

The influence of carbon dioxide on brain activity and metabolism in conscious humans

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A better understanding of carbon dioxide (CO₂) effect on brain activity may have a profound impact on clinical studies using CO₂ manipulation to assess cerebrovascular reserve and on the use of hypercapnia as a means to calibrate functional magnetic resonance imaging (fMRI) signal. This study investigates how an increase in blood CO₂, via inhalation of 5% CO₂, may alter brain activity in humans. Dynamic measurement of brain metabolism revealed that mild hypercapnia resulted in a suppression of cerebral metabolic rate of oxygen (CMRO₂) by 13.4% ± 2.3% (N=14) and, furthermore, the CMRO₂ change was proportional to the subject's end-tidal CO₂ (Et-CO₂) change. When using functional connectivity MRI (fcMRI) to assess the changes in resting-state neural activity, it was found that hypercapnia resulted in a reduction in all fcMRI indices assessed including cluster volume, cross-correlation coefficient, and amplitude of the fcMRI signal in the default-mode network (DMN). The extent of the reduction was more pronounced than similar indices obtained in visual-evoked fMRI, suggesting a selective suppression effect on resting-state neural activity. Scalp electroencephalogram (EEG) studies comparing hypercapnia with normocapnia conditions showed a relative increase in low frequency power in the EEG spectra, suggesting that the brain is entering a low arousal state on CO₂ inhalation.

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Introduction

Carbon dioxide (CO₂) is a potent vasodilator, and an increase of CO₂ in the inspired air is known to cause a number of vascular changes in the brain (Kastrup *et al.*, 1999; Rostrup *et al.*, 2000; Sicard and Duong, 2005), including increased cerebral blood flow (CBF), cerebral blood volume, as well as higher CO₂ and O₂ concentrations in the blood. The potential effect of CO₂ inhalation on neural activity is not clear. Existing literature mostly presumes that altering CO₂ partial pressure has no effect on brain tissue and the use of CO₂ inhalation or the carbonic anhydrase inhibitor acetazolamide can be considered

a purely vascular challenge to assess cerebrovascular reserve (de Boorder *et al.*, 2004) or to calibrate functional magnetic resonance imaging (fMRI) signal (Chiarelli *et al.*, 2007; Davis *et al.*, 1998; Kim and Ugurbil, 1997).

Despite the wide acceptance and application of these assumptions in cerebrovascular research, some evidence suggested that the influence of CO₂ on brain activity merits more research. An important marker for tissue function, cerebral metabolic rate of oxygen (CMRO₂), has been investigated under hypercapnia conditions. The results, however, were not consistent in the literature. A few studies (Barzilay *et al.*, 1985; Kety and Schmidt, 1948; Novack *et al.*, 1953) reported that CMRO₂ remains constant with hypercapnia, whereas other studies found a decrease (Kliefelth *et al.*, 1979; Kogure *et al.*, 1975; Sicard and Duong, 2005) or increase (Horvath *et al.*, 1994; Jones *et al.*, 2005; Yang and Krasney, 1995) in CMRO₂. More recent findings in brain slices and anesthetized animals provided evidence at the cellular level that higher CO₂ partial pressure can have a profound effect on neural tissue including reducing pH, elevating adenosine concentration, and

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suppressing synaptic potentials (Dulla *et al*, 2005; Gourine *et al*, 2005; Zappe *et al*, 2008b). However, these previous studies were largely performed under laboratory conditions, and it is unclear if the results were influenced by certain factors including the anesthetic agent, which by itself will reduce neural activity (Shulman *et al*, 1999). We are therefore interested in conducting measurements in conscious human subjects under physiologically relevant conditions.

The study of neural response to CO_2 pressure change is particularly challenging compared with studies using sensory, motor, or cognitive stimuli. Traditional tools such as PET, fMRI, and optical imaging are not readily usable, because these methods all use vascular response as a surrogate for neural function (Fox and Raichle, 1986). However, CO_2 is known to have a strong vascular effect that is present even if neural activity does not change (e.g., it has been shown to change vessel size even in dissected blood vessels). Therefore, an observation of altered blood flow, blood volume, or BOLD fMRI signal alone would not necessarily suggest a change in neural activity (Biswal *et al*, 1997). In fact, the vascular effect of CO_2 may be a nuisance in the study of tissue. It is perhaps for these reasons that the exact influence of CO_2 inspiration on neural activity is not fully characterized to date (Horvath *et al*, 1994; Jones *et al*, 2005; Kety and Schmidt, 1948; Kliefeth *et al*, 1979).

This study used three noninvasive techniques to assess the effect of breathing a CO_2 -enriched air (5% CO_2 , 21% O_2 , and 74% N_2) on brain tissue. We have recently developed an MRI technique to quantify CMRO_2 (Lu and Ge, 2008; Xu *et al*, 2009), allowing us to assess the effect of CO_2 on neural metabolism. In the first study, we compared the participant's CMRO_2 levels when breathing CO_2 -enriched gases (referred to as hypercapnia) to breathing normal room-air gases (normocapnia). In the second study, we investigated the spontaneous neural activity under normocapnia and hypercapnia conditions by performing functional connectivity MRI (fcMRI). Finally, neural electrical potential was investigated directly by scalp electroencephalogram (EEG) under room-air and CO_2 breathing conditions.

