Transplantation of Embryonic Motoneurons into Peripheral Nerve Combined with Functional Electrical Stimulation Restores Functional Muscle Activity in the Rat Sciatic Nerve Transection Model

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Short title: Motoneuron Integrated Striated Muscle with Functional Electrical Stimulation

Abstract

Reinnervation of denervated muscle by motoneurons transplanted into the peripheral nerve may provide the potential to excite muscles artificially with functional electrical stimulation (FES). Here we investigated if transplantation of embryonic motoneurons into peripheral nerve combined with FES restored functional muscle activity in adult Fischer 344 rats after transection of the sciatic nerve. One week after sciatic nerve transection, cell culture medium containing (cell transplantation group, n=6) or lacking (surgical control group, n=6) dissociated embryonic spinal neurons was injected into the distal stump of the tibial and peroneal nerves. Electrophysiological and tissue analyses were performed in the cell transplantation and surgical control groups twelve weeks after transplantation, as well as a in naïve control group (n=6) that received no surgery. In the cell transplantation group, ankle angle was measured during gait with and without FES of the peroneal nerve. Ankle angle at mid-swing was more flexed during gait with FES (26.6 ± 8.7°) than gait without FES (51.4 ± 12.8°, p=0.011), indicating that transplantated motoneurons in conjunction with FES restored ankle flexion in gait, even though no neural connection between central nervous system and muscle was present. These results indicate that transplantation of embryonic motoneurons into peripheral nerve combined with

FES can provide a novel treatment strategy for paralyzed muscles.

Keywords

Peripheral nerve, Motoneuron, Cell transplantation, Denervated muscle, Functional electrical stimulation, Regeneration, Muscle reinnervation, Gait analysis

1. Introduction

Cell transplantation therapies are a major focus of pre-clinical and clinical research as a strategy for the treatment of intractable diseases, and there has been a rapid surge in the number of clinical trials involving stem cell therapies over the last three to four years (Trounson et al., 2011). These trials are establishing the clinical pathways for regenerative medicine, especially in nervous system.

One experimental approach to rescue denervated muscles from lower motor neuron damage or axonal disconnection is transplantation of motoneurons into a peripheral nerve to provide a source of neurons for muscle reinnervation (Erb et al., 1993; Yohn et al., 2008). Since Erb et al. first reported the reinnervation of denervated muscle by embryonic motoneurons transplanted into a peripheral nerve, several studies have investigated the factors that improve motoneuron survival in peripheral nerves (Grumbles et al., 2005; Grumbles et al., 2009; Grumbles et al., 2002). Considering the simplicity of the neural network and the wide window of opportunity for treatment (Merrell et al., 2001), the peripheral nerve system provides an ideal target for neuronal replacement therapy.

Functional electrical stimulation (FES) has been used to treat chronic hemiplegia since the 1960s (Liberson et al., 1961), and can lead to paraplegic patients being able to stand, pedal bicycles, and walk short distances with support (Agarwal et al., 2003). FES has great restorative potential if the paralysis is of upper motor neuron origin and the paralyzed muscles have an intact lower motor neuron pathway from the spinal cord to the muscle endplate. However, if the paralysis is of lower motor neuron origin, irreversible muscle degeneration occurs, and application of electrical current fails to cause contraction of muscle after long-term denervation (Kern et al., 2004).

Transplantation of motoneurons into a peripheral nerve has been shown to provide a source of neurons for muscle reinnervation (Yohn et al., 2008). This transplantation strategy, which we term motoneuron integrated striated muscle (MISM), may provide the potential to artificially excite long-term denervated muscles when combined with FES, even though there is no neural connection between central nervous system and the muscle. The aim of this study was to examine whether MISM combined with FES restores functional muscle activity in the rat sciatic nerve transection model.

