoflurane. The latter is also supported by the monotonic decrease of CBF with decreasing $p\text{CO}_2$ in the anesthetized (but not awake) condition (Fig. 5). There are, however, some notable differences between the results of the two studies. In the mechanically ventilated rats under anesthesia, animals: (1) had lower arterial $p\text{O}_2$, (2) were less hypocapnic, and (3) showed a larger CBF decrease for similar hypoxic exposure, relative to spontaneously breathing rats under anesthesia. These quantitative differences could be explained by the differences in animal physiology arising from “spontaneous breathing” versus “mechanically ventilation”. Mechanical ventilation prevented the anesthetized animals from changing their respiration rate and volume, resulting in a lower $p\text{O}_2$ and a higher $p\text{CO}_2$ compared to the spontaneously breathing, anesthetized animals. Despite being less hypocapnic under mechanical ventilation (Duong et al., 2001a), CBF under mechanically ventilated anesthetized animals was lower compared to the spontaneously breathing and anesthetized animals herein, potentially due to the confounding effects of mechanical ventilation and anesthesia.

Interestingly, other studies have reported hypoxia-induced CBF increases in anesthetized animals. A hypoxia-induced CBF increase of $\sim 80\%$ (measured using butanol indicator-fractionation method) was reported in choral hydrate-anesthetized rats with an arterial $p\text{O}_2$ of 49 mm Hg (Shockley and LaManna, 1988). A hypoxia-induced CBF increase was reported in mechanically ventilated animals that were anesthetized with thiopental (Julien-Dolber et al., 2002). These differences in findings could be due to the use of different anesthetics, the duration of hypoxic exposure, or other methodological details. There is evidence that isoflurane interferes with cerebral autoregulation during hypoxia in a dose-dependent manner (Hoffman et al., 1991; Lee et al., 1994). This could partially account for the discrepancy across some laboratories.

3.3. CBF in awake conditions

In the awake conditions, CBF reduction was smaller under the awake state compared to the anesthetized state at low arterial $p\text{O}_2$ (< 50 mm Hg) (Fig. 4c) although the awake animals were more hypocapnic (Fig. 2). These comparisons suggest that a hypoxia-induced CBF increase was present but masked by the hypoxia-induced hypocapnia. This notion is also supported by the observation that the CBF leveled off with decreasing arterial $p\text{CO}_2$ in awake but not in anesthetized animals (Fig. 5). The hypoxia-induced hypocapnia has been reported previously (Marshall and Metcalfe, 1989; Bao et al., 1997; Poulin et al., 2002). Bao et al. (1997) reported a marked hypoxia-induced hypocapnia although they did not measure CBF. Poulin et al. (2002) reported hypoxia-induced hypocapnia and a decrease in CBF as measured by transcranial Doppler ultrasound. Interestingly, when CO_2 was added to the inspired gas to maintain physiological blood $p\text{CO}_2$, the CBF decrease was abolished (Poulin et al., 2002).

Hypoxia-induced CBF increases have also been reported in awake animals. CBF measured using (14C)iodoantipyrine technique increased 25–90% in awake rats exposed to 10% oxygen (Bereczki et al., 1993), and CBF measured using radiolabeled microspheres increased 200–250% in awake sheep with an arterial $p\text{O}_2$ of 40 mm Hg while the arterial $p\text{CO}_2$ was allowed to vary (Iwamoto et al., 1991). These results contradict our findings as well as those of others (Bao et al., 1997; Poulin et al., 2002). One possible explanation could be the different physiological conditions under which the animals were prepared. For example, basal inspired oxygen tension prior to hypoxic challenge affects the hypoxic response (Kannurpatti et al., 2002). When the basal inspired oxygen tension before apnea was 21% in mechanically ventilated and anesthetized rats, MABP decreased and CBF, as measured by Laser Doppler technique, markedly increased. When the basal inspired oxygen tension before apnea was 100%, MABP remained constant and CBF increased slightly. BOLD signal responses decreased when the basal inspired $p\text{O}_2$ before apnea was 21% but increased when the basal inspired $p\text{O}_2$ was 100% before apnea. These comparisons underscore the importance of specifying the experimental conditions as well as carefully monitoring physiological parameters.