Materials and methods

Participants

A total of 50 healthy subjects (27.9 \pm 6.5 years old, 32 males and 18 females) were studied. Each subject gave an informed written consent before participating in the study. The study protocol was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. These subjects were divided into four groups for different arms of the study. Fourteen subjects were studied to assess the effect of CO_2 inhalation on CMRO_2 . Ten subjects were studied in the CMRO_2 sham control study. Fourteen subjects were studied to assess the

effect of CO_2 on resting-state functional connectivity. Twelve subjects were studied to assess the effect of CO_2 on EEG spectra.

Carbon Dioxide Manipulation

Carbon dioxide delivery was achieved by using a gas delivery system created in our laboratory, with only nonmetallic components (see Supplementary Figure S1 for a diagram). With this system, a change in gas content can be made without stopping the scan or recording and without moving the subject. Other details of the CO_2 manipulation are described in Supplementary Text.

Measurement of Global Cerebral Metabolic Rate of Oxygen

Measurement of CMRO_2 was performed on a 3 Tesla MRI system (Philips Medical Systems, Best, The Netherlands) using a noninvasive technique (Lu and Ge, 2008; Xu *et al*, 2009). Briefly, CMRO_2 in units of $\mu\text{mol}/\text{min}$ was calculated using Fick principle:

$$\text{CMRO}_2 = \text{CBF}(Y_a - Y_v)C_a \quad (1)$$

where CBF is the blood flow in mL/min , Y_a is the arterial oxygenation, Y_v is the venous oxygenation, C_a is the amount of oxygen molecules that a unit volume of blood can carry, assumed to be $833.7 \mu\text{mol O}_2/100 \text{mL}$ blood based on physiology literature (Guyton and Hall, 2005). The CBF was measured with phase-contrast MRI, Y_a was determined by a pulse oximeter; Y_v was estimated with a recently developed technique, T2-relaxation-under-spin-tagging MRI (Lu and Ge, 2008). The details of the imaging parameters are described in Supplementary Text.

The experiment began with the subject breathing room-air for 4 minutes, establishing a normocapnic state, during which CMRO_2 was determined. The subject was then switched to the gas mixture containing 5% CO_2 and continued to breath for another 6 minutes. We waited for 2 minutes to allow for physiological stabilization in the hypercapnic state (Figure 1). The CMRO_2 was measured again in the last 4 minutes under stable hypercapnia condition.

The data processing procedures for T2-relaxation-under-spin-tagging MRI and phase-contrast MRI were based on an algorithm described previously (Lu and Ge, 2008; Xu *et al*, 2009) and are described in Supplementary Text and Supplementary Figure S2. The arterial blood oxygenation, Y_a , was recorded at 1 sample/s and the data corresponding to MRI acquisition period (about 4 minutes) were averaged to yield the final value.

Once Y_a , Y_v , and CBF were obtained, CMRO_2 was calculated from these variables using equation (1). The CMRO_2 values during normocapnia and hypercapnia were compared using a paired Student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

A sham control study was conducted in a group of healthy subjects ($N=10$, age 28.7 \pm 5.7 years, 6 males, 4 females), in which the subject was given the same instruction as the real CO_2 study (they were told that they

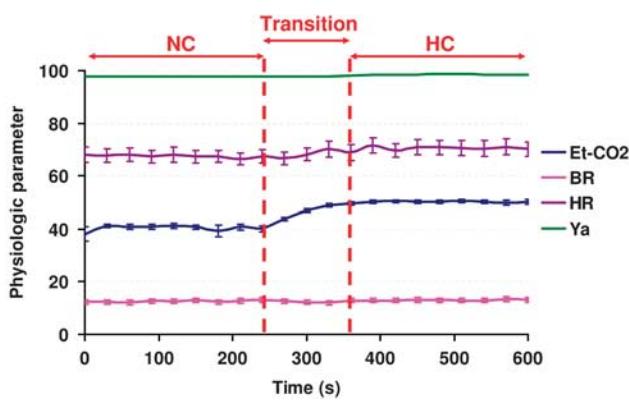


Figure 1 Time courses of physiologic parameters under normocapnia (NC) and hypercapnia (HC) conditions. End-tidal (Et) CO_2 , heart rate (HR), breathing rate (BR), and arterial oxygenation (Y_a) were monitored and recorded during the cerebral metabolic rate of oxygen (CMRO_2) experiments. After the gas valve was switched to the CO_2 mixture, the first 2 minutes were considered as a transition period, during which no magnetic resonance imaging (MRI) measurements were made. Error bars indicate standard errors of mean across subjects ($N = 14$). Hypercapnia resulted in an increase in Et- CO_2 , HR, and Y_a ($P < 0.05$). The BR did not show a significant change.

will inhale CO_2), but the gas valve was on room-air for the entire duration of the experiment. This was conducted to rule out the possibility that the observed CMRO_2 change in the CO_2 breathing task was due to mental stress or the subject becoming anxious, drowsy, and/or sleepy after being inside the magnet. All other procedures and MRI scans were identical to the real CO_2 study.

Functional Connectivity Magnetic Resonance Imaging

The fcMRI scans were performed while the subjects were fixated on a crosshair. For comparison, a visual-task fMRI using a flashing checkerboard was also performed. The details of the imaging parameters are described in Supplementary Text. Each scan was performed twice, first under a normocapnia condition and the other under a hypercapnia condition. Similar to the CMRO_2 studies, we waited for 2 minutes to allow a transition between normocapnic and hypercapnic conditions.

