Negative BOLD with Large Increases in Neuronal Activity

Blood oxygen level–dependent (BOLD) functional magnetic resonance imaging (fMRI) is widely used in neuroscience to study brain activity. However, BOLD fMRI does not measure neuronal activity directly but depends on cerebral blood flow (CBF), cerebral blood volume (CBV), and cerebral metabolic rate of oxygen (CMRO$_2$) consumption. Using fMRI, CBV, CBF, neuronal recordings, and CMRO$_2$ modeling, we investigated how the signals are related during seizures in rats. We found that increases in hemodynamic, neuronal, and metabolic activity were associated with positive BOLD signals in the cortex, but with negative BOLD signals in hippocampus. Our data show that negative BOLD signals do not necessarily imply decreased neuronal activity or CBF, but can result from increased neuronal activity, depending on the interplay between hemodynamics and metabolism. Caution should be used in interpreting fMRI signals because the relationship between neuronal activity and BOLD signals may depend on brain region and state and can be different during normal and pathological conditions.

Keywords: cerebral blood flow, epilepsy, fMRI, hippocampus, neuroimaging, neurovascular coupling

Introduction

The blood oxygen level–dependent (BOLD) contrast in functional magnetic resonance imaging (fMRI) has become one of the most popular tools in cognitive neuroscience, mainly due to its noninvasiveness and relatively high spatial–temporal resolution. Although electrophysiological recordings like electroencephalography (EEG), local field potentials (LFP), or multiunit activity (MUA) recordings have superior temporal resolution compared with fMRI, they all suffer from spatial undersampling and in the latter case are highly invasive, limiting its use in human research.

However, the BOLD signal is dependent on complex interplay between cerebral blood flow (CBF), cerebral blood volume (CBV), and the cerebral metabolic rate of oxygen (CMRO$_2$) (Ogawa et al. 1990), which are all related to neuronal activity. Furthermore, evidence suggests that the coupling between these parameters may be altered during aging and disease (D’Esposito et al. 2003). Therefore, a detailed understanding of the relationship between these parameters and the BOLD signal, and most importantly its neuronal correlate, is of paramount importance for the interpretation of BOLD fMRI experiments during normal as well as pathological brain functioning.

Although the positive BOLD response has been shown to be related to increased neuronal activity (Logothetis et al. 2001; Smith et al. 2002) accompanied by hemodynamic changes like increases in CBF and CBV (Huettel et al. 2004), the negative BOLD response is less well understood (Shulman et al. 2007). Sustained negative BOLD signals have been commonly observed (Allison et al. 2000; Harel et al. 2002; Shmuel et al. 2002; Stefanovic et al. 2004; Shmuel et al. 2006); however, detailed studies of its origin are sparse and its explanations controversial. So far, decreased BOLD signals have either been related to some sort of "vascular steal" phenomenon (Harel et al. 2002) or to decreases in neuronal activity (Allison et al. 2000; Gusnard and Raichle 2001; Shmuel et al. 2002; Stefanovic et al. 2004; Shmuel et al. 2006). However, a purely vascular steal-based mechanism cannot explain decreased hemodynamic responses in areas that have distinctly different blood supplies. A recent study that directly measured neuronal activity and fMRI simultaneously revealed that the negative BOLD signal mainly reflected a decrease in neuronal activity (Shmuel et al. 2006). There is another possible mechanism for decreased BOLD signals that has not been thoroughly investigated. Because the BOLD signal depends on the balance between oxygen delivery and consumption (Hyder 2004), it is possible for negative BOLD signals to occur in circumstances of very high neuronal activity, where changes in oxygen consumption may far exceed oxygen supply. Although transient negative BOLD signals (initial dip) have been attributed to this mechanism (Malonek and Grinvald 1996; Yacoub et al. 2001; Chen-Bee et al. 2007), the possibility that this contributes to sustained negative BOLD signals under some circumstances warrants further investigation.

Recent imaging experiments revealed that during general-ized seizures, the brain shows a mixed pattern of focal increased and decreased BOLD activity in humans (Archer et al. 2003; Aghakhani et al. 2004) and animals (Van Camp et al. 2003; Nersesyan, Hyder, et al. 2004), making seizures a suitable condition to study simultaneous negative and positive BOLD signals. In the present study, we therefore performed combined fMRI, CBV, CBF, and neurophysiological recordings, as well as CMRO$_2$ modeling, during bicuculline-induced general-ized seizures in rats to investigate how these different signals are related. We found that during seizures increases in the BOLD signal in the cerebral cortex corresponded to increases in hemodynamic, neuronal, and metabolic responses. At the same time, however, the BOLD signal in the hippocampus often showed prominent decreases or no changes from baseline, whereas hemodynamic, neuronal, and metabolic responses always increased. Our data show for the first time, to our knowledge, that negative BOLD responses can be associated with increased neuronal, metabolic, and hemodynamic
responses and suggest a complex nonlinear relationship between these variables.

