



# A Critical Period for Functional Motor Recovery After Peripheral Nerve Injury in the Mouse

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## Abstract

Repair of peripheral nerve injury in humans often results in poor functional motor recovery, depending on the location and the timing of surgery. This deficit in motor recovery has previously been attributed to the failure of axons to regenerate into the target muscle. However, we have recently reported that following sciatic nerve transection and immediate resuture in mice, regenerating axons are observed at the motor end plate even in animals with poor functional recovery. In this model, motor recovery reaches a plateau approximately 35 days post-injury, with no further recovery beyond this period. Based on this, we proposed that following axonal injury, there is a critical period during which the axon must reach the target muscle in order to form a functional neuromuscular junction. To test this, we have developed a mouse model of differential prolonged denervation, in which the proximal sciatic nerve is crushed repeatedly every 3 to 7 days, preventing regenerating axons from reaching the target muscle. This multiple crush model allows us to vary the period of denervation, by modifying the number of crushes. We performed 3, 4, or 5 crushes every 7 days (corresponding to ~24, 31, or 38 days of denervation, respectively) and assessed functional motor recovery using the toe-spreading score and modified sciatic function index. Motor recovery occurs after 3 or 4 multiple crushes (24 or 31 days of denervation) but not after 5 crushes (38 days). Immunostaining for alpha-bungarotoxin and neurofilament demonstrated axonal regeneration reaching the motor end plates in all 3 groups. Thus following prolonged denervation > 38 days, a functional motor deficit persists despite motor end plate reinnervation. Although the mechanism for the motor deficit requires investigation, these results suggest that functional neuromuscular junction reestablishment following nerve injury requires more than anatomical reinnervation of the motor end plate and that there is a time limit for functional synapse reformation.

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## **Glossary**

AchR: Acetylcholine receptor

BTX: Bungarotoxin

CHAT: Choline acetyltransferase

CMAP: Compound muscle action potential

NF: Neurofilament

NMJ: Neuromuscular junction

PBS: Phosphate-buffered saline

SYP: Synaptophysin

# 1 Introduction

## 1.1 Background

Mammalian peripheral neurons are capable of axonal regeneration after injury. In the clinical setting, however, peripheral nerve injuries often result in permanent deficits in motor function. Current medical treatment is inadequate despite advances in surgical techniques, such as the use of autologous and artificial nerve grafts, which have improved the outcome of peripheral nerve injury (Dahlin, 2009). Peripheral nerve surgeries are especially unsuccessful when there is prolonged delay between injury and repair, such as in delayed carpal tunnel surgery, and when there is extensive distance between nerve injury and the target muscle, such as in brachial plexus injuries (Hoke, 2006).

Compared to mouse models, axons of human motor neurons must regenerate significantly longer distances to reach their target at the neuromuscular junction (Griffin, 2010). Thus, in both proximal nerve injuries and delayed nerve repairs, there is a resulting prolonged period of muscle denervation. It has long been observed that prolonged muscle denervation is detrimental to functional motor recovery (Gutmann and Young, 1944), and the poor recovery has classically been attributed to muscle atrophy, the breakdown of motor end plates, or the inability of the muscle to accept reinnervation (Guttman and Young, 1944; Guttman, 1948).

More recently, the failure of peripheral regeneration has been attributed to the failure of regenerating axons to reach the target, and therefore, most studies have been focused on understanding cellular alterations in both Schwann cells and neurons that reduce axonal regenerative capabilities (Dahlin et al., 2009). It has been proposed that there is progressive degeneration of distal nerve sheaths, which contain Schwann cells supportive for axonal regeneration, and a corresponding change in the milieu that becomes progressively non-

permissive to growth (Fu and Gordon, 1995a). Key experiments that have led to this focus are summarized below.

## 1.2 Key Experiments

Fu and Gordon (1995a) performed a series of nerve cross-anastomosis experiments to tease out whether poor functional recovery after delayed nerve repair is due to changes associated with chronic axotomy of the injured neurons or to changes associated with chronic denervation of the distal nerve segment and muscle. They created a model of prolonged axotomy by transecting the tibial nerve, axotomizing it for up to 12 months, and then cross-suturing it to the distal stump of a freshly cut common peroneal nerve, allowing it to reinnervate the freshly denervated tibialis anterior muscle, which was previously innervated by the common peroneal nerve. They found that although there was a decrease in the number of axons reinnervating the muscle, there was an increase in the innervation ratio, the number of muscle fibers innervated by each motor neuron. This allowed for a total force generation similar to that after immediate cross-suture of the cut tibial nerve to the cut peroneal nerve. They concluded that prolonged axotomy results in a decreased capacity of axonal regeneration that can be compensated by the capacity to increase the innervation ratio.

Fu and Gordon (1995b) next created a model of prolonged denervation by cutting the common peroneal nerve, allowing the tibialis anterior muscle to remain denervated for up to 12 months before cross-suturing the freshly cut tibial nerve to the distal stump of the common peroneal nerve, allowing for reinnervation of the tibialis anterior muscle. They found that compared to prolonged axotomy, prolonged denervation results in a more severe decrease in the number of axons reinnervating the muscle. The increase in the innervation ratio is insufficient to compensate for the large decline in number of axons, and as a result, there is a decrease in the number of reinnervated muscle fibers and a decrease in the muscle force generation; denervation

atrophy of muscle fibers also contributed, albeit less significantly, to the functional defect. They also found impaired regeneration in another injury model involving suturing a cut nerve directly into muscle. Because of the similar deficits in the chronic denervation model and the direct neurotization model, they proposed that the functional defect after prolonged denervation is primarily due to progressive breakdown of the intramuscular nerve sheaths and a resultant decrease in the ability of the distal nerve sheath to support axonal outgrowth.