We used AFNI (NIMH Scientific and Statistical Computing Core, Bethesda, MD, USA) and in-house Matlab scripts for fcMRI and visual fMRI data processing. The processing of visual fMRI used standard procedures and is described in Supplementary Text. The steps of the fcMRI processing followed those described by Hong *et al* (2009). Briefly, each data set was preprocessed with slice timing correction, motion correction (realignment), removal of the linear trend, transformation to standard Talairach space (matrix = $61 \times 73 \times 61$, resolution = $3 \times 3 \times 3 \text{ mm}^3$), and smoothing by a Gaussian filter with a full width at half maximum of 6 mm. The fcMRI data were then analyzed using a seed-based approach to identify the resting-state networks. Low-pass filtering (cutoff frequency = 0.1 Hz) was applied to the preprocessed signal time course on a voxel-by-voxel basis. Cardiac and respiratory pulsation effects were removed

using the respiratory belt and pulse sensor that are integrated into the MRI system. The fluctuations of end-tidal CO_2 (Et- CO_2) and the whole brain averaged fMRI signal were also removed from the subject's voxel time course. For the default-mode network (DMN), seed regions of interest (ROIs) (size = 0.73 cm^3) were positioned at bilateral posterior cingulate cortices based on Talairach coordinates. The cross-correlation coefficient (cc) between these seed voxels and all other voxels was calculated to generate a correlation map. Then, a Fisher-z transform was used and a group analysis was performed on the z-maps of all subjects to identify the DMN (threshold: $P < 0.005$, cluster size $> 2.7 \text{ cm}^3$). The data sets from normocapnia and hypercapnia conditions were analyzed separately.

Three fcMRI indices were compared between the normocapnia and hypercapnia conditions. An index of volume of correlated clusters was defined as the total number of voxels that are significantly correlated with the seed ROI (excluding the voxels in the seed cluster). The cc between the seed voxels and the voxels delineated in the correlated clusters was calculated. To avoid the confounding effect of volume differences between normocapnia and hypercapnia, only the voxels that were detected in both conditions (i.e., overlapping voxels) were included in the cc analysis. As a third index, the amplitude of fcMRI signal fluctuation was calculated. As there is not a ubiquitously defined parameter for fcMRI amplitude, we used an index that is equivalent to the percentage signal change in conventional fMRI. The fcMRI signal, S_{fcMRI} , is defined as follows. Let x and y be the time courses of seed voxels and the voxels in the correlated clusters in fcMRI. Then, S_{fcMRI} is calculated by a linear regression on: $y' = S_{\text{fcMRI}} x'$, where $x' = (x - \text{mean}(x)) / (2 \parallel x - \text{mean}(x) \parallel / \sqrt{n_{\text{dyn}}})$ and $y' = (y - \text{mean}(y)) / \text{mean}(y)$. In this expression, 'mean' indicates the mean signal intensity of the time course, n_{dyn} is the number of points in the time course, and the operator $\parallel \bullet \parallel$ indicates 2-norm. A desirable feature of this index is that, if we apply this calculation to a conventional fMRI data set with the voxel time course as y and the fMRI paradigm as x , the resulting value would be exactly the same as BOLD percentage change obtained from a typical fMRI general linear model analysis. Using this index, the CO_2 effect on fcMRI signal can be feasibly compared with that on visual-evoked fMRI signal.

It should be noted that these hypercapnia-induced decreases in fcMRI signal may be caused by a combined effect of CO_2 on electrophysiological signal and on basal vascular state (Cohen *et al*, 2002), which has a modulatory effect on BOLD signal amplitude. Therefore, we defined a normalized fcMRI signal amplitude (calculated for each individual), $S_{\text{fcMRI},n} = S_{\text{fcMRI}} / S_{\text{fmri}}$, where a visual-evoked fMRI, S_{fmri} , is used as a reference to eliminate the vascular effect from fcMRI signal. This calculation assumed that differences in S_{fmri} between normocapnia and hypercapnia were primarily due to vascular effect of CO_2 . Previous studies have shown that visual-evoked EEG response is minimally affected by the CO_2 level (Jones *et al*, 2005; Zappe *et al*, 2008b). With the assumption that EEG response provides a reasonable indication of electrophysiological response, we hypothesize that visual-evoked electrophysiological response is also independent of CO_2 .

level. Thus, the visual fMRI signal difference comparing normocapnia and hypercapnia is solely due to the vascular effect. A more detailed description and justification of the normalization calculation is described in Supplementary Text.

In addition to the DMN, sensorimotor regions were also identified from the fcMRI data. The seed regions were positioned in the right motor cortex and the voxels correlating with the seed time course were identified. We have also tested using the left motor cortex for seed regions and the results were similar. Comparison was made between normocapnia and hypercapnia conditions.

Electroencephalogram

We used a 64-channel SynAmps II EEG system (Neuro Scan, Charlotte, NC, USA), which included an EEG cap on which the geometric locations of the electrodes are preset. Saline was applied in each electrode to ensure proper electrode conduction, the impedance of which is monitored on a computer screen. To avoid mechanical pressure on the electrodes, these experiments were conducted with subject sitting upright, unlike the MRI experiments in which the subjects were at supine position. We assumed that the CO_2 effect, if any, is comparable between these two body positions. The subjects were instructed to keep their eyes open and fixate on a white cross throughout the experiments. This control of eye fixation is important as it can change the EEG power spectra (Kiloh *et al.*, 1972).

The experiments were conducted for 17 minutes during which time the subjects were breathing room-air for 5 minutes, switched to the 5% CO_2 gas mixture for 7 minutes and then returned to room-air for the final 5 minutes. The EEG recordings were made continuously during this 17-minute period. The EEG data from the first and second normocapnia periods were combined to yield an interpolated signal that will be compared with the signal of the hypercapnia period. The interpolation computation was necessary to correct for linear trends, hysteresis, and rebound effects in the recordings (Zappe *et al.*, 2008b), which could cause a signal drift over time.