2. Materials and Methods

All experimental protocols and animal maintenance procedures used in this study were approved by the Animal Ethics Research Committee at Nagoya University. Adult (8-week-old) Fischer 344 rats (Japan SLC, Inc., Shizuoka, Japan) were assigned to one of three groups: Cell transplantation (n=6), surgical control (n=6) or naïve control (n=6). In the cell transplantation group, the sciatic nerves were transected and, 1 week later, medium containing dissociated embryonic spinal neurons was injected into the distal stump of the tibial and peroneal nerves. In the surgical control group, the sciatic nerves were transected and, 1 week later, medium alone was injected into the distal stump of the tibial and peroneal nerves. The naïve control group underwent physiological assessment and tissue analysis but did not receive any surgery (Fig. 1)

2.1. Cell preparation

Ventral spinal cord cells were obtained from Fischer 344 rat embryos (Japan SLC, Inc., Shizuoka, Japan). Fischer rats on day 14 of pregnancy were anesthetized with isoflurane (2% delivered by calibrated vaporizer through facial mask), and their ventral spinal cords were resected from the fetuses using a

surgical microscope and cut into small pieces in ice-cold Hanks' balanced salt solution. Ventral spinal neurons were dissociated using papain-containing separation solution (MB-X9901; Sumitomo Bakelite Co. Ltd, Tokyo, Japan) and were suspended in Neurobasal medium (Gibco) containing B27 supplement (Gibco), Glutamax (Gibco), and N-2 supplement (Gibco).

2.2. Surgical procedures and transplantation

All surgical procedures were performed under 2% isoflurane anesthesia. The sciatic nerves were completely transected at midthigh. The nerves were ligated on both ends and the proximal nerve stump was sutured into hip muscles to prevent regeneration. One week later, approximately 1 x 10^6 neurons contained within 10 µl of medium were injected into the distal stumps of the tibial and peroneal nerves of rats in the cell transplantation group using a Hamilton syringe with a 30G needle. The needle was inserted to as closer to the center of each nerve as possible. To confirm that the medium containing neurons was injected intraneurally, the nerve was examined under dissecting microscope during injection to visually confirm swelling of the nerve. The injection site was 20 mm proximal to the entry into the lateral gastrocnemius and anterior tibialis

muscles. Rats in the surgical control group underwent the identical surgeries but had only medium lacking neurons injected into the peripheral nerves.

2.3. Electrophysiological analysis

Electrophysiological analyses were performed in the cell transplantation group, the surgical control group and the naïve control group at 12 weeks after intraneural injection of neuron or plain medium. The compound muscle action potential (CMAP) of the lateral gastrocnemius muscle was measured at room temperature (24 ºC) under isoflurane anesthesia using a standard nerve evoked potential recording system (Neuropack MEB-5504; Nihon Kohden, Tokyo, Japan). Two stainless steel monopolar recording electrodes (H537A; Nihon Koden, Tokyo, Japan) were placed at the center of the belly of the lateral gastrocnemius muscle. The tibial nerve was carefully exposed and a bipolar stimulating electrode (UM2-5050; Nihon Koden) was placed around the nerve at 20 mm proximal to the entry into the lateral gastrocnemius. Electrical pulses (supramaximal intensity; 100 ms duration; 1 Hz frequency; square wave) were applied with an isolator (SS-201J; Nihon Koden) connected to the electronic stimulator.

2.4. Gait analysis

After the electrophysiological analysis, stainless steel wire electrodes (Bioflex wire AS633; Cooner wire, Chatsworth, CA, USA) were placed on the peroneal and tibial nerves and covered with silicone gel for insulation and immobilization. The other ends of wires were passed through the dorsal neck skin and connected to an electric generator (Neuropack MEB-5504; Nihon Kohden, Tokyo, Japan). After confirmation of plantar flexion and dorsiflexion of the ankle by stimulating both the peroneal and tibial nerves alternately, the rats emerged from anesthesia. The rats were placed on one end of a walking track that comprised an acrylic resin chamber, and were allowed to spontaneously walk toward the other end. For each rat in the cell transplantation group, gait trials were repeated until five satisfactory walks without pause were observed for each condition (stimulation, no stimulation). During walks with electrical stimulation, the peroneal nerve was stimulated at 60 Hz with 1 mA current. In naïve control group, walking was also repeated until five satisfactory walks were obtained. One HDR-CX12 video camera (Sony, Inc., Tokyo, Japan) was positioned in the front to give a direct lateral view and used to record the motion

