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Optical imaging of disrupted functional connectivity following ischemic stroke in mice

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Abstract

Recent human neuroimaging studies indicate that spontaneous fluctuations in neural activity, as measured by functional connectivity magnetic resonance imaging (fcMRI), are significantly affected following stroke. Disrupted functional connectivity is associated with behavioral deficits and has been linked to long-term recovery potential. FcMRI studies of stroke in rats have generally produced similar findings, although subacute cortical reorganization following focal ischemia appears to be more rapid than in humans. Similar studies in mice have not been published, most likely because fMRI in the small mouse brain is technically challenging. Extending functional connectivity methods to mouse models of stroke could provide a valuable tool for understanding the link between molecular mechanisms of stroke repair and human fcMRI findings at the systems level. We applied functional connectivity optical intrinsic signal imaging (fcOIS) to mice before and 72 hours after transient middle cerebral artery occlusion (tMCAO) to examine how graded ischemic injury affects the relationship between functional connectivity and infarct volume, stimulus-induced response, and behavior. Regional changes in functional connectivity within the MCA territory were largely proportional to infarct volume. However, subcortical damage affected functional connectivity in somatosensory cortex as much as larger infarcts of cortex and subcortex. The extent of injury correlated with cortical activations following electrical stimulation of the affected forelimb and with functional connectivity in somatosensory cortex. Regional homotopic functional connectivity in motor cortex correlated with behavioral deficits measured using an adhesive patch removal test. Spontaneous hemodynamic activity within the infarct exhibited altered temporal and spectral features in comparison to intact tissue; failing to account for these regional differences significantly affected apparent post-stroke functional connectivity measures. Thus, several results were strongly dependent on how the resting-state data were processed. Specifically, global signal regression alone resulted in apparently distorted functional connectivity measures in the intact hemisphere. These distortions were corrected by regressing out multiple sources of variance, as performed in human fcMRI. We conclude that fcOIS provides a sensitive imaging modality in the murine stroke model; however, it is necessary

to properly account for altered hemodynamics in injured brain to obtain accurate measures of functional connectivity.

Keywords

Functional connectivity; mice; stroke; functional recovery; global signal regression

1. Introduction

Stroke is a major health concern in the United States, where it is the fourth leading cause of death and the leading cause of adult disability[1]. Although tissue death from ischemic injury is often well localized, it is becoming increasingly clear that focal injuries affect distributed patterns of synchronized neural activity throughout the brain. Recent studies using resting-state functional connectivity magnetic resonance imaging (fcMRI) have demonstrated that intra- and inter-hemispheric connections are altered shortly after stroke in humans and predict performance in tasks related to the injury [2, 3]. In particular, disruption of functional connectivity between homotopic cortical regions appears to be a strong predictor of poor performance after injury in domains of both attention and motor tasks [2–5], findings which underscore studies reporting altered evoked responses in the affected brain regions of stroke patients[6–8].

FcMRI studies of stroke in rats have generally produced similar results to those in humans. Both stimulus-induced cortical responses [8–10], and functional connectivity[11] are reduced following focal ischemia, and correlate with behavioral deficits and subsequent recovery. However, interhemispheric homotopic connectivity and contralesional ipsilateral connectivity in somatosensory and motor regions in rats has been reported to subacutely increase[11]. These two latter results might suggest more rapid systems level reorganization in rats following focal ischemia than has otherwise observed in humans[12] or at the cellular level in other animal models of stroke recovery [13–15].

Because the size of the mouse brain has presented a more significant challenge than rats for fcMRI, to date, there have not been analogous hemodynamics-based studies of functional connectivity in mice subjected to ischemic injury. Establishing analogous functional imaging in both mouse and humans is one of the most promising strategies to providing clinical translation. It is important to extend functional connectivity methods to mouse models of stroke so that molecular studies in mice[16–19] can be related to human stroke fcMRI findings. To address this need, we have developed functional connectivity optical intrinsic signal imaging (fcOIS) in mouse models of healthy[20] and diseased [21] brain. The observed functional connectivity patterns are robust and reproducible across mice and reveal cross-species homologies with humans (e.g. compare Fig. 3 in[20] with Fig. 1 in [22]).

To establish fcOIS in the context of an acute ischemic stroke model, we performed fcOIS before and 72 hours after transient middle cerebral artery occlusion (tMCAO). Functional status of the mice was evaluated in a manner akin to human stroke studies. Mice were separated into three groups based on infarct size and location to determine if graded

ischemic injury incrementally impacts the relationship between functional connectivity and infarct volume, stimulus-related activations, and behavior. Determining how these relationships are affected after stroke will provide a more complete understanding of acute systems-level damage, but in a model capable of facilitating targeted studies of stroke recovery mechanisms using genetic and molecular approaches.

Because functional connectivity measures depend on a preprocessing strategy, as a secondary goal, we examined how alternative regression approaches affect observed functional connectivity measures. These investigations indicated that global signal regression (GSR) alone can lead to distorted functional connectivity measures, and that multiple regression of nuisance variables is necessary to obtain accurate results. Overall, we found that fcOIS is a useful tool for understanding functional disruption in a mouse model of focal ischemia, and for bringing a robust and efficient functional assay into mouse studies of stroke recovery.

2. Methods

Animal Preparation

Male ND4 Swiss Webster mice, aged to 6–10 weeks (22–32g), were used for experimentation. Mice were given *ab libitum* access to food and water. All experimental protocols were approved by the Animal Studies Committee at Washington University.

