Differential recovery of multimodal MRI and behavior after transient focal cerebral ischemia in rats

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Abstract

The association between recovery of brain function and behavior after transient cerebral ischemia in animals and humans is incompletely characterized. Quantitative diffusion- (DWI), perfusion- (PWI), T_2-weighted (T_2WI), and functional magnetic resonance imaging (fMRI) were performed before, during, and up to 1 day after 20-mins transient middle cerebral artery occlusion (tMCAO; $n = 6$) or sham operation ($n = 6$) in male Sprague–Dawley rats. Viability thresholds were employed to calculate diffusion, perfusion, and T_2 lesion volumes. Region of interest analysis was used to evaluate structural and functional MR signal changes within the sensorimotor network, which were then related to corresponding behavioral measures. Post-mortem 2,3,5-triphenyltetrazolium chloride (TTC) staining was performed 24 h after ischemia. Transient middle cerebral artery occlusion produced lesions on DWI and PWI, which fully recovered by 30 mins after reperfusion. Ipsilesional fMRI responses to hypercapnia and forepaw stimulation were significantly impaired after ischemia and did not fully normalize until 3 and 24 h after tMCAO, respectively. No abnormalities were observed on imaging or TTC at 24 h despite significant behavioral dysfunctions including contralesional forelimb impairment and ipsilesional neglect. No MRI, behavioral, or TTC anomalies were observed in sham-operated rats. There were no significant correlations between MRI parameters, behavior, and TTC in either group. Together, these results suggest that normal findings on diffusion, perfusion, and T_2 imaging shortly after transient ischemia may not indicate normal tissue status as indicated by fMRI and behavior, which may help explain the persistence of neurologic deficits in patients with normal conventional MRI after cerebral ischemia.

Keywords

CBF; DWI; fMRI; MCAO; PWI; sensorimotor

Introduction

Cerebral ischemia is a main cause of sensorimotor morbidity in modern society and substantially increases short- and long-term risks for adverse cardiovascular and cerebrovascular events (Daffertshofer *et al*, 2004). Yet despite persistent symptoms, many

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patients present with no abnormalities during the hyperacute stage on conventional T_1- and T_2-weighted magnetic resonance imaging (MRI). Attempts have recently been made to use diffusion-(DWI) and perfusion-weighted imaging (PWI) to evaluate potential brain pathology after transient focal ischemia both clinically and experimentally (Daffertshofer *et al*, 2004; Kidwell *et al*, 1999). Earlier studies conducted in our lab revealed ischemic changes on multimodal MRI after periods of transient middle cerebral artery occlusion (tMCAO) in the rat and correlated these findings with histopathologic outcomes in regions where imaging abnormalities were permanently reversible (Li *et al*, 2000*a*). It was found that resolution of DWI lesions does not necessarily indicate tissue salvage from ischemia as selective neural necrosis was seen in such regions. This observation may help explain the persistence of neurologic deficits in patients who have normal DWI and PWI results after cerebral ischemia (Ay *et al*, 1999).

Although histologic analysis is useful in animal modeling, it cannot be replicated in clinical studies and, along with conventional imaging, fails to assess the postischemic functional integrity of brain tissue *in vivo*. Alternate surrogate techniques have therefore been developed to better understand neural function after transient ischemia, including positron emission tomography (PET), magnetoencephalography (MEG), transcranial magnetic stimulation (TMS), electroencephalography (EEG), and functional MRI (fMRI) (Kim *et al*, 2005). However, only the latter enables simultaneous acquisition of high-resolution structural and functional information in a completely noninvasive manner and, for this reason, has been widely used to map brain responses to external stimuli after stroke (Dijkhuizen *et al*, 2001). Surprisingly, fMRI has not—to our knowledge—been employed to investigate brain activity after transient cerebrovascular insults characterized by *permanent* resolution of ischemic lesions. Repeated fMRI measurements performed acutely after brief ischemia could provide useful information about regional hemodynamic and metabolic processes, which may contribute to developing a means for monitoring and understanding recovery processes.

Measures of behavioral outcome are gaining widespread popularity in animal studies and are arguably necessary to better assess the full range of consequences of cerebral ischemia (Virley *et al*, 2000). A few experimental stroke studies have attempted to correlate behavioral recovery with restitution of MR parameters, but a clear causal link between the two has not been established (Dijkhuizen *et al*, 2003; Rogers *et al*, 1997). It has been postulated that such a relationship may be found if the employed behavioral tests inform exclusively on the brain regions being examined (Virley *et al*, 2000). This supposition is supported by clinical studies showing that postischemic clinical syndromes vary in patients depending on the location of cerebral infarction (Gavrilescu and Kase, 1995), which implies that the type of functional impairment is contingent on damage within a particular region of the brain and that a stronger relationship between restorations of brain activity and behavioral function may be found when neural and functional measures are matched more specifically. Several recent clinical stroke studies utilizing MRI have begun to address these issues (Baron and Warach, 2005), but similar progress in animal models is lacking and there remains a paucity of information in either field with respect to transient ischemia.

Thus, the goal of this study was to correlate changes in ischemic tissue injury, hemodynamics, and activity within structures of the cerebral sensorimotor network with changes in the specific behavioral functions that they subserve in rats acutely recovering from 20-mins tMCAO. It was hypothesized that (1) the extent of ischemic damage is linked to the degree of loss of brain functions and (2) recovery of brain functions within the ipsilesional sensorimotor network is correlated with recovery of sensorimotor behavioral deficits. To this end, (1) multiparametric MRI was used to map and dynamically measure signatures of tissue damage and function within the brain and (2) adhesive label removal and forepaw placement tests were employed to evaluate sensorimotor function with general neurologic status assessed via Bederson scoring.

Materials and methods

Animal Preparation

All procedures were in accordance with institutional guidelines. Twelve male Sprague–Dawley rats (Taconic Farms, Hudson, NY, USA) weighing 300 to 350 g were divided into Groups 1 ($n = 6$) and 2 ($n = 6$) undergoing 20-mins tMCAO with reperfusion or sham operation, respectively, using the intraluminal suture method (Li *et al*, 1998). After surgery, animals were immediately placed into the magnet for imaging. Anesthesia was induced with 5.0% isoflurane under spontaneous respiration and maintained at 2.0% during surgery and 1.0% during MRI at a flow rate of 1.5L/min (Shen *et al*, 2005). Needle electrodes were subcutaneously inserted into both forepaws and connected in series for forepaw stimulation during imaging (Liu *et al*, 2004; Shen *et al*, 2005; Sicard and Duong, 2005). Physiologic parameters (respiration rate (RR), heart rate (HR), arterial oxyhemoglobin saturation, and end-tidal CO_2 (EtCO$_2$)) were noninvasively monitored and recorded throughout imaging onto a computer via the Biopac System (Santa Barbara, CA, USA) (Sicard and Duong, 2005). Temperature was continuously monitored with a rectal probe and maintained at $37.0 \pm 0.5°C$ with a feedback-controlled heating pad (Sicard and Duong, 2005).