of the hindlimb of walking rats at a 60 Hz frame rate. Ankle angle was calculated as the angle subtending a line connecting the knee and ankle joints, and a second line connecting the ankle joint and the metatarsal head (Yu et al., 2001). With this measurements, lower values reflected greater flexion. Ankle angle at the mid-swing point of the gait cycle was calculated for stimulated and non-stimulated gait in cell transplantation group and non-stimulated gait in the naïve control group by taking the average of all strides in each of the five trials.

2.5. Tissue analysis

Tissue analyses were performed in the cell transplantation group, the surgical control group and the naïve control group. For fixation, the rats were perfused through the left ventricle with 100 ml of 0.9% saline, followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tibial nerves and gastrocnemius and anterior tibialis muscles were removed for immunohistochemical and histochemical analysis. An approximately 20-mm segment of the tibial nerve was harvested and divided into proximal and distal halves. The distal half of the harvested nerve was used to measure the number and the diameter of myelinated axons. The distal half of the tibial nerve was fixed

in 4% glutaraldehyde in phosphate buffer. The extracted nerve was embedded in Epon, 1-μm thick sections were stained with Toluidine blue (Sigma-Aldrich, Germany) for light microscopic examination, where the number and the diameter of myelinated axons were measured. The gastrocnemius muscle was dissected free from the origin and insertion and immediately weighed. Then it was embedded in paraffin, sectioned into 10-μm cross sections and stained with hematoxylin and eosin for light microscopic examination, where the mean muscle fiber cross-sectional area at the middle of the muscle belly was calculated. The five arbitrary points were automatically selected for calculation of the muscle fiber cross-sectional area using an all-in-one type fluorescence microscope BZ-8000 (Keyence, Osaka, Japan).

The proximal half of the harvested tibial nerve was used to confirm neuron survival. The tibial nerve was cryoprotected in sucrose, and then frozen in dry-ice-cooled isopentane. Tibial nerve sections (10 μm thick) were stained with the antibodies against SMI-32 (1:500, Covance, Emeryville, CA) and choline acetyltransferase (ChAT, 1:50; Millipore, Billerica, MA, USA) in order to assess the survival of motoneurons. SMI-32 was used to detect a nonphosphorylated epitope on the heavy and medium chain neurofilament

proteins expressed in soma of motoneurons (Breckenridge et al., 1997; Sternberger and Sternberger, 1983).

Anterior tibialis muscle sections (50 μm thick) were stained with antibodies against neurofilament H (1:500; Millipore, Billerica, MA) and α-bungarotoxin (1:200, Molecular Probes, Eugene, OR) in order to assess terminal muscle innervation and endplate morphology. To quantify disassembly of the motor endplate after long-term denervation, we used a previously described classification to characterize endplate morphology (Marques et al., 2000). Endplates were categorized as pretzel (mature with a weblike pattern including multiple perforations), plaque (immature and smaller size lacking perforations), and intermediate (morphology between that of plaque and pretzel) (Kummer et al., 2004; Shi et al., 2010). All sections were stained using a full automatic immunohistochemical staining system: Ventana HX system (Ventana, Yokohama, Japan) according to the manufacturer's instruction.

2.6. Statistics

ANOVA with Tukey or Games-Howell post hoc comparisons were used to compare outcome measures across groups, and paired t-test was used to

compare ankle angle between gait with and without electrical stimulation of the peroneal nerve in the cell transplantation group. All statistical analyses were conducted using the Statistical Package for Social Science version 19.0 software (SPSS Inc, Chicago, IL, USA). The significance level was set at 0.05. All data are reported as mean ± standard deviation.

