Muscle Fiber Type Changes in Lumbrical Muscles at Early Stages of Chronic Nerve Compression*

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Summary: Chronic nerve compression (CNC) neuropathy is a common disease in the clinic and provokes paraesthesia, or numbness at early stage. The changes in muscle fiber composition and motor nerve terminal morphology in distal muscles were studied in this study. A well-established CNC model was used to assess the changes in the muscles. Behaviors were measured by von Frey filament test. The myosin heavy chain isoforms and neuromuscular junctions (NMJs) were stained by immunofluorescence to show the muscle fiber types composition and motor nerve terminals morphologic changes in the flexor digitorum longus (FDL) and lumbrical muscle. The fiber cross-sectional areas of different muscle fiber types were measured. The small-fiber degeneration of cutaneous nerve fibers was examined by detecting the protein gene product 9.5 (PGP9.5) with immunofluorescence. At 2nd month after compression, the proportion of type Ⅰ and type ⅡB fibers was markedly decreased, and that of type ⅡA fibers was increased in the lumbrical muscle. There was no significant change in composition of muscle fiber types in FDL and NMJ morphology of FDL and lumbrical muscles. Intra-epidermal nerve fibre density (IENFD) declined at 2nd month after the compression. Our study reveals the morphological changes of the FDL and lumbrical muscle at an early stage of CNC. These findings may be helpful to understand muscle damage and pathophysiological development of the nerve compression, and provide new evidence for early treatment of CNC.

Key words: chronic nerve compression neuropathy; carpal tunnel syndrome; lumbrical muscles; intra-epidermal nerve fiber density; neuromuscular junctions

Chronic nerve compression (CNC) neuropathies, such as carpal tunnel syndrome (CTS) and cubital tunnel syndrome (CuTS) are very common clinically[1, 2]. CNC neuropathies result in many symptoms, such as neuropathic pain, numbness and weakness. Patients may present with skeletal muscle atrophy and motor dysfunction in the late-stage. Non-surgical treatments, such as injections, anti-inflammatory agents and splinting are effective[3, 4]. However, some clinical data have also shown that early surgical management of CTS has better efficacy and produces better long-term outcomes than non-surgical treatment[5–7]. Therefore, understanding the pathological changes at an early stage of CNC is helpful to identify more successful treatment options.

Skeletal muscle can be divided into two types according to its biological characteristics: type Ⅰ (slow-twitch) and Ⅱ (fast-twitch) muscle fibers. Type Ⅱ fibers include three subtypes that are type ⅡA, ⅡB, and ⅡX. Type Ⅰ fibers are more resistant to fatigue than type Ⅱ fibers, but produce less force. Type ⅡA fibers have smaller force and better anti-fatigue ability than type ⅡB fibers. The biological characteristics of type ⅡX is between type ⅡA and type ⅡB[8, 9]. Skeletal muscle fibers are dynamic structures that can alter their phenotype. Current studies have described muscles fiber type changes in response to denervation, electrical stimulation, exercise, age and unloading[10–12]. Patients with CTS usually complain about "tiredness" in their hand when they work[13]. Although fatigue and weakness are well-known clinical characteristics of CNC neuropathies, the changes of the muscle fiber remain non-studied. Moreover, in the current research of nerve and muscle lesion, large and proximal limb muscles in rodents, such as the gastrocnemius, soleus, flexor digitorum longus (FDL) and extensor digitorum longus (EDL), have often been studied in muscle

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function. The small and distal muscles are rarely mentioned after nerve injury. The rodent lumbrical muscles are located in the distal hindlimb and aid in metatarsophalangeal joint flexion and thus are required for paw clasping. These muscles originate from the ventral surface of the FDL tendon and are innervated by terminal branches of the tibial nerve[14, 15]. The size and relatively high surface area-to-volume ratio make the lumbrical muscle an ideal muscle to study the direct changes in fibers during CNC.