Hypercapnic Challenge and Forepaw Stimulation

Hypercapnic challenges tested cerebrovascular reactivity and consisted of a premixed gas of 5% CO_2 with 21% O_2 and balance N_2 equilibrated for ~3 mins before imaging initiation (Sicard *et al*, 2003; Sicard and Duong, 2005). Tissue function was assessed with previously optimized bilateral somatosensory forepaw stimulation parameters (6 mA current with 0.3 ms pulse duration at 3 Hz) designed to yield robust fMRI responses without inducing significant changes in HR, RR, blood pressure, and blood gases (Liu *et al*, 2004; Sicard and Duong, 2005). Two forepaw stimulation and hypercapnic challenge trials were performed per imaging block, each trial consisting of a 2 mins baseline period followed by 2 mins of forepaw stimulation or hypercapnia (Sicard and Duong, 2005).

Magnetic Resonance Imaging Measurements

Magnetic resonance imaging experiments were performed on a 4.7 T/40 cm horizontal magnet equipped with a Biospec Bruker console (Billerica, MA, USA) and a 20G/cm gradient insert (ID = 12 cm and rise time = 120 µs). A surface coil (ID = 2.3 cm) was used for brain imaging and an actively decoupled neck coil for perfusion labeling (Meng *et al*, 2004). Animals were imaged before and midway during occlusion, as well as 15, 30, 60, 90, 120, 150, 180 mins, and 24 h after reperfusion. A complete imaging block lasted 30 mins where the apparent diffusion coefficient (ADC) of water, basal cerebral blood flow (bCBF), as well as hypercapnia- and forepaw stimulation-induced changes in CBF and the blood oxygenation level-dependent (BOLD) fMRI signals were recorded. Basal cerebral blood flow and ADC were the only parameters recorded during occlusion because of time limitations. Lastly, T_2-weighted images (T$_2$WIs) were acquired at preocclusion, 180 mins, and 24 h time points.

Three ADC maps were separately acquired with diffusion-sensitive gradients applied along the *x*, *y*, or *z* direction (Shen *et al*, 2003). Single-shot, echo-planar images (EPI) were acquired over 2.5 mins with matrix = 64×64, spectral width = 200 kHz, time to repetition (TR) = 2 secs (90° flip-angle), time to echo (TE) = 37.5 ms, b = 4 and 1,170 secs/mm^2, Δ = 24 ms, δ = 4.75 ms, field of view (FOV) = 2.56×2.56 cm, seven 1.5 mm slices, and 16 averages. T_2-weighted imaging was performed using a fast spin-echo pulse sequence (Sicard and Duong, 2005) with matrix = 128×128, TR = 2 secs, effective TE = 20 or 104 ms, FOV= 2.56×2.56 cm, seven 1.5 mm slices, and 16 averages.

Simultaneous CBF and BOLD measurements were made using the continuous arterial spin-labeling technique with single-shot, gradient-echo, EPI acquisition (Sicard and Duong, 2005). Paired images were acquired alternately—one with arterial spin labeling and the other without. BOLD images were obtained from the nonlabeled images of the CBF measurements. Continuous arterial spin-labeling employed a 1.78 secs square radiofrequency pulse to the labeling coil in the presence of a 1.0 G/cm gradient along the flow direction to satisfy the condition of adiabatic inversion and the sign of the frequency offset was switched for nonlabeled images (Shen *et al*, 2005). Magnetic resonance parameters were as follows: matrix $= 64 \times 64$, TR = 2 secs (90° flip-angle), TE = 20 ms, FOV= 2.56×2.56 cm, seven 1.5 mm slices, and 60 pairs of images.

Magnetic Resonance Data Analysis

Image analysis employed codes written in Matlab (Math-Works Inc., Natick, MA, USA) and STIMULATE (University of Minnesota, Minneapolis, MN, USA) software (Sicard and Duong, 2005). Coregistration of images was performed using in-house software involving manual and automatic alignment without spatial interpolation (Liu *et al*, 2004). Quantitative average ADC maps, in units of $mm^2/secs$, were calculated using the three separately acquired ADC maps using the Stejskal–Tanner equation: $ADC = -\ln(S_1/S_0)/(b_1-b_0)$, where $b_1 = \gamma^2 G_1^2 \delta(\Delta - \delta/3)$, ln is the natural logarithm, and S_0 and S_1 are the signal intensities obtained with b_0 and b_1, respectively (Stejskal and Tanner, 1965). *B* values are proportional to the gradient strength (*G*), magnetogyric ratio (γ), duration (δ), and time between application (Δ) of gradient pulses. Quantitative T_2-maps, in units of ms, were constructed using a linear least-squares regression performed on the two acquired T_2WIs (Li *et al*, 2000a). Quantitative bCBF maps, in units of mL/g/min, were calculated using: $S_{CBF} = \lambda/T_1(S_C-S_L)/(S_L + [2\alpha-1]S_C)$, where S_C and S_L are the signal intensities of the control and labeled images, respectively, λ is the water brain–blood partition coefficient set to 0.9, α is the spin-labeling efficiency set to 0.75, and tissue T_1 set to 1.5 secs (Duong *et al*, 2000; Silva *et al*, 1999). *In vivo* ADC, bCBF, and T_2 lesion volumes were calculated utilizing viability thresholds and methodology previously derived for this model as described in detail elsewhere (Dijkhuizen *et al*, 2003; Shen *et al*, 2003). These thresholds were used to identify all pixels with abnormal ADC, CBF, or T_2 characteristics on each image slice at each time point, with corresponding lesion volumes calculated by summing the abnormal areas and multiplying by slice thickness.