3. Results

3.1. Electrophysiological analysis

CMAPs were not evoked in any rats from the surgical control group. CMAPs were evoked in rats from the cell transplantation group, even though no axonal connections to the central nervous system had been present for the preceding 12 weeks. CMAP latency was longer (1.77 ± 0.31 vs. 1.24 ± 0.14 ms, p=0.004), and amplitude was smaller (1.23 ± 0.69 vs. 13.21 ± 8.37 mV, p=0.017) in the cell transplantation group than in the naïve control group (Fig. 2A–B).

3.2. Gait analysis

None of the rats from the surgical control group were able to flex their ankle even with electrical stimulation. In contrast, in all rats injected

motoneurons, MISM restored plantar flexion and dorsiflexion of the ankle with alternate stimulation of the tibial and peroneal nerves (Fig. 3A–B). Gait analyses were performed in the cell transplantation and naïve control groups. In the cell transplantation group, the ankle at the mid-swing phase of gait was significantly more flexed during gait with electrical stimulation of the peroneal nerve (26.6 ± 8.7°) than during gait without stimulation (51.4 ± 12.8°, p=0.011; Figs. 3 and 4). Dorsiflexion angle during gait with electrical stimulation in the cell transplantation group was similar to that in the naïve control group (27.6 ± 3.4°, p=0.79). These results demonstrated that MISM with electrical stimulation restored functional muscle activity in the rat sciatic nerve transection model.

3.3. Tissue analysis

There were no myelinated axons in the tibial nerves of the surgical control group. The cell transplantation group showed a tendency to have fewer myelinated axons in the tibial nerves (1198 ± 1218) than the naïve control group (2510 ± 802, p=0.079). The cross-sectional area of the myelinated axons in the tibial nerve was smaller in the cell transplantation group (10.7 ± 2.8 μm^2) than in the naïve control group (16.2 ± 1.3 μm^2, p=0.008; Fig. 2C–D, 5).

The weight of the gastrocnemius muscle was greatest in the naïve control group (0.561 ± 0.020% BW, $p<0.001$), and greater in the cell transplantation group (0.158 ± 0.003% BW) than in the surgical control group (0.123 ± 0.008% BW, $p<0.001$; Fig 2E). Muscle fiber cross-sectional area was larger in the naïve control group (2873 ± 233 μm^2) than in the cell transplantation group (326 ± 91 μm^2, $p<0.001$) and surgical control group (176 ± 105 μm^2, $p<0.001$), but was similar between the cell transplantation and surgical control groups (326 ± 91 vs. 176 ± 105, $p=0.28$; Fig. 2F).

3.4. Motoneuron survival and neuromuscular junction formation

Transplanted motoneurons and those axons positive for SMI-32 and ChAT via immunostaining were observed in the tibial nerve of all rats in the cell transplantation group (Fig. 6A-B). Neuromuscular junction formations were also present in the anterior tibialis muscle of all rats that received cell transplants. Figure 6C shows endplates in a reinnervated anterior tibialis muscle at high magnification. Regenerating axons stained with antibodies against neurofilament reached into fields of motor end plates, indicated by α-bungarotoxin labeling of acetylcholine receptor clusters. In contrast, none of

neurons and axons were detected in the surgical control group.

The endplate types differed for normally innervated versus denervated muscles. The pretzel phenotype was predominant in the naïve control group (76.6 ± 9.19% pretzel vs 14.7 ± 7.65% intermediate vs 8.6 ± 1.53% plaque). In contrast, the plaque phenotype was predominant in the surgical control group (1.6 ± 1.93% pretzel vs 15.9 ± 2.96% intermediate vs 82.5 ± 4.36% plaque). The proportion of the dispersed endplates was reduced in the transplantation group (11.1 ± 2.93% pretzel vs 30.3 ± 4.57% intermediate vs 58.7 ± 4.25% plaque; p=0.01).

