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“Early Evaluation of Nerve Regeneration After Nerve Injury and Repair Using Functional Connectivity MRI”

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Abstract

Resting state functional connectivity magnetic resonance imaging (fcMRI) studies in rat brain show brain reorganization caused by nerve injury and repair. In this study, distinguishable differences were found in healthy, nerve transection without repair (R+) and nerve transection with repair (R–) groups in the *subacute* stage (two weeks after initial injury). Only forepaw on the healthy side was used to determine seed voxel regions in this study. Disturbance of neuronal network in the primary sensory region of cortex occurs within two hours after initial injury, and the network pattern was restored in R+ group in *subacute* stage, while the disturbed pattern remained in R– group. These are the central findings of the study. This technique provides a novel way of detecting and monitoring the effectiveness of peripheral nerve injury treatment in the early stage and potentially offers a tool for clinicians to avoid poor clinical outcomes.

Introduction

The brain is known to change its cortical organization in response to short-or long-term peripheral influence as the result of changes in peripheral input.¹⁻⁵ This reorganization occurs within minutes to hours and lasts for various time depending on the different

procedures.⁶⁻⁸ In order to maintain the normal function of neurons in the somatosensory and motor networks, the feedback stimulation from target organs is needed. Once this feedback signal is blocked due to the nerve injury or target organ malfunction, the neurons in these networks tend to be dysfunction. The adjacent cortical receptive fields then shift and expand their cortical representation toward the dysfunctional neurons and replace them at last.^{2,9,10} Electrophysiology has been major technique used to detect this cortical plasticity. It has many advantages but very invasive and cannot be used on patients under normal circumstances. Due to the lack of means of early evaluation and monitoring after neurosurgery, both patients and surgeons are losing precious time which otherwise could be used to avoid poor clinical outcome by simply redoing the surgery within subacute stage.

Functional magnetic resonance imaging (fMRI) has been applied to many clinical areas. The fMRI signal relies on the ratio of oxygenated versus deoxygenated hemoglobin. Oxygenated hemoglobin is diamagnetic but paramagnetic when deoxygenated. The change of magnetic field caused by hemoglobin will be detected and used as contrast to create MR images. Neuronal activation in the central nervous system has been shown to cause activity-related changes in the blood oxygen level-dependent (BOLD) fMRI signal. The fMRI signal intensity can also be quantitatively measured by the number of activated units or defined as voxels. On top of this technique, spontaneous low frequency fluctuations in the cortical blood flow were found.¹¹⁻¹³ Based on these, resting state functional connectivity magnetic resonance imaging (fcMRI) was developed in 1995 by Biswal at MCW,¹⁴ and later on was widely accepted and applied in many clinical fields. Functional connectivity (FC) is defined as the temporal correlation between spatially remote neurophysiological events and is measured by correlating the neuroimaging time series in distributed brain areas.¹⁵ It has the unique property of revealing functional networks in the central nervous system without task activation. These resting state networks are found to be consistent across healthy subjects.¹⁶ Both fMRI and fcMRI are non-invasive, and the later one could be used on non-cooperative or incapable subjects. The purpose of the current work is to investigate the difference of fcMRI manifestations in healthy, R- and R+ groups of rat in subacute stage. The results of this study could be used to distinguish rats among health, R- and R+ groups and have a great potential to be used on nerve injury patients as an early marker to avoid poor clinical outcomes.

Materials and methods

General methods

Thirty-six Sprague-Dawley rats (SD rats, Charles River Laboratories, Wilmington, MA, USA) (300-400g) were used and divided evenly into two groups: acute group and subacute group. In each group, rats were further divided into three subgroups. Rats in one group were used as healthy control. In the second group, the right side median nerve was transected without repair (R- group). In the third group, the right side median nerve was transected and repaired immediately (R+ group). fMRI and fcMRI scans were done on all these rats under the same parameters.

fMRI/fcMRI parameters

9.4 Tesla small-animal scanner (AVANCE, Bruker, Billerica, MA, USA) was used for imaging procedures. A rapid acquisition with relaxation enhancement (RARE) anatomical scan was acquired prior to every fMRI and fcMRI run. RARE parameters were set as followed: 35mm field of view (FOV), 10 contiguous 1-mm-thick slices, echo time (TE)=50ms, pulse repetition time (TR)=2.5s, and matrix size=256×256. Slice three was located directly over the anterior commissure, which is located −0.36mm from bregma. The best result from these anatomical scans was used as the template for all other fMRI and fcMRI scans.