However, recent findings in our laboratory showed that axonal regeneration to the target muscle does occur even after prolonged denervation. A recent study (Ma et al., 2011) showed that following nerve transection and resuture in mice, even among mice that did not have motor recovery, choline acetyltransferase (CHAT)-positive axon terminals and acetylcholine receptor-positive motor end plates were observed within the target muscles. In addition, behavioral studies showed that functional recovery reached a plateau after approximately 35 days post-injury with no improvement beyond this period.

These findings from Ma et al.'s study suggest that following axonal injury, there is a critical time period during which the axon must reach the target muscle in order to form a functional NMJ; it also suggests that failure of functional recovery is not due to failure of axonal regeneration but due to lack of timely reinnervation of the target muscle. Therefore, we hypothesized that the time-dependence of functional motor recovery does not lie solely in diminishing capability of axonal regeneration over time but also in a critical period for the axons and end plates to reestablish a functional NMJ.

### **1.3 Purpose of Inquiry**

We hypothesized that following injury to motor neurons, there is a critical period for the formation of a functional neuromuscular junction. That is to say, in order to have motor recovery after peripheral nerve injury, axons must not only regenerate along the entire length of the nerve



but also reinnervate the target muscle within a critical period in order to form a functional synapse.

The primary aim of this project was to determine whether such a critical period exists after peripheral nerve injury, and if it existed, to determine the length of the critical period. Based upon previous work, we predicted that the critical period would be around 35 days in mice. To accomplish this aim, we developed a new nerve injury model, called the multiple crush method, which allows for variable periods of denervation. I induced various periods of denervation and tested whether functional recovery occurred only when the period of denervation was shorter than this critical period. The second aim was to identify molecular mechanisms of the critical period. To work towards the second aim, I assessed for any histological correlates of functional recovery at the neuromuscular junction.

## **2 Materials and Methods**

### **2.1 Animals**

Male C57BL/6J mice were used for all experiments. Animal experiments were approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital.

### **2.2 Surgery**

Sciatic nerve crush was performed under anesthesia with inhaled isoflurane. The left sciatic nerve was exposed at the mid-thigh level, and nerve crush using a pair of fine angled forceps was performed at the level of external rotator muscles for a period of 30 seconds. The surgical incision was closed with wound clips.

### **2.3 Multiple Crush Method**

Due to the small size of our chosen model animal and the speed of regeneration, there is a short distance between the nerve injury site and the target muscle. As a result, a single crush injury of the sciatic nerve in mice results in regeneration to the target muscle in a relative short period of time—previous work in our lab has indicated that the first sign of recovery is observed after 10-14 days—much too short a timeframe for us to study the predicted critical period of approximately 35 days. Therefore, our lab designed a new method to prevent axons from reaching the muscle after nerve injury.

In the “multiple crush” model of prolonged denervation, the first sciatic nerve crush was performed at the level of the external rotator muscles. This results in axonal degeneration distal to injury site and denervation of the muscle, followed by axonal regeneration. Subsequent nerve crushes were performed before the axons could reach the target muscle. As a result, the muscle should remain denervated for the entire period between the multiple crushes. Furthermore, this method allows us to control the period of denervation by varying the number and frequency of nerve crushes.

Based on the time course of sensory recovery observed in our lab following a sciatic nerve crush, the axons are predicted to reach the distal hindpaw 10 days after the crush. Therefore, a sciatic nerve crush every 7 days would keep the muscle denervated for the entire time between the multiple crushes. After the final crush, it would take approximately 10 days for the axon to reach the muscle. Three different groups for 3 different periods of denervation, consisting of 2 shorter and 1 longer than the predicted critical period, were chosen as an experimental model (Figure 1). Three crushes results in denervation for approximately 24 days, 4 crushes for approximately 31 days, and 5 crushes for approximately 38 days. An additional group that received 5 crushes but within a shorter period of time was included to investigate whether multiple nerve crushes itself resulted in limited axonal regeneration.

#### **2.4 Behavioral Tests**

The behavioral test chosen to track motor recovery of the plantar muscles in the hindpaw following sciatic nerve injury was the toe spreading score. Toe spreading score is based on reflexive spread of the hindpaw toes, and animals were scored with 2 for full spread sustained for > 2 seconds, 1 for partial or transient spread, and 0 for no spread. All the behavioral tests were performed in a blinded manner.

#### **2.5 Nerve Conduction Studies**

Experiments were performed under terminal anesthesia with intraperitoneal urethane. The sciatic nerve was exposed at the mid-thigh level, and a cuff containing a stimulating electrode was applied. Compound muscle action potential (CMAP) of the plantar muscles was measured using bipolar needle electrodes. The experimental conditions were blinded for the examiner.

#### **2.6 Immunohistochemistry**

Animals underwent terminal anesthesia with 0.2 mL intraperitoneal injection of 25% urethane. The mice were intracardially perfused with 50 ml of cold phosphate buffered saline

(PBS) followed by 50 mL of 2% paraformaldehyde/1.5% dimethyl suberimidate in borate buffer. Both ipsilateral and contralateral plantar muscles (abductor digiti minimi) in the hindpaw and a segment of the sciatic nerve spanning from 5 mm proximal to the crush site to 20 mm distal were harvested. The tissues were placed in 30% sucrose solution overnight at 4° C and mounted them onto Optimal Cutting Temperature (OCT) Compound (Tissue-Tek). Longitudinal muscle sections of 100 µm were cut on a cryostat at -20° C and processed as free-floating sections. 10 µm cross-sections of sciatic nerve were cut, and the sections were placed directly on a microscope slide.

Plantar muscle sections were incubated with chicken anti-neurofilament-200 (Abcam;1:2000) primary antibodies for 2 days at room temperature. They were incubated with AlexaFluor® 568 anti-chicken secondary antibody and AlexaFluor® 488  $\alpha$ -bungarotoxin overnight at room temperature. For synaptophysin staining, 20 µm sections were incubated with rabbit anti-synaptophysin (1:500) and chicken anti-neurofilament-200 (Abcam; 1:5000) for 2 days followed by AlexaFluor® 568 anti-rabbit, antiAlexaFluor® 647 anti-chicken and AlexaFluor® 488  $\alpha$ -bungarotoxin for 1 hour. All incubations were completed at room temperature.