4. Discussion

The aim of this study was to examine whether MISM restores functional muscle activity in the rat sciatic nerve transection model. Our results demonstrated MISM reduced degeneration of neuromuscular junctions. Moreover, when combined with FES, MISM restored ankle motion during the gait cycle, even though there was no neural connection between the central nervous system and the muscle. MISM therefore appears to provide the potential to

artificially excite long-term denervated muscles via FES.

Although all rats in the cell transplantation group were able to flex their ankle with electrical stimulation and a large enough number of neurons was transplanted into the tibial nerve in the cell transplantation group, the cell transplantation group had less than one-half of the number of axons than the naive group. This is possibly because the endoneurial environment of a peripheral nerve may not provide suitable conditions for the survival of motoneurons. Grumbles et al. reported that exogenous addition of a mixture of neurotrophic factors into the endoneurial environment improved motoneuron survival and function of muscle in peripheral nerve (Grumbles et al., 2009). Transplanting motoneurons with glial cells, such as astrocytes, may promote their survival. Further studies are needed to clarify the role of central nervous system derived cells in improving the survival rate of transplanted neurons in peripheral nerves.

Muscle fiber cross-sectional area was similar in the cell transplantation and surgical control groups, but less than that in the naïve control group, indicating that MISM did not prevent muscle atrophy. Buffelli et al. reported that innervated but noncontracting (by means of a nerve conduction block) muscles

undergo a decline in mass, force, and fiber size indistinguishable from declines seen in denervated muscles (Buffelli et al., 1997). Before the connection between motoneurons and muscle was established, other treatment strategies, such as electrical stimulation and exercise, were hypothesized to prevent muscle atrophy, as evidenced by previous studies that reported improvement in muscle strength and muscle fiber cross-sectional area after therapeutic electrical stimulation of denervated muscles (Dow et al., 2004; Minzly et al., 1993).

Functional recovery is incomplete if the reinnervation of skeletal muscle is delayed. This is due to several factors including a progressive decline in the ability of neurons to sustain axon growth, chronic Schwann cell atrophy with subsequent failure to provide a supportive growth environment, and irreversible muscle degeneration (Bush et al., 1996; Chen et al., 2007; Gordon et al., 2011). However, the therapeutic time window for neuronal replacement in the peripheral nervous system is hypothesized to be much wider than that in the central nervous system (Okada et al., 2005; Okano, 2002). In clinical settings, although earlier reconstruction results in better outcomes, reconstruction of denervated muscle with peripheral nerve transfer still results in some functional recovery if done within nine months of denervation. (Millesi, 1977; Nagano et al., 1989).

Target muscles can be reinnervated in short-term using our MISM technique, because motoneurons are placed very close to the neuromuscular junction. These facts suggest that denervated muscle can be treated with MISM even more than six months after nerve transection or after the onset of lower motor neuron disorders.

Another advantage of MISM is that it requires a small number of transplanting cells. There are several studies of motor unit number estimation (MUNE), which can provide a numeric estimate of the number of innervating motor axons (Bromberg, 2007; McComas, 1995). The average MUNE values in thenar, biceps brachii, extensor digitorum brevis and tibialis anterior muscles were 230, 109, 143 and 256, respectively (Galea, 1996). These studies demonstrate that the number of motor axons that innervate each muscle is usually in the range of several hundred to several thousand, therefore far less than ten thousand motoneurons are required for neural transplantation into a peripheral nerve. In a clinical context, the small number of cells required to be transplanted with MISM could have the benefit of reducing the risk of tumorigenicity.

Functional electrical stimulation has great restorative potential if the

paralysis is of upper motor neuron origin. In contrast, if the paralysis is of lower motor neuron origin, progressive myofiber loss and replacement by adipose and fbrous connective tissue occur in denervated muscles. Once this has occurred, application of electrical current fail to cause contraction of muscle. Prolonged muscle denervation resulting from motoneurons damage leads to atrophy and degeneration of neuromuscular junctions, causing acetylcholine receptors to redistribute throughout the muscle fiber (Frank et al., 1975; Hartzell et al., 1972; Steinbach et al., 1981). In our study, transplantation of motoneurons into a peripheral nerve resulted in prevention of degeneration of neuromuscular junctions. Our result suggests that the MISM can broaden the range of application of FES.

