Development/Plasticity/Repair

Early Prediction of Functional Recovery after Experimental Stroke: Functional Magnetic Resonance Imaging, Electrophysiology, and Behavioral Testing in Rats

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Therapeutic success of treatment of cerebral diseases must be assessed in terms of functional outcome. In experimental stroke studies, this has been limited to behavioral studies combined with morphological evaluations and single time point functional magnetic resonance imaging (fMRI) measurements but lacking the access to understanding underlying mechanisms for alterations in brain activation. Using a recently developed blood oxygenation level-dependent fMRI protocol to study longitudinal and intraindividual profiles of functional brain activation in the somatosensory system, we have demonstrated activation reemergence in the original representation field as the basic principle of functional recovery from experimental stroke. No plastic reorganization has been observed at any time point during 7 weeks after stroke induction. Applying combined recording of fMRI and somatosensory evoked potentials, we observed a tight coupling of electrical brain activity and hemodynamic response at all times, indicating persistent preservation of neurovascular coupling. Identification of functional brain recovery mechanisms has important implications for the understanding of brain plasticity after cerebral lesions, whereas preservation of neurovascular coupling is important for the clinical translation of fMRI.

Key words: **fMRI; neurovascular coupling; functional reorganization; stroke; functional recovery; ischemia**

Introduction

Focal brain ischemia is a major cause of permanent disability. A better understanding of brain recovery and potential reorganization processes is essential to develop new therapeutic strategies after ischemic brain injury and to improve rehabilitation methods.

To date, longitudinal functional magnetic resonance imaging (fMRI) studies in patients with ischemic stroke are scarce because parallel concomitant disease factors make the interpretation in heterogeneous patient pools difficult. fMRI investigations in animals have the major advantage to combine the noninvasive imaging techniques with invasive methods such as direct recording of cellular activity (Logothetis et al., 2001) and histology (Dijkhuizen et al., 2001; Dijkhuizen and Nicolay, 2003) to provide better insight into mechanisms of fMRI signal changes and underlying pathophysiology. To date, studies in rodents subjected to focal cerebral ischemia (Abo et al., 2001; Dijkhuizen et al., 2001, 2003; Sauter et al., 2002) have, however, also not been performed longitudinally because of limitations of anesthesia. The studies performed so far used contrast-enhanced changes of cerebral blood volume (CBV) instead of blood oxygenation level-dependent (BOLD) MR imaging, to visualize brain activation and its recovery. Furthermore, in those reports, measurements of hemodynamic changes had not been combined with direct assessment of electrical activity to investigate mechanisms of neurovascular coupling, thus omitting the chance to systematically assess controversial reports about alterations of neurovascular coupling after focal brain ischemia in humans (Pineiro et al., 2002; Rossini et al., 2004; Krainik et al., 2005).

Recently, we developed a protocol allowing for repetitive independent sessions of noninvasive BOLD fMRI studies of rat forepaw stimulation (Ramos-Cabrer et al., 2005; Weber et al., 2006). Using this approach, we investigated for the first time longitudinal brain reorganization in the somatosensory system of rats subjected to focal ischemic brain injury and mechanisms of coupling between neural activity and hemodynamic response in the acute, the early and late chronic stage after focal brain ischemia. Design of our study was performed to investigate our three major hypotheses. (1) We questioned the validity of lesion volume as the predominant parameter determining clinical outcome and hypothesized that the temporal pattern of functional brain activity is a more reliable factor for the prediction of func-

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tional outcome. (2) We hypothesized that the neurovascular coupling remains preserved in experimental stroke during all phases after stroke induction, thus allowing the conclusion about functional brain activity to be extracted from fMRI results under these pathophysiological conditions. (3) We further hypothesized that recovery of the original representation field is the dominant mechanism of functional recovery in the somatosensory system, as opposed to the assumption of major plastic reorganization as reason for functional improvement.

In our present investigation, we induced stroke in rats and studied the animals for 7 weeks. This stroke-dependent long-term profile included data on morphological lesion evolution as well as information on electrical brain activity using somatosensory evoked potentials (SSEP) together with parallel recording of the hemodynamic response to the stimulus, measured with BOLD fMRI. The study was complemented with behavioral tests analyzing the somatosensory system.

Materials and Methods

All experiments were performed in accordance with the National Institutes of Health animal protection guidelines and were approved by the local governmental authorities.

Stroke induction. Twenty-two male Wistar rats (330–390 g; Harlan-Winkelmann, Borchen, Germany) were used; 19 of these were subjected to 60 min of transient middle cerebral artery occlusion (MCAO) by the intraluminal thread occlusion method (Longa et al., 1989) under 1% halothane anesthesia in 65% N_2O/35% O_2. The occlusion period and the successful reperfusion of the right MCA were controlled by ipsilateral laser-Doppler flowmetry. Four animals died in the first 24 postoperative hours attributable to massive brain edema.

Anesthesia and physiological monitoring. In full agreement with a recently published experimental protocol for longitudinal fMRI experiments (Weber et al., 2006), animals were sedated with subcutaneous application of medetomidine (0.05 mg/kg bolus and 0.1 $mg \cdot kg^{-1} \cdot h^{-1}$ infusion) during the combined fMRI and electrophysiological experiment. Respiration rate and transcutaneous pCO_2 were continuously recorded noninvasively, and body temperature was kept constant by a feedback-controlled heating pad (Ramos-Cabrer et al., 2005). After the experiment, sedation was antagonized with an intraperitoneal injection of atipamezole (0.1 mg/kg). No animal died in the follow-up period.