Beirowski indicated that Wallerian degeneration progresses retrogradely after nerve crush injuries. In contrast to anterograde degeneration after nerve transaction, axons were partially fragmented in the distal tibial nerve but not at the proximal end of the distal stump at the investigated time points[16]. This suggests that the pathological process of nerve injury is different between nerves that have an intact basal lamina scaffold and those that have undergone axotomy. Similar to the crush injury, integrity of epineurium is maintained in CNC neuropathies. Therefore, we suspect that muscle fiber composition in the lumbrical muscles may be affected by nerve compression earlier than that in the FDL muscle. In the current study, we use a CNC model[17, 18], mimicking CTS or CuTS in clinic, to clarify the types of myofibers in the FDL and lumbrical muscles.

1 MATERIALS AND METHODS

1.1 Ethical Approval

The animals care complied with the guidelines of the authors' institution and any national law on the care and use of laboratory animals. Animals were housed under a 12/12 h light-dark cycle and provided with food and water *ad libitum*.

1.2 Animals and Surgical Procedures

Adult male Sprague-Dawley (SD) rats (12 weeks old) were used for the experiments. The rats were anesthetized with pentobarbital sodium (Abbott Laboratories, USA) at a dose of 40–50 mg/kg. A reported model of CNC[17] was applied. The right sciatic nerve was gently mobilized from the gluteus muscle. A 2-cm piece of non-constrictive silastic tubing with an inner diameter of 1.3 mm was placed around the segment of the sciatic nerve. The left sciatic nerve was mobilized and then returned to the host bed as a sham-operation. Each wound was closed with 3-0 polyester suture upon completion of the procedure. The rats were sacrificed for histological studies by anesthetic overdose (100 mg/kg) of pentobarbital sodium. Sciatic nerves and muscles were harvested from surgery and sham-operation sides.

1.3 Von Frey Filament Test

To determine the evoked reflex responses to mechanical stimuli, a normally non-noxious mechanical stimulus was measured by means of an electronic Von Frey apparatus (IITC Life Science Inc., USA). Rats were placed on a wire net platform in plastic chambers 10 min before the experiment for habituation. We measured the threshold force for withdrawal of the hind paw before and 2 weeks, 1 and 2 months after surgical operation. The von Frey filaments were applied to the plantar surface of the foot in ascending order. The threshold was defined as the minimum stimulus necessary to evoke total paw withdrawal, and it was recorded as the paw withdrawal threshold (PWT) in grams (g).

1.4 Electrophysiological Studies

Rats were anesthetized with pentobarbital sodium (40–50 mg/kg) before electrophysiological testing. The nerve conduction velocity (NCV) of the sciatic nerve was measured using an NID-092 electronic medical instrument (Haishen Inc., China) before and 2 weeks, 1 and 2 months after surgical operation. The stimulating electrode was placed on the proximal or distal side of the compression area of the sciatic nerve (stimulation duration: 0.1 ms; frequency: 1 Hz; stimulus intensity: 5 mA), and the recording electrode was placed on the dorsum of the foot. Nerve conduction latency was evaluated using the data obtained directly from the sciatic nerves.

1.5 Immunofluorescence of Footpad Skin

The footpads were removed from both sides of the hindpaws of rats 1 and 2 months after sciatic nerve compression. The footpads were fixed in 4% paraformaldehyde overnight and dehydrated in a 25% solution of sucrose for 10 h and were then cut into 40-μm thick sections with a cryostat.

The skin sections (40 μm) were incubated overnight with a primary polyclonal rabbit IgG antibody against PGP 9.5 (1:100, Abcam, USA). 12 h later, the sections were incubated in the anti-rabbit Alexa 488 secondary antibody (1:200, Abcam) for 1 h at 37°C. All the samples were visualized with a confocal laser-scanning microscope. The intra-epidermal nerve fibre density (IENFD) was derived as the number of fibers per millimeter of epidermal length (fibers/mm). The IENFD was quantified according to the guidelines by Hsieh[19], and counted in 4 randomly selected sections from each skin specimen. PGP9.5-immunoreactive (PGP 9.5-ir) nerve fibers in the intra-epidermis of each section were counted at a magnification of 40× under a fluorescence microscope (E800, Nikon, Japan).