In accord with our previously published animal preparation protocol for fcOIS imaging[20], anesthesia was initiated via i.p. injection with a bolus of ketamine-xylazine (1x dose: 86.9 mg/kg ketamine, 13.4 mg/kg xylazine) and animals were allowed 15 minutes for anesthetic transition. After induction, the animal was placed on a heating pad maintained at 37°C via feedback from a rectal probe (mTCII, Cell Microcontrols) and its head secured in a stereotactic frame. The head was shaved and cleaned, a midline incision was made along the top of the head to reflect the scalp and the skull was kept intact. To facilitate longer imaging times, after the initial bolus, mice were infused (i.p.) with a saline-ketamine cocktail (34.8 mg/kg/hr ketamine) during the imaging sessions.

Imaging system

Sequential illumination was provided at four wavelengths by a ring of light emitting diodes (LEDs) placed approximately 10 cm above the mouse's head. Our field of view included most of the cerebral cortex (approximately 1cm²). Diffuse reflected light was detected by a cooled, frame-transfer EMCCD camera (iXon 897, Andor Technologies); the LED ring and the camera were time-synchronized and controlled via computer using custom-written software (MATLAB, Mathworks) at a full frame rate of 30 Hz.

Imaging

Mice were imaged 7–14 days prior to and 3 days after tMCAO. Thirty minutes of activation data (15 min each paw, 18 stimulus presentations per paw) and up to 45 minutes of resting state data were collected for each mouse in 5 minute data sets (75 min of data total per mouse). The skull was kept moist with mineral oil during imaging.

Forepaw Stimulation

Needle electrodes were inserted into the dorsal and ventral sides of the left and right forepaws between digits 2 and 3. The stimulation paradigm consisted of 5 seconds of rest, followed by a 10 second stimulus train, then 35 seconds of rest administered in a block design. Electrical stimuli were 0.3ms pulses delivered at 3Hz at an amplitude of 1.5mA driven by a constant current stimulus isolation unit (World Precision Instruments). Fifteen minutes of data were collected for each paw (18 trials per paw total).

Image processing

Data from all mice were subject to an initial quality check prior to spectroscopic analysis. Data runs (5 minutes) in which reflected light level intensity (mean value over the brain) varied as a function of time by greater than 1% for any wavelength were excluded from further analysis. This preliminary quality control yielded 45–75 minutes of data per mouse. For subsequent analysis, image light intensity at each wavelength was interpreted using the Modified Beer-Lambert Law, usually expressed as: $\Phi(\mathbf{r},t) = \Phi_0 \cdot \exp(-\mu_a(\mathbf{r},t) \cdot L)$. Here, $\Phi(\mathbf{r},t)$ is the measured light intensity, Φ_0 is the baseline light intensity, $\mu_a(\mathbf{r},t)$ is the change in absorption coefficient due to hemodynamic changes, and L is the optical path length factor for photons in the tissue[23]. As there is no pre-stimulus baseline in resting-state experimentation, we normalized relative to the average light intensity at each pixel, resulting in differential measures of absorption at each wavelength at each pixel: $\mu_{a,\lambda}(\mathbf{r},t) = -\ln(\Phi_{\lambda}(\mathbf{r},t)/\langle \Phi_{0\lambda}(\mathbf{r},t) \rangle)/L_{\lambda}$. Absorption coefficient data were converted to hemoglobin (Hb) concentration changes by inverting the system of equations, $\mu_{a,\lambda}(\mathbf{r},t) = E_{\lambda,i} [\text{Hb}_i](\mathbf{r},t)$ (where E is the extinction coefficient matrix[24], and i runs over hemoglobin species). This inversion was performed using least-squares methods, yielding changes in oxygenated hemoglobin (HbO) and deoxygenated hemoglobin (HbR) at each pixel at each time point. Differential changes in hemoglobin concentration were filtered to retain the functional connectivity band (0.009–0.08 Hz) following previous human functional connectivity algorithms[25]. After filtering, each pixel's time series was downsampled from 30 Hz to 1 Hz, and all further analysis was performed only on those pixels labeled as brain using a manually-constructed brain mask. Temporal frequency power spectra were calculated by a Fast Fourier Transform (FFT). Image sequences during forepaw stimulation trials were coregistered and averaged over the 18, 50-second blocks presented to each paw. For all mice, responses from 13–15 seconds (corresponding to peak response in the Control group) were averaged to obtain activation maps for each paw. To account for inter-mouse variability in response amplitude and location, maps for each mouse were normalized by the amplitude at the coordinate corresponding to peak response in the control group divided by the peak response in a 20×20 pixel region (~2.4mm² area) around that coordinate. All analyses were performed using HbO unless otherwise stated.

Image coregistration

Image sequences of each mouse (as well as the brain mask for each mouse) were affine-transformed to a common atlas space determined by the positions of the junction between the coronal suture and sagittal suture (posterior to the olfactory bulb and cerebrum along midline) and lambda[26]. Bregma was not visible in all mice, and was calculated based on

the above two anatomical landmarks. The anterior-posterior stretch was set equal to the medial-lateral stretch, and all transformed images were centered at bregma. The intersection of every brain mask was calculated and made symmetric by reflection across the midline allowing all subsequent comparisons to be performed on shared brain areas across all mice. Brain slices were reassembled and aligned after TTC staining and registered with image sequence data using bregma and lambda. The infarct was segmented from the rest of the brain using the contrast provided by TTC staining of ischemic and non-ischemic brain tissue.