Crosscorrelation analysis was performed on fMRI data to calculate CBF and BOLD activation maps used to determine hypercapnia ($\Delta BOLD_{CO_2}$ and ΔCBF_{CO_2}) and forepaw stimulation ($\Delta BOLD_{FS}$ and ΔCBF_{FS}) evoked signal changes relative to baseline (Liu *et al*, 2004; Shen *et al*, 2005; Sicard and Duong, 2005). Reported $\Delta BOLD_{CO_2}$ and ΔCBF_{CO_2} values are averages of two hypercapnia trials performed during each imaging block; the same is true for reported $\Delta BOLD_{FS}$ and ΔCBF_{FS} values.

Region of Interest Analysis of the Sensorimotor Network

Regions of interest (ROIs) were positioned bilaterally on MR maps and encompassed structures of the cerebral sensorimotor network, namely: forepaw region of primary somatosensory cortex (Sf1), secondary somatosensory cortex (S2), primary motor cortex (M1), ventral thalamus (thalamus), and caudatoputamen (CPu) (Dijkhuizen *et al*, 2001; Sicard and Duong, 2005; Virley *et al*, 2000; Weiller, 1998). ROIs were drawn carefully and conservatively with regard to anatomic images and a stereotaxic atlas of the rat brain (Paxinos and Watson, 1997) to ensure correct placement and to minimize partial-volume effects (Liu *et al*, 2004; Sicard *et al*, 2003; Sicard and Duong, 2005). ROIs of the ischemic hemisphere were obtained by symmetrically reflecting the contralesional ROIs along the midline (Shen *et al*, 2005). This approach was used to measure ADC, bCBF, $\Delta BOLD_{CO_2}$, ΔCBF_{CO_2}, $\Delta BOLD_{FS}$, and ΔCBF_{FS} values within

ROIs, with the latter two parameters investigated solely within Sf1 (Liu *et al*, 2004; Sicard and Duong, 2005).

Neurologic and Histologic Evaluation

Animals underwent objective adhesive removal and forepaw placement sensorimotor behavioral testing before, 5 and 24 h after tMCAO or sham operation as fully described elsewhere (De Ryck *et al*, 1992; Virley *et al*, 2000). Briefly, the bilateral adhesive label removal test assessed contralesional neglect and ipsilesional bias by recording latency to contact and remove labels (in secs) as well as order of label contact and removal. The forepaw placement test examined sensorimotor integration scored on the following scale: 0 if the placing response was immediate and normal, 1 if the response was slow or delayed, and 2 if the response did not occur within 2 secs. These specific behavioral measures were used because they are fulfilled by the anatomic structures probed by MRI, and also because they reflect a quantitative assessment, which makes them more appropriate parameters to correlate with quantitative MR changes for investigating the relationship between functional outcome and brain pathology/ function (Virley *et al*, 2000). Lastly, general neurologic status was monitored via Bederson scoring as this qualitative grading system is commonly used to assesses behavior in experimental models of cerebral ischemia (De Ryck *et al*, 1992).

At 24 h after tMCAO, subsequent to MRI and behavioral testing, animals were killed under anesthesia and their brains sectioned coronally into seven 1.5 mm-thick slices (corresponding to the MR slices), which were then stained with 2,3,5-triphenyltetrazolium chloride (TTC) for postmortem infarct volume calculation with edema correction (Meng *et al*, 2004).

Statistical Analysis

Data are presented as mean $\pm$ s.d. unless otherwise indicated, and were analyzed with SigmaStat software (Rockware Inc., Golden, CO, USA). Statistical comparisons were performed using analysis of variance (with repeated measures where appropriate) with *post hoc* Dunn's or Tukey's test for multiple comparisons and two-tailed paired or unpaired Student's *t*-test, where appropriate. Correlation analyses employed Pearson's product moment- or the Spearman rank-order tests. $P < 0.05$ was considered significant.

Results

Physiologic Measurements

All basal physiologic parameters did not significantly differ between groups or before and after tMCAO/sham operation ($P > 0.05$; data not shown), and were consistent with previous data obtained in normal isoflurane-anesthetized rats respiring spontaneously (Sicard *et al*, 2003). There were no statistically significant changes in recorded measures during forepaw stimulation in both groups at all time points ($P > 0.05$). Transient increases in RR and EtCO$_2$ were recorded during hypercapnic challenge ($P < 0.05$), the magnitudes of which being similar between groups and time points and consistent with changes observed in normal rats under similar experimental conditions (Sicard and Duong, 2005). All proceeding shown data are exclusive to the tMCAO group.

Ischemic Damage

CBF and ADC lesions (197 ± 30 and 110 ± 26 mm^3 volumes, respectively) were present during occlusion in the right MCA territory as shown in Figure 1. The temporal evolution of local quantitative bCBF and ADC changes in tMCAO rats is given in Figure 2. During tMCAO, bCBF and ADC values within investigated anatomic areas were significantly and heterogeneously reduced compared with corresponding preocclusion or contralesional regions

($P < 0.05$), with the greatest decreases found in subcortical structures such as CPu and milder perturbations occurring within lateral borderzone regions such as primary somatosensory cortex, which obtain collateral perfusion from the anterior communicating artery (Shen *et al*, 2005). All perfusion and diffusion abnormalities fully resolved by 15 and 30 mins postreperfusion, respectively, and no rats developed secondary lesions. At all postocclusion time points, ADC and bCBF values within contralesional regions did not significantly differ from preocclusion or sham-group values of corresponding localities ($P > 0.05$). Swelling or lesions were not present on T_2 and TTC in either hemisphere 24 h after tMCAO (Figure 1). In sham-operated rats, there were no statistically significant differences in ADC, bCBF, and T_2 values between hemispheres throughout the period of observation ($P > 0.05$), nor were TTC anomalies present.

Functional Magnetic Resonance Imaging

Hypercapnic challenges were employed to assess cerebrovascular reactivity. Hypercapnia fMRI maps and region-specific temporal evolution of ΔCBF_{CO_2} and $\Delta BOLD_{CO_2}$ responses of tMCAO rats are shown in Figures 1 and 3, respectively. Before occlusion, hypercapnic challenge produced robust increases in BOLD and CBF, which did not significantly differ between ROIs intra- or interhemispherically ($P > 0.05$). Shortly after ischemia, $\Delta BOLD_{CO_2}$ and ΔCBF_{CO_2} were nearly zero in ipsilesional ROIs and thereafter showed a differential recovery rate across localities with, for example, Sf1 and CPu renormalizing first (90 mins) and last (180 mins), respectively. There were no significant differences in $\Delta BOLD_{CO_2}$ and ΔCBF_{CO_2} across ROIs, hemispheres, or time in sham animals ($P > 0.05$).