3.4. BOLD responses

BOLD signals decreased under hypoxia for both awake and anesthetized conditions as expected. BOLD changes largely paralleled CBF changes. There are, however, some differences in the BOLD responses to hypoxia between awake and anesthetized conditions. The BOLD signals under anesthetized conditions decreased more significantly at the same hypoxic conditions even after adjusting for blood gas $p\text{O}_2$ values (Fig 4c). Quantitative interpretation of BOLD signals is, however, complicated because of (1) the potential loss of cerebral autoregulation under anesthesia, (2) strong perturbation of vascular coupling leading to non-linearity of the BOLD signals under hypoxia, and (3) alkaline-induced changes in the affinity of hemoglobin for oxygen.

3.5. CMRO₂ changes

CMRO₂ is an important physiological parameter. Davis' biophysical model uses CBF and BOLD measurements to derive CMRO₂ (Davis et al., 1998). This derivation relies on the constants α and β which are pixel-specific. However, Davis et al. showed that CMRO₂ is weakly dependent on α and β . The calculated CMRO₂ changes herein were relatively independent for β ranging from 1 to 1.5 (data not shown). Further improvements in Davis' CMRO₂ MRI model can be made by validating the assumptions in the CMRO₂ formalism, demonstrating the reproducibility of the CMRO₂ maps, and cross-validating with PET-CMRO₂ measurements. Nonetheless, CBF and BOLD hypoxia data superimposed on the iso-CMRO₂ contour plots provided another perspective to analyze the hypoxia data. Our analyses suggest mild hypercapnia and hypoxia did not significantly change CMRO₂. Severe hypoxia (9% O₂) and severe hypercapnia (10% CO₂), however, decreased CMRO₂ under anesthetized conditions but not under awake conditions, suggesting that isoflurane anesthesia could be a confounding factor for energy metabolism under hypoxia. An alternative explanation is that strong physiological perturbations could render the CMRO₂ biophysical model inaccurate. Cross-validation studies are needed.

In summary, BOLD and CBF responses to identical hypoxic exposure are remarkably different between awake and anesthetized rats. In contrast to the anesthetized condition, compensatory mechanisms in the awake state allow blood pressure to be maintained, heart rate and respiration rate increase. CBF and tissue oxygenation in awake animals decrease to a lesser extent and appear to level off during hypoxia whereas CBF and tissue oxygenation in anesthetized animals decrease monotonically. CMRO₂ appears to decrease under severe, but not mild, hypoxia, although alternative explanation is possible. This study also further supports the feasibility of BOLD and CBF fMRI of conscious and restrained animals in a serial and non-invasive manner, and underscores the marked differences in BOLD and CBF responses to hypoxia between awake and anesthetized models for fMRI studies.

4. Experimental procedures

4.1. Animal preparation

These studies were approved and closely monitored by Institutional Animal Care and Use Committee (IACUC). Seven Sprague–Dawley rats (250–300 g) were imaged under awake and anesthetized (2% isoflurane) conditions in the same animals. Rats were initially anesthetized with 2% isoflurane. A femoral artery was catheterized for monitoring the mean arterial blood pressure (MABP), heart rate (HR), and for sampling blood gases. Topical anesthetics (2% lidocaine) was applied to the surgical site which was sutured close. Respiration rates (RR) and waveforms were continuously monitored via a force transducer. MABP, HR, and RR were recorded onto a PC using the BIOPAC system (MP100 model, Santa Barbara, CA). The rectal temperature was monitored and maintained at 37 ± 1 °C via a feedback-regulated,

circulating-water pad. Rats were immobilized in a rat restrainer with ear-, nose-, tooth-, and shoulder-bars, and a body restraining tube (Sicard et al., 2003). All rats respired spontaneously without mechanically ventilation.

4.2. Hypoxia

For each animal, 17%, 12%, and 9% O₂, (balance N₂) with air (21% O₂) as the baseline in a random order were studied under awake and anesthetized conditions (randomized). The randomized acquisition minimized potential habituation effects related to restraint and/or repeated hypoxia. In a typical trial, the animal was exposed to 1:00 min air, 1:30 min hypoxia, 2:00 min air, 1:30 min hypoxia, and 0:45 min air. At least 15 min elapsed between different hypoxia exposures and between awake and anesthetized conditions.

