Effects of multiple nerve crushes on functional recovery of the sciatic nerve

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INTRODUCTION
The study for the sciatic nerve injury by single crush is performed enough, but there are few reports about repeated crush injuries of sciatic nerve. Thus, it is unclear how the motor function is restored by repeated crush injuries of sciatic nerve. The present study was undertaken to investigate the influence on motor function, thickness of the muscle fiber, and muscle fiber reinnervation by repeated sciatic nerve crush of adult rats. In addition, we measured a diameter of the muscular fiber, and assessed a quantification of muscle fiber reinnervation by immunohistochemistry using synaptophysin, a transmembrane protein in synaptic vesicles, and α-bungarotoxin, acetylcholine receptor antagonist.

MATERIALS AND METHODS
Animals: The experiments were carried out on adult female Wistar 124 rats (220-260g body weight: Japan SLC Inc., Hamamatsu, Shizuoka, Japan). All procedures were conducted in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by our Institutional Animal Care and Use Committee.

Crush on the sciatic nerve and classification of the rats: Under general anesthesia, the unilateral left sciatic nerve of the rats was exposed just behind the greater trochanter (GT). The nerve expose to distal was performed by width of 15mm (Figure1a&b). One time nerve crush was carried out for 180 seconds with fine forceps. The animals were divided into five groups. Rats in A group (n=5), consisted of surgical control group, received only neurolysis not to damage the nerve. Rats in B group (n=52) received one crush at a position of GT level (single nerve crush). Rats in C group (n=6), received the first crush at a position of 7.5mm distal of GT level, and the second crush at a position of GT level 1 week later (double nerve crush). Rats in D group (n=55) and E (n=6) groups received the first crush at a position of 15mm distal of GT level, the second crush at a position of 7.5mm distal of GT level and the third crush at a position of GT level at 1 week (D group) or 4 weeks (E group) intervals (triple nerve crush). In the nerve-crushed groups, the final crush was designed to perform at a position of GT level of each animal.

Functional assessment by static sciatic index (SSI): To assess functional loss following crush to the sciatic nerve, footprints were taken every week until 8 weeks after the last crush for all groups (A group, n=5; B group, n=5; C group, n=6; D group, n=8; E group, n=6).

Tissue preparation: At 2, 3 and 4 weeks after the last crush, rats in B (2 weeks, n=14; 3 weeks, n=16; 4 weeks, n=18) and D (2 weeks, n=14; 3 weeks, n=16; 4 weeks, n=16) groups were perfused. Longitudinal sections of the tibialis anterior muscle were cut serially into 50 μm slices on a freezing microtome, and collected at 200 μm intervals. As the control, rats in A group (n=5) were perfused with a fixative after the last assessment of the sciatic motor function by SSI, followed by removal and preparation of the 50 μm-thick frozen section of the tibialis anterior muscle in the same manner.

Measurement of muscle fiber diameter: Longitudinal sections of the tibialis anterior muscle were mounted on coated slides and stained with hematoxylin-eosin. The diameters of 50 muscle fibers in the thickest part near the middle of the muscle were measured using microscope. The average of the muscle fiber diameters were calculated for each animal and group.

Neuromuscular junctions: Longitudinal sections of the tibialis anterior muscle were stained on a shaker at room temperature. The sections were immunostained for nerve terminals using anti-synaptophysin antibody and stained for acetylcholine receptors (AChRs) using α-bungarotoxin. For synaptophysin immunostaining, they were incubated with biotinylated anti-rabbit immunoglobulins for 2h. After washing, they

incubated with streptavidin-peroxidase for 2h, and the peroxidase reaction product was visualized with the Metal Enhanced DAB Substrate Kit. To evaluate the neuromuscular junctions, the numbers of synaptophysin-positive nerve terminals and α-bungarotoxin-positive AchRs were counted in the serial sections. The most numerous five sections were selected and total numbers of synaptophysin-positive nerve terminals and α-bungarotoxin-positive AchRs were calculated for each animal. The ratios of the number of synaptophysin-positive nerve terminals to that of α-bungarotoxin-positive AchRs were used to determine the degree of reinnervation of the neuromuscular junctions.

RESULTS
The SSI scores in the rats with the triple nerve crush injuries every week did not recover to normal range up to 8 weeks (Figure 2a&b). Comparisons between the single and triple crush injuries every week were made with muscle fiber diameter and reinnervation of the tibialis anterior muscles. In the rats with the triple nerve crush injuries, the average diameters of 50 muscle fibers were significantly smaller than those with the single nerve crush injury at the point of 2, 3 and 4 weeks after the last crush injury (Figure 3a&b).

DISCUSSION
In the rats with repeated sciatic nerve crush injuries at short intervals before recovering motor function, the function may not recover completely because of delay of the reinnervation and the atrophy of the muscles.

SIGNIFICANCE
This study may help clinical condition elucidation of “double crush lesion” such as radicular and peripheral nerve injury at the same time.