**Materials and Methods**

**Animals and General Procedures**

A total of 79 experiments were performed in 39 adult, female, Wistar rats (Charles River Laboratories, Wilmington, MA) with an average weight of 262 ± 4.5 g (mean ± standard error of the mean [SEM]). Fourteen rats were used for the fMRI experiments, and 25 rats were used for combined neurophysiological and laser Doppler flowmetry recordings. All experiments were in accordance with local guidelines, approved by the ethical committee of Yale University School of Medicine, Institutional Animal Care and Use Committee and in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Anesthesia was induced using 1.5% halothane (Halocarbon Products Corp., River Edge, NJ) and later switched to intravenous (i.v.) fentanyl (40 μg/Kg, Hospira, Inc., Lake Forest, IL) and intraperitoneal (i.p.) haloperidol (1 mg/Kg, Bedford Labs, Bedford, OH). Rats were tracheotomized and artificially ventilated (70% N2O and 30% O2). To prevent any movements during the experiments and to facilitate artificial breathing, rats were paralyzed by repeated i.v. injections of 0.5 mg/Kg α-tubocurarine chloride (Sigma-Aldrich, Inc., St. Louis, MO). A femoral artery was cannulated (Intramedic PE50 tubing, Becton Dickinson and Company, Sparks, MD) for continuous arterial blood pressure monitoring and periodic blood sampling for measurements of blood gases and pH using either an i-STAT 1 (i-STAT Corporation, East Windsor, NJ) or an ABL 5 blood gas analyzer (Radiometer Copenhagen, Copenhagen, Denmark). All physiological values (blood gases, mean arterial blood pressure [MABP], and pH) were maintained within physiological range throughout the experiments: pCO2 = 37.5 ± 0.48 mmHg, pO2 = 136.29 ± 2.53 mmHg, MABP = 117.5 ± 2.59 mmHg, pH = 7.37 ± 0.01 units (mean ± SEM). One femoral vein was cannulated and an i.p. line was placed (Intramedic PE10 tubing) for the injections of anesthetics and paralyzing agents. For flow-related CBV fMRI experiments, rats were also injected with iron oxide contrast agent (10–12 mg/kg body weight) (Combidex [ferumoxtran-10], Advanced Magnetics Inc., Cambridge, MA). The other femoral vein was cannulated for the i.v. administration of the γ-aminobutyric acid A (GABA-A) antagonist bicuculline (0.5 mg/Kg, Sigma-Aldrich, Inc.) to induce tonic-clonic seizures. An extra dose of α-tubocurarine was given prior to each bicuculline injection. The body temperature of the rat was monitored with a rectal probe and kept constant around 37°C using a heating pad. At the end of the experiment, rats were euthanized by either an overdose of halothane or by intracardiac injection of Euthasol (Virbac, Hyder, et al. 2004). The wires were secured using tissue glue. All skin and tissue of the rat’s head were left intact except for small insertion of carbon electrodes to minimize distortion of the MRI due to bleeding or fluids. To minimize mechanical transmission of acoustic vibrations from the MRI scanner and to reduce artifacts in the EEG caused by any wire movements in the high magnetic field, the wires were fixed to the lateral surface of the holder and bound together using plastic adhesive tape. The EEG signals were amplified and filtered (1–100 Hz) using a Grass Data Recording System Model 79D (Grass Instruments Co., Quincy, MA). EEG signals were digitized and recorded (sampling rate 1000 Hz) using a CED Micro 1401 and Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

All MRI experiments were acquired on a 9.4-Tesla Bruker horizontal-bore (16-cm-diameter working space) spectrometer (Bruker Avance, Billerica, MA), equipped with passively shielded shim/gradient coils (47.5 G/cm) operating at 400.5 MHz for 1H. The transceiver system consisted of a 1H resonator radio frequency surface coil (15.18 mm diameter) for transmission of radio frequency pulses and receiving. To optimize the homogeneity of the static magnetic field, the system was shimmed before each experiment using either global manual shimming or local FASTMAP (Gruetter 1993).

High spatial resolution anatomical images for each animal were acquired with 11 interleaved slices in the coronal plane using the fast, low-angle shot (FLASH) sequence with time repetition (TR) 100 ms; time echo (TE) 6 ms; flip angle = 30°; field of view (FOV) 25 × 25 mm; 256 × 256 matrix and in-plane resolution of 98 × 98 μm; and slice thickness 1000 μm. BOLD and flow-related CBV fMRI data were obtained in the same planes as anatomical images using single-shot spin echo, planar imaging with the following parameters: TR = 1000 ms, TE = 25 ms; excitation flip angle 90°; inversion flip angle 180°; FOV = 25 × 25 mm; 64 × 64 matrix; and in-plane resolution of 300 × 300 μm; and slice thickness 1000 μm. The fMRI image acquisitions of 11 slices were obtained continuously with 2 s in between image acquisitions, resulting in a total of 600 image acquisitions per 30-min experiment.

**Neurophysiological Experiments and Laser Doppler Flowmetry Recordings**

A total of 45 experiments were performed in 25 rats. Five rats were recorded once, whereas the other 20 rats were recorded twice. No rat underwent more than 2 bicuculline injections (experiments). A detailed summary of how many experiments and rats were used for each individual parameter can be found in Table 1.

Rats were mounted in a stereotactic frame (David Kopf Instruments, Tujunga, CA) placed on a vibration-free table inside a Faraday cage. The skin and underlying tissue over the skull was removed and small burr holes were drilled for the insertion of combined microelectrode/laser-Doppler flowmetry probes (Nersesyan, Herman, et al. 2004) in the barrel cortex (S1BF) (A-P: -2.3 mm; M-L: + 5.02 mm; 30° angle) and dorsal hippocampus (A-P: -3.8 mm; M-L: +2.8 mm). All coordinates were determined according to (Paxinos and Watson 1998) (Bregma zero-zero, skull surface flat). Duratome was used to facilitate microelectrode/probe insertion, and the recording probes were lowered under auditory and visual guidance until touching the cortex, which marked the zero point for microelectrode/probe insertion. The
probes were then slowly advanced perpendicular to the cortex to a final depth between 600 and 1600 μm for S1BF and 2000 and 3500 μm for hippocampus. Special care was taken not to perturb the vasculature during insertion. All microelectrode/probe placements were verified histologically at the end of the experiment and nonconfirmed experiments were discarded from the analysis.