The data were recorded with a reference electrode located near the vertex, resulting in small amplitudes over the top of the head. To eliminate this effect, the data were rereferenced to the average potential over the entire head, which approximates the voltages relative to infinity. To reduce a slight bias in the electrode-based average reference, spherical splines were fit to the data and used to compute the average. Other details of the EEG processing procedures are described in Supplementary Text.

Power spectra of three frequency bands were investigated based on well-established frequency definition: 1 to 3 Hz for δ band, 4 to 7 Hz for θ band, and 8 to 13 Hz for α band (Kiloh *et al.*, 1972). The analysis was performed for each of the channels as well as for the average of all channels.

Results

Inhalation of 5% CO_2 increased the subjects' Et- CO_2 from 41.3 ± 0.8 mm Hg ($N=14$, 9 males and 5 females,

$\text{mean} \pm \text{s.e.m.}$) to 50.1 ± 0.7 mm Hg ($P<0.001$). Figure 2A shows representative T2-relaxation-under-spin-tagging MRI data for the normocapnia and hypercapnia conditions. The MR T₂ signal decay is much slower during hypercapnia, suggesting a longer venous blood T₂. Accordingly, venous oxygenation, Y_v , was found to increase significantly (paired *t*-test, $P<0.001$) from normocapnia to hypercapnia (Table 1). Arterial oxygenation (Y_a) measured by pulse oximeter showed a negligible change between the two conditions (Table 1). Thus, the oxygen extraction fraction, i.e., $Y_a - Y_v$, reduced considerably (Table 1). Figure 2B shows representative phase-contrast MRI results. One can see a clear increase of flow velocity as indicated by darker pixel intensity (arrows). Quantitative analysis revealed that CBF increased significantly from normocapnia to hypercapnia ($P<0.001$). Table 1 summarizes the values of the physiologic parameters during normocapnia and hypercapnia conditions. The relative changes are plotted in Figure 2C. Using equation (1), CMRO_2 was estimated and found to show a reduction of $13.4\% \pm 2.3\%$ comparing hypercapnia with normocapnia ($P<0.001$). That is, in addition to the pronounced vascular effect, high CO_2 content also modestly reduces tissue's metabolic rate. In the sham study, CMRO_2 values during the baseline and sham control periods were $977.6 \pm 88.5 \mu\text{mol}/\text{min}$ and $995.4 \pm 89.4 \mu\text{mol}/\text{min}$, respectively, and did not show a difference ($P=0.34$, paired *t*-test, $N=10$) (see Supplementary Table S1).

We further hypothesized that CO_2 has a dose-dependent effect on CMRO_2 where an individual with a smaller Et- CO_2 change during the hypercapnic phase will have a smaller change in CMRO_2 and vice versa. Figure 3 shows a scatter plot between Et- CO_2 change and CMRO_2 change across the 14 subjects studied. A significant correlation ($P=0.014$) was observed.

Figure 4 shows DMN in both the normocapnia and hypercapnia conditions identified from the fcMRI data ($N=14$). The DMN under normocapnia condition (Figure 4A) clearly illustrates the connectivity among posterior cingulate cortex (seed region), bilateral inferior parietal regions, medial prefrontal cortex, and medial temporal lobe. In contrast, cluster volume under hypercapnia condition (Figure 4B) shows a drastic reduction. Similarly, the cc values between the seed region and the other DMN regions were reduced significantly (paired *t*-test $P=0.024$) comparing hypercapnia with normocapnia. The amplitude of fcMRI signal also showed a reduction by 37.2% due to hypercapnia ($P=0.001$). The application of similar analysis to the visual fMRI data revealed that the activation volume, cc, and fMRI signal amplitude were reduced by 13.5%, 2.4%, and 22.1%, respectively, when comparing results under hypercapnia with that under normocapnia (Table 2). All indices showed smaller hypercapnia-induced changes compared with those in the fcMRI data. The normalized fcMRI signals were

