Altered excitability of motor neuron pathways after stroke: more than upper motor neuron impairments

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ABSTRACT

Background Previous studies have suggested that impairment occurs in the lower motor neuron (LMN) pathway after stroke, but more research remains to be supported.

Objective In this study, we tested the hypotheses: (1) both motor cortex and peripheral nerve pathways have decreased excitability and structural damage after stroke; (2) parameters of transcranial magnetic stimulation motor evoked potentials (TMS-MEP) can be used as predictors of motor function and stroke prognosis.

Methods We studied five male cynomolgus monkeys with ischemic stroke. TMS-MEP, cranial MRI, behavioural assessment, neurological scales and pathology were applied.

Results Elevated resting motor threshold (RMT) (p<0.05), decreased TMS-MEP amplitudes (p<0.05) and negative RMT lateralisation were detected in both the affected motor cortex (AMC) and the paretic side median nerve (PMN) at 2 weeks poststroke. Disturbed structure and loose arrangement of myelin sheaths were observed in the PMN through HE staining and LFB staining at 12 weeks poststroke. The Primate Rankin Scale (used for assess the stroke prognosis) scores at 2–12 weeks after middle cerebral artery occlusion were [1, 1; 3], [1, 1; 2], [1, 1; 1.5] and [1, 1; 1.5], respectively. The RMT and RMT lateralisation (AMC) were predictors of stroke prognosis, and the RMT lateralisation of PMN and latency of AMC were predictors of motor impairment.

Conclusions Both upper motor neuron (UMN) and LMN pathway excitability is reduced after stroke, and structural damage in median nerve 12 weeks after stroke occur. In addition, RMT and RMT lateralisation are predictors of stroke prognosis and motor impairment.

INTRODUCTION

As we know, transcranial magnetic stimulation motor evoked potentials (TMS-MEP) is a safe, effective and non-invasive examination technique aimed at assessing cortical and corticospinal motor conduction bundle function, which provides a quantitative representation of cortico-spinal excitability during stimulation.1–7 Previous studies have reported the application of transcranial electrical stimulation for the acquisition of motor evoked potentials in non-human primates (NHPs).8–13 Some experiments were performed on monkeys and cats in which electrical stimulation was applied to the exposed cortical surface and the D-wave of action potentials descending through the cone bundle to the spinal cord and the I-wave of downward projection waves from the spinal cord were collected. However, most electrophysiological studies focus on the motor cortex, while the nerve function distant from the brain lesion dose not receive sufficient attention. Annette et al once proposed that upper motor neuron (UMN) lesions lead to functional changes in the lower motor neuron (LMN) and that the amplitude of compound muscle action potentials of the ulnar nerve in patients with severe ischaemic stroke decreased.14 Moreover, our previous studies have discovered secondary damage in the anterior spinal cord root in cynomolgus monkeys with ischaemic stroke.15 Thus, we propose the following hypothesis:

WHAT IS ALREADY KNOWN ON THIS TOPIC?

⇒ Subsequent damage to the distal site of infarct lesion after stroke has been found in animal models, most of which focus on the intracerebral region. In order to further investigate if there was secondary damage to the peripheral nerves far from the primary lesion, we applied magnetic stimulation of motor evoked potentials combined with pathological assessment in a cynomolgus monkey stroke model.

WHAT THIS STUDY ADDS?

⇒ Excitability of both upper and lower motor neuron pathways decreased after stroke in cynomolgus monkeys, and myelin loss was observed in the median nerve 12 weeks poststroke. The prognosis of stroke and motor impairment could both be predicted by applying transcranial magnetic stimulation motor evoked potentials-related indicators.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY?

⇒ These findings may provide neurophysiological insights for future studies on non-human primates stroke models, and may also contribute to decision making in the diagnosis and treatment of clinical stroke.
(1) the excitability of both UMN and LMN pathways is reduced after stroke; (2) structural damage on LMN pathway is possible and (3) some parameters of TMS-MEP could predict the motor function impairment and prognosis of stroke.