MRI and stimulation protocol. Repetitive MRI experiments were conducted on a 7.0 T Biospec animal scanner (Bruker BioSpin, Ettlingen, Germany) during the day cycle of the animals, 1 week before stroke induction and 2 d, 1 week, 2 weeks, 4 weeks, and 7 weeks after MCAO. After positioning the animal's head, quantitative T_2 measurements were performed with a multislice spin-echo sequence (Eis et al., 1994) [repetition time (TR)/echo time (TE), 3000/7.5 ms; 16 slices of 1 mm thickness; 16 echoes] to assess T_2 relaxation times and T_2 lesion volume.

Functional MRI was achieved using BOLD contrast, starting at least 1 h after the induction of the sedation. Multislice spin-echo echo-planar images (five slices of 2 mm thickness), centered at 4.7 mm caudal to the rhinal fissure, were acquired (TR/TE, 3000/30 ms).

Electrical forepaw stimulation was performed using rectangular pulses (2 mA, 3 Hz, 0.3 ms) in a paradigm of five blocks consisting each of 45 s resting period and 15 s activation period, ending with an additional 45 s resting period. BOLD fMRI was conducted alternating at least three times between each hemisphere, and animals were allowed to rest for at least 5 min between stimulation sessions. Statistical parametric activation maps were constructed with the software STIMULATE (Strupp, 1996). The time course of each pixel during forepaw stimulation was examined using a paired Student's *t* test ($p < 0.01$). Only clusters that included at least four adjacent activated pixels were considered as positive activation areas (Forman et al., 1995).

Electrophysiology. Directly after the fMRI measurement, sedated animals ($n = 9$) were transferred to a Faraday cage, and short-latency SSEPs were recorded bilaterally from the primary somatosensory cortices (3.5 mm lateral at bregma level) of the intact skull, using subcutaneously inserted steel needle electrodes. Reference and ground electrodes were placed at the nose and neck, respectively. Because of the reference electrode on the nose, independent SSEPs are recorded separately from each hemisphere. Unilateral forepaw stimulation resulted in an SSEP signal in the forelimb area of the primary somatosensory cortex (S1FL) contralateral to the stimulated paw. SSEPs were constructed with the program DasyLab (DATALOG, Mönchengladbach, Germany) by averaging the signal recorded of 100 triggered rectangular pulses (2 mA, 3 Hz, 0.3 ms), amplified 1000-fold and bandpass filtered between 5 and 1000 Hz. SSEP recording was repeated at least two times for each hemisphere. SSEP latencies and amplitudes were considered significantly abnormal if they differed from the corresponding mean value of the baseline values of each hemisphere by >2 SDs.

Behavioral testing. The adhesive tape removal test was used to evaluate sensory and motor deficits (Schallert et al., 2002). Two rectangular stripes of tape (18 × 12 mm) were applied, in random order and with equal pressure, to the saphaneous part of the forepaws. Animals were observed while removing the tapes in their home cages. Latency to contact and to remove the right and left tape were recorded in three trials per session, and the mean value of the three trials was calculated. At least 5 min of rest was allowed between each trial. Healthy animals were trained daily, 3 d before baseline session, which was performed at the day before MCAO. After MCAO, the sessions were performed the day before the combined MRI–electrophysiology experiments during the day cycle of the animals.

Histology. Animals were killed after the last fMRI–electrophysiology experiment under deep halothane anesthesia by perfusion fixation with PBS and 4% paraformaldehyde (PFA). Brains were removed and post-fixed overnight in 4% PFA, followed by immersion in a 30% sucrose solution for 3 d. Coronal 40-μm-thick sections were cut on a freezing microtome (Leica, Nussloch, Germany) and stored free floating. Conventional hematoxylin–eosin and luxol–cresyl-violet stainings were performed on every 12th section. Immunohistochemical staining against the neuronal-specific nuclear protein antibody NeuN (mouse anti-NeuN, 1:100; Millipore, Billerica, MA) was performed on adjacent sections. The Vectastain ABC Method (Vector Laboratories, Burlingame, CA) and 3′,3′-diaminobenzidine/$NiCl_2$ were used for visualization of primary antibody binding. All sections were analyzed and digitally photographed using Leica MZ FL III and Leica DM RB microscopes, both equipped with a CCD camera.

Grouping of animals for data analysis. All animals were treated the same and underwent the same experimental protocol. Only during data evaluation were they retrospectively grouped, based on functional criteria instead of using purely morphological lesion volume determination. The first criterion was successful induction of ischemic lesion, observed on T_2-weighted MR images, 24 h after stroke induction. Then, animals were grouped depending on their long-term capacity for functional restoration. This led to the following classification at the end of the survival period for the whole group. Healthy animals served as control group (group C; $n = 3$). Animals with a clear MCAO-induced ischemic lesion but without detectable functional deficits were collected in group 1 ($n = 4$). Primary occurrence of a functional deficit in all three investigative criteria (fMRI, SSEP, and behavior) formed groups 2 and 3. Group 2 ($n = 6$) consisted of animals with a primary, but only transient functional deficit (observable on all three investigative criteria; compare with above) followed by partial recovery of the studied functions. Group 3 ($n = 5$) included animals with an unchanged, persistent functional deficit over the whole observation period (7 weeks).

Statistical analysis. All values are expressed as mean ± SEM, unless stated otherwise. Because of sample sizes, statistical comparisons of T_2 lesion volume, BOLD percentage change in the left and right primary somatosensory cortex, and behavioral testing at each time point were performed using Kruskal–Wallis ANOVA and exact testing, followed by the Mann–Whitney *U* test when appropriate.

The statistical comparison of SSEP amplitudes between the left and right primary somatosensory cortex in healthy animals at baseline was performed using a paired Student's *t* test. SSEP latencies and amplitudes were considered abnormal if they differed from the corresponding mean value of the baseline values of each hemisphere by >2 SDs.

For correlation analysis between BOLD percentage change and SSEP amplitude and between T_2 lesion volume and histologically determined infarct size, the Spearman's rank order correlation test was used. A p value of <0.05 was considered significant.