1.6 Immunofluorescence of Muscles

FDL muscles and the second lumbrical muscles (the second deep lumbrical muscle) receive innervation from the medial plantar nerve only[20]. The muscles were harvested at 1st and 2nd month after CNC on both sides. The whole muscles were quickly frozen at resting length in isopentane cooled to –160°C with liquid nitrogen. The muscle samples were cut into 10-

μm thick cross-sections and 20-μm thick longitudinal sections in a cryostat for the immunofluorescence analysis of muscle fiber types and neuromuscular junctions (NMJs).

To characterize the fiber composition of the skeletal muscles, 10-μm cross-sections were immunolabeled with the following mouse monoclonal antibodies [Developmental Studies Hybridoma Bank (DSHB), USA]: anti-type Ⅰ (SC-71, mouse IgG1), anti-type ⅡA (BA-D5, mouse IgG2B), and anti-type ⅡB (BF-F3, mouse IgM). The sections were incubated for 1 h at 37°C in a mixture of BA-D5, SC-71, and BF-F3 antibodies (1:100). Then, the sections were incubated with secondary antibodies (1:100, Life Technologies, USA) for 1 h at 37°C: Alexa Fluor 350-labeled donkey anti-mouse IgG2B (for BA-D5), Alexa Fluor 594-labeled donkey anti-mouse IgG1 (for SC-71) and Alexa Fluor 488-labeled donkey anti-mouse IgM (for BF-F3). The images of muscle fiber types composition were obtained via confocal microscopy. Red labeled fibers were identified as type Ⅰ, blue labeled fibers as type ⅡA, purple labeled fibers as hybrid type Ⅰ/ⅡA, and green labeled fibers as type ⅡB, and the unlabeled fibers as type ⅡX. The fiber cross-sectional areas (FCSAs) of different muscle fiber types were measured from confocal laser-scanning photographs using Image-Pro Plus 6.0 image analysis software (Media Cybernetics, USA).

NMJs in the FDL and lumbrical muscles were immunostained with neurofilament (NF) and synaptophysin to detect pre-synaptic neuronal compartments and with α-bungarotoxin (α-BTX) to detect acetylcholine receptors at the muscle endplates. Twenty-micrometer longitudinal sections were incubated with the primary antibody solutions containing mouse anti-synaptophysin (1:200, Abcam) and anti-NF (1:200, Sigma, USA) overnight at 4°C. 12 h later, the samples were incubated for 1 h at 37°C in a mixture of secondary antibodies containing anti-mouse Alexa Fluor 488 (1:200, Abcam) and Alexa Fluor 647-conjugated α-BTX (1:1000, Molecular Probes).

1.7 Statistical Processing

All values are presented as the mean±standard deviation (SD). Statistical significance was analyzed by one-way or two-way ANOVA with repeated measures over time, and Dunnett's post hoc test was used for multiple comparisons. A P-value <0.05 was considered significant.

2 RESULTS

2.1 Changes in Mechanical Sensitivity

In the CNC hand paws (n=5), no significant change was detected in the PWT of von Frey filament testing before (20.4±0.2 g) and 2 weeks after surgery (20.9±0.4 g, P>0.05). The PWT was significantly increased at 1st (24.7±0.5 g, P<0.001) and 2nd month (27.5±0.8 g, P<0.001) after compression as compared with that pre-operation (fig. 1).