## **2.7 Neuromuscular Junction Reinnervation**

High-power images of neuromuscular junctions were obtained at 63x magnification using confocal microscopy. Motor end plate reinnervation was scored based on overlap of anti-neurofilament 200 and  $\alpha$ -bungarotoxin signals on maximum intensity projection images obtained from confocal image z-stacks. The experimental conditions were blinded for the manual scoring.

## **2.8 Axon Regeneration**

The number of axons positive for NF staining 20 mm distal to the crush site was quantified using ImageJ software.

## 2.9 Statistical Analysis

Data are shown as mean +/- standard error of the mean. Student's t-test was completed compared to the relevant control group, and statistical significance was set at  $p < 0.05$ .

## 3 Results

### 3.1 The sciatic nerve can be crushed multiple times to create a variable period of muscle denervation

To validate the multiple crush method, we verified whether the motor end plates remain denervated during the 7 days between successive nerve crushes. Immunohistochemistry of axon terminals was performed with anti-neurofilament antibody and motor end plates were labeled with  $\alpha$ -bungarotoxin. Seven days after the first crush, there were no axons present in the plantar muscle sections (Figure 2). In the case that numerous nerve crushes would cause a preconditioning effect, we verified that the muscle was still denervated at 7 days post-crush after 5 multiple crushes..

### 3.2 Axonal regeneration occurs even after multiple crushes

To further validate the multiple crush method, the axons were also assessed for its regenerative capacity. Cross-sections of the tibial nerve, 20 mm distal to the nerve crush site, harvested 60 days after the last crush injury, shows neurofilament-containing axons that have regenerated along the nerve after 3, 4, or 5 crushes (Figure 3).

### 3.3 There is a deficit in functional motor recovery after 38 days of denervation

To determine whether functional motor recovery is limited to a critical time period after nerve injury, multiple nerve crushes resulting in denervation for 24, 31, or 38 days were performed. Motor recovery was assessed by toe spreading to see whether motor recovery occurs only within a certain time period. At 54 days after the last crush, 24-day and 31-day prolonged denervated animals showed good motor recovery (mean = 1.43 +/- 0.20, 1.86 +/- 0.38; n = 7, n = 7), whereas the 38-day prolonged denervated animals showed minimal recovery (mean = 0.33 +/- 0.33; n = 6) (Figure 4). In contrast to the 38-day group, the 22-day denervated animals, which

also received 5 nerve crushes but within a shorter time period, showed full motor recovery (mean = 2.0 +/- 0, n = 7).

### **3.4 There is a deficit in compound action potential after 24-38 days of denervation**

Compound muscle action potential (CMAP), which represents an integration of the number of functional motor units and the size of each motor unit, was measured to quantify the amount of recovery in the plantar muscle. At 60 days after the last crush, the recovery of CMAP in the plantar muscle was low across all three groups when compared to the CMAP of the contralateral muscle for each animal (mean = 41.6% +/- 17.7%, 37.9% +/- 17.8%, 28.3 +/- 12.9%, n = 5, 4, 4 for the 24-day, 31-day, and 38-day groups respectively) (Figure 5).

### **3.5 Regenerating axons reach the motor end plate after 24-38 days of denervation**

To determine whether motor end plate reinnervation occurs after a period of denervation, plantar muscles were stained with anti-neurofilament antibody (NF) to label axon fibers and  $\alpha$ -bungarotoxin (BTX) to label motor end plates 60 days after the final crush. Maximum intensity projection images obtained from confocal microscopy showed that in each of the 24, 31, and 38-day denervation groups, axons have regenerated to the motor end plates (Figure 6).

Manual scoring for NMJ reinnervation showed reduction in the percentage reinnervation in the 38-day group compared to all other groups (82% +/- 3.2% in the 38-day group vs. 97.2% +/- 2.8%, 96.3% +/- 2.4%, 92.5% +/- 3.2% in the uninjured group, 24-day group, and 31-day group, respectively. n = 4 animals per group) (Figure 7).

### **3.6 Neuromuscular junctions have altered appearance after 24-38 days of denervation and subsequent reinnervation**

Although the majority of end plates were reinnervated in all groups, NMJs had an abnormal appearance when reinnervation occurred after 24-38 days of denervation, compared to NMJs in uninjured muscle or to NMJs that were reinnervated after 22 days of denervation. Confocal images show fragmentation of the end plates based on bungarotoxin staining and

abnormal trajectory of reinnervating axons (Figure 8). Based on unblinded observation, these abnormalities appear more common and more severe with longer periods of denervation.

### **3.7 Axon terminals that have regenerated after 24-38 days of denervation contain pre-synaptic components**

To determine whether the axons observed in the re-innervated endplates could represent potentially functional synapses, plantar muscles were stained for synaptophysin, a component of the pre-synaptic mechanism. Synaptophysin was present in NMJs in the plantar muscle in the 38-day denervation group (Figure 9).



## 4 Discussion

### 4.1 Conclusions

*Multiple nerve crushes provides a new method for inducing axotomy and denervation for variable periods of time.* At the same time, the repetitive injury does not prevent axons from regenerating along the nerve. The advantages of this method are that the period of denervation can be controlled precisely by varying the number and frequency of crushes, it is technically less challenging than other models of prolonged denervation, such as transection and resuture, and the nerve sheaths are preserved in this type of injury.