Results

Ischemic lesion induction by MCAO

Invariably, 60 min occlusion of the right MCA led to a lesion area in the acute phase that always encompassed the striatal territory and often extended into the cortex. Despite the standardized, well established and tightly controlled model, there was distinct interindividual variability in lesion size (see Figs. 1, 5).

Temporal profile of functional brain activation patterns after focal brain ischemia

fMRI

Alternating unilateral electrical stimulation of the right and the left forepaw in healthy Wistar rats resulted in a significant and reproducible BOLD response in the forelimb area of the contralateral primary somatosensory cortex [forelimb area, S1FL (according to the rat brain atlas of Paxinos and Watson, 1998)] in all animals.

Transient occlusion of the right middle cerebral artery for 60 min did not alter the activation patterns in the S1FL of the left, unaffected hemisphere both in the subacute (day 2) and chronic (weeks 1–7) stages for all study groups (Figs. 1, 2*a*) but resulted in three different activation patterns in the right, affected hemisphere (Figs. 1, 2*b*). In the first group of animals

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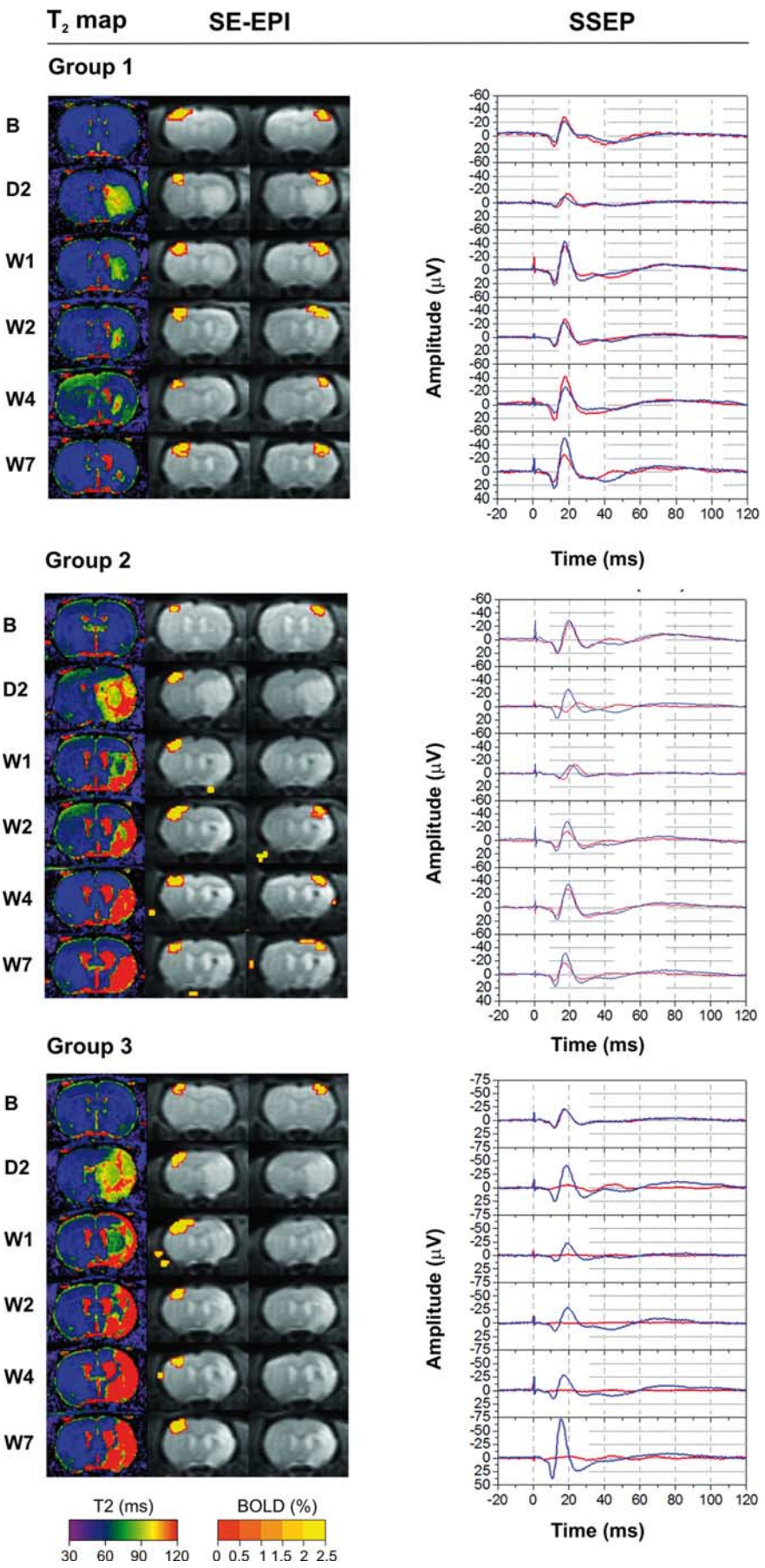

Figure 1. Combined structural brain imaging, functional brain imaging, and SSEP recording after focal ischemic stroke. T_2 maps (first column), BOLD spin-echo echo-planar images (SE-EPI) during right (second column) and left (third column) forepaw stimulation, and the corresponding SSEP signals from S1FL (fourth column; left hemispheric SSEP signal in blue; right hemispheric SSEP signal in red) from representative animals with no loss (group 1), transient loss (group 2), and permanent loss (group 3) of BOLD activation in the right primary somatosensory cortex. BOLD activation and normal SSEP signals can be observed in both primary somatosensory cortices in all animals during baseline measurements (***B***), i.e., before transient MCAO. No loss of BOLD activation and normal SSEP signals are found in animals with subcortical infarction (group 1) at all time points during the 7 week observation period. In animals with a transient loss of BOLD activation in the right S1FL area (group 2), the right SSEP signal is delayed and diminished 2 d after MCAO (D2) and delayed 1 week after MCAO (W1). In parallel with reemergence of BOLD activation 2 weeks after MCAO (W2), a normal SSEP signal is observed again. In animals with permanent loss of BOLD activation in the right S1FL area (group 3), no restoration of the SSEP signal is observed over time. Note that the first spike at time point 0 represents the electrical stimulus in the SSEP recording.