Signal Regression

Signal regression is a common method used to remove sources of variance from a given voxel's time series[27–30]. For a particular voxel, i , the time series detected $S_i(t)$ (in this case the pixel-wise OIS data) can be written as

$$S_i(t) = g(t) * \beta_i + x_i(t) \quad (1)$$

where $g(t)$ is a regressor, β_i is the regression coefficient, and $x_i(t)$ is the signal within the i th pixel after regression. For control mice and those with subcortical infarcts (Group 1), global signal regression (GSR) was used. Time traces of all pixels defined as brain were averaged to create a global brain signal, $g_{GSR}(t)$ and regressed from every pixel labeled as brain, where

$$g_{GSR}(t) = \frac{1}{N} \sum_{j=1}^N S_j(t) \quad (2)$$

and N is the number of all brain pixels ($N \sim 10,000$). For mice with cortical and subcortical infarcts (Groups 2 and 3) we used a multiple signal regression (MSR) approach where the time traces of all pixels within the infarct and those outside of the infarct were averaged separately to create two separate regressors,

$$g_{Infarct}(t) = \frac{1}{N_{Infarct}} \sum_{j=1}^{N_{Infarct}} S_{j,Infarct}(t) \quad (3)$$

$$g_{Non-infarct}(t) = \frac{1}{N_{Non-Infarct}} \sum_{j=1}^{N_{Non-Infarct}} S_{j,Non-Infarct}(t) \quad (4)$$

which were then regressed from all brain pixels simultaneously:

$$S_i(t) = g_{MSR}(t) * \beta_{i,MSR} + x_{i,MSR}(t) \quad (5)$$

$$g_{MSR}(t) = [g_{Infarct}(t) \ g_{Non-infarct}(t)] \quad (6)$$

Functional connectivity measures

To investigate the strength of network connections, 10 seed locations were positioned at coordinates expected to correspond to the left and right olfactory, motor, somatosensory, retrosplenial, and visual cortices using a histological atlas[26] as described previously[20, 21]. Seed time-traces were calculated by averaging the time traces within 0.25 mm of a seed locus (containing approximately 30 pixels). Functional connectivity analysis was performed by correlating these time courses against the time course of every pixel in the brain, producing 10 functional connectivity maps over the mouse brain. In addition, a 10×10 seed-to-seed correlation matrices was computed by correlating each of the 10 seed time traces. To further examine homotopic functional connectivity across the entire brain, three 5-minute data runs from each mouse were selected at random, concatenated, and every pixel in the mouse brain was used as a seed. Each pixel's time trace was correlated with its contralateral homologue, resulting in a homotopic functional connectivity map for each mouse. Functional connectivity maps were averaged within each group to obtain group-level results.

Adhesive tape removal test

A small square piece of adhesive tape (an Avery® label cut to 0.3cm x 0.3cm) was placed over the ventral hairless region of the right and left forepaws, and the time taken to remove the tape was measured for each forelimb with a maximum allowed time of 120 seconds per trial. Five days before the first imaging session all mice were trained to remove adhesive patches from the right and left paws within 5 seconds; reported post-stroke times are the average adhesive removal times at 48 and 72 hours after tMCAO. All imaging was performed after behavioral tests.

Transient middle cerebral artery occlusion (tMCAO)

tMCAO was performed using a nylon suture coated with silicone for 60 or 90 min, as described [31]. Restoration of blood flow through the internal carotid artery by removal of the intraluminal suture was directly confirmed by inspection via an operating microscope and more distally by laser Doppler. After the procedure, animals recovered in an incubator before being transferred to their home cages.

Histology and group designations

After the final imaging session, brains were sectioned and stained with 2,3,5-triphenyltetrazolium chloride (TTC) to assist in characterizing the infarcts. After all mice were imaged, infarct volumes were ranked from smallest to largest. All statistical regression analysis was performed across all of the mice allowing infarct volume to vary continuously. For group analysis, the mice were classified into 3 groups post-hoc based on infarct location and infarct volume - group 1: mice with only subcortical infarcts (N=14, infarct volume ranging from 7.3 mm³ to 54.3 mm³); group 2: mice with infarcts involving subcortex and small lateral portions of cortex (N=20, infarct volume ranging from 32.9 mm³ to 71.6 mm³); group 3: mice with infarcts involving subcortex and large portions of cortex (N=12, infarct volume ranging from 90 mm³ to 169.3 mm³).

Statistical analysis

Pearson r values were Fisher z -transformed ($z(r) = \text{arctanh}(r)$) prior to all statistical comparisons. Statistical significance was determined using unpaired, two-tailed t -tests assuming unequal variance unless otherwise stated. All statistical analyses were performed using MATLAB, and values were accepted as significant if $p < 0.05$.

3. Results

To provide a basis for examining the interaction between functional connectivity deficits, ischemic injury, stimulus-induced responses, and behavior, the mouse data were separated into three groups according to infarct size and location. Group 1 had only subcortical infarcts (N=14); group 2 had moderate infarcts involving lateral/ventral cortex and subcortex (N=20); and group 3 had large infarcts involving substantial portions of both the cortex and subcortex (N=12) (Fig. 1A). Infarct volumes were statistically different for all group comparisons (Fig. 1B), and were estimated by analysis of brain slices stained with TTC (Fig. 1C: representative slices; Fig. 1D: reorganized slices to show representative infarcts within our field-of-view). The functional connectivity analyses examined brain regions within the MCA territory (motor, somatosensory, and visual), perilesional cortex (retrosplenial), and a control region (olfactory) as determined by their atlas designations (Fig. 1D).