NHPs are often selected for preclinical testing because their anatomical and physiological characteristics are very close to those of humans. Long-term studies have shown that it is safe, effective, and feasible to develop human disease models and conduct research with NHP. Furthermore, the Academic-Industry Roundtable on Stroke Therapy group recommends the use of large animals, including NHP, to establish ischaemic stroke animal models. Therefore, cynomolgus monkeys were included in our study for TMS-MEP testing as well as motor function assessment and histopathological examination.

MATERIALS AND METHODS

Animals

A total of five male cynomolgus monkeys (5–7 years, 4.5–7.5 kg, provided by CongHua HuaZhen Animal Farm, Guangdong, China) were included in the study. Monkeys were assigned to middle cerebral artery occlusion (MCAO) operation as reported in detail in the previous literature (online supplemental material 1).

Skeletal muscle coordination assessment

Motor function in the cynomolgus monkeys was assessed by the Skeletal Muscle Coordination Scale (SMCS). Two neurologists who were blinded to the study measured the SMCS by observing cynomolgus monkeys moving freely in a spacious cage before the operation, at 2 and 4 weeks after the operation, respectively. (online supplemental material 1)

primate Rankin Scale score

primate Rankin Scale (pRS) was scored at 4 weeks postoperatively by two neurologists and a veterinarian independently who were blind to the experiment protocol. The pRS is designed by Di Wu et al to assess the degree of disability and prognosis of monkeys depending on the daily activities of stroke monkeys as compared with the modified Rankin Scale for patients who had a stroke (online supplemental material 1).

MRI scanning

All cynomolgus monkeys underwent cranial MRI scans with Siemens’ 3.0T Trio system (Siemens, Germany) before and 2 weeks after the surgery to clarify the site and severity of cerebral infarction. T1-weighted three-dimensional magnetisation prepared rapid acquisition gradient echo (TIWI-3D-MPRAGE) sequences and T2-weighted sequences were scanned to assess brain parenchyma, and the specific scan parameters are detailed in our previous study. Brain infarct volume was measured on TIWI-3D-MPRAGE images of each monkey 2 weeks after MCAO. In summary, infarct areas and the ipsilateral hemisphere (region of interest) were manually mapped and then measured using ITK-SNAP. The infarct volume ratio was computed by the formula: (infarct volume/ipsilateral hemisphere volume) ×100% (online supplemental material 1).

Behaviour assessment

All cynomolgus monkeys were trained to become accustomed to the stairs of Hill and Valley Task and proficient in using the corresponding hands to properly reach for food through the round hole before the formal testing (figure 1). Testing on behaviour tasks was conducted preoperatively, and at 2 and 4 weeks postoperatively (online supplemental material 1).

TMS-MEP protocol

Cynomolgus monkeys were fasting for food and liquids for 6 hours before the examination. As we know, anaesthetic and inotropic drugs can affect the outcome of MEP to varying degrees, mainly in terms of amplitude. In order to reduce the effect of sedative drugs on the results, we applied very small doses on the monkeys. They were sedated with ketamine (2.5 mg/kg, intramuscular) combined with Serazine hydrochloride (0.0125 mL/kg, intramuscular). Atropine (0.0625 mg/kg, intramuscular) was used to reduce salivation. In addition, all monkeys were injected with sedative drugs according to a uniform standard as mentioned above at all time points, so the MEP results obtained were still reliable although the effect of sedative drugs could not be completely excluded. TMS was performed on monkeys with Wuhan YiRuDe (Wuhan, Hubei, China) CCY-IA type magnetic field stimulator with an 8-shaped coil. Single-pulse stimulation protocol was set, with biphasic anterior–posterior and posterior–anterior MS pulse waveforms in the coil. And the handheld coil was placed on the target motor cortex with the handle angled backward at 45°. Orientation of the induced current in the brain is determined by the shape of the coil. The 8-shaped coils are formed by overlapping two small circular coils to produce two currents with opposite directions, with the greatest current generated at the point where the two coils intersect. The stimulation frequency can be adjusted continuously from 0 to 100 Hz, and its intensity can be adjusted continuously from 1.5 to 6
Resting motor threshold and RMT lateralisation