Two fcMRI acquisitions were performed without any stimulation prior to fMRI experiment. T_2^* -weighted echo-planar imaging (EPI) sequences were used for all experiments. The parameters were TR=2s, TE=18.76ms, FOV=35mm, matrix size=96×96 (zero filled to 128×128 matrix), with the same slice geometry as the RARE images. One hundred ten images were obtained during each fMRI/fcMRI experiment for a total acquisition time of 3 min 40s. Four minutes' recovery time was given after each acquisition.

fMRI data analysis

EPI acquisitions were registered to an ideal anatomy using the Oxford Center for Functional Magnetic Resonance Imaging of the Brain (FMRIB) Linear Image Registration Tool (FLIRT) software¹⁷ with 6 degrees of freedom at each direction. It should be pointed out that fMRI rat imaging uses a slightly different coordinate system than clinically used human MRI, which left hemisphere shows on the left side of the MRI image. Data for each nerve and stimulation protocol were averaged and masked using Analysis of Functional NeuroImages (AFNI) software. Activation was determined by an F test with a P-value threshold of 0.005.¹⁸ Voxels were classified as active if the F statistic was above threshold. Regions of interest (ROI) were drawn by consulting the activation map and cross-referencing it with the Paxinos and Watson rat atlas.¹⁹ In this study, ROI was chosen as primary sensory area of forelimb in both hemispheres (S1FL). They are “mirrored” areas in the central nervous system. With this method, the degree of cortical activation in different functional areas can be quantified by the area (or number) of activated voxels.

fcMRI data analysis

Two resting-state fcMRI acquisitions were done on each animal, and the results in each group were combined to build a new data set. Group fcMRI results were created by averaging all the individual fcMRI results within same groups. For this study, ROI was chosen as primary sensory cortex (S1), primary and secondary motor cortex (M1/M2) for both hemispheres. Two fcMRI techniques were used in this study. In the seed voxel based fcMRI technique, the most activated voxels when healthy left forepaw was stimulated in fMRI acquisition was selected as the seed voxel. A band-pass filter with a cutoff at 0.1Hz and 0.01 Hz was applied to all voxel time courses on a voxel-by-voxel basis covering the entire brain.²⁰ An averaged time course was made by averaging the time course of this seed voxel and six adjacent voxels during resting-state scan. This averaged time course was cross-correlated with each voxel time course in the whole brain to form the FC (r values) map for individual subjects. All r values in the individual map will be then transformed to

Fisher's Z scores by using the relationship between Z score and r value: $z = \frac{1}{2} \ln \frac{1+r}{1-r}$, which yields variable Z that is approximately normal distributed. All voxels that passed threshold of $p < 0.05$ after Fisher's transform were considered significant. In order to study the sign of early recovery, we also took all the voxels from the fourth layer of the cortex in S1FL area of both sides in two consecutive slices from the resting state scan.

In order to further reveal the connectivity change, two-way ANOVA analysis was later performed comparing fcMRI result from all three groups at 2 weeks post-operation with results acquired from the acute stage. P-value was set to 0.05 for significant changes. Although for both fMRI and fcMRI studies, only minimum head movement can be detected, motion correction was still performed using AFNI (3dvolreg). All the images were smoothed with FWHM of 1mm.

For both fMRI and fcMRI experiment, due to the administration of pancuronium bromide and tracheotomy procedure, the rats were securely mounted to the customized cradle. Meanwhile, the respiration rate for all animals was maintained at 55/minute. By adjusting the ventilator, the PaO_2 was maintained at about 95% for all animals during the scan. The heart rate of the animals was recorded and later on filtered out from the time course after Fourier transform. For all the fMRI and fcMRI analysis, multiple comparisons was performed and the p was set to 0.005.