EEG recordings were acquired in differential mode using 2 carbon wires (Biopac Systems, Inc., Goleta, CA) placed laterally on the right side of the frontal and occipital areas between the scalp and the outside surface of the skull, using the same technique as in MRI experiments. The EEG signal was amplified and filtered (1–30 Hz) using a Grass CP 511 AC amplifier (Grass Instruments Co.). LFP, MUA, and CBV signals were recorded simultaneously in cortex (S1BF) and hippocampus using tungsten microelectrodes (UEWMGGsedNNM; FHC, Bowdoinham, ME) with an impedance of 2–4 MΩ (measured with a 1 kHz, 10 nA sine wave current) attached to an Oxyflow MNP110 XP needle laser Doppler flow probe (outer diameter 0.5 mm) connected to an Oxyflow 4000 (Oxford Optronix, Oxford, UK) laser Doppler flowmetry monitor to measure local CBF. The tip of a microelectrode was placed approximately 200–400 μm below the laser Doppler probe to ensure that the sampling volumes of all measurements were overlapping. Signals from the microelectrodes were first acquired broadband (1 Hz to 10 kHz) and amplified using a microelectrode amplifier Model 1800 (A-M Systems, Carlsborg, WA). The signals were then fed into a hardware dynamic filter Model 3364 (Krohn-Hite Corporation, Brockton, MA) and split into LFP (1–100 Hz) and MUA (400 Hz to 10 kHz) using a Butterworth filter (24 dB/oct attenuation). All signals were digitized and recorded with sampling rates of 1000 Hz (EEG, LFP, and CBV) or 20 kHz (MUA) using either a CED Power 1401 or a CED Micro 1401 and Spike 2 Software.

For EEG, LFP, and MUA, the power ($V_{rms}^2$) of each signal was estimated by squaring the root-mean-square (rms) signal for consecutive nonoverlapping 3-s intervals (a 3-s window was used to equal the time resolution of fMRI acquisitions). Before processing, MUA signals underwent additional low-pass filtering at 5 kHz using the digital filter function from Spike 2 to minimize influences of noise on power estimates. For MUA, in addition to calculating signal power, we also performed spike sorting using a template-matching algorithm (Nersesyan, Herman, et al. 2004) to measure spike frequency. This produced similar results to MUA power as reported previously (Logothetis et al. 2001; Shmuel et al. 2006). However, because unit activity during seizures took the form of synchronized population spikes, which differed in shape and amplitude from spikes identified at baseline, we used MUA power as a more reliable estimate of total action potential firing for all statistical calculations.

The CBF data were analyzed using the same 3-s intervals as for the other data sets.

### Data Analysis and Statistics

All EEG signals from MRI experiments were first processed using Spike 2 software to reduce residual artifacts in the EEG resulting from magnetic field gradients by digital filtering as described previously (Nersesyan, Hyder, et al. 2004) (Fig. 1A,F). Because seizures are dynamic events and their onset timing after bicuculline injections was slightly variable between experiments and rats, all data were first
realigned according to individual seizure onset time as determined from the EEG recordings for all experiments (MRI and neurophysiology).

MRI images were processed using our own software running on a MATLAB platform (The MathWorks, Inc., Natick, MA) written by 2 of the authors (E.H. and J.E.M) and by Miro Enev. Although rats were completely paralyzed during experiments, all fMRI series were first screened for movement artifacts using a MOVIE function and center of mass analysis. Runs that exhibited significant movement (more than 20% of a pixel in either plane of the center of mass image) were removed from analysis. The center of mass analysis was restricted to voxels within the brain boundaries. For BOLD and flow-related CBV experiments, t-maps were calculated continuously for groups of 10 consecutive images (30 s) from seizure onset onwards, compared with the same number of completely artifact-free baseline images directly preceding the injection of bicuculline (see Fig. 1B for a schematic overview). The t-maps were thresholded for $P < 0.0005 \ (t = 5)$ to help control for multiple comparisons, and images were superimposed onto corresponding high-resolution anatomical images for each slice and from each individual animal.

For flow-related CBV measurements, an increase in volume causes an increase in the amount of the paramagnetic contrast agent in any given voxel causing a decrease in T2* and a drop in the fMRI signal. Thus, negative signal changes are associated with increased CBV, and positive signal changes are related to decreased CBV. However, for better comparison with other signals, we inverted the CBV signal in all analyses and figures, and hence, increases in the signal portray increases in CBV and vice versa.

To analyze and compare the temporal relationship between the signal changes from baseline for the BOLD and CBV experiments with those from neuronal activity (LFP and MUA) and local CBF recorded from cortex (SI1BF) and dorsal hippocampus, corresponding regions of interest (ROIs) were chosen for the MRI experiments based on high-resolution anatomical images in each animal and a standard rat brain atlas (Paxinos and Watson 1998) (Fig. 4.A,B). For all MRI and neurophysiological experiments, signal changes were calculated as percentage change from the mean of the 30-s baseline signal before the injection of bicuculline ([signal–mean baseline]/mean baseline] × 100 for consecutive nonoverlapping 3-s intervals.

To test if signal changes were significantly different between uninterrupted preinjection recordings and seizure for cortex (SI1BF) and hippocampus, or between these 2 brain regions during seizures, the mean percent signal change for the first 150 s after seizure onset as well as the mean percent signal change during the uninterrupted preinjection recording were calculated. The 150-s interval was chosen because it represented the main phase of maximal seizure activity across all experiments and animals. Paired t-tests were used to analyze differences for signal changes during seizures compared with uninterrupted preinjection recordings, and unpaired t-tests were used to test for putative differences in signal intensities between ROIs during seizures. For all analyses, a P value of 0.05 was regarded as significant. All graphs are plotted as mean ± SEM over the time range of the experiments in 3-s intervals and synchronized to seizure onset; and all statistics and data analysis are based on the number of experiments (seizure recordings), except where otherwise noted.