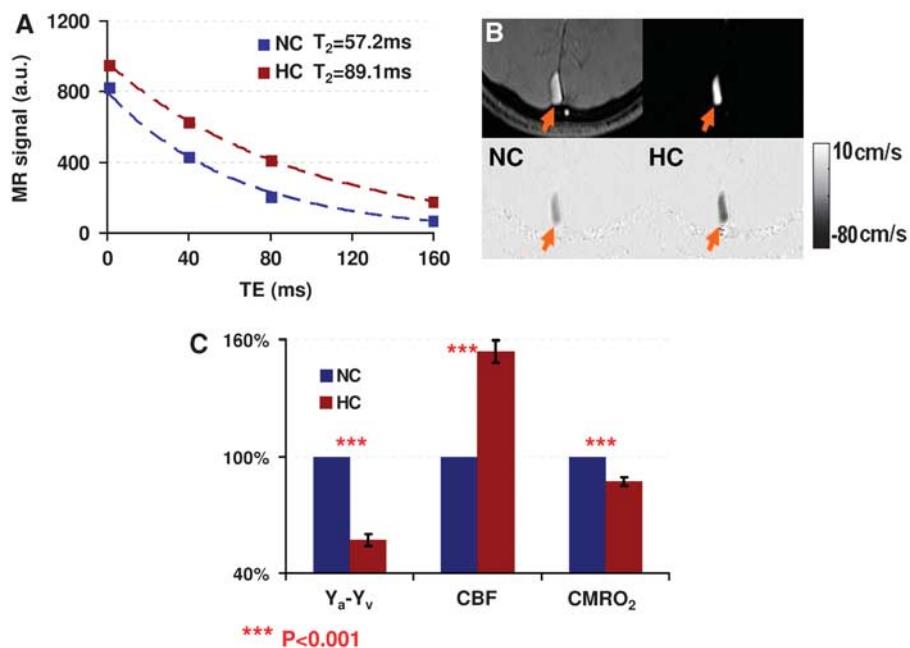


Figure 2 Magnetic resonance imaging (MRI) measurement of cerebral metabolic rate of oxygen (CMRO_2) under normocapnia (NC) and hypercapnia (HC) conditions. **(A)** Venous oxygenation, Y_v , used in the CMRO_2 calculation was measured with a T2-relaxation-under-spin-tagging (TRUST) MRI technique. The MR signal in the venous blood was measured as a function of TE. The decay time constant is the blood T_2 , which can be converted to blood oxygenation. The time constant during hypercapnia (HC, red) is longer than that in normocapnia (NC, blue). **(B)** Cerebral blood flow (CBF) used in the CMRO_2 calculation was measured with phase-contrast MRI applied in the sagittal sinus. The four images are: raw image, magnitude image, velocity map during NC, and velocity map during HC. Arrows indicate the sagittal sinus. In the velocity maps, the darker color indicates higher outflow velocity. **(C)** Summary of CO_2 -induced changes in $Y_a - Y_v$, CBF, and CMRO_2 ($N = 14$). The parameter values under normocapnia were set as 100%. The values under hypercapnia condition were presented relative to the normocapnia values.

Table 1 Summary of vascular and metabolic parameters under normocapnia and hypercapnia conditions

	Y_v	Y_a	$Y_a - Y_v$	CBF (mL/min)	CMRO_2 ($\mu\text{mol/min}$)
Normocapnia	0.624 ± 0.017	0.979 ± 0.003	0.355 ± 0.067	352.8 ± 16.8	1042.3 ± 76.1
Hypercapnia	0.782 ± 0.014	0.985 ± 0.002	0.203 ± 0.055	547.1 ± 35.6	899.0 ± 66.1
Relative change	26.1%	0.6%	-42.6%	54.5%	-13.4%
<i>P</i> value (paired <i>t</i> -test)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

CBF, cerebral blood flow; CMRO_2 , cerebral metabolic rate of oxygen.

0.29 ± 0.03 and 0.23 ± 0.02 for normocapnia and hypercapnia, respectively, showing a 20.1% reduction ($P = 0.020$). In addition to the DMN brain regions, we have studied the sensorimotor regions, which also have a well-defined resting-state network (Biswal *et al*, 1995; Xiong *et al*, 1999). Reductions similar to that observed for the DMN were observed comparing hypercapnia with normocapnia (see Supplementary Figure S3 and Supplementary Table S2 for details).

The EEG recordings with scalp electrodes were compared between normocapnia and hypercapnia conditions ($N = 12$). Table 3 shows the EEG signal power in δ band (1 to 3 Hz), θ band (4 to 7 Hz), and α band (8 to 13 Hz) under normocapnia and hypercapnia conditions. The signal power was significantly

increased in the δ band ($P = 0.049$), accompanied by a significant decrease in the α band ($P = 0.003$) (Figure 5). The θ band power did not show a significant change. Therefore, the relative EEG power was shifted toward the lower frequency range, a typical pattern of reduced brain arousal state (Kiloh *et al*, 1972). Figure 6 shows the topographic maps of EEG power in different frequency bands during normocapnia and hypercapnia conditions, as well as the power ratio. The data suggest that the slowing of the EEG signal appeared to be present in all electrodes across the entire brain. We have also studied frequency ranges beyond 13 Hz (e.g., β band, γ band), but the signal was small and statistical comparisons did not yield significant differences between the two conditions.

Discussion

This study investigated the effect of blood CO_2 levels on brain activity from a number of perspectives. We first showed that hypercapnia reduced metabolic activity in the brain. Second, neural activity was

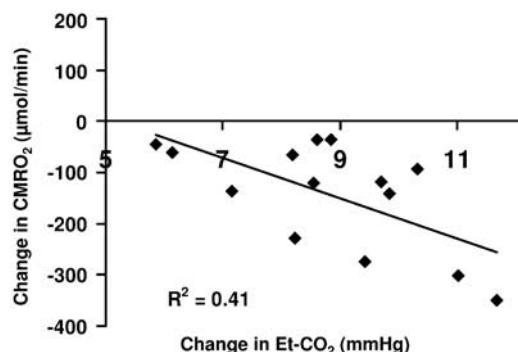


Figure 3 Scatter plot between hypercapnia-induced end-tidal CO_2 (Et-CO_2) change and cerebral metabolic rate of oxygen (CMRO_2) change across subjects ($N = 14$). Inhalation of 5% CO_2 caused an increase in Et-CO_2 and a decrease in CMRO_2 in all subjects studied. Different subjects have slightly different Et-CO_2 change. Individuals with a greater Et-CO_2 change tended to have a greater CMRO_2 change ($P = 0.014$), suggesting a dose-dependent effect of CO_2 on brain metabolism.

assessed indirectly by BOLD fMRI and the data suggested that CO_2 inhalation caused a decrease in spontaneous brain connectivity. Finally, EEG was used as a direct measure of neural activity and the results showed that hypercapnia caused a relative increase in lower frequency power spectra. Overall, our data showed a suppressive effect of CO_2 on brain activity.