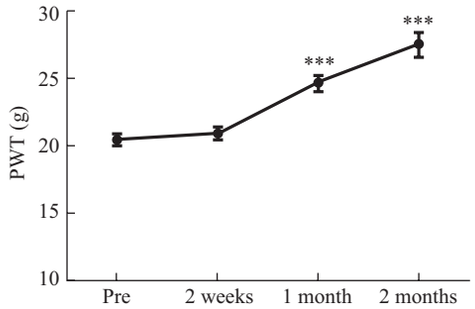

Fig. 1 Paw withdrawal threshold (PWT) analysis of von Frey filament test (n=5)
The data are presented as the mean±SD; $^{***}P$<0.001 *vs.* pre and 2 weeks. Pre: preoperation

2.2 Electrophysiological Analysis

In the CNC group (n=5), the NCV did not exhibit a statistically significant change at 1st month (52.4±1.9 m/s) and 2nd month (50.4±2.4 m/s) post-operation compared to the sham operation-side NCV (53.1±2.0 m/s and 54.7±2.4 m/s). The data are consistent with those reported previously[17] (fig. 2).

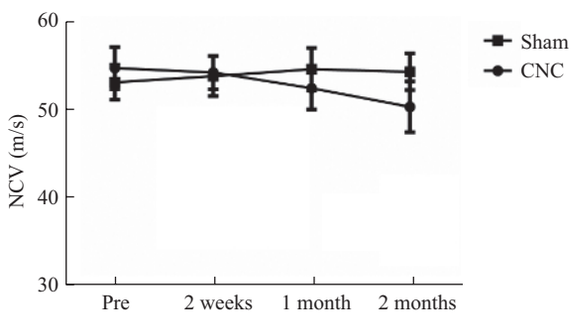

Fig. 2 NCV analysis of the sciatic nerve (n=10)
Both compressed and un-compressed sides were tested. No significant difference in NCV preoperation and at 2nd week, 1st month and 2nd month was found between sham operation group and CNC group. All data are represented as the mean±SD.

2.3 Immunofluorescence of Nerve Fibers in Footpad Skin

In the footpad, the epidermis was richly innervated by nerve fibers, and showed a continuous linear staining pattern in sham operation side (n=5). The IENFD in the sham operation side was 18.51±1.82 fibers/mm. The IENFD was markedly decreased in the epidermis at 1st month (11.67±1.46 fibers/mm, P<0.001) and 2nd month (7.33±1.92 fibers/mm, P<0.001) after compression (n=5) (fig. 3).