The resting motor threshold (RMT) was determined by adjusting the stimulus intensity. RMT (%) was defined according to the International Federation of Clinical Neurophysiology guidelines as the lowest intensity that elicited MEP ≥50 μV in at least 50% of the trials. RMT lateralisation was calculated: \[ \text{RMT}_{\text{lat}} = 100 \times (\text{RMT}_\text{contra} - \text{RMT}_\text{ipsi}) / (\text{RMT}_\text{contra} + \text{RMT}_\text{ipsi}) \], in which zero values represent symmetry and negative values reflect ipsilateral hypoexcitability. Here the ipsilateral hemisphere (short for ‘ipsi’ in the above formula) referred to the infarcted lateral hemisphere, whereas the contralateral (short for ‘contra’ in the above formula) hemisphere referred to the opposite hemisphere of the infarct.

MEP amplitude and latency

Five consecutive stimuli were given, each at an interval of 15 s. Peak-to-peak amplitudes (peak to trough) and latencies (from stimulus onset to MEP onset) were recorded and averaged over five consecutive responses (online supplemental material 1).

Median nerve magnetic stimulation MEP protocol

To measure the excitability of the median nerve pathway, we performed the median nerve magnetic stimulation MEP protocol as the follow: The position of the surface recording electrodes was unchanged and the stimulation site was the bilateral elbow muscles where the median nerve went through (figure 2). Magnetic stimulation of the median nerve region was administered from the RMT, and gradually increased until the maximum MEP amplitude appeared. It was observed that the maximum MEP was detected in most monkeys when the stimulation intensity was in the range of 20%–50% MSO. Thus, the magnetic stimulation output was set to 20%–50% MSO. The maximum MEP stimulus intensities for the five monkeys were as follows: monkey A: left 28% MSO, right 28% MSO; monkey B: left 27% MSO, right 31% MSO; monkey C: left 31% MSO, right 28% MSO; monkey D: left 28% MSO, right 28% MSO; monkey E: left 32% MSO, right 28% MSO. MEP testing was performed before surgery, 2, 4, 8 and 12 weeks after the operation, respectively. Complete disappearance of waveforms or a 50% or greater reduction in amplitude, or even the presence of these changes when stimulus intensity is increased by 20% or more, is considered a warning criterion indicating impaired motor pathways.

Animal sacrifice and histopathology

As we know, a typical time point for clinical assessment of stroke prognosis or other clinical event endpoint is 12 weeks (or 3 months/90 days). And it has been demonstrated that damage to distal sites does not occur immediately after stroke, but often occurs weeks or months after stroke. In addition, pathological changes have been observed in the thalamus and hippocampus away from the infarct area 12 weeks after stroke in cynomolgus monkeys. Thus, the monkeys were sacrificed 12 weeks after surgery and the bilateral median nerve was removed to prepare paraffin sections for pathological analysis (online supplemental material 1).

Double-labelling immunofluorescence

Myelin and axonal alterations of the median nerve were detected bilaterally using double-labelled immunofluorescence staining. Five images of non-overlapping areas were acquired at ×400 lens for each slice, and image J software (National Institutes of Health, Bethesda, USA) was applied to calculate the mean fluorescence intensity (MFI) of the images according to the method described in the previous literature (online supplemental material 1).

Statistical analysis

All results were statistically calculated by applying IBM SPSS Statistics V.25 (SPSS). Data matching the normal distribution are expressed as mean±SD, and data not matching the normal distribution are expressed as median (quartile−25%; quartile−75%). Statistical significance was considered when the p value was less than 0.05 (online supplemental material 1).