We chose the number of experiments and not the number of animals as the sample size because we wanted to investigate how the different signals (BOLD, CBV, LFP, MUA, CBF, and CMRO2) are related during seizures and due to the fact that seizures itself are dynamic events that can be variable between experiments (recordings) and rats. However, either method has its merits and the potential of biasing the data. Therefore, we also analyzed our data using the number of rats as the sample size and taking the mean of the repeated experiments as a single value for a particular rat where necessary. The outcomes of this analysis yielded the same results (data not shown).

Estimation of CMRO2 Change

Changes in the CMRO2 consumption during seizures were estimated separately for cortex (SI1BF) and hippocampus, using the known general relationship between oxygen consumption and BOLD, CBV, and CBF at steady state as described in equation (1),

\[ \Delta S/S = \Delta \cdot \left( \frac{\text{ACBF/CBF} - \text{ACMRO2/CMRO2}}{1 + \text{ACBF/CBF} - \text{DCBV/CBV}} \right) \]

where \( \Delta S/S \) represents changes in the BOLD signal relative to baseline, and \( \Delta \text{CBF/CBF}, \Delta \text{CBV/CBV}, \) and \( \Delta \text{CMRO2/CMRO2} \) are changes in CBF, blood volume, and oxygen consumption, respectively (Hyder et al. 2002). CMRO2 values were calculated over time in intervals of 3 s. \( \hat{A} \) is a BOLD calibration parameter, a measurable constant that depends on baseline physiology and static magnetic field strengths and might also be region specific (Shulman et al. 2002; Wu et al. 2002; Uludag et al. 2004). Because \( \hat{A} \) and \( \Delta \text{CMRO2/CMRO2} \) are not known, hypercapnia experiments are usually performed under the assumption that CMRO2 is constant under this condition (Davis et al. 1998) leaving \( \hat{A} \) as the only unknown to be solved in the equation. However, recent evidence challenges the assumption of stable CMRO2 values and unaltered neuronal activity under hypercapnia (Martin et al. 2006), and it has been suggested that during hypercapnia some uncoupling of oxygen delivery and consumption can occur (Hyder et al. 2001); and CO2 can become an important energy source itself (Siesjo 1978). Therefore, we did not perform hypercapnia experiments to estimate \( \hat{A} \) but instead calculated \( \Delta \text{CMRO2/CMRO2}(t) \) using different values of \( \hat{A} \) determined in our laboratory based on previous measurements (Kida et al. 2000; Hyder et al. 2001). Using a range of 3 different values (0.4, 0.5, and 0.6) for \( \hat{A} \) that are physiologically meaningful allowed us to analyze the dependence of our CMRO2 calculations on \( \hat{A} \) for the cortex and hippocampus and also to control for any uncertainties regarding \( \hat{A} \) (Uldag et al. 2004).

To calculate the time course of ACMRO2/CMRO2(t), we used values from individual experiments for \( \Delta S/S(t) \), but we used the average values of \( \Delta \text{CBV/CBV}(t) \) and \( \Delta \text{CBF/CBF}(t) \). We used averages instead of individual values for \( \Delta \text{CBV/CBV}(t) \) and \( \Delta \text{CBF/CBF}(t) \) because we did not acquire CBV measurements in all experiments and performed separate measurements of CBF using laser Doppler flowmetry instead of arterial spin labeling (ASL) MRI. Although optical imaging techniques like laser Doppler flowmetry measure CBF qualitative and vary spatiotemporally as well as in their underlying physical mechanisms from ASL-MRI (He et al. 2007), they have been proven useful in reliably calculating CMRO2 and to be comparable to CBF acquired using ASL-MRI (Mandeville et al. 1999; He et al. 2007). Furthermore, our measured increases in CBF during seizures are in line with earlier observations (Nersesyan, Herman, et al. 2004; Trubel et al. 2004; Kida et al. 2007).

To calculate uncertainties for our CMRO2 estimates, we propagated errors in ACMRO2/CMRO2(t) as shown in equations 2-5 using partial derivatives,

\[ \frac{\partial O}{\partial B}(t) = -1 \cdot \frac{\Delta}{\hat{A}} \cdot F(t) \cdot \hat{A}, \]

\[ \frac{\partial O}{\partial F}(t) = (1 - B(t) \cdot \hat{A}) \cdot \frac{\Delta}{\hat{A}} \cdot V(t), \]

\[ \frac{\partial O}{\partial V}(t) = -1 \cdot F(t), \quad \text{and} \]

\[ \sigma^2(t) = \left( \frac{\partial O}{\partial B}(t)^2 \cdot \text{SEM}_B(t)^2 + \left( \frac{\partial O}{\partial F}(t)^2 \cdot \text{SEM}_F(t)^2 + \left( \frac{\partial O}{\partial V}(t)^2 \cdot \text{SEM}_V(t)^2 \right)^2 \right) \right)^{1/2}, \]

where \( \sigma(t) = \Delta \text{CMRO2/CMRO2}(t), B(t) = \Delta S/S(t), F(t) = \Delta \text{CBF/CBF}(t), V(t) = \Delta \text{CBV/CBV}(t), \text{SEM}_B(t) = \text{SEM}_F(t) = \text{SEM}_V(t) = \text{SEM}_O(t) \) is the SEM for \( \Delta S/S(t), \text{SEM}_O(t) \) is the SEM for \( \Delta \text{CBF/CBF}(t), \text{SEM}_B(t) \) is the SEM for \( \Delta \text{CBV/CBV}(t), \) and \( \sigma(t) \) is the uncertainty for \( \Delta \text{CMRO2/CMRO2}(t). \)

Results

Ictal BOLD Signals Can Show Prominent Decreases in Hippocampus

In a first step, we acquired BOLD fMRI images to characterize areas that are involved during seizure activity. Animals were injected systemically with the GABA-A antagonist bicuculline. Shortly after injection, paroxysmal discharge patterns could be
easily identified in the EEG after removal of MRI-related artifacts (Fig. 1). The ictal EEG was characterized by high amplitude, high-frequency oscillations during the beginning of the seizure and progressed into clonic paroxysmal discharges of lower frequency toward the end. The EEG after seizure end showed the typical postictal depression, often interspersed with recurrent brief paroxysmal episodes. Although the main phase of ictal activity usually occurred during the first 150 s, the total time until no paroxysmal activity was visible in the EEG was $480.67 \pm 26.46$ s (mean ± SEM).