2.4 Immunofluorescence of Muscle Fiber-type in Muscles

In the lumbrical muscles, five muscle fiber-type

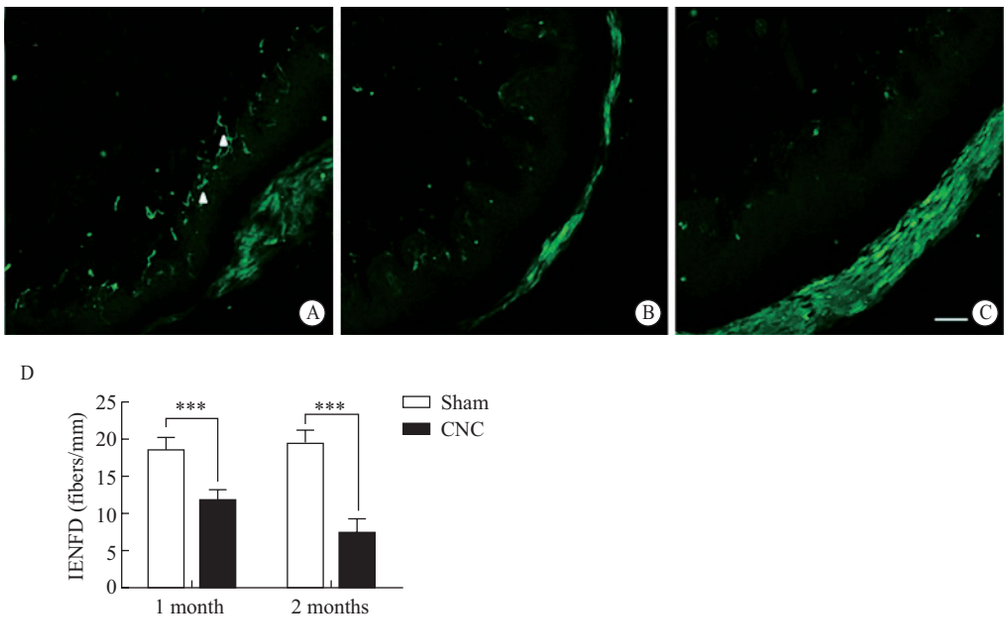

Fig. 3 Immunostaining of PGP 9.5 protein in the footpad skin from the hindpaws
A: The intra-epidermis was richly innervated by PGP 9.5-ir fibers in the footpads of naïve rats. B and C: A marked reduction in the PGP 9.5-ir fibers was observed in the CNC footpads at 1st month post-operation (B) and 2nd month post-operation (C). Scale bar=100 μm. D: The IENFD was significantly decreased on CNC side (gray bar) compared to the uncompressed side (black bar). Data are presented as the mean±SD. n=5 in each group. $^{***}P<0.001$

categories were type Ⅰ, type Ⅰ/ⅡA, type ⅡA, type ⅡB, and type ⅡX fibers. In the type ⅡB myofibers, the FCSAs were significantly larger than in the other muscle fiber types. At 1st month after compression (1MCNC), the percentages of type Ⅰ and type ⅡB fibers declined markedly; in contrast, the percentages of type ⅡA and type ⅡX fibers increased compared with those on the sham side (1MSham). The type Ⅰ/ⅡA hybrid fibers did not change (fig. 4D). There were no significant changes in the FCSAs within the different muscle fiber types (fig. 4E). At 2nd month after compression (2MCNC), the percentages of type Ⅰ and type ⅡB fibers further declined compared with 1MCNC. The percentages of type Ⅰ/ⅡA hybrid fibers declined. The percentages of type ⅡA and type ⅡX fibers increased compared with 1MCNC (fig. 4D). There were no significant changes in the FCSAs within the different muscle fiber types (fig. 4E). The cross-sectional area (CSA) of the lumbrical muscle revealed no significant difference between the sham side and the CNC side (n=5).

In FDL muscle, the proportions of the muscle fiber components did not change significantly at 1st and 2nd month after compression (fig. 5D). There was no significant change in FCSAs of different muscle fiber types (fig. 5E). The CSA of the FDL revealed no significant difference between two sides.

In both FDL and lumbrical muscle, the NMJs remained structurally intact, and both presynaptic nerve terminals and postsynaptic motor endplates were well organized and compact at 2nd month post-operation (fig. 6).

3 DISCUSSION

CNC neuropathy is a common problem in the clinic that often results in significant morbidity, including loss of motor and sensory function[1, 2]. Early treatment of CNC has good efficacy and produces good long-term outcomes[21]. In muscle atrophy patients with CNC, the effect of surgery is not satisfactory. Motor function recovery is rare if muscle atrophy is present for more than one year prior to surgery[22]. After decompression surgery, paresthesia was often restored, however, motor functional recovery was poor even several years later. Half of the patients complain that hand muscle strength is not improved after decompression[23, 24]. The motor functional recovery of the intrinsic muscles after an ulnar nerve injury is extremely poor, and often results in finger clawing and hand clumsiness. Lumbrical muscles are located in the hand and are involved in proximal interphalangeal and distal interphalangeal joint extension in humans. The lumbrical muscles play an important role in motion feedback and fine movement coordination[25, 26].

In the present research, we observed a decrease in type Ⅰ muscle fibers, and an increase in type Ⅱ fibers in lumbrical muscles at 1st month after compression. The type Ⅰ-to-type Ⅱ shift was commonly present when nerves injury. The model of loose ligation of sciatic