RESULT

Baseline characteristics

All cynomolgus monkeys can trigger normal waveforms and values of MEP before the surgery. Most of the MEP waveforms were bidirectional with well-stabilised waveforms. The results showed no statistical difference in amplitude, latency and RMT between the bilateral motor cortex and bilateral median nerve in the preoperative period. All cynomolgus monkeys scored 0 on the preoperative neurological function score and motor function score.
Infarct volume

The mean infarct volume was 6.47% (6.47±2.45%), while the correlation analysis revealed that infarct volume was independent of RMT, amplitude and latency of MEP. As shown in figure 3, the T2-weighted of cranial MRI after stroke showed the infarct lesions with high-signal distributed in the temporal lobe, frontal cortex and subcortex, but did not involve the ipsilateral primary motor cortex.

pRS and motor function impairment

Compared with the preoperative period, pRS score was elevated from 2 to 12 weeks poststroke, with the highest score at 2 weeks (p<0.05, figure 4A). Skeletal muscle coordination was the worst at 2 weeks postoperatively with the highest score (p<0.05, figure 4B). The affected hands showed varying increases in completion time on both the hill task and the valley task postoperatively, peaking at 2 weeks (p<0.05, figure 4C,D).

Alteration of TMS-MEP on the motor cortex

Once a reproducible MEP with good waveform was elicited, the location was marked with a black marker and each subsequent TMS was always performed at the same location on the motor cortex for each MEP acquisition. For the affected motor cortex (AMC) MEP, two cynomolgus monkeys failed to evoke the waveform at 2 and 4 weeks postoperatively despite the stimulation intensity has reached 100% MSO, and one cynomolgus monkey was unable to elicit the MEP waveform of the unaffected motor cortex at 4 weeks postoperatively, which indicated impairment of the corresponding motor pathway according to the warning criteria for motor pathway impairment. MEP waveforms were shown in figure 5.

The amplitude of the AMC decreased at 2 (0.64±0.65 mV, p<0.05), 4 (1.10±1.16 mV, p<0.05) and 8 weeks (1.47±1.15 mV, p<0.05) postoperatively compared with the preoperative period (2.24±1.44 mV), except for 12 weeks (2.27±2.40 mV) postoperatively. Compared with the preoperative period, the decrease in amplitude of the AMC was more than 50% at 2 and 4 weeks after MCAO, suggesting an impaired motor pathway on that side. And the amplitude of the AMC at 2 weeks postoperatively was also lower than that on the unaffected side [1.44 (1.00; 3.55) mV, p<0.05]. Amplitude of the unaffected motor cortex at 2, 4, 8 and 12 weeks postoperatively were not statistically different from those before the surgery.

The RMT in the AMC was higher at 2 weeks postoperatively [100% (27%; 100%), p<0.05] than preoperatively (24%±11%), and also higher than that of the unaffected motor cortex (20%±13%) 2 weeks after stroke. No statistical differences were found in RMT of the AMC at other postoperative time points, and similarly no statistical differences were found in the comparison of preoperative and postoperative levels of RMT in the unaffected motor cortex.

For the RMT lateralisation in the AMC, it became negative from 2 weeks to 12 weeks after surgery, and the degree of negative direction gradually declined. And the differences in RMT lateralisation were statistically significant at both 2 (−0.522±0.212, p<0.05) and 12 weeks (−0.196±0.260, p<0.05) postoperatively compared with the preoperative period (0.029±0.188).

The latency of bilateral motor cortex was not significantly altered before and after MCAO. And there was no significant difference in the latency of the AMC compared with the unaffected side at all time points.
Alteration of TMS-MEP on median nerve

The amplitude in the paretic side median nerve (PMN) decreased significantly at 2 weeks postoperatively (3.74±2.12 mV, p<0.05) and gradually increased from 2 to 12 weeks. And the amplitude of the non-paralysed side median nerve also showed a slight decrease at 2 weeks postoperatively (5.21±2.61 mV) but did not reach a statistical difference.