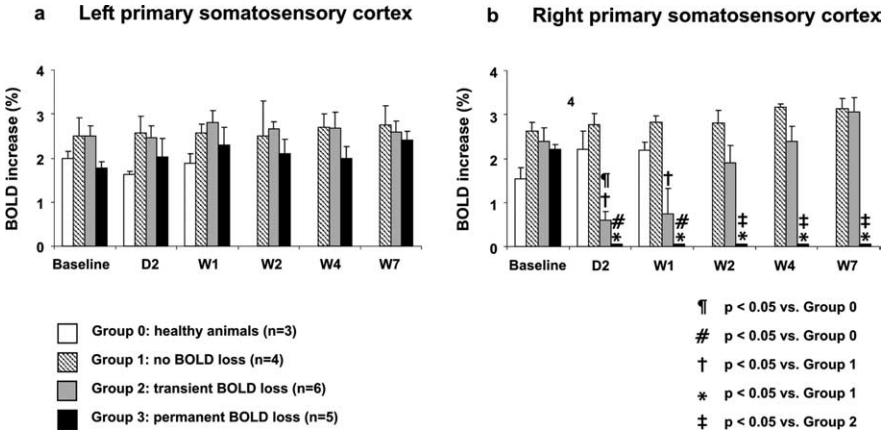

Figure 2. BOLD percentage change in the left (***a***) and right (***b***) primary somatosensory cortex during electrical forepaw stimulation before (baseline) and 2 d (D2), 1 week (W1), 2 weeks (W2), 4 weeks (W4), and 7 weeks (W7) after MCAO. Healthy animals were only studied at the first three time points. No positive BOLD percentage change was observed in animals with permanent BOLD loss after MCAO in the right primary somatosensory cortex. Data represent mean ± SEM.

(group 1; $n = 4$), a significant BOLD response was invariably observed in the right S1FL at all follow-up fMRI measurements, indistinguishable from the control group (Fig. 2*b*). The animals of group 1 either developed selective neuronal death ($n = 2$) or infarction ($n = 2$) of the right striatum.

In the second group of animals (group 2; $n = 6$), three rats developed an infarction of the right striatum, whereas three animals had a combined infarction of the right striatum and parietal cortex. Stimulation of the left forepaw in this group resulted in either a transient decrease or transient loss of the BOLD response in the subacute and early chronic stage after MCAO. At day 2 after stroke induction, this reduction of BOLD response was significant relative to the control group ($p = 0.012$) and relative to group 1 (no loss of BOLD activity; $p = 0.001$), whereas during the following weeks a clear trend toward normalization was observed (Fig. 2*b*). Complete reemergence of normal activation in the right S1FL occurred between 1 and 4 weeks after MCAO in all animals. Functional reorganization in remote brain areas was not observed at any time point.

In the third group (group 3; $n = 5$), all animals developed a combined infarction of the right striatum and parietal cortex. Stimulation of the left forepaw after transient MCAO did not result in a statistically significant BOLD response in the right S1FL or any other brain area during the following 7 weeks in this group (Figs. 1, 2*b*).

Electrical activity and hemodynamic response after stroke

Combined electrophysiological recording of short-latency SSEPs and BOLD fMRI were used to investigate the neuronal activity and hemodynamic response before and after stroke induction (Figs. 1, 2). This combined dataset over time was exploited to investigate potential disturbances of the neurovascular coupling during stimulation and after stroke.

SSEP recording of the primary somatosensory cortex in healthy animals revealed a typical waveform with a first positive peak (P1) followed by a first negative (N1) and a second positive peak, similar to the SSEP signals recorded from intracranially or transcranially implanted electrodes (Sakatani et al., 1990; Ogawa et al., 2000; Gsell et al., 2006). No significant interhemispheric differences for latency of P1 (mean ± SD; $P1_{left}$, 12.4 ± 0.6 ms; $P1_{right}$, 12.3 ± 0.5 ms; $p = 0.62$), for N1 latency ($N1_{left}$, 18.3 ± 0.8 ms; $N1_{right}$, 18.2 ± 1.3 ms; $p = 0.71$), or for the amplitude between these two peaks ($amplitude_{left}$, 31 ± 9.3 μV; $amplitude_{right}$, 33.8 ± 9.7 μV; $p = 0.21$) were observed. Latencies and amplitudes remained unchanged at all time points after MCAO for the S1FL recording site of the left, unaffected hemisphere in all animals, regardless of the extent of brain ischemia.

After transient MCAO, SSEP recording in animals with preserved BOLD response in the right S1FL (groups C and 1) showed changes in neither the peak latencies nor the amplitudes at both S1FL recording sites. SSEP recording in animals with transient loss of BOLD activation (group 2) revealed a significant amplitude reduction and/or prolonged latencies for P1 and N1 when no significant BOLD response was present in the right S1FL (Table 1). Reemergence of a significant BOLD response was always connected with the restoration of normal SSEP latencies and amplitudes.

Animals presenting a permanent loss of the BOLD response in the right S1FL (group 3) had no recordable SSEP signal or a marked abnormal amplitude reduction over the right S1FL. No restoration of the SSEP signal occurred during the complete study period in these animals (Table 1). A SSEP signal in the left (ipsilateral) S1FL during electrical stimulation of the left, affected forepaw (that would have indicated interhemispheric reorganization) was not observed at any time point.