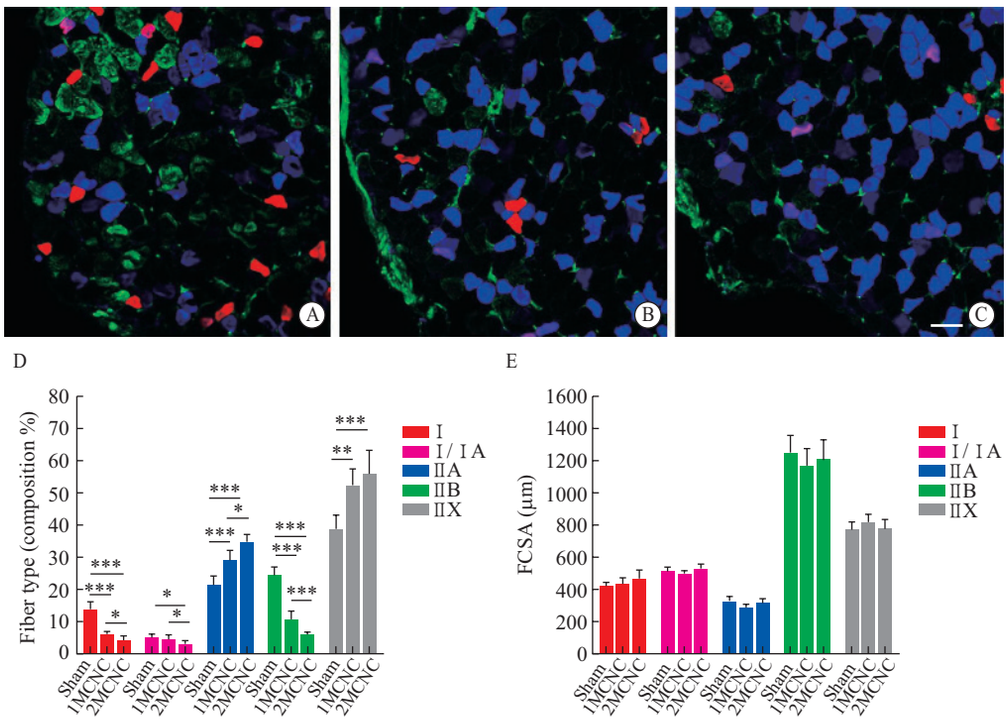

Fig. 4 Immunofluorescence of the lumbrical muscle fibers (n=5)

The lumbrical muscle fiber types were analyzed with immunofluorescence: type Ⅰ (red), type ⅡA (blue), type Ⅰ/ⅡA (purple), type ⅡB (green) and type ⅡX (black). The composition of the lumbrical muscle fibers changed significantly at 1st month (B) and 2nd month (C) post-operation on the CNC side as compared with that on the sham-operation side (A). Scale bar=200 μm. The bar graphs are presented as the fiber type percent composition (D) and FCSA (E). The percentages of type Ⅰ/ⅡA hybrid fibers declined until 2 months post-operation. 1MSham: sham group at 1st month; 1MCNC: CNC group at 1st month; 2MCNC: CNC group at 2nd month. The data are presented as the mean±SD. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001