For the PMN RMT, it was higher than preoperatively [10% (4%; 13%)] at 2 weeks [15% (12%; 16%)] and 4 weeks [15% (7%; 17%)] postoperatively, but did not reach statistical significance. Overall, the RMT of the median nerve on the paralysed and non-paralysed sides did not show statistically different changes postoperatively.

For the latency, the median nerve on both the paralysed and non-paralysed sides preserved good stability and consistency after the surgery, a result considered to be related to the relatively uniform body length among these cynomolgus monkeys.

Compared with the preoperative period, the RMT of the median nerve on both the paralysed and non-paralysed sides showed a trend of first increase and then gradual decrease from 2 to 12 weeks after the surgery, but still did not reach a statistical difference.

In addition, the results showed that the RMT lateralisation of the PMN was positive preoperatively (0.02±0.35) and became negative at 2 (−0.26±0.37), 4 (−0.15±0.32) and 12 weeks (−0.01±0.45) postoperatively, suggesting low excitability.

Morphological alterations on the median nerve

PMN damage was observed in MCAO monkeys, HE staining and LuxolFastBlue (LFB) staining results showed that the structure of the myelin sheath in PMN was disorganised and loosely arranged, as shown in figure 6. Conversely, the non-paretic side median nerve showed relatively normal myelin structure with tight alignment. LFB score was used to semiquantitatively investigate the myelin sheath damage. The LFB scores for the non-paralysed side of the median nerve in both monkeys with MCAO were 0, while that for the paralysed side of the median nerve was 1.

Expression of myelin sheaths and axons in median nerve

To further accurately and quantitatively assess myelin and axonal damage, immunofluorescence double-labelling was performed. For MBP expression, the MFI of the PMN (212.27±7.30, p<0.05) was lower than the that of the non-paretic side median nerve (231.72±6.09, figure 7A). The MFI of NF200 in the PMN (229.75±6.35) was lower than that of non-paretic side median nerve (240.65±7.56), but the difference between them did not meet statistical significance (figure 7B).
Correlation analysis and automatic linear modelling

Correlation analysis showed that Hill task time had a positive correlation with TMS-RMT (%) of affected side (2 weeks: r=0.894, p=0.041; 4 weeks: r=0.949, p=0.014; 8 weeks: r=0.898, p=0.039, figure 8A, B, C) and a negative correlation with amplitude (4 weeks: r=−0.895, p=0.040, figure 8D). No correlation was found between valley task time and amplitude, latency or TMS-RMT (%). To explore the relationship between TMS-MEP parameters and the prognosis of stroke and skeletal muscle motor function, we performed automatic linear regression modelling analysis. The results showed that the TMS-RMT (%) of AMC (R²=0.992, p<0.001, figure 8E) and TMS-RMT lateralisiation (R²=0.954, p=0.004, figure 8F) at 4 weeks on the AMC could be predictors of the pRS scale at 12 weeks. In other words, the higher the 4 week TMS-RMT (%), the higher the pRS scale score at 12 weeks and the worse the stroke prognosis, while the higher the 4 week TMS-RMT lateralisiation, the lower the pRS scale score at 12 weeks and the better the stroke prognosis. And the PMN-RMT lateralisiation (R²=0.952, p=0.005, figure 8G) of the PMN at 4 weeks could be a predictor of skeletal muscle motor coordination, which implies that the higher the lateralisation of PMN-RMT (%), the better the skeletal muscle motor coordination. In contrast, the predictor of skeletal muscle motor coordination (R²=0.999, p=0.017, figure 8H) at 2 weeks is the latency of the AMC, suggesting that the longer the 2 week latency of stroke, the worse the skeletal muscle motor coordination.

DISCUSSION

In this study, we investigated the excitability of the UMN and LMN pathways in cynomolgus monkeys with stroke for the first time. For the AMC, excitability reached lowest level at 2 weeks after stroke with a trend of gradual recovery from 2 to 12 weeks after stroke, which were consistent with that in humans. The RMT or MEP amplitude can represent an output of cortical excitability, but RMT seems to be the most reliable and standardised parameter across time and physiological states.
The parameters of the magnetically stimulated MEP of peripheral nerves are not quite the same as the TMS-MEP of motor cortex. According to the preoperative MEP characteristics, the threshold and latency of bilateral median nerve MEP were lower and the wave amplitude was markedly higher compared with motor cortex MEP.