Results

Figure 2 shows the fMRI results of right median nerve stimulation in the acute stage right after the initial surgery for all three groups. In the healthy control group (Figure 2A), distinct and localized activation could be seen in primary sensory region of forelimb (S1FL) of the left side hemisphere, which was circled in red. This activation was eliminated in both acute R- (Figure 2B) and acute R+ (Figure 2C) groups. Thalamic activation, cingulate activation and basal ganglia activation were observed in these two groups. They are natural brain responses to acute injury and also may be related to stress.²¹⁻²³ Either way, these physiological changes are beyond the scope of this study, and as a stress response, these activations disappear within two weeks. Significant statistical difference in activated voxel numbers was found between healthy control group (179 ± 27) and R-/R+ groups (0 ± 0). Negative BOLD response is apparent in both sides of the caudate putamen in all three groups (shown in blue color). They are mostly symmetric, and the underlying mechanism remains unknown. From our previous study results, this is a general marker of brain plasticity and it always decreases as long as dramatic brain plasticity occurs, which is also shown later in the study. This BOLD fMRI response could be seen in slices 1 through 4, with the most intense activation in slice 3. This correlates well with the description in the Paxinos and Watson atlas.

Figure 3 shows representative BOLD fMRI results of subacute stage groups (2 weeks postoperatively) when right side median nerve was stimulated. In all groups, the stress related basal ganglia and thalamic activation were eliminated. In the sham operation group (Figure 3A), localized S1FL activation can be observed. The voxel counting (135 ± 36)

revealed minute difference when it was compared to the healthy control group in the acute stage (Figure 2A). It might be the result of scar tissue created by the previous surgery compressing the nerve trunk. This S1FL activation completely disappeared in the subacute R–group (Figure 3B, voxel counting 0 ± 0), but somehow well restored in the subacute R+ group (Figure 3C, voxel counting 33 ± 17). Significant difference of activated voxel numbers were found among all three subacute groups (Figure 3D, $p<0.01$). The fMRI results from healthy control groups of both acute and subacute groups are consistent with our previous acquired fMRI map of nerve representation.²⁴

Seed voxel applied to resting state fcMRI scans was chosen based on BOLD fMRI while healthy left forepaw was stimulated. Figure 4 shows the fcMRI result of acute stage. In the healthy control group, by choosing the seed voxel from the healthy left forepaw representation area (S1FL), the whole primary sensory area (S1) on both hemispheres were revealed. This resting state fcMRI has distinct patterns of wide spread and symmetric. The fcMRI signal intensity of the whole system varies according to the strength of internal connections within S1 area. In R– (Figure 4B) and R+ (Figure 4C) groups of acute stage, this pattern was disrupted. In both these two groups, although seed voxel was the same as the control group, the fcMRI was greatly suppressed. Not only the symmetric property was eliminated, but also widely spread pattern was replaced by localized presentation. The histogram of fcMRI result was plotted for all three groups to reveal the influence of nerve injury to the resting state connectivity in S1 area (Figure 4D). After injury, for both R– and R+ groups, the fcMRI network in S1 area was greatly suppressed in both amplitude and the sum of voxel counts. There is no significant difference between R– and R+ groups at this time point since the repaired nerve hasn't initiate regeneration. The threshold of this result was set as $p<0.05$ after Fisher's transform. It shows most of the suppression happens at p between 0.05 and 0.02 range.

Figure 5 shows the result of fcMRI in *subacute stage*. The same widely spread and symmetric activation pattern was well remained in the healthy control group (Figure 5A). Distinct “mirrored” activation was seen which revealed entire S1 area. Result from *subacute R– group* (Figure 5B) was comparable to the result of acute R– and R+ group (Figure 4B and 4C). The symmetric and widely spread pattern remained being disturbed. The fcMRI result in this group kept localized with only part of S1 area activated in all slices. While in the *subacute R+ group* (Figure 5C), the normal fcMRI pattern was clearly restored, especially in the first slice. The newly restored fcMRI has the same property of wide spread and symmetric comparing to the normal group (Figure 4A, 5A). However, the activation appears to be the same in intensity, which leads to the uniform activation. The histogram of fcMRI result was also plotted for all three groups to reveal the influence of nerve injury to the resting state connectivity in S1 area in the subacute stages (Figure 5D). For the healthy control group, the result is highly comparable with acute stage with majority of the voxels detected within p of 0.05 to 0.02 range. For nerve injury group, this fcMRI network was greatly suppressed in both amplitude and total number of voxels. There is only few voxels acquired from $p<0.01$ range comparing to the other two groups. While in the nerve repair group, the fcMRI network was restored and become over compensated with more voxels detected almost at every p value. Most of the voxels are in p between 0.035 to 0.015 range