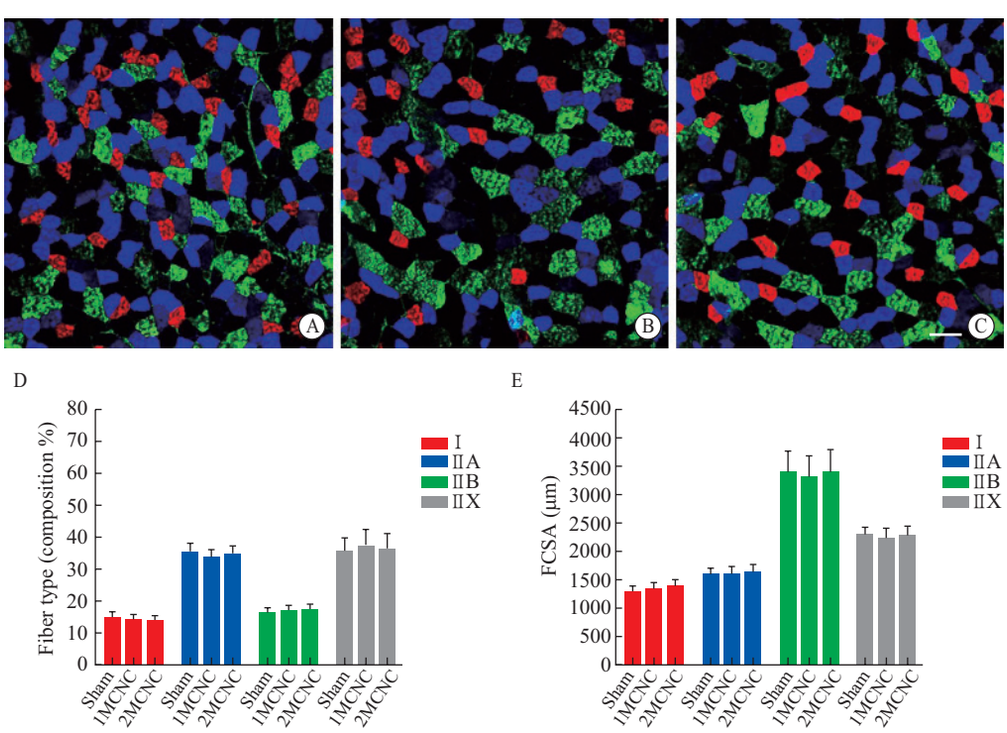

Fig. 5 Immunofluorescence of the FDL muscle fibers (n=5)

The FDL muscle fiber types were analyzed with immunofluorescence: type Ⅰ (red), type ⅡA (blue), type ⅡB (green) and type ⅡX (black). The composition of the FDL muscle fibers unchanged at 1st month (B) and 2nd month (C) post-operation on the CNC side as compared with that on the sham-operation side (A). Scale bar=100 μm. The bar graphs are presented as the fiber type percent composition (D) and FCSA (E). 1MSham: sham group at 1st month; 1MCNC: CNC group at 1st month; 2MCNC: CNC group at 2nd month. The data are presented as the mean±SD.

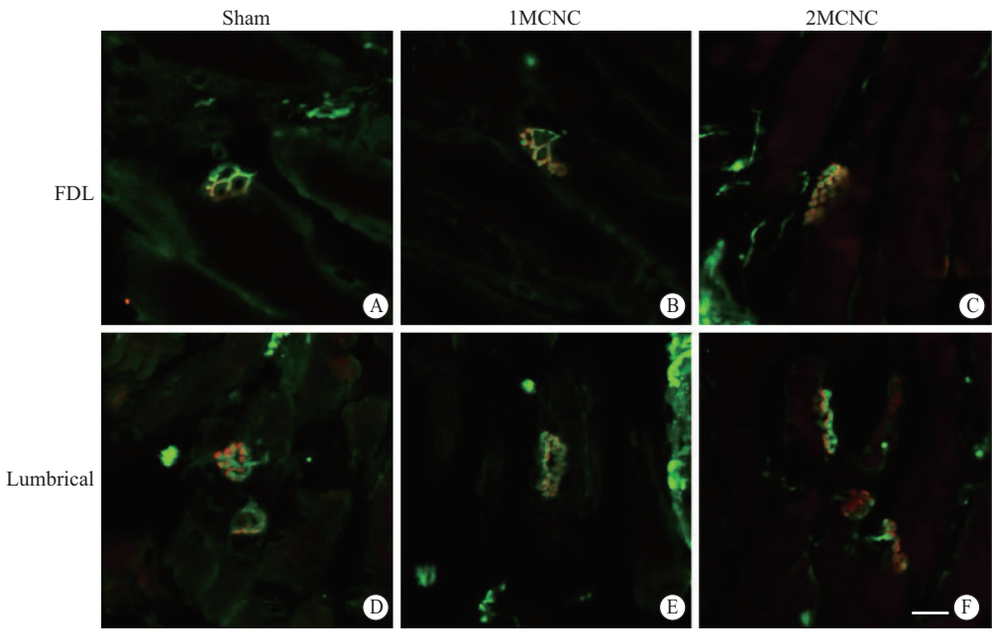

Fig. 6 Immunofluorescence of NMJs in the FDL (A–C) and lumbrical muscles (D–F)
The NMJs were immunostained with NF, synaptophysin (green) and α-BTX (red). The NMJs are intact on both the sham-operation side and CNC side at 1st and 2nd month post-operation. Scale bar=20 μm

