Original Article

Asynchronization in Changes of Electrophysiology and Pathology of Spinal Cord Motor Neurons in Rats Following Middle Cerebral Artery Occlusion

Nan Lin, Ming-Sheng Liu, Si-Yuan Fan, Yu-Zhou Guan, Li-Ying Cui

Department of Neurology, Peking Union Medical College Hospital, Beijing 100730, China

Abstract

Background: Motor dysfunction is common in stroke patients. Clinical electrophysiological studies suggest that transsynaptic degeneration occurred in the lower motor neurons, while pathological evidence is lacked. This study aimed to combine the electrophysiological and pathological results to prove the existence of transsynaptic degeneration in the motor system after stroke.

Methods: Modified neurologic severity score, electrophysiological, and pathological assessments were evaluated in rats before middle cerebral artery occlusion (MCAO), and at 24 hours, 7 days, and 14 days after MCAO. Paired and independent-sample *t*-tests were applied to assess the changes of electrophysiological and pathological data.

Results: Compound motor action potential amplitude in the paretic side was significantly lower than the nonparetic side at both 24 hours (61.9 ± 10.4 vs. 66.6 ± 8.9, $P < 0.05$) and 7 days (60.9 ± 8.4 vs. 67.3 ± 9.6, $P < 0.05$) after MCAO. Motor unit number estimation of the paretic side was significantly less than the nonparetic side (379.0 ± 84.6 vs. 445.0 ± 89.5, $P < 0.05$) at 7 days after MCAO. Until 14 days after stroke, the pathological loss of motor neurons was detected. Motor neurons in 14-day MCAO group were significantly decreased, compared with control group (5.3 ± 0.7 vs. 7.3 ± 1.8, $P < 0.05$).

Conclusions: Both electrophysiological and pathological studies showed transsynaptic degeneration after stroke. This study identified the asynchronization in changes of electrophysiology and pathology. The abnormal physiological changes and function impairment can be detected in the early stage and recovered quickly, while the pathological loss of motor neuron can be detected only in a later stage.

Key words: Middle Cerebral Artery Occlusion; Electrophysiology; Lower Motor Neurons; Pathology; Stroke

Introduction

Stroke is the leading cause of disability in adult life.[1] Motor dysfunction is the major neurological deficits after stroke, with 50% of patients suffering from hemiparesis and 30% remaining unable to walk independently.[2] It is common that stroke patients have muscle atrophy in the paretic limb. Transsynaptic degeneration of lower motor neurons secondary to upper motor neurons injury plays a role in muscle mass loss.

It has been demonstrated that transsynaptic degeneration occurred wildly in nervous system after cerebral cortex injury, such as cerebellum,[3] thalamus,[4-9] and substantial nigra,[4,10-12] whereas transsynaptic degeneration in motor system remains controversial. Clinical electrophysiological studies have shown that neurogenic injury could be detected in paretic limbs of patients with stroke, suggesting that there is lower motor neuron injury. Abnormal spontaneous activity (SA, including fibrillation potentials, and positive sharp waves) could be observed in hemiplegic muscles 2–3 weeks after stroke,[13] lasting up to 1 year.[14-16] Hara and associates reported that motor unit number estimation (MUNE) and the mean compound muscle action potential (CMAP) amplitude on the paretic side was significantly lower in a period of 9 days to several months, after a unilateral cerebrovascular accident.[14,17,18] Arasaki

Address for correspondence: Dr. Ming-Sheng Liu, Department of Neurology, Peking Union Medical College Hospital, Beijing 100730, China
E-Mail: pumclms@126.com

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et al.[19] observed that MUNE and the maximal CMAP amplitude were decreased as early as 4 hours after cerebral infarction. These studies indicate that upper motor neuron lesions may lead to lower motor neuron abnormality.

Pathological evidence of anterior motoneurons degeneration in patients or rats after stroke is insufficient, and few studies had directly proved the degeneration of lower motor neurons. Terao *et al*.[20] and Qiu *et al*.[21] both studied on the spinal cord autopsy of the hemiparetic stroke patients. They observed inflammation[20] and degeneration of myelinated corticospinal axons in the spinal cord.[21] But neither study showed the number of anterior horn cells decreased. Experiments of Wu and Ling reported that in rats undergoing middle cerebral artery occlusion (MCAO), all ventral horn motoneurons remained structurally intact in 3 and 5 days after MCAO.[22]

Our work combined the electrophysiological and pathological assessments to investigate the existence transsynaptic degeneration in the motor system, and the relationship between electrophysiology and pathology.

Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Peking Union Medical College Hospital (PUMCH). The protocol was approved by the Ethics Committee of the PUMCH. This experiment was performed in the Animal Center of PUMCH.

Animals

Sprague-Dawley male rats were obtained from the Animal Center of PUMCH, weighing between 250 g and 380 g. Animals were specific pathogen free, and raised in 20–25°C room temperature. We monitored the status of the animals at least 1 time/day. Subjects were randomly grouped by a random number generated by a computer. Control group contains nine rats. During the experiment, 56 rats were performed MCAO surgery. Seventeen subjects were died of anesthesia accidents and cerebral hernia after MCAO, and another nine subjects were found had subarachnoid hemorrhage and intracranial hemorrhage after sacrifice. All of their data were excluded. Thirty rats were presented unilateral permanent MCAO. Every 10 MCAO rats were sacrificed for pathology tests, at 24 hours, 7 days, and 14 days after operation separately. All surviving subjects were evaluated by electrophysiology assessments and modified neurological severity score (mNSS)[23] at 0 day, 24 hours, 7 days, and 14 days after surgery.

Surgical procedures

The permanent MCAO procedure was based on the Longa MCAO logi[24] After anesthesia with 3–4 ml/kg i.p. chloral hydrate, the rat was laid on the operation table and disinfected. Following midline incision on the neck, the right common carotid artery was exposed. The internal carotid artery was also isolated and separated carefully from the adjacent vagus nerve, and the external carotid artery was ligated. Monofilament nylon suture (diameter: 2.6–3.0 mm) was introduced into the common carotid artery lumen through a puncture and advanced gently to the internal carotid artery lumen. After about 18 mm length of nylon suture had been inserted into artery lumen, resistance was felt, indicating that the suture had passed the middle cerebral artery origin and occluded the artery. After all possible bleedings were stopped, the skin was sutured. Rats were under lamplight to maintain warmth during operation and anesthesia recovering.

Electrophysiological assessments

All electrophysiology tests were performed using a portable electromyography (EMG) machine (10CH Medelec Synergy, Natus Europe GmbH, Germany). Rats were placed on the operation table after anesthesia, with bilateral lower limbs spread at an approximately 45° angle to the spine [Figure 1a], under lamplight to stay warm. We chose the internal posterior tibial muscle group and sciatic nerve for both EMG and motor nerve conduction studies (MNCS).

EMG recording was performed bilaterally using a concentric needle electrode. Four points in the muscle group were selected to investigate SA, with more than 10-second observation at each point. Sweep duration was 10 ms/division, sensitivity was 100 μV/division, and filter settings were 20 Hz–10 kHz.

MNCS was performed after EMG on the bilateral sciatic nerve. The sciatic nerve was stimulated at the root of the hind limb, and the anode of the stimulating electrode was placed at anterior superior iliac spine. CMAPs [Figure 1b] were recorded by surface electrodes. The active electrode was placed over the belly of the internal posterior tibial muscle group, while the reference electrode was positioned on the ankle. Sweep speed was 2 ms/division, and sensitivity was 20 mV/division.

The set-up of MUNE was identical to CMAPs. MUNE [Figure 1c] was performed by the standard incremental method after careful training.[25] Stimulus intensity kept increasing from zero to get 10 potentials

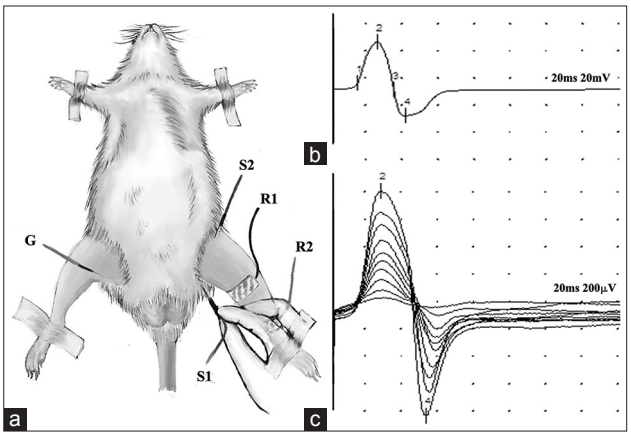

Figure 1: (a) Schematic representation of the electrophysiology tests. R1: The active recording electrode; R2: The reference recording electrode; S1: The cathode of the stimulating electrode; S2: The anode of stimulating electrode; G: The ground electrode. (b and c) Compound muscle action potential and motor unit number estimation examples.