Correlation analysis between BOLD percentage change and SSEP amplitude in the right S1FL during left forepaw stimulation showed a correlation coefficient of 0.884 ($p < 0.0001$) for all time points (Fig. 3). A significantly detectable, but very weak BOLD percentage change (0.3%) was only detected in one animal with an SSEP amplitude <14 μV. SSEP amplitudes >50 μV did not result in a higher BOLD percentage change than 3.8%.

Behavioral testing for functional outcome

To study functional outcome, behavioral tests were always performed on the day before the combined fMRI–electrophysiology experiments (Fig. 4). To assess the somatosensory pathway, we focused on the latency-to-contact of both forepaws with the adhesive tape removal test (Schallert et al., 2002).

All animals during baseline testing and animals developing no ischemic injury (group C) demonstrated no significant differences in their latency to contact the tape between the left and the right paw. Whereas the latency to contact the tape on the unimpaired right forepaw was not significantly affected by the transient MCAO at any time point (Fig. 4*a*), the latency to contact the tape on the impaired left forepaw was prolonged in all animals presenting an infarction of the right hemisphere (Fig. 4*b*), yet differences in both the degree of impairment and recovery were obvious between the three different groups.

Animals with preservation of their BOLD response (group 1) showed only a minor and nonsignificant prolongation of the latency to contact the left tape 2 d after MCAO, which remained prolonged at the same level at all follow-up time points.

Animals with a BOLD reemergence (group 2) presented a significant prolongation to contact the tape on the left, affected paw when compared with healthy animals 1 week after MCAO ($p = 0.025$) (Fig. 4*b*). These animals constantly improved during edema resorption toward week 7, although not reaching preischemic latencies or amplitudes of animals with no loss of BOLD response or healthy animals.

Table 1. SSEP amplitudes (μV) and first positive peak latency (P1; ms) in the right (ischemic) hemisphere during stimulation of the contralateral forepaw at preischemic (Baseline) and postischemic time points

Time point		Baseline		Day 2		Week 1		Week 2		Week 4		Week 7	
Animal	Group	SSEP amplitude	P1 latency	SSEP amplitude	P1 latency	SSEP amplitude	P1 latency	SSEP amplitude	P1 latency	SSEP amplitude	P1 latency	SSEP amplitude	P1 latency
22058	1	44	13.0	47	13.0	55	12.5	n.d.	n.d.	55	12.5	49	12.0
22059	1	33	12.0	30	12.5	32	13.0	n.d.	n.d.	35	12.0	29	12.0
22060	1	45	12.0	22	13.5	66	12.0	41	12.0	65	12.0	41	11.5
22080	1	29	12.0	15	13.5	29	11.5	41	12.0	64	12.0	74	12.0
22067	2	45	13.0	8[a]	14[a]	14[a]	11.0	8[a]	11.5	25	11.5	32	12.0
22081	2	29	12.5	22	16.5[a]	14[a]	17.5[a]	24	12.0	40	12.0	49	11.5
22068	3	31	12.0	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	7[a]	11.5
22069	3	15	12.0	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]
22070	3	34	12.0	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]

[a]Abnormal amplitude reduction and abnormal prolongation of P1 latency (≥2 SDs from baseline value).

[b]No SSEP signal was recorded on these post-ischemic time points.

n.d., SSEP signal has not been measured in these two animals 2 weeks after MCAO.

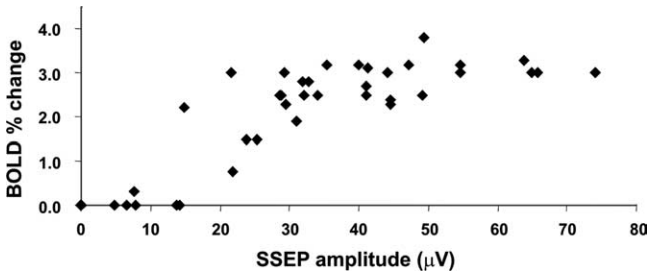

Figure 3. Correlation between BOLD percentage change and SSEP amplitude in the right affected primary somatosensory cortex (S1FL) during electrical forepaw stimulation of the left forepaw. Data are compiled for all animals and at all time points (Spearman's $r = 0.884$; $p < 0.0001$). Note the symbol at the coordinate system zero represents 13 measurements without BOLD and SSEP signals.

Animals with permanent loss of their BOLD response (group 3) showed a significant prolongation to contact the tape on the left paw when compared with healthy animals, both 2 d and at 1 week after MCAO ($p = 0.024$ and $p = 0.025$, respectively). These animals also improved during edema resorption but slightly deteriorated again from week 2 to week 7 after MCAO (Fig. 4*b*). This group performed worst during the chronic observation period.

Temporal profile of morphological lesion after focal brain ischemia

Analysis of quantitative T_2 maps showed a T_2 relaxation time of 55.4 ± 1.0 and 51.3 ± 0.4 ms in normal cortex and striatum, respectively. This value remained indistinguishable between preischemic values and postischemic values of the healthy hemisphere at all times. Two days after stroke induction, the ischemic lesion was clearly visible as a hyperintense area on T_2-weighted MR images. The ischemic T_2 value increased to 76.6 ± 1.6, 88.3 ± 3.9, and 94.5 ± 1.1 ms in groups 1, 2, and 3, respectively. During the following 7 weeks, T_2 values of group 1 remained essentially stable at this level (Fig. 4) (T_2 at 7 weeks, 75.6 ± 12.7 ms). In groups 2 and 3, conversely, a continuous increase in T_2 was observed. In group 2, this increase resulted in an ischemic T_2 value of 126.9 ± 11.7 ms after 7 weeks, whereas the corresponding increase in group 3 was very pronounced (T_2 at 7 weeks, 168.1 ± 14.5 ms).