3.1 The effect of ischemic injury on functional connectivity

Resting-state functional connectivity was evaluated as the Pearson correlation between a seed and other cortical locations. Correlations were calculated for each mouse and then averaged in mouse atlas space within each group (controls and infarct groups 1–3) for both pre- and post- stroke analyses. Prior to tMCAO, all of the mice revealed distinct, symmetric resting-state functional connectivity for each of the investigated seeds (Figs. 2, 3 control, top row). The pre-stroke maps exhibited symmetric patterns with positive correlations between adjacent cortex and functionally-related regions (e.g., motor and somatosensory cortex) and anti-correlations (negative r , blue), between functionally-opposed regions (e.g., somatosensory and retrosplenial cortices). Graded disruption of interhemispheric functional connectivity was observed primarily within the MCA territory (somatosensory, motor, visual cortices), depending on infarct volume for seeds placed in either hemisphere. Substantial change in functional connectivity in perilesional (retrosplenial) cortex was observed in groups 2 and 3. Moderate disruption in distant (olfactory) regions was also observed in group 3. Interestingly, seeds placed in the right (contralateral) hemisphere (Fig. 2) showed very little change in local, intrahemispheric functional connectivity as a function of injury. In contrast, many of the seeds placed in the left (ipsilesional) hemisphere (Fig. 3) show graded decline in intrahemispheric functional connectivity with the most notable changes occurring in somatosensory and visual cortices. In general, functional connectivity maps calculated for HbR (Fig. S1) and total hemoglobin (Fig. S2) exhibited similar trends as those calculated using oxygenated hemoglobin and were not further analyzed.

Connections between homotopic regions appear to be those most affected by ischemia, and decline in homotopic functional connectivity is strongly correlated with behavioral deficits and recovery [3, 4, 11]. To examine the topography of this measure in the mouse, every

pixel within our field of view was used as a seed center and its time trace was correlated with its contralateral homolog, producing a brain-wide map of homotopic functional connectivity within each cohort (Fig. 4). (Because a map of homotopic functional connectivity is necessarily symmetric about midline, we chose to visualize this measure in the ipsilesional hemisphere to illustrate how reductions in homotopic connectivity are spatially related to the functional domains perfused by the left MCA.) After tMCAO, functional connectivity in group 1 was dramatically reduced in somatosensory cortex and other lateral regions; decline in surrounding cortical areas becomes more apparent with larger infarcts. Thus, the spatial extent of reduced homotopic functional connectivity increased in proportion to infarct severity. In the most affected group, this measure trended towards zero (no correlation) over the entire field of view, indicating that the temporal synchrony between homotopic brain regions is globally affected by ischemia.

3.2 Quantitative relations between functional connectivity and lesion size

Figure 5 reports functional connectivity in relation to infarct size for mice following tMCAO. As suggested by the qualitative post-stroke results (Figs. 2, 3, 4), infarct size correlated most strongly with functional connectivity reductions in the motor and retrosplenial cortices. In contrast, infarcts of all sizes had similarly strong effects on homotopic functional connectivity in the somatosensory and visual regions. Ipsilesional somatomotor functional connectivity, calculated by correlating time traces from left somatosensory cortex with those from left motor cortex, was also invariant to injury (Fig. 5B, left somatomotor). Finally, homotopic functional connectivity in the olfactory region was preserved in mice with small stroke volumes and only minimally declined with increasing stroke volume (these effects are further illustrated in Fig. 9, which also includes control mice, see below).

3.3 Stimulus induced responses are incrementally affected by stroke severity

OIS imaging was used to map stimulus-induced responses to electrical forepaw stimulation before and after tMCAO in a subset of mice (control: N=16; group1: N=12; group 2: N=10; group 3: N=6). Responses to forepaw stimulation in control mice (Fig. 6, control) were well localized to somatosensory cortex, as defined by our atlas (Fig. 1D). Following stroke, responses to contralesional (right) limb stimulation declined significantly in the left hemisphere with increasing infarct severity (Fig. 6 bottom row, Fig. S3), and strongly correlated with functional connectivity decline in somatosensory cortex (Fig. S4). Responses in the affected hemisphere in group 3 had poorer signal to noise, and did not statistically differ from zero. While electrical stimulation of the unaffected (left) limb appears to produce a stereotypical response in all groups qualitatively similar to that as observed in the controls (Fig. 6, top row), we did observe very weak correlation between response magnitude in the unaffected (right) hemisphere and homotopic functional connectivity in somatosensory cortex (Fig. S4).

3.4 Behavioral measures in relation to homotopic functional connectivity

Sensorimotor function was assessed in 23 mice using an adhesive removal test before and after focal ischemia (15 mice in the control group; 3 mice in group 1, 10 mice in group 2, and 3 mice in group 3). Over the 5 days prior to the first imaging session, the mice were

trained to remove adhesive tape from the left and right forepaws within 5 seconds, and then were retested 48 and 72 hours after stroke. Removal times for the affected limb after stroke (Fig. 7A) significantly increased in each group compared to controls. A subset of those mice tested were imaged pre and post stroke and had their removal times evaluated against functional connectivity pre and post ischemia (6 mice in the control group; 3 mice in group 1, 7 mice in group 2, and 2 mice in group 3). Mice with removal times longer than 120s were not included (i.e., 3 mice from group 2 and 1 mouse from group 3 were removed from this analysis). Strong correlation was found between removal times for the contralesional limb and homotopic functional connectivity (Fig. 7B) in the motor region. Interestingly, a strong correlation was also found between affected limb removal times and homotopic functional connectivity in the retrosplenial cortex, a brain region not typically associated with this behavioral task.