During the beginning of seizures, BOLD activation maps showed not only a mixed pattern of widespread increases throughout the cortex and subcortical structures but also prominent focal decreases, especially in the hippocampus (Fig. 2A). Toward the end of seizures, the increases became less prevalent and decreases became more widespread throughout the brain (Fig. 2B). Although the maps always showed increased BOLD activity in the cortex during seizures, the pattern was more variable for the hippocampus; decreases were prominent in the hippocampus, but some maps also revealed no changes (from the baseline) or small increased BOLD signals (see Supplementary Fig. 1 online).

**Ictal Flow-Related CBV Always Increases throughout the Brain**

One possible explanation for a negative BOLD signal is a decrease of blood flow due to either vascular steal or vascular spasm during seizures. Reduced blood flow would produce less oxygen supply to the area, causing a negative BOLD response (Harel et al. 2002). Another possible explanation for negative BOLD would be a local decrease in neuronal activity, with a concomitant excessive decrease in blood supply (Shmuel et al. 2002, 2006). If either of these 2 mechanisms played a role, there should be a decrease in blood supply to the hippocampus.
during seizures. To test this, we injected an exogenous paramagnetic contrast agent to measure changes in the flow-related CBV during seizures in the same animals. Figure 3A,B show representative activation maps for a CBV experiment from the same rat as BOLD data in Figure 2. In contrast to the BOLD response, the CBV activation maps always showed prominent increases throughout the whole brain, including the hippocampus, and stayed elevated even toward seizure end. Unlike the BOLD fMRI signal, where decreases were often seen in the hippocampus during seizures, with CBV contrast there were always increases in the hippocampus (Supplementary Fig. 1 online). This finding suggests that negative BOLD signals in the hippocampus are not due to a decrease in blood supply during seizures compared with baseline (through either hemodynamic steal, spasm, or due to reduced neuronal activity), so another explanation must be sought.

**Time Courses of BOLD and CBV Changes**

To further characterize this heterogeneous pattern of regional increases and decreases and to compare neuroimaging changes to direct measurements of neurophysiology, we analyzed the time courses of the BOLD and CBV signals for the cortex and hippocampus. We chose the somatosensory barrel field cortex (S1BF) and the dorsal hippocampus as representative ROIs. In accordance with the expectations from the BOLD activation maps in Figure 2, the BOLD signal showed a prominent increase in cortex directly after seizure onset followed by a return to baseline and ending in a postictal undershoot (Fig. 4A). In contrast, the hippocampus often revealed a prominent negative BOLD signal during the entire seizure (Fig. 4B). The mean BOLD responses averaged over 20 experiments from 14 rats are shown in Figure 4C,D. On average, the cortex showed an increased BOLD response.

**Figure 3.** Example of uniform increases in the flow-related CBV response during bicuculline-induced generalized seizures. (A) In contrast to the BOLD response, the CBV showed prominent and homogenous increases throughout the whole brain (including hippocampus) and stayed elevated even toward the end of the seizure (B). In (A) and (B), t-maps are shown for 30 s of data (10 consecutive fMRI images acquired every 3 s) during seizure compared with 30 s of baseline (see Fig. 1). Maps are superimposed on high-resolution anatomical images. Slices are shown from anterior to posterior with approximate coordinates relative to bregma (Paxinos and Watson 1998). Color bars indicate t-values for increases (warm colors) and decreases (cold colors) in CBV. Note that in reality, exogenous paramagnetic contrast causes a decreased signal with increased CBV; however, for clarity, we have inverted the scale and used warm colors to represent increased CBV.
during the seizure followed by a gradual decrease toward seizure end (Fig. 4C). The cortical BOLD signal increased prominently in all experiments; however, the hippocampus showed more variable responses, including decreases or relatively small changes (Supplementary Fig. 1 online). Therefore, unlike the cortex, the average BOLD signal in the hippocampus showed a rather flat response without any noticeable increases during seizures (Fig. 4D). Both regions showed a prominent postictal undershoot of the BOLD signal.

To test if signal changes were significantly different between uninterrupted preinjection recordings and seizure for cortex and hippocampus or between the 2 brain regions during seizures, we calculated the mean percent signal change for the first 150 s following seizure onset as well as the mean percent signal change during the uninterrupted preinjection recordings for each experiment (Supplementary Figure 1 online). Generally, the first 150 s represented the period of most intense seizure activity across all experiments and animals. We found that although the BOLD signal showed significant increases during seizures for cortex, the hippocampus signal was not different compared with preinjection (Table 1).