Correspondingly, the evolution of the apparent T_2 lesion volume differed. In group 1, the T_2-defined lesion volume was highest at 2 d after stroke induction (63.3 ± 15.2 mm^3) and then continuously reduced to reach 8.8 ± 4.2 mm^3 after 7 weeks. In group 2, T_2 lesion volume was 124.1 ± 28.5 mm^3 at day 2, followed by a continuous reduction to 55.2 ± 23.3 mm^3 after 7 weeks. In group 3, T_2 lesion volume was 198.2 ± 12 mm^3 after 2 d and slowly decreased to 143.0 ± 15.3 mm^3 after 7 weeks (Fig. 5).

Correlation between T_2-based lesion and functional deficit

To assess the interpretation of T_2-weighted MRI hyperintensity as representing the true lesion size, we determined the lesion area on histological sections through the center of the lesion. This lesion area was compared with the area of elevated T_2 value on the T_2 maps of the last session, at 7 weeks, directly before killing the animals for histological analysis. Small lesions will not necessarily lead to pannecrosis but only to selective neuronal death (group 1), which can lead to normalization of T_1 and T_2 relaxation times during the chronic stroke period (Wegener et al., 2006).

The correlation analysis between T_2 lesion volume and histologically determined infarction size was therefore limited to the data of groups 2 and 3 in which pannecrosis was always detectable on histological sections. This resulted in a good linear correlation between T_2-based lesion size and histological lesion determination with a correlation coefficient $r = 0.85$, indicating that the T_2-visible lesion does reflect the necrotic tissue area during the chronic phase of the lesion evolution in these two groups.

We further analyzed whether T_2-based lesions allow a morphological prediction of functional deficit or outcome. However, although T_2 lesion volume was significantly different in the chronic phase between animals with a transient and permanent BOLD loss (a consequence of the mixture of pure subcortical infarcts and subcortico-cortical infarcts in the group with transient BOLD loss), when investigating the BOLD pattern in individual animals, it became obvious that no connection exists between lesion size and functional brain activation potential. As a matter of fact, extreme cases were observed in which functional brain activation still persisted despite very large T_2 lesions, whereas in other cases smaller lesions led to a permanent inhibition of functional activation (Fig. 6).

Discussion

Intraindividual longitudinal fMRI monitoring during lesion evolution

To our knowledge, this is the first animal fMRI study permitting an intraindividual temporal profile. Previous fMRI studies used α-chloralose in which functional–metabolic coupling is well preserved (Ueki et al., 1988). However, severe side effects of α-chloralose (Silverman and Muir, 1993; Hedenquist and Hellebrekers, 2003) make it unsuitable for longitudinal fMRI studies,

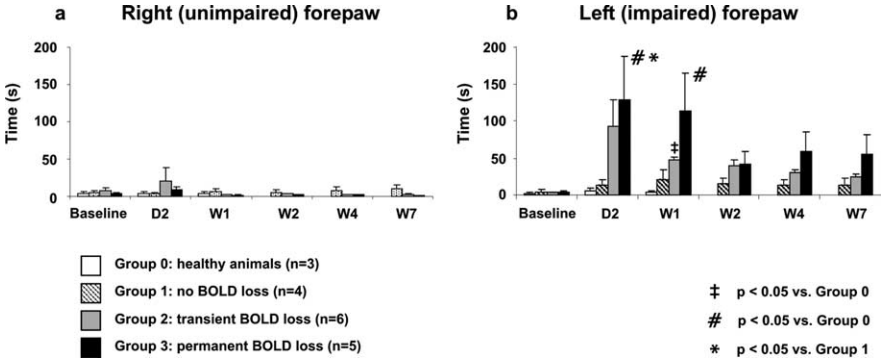

Figure 4. Behavioral testing after stroke. Time to first contact of the right, unimpaired forepaw (***a***) and left, impaired forepaw (***b***) in the adhesive tape removal test before (baseline) and 2 d (D2), 1 week (W1), 2 weeks (W2), 4 weeks (W4), and 7 weeks (W7) after MCAO. Healthy animals were tested only at the first three time points. Data represent mean ± SEM.

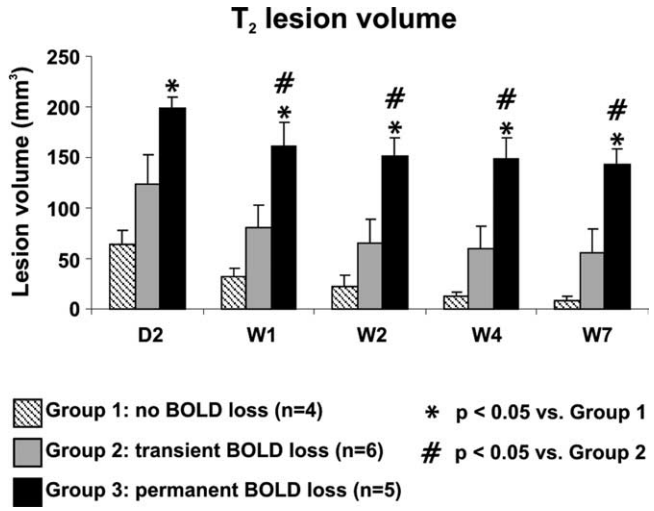

Figure 5. T_2 lesion volume 2 d (D2), 1 week (W1), 2 weeks (D2), 4 weeks (W4), and 7 weeks (W7) after MCAO. Data represent mean ± SEM. $^{*}p < 0.05$ vs group 1; $^{\#}p < 0.05$ vs group 2.

but longitudinal fMRI studies become more and more important for assessing functional deficits and improvement in the course of a neurological disease and treatment. As a compromise, earlier fMRI studies after experimental stroke dealt with separate groups of animals for separate survival times (Dijkhuizen et al., 2001, 2003; Kim et al., 2005). As we show here, there is non-negligible interindividual variation during recovery from ischemic stroke concerning functional deficit and recovery chances despite the standardized MCAO stroke model. Although maybe not relevant for the characterization of pathophysiological and/or morphological lesion evolution, these variations in functional brain activation make the interpretation of group averages at separate survival times especially in the acute and early chronic stroke phase difficult. The present investigation deals with most of those challenges by applying a noninvasive medetomidine anesthesia protocol (Ramos-Cabrer et al., 2005; Weber et al., 2006).