**Figure 8** Correlation analysis.
Note: A: positive correlation between RMT of affected motor cortex and Hill Task of right hand at 2w; B: positive correlation between RMT of affected motor cortex and Hill Task of right hand at 4w; C: positive correlation between RMT of affected motor cortex and Hill Task of right hand at 8w; D: negative correlation between amplitude of affected motor cortex and Hill Task of right hand at 4w; E: automatic linear modeling of RMT of affected motor cortex (4w) and pRS (12w) with positive correlation; F: automatic linear modeling of RMT lateralization (4w) and pRS (12w) with negative correlation; G: automatic linear modeling of RMT lateralization (4w) and SMCS (4w) with negative correlation; H: automatic linear modeling of latency of affected motor cortex (2w) and SMCS (2w) with positive correlation. RMT, resting motor threshold. pRS, primate Rankin Scale. SMCS, Skeletal Muscle Coordination Scale.
And the decrease in amplitude of the paretic median nerve was also most pronounced at 2w after stroke, whereas the excitability had largely returned to preoperative levels by 4–12 weeks after stroke, suggesting a transient functional decrease in the excitability of the paretic median nerve pathway within 2 weeks after stroke. These results support the hypothesis that peripheral nerves distant from the brain lesion show a functional decline after stroke and that such changes can be detected by the amplitude of MEP. Histopathology at 12 weeks after stroke demonstrated mild demyelination changes in the paretic median nerve, but no significant changes in TMS-MEP parameters were observed. Animal studies have shown that the MEP parameters did not change significantly when the nerve fibres were damaged in degree I (mild), but the swelling of myelin sheath, bundle membrane and endothelium could be observed pathologically. When the nerve fibres were damaged in degree II, transient demyelination may occur, and the latency of MEP may appear to be prolonged. And when the injured nerve fibre entered the recovery period, the latency of MEP can recover to normal as the damaged myelin sheath was partially repaired. We assume that the mild myelin morphological changes are not sufficient to cause a decrease in nerve conduction function. Unfortunately, we do not have a 2-week poststroke pathology specimen to further clarify the histological changes when the nerve conduction function decreases.

The results of the correlation analysis suggest that RMT and amplitude may reflect the impairment of motor function in the paretic limb after stroke. Many pieces of literature reported that the amplitude in the AMC can be considered as a surrogate marker of motor impairment in the pathological conditions of stroke. It has been observed that in patients with focal subcortical lesions, MEP amplitude has a close relationship with functional impairment, meaning that MEP amplitude does closely track the degree of impairment.

Early studies have proposed the use of TMS to assess the prognosis of stroke patients by measuring the level of motor recovery. In a recent cross-sectional study that included 341 cases, the authors found that RMT was associated with prognosis by integrating a multivariate analysis of data, which is in line with the results of this study.

MEP is a complex electrophysiological technique and there are insufficient data on electrophysiological studies in NHP. Future studies are recommended to continue to increase the number of animals and design a more complete research programme to explore the neurophysiological characteristics of NHP.

CONCLUSIONS
This study is the first to apply TMS-MEP to detect the excitability of upper and LMN pathways in cynomolgus monkeys with stroke combined with histopathology analysis. Excitability of both upper and LMN pathways decreased after stroke in cynomolgus monkeys, and myelin loss was observed in the median nerve 12 weeks poststroke. The prognosis of stroke and motor impairment could both be predicted by applying TMS-MEP-related indicators. These findings may provide neurophysiological insights for future studies on NHP stroke models, and may also contribute to decision making in the diagnosis and treatment of clinical stroke.