*There is a critical period for the re-establishment of a neuromuscular junction such that if the regenerating axons reach the motor end plates after the critical period has ended, there is no functional motor recovery.* In the 24- and 31-day denervated animals, there was eventual motor recovery. In the 38-day denervated animals, there was poor recovery. Therefore, I concluded that the critical period is between 24 and 38 days. The higher number of nerve crushes is not the cause of the motor recovery, since animals that received 5 crushes within a shorter denervation period had good motor recovery.

*Axonal regeneration into the muscle is not sufficient for motor recovery after nerve injury.* Axons regenerate into the target muscle and reach the motor end plates after 24, 31, and 38 days of denervation, even in animals with poor motor recovery. Although there was a lower rate of end plate reinnervation in the 38-day denervation group, the 80% decrease in innervation rate is not likely to account for the drastic deficit in motor function.

*There appears to be degeneration of the end plates and abnormal axon trajectory within the end plate when there is reinnervation after 24-38 days of denervation.* Compared to uninjured muscle and the 22-day denervation group, muscles in the 24-, 31-, and 38-day denervation groups show abnormal NMJ morphology with fragmentation of the end plate and

loss of a normal contour with bright edges. Some axons that reached the end plate did not appear to follow the contours of the bungarotoxin staining. Based on unblinded observation, there appeared to be more abnormal morphology in the 38-day group compared to the 24- and 31-day denervated groups. Therefore, it is possible that end plate degeneration occurs more quickly than at the months-long timescale previously thought (Gutmann, 1944).

Based on the above observations, it appeared possible that end plate degeneration could be the cause of poor motor recovery. Chao et al. (2013) studied the role of acetylcholine receptor declustering in functional recovery after prolonged nerve injury. Agrin is a molecule that is known to trigger clustering of acetylcholine receptors during development (McMahon, 1992), and agrin levels after development is controlled by the expression of matrix metalloproteinase 3 (MMP3) secreted by perisynaptic Schwann cells. Using MMP3 knockout mice, which lack the ability to break down agrin, they created prolonged denervation models using the cross-anastomosis similar to that reported by Fu and Gordon (1995b). They found that preservation of agrin levels was associated with improved stability of the motor end plates after prolonged nerve injury and improved functional recovery after nerve repair. Thus they concluded that prevention of motor end plate degradation following nerve injury could improve functional recovery.

However, we have seen in the transection and resuture experiments (Ma et al., 2011) and have confirmed in later experiments that motor recovery sometimes does not occur even when there is a normal-appearing end plate that has been reinnervated. The difference in the appearance of NMJs in the transection and resture and multiple crush models has not yet been resolved, and therefore, I have been yet been able to find a consistent histological correlate of poor motor recovery.

## 4.2 Limitations

It is possible that the multiple crush method introduces new variables other than the prolonged period of denervation. As the muscle remains denervated for the entire duration between the first and last nerve crush, we assumed that the number of nerve crushes would have no effect on the muscle's ability to accept reinnervation and reestablish neuromuscular junctions. It is possible that the repeated axotomies have an effect on the axonal regenerative capabilities of the axons, but we have shown that axons can regenerate into the muscle after as many as 5 nerve crushes.

Muscles harvested 24 days after the last crush in the 38-day group (not shown) show that axons have regenerated to the intramuscular nerve sheaths by this time, but they have not yet reached the motor end plates. Since after a single crush, mice begin to show motor recovery in 10-14 days, this represents a delay in reinnervation. This could represent a decreased rate of axonal regeneration along the nerve as a result of the multiple crushes, or it could simply represent a decreased rate of regeneration along the intramuscular nerve sheaths, perhaps one of the consequences prolonged denervation. To eliminate the former cause as a possibility—a potential weakness of the model—we would need to obtain axon counts along the nerve at various time points to determine the rate of axonal regeneration along the nerve.

The time course of functional motor recovery was not as expected, where there would be full recovery in some groups and no recovery in other groups, as well as a clear time point beyond which no motor recovery occurred. Instead, there was an intermediate degree of recovery in the group that was exposed to an intermediate period of denervation. It could be that this group was exposed to a period of denervation that was approximately equal to the critical period, so that some animals or some neuromuscular junctions within a given animal were reinnervated, but it could also show that the critical period for motor recovery is not an all-or-none phenomenon but a gradual decline in the ability to reform functional NMJs.

Toe spreading score may not be the most sensitive behavioral test to identify motor deficits. By toe spreading score, some animals had good motor recovery while others did not; on nerve conduction studies, 24-, 31-, and 38-day denervation groups all had reduced CMAP with no statistical significance between the groups. Use of a second behavioral test, such as the sciatic functional index (SFI), which measures a continuous variable based on footprint image analysis rather relying on manual scoring, may identify more subtle defects.

### **4.3 Future Work**

To further explore the histologic abnormalities at the motor end plate, blinded scoring of NMJs will be necessary to determine whether there is truly degradation of end plates and abnormal morphology after the periods of denervation studied. Electrophysiological studies made by recording muscle fiber depolarization following the application of acetylcholine will also be necessary to determine whether the motor end plates are functional despite their abnormal appearance.

After further validating the multiple crush method through the experiments identified in the previous section, the method will need to be tested on other muscles and in other animals. This would allow us to see whether the critical period is a universal phenomenon underlying poor motor recovery after prolonged denervation.

The next major research direction would be to determine the molecular mechanism underlying the critical period and to understand why functional neuromuscular junction reestablishment is not possible following this critical period. To work towards this goal, performing microarrays of muscle fibers harvested before and after the critical period would show changes in the expression profile associated with the end of the critical period. Understanding the molecular mechanism of the critical period would have great clinical

implications, as pharmacologic interventions to slow down the critical period would improve the outcome of peripheral nerve repairs.