3.5 Spectral and temporal features of injured and healthy hemisphere signals

Ischemic stroke produces focal lesions that alter regional spontaneous activity. An initial examination of resting-state hemodynamics compared the average time traces of infarct and non-infarct regions in group 2 and 3 mice (Fig. 8A); distinct temporal and spectral features were found over the functional connectivity band. For example, time traces within the infarct region (Fig. 8B, red trace) exhibit a “low-pass-filtering” characteristic compared with the non-infarcted tissue (Fig. 8B, black trace). Average power in frequencies above 0.03 Hz in the group 3 mice (Fig. 8C, red trace) was significantly lower in damaged tissue when compared to non-infarcted regions. Cross-correlation analysis of left and right homotopic regions within each group revealed peak time shifts ranging from 0 to -3.5 seconds across groups (Fig. 8D). Here, negative time shifts correspond to left hemisphere delay and motivated the implementation of the MSR analysis. In the following section, we show that failing to separately account for shared variance within the healthy and injured tissue significantly affects quantitative estimates of functional connectivity.

3.6 Multiple nuisance signal regression versus global signal regression

Homotopic functional connectivity was quantified using both MSR (Fig. 9A) and GSR (Fig. 9B). Results from both methods reveal altered functional connectivity following stroke. However, GSR produced homotopic anti-correlations with much of the lesioned hemisphere, including homotopic motor, somatosensory, and visual cortices in mice of groups 2 and 3 (Figs. 9B, 10, S5). In contrast, following MSR, these correlations approached zero (e.g. Fig. 9A, motor; Fig. 10, S5) for seeds placed in either hemisphere. Additionally, following MSR, intrahemispheric functional connectivity contralateral to the infarct between right motor cortex and right somatosensory cortex was preserved across all three groups (Fig. 9A, right somatomotor) and was not significantly dependent on infarct size (not shown). In contrast, correlations within either hemisphere incrementally increased with stroke severity following GSR (e.g. Fig. 9B, right and left somatomotor, and Figs. 10, S5).

4. Discussion

In this study, we used novel functional neuroimaging, fcOIS, and graded degrees of ischemia in mice to evaluate the relationships between functional connectivity and infarct

size, cortical responses to forepaw stimulation, and behavior. Establishing the degree of functional disruption in a mouse model of ischemia will enable further studies aimed at understanding network damage and repair in the context of human stroke where infarct size and location vary widely. Because the mouse brain exhibits many of the resting-state networks observed in humans, the mouse model provides a platform on which to study molecular mechanisms of repair [16, 17, 32, 33].

4.1 Effects of stroke on functional connectivity

Control mice (ND4 Swiss Webster) exhibit strong, brain-wide correlation between homotopic regions (Fig. 4, Control) similar to other wild-type (B6C3) mice that have previously been imaged [21]. Regional differences in homotopic functional connectivity are apparent; motor and retrosplenial cortices exhibit higher correlation than, for example, somatosensory cortices. These regional inhomogeneities suggest an inherent difference in the temporal coherence of spontaneous activity within and across different brain networks of the mouse brain. Overall, following tMCAO, functional connectivity between homotopic regions declined towards zero with increasing infarct severity. Significant disruption in functional connectivity was observed in motor, somatosensory, and visual cortices in all three groups, and in the retrosplenial cortex for moderate and large infarcts (Figs. 2, 3, 4, 5, 9A). In motor and retrosplenial cortices, we see significant correlation between functional connectivity and infarct volume (Fig. 5) and adhesive removal time and functional connectivity (Fig. 7). These data illustrate the specificity of correlation between functional connectivity and infarct volumes and behavior within specific brain regions (representing different networks). Lesion size correlated most strongly with the degree of functional connectivity disruption in motor cortical regions (Fig. 5). A different picture was found in the olfactory, somatosensory, and visual regions. Functional connectivity in the olfactory region remained high, near control values in all infarcted mice. Subcortical infarcts resulting from MCAO affected homotopic functional connectivity in somatosensory and visual cortices to a much greater extent than other cortical regions (e.g. motor). The dorsal branch of the MCA in the mouse directly perfuses tissue within somatosensory cortex, whereas both the MCA and the anterior carotid artery (ACA) supply blood to motor cortex [34]. During an MCA occlusion, the ACA might provide some, though insufficient, collateral flow to motor cortex while somatosensory cortex remains largely ischemic. The impaired reperfusion to motor cortex might explain why local ipsilesional connectivity around the motor seed was relatively preserved across groups while somatomotor connectivity in the ipsilesional hemisphere was not. Additionally, subcortical lesions involve both white matter tracts and the striatum [35]. Although the striatum receives excitatory input diffusely from the cerebral cortex, cortical afferents from somatosensory and visual cortices to intra- and inter-hemispheric targets seem to be preferentially damaged compared to motor cortex following tMCAO in the mouse. Somatomotor functional connectivity in the ipsilesional hemisphere was significantly reduced in all mice with infarcts of any size, however, intrahemispheric functional connectivity between right motor and right somatosensory cortex did not show a statistically significant decline for any infarct size. (Fig. 9). This result, which has not been observed in the rat, provides additional support for the implementation of an MSR approach to calculating functional connectivity post ischemia.