4.3. MR experiments

All MRI experiments were performed on a 4.7-T/40-cm horizontal magnet a 20-G/cm magnetic field gradient insert (Biospec Bruker, Billerica, MA). Animals were placed in a restrainer (Sicard et al., 2003) that had a built-in, actively decoupled brain imaging surface coil (2.3-cm ID) and a neck coil for CBF labeling. Coil-to-coil electromagnetic interaction was actively decoupled. Anatomical images were acquired using the fast spin-echo pulse sequence (RARE) with TR=2 s, effective TE=65 ms, matrix=128×128, FOV=2 cm×2 cm, six 1.5-mm slices, and 4 averages.

Simultaneous CBF and BOLD measurements were made using the two-coil continuous arterial spin-labeling method (Silva et al., 1999; Duong et al., 2000) with single-shot, gradient-echo, echo-planar-imaging (EPI) acquisition. Paired images were acquired alternately—one with arterial spin labeling and the other without spin labeling. The MR parameters were: data matrix=64×64, FOV=2 cm×2 cm, six 1.5-mm slices, TE=17 ms, and TR=2 s. For each trial, 100 pairs of images (~6 min 45 s) were acquired. The entire study took ~3 h. The EPI image has a SNR of at least 30 (without signal averaging).

4.4. Data analysis

Image analysis employed codes written in Matlab (MathWorks Inc, Natick, MA) and STIMULATE software (Univ. of Minnesota). BOLD image was obtained from the control images of the CBF measurement. CBF image (S_{CBF}) with intensity in unit of ml/g/min was calculated pixel-by-pixel using (Silva et al., 1999; Duong et al., 2000), $S_{CBF} = \lambda / T_1^* (S_C - S_L) / [S_L + (2\alpha - 1)S_C]$, where S_C and S_L are signal intensities of the control and labeled images, respectively. λ is the water brain–blood partition coefficient, T_1 is the water spin-lattice relaxation time of tissue, and α is the arterial spin-labeling efficiency. λ was taken to be 0.9 (Herscovitch and Raichle, 1985). T_1 and α were measured to be 1.5 s and 0.75, respectively.

BOLD and CBF percent-change maps were also calculated. BOLD and CBF data were correlated with physiological measurements. Quantitative CBF and relative CBF and BOLD changes were analyzed with whole-brain region of interests (ROI), avoiding surface vessels, without statistical thresholds to avoid bias. The same ROIs were used for awake and anesthe-

tized conditions in the same animal. All statistical tests employed the two-tail paired t-test and all reported values are in mean \pm SD. Error bars on graphs are standard errors of the means.

To evaluate the possible CMRO₂ changes associated graded hypoxia, the awake and anesthetized hypoxia data were overlaid onto iso-CMRO₂ contour plots for comparison. The iso-CMRO₂ contour plots were calculated using (Davis et al., 1998; Hoge et al., 1999),

$$\frac{\Delta\text{BOLD}}{\text{BOLD}_0} = M \left(1 - \left(\frac{\text{CMRO}_2}{\text{CMRO}_{2_0}} \right)^\beta \left(\frac{\text{CBF}}{\text{CBF}_0} \right)^{\alpha-\beta} \right) \quad (1)$$

where M is the proportionality constant and parameters with subscript zero indicate baseline values. α is the Grubb's factor in $\text{CBV} = \text{CBF}$ and a value of 0.4 was used (Eichling et al., 1974; Grubb et al., 1974; Mandeville et al., 1999). β is a proportionality constant relating deoxyhemoglobin concentration to the BOLD signals and a value of 1.2 was used (Boxerman et al., 1995; Davis et al., 1998). The proportionality constant M relating BOLD and CBF changes reflects the maximum BOLD response that can be expected from a given region. An average M value of 0.16 was used (Liu et al., 2004). Published data from hypercapnia and hypoxia and functional stimulation experiments obtained under essentially identical experimental protocols (Sicard et al., 2003; Liu et al., 2004; Sicard and Duong, 2005; Schmidt et al., 2006) were also overlaid for comparison.

Temporal dynamics of the fMRI responses were not analyzed because the stimulus onsets and flow rates of the gas challenges were manually controlled. The temporal dynamics between awake and anesthetized conditions was addressed using somatosensory stimulation previously and we found that the onset times of the awake conditions were markedly shorter (Sicard et al., 2003), consistent with isoflurane being a potent vasodilator.

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