## Summary

Multiple nerve crushes provides a new method to induce variable periods of prolonged denervation. These experiments demonstrate that there is a critical period for the formation of the functional neuromuscular junction after peripheral nerve injury. It appears that after a denervation period greater than the critical period, there is no functional motor recovery. Based on our experiments, denervation-associated changes may occur more quickly than previously thought. Further studies will be necessary to explore the histological abnormality at the motor end plate after denervation and to determine the molecular cause of the functional motor defect. Understanding the mechanism of the critical period will be the next step to finding pharmacological therapies to extend the critical period; this therapy has the potential to improve the outcome of peripheral nerve repairs. The existence of a critical period also indicates that therapies to accelerate axonal regeneration will also improve the ultimate clinical outcome after peripheral nerve injuries.

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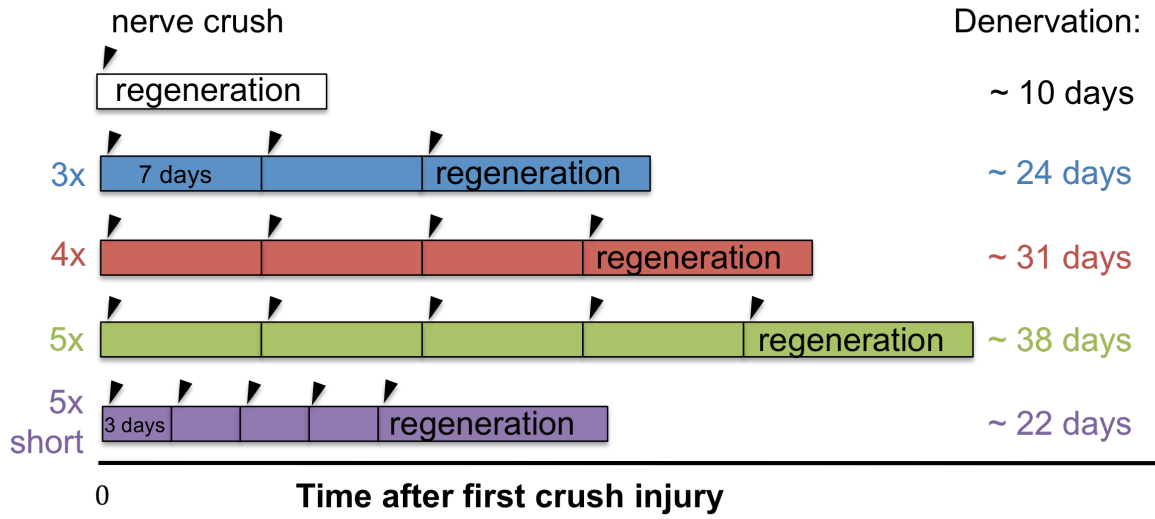
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## Tables and Figures

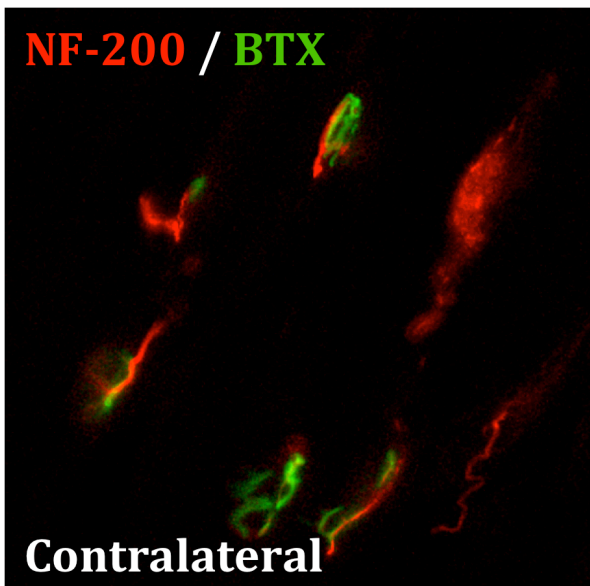
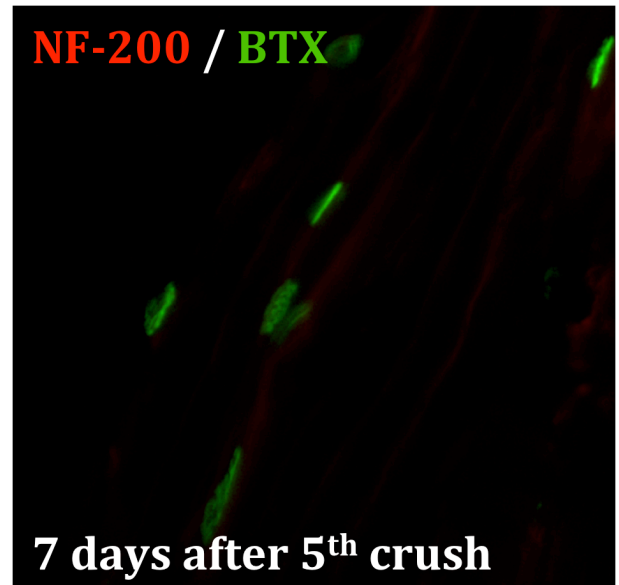
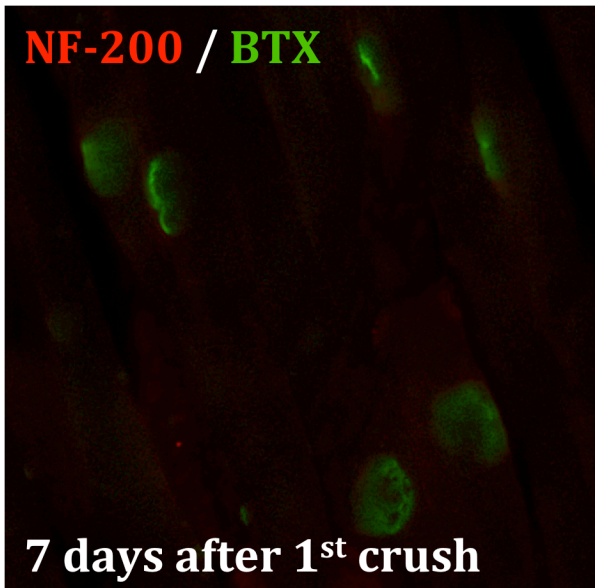
### Figure 1: Multiple crush method for prolonged denervation

Timing of repeated crushes of the sciatic nerve to control the period of denervation.