Forepaw stimulation fMRI maps and the temporal profile of ΔCBF_{FS} and $\Delta BOLD_{FS}$ responses within Sf1 are shown in Figures 1 and 4, respectively. Before tMCAO, there were no significant interhemispheric differences in $\Delta BOLD_{FS}$ (2.5% ± 0.1% (ipsilesional) versus 2.4% ± 0.2% (contralesional); $P > 0.05$) and ΔCBF_{FS} (0.70 ± 0.20 mL/g/min (ipsilesional) versus 0.68 ± 0.17 mL/g/min (contralesional); $P > 0.05$). However, immediately after transient ischemia, ipsilesional ΔCBF_{FS} and $\Delta BOLD_{FS}$ were negligible and differed significantly from preocclusion and contralesional values ($P < 0.05$), suggesting an uncoupling of metabolic activity and blood flow. ΔCBF_{FS} and $\Delta BOLD_{FS}$ responses were found to be normal at the 24 h time point. Notably, recovery of activation responses to somatosensory forepaw stimulation lagged significantly behind that of hypercapnic challenges within ipsilesional Sf1. Contralesional ΔCBF_{FS} and $\Delta BOLD_{FS}$ activations did not significantly differ between pre- and postocclusion time points, nor from corresponding sham values ($P > 0.05$). In sham-operated rats, there were no statistically significant differences in $\Delta BOLD_{FS}$ and ΔCBF_{FS} between sides and time points. Lastly, in both groups, occasional activations during forepaw stimulation were observed in M1, S2, and subcortical areas at time points before and after reperfusion as is typical for rats under isoflurane anesthesia (Dijkhuizen *et al*, 2001; Sicard and Duong, 2005).

Behavioral Status

Figure 5 summarizes behavioral test results of tMCAO rats. Groups did not differ significantly in performance before surgery ($P > 0.05$; data not shown), indicating that they were matched for functional ability (Virley *et al*, 2000). At 5 h after tMCAO, rats displayed side-specific sensorimotor abnormalities relative to sham and preocclusion testing, which remained significant at 24 h ($P < 0.05$). Specifically, adhesive removal testing revealed increases in latency to contact and remove labels from the contralesional forepaw, indicating contralesional neglect (Virley *et al*, 2000). This test also showed a significant group × side × time interaction for order of label contact and removal, which further reflected a postischemic contralesional neglect and ipsilesional bias to sensory stimuli (Virley *et al*, 2000). The forepaw placement test showed preserved ipsilesional function with significantly increased contralesional limb

placing scores. Lastly, Bederson scoring was normal (score = 0) at all investigated time points, which is not surprising given this test grades generalized behavior shown to be largely unaffected by short periods of transient ischemia (De Ryck *et al*, 1992). In sham rats, there were no statistically significant differences in behavioral parameters between sides and across time ($P > 0.05$).

Correlation Analyses

We attempted to compare behavioral responses versus a wide array of functional (ΔCBF_{CO_2}, $\Delta BOLD_{CO_2}$, ΔCBF_{FS}, and $\Delta BOLD_{FS}$) and structural (ADC, bCBF, and T_2) MRI parameters within ROIs after tMCAO. There were no significant correlations between MRI values within ipsilesional ROIs with any contralesional behavioral test measure at all individual time points and when temporally matched data were grouped together (*R* ranging from −0.41 to 0.79; *P* > 0.05 for all).

Discussion

This study serially tracked regional changes in the spatiotemporal evolution of quantitative ADC, bCBF, T_2, and fMRI tissue signatures after transient MCAO in the rat and correlate these with appropriately specific sensorimotor behavioral measures. The major results of this study are: (1) 20 mins of transient focal cerebral ischemia produces ADC and CBF lesions that resolve permanently by 30 mins after reperfusion with no significant changes on T_2 or TTC, (2) ipsilesional cerebrovascular reactivity and activation responses to somatosensory stimulation —assessed via fMRI—remain significantly perturbed in otherwise normal-appearing tissue for up to 3 and 24 h after reperfusion, respectively, (3) behavioral dysfunctions persist at 24 h despite complete renormalization of all MR parameters, and thus (4) there appears to be no significant association between the normalization of imaging modalities and recovery of behavior acutely after 20 mins of tMCAO.

Ischemic Damage

Perfusion-weighted imaging is widely used to measure cerebral blood flow during and after ischemia (Li *et al*, 2000*a*; Shen *et al*, 2005) with the technique used herein being sufficiently accurate and sensitive to quantitatively estimate bCBF reductions during and after transient and permanent ischemia produced by the employed MCAO model (Li *et al*, 2000*a*; Shen *et al*, 2005). Our reported region-specific variability in the magnitude of bCBF reduction during occlusion has been recognized in experimental focal ischemia (Dijkhuizen *et al*, 1998; Hakim *et al*, 1992; Marcoux *et al*, 1982), with perfusion deficits below a critical threshold producing metabolic energy failure and subsequent cellular swelling (Shen *et al*, 2005). The permanent renormalization of bCBF subsequent to reperfusion is also in agreement with previous studies (Dijkhuizen *et al*, 1998; Li *et al*, 2000*a*) and suggests that any postischemic injury in this study may not be because of a secondary compromise of blood flow.

Diffusion-weighted imaging is capable of mapping reversible and irreversible injury from the associated reduction of ADC and is widely recognized as a powerful tool for early detection and evaluation of transient cerebral ischemic damage in both animal models and humans (Coutts *et al*, 2005; Li *et al*, 2000*a*). Prior studies indicate that ADC lesions are potentially reversible when reperfusion is performed quickly after ischemia, with the permanency of reversal dependent on the duration of ischemia (Li *et al*, 2000*a*). Indeed, rats undergoing less than 30 mins of ischemia typically have complete reversal of initial ADC lesion within 15 to 60 mins after reperfusion (Li *et al*, 2000*a*). The present study corroborates this finding and further showed that certain brain regions appear more susceptible to ischemic insult than others. For example, the severity of ADC decline within the CPu during occlusion was significantly greater than in cortical regions. This selective vulnerability is likely because of heterogeneities

in local bCBF during tMCAO (Dijkhuizen *et al*, 1998; Pulsinelli *et al*, 1982). However, other factors such as differences in postsynaptic organization (Mitani *et al*, 1992) as well as variations in mitochondrial capacity (Ter Horst *et al*, 1994) and the activity of free radical defense systems (Dijkhuizen *et al*, 2003) may also be involved.