The findings from this study are in general agreement with earlier studies conducted in animals. In a study of macaque monkeys under anesthesia, Zappe *et al* (2008b) reported that hypercapnia induced a 15% reduction in resting-state multiunit neural activity in the primary visual cortex. Although the authors did not investigate other brain regions in the study (personal communications), previous literature suggests that this depression effect is likely to be global (Kliefoth *et al*, 1979). Our study represents the first systematic investigation of this effect in conscious humans and suggests that resting-state neural activity is reduced due to mild hypercapnia. Further, we showed that this suppression of neural activity is accompanied by a reduction in metabolic activity of similar amplitude.

A number of studies in the literature have compared CMRO_2 between normocapnia and hypercapnia conditions. Our finding of a 13% decrease is

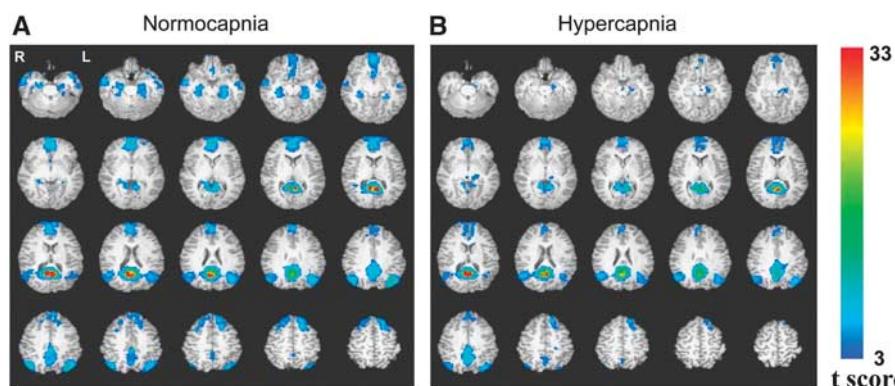


Figure 4 Resting-state default-mode network (DMN) under normocapnia (A) and hypercapnia (B) conditions ($N = 14$). The DMN regions consist of posterior cingulate cortex, bilateral inferior parietal cortex, medial frontal cortex, and bilateral medial temporal lobe, as defined by many previous studies. Hypercapnia resulted in a significant reduction in the size of the cluster volume.

Table 2 Summary of resting-state default-mode network parameters during normocapnia and hypercapnia

	Et-CO_2 (mm Hg)	Cluster volume (mL)		Correlation coefficient		Signal amplitude (%)		
		DMN fcMRI	Visual fMRI	DMN fcMRI	Visual fMRI	DMN fcMRI	Visual fMRI	Normalized fcMRI
Normocapnia	40.2 ± 0.7	120.4	206.9	0.67 ± 0.03	0.69 ± 0.04	0.29 ± 0.02	1.02 ± 0.07	0.29 ± 0.03
Hypercapnia	47.4 ± 0.7	54.3	178.9	0.55 ± 0.05	0.68 ± 0.03	0.18 ± 0.02	0.80 ± 0.08	0.23 ± 0.02
Relative change	18.0%	-54.9%	-13.5%	-17.6%	-2.4%	-37.2%	-22.1%	-20.1%
P value (paired <i>t</i> -test)	< 0.001	N/A ^a	N/A ^a	0.024	0.30	0.001	0.018	0.020

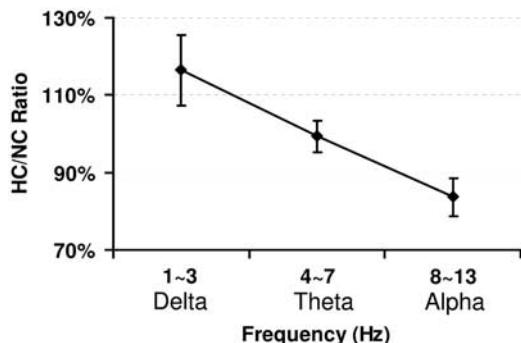
DMN, default-mode network; Et-CO_2 , end-tidal carbon dioxide; fcMRI, functional connectivity magnetic resonance imaging. For comparison, visual-evoked fMRI results are also shown.

^aThis index was obtained from a group analysis, thus the value does not have a s.d. or an associated *P* value.

Table 3 EEG power in different frequency bands under normocapnia and hypercapnia conditions

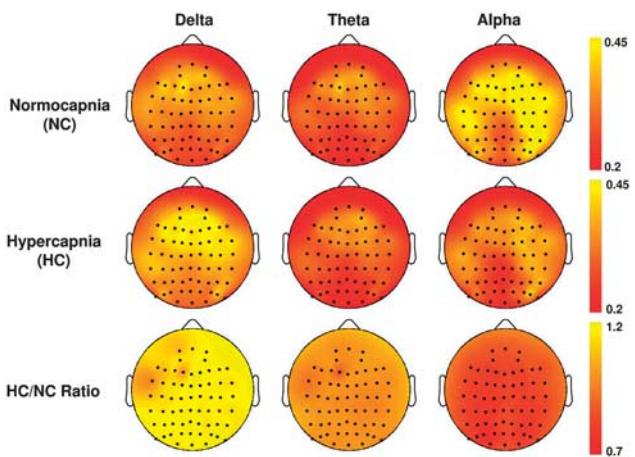
Frequency band	δ	θ	α
Frequency range	1–3	4–7	8–13
Normocapnia power (μV^2)	6.4 ± 0.7	6.4 ± 0.9	10.2 ± 2.1
Hypercapnia power (μV^2)	7.6 ± 1.1	6.3 ± 1.0	8.1 ± 1.8
Ratio between hypercapnia power and normocapnia power	1.16	0.99	0.84
P value	0.049	0.440	0.003

EEG, electroencephalogram.