Lack of correlation between lesion size and functional deficit

Past experimental and clinical studies assessed ischemic lesion severity based on lesion volume as the determining parameter (Palmer et al., 2001). Concerning metabolic and hemodynamic alterations and recovery, this approach may be valid because it characterizes the tissue volume affected by the ischemic condition. However, as shown here, lesion volume alone will at best provide a statistically relevant trend across group comparisons but will not permit reliable prediction of long-term functional outcome on an individual level. Spontaneous recovery of functional brain activation exists in cases of almost hemispheric ischemic involvement, whereas functional deficit may persist in equivalent or even smaller lesions (Fig. 6).

Functional outcome is reflected in BOLD fMRI activation pattern and confirms reemergence of activation as basic principle of brain reorganization

Our intraindividual time profile studies showed a stable pattern: those animals with a chance of recovery gained significant BOLD activation in the primary somatosensory cortex 1–4 weeks after stroke at the latest, independent of lesion size. Animals that did not recover within the first 4 weeks did not show significant BOLD activation over the next 6 months (unpublished data; Ramos-Cabrer and Justicia) and presented the worst functional outcome in behavioral testing after edema resorption. The finding that reemergence of activation in the primary somatosensory cortex is the basic principle of brain reorganization in the somatosensory system is in agreement with recent functional imaging reports in both rats (Dijkhuizen et al., 2001, 2003) and humans (Wikstrom et al., 2000; Carey et al., 2002; Oliviero et al., 2004).

In contrast to previous reports in rats (Abo et al., 2001; Dijkhuizen et al., 2001, 2003), no signs of brain plasticity in both the affected and unaffected hemisphere were observed here. Even in severely affected animals with no restoration of electrical and BOLD activation in the primary somatosensory cortex, no BOLD activation was seen in other brain regions. Dijkhuizen and colleagues reported activation in the unaffected cortex 3 d after stroke induction. This trans-hemispheric shift resolved when activation was restored in the primary somatosensory cortex of the affected hemisphere after 14 d (Dijkhuizen et al., 2001, 2003). In a study in which the primary somatosensory cortex was directly lesioned (Abo et al., 2001), activation of the sensorimotor cortex of the unaffected hemisphere and activation lateral to the lesion was observed 3 weeks after stroke induction, at a time when all animals showed complete functional recovery. Whereas the aforementioned authors measured changes of CBV after injection of an intravascular contrast agent, we used the BOLD fMRI method, because it is noninvasive, can be easily repeated in a longitudinal study, and is used in the vast majority of human fMRI studies.

The BOLD fMRI signal depends on blood volume, blood flow, and oxygenation (Heeger and Ress, 2002; Logothetis and Wandell, 2004). It was reported that activation-induced CBV changes are more sensitive compared with BOLD signal changes in rats subjected to transient MCAO (Kim et al., 2005), but the interpretation of CBV-based fMRI is also critically discussed in the literature (Smirnakis et al., 2007). The lower sensitivity of BOLD fMRI could account for the lack of detection of brain plasticity in our study. Recently, an electrophysiological threshold for the coupling of neuronal activity and cerebral blood flow in the somatosensory cortex of healthy rats was reported (Norup Nielsen and Lauritzen, 2001). Figure 3 shows lack of BOLD for SSEPs smaller than 14 μV, but, because of our experimental setup, SSEP amplitudes below 10 μV are not significantly above the noise level. Thus, we may not have been able to detect electrical activation during forepaw stimulation below this threshold value of 14