Brain Function

Functional magnetic resonance imaging is gaining wide acceptance as a method for assessing the spatial and temporal dynamics of tissue function and how it relates to behavioral recovery and pathophysiology after ischemic insult to the mammalian brain, yet a clear relationship between these parameters remains unknown (Dijkhuizen *et al*, 2001, 2003; Nhan *et al*, 2004; Shen *et al*, 2005). Here, we found that transient focal ischemia led to dysfunctions of cerebrovascular responsivity to CO_2 and neurovascular coupling response to somatosensory stimulation that were associated with perfusion/diffusion declines but remained abnormal far longer. These findings are in accord with previous animal studies reporting loss of stimulus-evoked cerebral hemodynamic responses that slowly recovered but were still significantly depressed up to 24 h after full renormalization of ADC (Reese *et al*, 2000; Schmitz *et al*, 1997). They are also supported by a human study showing persistently dysfunctional metabolic activity within the affected hemisphere of patients with clinically diagnosed transient ischemic attack (TIA) subsequently lacking ischemic lesions or hypoperfusion (Bisschops *et al*, 2002). Together, our findings suggest that fMRI is capable of identifying dysfunctional neural tissue that appears completely normal on diffusion-, perfusion-, and T_2-weighted imaging and, thus, could potentially serve as a more sensitive and complementary indicator to such techniques for detecting ischemic brain injury.

Different, potentially interacting pathophysiologic mechanisms may be responsible for the recalcitrance of functional recovery within the sensorimotor network, including damage to its efferent and/or afferent projections (Andrews, 1991), tissue injury secondary to edematous swelling (Fagan *et al*, 2005), alterations in F-actin filaments in vascular smooth muscle cells (Kwon *et al*, 2002), selective neuronal necrosis (Li *et al*, 2000*a*), and ischemia below the threshold for irreversible damage but above the threshold for neuronal dysfunction (Astrup *et al*, 1981). The former two possibilities are remote given the apparently normal MR findings in nonsensorimotor network brain regions and no evidence of brain swelling on TTC or MRI, respectively. However, the latter three remain viable candidates. For instance, cytoskeletal disruption of vascular smooth muscle F-actin has been shown to occur after 15 or 45 mins transient ischemia in a duration-dependent manner (incapacitating its ability to maintain function in response to extrinsic stimuli) and tended to recover within 3 h after reperfusion (Kwon *et al*, 2002), potentially explaining the temporal profile of fMRI responses acutely after 20 mins tMCAO. Lastly, on visual inspection of fMRI maps, we did not find altered perilesional or contralesional activity thought to be associated with behavioral recovery as reported in some human and animal stroke studies (Dijkhuizen *et al*, 2003; Rossini and Pauri, 2000). This is plausibly owing to an interstudy difference in ischemic duration (among other methodological considerations), which is known to produce differences in postischemic pathologic processes (Dijkhuizen *et al*, 2003; Garcia *et al*, 1995) or such phenomena may simply occur after the end point of this study (Jones and Schallert, 1992).

Interestingly, there was a differential recovery rate in cerebrovascular reactivity to CO_2 and neurovascular coupling response to forepaw somatosensory stimulation within ipsilesional Sf1; that is, at several time points, the structure responded normally to CO_2 challenge but not to forepaw stimulation. This suggests vasoreactivity must be preserved for neural activity-coupled vascular responses to occur. Given this, one explanation for the lagging recovery of hemodynamic responses to forepaw stimulation in Sf1 is that the tissue itself was functionally silenced or subtly damaged by ischemia. Another possibility is that the thalamus or its afferent

projections to Sf1 were damaged, resulting in a 'secondary' silencing of an otherwise uninjured cortex. These scenarios are not mutually exclusive and both may have contributed to the observed disconnect in recovery rates, as supported by a prior study (Li *et al*, 2000*b*) showing subtle histologic damage in both cortical and subcortical structures after brief focal cerebral ischemia.

Behavioral Status

Functional outcome is necessary to assess the consequences of cerebral ischemia and it is arguable that neurologic status is more relevant than underlying pathophysiology when predicting 'quality of life' (Virley *et al*, 2000). However, no animal experimental studies to date have attempted to relate performance on specific behavioral tasks with pathologic and functional changes occurring within their cerebroanatomic correlates as visualized by multimodal MRI. It has been postulated that such an analysis could yield valuable information on the exact interactions between postischemic events occurring in the brain and outward behavior (Dijkhuizen *et al*, 2003; Virley *et al*, 2000). For these reasons, the battery of behavioral tests used herein was chosen as it is sensitive to the effects of tMCAO for up to a period of 90 days (Lindner *et al*, 2003), models the impairments seen in human stroke patients (Rose *et al*, 1994), and informs exclusively on the observable activities subserved by the sensorimotor network, thereby permitting a more precise investigation of the relations between changes in MRI and behavioral parameters (Virley *et al*, 2000).

The persistent contralesional neglect and ipsilesional bias found after tMCAO is consistent with a prior animal study employing similar tests and experimental conditions (Virley *et al*, 2000) as well as in patients recovering from cerebral ischemia. Surprisingly, however, no significant associations were found between contralesional behavioral parameters and ipsilesional MR findings at all investigated time points; these results are similar to those of prior studies (Dijkhuizen *et al*, 2001, 2003; Kim *et al*, 2005) and show that a clear causal relationship between changes in brain and behavior remains to be found. We believe the lack of correlation between MR parameters and neurologic status reported herein to be valid but this finding could be at least partly attributable to other factors. Firstly, MRI may have been unable to detect subtle pathologic changes occurring after brief focal ischemia owing to partial volume effects resulting from inadequate spatial resolution (Li *et al*, 2000*a*). Other imaging techniques may be able to detect these changes of cellular integrity, including PET mapping of benzodiazepine receptors (Sette *et al*, 1993) or a combination of T_1 and T_2 MRI at 1 week after the onset of ischemia (Fujioka *et al*, 1999). Secondly, long-term postischemic plastic changes in the brain such as dendritic outgrowth and synaptogenesis (Weiller, 1998) may not have occurred within the time frame of this study as they require days to weeks to develop (Jones and Schallert, 1992). Thus, a correlation between fMRI and behavior may be found chronically after 20 mins tMCAO.