with the smallest step. MUNE was equal to the value of the maximal CMAP amplitude divided the average of the 10 potentials amplitudes. Sweep speed was 2 ms/division, and sensitivity was 100 μV to 20 mV/division.

Histological and immunohistochemical assessments

After anesthesia, rats were perfused with normal saline and 0.4% paraformaldehyde sequentially. The brain and spinal cord were removed. Gross observation and hematoxylin and eosin staining were performed to identify the cerebral infarction. Rats with cerebral infarction were chosen to the following steps. The lower lumbar spinal cord (L4–S1) was cut into 3-μm thick slides at L4, L5 and S1 levels. Neuron-specific nuclear protein (NeuN) that is nuclear protein specifically expressed by mature neuron was used here to mark neurons.[26] B-cell lymphoma/leukaemia-2 (Bcl-2) protein can prevent cell apoptosis,[27] while Bcl-2 associated X (Bax) protein promotes apoptosis and has an antagonistic effect on Bcl-2.[28] Neurons were stained with Bcl-2 and Bax to express their conditions, trend to alive or apoptosis.

Only anterior motoneurons with visible staining nucleus and nucleolus were counted. We also recorded the number of NeuN positive neurons in the posterior horn. Three nonoverlapping regions in the posterior horn were chosen to count NeuN positive cells, and the average number represented the number of neurons unit area in the posterior horn. Glial fibrillary acidic protein (GFAP) immunostaining was performed to mark active astrocyte. The GFAP positive astrocytes in anterior horn were counted in the same way of posterior horn neurons counting.

Statistical analysis

We performed the statistical analysis of our data using SPSS for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). All data were expressed in the form of mean ± standard deviation (SD). The data were detected with Shapiro-Wilk test and Levene test for normality and homogeneity of the variance respectively first. If the normality or homogeneity was not met, we adopted nonparametric statistical tests. Otherwise, we used paired *t*-test to compare bilateral electrophysiological and pathologic data, and independent-sample *t*-test for pairwise comparison in groups of pathologic data. Analysis of variance (ANOVA) for repeated measurement data, or one-way ANOVA was used for comparison of data over time. Group *t*-test was used to analyze the difference of MCAO and control groups. The frequency of SA in different mNSS groups was analyzed by the Fisher's exact test. *P* values < 0.05 were considered statistically significant.

Results

Electrophysiological assessments

Abnormal SA were detected in the paretic hind limb of 16.7% (5/30) MCAO rats, including 3 at 24 hours after MCAO (mNSS: 9, 10, 11), 1 at 7 days after MCAO (mNSS: 4), and 1 at 14 days after MCAO (mNSS: 9). No SA occurred on the nonparetic side. We divided each group into two subgroups according to mNSS (mNSS ≤7 and mNSS >7). The frequency of SA in mNSS ≤7 group was 7.1% (1/14), while the frequency in mNSS >7 group was 25.0% (4/16) ($P = 0.209$). These results showed SA was more likely to occur in rats with a higher mNSS, although there was no significant difference between the two groups.

Changes of CMAP amplitude are showed in Table 1 and Figure 2a. In the control group, there was no significant difference between bilateral CMAP amplitudes and among CMAP amplitudes over time. In MCAO groups, CMAP amplitude on paretic side was significantly lower than nonparetic side, at 24 hours and 7 days after operation (24 hours: 61.9 ± 10.4 vs. 66.6 ± 8.9, $P < 0.05$; 7 days: 60.9 ± 8.4 vs. 67.3 ± 9.6, $P < 0.05$) [Table 1 and Figure 2a]. The CMAP amplitudes over time showed no significant difference, neither on the paretic side nor the nonparetic side. At the four given time points, there were no significant CMAP amplitude differences between the control group and MCAO groups. We further divided the 7 days group into two subgroups (paretic CMAP amplitude >60.60 mV and ≤60.60 mV). The average mNSS was 5.7 in group with CMAP amplitude >60.60 mV ($n = 11$), and 6.9 in group with CMAP amplitude ≤60.60 mV ($n = 10$). There was no significant difference in mNSS between the two groups.