Contributors JZ: designed research protocols and reviewed the manuscript, responsible for the overall content as guarantor. PL: performed all experimental operations, analysed statistical data, and wrote the manuscript. CC: performed animal behaviour training and some laboratory operations, collected experimental data. BH: assisted with animal training and provided care for animals. JZ: collected experimental data. JW: collected experimental data.

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Patient consent for publication Not applicable.

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Supplementary Material

Animals

Monkeys were housed in the *** indoors individually at a constant temperature of 24°C on a 12-h light/dark cycle by professional breeders. All monkeys were fed twice a day and had free access to clean drinking water. Intravenous 0.9% saline was administered throughout the operation in case of fluids lost. The monkeys were placed on a warm operating table, and a craniotomy was performed from the left frontotemporal pterion. Then the left middle cerebral artery (MCA) was gradually exposed, and the distal M1 branch of MCA was occluded with bipolar electrocoagulation. Penicillin (0.4 million IU, intramuscular, per day) was administered to prevent infection, and tramadol (50-100mg, intramuscular, per day) was administered to relieve pain during the 3 days after the surgery. 25ml of 20% mannitol was administered intravenously daily to relieve cerebral edema, for monkeys with severe consciousness deficits, mannitol was administered twice a day.

Skeletal Muscle Coordination Assessment

The scale is scored from 0 to 18, where the higher the score the poorer the skeletal muscle coordination and the poorer the motor function. A score of 0 is considered normal walking and a score of 18 shows a complete absence of movement, suggesting a loss of motor function.
Primate Rankin Scale (pRS) Score

The pRS scores include 0-5 points. 0 points indicated no symptoms, 1 point indicated no significant disability, 2 points indicated slight disability, 3 points indicate moderate disability, 4 points indicate moderately severe disability, and 5 points indicated severe disability or death.

Behavior Assessment

Hill Task unit consists of two staircases each with 5 compartments in the forward direction, while the staircases in the Valley Task unit are in the reverse direction. A small piece of carrot or apple, about 14×14×14 mm in size, was placed on each staircase. The monkeys were allowed to retrieve all the food within 5 minutes and the time spent retrieving each side of the stairs was recorded by observers. Only successful retrieval of food through the square holes on both sides of the Hill Task device or through the round hole in the middle of the Valley Task device using the correct hand was recorded. If the monkey could not successfully retrieve the food after several attempts for a total time of more than 5 minutes, the time was recorded for that side of the hand as three times the average of the corresponding hand before surgery.

MEP Amplitude and Latency

The relationship between stimulus intensity and MEP amplitude is in
the form of an S-shaped curve, generally referred to as the “stimulus-response curve” or “recruitment curve”. The initial segment of this curve was relatively flat, with a near-linear increase in MEP amplitude at stimulus intensities between 120% and 140% of RMT, while at higher stimulus intensities the curve approached a plateau, at which point the MEP amplitude did not increase further even when the stimulus intensity was increased. In many studies, only a single intensity MEP was assessed due to experimental limitations, rather than assessing the entire stimulus-response curve. In that case, TMS intensity is usually set to 115%-125% of the individual RMT to ensure that the experiment detects the MEP size in the rising phase of the stimulus-response curve. However, in clinical diagnosis, the primary goal of TMS-MEP is to elicit the maximal response of motor cortex. Therefore, the intensity of TMS should be high enough. In our study MEP with multiple stimulus intensities was performed. According to the International Federation of Clinical Neurophysiology guidelines, TMS stimulation intensity is usually expressed as a single motor threshold or as a percentage of the maximum stimulator output (MSO). The stimulus intensity was gradually increased from RMT until the MEP no longer increased, at which point the maximum MEP amplitude was obtained. In the TMS-MEP on motor cortex, the range of stimulus intensities where maximum MEP amplitude was between 60% MSO and 80% MSO in our
experiment. Thus, the magnetic stimulation output was set to 60-80% MSO to obtain the stable and maximum MEP amplitude. It is known that the variability of the MEP amplitude excited by 100% RMT TMS was small when the muscle was completely relaxed, generally 0-0.5mV in absolute value. Therefore, the maximum amplitude in the pre-stimulation period generally did not exceed 0.5mV in absolute value when excited by 100% RMT. In general, the muscles in sedated monkeys were presumed to be relaxed, yet further confirmation by electromyography would have been preferable. Since electromyography could not be performed due to the instrumentation limitations, no pre-stimulus muscle activities were confirmed by baseline mean amplitude before the stimulation. And it was possible to judge whether a waveform was an artifact based on the location, shape, etc. Due to the numerous factors influencing the flatness of the MEP baseline, slight fluctuations in the baseline have little effect on the assessment of MEP parameters when the animal’s muscles stayed relaxed.