(58%) comparing to control group (44%) which results in the uniform fcMRI network pattern. Comparing the results of fcMRI in subacute stages between R- and R+ groups (Figure 5 B vs. C), it is obvious that fcMRI network analysis can differentiate non-regenerated nerve injury (R- group) from regenerated nerve pair (R+) early in the subacute stage with the restored uniform and wide spread network pattern. At this time point, normal BOLD fMRI acquired by direct nerve stimulation only shows extremely limited signs of nerve regeneration for R+ group (Figure 4C) and it is hard to distinguish it from non-regenerated nerve injury (Figure 4B).

Figure 6 demonstrates the sensorimotor network change caused by nerve injury and repair over two weeks' period ($p < 0.05$). In the control group (A), it is clear that the connectivity network in the S1 area was affected by the sham operation procedure. Although the mirrored pattern remained, the sensory network diminished significantly from both sides, with the left brain (contralateral to the sham operation side) being most affected. Motor network (M1) was slightly involved from the left brain. In the R- group (B), both sensory and motor network are affected. After the immediate sensory network disturbance, the sensory network on the left side of brain kept on breaking down yielding a negative signal. Different from the control group, the sensory network change was not significant in the right side brain, which is in charge of the sensory function of the healthy limb. The S1 network in both sides of the brain in the R- group became detached from each other and resulting in a non-mirrored pattern. Interestingly, significant reorganization happened equally to the motor networks (M1) on both sides. This motor network reorganization is quite unique that only showed in R- group. For animals of R+ group, the fcMRI change is almost reversed comparing the control group (Figure 6C). The sensory network from the left brain was widely restored. Similar as animals from control group, the M1 network was only slightly involved in this process.

Discussion

The clinical outcome of nerve repair remains unsatisfied. There is a big portion of poor results remains after surgical repair. Scientists and clinicians are trying every possible ways to save those patients with poor results. Aside from the different techniques and neuronal factors developed in the last few decades, there is no reliable ways that can be used on patients to early monitor the therapeutic result, pick out those patients with poor result in time and give them a second chance to correct this bad outcome. As we mentioned and showed previously in Figure 3B, without a proper treatment, the neurons and networks will break down in the *subacute stage*, which will lead to the non-recoverable damage to the patient clinically. Electrophysiological test could certainly detect this change, but it is too invasive and could not be applied on all patients. The fMRI early evaluation is another way (Figure 3C), but it need to stimulate the damaged nerve precisely and is also hard to perform on patients. In this study, we introduce this novel way to early distinguish poor/none regeneration after nerve repair that only requires the sensory stimulation of the healthy limb.

From the fcMRI results of both *acute* and *subacute* healthy control group (Figure 4A and 5A), we can summarize the fcMRI pattern of the primary sensory area in healthy rats as: symmetric, widely spread activation across the entire S1 area in both hemispheres. In the

non-recovery group after nerve injury (Figure 4B, 4C and 5B), although there are minute differences, the activation pattern was switched to: asymmetric, localized activation that occupies part of S1 area. This distorted pattern is the result of distorted neuronal network that is caused by non-regenerated nerve damage. In the nerve repair group (Figure 5C), the symmetric and wide spread activation of entire S1 area was restored. “Mirrored” activation was again observed other from normal healthy control groups. The only difference in this restored fcMRI pattern comparing to the original healthy fcMRI pattern is that it is uniform distributed with less detail of contouring information. The fMRI task activation revealed clearly that the cortical integrity was preserved in the nerve repair group by showing cortical BOLD activation, which did not exist in non-repaired groups. The same cortical integrity was shown using fcMRI in another way without any task requirement to the damaged limb in this study.