The MUNE data are showed in Table 2 and Figure 2b. In the control group, there was no significant difference in MUNE between bilateral limbs at different duration. In MCAO groups, however, MUNE on paretic side was significantly less than that on nonparetic side, at 7 days after operation (379.0 ± 84.6 vs. 445.0 ± 89.5, $P < 0.05$) [Table 2 and Figure 2b]. There was no significant difference in MUNEs of paretic limbs among

Table 1: The summary of CMAP amplitude data in control and MCAO rats (mean ± SD)

Time after MCAO	Control			
	Left	Right	*t*	*P*
0 day ($n = 9$)	62.4 ± 10.0	61.4 ± 10.2	0.406	0.695
24 hours ($n = 6$)	67.6 ± 16.5	57.5 ± 13.9	1.746	0.141
7 days ($n = 6$)	61.2 ± 8.5	61.3 ± 14.5	−0.035	0.973
14 days ($n = 3$)	61.7 ± 11.5	60.8 ± 9.1	0.358	0.755
Time after MCAO	**MCAO**			
	Left	**Right**	***t***	***P***
0 day ($n = 30$)	61.9 ± 10.6	62.2 ± 7.7	−0.160	0.874
24 hours ($n = 30$)	61.9 ± 10.4	66.6 ± 8.9	−2.249	0.032
7 days ($n = 20$)	60.9 ± 8.4	67.3 ± 9.6	−3.382	0.003
14 days ($n = 10$)	61.5 ± 12.7	63.0 ± 10.7	−0.866*	0.386*

*Wilcoxon test. SD: Standard deviation; CMAP: Compound muscle action potential; MCAO: Middle cerebral artery occlusion.