**Figure 5** Relative changes in electroencephalogram (EEG) signal power comparing hypercapnia with normocapnia. The powers under normocapnia condition were set as 100%. Hypercapnia resulted in a slowing of the EEG power spectra, with δ band (1 to 3 Hz) showing a power increase and α band (8 to 13 Hz) showing a decrease.

in general agreement with the reports by Kogure *et al* (1975) (−15%), Sicard and Duong (2005) (−10%), Barzilay *et al* (1985) (−15%), and Kliefeth *et al* (1979) (−30%), but is inconsistent with those reported by Kety and Schmidt (1948) (+3%), Novack *et al* (1953) (0%), Yang and Krasney (1995) (+17%), and Horvath *et al* (1994) (+35%). Possible reasons for the discrepancy include different measurement techniques, different species and the effect of anesthetic agent. Specifically, only the earlier studies by Kety and Schmidt (1948) and Novack *et al* (1953) used human subjects, and the more recent studies all used animal subjects under anesthesia. It is known that anesthetic agent itself may affect neural activity and metabolism (Shulman *et al*, 1999).

A change in CMRO_2 because of hypercapnia also has important implications for calibrated fMRI. Calibrated fMRI is an approach to obtain quantitative changes in brain metabolism during neural activation (Chiarelli *et al*, 2007; Davis *et al*, 1998; Kim and Ugurbil, 1997). This technique often uses CO_2 inhalation to determine the calibration factor and assumes that mild hypercapnia does not alter CMRO_2 . In the case that CO_2 does cause a decrease in CMRO_2 , the theoretical framework of calibrated fMRI will remain valid but the processing of experimental data should be adjusted slightly. Specifically, the M factor in calibrated fMRI will

**Figure 6** Topographic maps of electroencephalogram (EEG) signals under normocapnia and hypercapnia conditions ($N = 12$). The EEG signal power was calculated for each frequency band. In the topographic maps, the nose of the subject is on the top, illustrated by the triangle. The ears are illustrated by the rectangles. Each black dot indicates one electrode. The bottom row shows the ratio between hypercapnia and normocapnia signals. It can be seen that the δ power increased during hypercapnia, whereas the α power decreased. The θ power remained unchanged. From the ratio map, it can also be seen that hypercapnia-induced changes affected all electrodes. The maps shown were averages of all subjects studied. Before averaging, the EEG power of each subject was normalized to the electrode-averaged power under normocapnia, so that the variations in EEG signal power (e.g., because of differences in scalp thickness) between subjects are accounted for.

become lower, the estimated CMRO_2 will be smaller, and the CBF/CMRO_2 coupling factor n will be greater. Interestingly, these trends will shift the MRI results to become more comparable to earlier reports using PET (Fox and Raichle, 1986).

Our observation of a reduced functional connectivity during CO_2 breathing is consistent with previous reports that light sedation (Greicius *et al*, 2008) or anesthesia (Deshpande *et al*, 2010) decreases connectivity in DMN regions, and is also in line with the suggestions that CO_2 has a mild sedative effect (Fukuda *et al*, 2006). The CO_2 -induced reduction in sensorimotor connectivity is also in good agreement with a previous report by Biswal *et al* (1997). In our study, we have taken special precaution to minimize the influence of basal vascular state on BOLD signals, which were not accounted for in previous studies comparing fMRI data across physiologic states (Biswal *et al*, 1997; Deshpande *et al*, 2010; Greicius *et al*, 2008; Horovitz *et al*, 2009). We have used visual-evoked fMRI signal as a reference with the assumption that the alterations in fMRI signal is solely due to a change in vascular state (Jones *et al*, 2005; Zappe *et al*, 2008a). Although this referencing strategy is still based on assumptions and biophysical models (Davis *et al*, 1998; Jones *et al*, 2005; Zappe *et al*, 2008a), it nonetheless provides an additional level of control

for nonneuronal effect and is the best available technique to our knowledge. Our results demonstrated that the 'normalized' fcMRI signal still showed a significant decrease because of hypercapnia, suggesting that some of the fcMRI signal reduction is of neuronal origin. We have also applied similar normalization procedures to the activation volumes and cc values, and the results showed that the normalized cc showed significant hypercapnia-related reduction ($P=0.03$), although the cluster volume did not show significant changes.

In this study, we found a relative increase in low frequency EEG power spectra on inducing hypercapnia. It is known that EEG frequency is highly dependent on the physiologic state of an individual (Kiloh *et al*, 1972). If the brain is in a high arousal state (e.g., conversation, thinking), the EEG power spectra show a relative increase in higher frequencies, whereas if the brain is in a low arousal state (e.g., relaxing, drowsy, sleepy, day dreaming) lower frequencies predominate. Our data suggest that during hypercapnia, the brain's electrical activity resembles lower arousal states. This finding is consistent with reports that mild hypercapnia may improve the duration and efficiency of sleep (Fraigne *et al*, 2008). This study has focused on the effect of CO_2 inhalation on resting-state EEG. Using intracortical recordings in anesthetized monkeys, Zappe *et al* (2008a) suggested that relative changes in visual-evoked EEG are not affected by CO_2 inhalation. Further work is needed to determine whether this finding is applicable for absolute EEG signal changes in awake humans.