Experimental Limitations

Although this study contains several novel findings, it has some unavoidable design limitations, which will be mitigated by future studies. The persistence of behavioral deficits at 24 h despite normal MR findings suggests that cytologic damage may be present as found by other investigators (Li *et al*, 2000*a*), but the current study design provides little insight into this point as TTC staining was performed in place of more sensitive histologic stains. However, this choice was intentional as TTC is an excellent method for correlating *in vivo* with post-mortem infarct volumes (Chen *et al*, 2005) as well as for calculating viability thresholds (Bardutzky *et al*, 2005; Meng *et al*, 2004; Shen *et al*, 2003). Now that the MR and TTC results have been shown to be in good agreement, a histologic analysis (i.e., microscopic evaluation of hematoxylin–eosin stained brain tissue) more capable of detecting subtle postischemic pathology is currently being performed serially from 24 h to 21 days after tMCAO. Lastly, the

purpose of this study was to examine the evolution of changes in multimodal MRI and behavioral parameters during hyperacute to acute time points after transient cerebral ischemia, which is why a longer experimental endpoint was not chosen. Retrospectively, however, our findings warrant a lengthier observation period that may enhance the ability to find significant correlations between MRI and behavior as well as show whether imaging parameters and brain pathology remain—or do not remain—negative during more chronic stages.

Although consistent with many established methodologies, the functional imaging performed in this study could be subject to several deficiencies and errors. First, bilateral—rather than unilateral—forepaw stimulation was chosen, making it relatively more difficult to assess contralesional activation responses in response to stimulation of a particular forepaw. However, bilateral stimulation is appropriate for functional imaging conducted hyperacutely after ischemia as it enables an equal number of trials to be performed in a shorter duration, thereby enhancing temporal resolution, and also reduces the likelihood of misinterpreting activation data that may be confounded by fluctuations in basal physiologic conditions (Sicard and Duong, 2005) known to occur during and shortly after ischemia (Shen *et al*, 2005). Secondly, no MRI measurements were made between 3 and 24 h, making it impossible to determine when fMRI responses to forepaw stimulation first renormalized. However, most MRI experiments do not continue past several hours postischemia because of increased mortality associated with prolonged anesthesia (Forrest *et al*, 1992)—therefore, we opted to terminate imaging at 3 h to improve next-day survivability for TTC staining and follow-up MRI. Lastly, the accuracy of the employed CBF technique may be subject to errors from magnetization transfer, transit time, and water exchange (Zhou *et al*, 2001), although these effects are negligible in setups such as ours utilizing actively decoupled two-coil systems and small animals (Shen *et al*, 2005). Even with this potential error, our quantitative fMRI responses arguably serve as better measures considering alternative techniques that index relative percent-changes, which may incorrectly reflect neural activity in light of aforementioned potential changes in baseline physiology.

Clinical Implications

Irrespective of its limitations, this study may provide clinicians with at least two pieces of important information. First, the data seem to uphold the notion that time is critical in the context of cerebrovascular disease (Coutts *et al*, 2005; Hacke *et al*, 2004). Acute brain imaging has been proven to be useful in the differential diagnosis, triage, and management of patients with TIA or stroke (Coutts *et al*, 2005; Hacke *et al*, 2004). However, this study suggests that the utility of acute imaging may be compromised by the passage of time periods as short as half an hour in, for example, cases where clinically significant cerebral ischemia results in readily discernible yet transient alterations in ADC. Secondly, the data argue that normal DWI, PWI, and T_2 imaging after brief periods of ischemia may not indicate normal tissue status as fMRI and behavior remain impaired and, for this reason, fMRI may be a useful adjunct for evaluating the efficacy of potential neuroprotective therapies. Lastly, this model may help explain persisting neurologic deficits in subsets of patients with a normal MRI battery after cerebral ischemia (Ay *et al*, 1999).

In conclusion, the present study shows that 20 mins tMCAO produces sensorimotor behavioral deficits that continue despite recovery of multimodal MR parameters in their cerebroanatomic correlates. These findings may be relevant to a clinical subset of patients with negative MRI results but persistent neurologic morbidities.