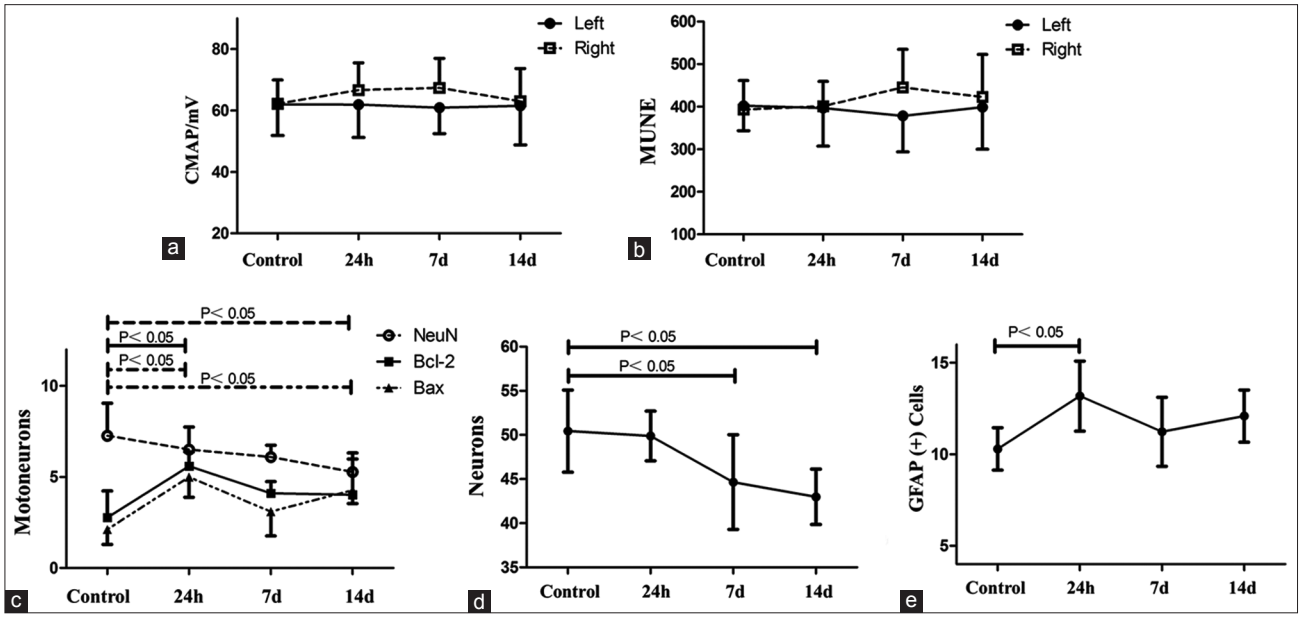

Figure 2: (a and b) The max difference of bilateral compound muscle action potential (CMAP) amplitude and motor unit number estimation (MUNE) showed at 7 days after the operation. (c-e) The number of anterior/posterior horn neurons was decreasing over the time. Active astrocytes showed in the early time after middle cerebral artery occlusion. Vertical bars represented standard deviation. GFAP: Glial fibrillary acidic protein.

Table 2: The summary of MUNE data in control and MCAO rats (mean ± SD)

Time after MCAO	Control			
	Left	Right	*t*	*P*
0 day (*n* = 9)	381.0 ± 114.3	373.0 ± 96.7	0.272	0.793
24 hours (*n* = 6)	392.0 ± 76.5	365.0 ± 134.5	0.490	0.645
7 days (*n* = 6)	369.0 ± 86.3	355.0 ± 90.3	0.956	0.383
14 days (*n* = 3)	369.0 ± 48.6	402.0 ± 83.4	–0.810	0.503
Time after MCAO	**MCAO**			
	Left	**Right**	***t***	***P***
0 day (*n* = 30)	402.0 ± 58.6	393.0 ± 68.4	0.733	0.469
24 hours (*n* = 30)	397.0 ± 89.8	401.0 ± 58.1	–0.266	0.792
7 days (*n* = 20)	379.0 ± 84.6	445.0 ± 89.5	–3.452	0.003
14 days (*n* = 10)	399.0 ± 99.1	423.0 ± 99.9	–1.775	0.110

MCAO: Middle cerebral artery occlusion; SD: Standard deviation; MUNE: Motor unit number estimation.

four given time points, as well as a nonparetic side. There was no significant difference in MUNEs between control and MCAO group. The 7-day group was also divided into two subgroups (paretic MUNE >381 and ≤381). The average mNSS was 5.6 in group with MUNE >381 (n = 10), and 6.9 in group with MUNE ≤381 (n = 10). Again, there was no significant difference between them.

CMAP amplitude on paretic side was significantly lower than on the nonparetic side, at both 24 hours and 7 days after MCAO, and MUNE on paretic side was significantly less than on nonpareticside at 7 days after MCAO. These results revealed that cerebral infarction could cause spinal motor neurons abnormalities, and loss of functional anterior motor neurons. The electrophysiology data prompted us to investigate pathologic data.

Histological and immunohistochemical assessment

Compared with control group, the number of NeuN-positive anterior motor neurons [Figure 3a] declined over time, and was significantly decreased in 14-day MCAO group (5.3 ± 0.7 vs. 7.3 ± 1.8, $P < 0.05$) [Table 3 and Figure 2c]. The expression of Bcl-2 and Bax in anterior motor neurons [Figure 3b and 3c] at 24 hours after MCAO was significantly increased (Bcl-2: 5.6 ± 0.8 vs. 2.8 ± 1.5, $P < 0.05$; Bax: 5.0 ± 1.1 vs. 2.1 ± 0.8, $P < 0.05$), while in 14 days MCAO group, Bax expression increased significantly again (4.3 ± 0.7 vs. 2.1 ± 0.8, $P < 0.05$), with Bcl-2 expression remained normal [Table 3 and Figure 2c]. These results revealed that in the early time after stroke, apoptosis of the spinal motor neurons were induced and seemed to be reversible for high expression of Bcl-2. After 14 days, high expression of Bax alone and significantly decreased a number of anterior neurons indicated that anterior motor neurons were degenerated.

The number of NeuN positive posterior horn neurons [Figure 3d] in 7 days and 14 days MCAO group was significantly less than control (control vs. 7 days: 50.4 ± 4.7 vs. 44.7 ± 5.4, $P < 0.05$; control vs. 14 days: 50.4 ± 4.7 vs. 43.0 ± 3.1, $P < 0.05$) [Table 3 and Figure 2d]. There were more GFAP positive astrocytes [Figure 3e] in 24 hours group than in control (P = 0.050) [Table 3 and Figure 2e]. The rate of posterior neurons decline was faster than anterior neurons. And astrocytes were activated at 24 hours, suggesting that the injury and inflammation occurs in the early time.