4.2 Stroke affects spontaneous and stimulus-induced activity

Following tMCAO, resting state signals within the infarct and non-infarct tissue revealed different temporal and spectral characteristics (Fig. 8B, C). Spontaneous hemodynamic fluctuations within the infarct exhibited significant attenuation over most of the functional connectivity band while the remaining components displayed delayed correlations with contralateral brain regions. These observations are consistent with several other studies reporting alterations in neurovascular coupling following stroke[36] in animals and humans where focal ischemia produced regional changes in basal perfusion[37–39] and cerebrovascular reactivity[40] within lesional and perilesional tissue. Such physiological effects do not exclude the possibility that viable neuronal tissue may still be present within the ischemic core and surrounding regions. Two recent fcMRI studies have analyzed the temporal shift between all brain voxels and the global signal in a manner similar to the cross correlation analysis between homotopic regions reported herein[41, 42]. In these studies, the authors found that in non-hemorrhagic, post stroke patients, a delay in time-to-peak correlation with the global signal was observed in subjects with chronic hypoperfusion[41, 42] and without neurologic impairment[42]. That is, a temporal delay in spontaneous activity corresponds to a vascular, but not necessarily a neuronal, deficit. Hypoperfused regions may still contain viable neuronal networks, but quantitative comparisons between temporal delay, functional connectivity changes, and tissue viability are beyond the scope of this manuscript (animals in the current study were imaged following complete reperfusion as determined by laser Doppler). We suggest that such studies would be better informed using multiple signal regression techniques, as presently described.

Reduced stimulus-induced responses in somatosensory cortex within the ipsilesional hemisphere (Fig. 6) agree with similar previously published studies of responses to forelimb stimulation in mice [43] and rats[10, 44]. Additionally, decreases in the blood oxygen level dependent (BOLD) response in humans have been observed in lesioned somatomotor regions, and impaired cerebrovascular reactivity has predicted weakened motor-related BOLD responses to contralateral movements[40]. We found that the cortical responses in the affected limb correlated significantly with functional connectivity decline in somatosensory cortex, in good agreement with several studies in rats and humans following stroke [2–5, 8–11].

4.3 Disrupted functional connectivity as a behavioral correlate

Behavioral recovery has been associated with alterations in perilesional cortex excitability[16], changes in local connectivity[45], and ultimately, cortical remapping [43, 46]. Ischemic injury has been shown to also affect neuronal connections more broadly at the network level - damage to one node can cause functional anomalies in distant nodes of the network, even those appearing anatomically normal. Recent fcMRI studies have shown that resting state networks are altered shortly after stroke in humans and predict performance in tasks related to the injury [2, 5, 8]. In particular, the disruption of interhemispheric homotopic functional connectivity has been shown to be predictive of poor behavioral recovery[4, 5]. Post stroke, we found that that subcortical lesions restricted to the white matter tracts and the striatum impaired somatosensory functional connectivity to the same extent as larger cortical infarcts (e.g., Figs. 2, 3, 4, 5, 9A). Thus, somewhat surprisingly, we

found that functional connectivity in somatosensory cortex, one of the brain regions typically associated with this somatomotor test of asymmetry, was not predictive of post stroke removal times ($R^2=0.10$, $p=0.19$, Fig. 7), even though the responses to forepaw stimulation did show a graded deficit that correlated with infarct volume (Fig. S3) and homologous functional connectivity (Fig. S4). Interestingly, motor and retrosplenial cortices were the only two regions where functional connectivity significantly correlated with adhesive removal time in the affected limb (Fig. 7B). While correlation between removal times and motor functional connectivity might be expected (mice remove the patches by bringing their forepaws to their mouths and pulling them off with their teeth), the correlation of this behavioral task with functional connectivity in retrosplenial cortex was not anticipated. One plausible explanation for this finding is that the mice must learn how to perform this task, a process that involves retrosplenial cortex for working memory and retrieval [47, 48]. A lesion of this region could disrupt normal memory pathways, potentially causing the mouse to forget how to remove the adhesive.

4.4 The effect of regression strategy on functional connectivity measures

GSR, that is, removal by regression of the timeseries averaged over the whole brain, was first used in application to human task-fMRI in 1998 [27], and continues to be primarily used as a preprocessing step in human resting state fcMRI [49]. GSR removes widely shared variance, much of which, in human fcMRI, is artifact attributable to head motion [50, 51] and fluctuating arterial pCO_2 [52], although part of the global signal undoubtedly reflects neural activity [53]. Within the preprocessing stream of human fcMRI, the global signal is only one of several regressors used to reduce artifact in functional connectivity patterns [54–56]. The primary virtue of including the global signal in the set of nuisance regressors that this greatly increases the specificity of computed correlations [57]; the primary drawback is that all subsequently computed correlations are negatively biased [30, 58]. Thus, physiology cannot be reliably inferred from the sign of an observed correlation following GSR. However, absent GSR, all parts of the brain appear to be positively correlated [56, 59], which also is biologically implausible [25, 60, 61].

In mouse fcOIS, head motion is not an issue generally because the animals are anesthetized. Nevertheless, GSR is required to remove widely shared variance attributable to cardiac pulsations [20]. But GSR alone is insufficient in this study, because spectrally altered and delayed activity is present over the infarcted hemisphere (Fig. 8B). It can be shown that a delay between two identical signals will generate anticorrelations following GSR [30]. This effect is prominently illustrated in group 2 and 3 mice in homotopic motor, somatosensory, and visual cortices (Figs. 9B, 10, and S5). The average delay between the infarcted and non-infarcted hemispheres in these animals was ~ 1.5 s, which corresponds to a phase shift of $\sim \pi/30$ to $\pi/4$ radians in the frequency band retained in our data (0.009 – 0.08 Hz).