Acknowledgments

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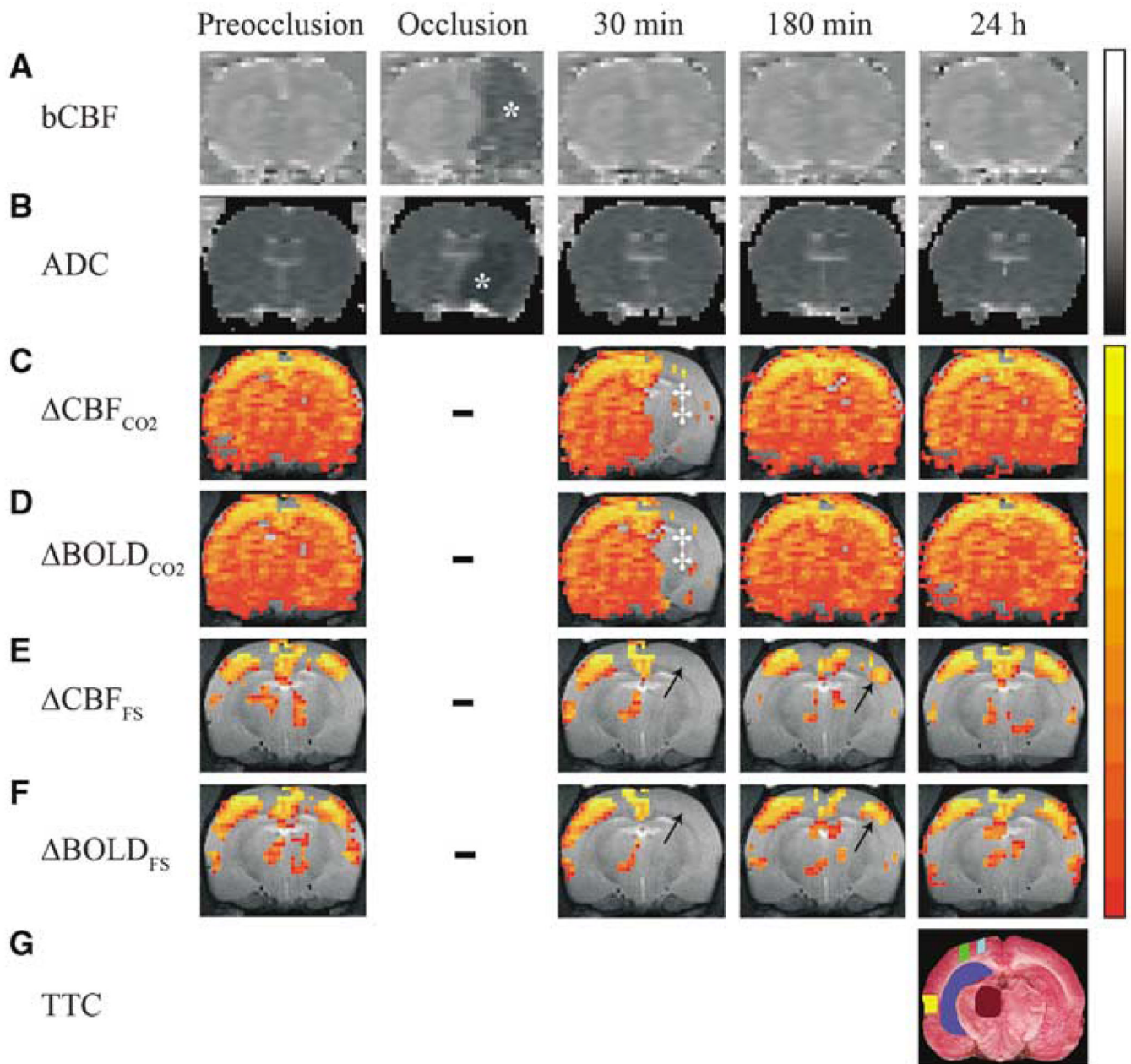
Figure 1.
Representative magnetic resonance (MR) and histological images of a 20-mins transient middle cerebral artery occlusion (tMCAO) rat at selected time points showing (**A**) basal cerebral blood flow (bCBF) (**B**) apparent diffusion coefficient (ADC), (**C**) ΔCBF_{CO_2}, (**D**) $\Delta BOLD_{CO_2}$, (**E**) ΔCBF_{FS}, and (**F**) $\Delta BOLD_{FS}$ maps as well as (**G**) 2,3,5-triphenyltetrazolium chloride (TTC) staining. CO_2 and FS subscripts refer to functional magnetic resonance imaging (fMRI) signal changes evoked by hypercapnic challenge and forepaw stimulation, respectively. Gray-scale bar indicates ADC ranging from 0 to 0.001 mm²/sec and bCBF from 0 to 3 mL/g/min. Color-scale bar indicates ΔCBF ranging from 10 to 200% and $\Delta BOLD$ from 1% to 5%. *ADC and CBF lesions. ‡Area of impaired response to 5% CO_2. Arrows indicate impaired response to forepaw stimulation. Dashes indicate fMRI not performed during occlusion. Activation maps