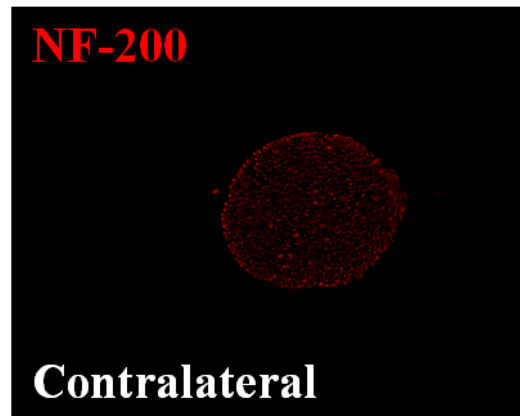
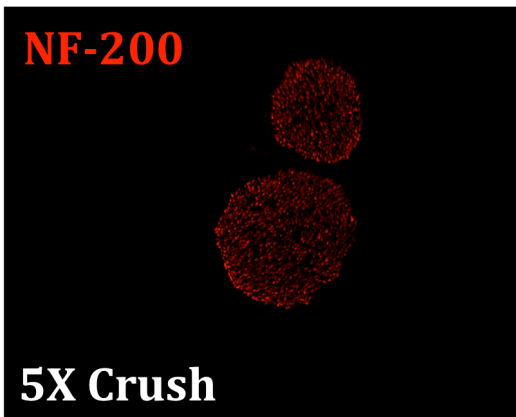
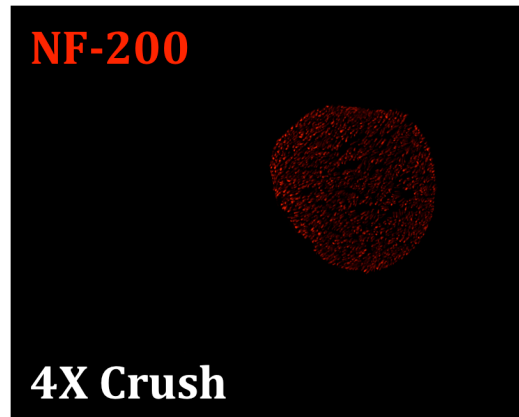
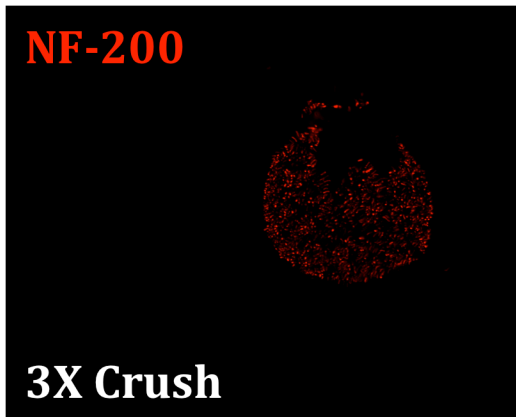
**Figure 2: Plantar muscle denervation 7 days after nerve crush**

Plantar muscle sections from the hindpaw harvested 7 days after 1 sciatic nerve crush and after 5 sciatic nerve crushes 7 days apart. Labeled with NF-200 (red) and  $\alpha$ -bungarotoxin (green). At 7 days after the 1<sup>st</sup> and 5<sup>th</sup> nerve crush, the muscle remains denervated.



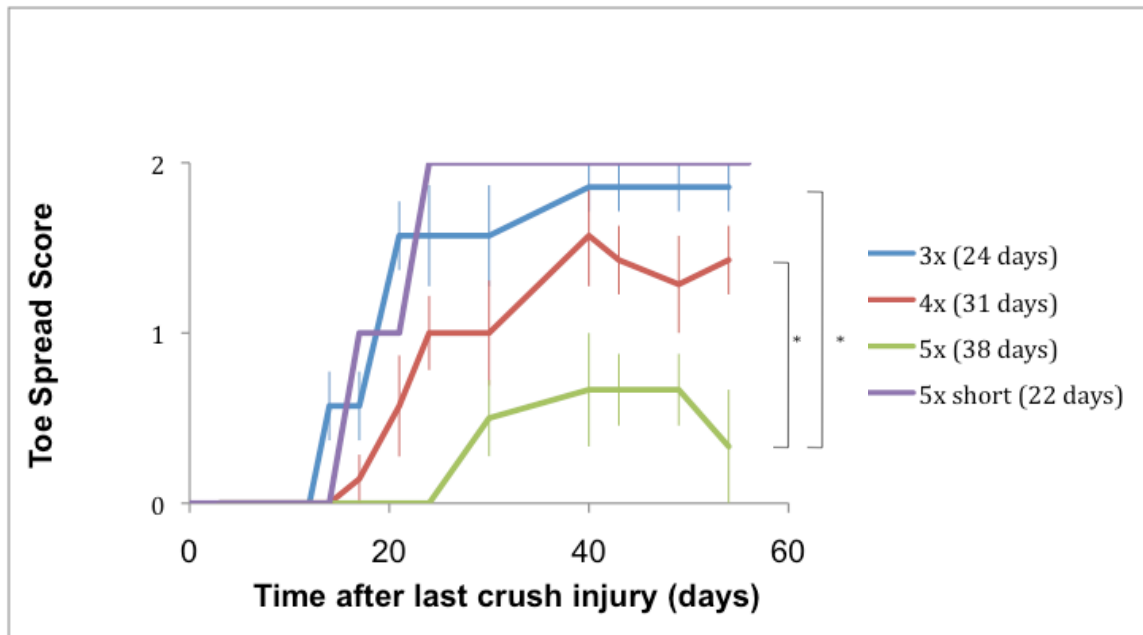
**Figure 3: Axonal regeneration after multiple crushes**

Cross-sections of distal tibial nerve 20 mm distal to the sciatic nerve crush site harvested 60 days after the last injury. Labeled with NF-200 (red). Axonal regeneration occurs after 3, 4, or 5 sciatic nerve crushes 7 days apart.