Discussion

This study showed that abnormal SA, decreased MUNE

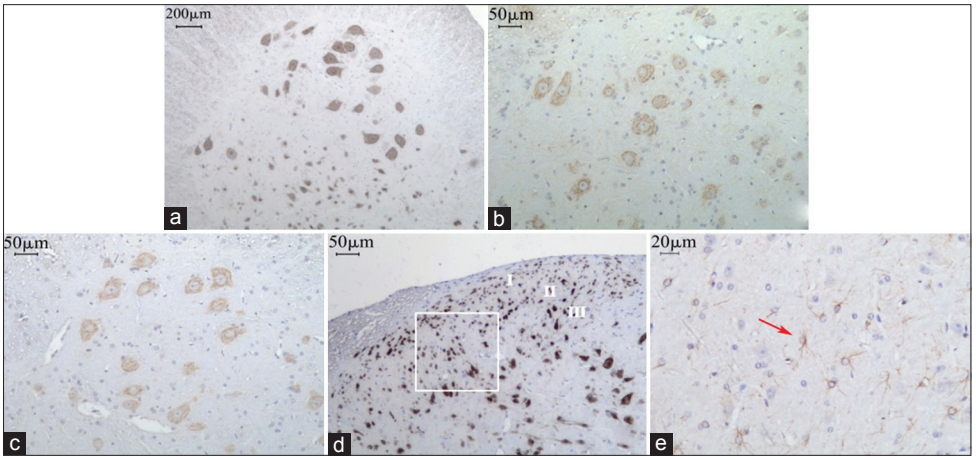

Figure 3: (a-c) Motoneurons with neuron-specific nuclear protein (Immunohistochemical [IHC] staining, original magnification ×40), B-cell lymphoma/leukaemia-2 (IHC, ×100), B-cell lymphoma/leukaemia-2 associated X expressions (IHC, ×100), respectively. (d) Neuron-specific nuclear protein staining neurons in the posterior horn. Neurons of Rexed layers I–III (white Rome number) in three nonoverlapping areas (white rectangle) were counted (IHC, ×100). (e) Glial fibrillary acidic protein positive astrocytes (red arrow) (IHC, ×200).

Table 3: The summary of histological data in control and MCAO rats (mean ± SD)

Variables	Control	MCAO (n = 6)	t	P
NeuN (anterior horn)	7.3 ± 1.8 (n = 9)			
24 hours		6.5 ± 1.3	0.909	0.380
7 days		6.1 ± 0.7	1.521	0.152
14 days		5.3 ± 0.7	2.567	0.023
Bcl-2	2.8 ± 1.5 (n = 9)			
24 hours		5.6 ± 0.8	–4.316	0.001
7 days		4.1 ± 0.6	–2.084	0.057
14 days		4.0 ± 2.3	–1.298	0.217
Bax	2.1 ± 0.8 (n = 9)			
24 hours		5.0 ± 1.1	–5.778	0.000
7 days		3.1 ± 1.3	–1.774	0.099
14 days		4.3 ± 0.7	–5.202	0.000
NeuN (posterior horn)	50.4 ± 4.7 (n = 9)			
24 hours		49.9 ± 2.8	0.255	0.803
7 days		44.7 ± 5.4	2.217	0.045
14 days		43.0 ± 3.1	3.410	0.005
GFAP	10.3 ± 1.2 (n = 3)			
24 hours		13.2 ± 1.9	–2.360	0.050
7 days		11.2 ± 1.9	–0.776	0.463
14 days		12.1 ± 1.4	–1.875	0.103

MCAO: Middle cerebral artery occlusion; SD: Standard deviation; NeuN: Neuron-specific nuclear protein; Bcl-2: B-cell lymphoma/leukaemia-2; Bax; Bcl-2 associated X; GFAP: Glial fibrillary acidic protein.

and CMAP appeared on the paretic side in MCAO rats, consistently with previous clinical studies, suggesting that lower motor neurons injury happened in the early time after stroke. And the pathology results showed that anterior motor neurons significantly decreased, indicating transsynaptic degeneration happened. However, we also found the asynchronization of electrophysiology and pathology changes.