nerve in rats has demonstrated a shift from slow to fast fibers because of denervation and/or pain[27]. In this sutdy, we noted hypoalgesia to mechanical stimulation and intraepidermal nerve fiber degeneration in the hindpaw, but the NMJs in the FDL and lumbrical muscle were intact and NCV was stable at 2nd month post-operation. In addition, the FCSA of lumbrical muscle was unaltered. We suggest that the fiber composition changes in lumbrical muscles may relate to paresthesia-related control of limb movement rather than motor denervation. Sensory feedback from the fingertips plays an important role in the coordination of movement and grip force in the hand[28, 29]. Some studies found that CTS-induced deficits in tactile sensitivity interfere with sensorimotor control. Force modulation to object weight was less accurate in CTS, which might be particularly detrimental for tasks that require fine regulation of fingertip forces[30, 31]. This study suggests that loss of sensory function in the skin interferes with sensorimotor control of hindpaw. The lumbrical muscles play an important role in motion feedback and fine movement coordination[25, 26]. To adapt to the change of movement coordination, the fiber composition changes in lumbrical muscles.

In our study, we observed a decrease in type Ⅰ and type ⅡB muscle fibers, an increase in type ⅡA and type ⅡX fibers and the percentage of hybrid type Ⅰ/ⅡA fibers was unchanged in lumbrical muscles at 1st month after compression. The percentage of Ⅰ/ⅡA fibers declined at 2nd month after compression. The role of hybrid fibers is considered as intermediates in fiber type transformations. In some cases hybrids play a role as intermediate fibers during fiber type transitions[32]. The transformation from type Ⅰ → Ⅰ/ⅡA→ ⅡA is in equilibrium at 1st month after compression. As compression went on, the equilibrium is off when the amount of type Ⅰ fibers got less and less. It is well known that type Ⅰ fibers (slow fibers) are more resistant to fatigue than type Ⅱ fibers (fast fibers). Type ⅡA fibers produce higher contraction force and power than type Ⅰ fibers. Type ⅡA fibers have slower contraction speed, smaller contraction force and better anti-fatigue than type ⅡB fiber. The biological characteristics of type ⅡX is between type ⅡA and type ⅡB. The reduction of type Ⅰ fiber may make muscle more fatigable. The transformation of type ⅡB→ ⅡX→ ⅡA leads to the muscle slower contraction speed after CNC injury. Some studies demonstrated that patients with CTS usually complaint about fatigue in their hand when doing manual tasks[13], and the patients applied excessive grip force and longer time to peak of grip force than healthy controls during object manipulation[33]. We suggest that the rats modify the muscle fibers type in lumbrical muscles to adapt the change of movement coordination in the hindpaws after CNC. The direction of transformation is type Ⅰ → Ⅱ and type ⅡB→ ⅡX→ ⅡA.

We did not observe any changes in muscle fiber composition or morphology in the FDL. In addition, the MNJs were intact in the FDL. The existing literatures have indicated that NMJs were intact in the FDL at an early stage of CNC[34]. Our research shows that NMJs in lumbrical muscles are also intact. Although we did not find retrograde motor denervation at an early stage

of CNC, these data indicate that the distal lumbrical muscle is more vulnerable than the proximal FDL in chronic sciatic nerve compression. The changes in distal lumbrical muscle composition occurred earlier than those in motor NCV. Clinically, we can identify the patients who meet the clinical criteria (e.g., numbness and fatigue) for CTS but have negative electrodiagnostic tests. The current study suggests that early intervention is needed when the nerve compression clinical criteria are met. Additionally, the lumbrical muscle should be considered as a target muscle in future muscle studies. Although the mechanism of muscle fiber type change is not well understood, these findings would be helpful to understand muscle damage and pathophysiology development of the nerve compression, and provide new evidence for early treatment.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest or financial disclosures to report.

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