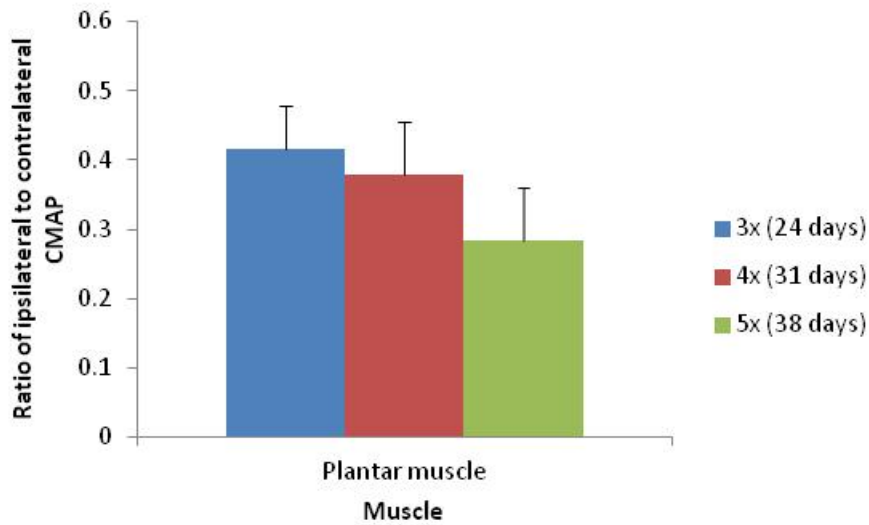
#### Figure 4: Motor recovery after 24-38 days of denervation

Toe spreading score after 3, 4, and 5 sciatic nerve crushes 7 days apart measured as a function of time after the last nerve crush. Score of 2 = full, sustained toe spreading, 1 = partial or transient toe spreading, 0 = absent toe spreading. (n = 7 animals per group). The 24- and 31- day denervated groups showed good motor recovery, but the 38-day denervated group had poor motor function.



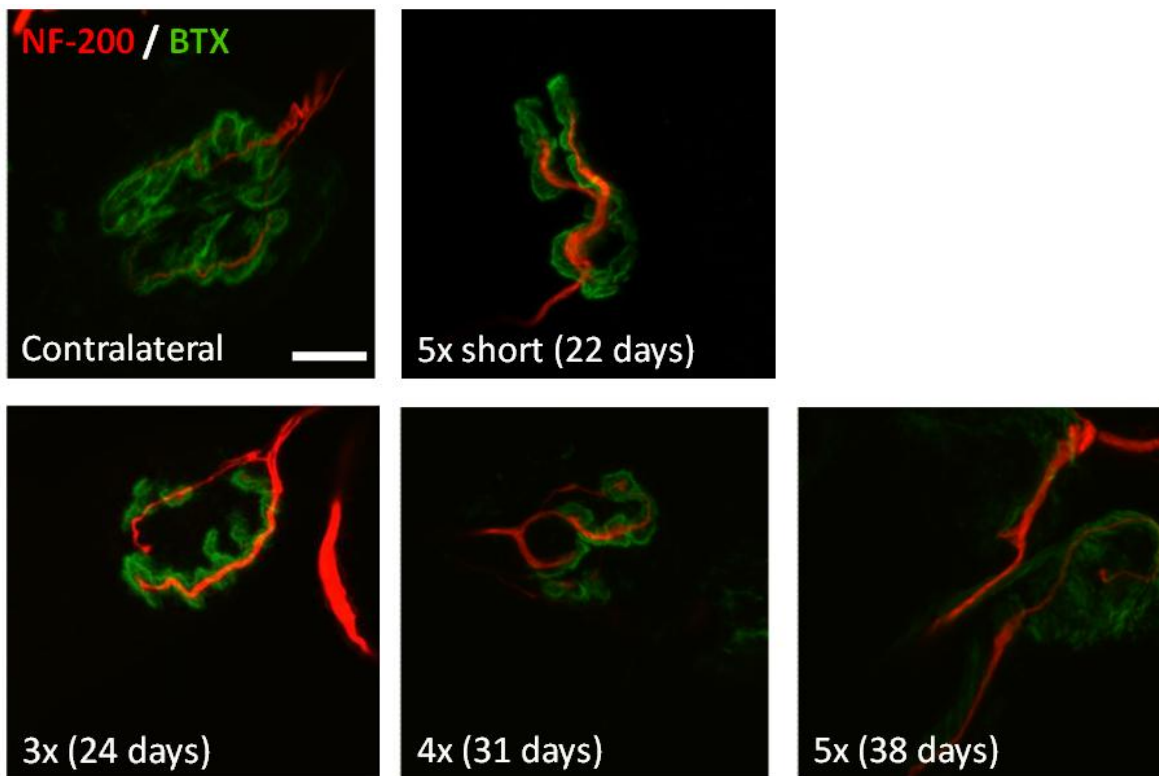
### Figure 5: Compound muscle action potential after 24- 38 days of denervation

Recovery of compound muscle action potential at the plantar muscle at 60 days after the last crush. Results are presented as the ratio of ipsilateral to contralateral CMAP. (n = 4 animals per group). CMAP recovery was low in all three groups.