At 24 hours after cerebral infarction, SA appeared, CMAP amplitude on paretic side declined, while Bcl-2, Bax, and GFAP expressions were increased, with normal anterior motor neurons counting. These results showed that the lower motor neurons had a functional impairment. Arasaki *et al*. had reported that abnormal electrophysiology could be appeared in the paretic limb as early as 4 h after stroke, which may due to the cut-off of corticospinal nerve fibers and inflammation.[19] Our study provided pathology evidence to this speculation. At 7 days after cerebral infarction, electrophysiology showed a significant decrease in paretic MUNE and CMAP, while pathology results showed almost normal after the early stress reaction. At 14 days after cerebral infarction, the electrophysiology was back to normal, while anterior horn neurons decreased significantly with high expression of Bax.

Our study provided the pathology evidence of lower motor neurons dysfunction and degeneration after stroke, and the variation characteristics of electrophysiology and pathology. These results revealed that abnormal electrophysiology could be detected in the early time, when motor neurons were functionally impaired, suggesting electrophysiology was more sensitive than pathology. However, when lower motor neurons degenerated in the later stage, the abnormal electrophysiological changes could not be detected. The reason for this phenomenon might be complicated. We suspected that, the rats have strong self-repair ability and quick recovering from cerebral infarction, although there was decrease in motor neurons due to transsynaptic degeneration in pathological studies at the later stage, the denervated muscle fibers of those motor neuron may be reinnervated by the survived motor neurons, which may lead to the increase in CMAP amplitude and MUNE. Clinical symptoms presented only when the number of lower motor neurons loss over 40%,[29] while our study showed only about 20% loss of motor neurons. These results also indicated that electrophysiology may be more liable to be affected by dysfunction of motor

neurons instead of the number of motor neurons at an early stage after MCAO.

We also found, significantly higher expression of Bcl-2 and Bax in anterior motoneurons, and GFAP in astrocytes was observed at 24 hours after cerebral infarction. These results revealed that in the early time after stroke, apoptosis of the spinal motor neurons were induced and astrocytes were activated, suggesting that injury and inflammation have occurred, which may initiate the lower motor neurons impairments. Posterior horn neurons also declined, and even faster than anterior neurons, illustrating that transsynaptic degeneration also existed in the sensory system, which was consistent with the previous study.[22] It was speculated that the anterior motor neurons received multiple inputs from other interneurons postsynaptic, which abating the influence from impaired corticospinal projections. Posterior horn neuron did not have many contacts with other cells, which made it more sensitive.[30]

However, some results need to be further discussed: (1) Previous studies showed that SA appeared in hemiparetic stroke patients as early as 2 weeks after stroke,[13] while in our experiment, SA appeared at 24 hours after MCAO, which could be related to the short length of peripheral nerve in rats. The onset duration of SA is dependent on the distance of peripheral nerve lesion and the target muscle. It has been reported that SA could be observed at 36 h after cutting the sciatic nerve at the sciatic notch in rats.[31] In previous research, MUNE decreasing started as early as 4 h after stroke in a clinical study.[19] When anterior motor neurons were functional impaired, the neuromuscular junctions were abnormal, which might lead to spontaneous exciting of the muscle fiber. It may be another reason of SA early occurrence. But this hypothesis need to be further verified. (2) There was no significant difference in changes of CMAP amplitude and MUNE among different duration after MCAO, neither in CMAP amplitude and MUNE between control and MCAO groups. The electrophysiology could be affected by multiple factors, such as individual differences, electrode differences, and environmental temperature difference, as well as good recovering capacity in rats. Fortunately, bilateral sides will not be affected by these factors, that was more reasonable and powerful for detecting the electrophysiological changes.

In conclusion, in the early time after cerebral infarction, functional disturbance in the lower motor neurons caused the abnormal electrophysiology results. In the later stage, anterior motor neurons declined, which provide the evidence of transsynaptic degeneration in the motor system. Our study combined the electrophysiology and pathology to provide the evidence of transsynaptic degeneration in the motor system. We also showed the asynchronous relation between the electrophysiology and pathology. More researches were necessary for further exploring the mechanisms of the transsynaptic degenerations in the motor system.

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Conflicts of interest

There are no conflicts of interest.

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