**Animal Sacrifice and Histopathology**

Intramuscular ketamine overdoses were given to anesthetize the monkeys (n=2) at 12 weeks after MCAO. Saline cardiac perfusion was performed after the pain reflexes had completely disappeared, followed by 4% paraformaldehyde perfusion. The skin and subcutaneous tissues of both upper limbs were incised layer by layer, and the bilateral median nerves
were removed and soaked in 4% paraformaldehyde for post-fixation. After 24-48 hours of fixation, dehydration and paraffin embedding were performed and cut into paraffin sections of 3μm thickness. The slices were stained with a HE staining kit (Servicebio, G1003) and a Luxol Fast Blue staining kit (Servicebio, G1030) according to the manufacturer’s instructions. Morphological evaluation of the median nerve was performed according to LuxolFastBlue (LFB) staining, and the evaluation criteria were: grade 0: normal; grade 1: structural disorder of nerve fibers; grade 2: obvious vacuole formation; grade 3: disappearance of myelinated nerve fibers.

Double-labeling Immunofluorescence

Cross sections (3-μm thick) were cut, and every 20th cross section was selected for fluorescence staining, for a total of 3 sections. Areas where corresponding fluorescence could be detected were defined as regions of interest. A negative control was placed on each section without the adding of the corresponding primary antibody. For double-labeled immunofluorescence staining, paraffin sections were incubated overnight at 4°C with the following primary antibodies after dewaxing to water, antigen repair and blocking steps: rabbit anti-myelin basic protein (MBP; myelin marker, 1:200, Cell Signaling Technology, #78896) and mouse monoclonal anti-neurofilament 200 antibody (NF-200; 1:100, Sigma-Aldrich, N5389). Sections were then incubated with the following
fluorescent secondary antibodies for 1 hour at room temperature shielded from light: Alexa Fluor 555-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti rabbit IgG (1:200, Cell Signaling Technology). Fluorescence images were acquired by a Leica DMi8 fluorescence confocal microscope.

**Statistical Analysis**

All data were first tested for normal distribution by the Shapiro-Wilk method. Data that did not meet the normal distribution were analyzed with the paired Wilcoxon test. Data collected at different time points for the same indicator that matched the normal distribution, equal variances and Mauchly’s test of sphericity were analyzed with repeated measures ANOVA. The pRS and SMCS scores at multiple time points were analyzed by one-way repeated measures ANOVA. MEP parameters between the affected and unaffected side at multiple time points were analyzed by two-way repeated measures ANOVA. The MFI data of bilateral median nerve sections was tested for normality with Shapiro-Wilk test. Then paired t-test was applied if they met the normality distribution, otherwise non-parametric test (paired Wilcoxon test) was conducted. Correlation analysis was performed between the Hill and Valley Task, pRS and SMCS dataset with parameters of TMS-MEP/median nerve magnetic stimulation MEP, respectively. Pearson correlation test was used for data conforming to normal
distribution, and non-normal distribution data was analyzed with Spearman correlation test. The relationships between these datasets were then explored with automatic linear regression modeling. The Hill and Valley Task, pRS and SMCS dataset were modeled separately, with predictors RMT, RMTlat, latency, and amplitude of TMS-MEP and median nerve MEP.