The change of fcMRI network over 2 weeks period is a complicated process. It is clear that sham surgery will cause fcMRI change. This might come from the scar tissue that compresses the nerve or the small damage on the nerve when it was isolated. With this small damage, S1 network from both sides are involved since the network in the left brain was calculated through seeds from the right brain. This fcMRI change remains more obvious on the left brain which directly in charge of the function of the limb that went through operation (Figure 6A). Only limited motor network (M1) was involved in this network reorganization. It is very obvious that the result from R+ group (Figure 6C) shares many common characteristics with the control group: 1) Sensory network reorganization happened bilaterally with the left brain being more involved. 2) fcMRI network change mainly happened in S1 with very limited M1 component involved. The major difference between these two groups was for the R+ group, the fcMRI network was being restored after anastomosis which yielded a positive result in Figure 6C. While in the control group, the same network was affected by the surgery manipulation, and might need more time to be fully recovered. On the other hand, it shows the great sensitivity of fMRI and fcMRI technique in nerve injury research that a simple sham operation procedure is enough to cause distinguishable changes.

fcMRI statistic analysis shows brain reacts significantly differently to the non-repaired nerve injury (Figure 6B). In the R– group, the sensory network from both side of the brain became detached from each other. The disturbed and suppressed fcMRI pattern remained for both S1 areas resulting in no significant change in the ANOVA analysis in the right brain. While on the left side, the S1 network kept on breaking down and resulted in a negative result. Unique network remodeling happened to the M1 area of both sides in the R– group. Both M1 areas are almost equally involved. Since the seeds for analysis came from the right brain, the result suggested that M1 network from the left brain became more attached to the right brain. Previous studies have demonstrated that inter-hemispheric reorganization happens to S1 when total deafferentation was performed.^{5,10,25} Nerve injuries have various sources like trauma, neuropathy, tumor, poisoning et al. The clinical outcome of nerve repair could be divided into three levels: good, fair and poor. Due to the complexity of neural surgery and lack of early monitoring, poor clinical outcome with persisting sensory and motor dysfunction consists a big portion of the whole patient population.²⁶ Built on top of these knowledge, our study suggests when a limited nerve injury happens, the fMRI results

remains unilateral no matter whether the anastomoses was done or not. The fMRI result of anastomoses is very limited at subacute stage even when the nerve directly below the repair site is electrically stimulated. When the nerve injury was left untreated, no fcMRI network restoration can be seen. Both M1 areas are heavily remodeled and attached to the right brain.

It is interesting that we did not find BOLD activation in the S1FL area of R+ and R- groups. It has been proved that task induced BOLD signal is usually less than 5% higher than it is in the resting state.^{27,28} After acute peripheral injury, neurons in the dorsal root ganglia constantly fire due to the nerve lesion,²⁹⁻³¹ which could cause pain and stress. This firing pattern is extremely potent and could easily override the small signal variation caused by task stimulation. This constant firing pattern will disappear along with the reorganization of the neuronal network.

Neuropathic pain caused by nerve transection has been well documented. Results from human studies suggest that the neuropathic pain alters the functional connectivity by disrupting thalamic feedback.^{32,33} Previous study from our group also revealed the breakdown of cortico-thalamic network in rats when total deafferentation was performed.²⁵ However, with a limited nerve injury and repair model used in this study, the change in cortico-thalamic network was not consistent across animals in the same group. There might be many reasons behind this, including the inter-subject variation that resulting in each animal reacting slightly differently to the nerve injury in the thalamic pathway. 1mm slice thickness used in this study is not enough to show this difference given the small size of the rat brain and nucleus.