were overlaid on anatomic images, and a select slice (i.e., fourth-most anterior, corresponding to −2.12 mm from bregma) and percent-thresholds were chosen for visual clarity. ROIs overlaid on TTC are CPu (dark blue), M1 (light blue), thalamus (brown), Sf1 (green), and S2 (yellow).
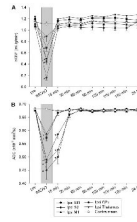
Figure 2.
Temporal evolution of (**A**) basal cerebral blood flow (bCBF) and (**B**) apparent diffusion coefficient (ADC) within ROIs in 20-mins transient middle cerebral artery occlusion (tMCAO) rats ($n = 6$; mean ± s.e.m.). Contralesional bCBF and ADC did not differ significantly between ROIs or time points before and after ischemia and were averaged into their respective contralesional means (Contra mean). $*P < 0.05$ versus ipsilesional (Ipsi) preocclusion (pre).
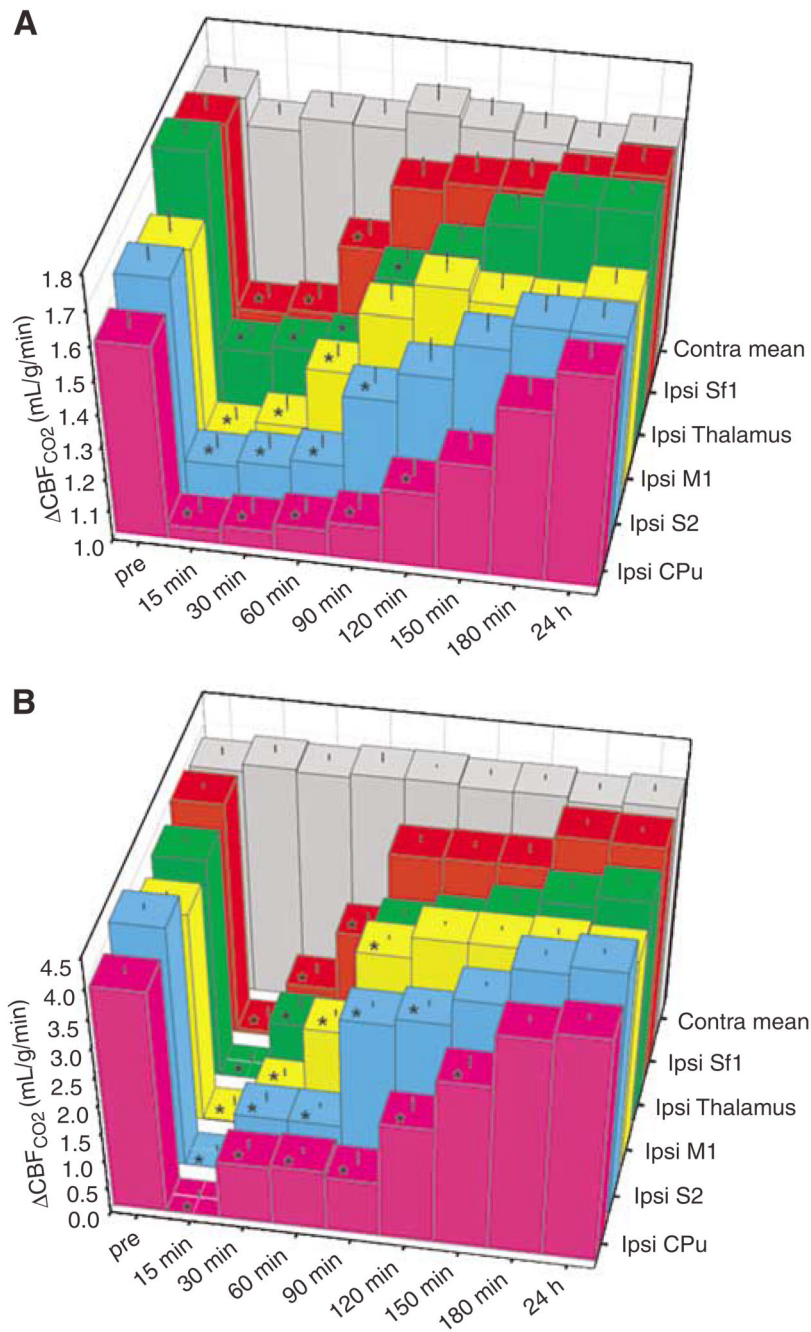
Figure 3.
Temporal evolution of cerebrovascular reactivity in terms of hypercapnia-evoked (**A**) ΔCBF_{CO_2} and (**B**) $\Delta BOLD_{CO_2}$ within ROIs in 20-mins transient middle cerebral artery occlusion (tMCAO) rats ($n = 6$; mean + s.e.m.). All ipsilesional (Ipsi) regions showed significant reductions of ΔCBF_{CO_2} and $\Delta BOLD_{CO_2}$ after reperfusion, which recovered between 90 to 180 mins. Contralesional ΔCBF_{CO_2} and $\Delta BOLD_{CO_2}$ did not differ significantly between ROIs or time points before and after ischemia and were averaged into their respective contralesional means (Contra mean). $*P < 0.05$ versus ipsilesional (Ipsi) preocclusion (pre).
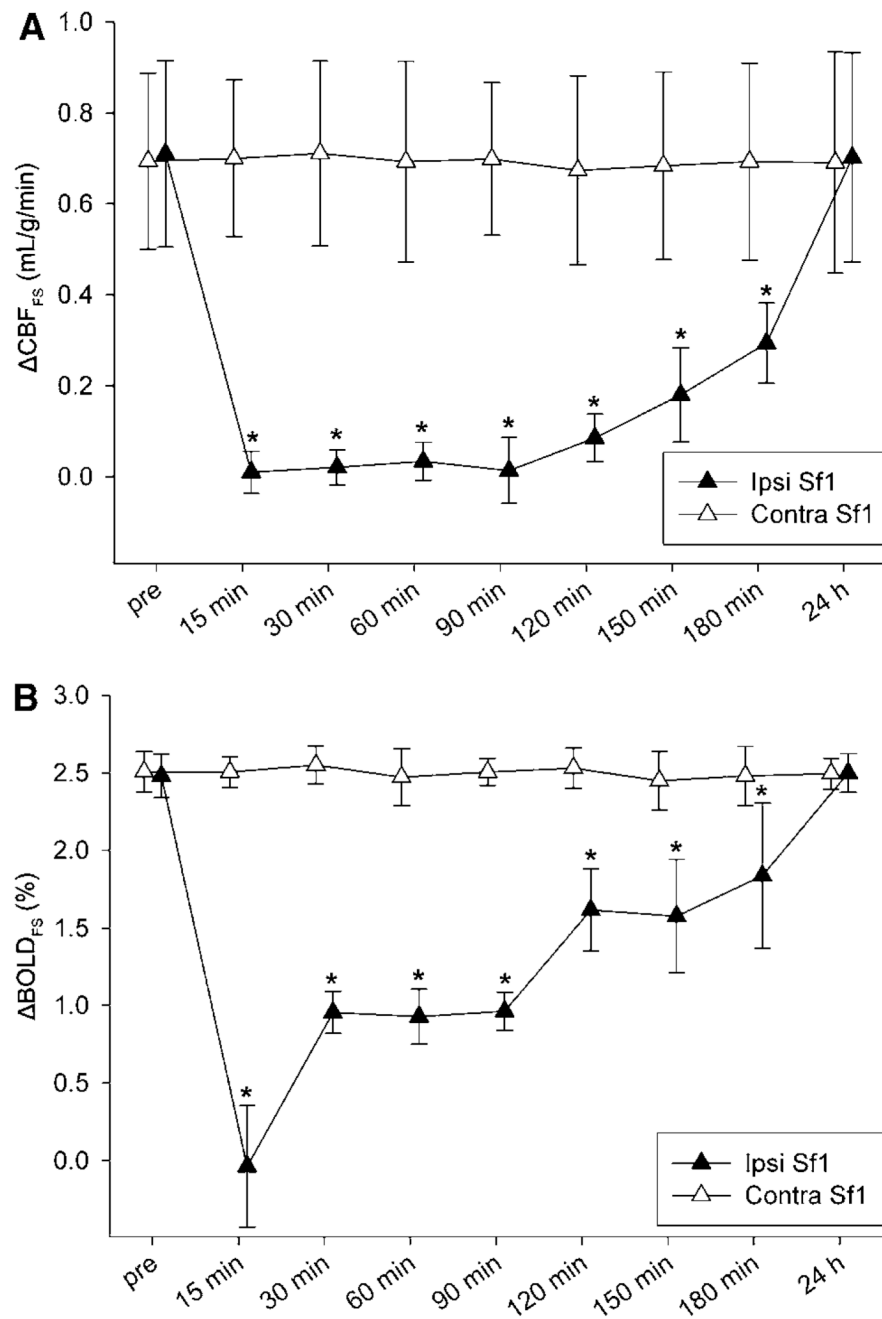
Figure 4.
Temporal evolution of forepaw stimulation-evoked (**A**) ΔCBF_{FS} and (**B**) $\Delta BOLD_{FS}$ within ipsilesional (Ipsi) and contralesional (Contra) Sf1 in 20-mins tMCAO rats ($n = 6$; mean ± s.d.). *$P < 0.05$ versus preocclusion (pre).
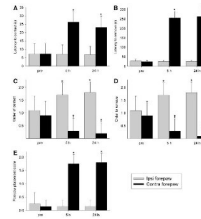
Figure 5.
Mean latency to (**A**) contact and (**B**) remove as well as order to (**C**) contact and (**D**) remove adhesive labels placed around ipsilesional (Ipsi) and contralesional (Contra) forepaws, and (**E**) forepaw placement scores in 20-mins transient middle cerebral artery occlusion (tMCAO) rats ($n = 6$; mean + s.e.m). *$P < 0.05$ versus preocclusion (pre) Contra forepaw and †$P < 0.05$ versus pre Ipsi forepaw.