We next analyzed the time courses of the CBV signal for the same ROIs as for the BOLD signal (Fig. 4A,D). In general the CBV signal followed the BOLD signal in cortex, showing prominent increases after seizure onset that gradually decreased after seizure end (Fig. 4A). Interestingly, we observed a comparable CBV response, albeit of smaller amplitude, in the hippocampus. This increase in CBV was in marked contrast to the prominent negative BOLD response often observed in the same region (Fig. 4B).
The mean CBV signal averaged over 14 experiments in 10 rats (Fig. 4C,D) showed the same pattern as the individual time courses. In all experiments and in both ROIs (cortex and hippocampus), the CBV signal increased during seizures (Supplementary Fig. 1 online). The average CBV signal in the cortex was, however, of higher amplitude compared with the hippocampus. Furthermore, although the CBV in the cortex showed a gradual decrease toward the end of the recordings (Fig. 4C), it stayed elevated in the hippocampus throughout the recording (Fig. 4D). However, unlike the BOLD signal, the CBV did not show a postictal undershoot in either one of the structures. To test if flow-related CBV signals were different between uninterrupted preinjection recordings and seizure for cortex and hippocampus, or between the 2 regions during seizures, we again analyzed mean signal changes for the first 150 s of the seizures. Like the BOLD signal, the CBV signal was significantly increased in the cortex. However, unlike the BOLD signal, CBV in the hippocampus was significantly increased as well (Table 1; Supplementary Fig. 1 online).

A Local Dissociation between BOLD Signal, Hemodynamic Changes, and Neuronal Activity

In a second set of experiments, we performed simultaneous laser Doppler flowmetry and neurophysiological recordings in cortex and hippocampus to investigate changes in local CBF and neuronal activity during seizures. The experimental setup was the same as during the MRI experiments, except that recordings were done outside the magnet, facilitating recordings with high-impedance local microelectrodes in S1BF and hippocampus. Immediately after injection of bicuculline, all animals showed the typical ictal EEG pattern as described for fMRI recordings (Fig. 1B). The paroxysmal activity in the EEG was always paralleled by synchronized high-amplitude, high-frequency oscillations in LFP accompanied by increased firing in MUA recordings during the beginning of the seizure, progressing into clonic paroxysmal discharges of lower frequency toward the end. To quantify these signals, we filtered the raw voltage and calculated mean power ($V_{rms}^2$) over different frequency ranges to determine the relative contributions of synaptic activity (LFP; 1–100 Hz) and action potentials (MUA; 400 Hz to 5 kHz), as described previously (Logothetis et al. 2001; Shmuel et al. 2006). To further quantify MUA, we also performed spike sorting using a template-matching algorithm (Nersesyan, Herman, et al. 2004), producing similar results to the filtering. However, this was less reliable because during seizures unit activity took the form of synchronized population spikes (data not shown).

Neuronal activity as measured by LFP and MUA showed large increases during seizures in both the cortex and hippocampus in all cases (Fig. 5A–D). This suggests that decreased BOLD in the hippocampus is not due to decreased neuronal activity. In fact, very large increases in neuronal activity were observed in both regions, as expected during seizures. The increased neuronal activity in cortex and hippocampus was accompanied by increased local CBF in both regions as measured by laser Doppler flowmetry (Fig. 5E,F). This further confirms that negative BOLD signals in the hippocampus are not related to reduced blood flow during seizures, as shown by the CBV data (see above).

In the group data, mean signal changes during the most intense part of seizures (first 150 s) in the cortex showed a significant local increase in LFP power, MUA power, and CBF (Table 1; Supplementary Fig. 2 online). The MUA also revealed a marked decrease in power after the termination of the seizure, along with the postictal depression (Fig. 5C). Interestingly, hippocampal recordings showed also intense signal increases in LFP, MUA, and CBF during seizures (Table 1; Supplementary Fig. 2 online) that were in line with our observed increases in CBV but in marked contrast to the absence of any significant BOLD changes or the often observed negative BOLD responses, for this area (Fig. 4B,D). As in the cortex, the MUA in the hippocampus also showed the typical postictal depression pattern. Although there were no differences in power for LFP and CBF between cortex and hippocampus, the MUA power in hippocampus exceeded by far the MUA power in cortex (Fig. 5C,D, Supplementary Fig. 2 online; $P = 0.004$, 2-tailed unpaired t-test, $n = 43$). These findings suggest that small or negative changes in BOLD signals can occur under some circumstances despite large increases in neuronal activity. We next investigated whether an imbalance of oxygen consumption and delivery in the face of large neuronal activity increases could explain the results.

CMRO$_2$ and CBF Mismatch Can Account for BOLD Decreases

In a third step, we estimated the changes in CMRO$_2$ from the BOLD, CBF, and CBV (Kida et al. 2000; Hyder et al. 2001) data. CMRO$_2$ is the most direct physiological correlate of neuronal activity due to its known proportionality to changes in energy consumption associated with changes in neuronal activity (Hyder et al. 2002; Shulman et al. 2002). CMRO$_2$ can be estimated from BOLD, CBF, and CBV signals because of their known relationship as described in equation (1) (see Materials and Methods). Figure 6A,B shows the calculated mean percent CMRO$_2$ signal change over time for 3 physiologically relevant values of the BOLD calibration parameter $A$ for cortex (S1BF) (Fig. 6A) and hippocampus (Fig. 6B). In both structures, CMRO$_2$ showed a significant increase during seizures (Table 1) and a slow return to baseline after seizure end, changing only slightly with different $A$ values. CMRO$_2$ in the hippocampus was significantly higher than in cortex for all $A$ values ($A = 0.4$, $P = 3.21 \times 10^{-6}$; $A = 0.5$, $P = 3.29 \times 10^{-6}$; $A = 0.6$, $P = 3.37 \times 10^{-6}$; in all cases 2-tailed unpaired t-test, $n = 20$, see Table 1 for mean ± SEM). The CMRO$_2$ in hippocampus was on average 46% higher compared with CMRO$_2$ in the cortex. The stronger increase in CMRO$_2$ in the hippocampus compared with the cortex was in line with our observations for MUA but in contrast to the BOLD signal (see also Supplementary Figs 1 and 2 online).