This work demonstrates the different cortical fcMRI patterns in the *acute* and *subacute* stages of healthy, R- and R+ groups. The healthy groups in both stages show symmetric widely spread activations across S1 area in the cortex. The groups without nerve regeneration in both stages show highly distorted, asymmetric and localized fcMRI pattern that only activates partial S1 area. The group in *subacute* stage with nerve regeneration shows unique symmetric, uniformly wide spread activation pattern across S1 area. Here, we demonstrate for the first time that non-regenerative nerve damage can be detected in rats as early as within *subacute* stage in non-invasive way while cortical networks are still functional. Both direct fcMRI network map and the ANOVA analysis can be used as detection tool to reveal the non-repaired nerve damage as early as two weeks after surgery. The same technique can be applied to human and greatly increase the clinical outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Group information and electrode placement. In all six groups, right side median nerve was isolated, and electrode was attached at the level of 1cm above elbow. (A) Healthy control groups: no further damage was done to the nerve. (B) R- groups: right median nerve was transected at the level of 1cm above elbow. No repair was done. (C) R+ groups: right median nerve was first transected at the level of 1cm above elbow, and then was repaired under microscope immediately. In all the groups, the electrodes were well isolated and no adjacent nerve was stimulated.

Figure 2.

BOLD fMRI results of right median nerve stimulation in acute stage. (A) S1FL BOLD fMRI activation induced by right median nerve stimulation in healthy control group. S1FL was circled in red. This slice is 0.36mm anterior to the bregma. BOLD fMRI activation of R- (B) and R+ (C) groups show complete loss of S1FL activation. Negative BOLD signal can be observed in CPu. (D) Voxel counts for all three groups. * $p < 0.01$

Figure 3.

BOLD fMRI results of right median nerve stimulation in subacute stage. (A) S1FL fMRI activation (135 ± 36) was well demonstrated in healthy control group.

(B) No S1FL fMRI activation (0 ± 0) observed in R- group.

(C) S1FL fMRI activation was restored (33 ± 17) in R+ group.

(D) Voxel counts for all three groups. Significant difference can be found across all three groups. * $p < 0.01$

Averaged fcMRI results in acute stage (0 week) of each group

Figure 4.

Averaged fcMRI results in acute stage (0 week) of each group.

(A) Averaged fcMRI results of healthy control group. Symmetric, wide spread activation with variable intensity across whole S1 area (circled in red) of both hemispheres was observed. The healthy fcMRI pattern was disturbed in R- (B) and R+ (C) groups. fcMRI of these two groups became asymmetric and localized which only covered part of S1 area. (D) Comparison of histograms of fcMRI networks in S1 acquired from all groups with the threshold of $p < 0.05$ after Fisher transform. The area under the curve represents the sum of the voxel counts observed in S1 area fcMRI network, which is demonstrated numerically on the right side. After nerve injury, in both nerve injury and nerve repair groups, the fcMRI network in S1 area was greatly suppressed in both amplitude and the sum of voxel number.

Averaged fcMRI results in *subacute* stage of each group

Figure 5.

Averaged fcMRI results in subacute stage (2 weeks postoperatively) of each group.

(A) Averaged fcMRI results of healthy control group. Symmetric, wide spread activation with variable intensity across whole S1 area (circled in red) of both hemispheres was still observed. The result is highly comparable to the acute stage in Figure 4A. The healthy fcMRI pattern was still disturbed in R- (B). fcMRI of this group remained the same pattern of asymmetric and highly suppressed which only covered part of S1 area. (C) Symmetric, uniformly spread fcMRI network across the whole S1 area was detected in the subacute R+ group. Note the most obvious difference comparing to the healthy control group is the uniformed distribution pattern. (D) Comparison of histograms of fcMRI networks in S1 acquired from all groups with the threshold of $p < 0.05$ after Fisher transform. The area under the curve represents the sum of the voxel counts observed in S1 area fcMRI network, which is demonstrated numerically on the right side. After nerve injury, in nerve injury group, the fcMRI network in S1 area was still greatly suppressed in both amplitude and the sum of voxel number. While in the nerve repair group, this network was restored with more voxels detected almost at every p value, showing signs of uniformed, over compensated fcMRI network.

ANOVA analysis of fcMRI showing connectivity change

Figure 6.

Two-way ANOVA analysis comparing fcMRI result from all three groups at 2 weeks post-operation with results acquired from the acute stage. P-value was set to 0.05 for significant changes. (A) control group (B) Nerve injury group (R-) (C) Nerve repair group (R+).