The MSR approach eliminated apparent anticorrelations between homotopic regions in mice within groups 2 and 3. For mice with cortical infarcts, correlation values closer to zero, as obtained using MSR (Figs. 2, 3, 9A, S5), are plausible because infarcted tissue should not be capable of generating neural signals; MSR also eliminated the appearance of increased somatomotor functional connectivity in both the left (Figs. 3, 10) and right (Figs. 2, S5)

hemispheres, and instead resulted in statistically significant decreases in functional connectivity with a seed placed in the ischemic core (Figs. 3, 9, and 10). In contrast, the GSR correlations suggest that the within-hemisphere connectivity somehow increased with stroke severity for both the affected and unaffected hemispheres (e.g. Fig. 9B, right and left somatomotor, Figs. 10, **S5**) with the infarct tissue having the highest positive correlation with surrounding brain regions. Apparent increased contralesional intrahemispheric connectivity would be inconsistent with prior animal studies showing no change in dendritic morphology in the intact hemisphere following unilateral stroke [13, 14]. And, while significant increased anatomical connectivity has been observed in the intact hemisphere using manganese-enhanced MRI [62–64], unlike the present acute study, those studies were performed up to 10 weeks after tMCAO; time points when remodeling and cortical remapping would have either been initialized or more fully matured. Therefore, the GSR estimate of increased connectivity with increased stroke severity appears to be an artifact. MSR in functional connectivity studies during stroke recovery has yet to be done and may provide new insights on cortical restructuring and functional recruitment after stroke. A better grounding of regional pair-wise correlations will likely also provide more robust input data input to novel graph measures aimed at understanding large-scale network reorganization after stroke [65]. While the differences between MSR and GSR can be modest for some regions (e.g. Fig. 9, retrosplenial and group 2 motor; Fig. 10, group 2 visual) overall, the present MSR strategy yields less distorted functional connectivity measures than GSR alone and simplifies the interpretation of animal studies of unilateral stroke using both fcOIS and fcMRI [11, 65]. Outside of the context of stroke, our MSR approach would also prove amenable to measuring functional connectivity immediately following cortical spreading depression where spontaneous activity would be very incoherent between hemispheres. [66]

4.5 Limitations of the Current Study

One physical limitation of OIS (due to light scattering) is the restriction of the field of view to the cortical surface (<1mm), which precludes direct mapping of deep brain structures, and in the current context, segmentation of lateral and subcortical infarcts. This limitation is most apparent when calculating functional connectivity values for mice in Group 1 where GSR is used, despite potential temporal differences occurring over the cortex between homotopic regions (Fig. 8D). It was recently shown in human stroke patients that the degree of functional disruption following ischemia has to do more with lesion location and less with lesion extent. So, while the physics of light transport through biological tissue may limit fcOIS to the cortical surface, fcOIS is still sensitive to the effects subcortical infarcts, as clear functional disruption is observed within our field of view from distant lesions (as seen in the functional connectivity patterns of group 1 mice in Figs. 2 and 3). Whole-brain imaging is still possible using diffuse optical tomography methods, and the ability of assessing functional disruption in deep brain structures would be useful for understanding how damage to subcortical structures might affect the entire mouse brain.

5. Conclusions

In this study, we applied fcOIS imaging to mouse models of ischemic stroke to evaluate how graded ischemic injury incrementally impacts the relationship between functional connectivity, forepaw responses, infarct volume, and behavior in the mouse before and 72 hours after tMCAO. Disruption of cortical activations and behavior in the affected limb were proportional to infarct size, and correlated with regional deficits in functional connectivity. Additionally, because spontaneous hemodynamic activity within the infarct exhibited altered temporal and spectral features, our data suggest that accounting for the separate hemodynamics occurring in the healthy and damaged hemispheres might provide a more accurate method for calculating functional connectivity after stroke. Because homotopic functional connectivity has been strongly associated with behavioral and functional outcome in post-stroke patients, accounting for regional hemodynamic differences in functional connectivity analyses could aid interpretation of functional mechanisms responsible for behavioral recovery after injury. Given that resting-state functional connectivity measures have provided valuable information regarding functional organization in the human brain, we are now in a unique position to probe questions about post-stroke recovery mechanisms and the role of ipsilesional and contralesional functional connectivity on recovery using fcOIS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MRI	magnetic resonance imaging
fMRI	functional magnetic resonance imaging
fcMRI	functional connectivity magnetic resonance imaging
OIS	optical intrinsic signal imaging
fcOIS	functional connectivity optical intrinsic signal imaging
GSR	global signal regression
MSR	multiple signal regression
EMCCD	electron multiplying charge coupled device

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Figure 1. Infarct volume characterization

Infarct volumes were sorted in ascending order and mice were grouped according to infarct location and volume. (A) group 1: mice with only subcortical infarcts (N=14, infarct volume ranging from 7.3 mm³ to 54.3 mm³); group 2: mice with infarcts involving subcortex and small lateral portions of cortex (N=20, infarct volume ranging from 32.9 mm³ to 71.6 mm³); group 3: mice with infarcts involving subcortex and large portions of cortex (N=12, infarct volume ranging from 90 mm³ to 169.3 mm³). (B) Direct infarct volume quantification, mean $\pm$ S.D., # = $p < 0.00001$. (C) Representative coronal TTC slices, and (D) image histological atlas assignments of regions of interest with rearranged brain slices depicting infarct locations within the image field.