Comparing the time courses of CBF and calculated CMRO$_2$ reveals the probable explanation for the observed relatively small changes or even decreases in BOLD in the hippocampus (Fig. 6C,D). In the cortex, the increase in CBF during seizures was nearly double that of the increase in the calculated CMRO$_2$, which would result in an oversupply of oxygen in capillaries, venules, and veins and a concomitant increase in the BOLD signal. The latter was indeed observed in all our cortical BOLD fMRI recordings (Fig. 6C, Supplementary Fig. 1 online). In the hippocampus, however, the increase in CMRO$_2$ during seizures nearly matched the increase in CBF. This observation suggests that oxygen consumption in the hippocampus might at certain times exceed its supply, resulting in a weak or even decreased BOLD signal due to increased deoxygenated hemoglobin content, despite strong increased neuronal activity. This
implies, albeit indirectly, that adenosine triphosphate (ATP) generation to support regional changes in neuronal activity during seizures may be oxidative in cortex but nonoxidative in hippocampus (Shulman et al. 2001). Such an assumption is in line with our fMRI measurements in hippocampus, which often showed decreases in the BOLD response (Supplementary Fig. 1 online) and on average no change from baseline (Fig. 6D; Table 1).

The mismatch in the hippocampus between neuronal activity and CMRO₂ on the one hand and hemodynamics...
CBF and CBV) on the other hand is also summarized in Figure 7, which shows the mean percent signal changes during seizures for all variables normalized to the cortex (S1BF). Although CBV, CBF, and CMRO2 always increased together with neuronal activity (LFP and MUA) in cortex and hippocampus during seizures, the increase in neuronal activity (MUA) and its concomitant rise in CMRO2 in the hippocampus exceeded those observed in the cortex (Fig. 7). This large increase in neuronal activity and CMRO2 in the hippocampus compared with the cortex was, however, not met by an equally pronounced increase in CBF or CBV in the hippocampus, which was comparable between the 2 areas, or even lower regarding CBV. These changes could explain a small or even decreased BOLD response in the hippocampus despite increased neuronal activity (Fig. 7).

**Discussion**

Using combined fMRI and neurophysiological recordings during bicuculline-induced seizures, we show that decreases in the BOLD signal can be associated with increases in neuronal, hemodynamic, and metabolic activity. We also show that although the cortical BOLD signal always increased during seizures, on average it did not change in the hippocampus, and often even decreased in the latter. Meanwhile, neuronal and hemodynamic signals were always increased in both regions. These findings demonstrate that increases, decreases, as well as no changes in the BOLD response can all occur during increases in neuronal activity in some circumstances.

The BOLD signal results from the paramagnetic properties of deoxygenated hemoglobin and is therefore positively correlated with the local oxygen content in the blood (Logothetis 2002; Huettel et al. 2004). The latter depends on CBF, CBV, and CMRO2, which are themselves related to neuronal activity (Huettel et al. 2004). Because of these relationships, there are at least 3 different scenarios that can cause a negative BOLD signal. One explanation might be a reduction in CBF, which is generally ascribed to some sort of "vascular steal" phenomenon or vascular spasm (Harel et al. 2002). This mechanism is unlikely to explain our results because, regardless of the sign of the BOLD response, we always observed increases in CBV and CBF in both cortex and hippocampus. Furthermore, the
hippocampus and cortex do not share the same vascular network for blood supply (Scremin 1995). A second explanation for the negative BOLD signal might be a decrease in neuronal activity as was recently shown in simultaneous fMRI and neurophysiological recordings in monkey visual cortex (Shmuel et al. 2006). Although our data support a neuronal origin for the negative BOLD response, we never observed any decreases in neuronal activity during seizures. In fact, LFP and MUA power showed pronounced increases in cortex and hippocampus. Interestingly, the MUA power in the hippocampus greatly exceeded the MUA power in the cortex, which is in marked contrast to the BOLD decreases often observed in the hippocampus.

A third possibility for a negative BOLD response might be an increase in neuronal activity and CMRO$_2$ that exceeds the concomitant increase in CBF, resulting in a net increase of deoxygenated hemoglobin and a negative BOLD signal (Harel et al. 2002). Indeed, transient negative BOLD signals like the initial dip have been ascribed to this mechanism (Malonek and Grinvald 1996; Yacoub et al. 2001; Chen-Bee et al. 2007). Although this situation has not been observed for sustained negative BOLD signals with large increases in neuronal activity. The histogram displays the mean percent signal change during the first 150 s after seizure onset (vs. 30-s baseline) for BOLD, CBV, CBF, CMRO$_2$ ($A = 0.6$), LFP power, and MUA power in cortex and hippocampus. All values are displayed as mean ± SEM normalized to cortex (S1BF).

Although we did not perform separate hypercapnia calibration to determine the value of the BOLD calibration parameter $A$ (Hoge and Pike 2001), we modeled CMRO$_2$ using a range of physiologically relevant values for $A$ based on prior measurements in our laboratory (Kida et al. 2000; Hyder et al. 2001). Recent experiments show that the assumption of stable CMRO$_2$ values and unaltered neuronal activity under hypercapnia may not necessarily be valid under all conditions (Martin et al. 2006). Furthermore, during hypercapnia some uncoupling of oxygen delivery and consumption can occur (Hyder et al. 2002; Shulman et al. 2002), and CO$_2$ can become an important energy source itself (Siesjo 1978).