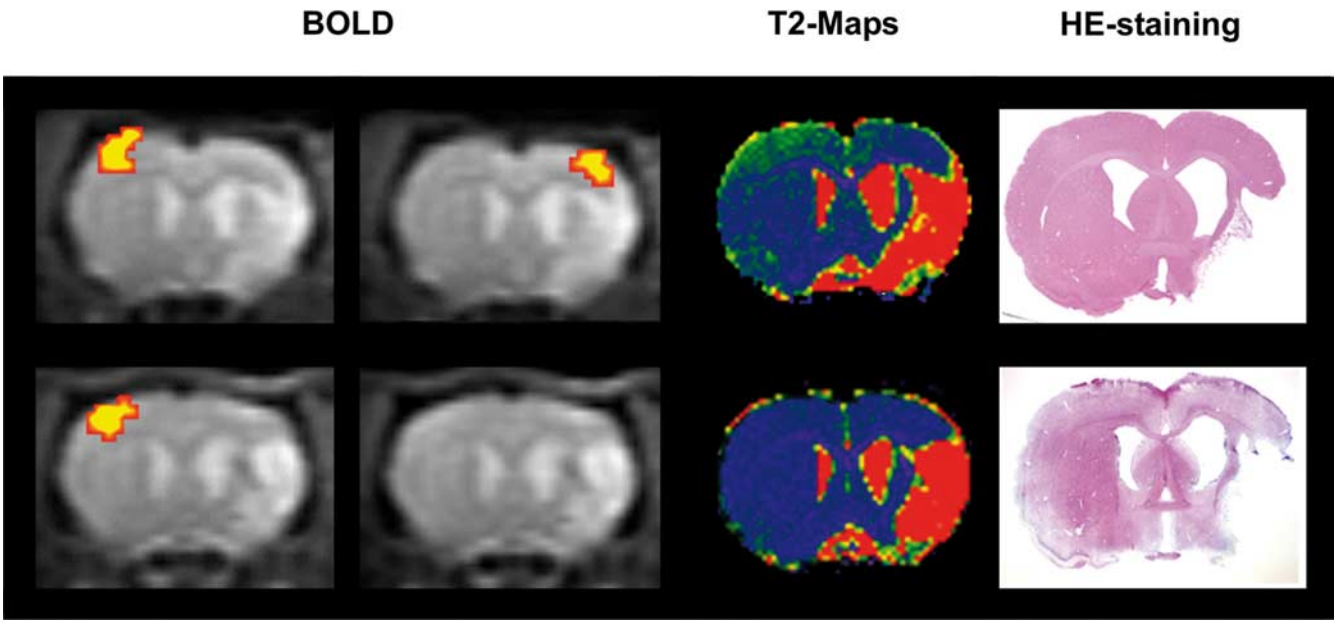

Figure 6. Comparison of lesion size and functional deficit condition at 7 weeks after MCAO. The first two images show the BOLD response after stimulation of both forepaws. The T_2 image demarcates the T_2 lesion size, in good agreement with the hematoxylin–eosin (HE) staining. The top row shows an animal with a rather large cortico-subcortical lesion but full restitution of BOLD fMRI signal in the ischemic hemisphere at 7 weeks. The bottom row presents an animal with a somewhat smaller lesion but with persistent functional deficit at 7 weeks (i.e., no BOLD recovery of the ischemic hemisphere).

μV in both the primary somatosensory cortex and remote brain areas resulting in a significant BOLD response in our study.

Furthermore, differences in the MCAO stroke models (90 or 120 min MCAO vs 60 min MCAO) may have lead to a gradual difference in lesion severity and induction of transient brain reorganization in more affected animals. Because our new anesthesia protocol for fMRI has only been compared with α-chloralose in healthy animals so far (Ramos-Cabrer et al., 2005; Weber et al., 2006), we cannot rule the possibility that α2-noradrenergic agonists such as medetomidine could alter or even suppress brain plasticity after stroke (Feeney et al., 2004).

Preservation of neurovascular coupling after stroke

By longitudinally combining noninvasive functional imaging and electrophysiological recording of SSEPs, the underlying causes of activation loss and restoration are better characterized. Activation reemergence in the somatosensory cortex was only registered by BOLD fMRI, when electrical activity was restored 1–4 weeks after induction of stroke. During the period of loss of electrical activity to the somatosensory stimulus, brain edema formation, followed by gradual reabsorption (Rossini et al., 2003), and impaired neurotransmission (Aoyagi et al., 1998) may be regarded as responsible for the transient dysfunction of the electrical activity. Furthermore, activation restoration was only observed when the primary somatosensory cortex was not directly affected by the ischemia.

The correlation of fMRI results with electrophysiological data are important. Although BOLD fMRI is proportional to the SSEPs in anesthetized rats (Brinker et al., 1999) and awake humans (Arthurs et al., 2000) under normal physiological conditions, this coupling between electrical activity and hemodynamic response may be disturbed or even inhibited during pathophysiological conditions. After resuscitation after cardiac arrest in the rat, the cerebral vasculature is transiently paralyzed, mainly because of extracellular acidosis acting as a potent vasodilator (Waltz, 1969; Hossmann, 1997). During the loss of vascular reactivity, electrical activity to somatosensory stimulation was recovering already, leading to a transient neurovascular decoupling (Schmitz et al., 1998). Therefore, a parallel recording of SSEP and BOLD fMRI during the chronic poststroke phase permits assessment of the long-term characterization of neurovascular coupling. Here, we show that, during the first 7 weeks after 1 h of MCA occlusion, the neurovascular coupling remains preserved at all times.

Kim et al. (2005) discussed stroke-related alterations in hemodynamic coupling, which were controversially reported in human BOLD fMRI studies of sensorimotor stroke recovery (Pineiro et al., 2002; Rossini et al., 2004; Krainik et al., 2005). Two of these human studies reported a decreased BOLD signal in both affected and unaffected sensorimotor cortices of stroke patients compared with age-matched controls (Pineiro et al., 2002; Rossini et al., 2004). In contrast to these observations, we did not observe uncoupling of neuronal activity and BOLD response in both hemispheres but a coupled alteration of both neuronal activity and hemodynamic response. A distorted or absent neuronal activity resulted in decreased or absent hemodynamic response. During restoration of normal neuronal activity, restoration of normal hemodynamic response occurred. A major difference between human and animal stroke fMRI studies are concomitant factors in human stroke patients, which can influence the neurovascular coupling. In both human studies, all reported patients had at least one cardiovascular risk factor, such as arterial hypertension, smoking, and hypercholesterolaemia, and were treated with various medications. All these factors can have marked influence on the vascular reactivity and therefore may induce uncoupling of neuronal activity and hemodynamic response in both the affected and unaffected hemisphere of stroke patients. Under controlled experimental stroke situations in rats, these confounding factors can safely be excluded and are not influencing the hemodynamic coupling.

In summary, our studies reveal the following aspects on functional brain activation in relation to experimental stroke in rats using BOLD fMRI: our data confirm previous studies that acti-

vation reemergence in the original cortical representation field is the basic principle of functional recovery after stroke in the somatosensory system. Our findings of continuing preservation of neurovascular coupling in both the early and late chronic phase after experimental stroke have important implications for the clinical translation: the coupling preservation indicates that BOLD fMRI may safely be applied as a surrogate marker for functional loss and its restoration in such animal studies.

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