Figure 2. Functional connectivity maps for seeds placed in the right (unaffected) hemisphere
 Group-averaged functional connectivity patterns in mice before (control) and 72 hours after tMCAO using HbO. Functional connectivity maps for seeds in right (unaffected) olfactory, somatosensory, motor, retrosplenial, and visual cortices (black circles) exhibit changes in connectivity patterns commensurate with injury, indicating a loss of temporal synchrony between brain networks. Note that connectivity patterns of regions within the MCA territory (motor, somatosensory, visual) are more affected than perilesional regions (retrosplenial) and those far from the insult (olfactory).

Figure 3. Functional connectivity maps for seeds placed in the left (affected) hemisphere
 Group-averaged functional connectivity patterns (calculated using HbO) in mice before (control) and 72 hours after tMCAO. Functional connectivity maps for seeds in left (affected) hemisphere for olfactory, somatosensory, motor, retrosplenial, and visual cortices (black circles) exhibit changes in connectivity patterns commensurate with injury. Note that the seed placed in left somatosensory cortex is effectively in the ischemic core and shows a near lack of correlation with any part of the brain as one would expect from dead tissue.

Figure 4. Homotopic functional connectivity within the ipsilesional hemisphere

Group-averaged correlation maps of interhemispheric homotopic functional connectivity for all pixels within our field of view reveal regional differences in observed connectivity after focal ischemia that depend on stroke severity. Note the overall trend that the brain becomes gradually less connected (connectivity goes to zero) as the injury becomes larger. Functional connectivity values are Fisher z-transformed Pearson correlations.

Figure 5. Functional connectivity vs. degree of injury

Post-stroke homotopic functional connectivity correlated strongly with infarct volume in motor and retrosplenial cortices. Homotopic functional connectivity in somatosensory and visual regions, and left intrahemispheric somatomotor functional connectivity was much more uniformly depressed in mice with infarcts of all sizes. Homotopic functional connectivity in olfactory cortex was nearly unaffected by infarcts and maintained values similar to those in pre-stroke mice. Functional connectivity values are Fisher z-transformed Pearson correlations. Control mice are not included in this figure. See Figure 9A for comparison of infarcted mice vs. controls.”

Figure 6. Reduced activations in affected forepaw

Cortical activation maps for electrical forelimb stimulation of left (unaffected) forepaw (**top row**) and right (injured) forepaw (**bottom row**). The right limb exhibits reduced response amplitude as a function of stroke severity (see Fig. S3), while the induced response in the unaffected left limb is similar to the control group. Pixels having a response amplitude within 75% of maximum response are overlaid on a representative white light image of the brain.

Figure 7. Sensorimotor performance in affected limb is significantly correlated with homotopic functional connectivity in motor and retrosplenial cortices

(A) During the 5 days before tMCAO, all mice were trained to remove pieces of adhesive tape from the right and left paws within 5 seconds. Post-stroke removal times (which represent the average adhesive removal times at 48 and 72 hrs.) are incrementally disrupted, mean $\pm$ S.D., * = $p < 0.05$, ** = $p < 0.01$ calculated using an unpaired, one tailed t-test and (B) strongly correlate with functional connectivity in motor and retrosplenial cortex. Functional connectivity values are Fisher z-transformed Pearson correlations.

Figure 8. Altered hemodynamics and homotopic temporal coherence following ischemic stroke (A) Representative segmentation for mice with cortical infarcts. All pixel time traces within infarct or non-infarct regions were averaged to create the two regressors in Eqn. (6) (B) Spontaneous activity occurring within the infarct (red) and non-infarct (black) tissue exhibits marked differences in hemodynamic fluctuations (representative traces from mice in Group 3). (C) Power spectra of spontaneous activity within infarct (red) tissue for all mice in Group 3 show significant attenuation in all frequencies above 0.03 Hz compared with non-infarct tissue (black). (D) Time shifts in the ipsilesional hemisphere were estimated by cross-correlating every pixel in the left hemisphere with its contralateral homologue and measuring the time shift associated with peak correlation. Across all three groups, time-to-peak correlation between homotopic brain pixels is gradually delayed within the MCA territory and surrounding areas.

Figure 9. Global signal regression overestimates functional connectivity after stroke
Seed-to-seed homotopic connectivity in olfactory, somatosensory, motor, retrosplenial, and visual cortices and intrahemispheric connectivity between somatosensory and motor cortices in the right and left hemisphere for (A) multiple signal and (B) global signal regression methods quantified as Fisher Z scores. With increasing stroke severity, homotopic functional connectivity switches sign using global regression and increases, but decreases using multiple signal regression. Intrahemispheric somatomotor connectivity does not increase with larger infarcts in either hemisphere using multiple signal regression. Values represent mean $\pm$ S.D., * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$; # = $p < 0.00001$; n.s.=not significant. Functional connectivity values are Fisher z-transformed Pearson correlations.

Figure 10. Measured functional connectivity following ischemic stroke depends on regression method (seeds in affected hemisphere)

Functional connectivity patterns for seeds (black circles) in left (affected) hemisphere show graded decline in inter- and intra-hemispheric functional connectivity using multiple signal regression (MSR). Global signal regression (GSR) results in the largest changes in connectivity, with contralateral regions becoming increasingly anticorrelated in groups 2 and 3. This increased connectivity (both positive and negative correlations) following GSR is a result of the temporal delay between infarct and non-infarct tissue and is an artifact. Note with MSR that correlations with dead tissue (seed in left somatosensory cortex) approach zero as one might expect.