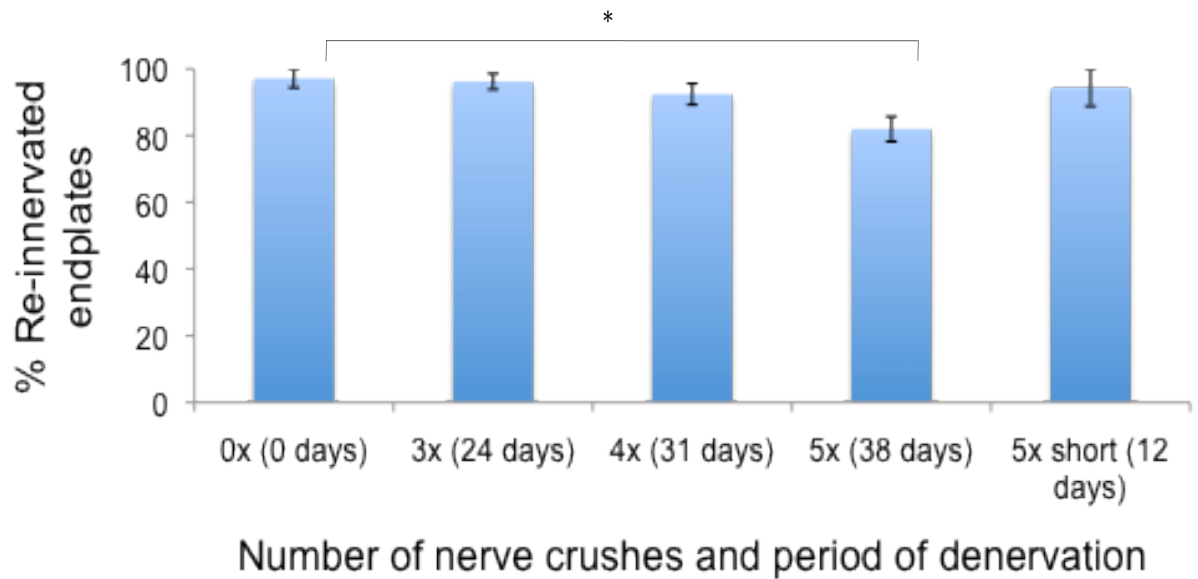
**Figure 6: Axonal regeneration to motor end plates after 24-38 days of denervation**

Maximum intensity projection images from confocal microscopy of neuromuscular junctions in plantar muscles harvested 60 days after the last injury. Labeled with NF-200 (red) and  $\alpha$ -bungarotoxin (green). Scale bar = 0.02  $\mu$ m. Axons are present in or near the endplates in all groups.



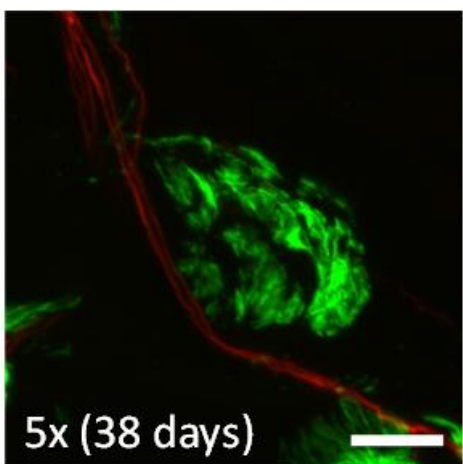
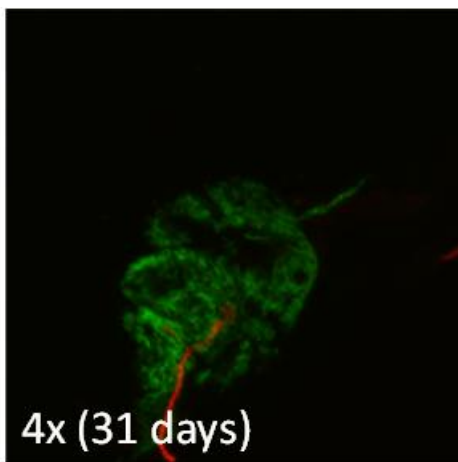
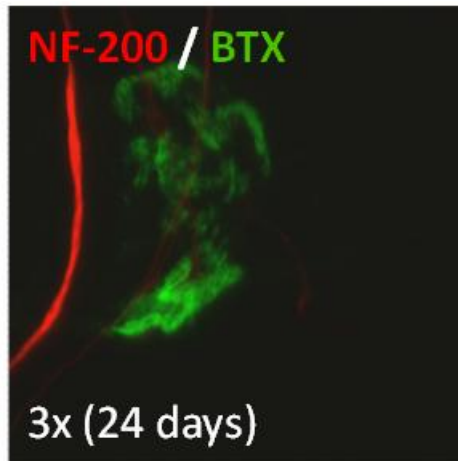
### Figure 7: Motor end plate reinnervation after 24-38 days of denervation

Percentage motor end plate reinnervation at 60 days after 3-5 crushes based on manual scoring of neuromuscular junction maximum intensity projection images from confocal microscopy. (n = 4 animals per group). There was a reduction in the percentage reinnervation in the 38-day denervated group.



**Figure 8: Neuromuscular junction degeneration after 24-38 days of denervation**

Maximum intensity projection images from confocal microscopy of neuromuscular junctions in plantar muscles harvested 60 days after the last injury. Labeled with NF-200 (red) and  $\alpha$ -bungarotoxin (green). Scale bar = 0.02  $\mu$ m. Fragmentation of end plates was observed in some images in each of the groups.





**Figure 9: Synaptophysin in the NMJ after 38 days of denervation and reinnervation**

Maximum intensity projection images from confocal microscopy of neuromuscular junctions in plantar muscles harvested 60 days after 5 nerve crushes. Labeled with synaptophysin (red) and  $\alpha$ -bungarotoxin (green). Scale bar = 0.02  $\mu$ m. Synaptophysin is present at the axon terminals after 38 days of denervation and subsequent reinnervation.