Our data revealed higher metabolic rates in the hippocampus compared with the cortex, regardless of $A$, suggesting that our CMRO$_2$ estimates were not confounded by regional difference in the scaling factor (Liu et al. 2004). This observation is in agreement with earlier experiments, showing that CMRO$_2$ can be reliably calculated using a range of values for $A$ instead of performing hypercapnia experiments and that the estimate of CMRO$_2$ is relatively stable regardless of $A$ chosen (Uludag et al. 2004). We performed separate measurements for CBF, using laser Doppler flowmetry instead of ASL-MRI. Although optical imaging techniques like laser Doppler flowmetry are qualitative measurements that vary spatiotemporally as well as in their underlying physical mechanisms from ASL-MRI (He et al. 2007), they have been proven useful in reliably calculating CMRO$_2$ and to be comparable to CBF acquired using ASL-MRI (Mandeville et al. 1999; He et al. 2007). Furthermore, our observed increases in CBF matched the increases in our flow-related CBV measurements and agree with earlier observations (Mueggler et al. 2001; Nersesyan, Herman, et al. 2004; Trubel et al. 2004; Kida et al. 2007).

Although the cortex and hippocampus showed different BOLD responses during seizures, both regions showed a prominent postictal undershoot in the BOLD signal. The postictal undershoot was associated with a return of the CBF as well as CMRO$_2$ to baseline and a profound postictal depression of all
neuronal activity in both regions. However, the CBV signal returned more slowly and remained elevated during the entire recording, especially in the hippocampus, suggesting that the postictal undershoot of the BOLD signal might in part also be ascribed to CBV effects (Buxton et al. 2004). Our observations suggest a complex, nonlinear relationship between the BOLD signal on the one hand and hemodynamic and neuronal activity on the other hand, with metabolic processes determining if an increase or decrease in neuronal activity results in a positive or negative BOLD response. Our data also indicate that CBF and CBV might be better indicators of changes in neuronal activity than the BOLD signal. Although CBF and CBV always increased together with neuronal activity in both cortex and hippocampus, the decreased BOLD signal misleadingly might have implied a decrease in neuronal activity in the hippocampus. It has to be mentioned, however, that these parameters alone also do not lend themselves for a direct interpretation of the underlying neuronal activity. Although MUA during seizures was stronger in the hippocampus than in the cortex, the CBV increase in the hippocampus was smaller than in the cortex. Likewise, despite this difference in MUA, the CBF responses between both structures were the same. Therefore one should be careful in making inferences of altered neuronal activity based on any one of these parameters in isolation, and especially based on BOLD signal changes, alone; even more so in individuals where metabolism or hemodynamics may be compromised due to disease or drugs (D’Esposito et al. 2003).

Our data suggest that the hippocampus may suffer from temporary hypoxia during seizures, possibly contributing to the observed changes in hippocampal tissue related to epileptogenesis (Sloviter 1999). That the enormous increase in oxygen metabolism during seizures can exceed its supply has been suggested earlier (Dymond and Crandall 1976; Ingvar and Siesjo 1983; Pereira de Vasconcelos et al. 2002; van Eijsden et al. 2004). However, the temporal resolution of the methods used so far is usually poor and a mismatch between increased metabolism and a decrease in blood flow were usually observed toward the end of prolonged seizures or status epilepticus activity. In our study, however, decreases in the hippocampal BOLD signal occurred even toward the beginning of relatively brief seizures. Our data are compatible with a high vulnerability of the hippocampus to hypoxic damage during ictal events (van Eijsden et al. 2004) and show that decreases in the BOLD signal may contain clinically relevant information. If this vulnerability is due solely to a difference in seizure severity between the cortex and hippocampus as may be inferred from MUA, or if it is also due to hemodynamic reasons as indicated by smaller CBV increases, would be an interesting topic for future research. One could speculate that if seizure severity is the main mechanism, then sufficiently strong seizures and coconcomitant increased CMRO₂ in the cortex might also result in unchanged or decreased BOLD responses and possible hypoxia under some circumstances. Accordingly, decreases in tissue oxygenation were recently shown in highly localized experimental focal seizures in the cortex despite increased CBV (Bahar et al. 2006). However, it could be that in general the upper limit of the blood supply may also be higher in the cortex than in the hippocampus; as the stronger increase of CBV in the cortex compared with hippocampus tentatively does suggest. Glucose is the main energy source for the brain and its oxidation yields a far greater amount of ATP than its alternate nonoxidative path (Siesjo 1978). Thus total glucose consumption (CMRglc) includes oxidative (CMRglc(ox)) and nonoxidative (CMRglc(nonox)) components,

\[
 CMRglc = CMRglc(ox) + CMRglc(nonox)
\]

and the oxygen-to-glucose index (OGI),

\[
 OGI = \frac{CMR_{O_2}}{CMR_{glc}}
\]

shows the ratio of oxygen-to-glucose metabolism. In the cortex, the OGI during seizures may be as low as 4 because of high CMRglc(nonox), whereas at rest it can be close to the theoretical maximum value of 6 where CMRglc(nonox) can be quite negligible (Siesjo 1978). OGI values less than 6 would indicate the presence of nonoxidative metabolism, which has been observed by measurement of lactate with magnetic resonance spectroscopy (MRS) during similar seizure activity (Patel et al. 2004). Although we have not measured CMRglc, we propose that the OGI between the hippocampus and cortex during seizures would be significantly different. Future MRS studies linking the lactate dynamics to temporal changes in CMRO₂ would provide a relative time course of the nonoxidative ATP source during seizure activity.

To conclude, our data support a neuronal origin for the negative BOLD response as demonstrated recently (Shmuel et al. 2006). However, we show for the first time, to our knowledge, that a sustained decreased BOLD signal does not unequivocally imply decreased neuronal activity, but can also result from increased neuronal activity, depending on the complex interplay between hemodynamics and metabolism. Furthermore, whether a negative BOLD results from an increase or decrease of neuronal activity might depend on brain region and state. Therefore, the interpretation of neuroimaging signals should proceed with caution and may be different during normal and pathological conditions as well as for different regions in the brain.

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Supplementary Material
Supplementary materials can be found at: http://www.cercor.oxfordjournals.org/.

Notes
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