The findings from the three complementary techniques are in general agreement with each other. Our observation that a lower metabolic state corresponds to a slower EEG signal is in agreement with reports by Maandag *et al* (2007) that neurons under low metabolic state have a slower oscillation frequency. The total EEG power in our data did not change from normocapnia to hypercapnia ($P=0.36$). Although this observation seems to be contradictory to the reduced metabolic rate, a number of previous studies have shown that the relationship between metabolism and EEG signal is frequency dependent (Alper *et al*, 2006; Buchan *et al*, 1997). Buchan *et al* (1997) compared EEG and oxygen metabolism in Alzheimer's patients and found that EEG and metabolic rate were positively correlated in frequency components above 8 Hz but they were negatively correlation in frequencies lower than 6 Hz. These findings are consistent with our observations that decreased metabolic rate is accompanied by decreased α band (8 to 13 Hz) power and increased δ band (1 to 3 Hz) power. The relationship between fcMRI and EEG signals has also been studied by a number of investigators, and the majority of the reports appear to suggest that fcMRI signal has a correlation with α power in the EEG (Mantini *et al*, 2007; Moosmann *et al*, 2003). Thus, our observation of a concomitant reduction in fcMRI signal and α power during hypercapnia is in general

agreement with this notion. Another evidence of a link between fcMRI and EEG comes from studies using simultaneous EEG-fcMRI recordings during sleep (Horovitz *et al*, 2009). It has been shown that a slowing of EEG (i.e., greater δ power) is accompanied by reduced functional connectivity among selective DMN regions (Horovitz *et al*, 2009). However, it is not straightforward to directly compare our findings of CO_2 effects to deep sleep, because the neural activity changes during sleep are likely more complex than those because of CO_2 inhalation.

The mechanism of the observed suppression of CMRO_2 is not clear. A few *in vitro* studies suggested that it may be related to tissue acidosis when carbonic acid is formed from CO_2 and water. In a study using brain slices, Dulla *et al* (2005) found that increased CO_2 pressure reduces pH in the extracellular space, which increases the extracellular adenosine concentration. Gourine *et al* (2005) suggested that ATP release may be involved in the CO_2 sensing pathways.

The reduction in spontaneous neural activity as shown by the fcMRI data is also consistent with reports that exposure to hypercapnia can terminate epileptic bursting. In laboratory settings, CO_2 breathing has also been shown to terminate some epileptic seizure activity (Dulla *et al*, 2005). In addition, acetazolamide, sold under the trade name 'Diamox', is a carbonic anhydrase inhibitor and increases CO_2 pressure in the blood. This drug is commonly used as a vasodilator in the assessment of vascular reserve and cerebrovascular function. Interestingly, acetazolamide is also an antiepileptic drug that is used to treat certain types of epilepsy (e.g., absence epilepsy, myoclonic seizure, catamenial epilepsy) (Levy *et al*, 1995). Intake of acetazolamide and inhalation of CO_2 have virtually the same effect and are often considered the same physiologic challenge in the cerebrovascular literature. Therefore, it is reasonable to expect that CO_2 inhalation may also have an antiepileptic effect. Another line of supporting evidence for the CO_2 effect is that hyperventilation causes hypocapnia, and has been known to induce interictal spike-and-wave activity (Niedermeyer and da Silva, 2005).

In the previously mentioned calculation of CMRO_2 , we have only considered oxygen bound to hemoglobin but did not account for oxygen that is dissolved in the plasma. This assumption is considered reasonable in our experiments because the majority (>98%) of the oxygen molecules carried by the blood is in the form of hemoglobin-bound oxygen. To confirm this point, we have incorporated an oxygen sensor in our gas apparatus and measured Et-O_2 pressure in four subjects outside the MRI room. This showed that Et-O_2 under normocapnia and hypercapnia conditions were 114.7 ± 3.1 mm Hg and 136.6 ± 2.3 mm Hg, respectively. The difference is presumably due to hyperventilation when the subject breathes CO_2 . By estimating the amount of dissolved oxygen from Et-O_2 (Guyton and Hall,

2005), CMRO_2 during normocapnia and hypercapnia was recalculated and the results still showed a reduction of CMRO_2 by $10.4\% \pm 2.2\%$.

The normalization calculation in fcMRI signal involved the division of two small numbers, thus errors may propagate nonlinearly. To assess the possibility of false-positive findings, Monte Carlo simulations were performed in which random noise was added to a set of simulated data assuming no differences between normocapnia and hypercapnia. The results showed that the false-positive rate was $\sim 5.3\%$, consistent with the P threshold used ($P=0.05$). Thus, the nonlinear calculation did not seem to cause excessive false-positive rate. The simulations also suggested that the potential errors in the reported percentage changes (-20.1%) were about $\pm 12.5\%$.

The T2-relaxation-under-spin-tagging technique determines venous oxygenation based on a T2-preparation sequence. This sequence uses nonselective pulses to avoid the effect of blood flow on the T2 estimation, as discussed in our previous reports (Lu and Ge, 2008; Xu et al, 2009). Blood flow under hypercapnia is faster, thus additional considerations are needed. Assuming a flow velocity of 46 cm/s in the sagittal sinus under hypercapnia, the travel distance under our T2-preparation duration (160 milliseconds) is estimated to be 7.4 cm. This is well within the coverage of the body coil of our MR system.

In summary, our findings suggest that increased CO_2 levels cause the brain to reduce metabolism and spontaneous neural activity, and enter a lower arousal state.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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