MISM provides the potential to artificially excite long-term denervated muscles via FES, even if the paralysis is of lower motor neuron origin. Recent advances in stem cell research have opened up possibilities for the clinical application of MISM. MISM with FES can provide a novel treatment strategy for paralyzed muscles.

Disclosure/conflict of interest

None

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References

Agarwal S, Kobetic R, Nandurkar S*, et al.* 2003; Functional electrical stimulation for walking in paraplegia: 17-year follow-up of 2 cases. J Spinal Cord Med **26**: 86-91.

Alper J. 2009; Geron gets green light for human trial of ES cell-derived product. Nat Biotechnol **27**: 213-214.

Blum B, Benvenisty N. 2008; The tumorigenicity of human embryonic stem cells. Adv Cancer Res **100**: 133-158.

Bonnel. 1984; Microscopic anatomy of the adult human brachial plexus: an anatomical and histological basis for microsurgery. Microsurgery **5**:107-118.

Breckenridge LJ, Sommer IU, Blackshaw SE. 1997; Developmentally regulated markers in the postnatal cervical spinal cord of the opossum Monodelphis domestica. Brain Res Dev Brain Res **103**: 47-57.

Bromberg MB. 2007; Updating motor unit number estimation (MUNE). Clin Neurophysiol **118**: 1-8.

Bush MS, Tonge DA, Woolf C, *et al.* 1996; Expression of a developmentally regulated, phosphorylated isoform of microtubule-associated protein 1B in regenerating axons of the sciatic nerve. Neuroscience **73**: 553-563.

Buffelli M, Pasino E, Cangiano A. 1997; Paralysis of rat skeletal muscle equallyaffects contractile properties as does permanent denervation. J Muscle Res Cell Motil **18**: 683-695.

Chen ZL, Yu WM, Strickland S. 2007; Peripheral regeneration. Annu Rev Neurosci **30**: 209-233.

Dow DE, Cederna PS, Hassett CA, *et al.* 2004; Number of contractions to maintain mass and force of a denervated rat muscle. Muscle Nerve **30:** 77-86.

Erb DE, Mora RJ, Bunge RP. 1993; Reinnervation of adult rat gastrocnemius muscle by embryonic motoneurons transplanted into the axotomized tibial nerve. Exp Neurol **124:** 372-376.

Frank E, Gautvik K, Sommerschild H. 1975. Cholinergic receptors at denervated mammalian motor end-plates. Acta Physiol Scand 95: 66-76.

Frantz S. 2012; Embryonic stem cell pioneer Geron exits field, cuts losses. Nat Biotechnol **30:** 12-13.

Galea. 1996; Changes in motor unit estimates with aging. J Clin Neurophysiol **13**: 253-260.

Gordon T, Tyreman N, Raji MA. 2011; The basis for diminished functional recovery after delayed peripheral nerve repair. J Neurosci **31**: 5325-5334.

Grumbles RM, Casella GT, Rudinsky MJ, *et al.* 2005; The immunophilin ligand FK506, but not the P38 kinase inhibitor SB203580, improves function of adult rat muscle reinnervated from transplants of embryonic neurons. Neuroscience **130**: 619-630.

Grumbles RM, Sesodia S, Wood PM, *et al.* 2009; Neurotrophic factors improve motoneuron survival and function of muscle reinnervated by embryonic neurons. J Neuropathol Exp Neurol **68**: 736-746.

Grumbles RM, Wood P, Rudinsky M, *et al.* 2002; Muscle reinnervation with delayed or immediate transplant of embryonic ventral spinal cord cells into adult rat peripheral nerve. Cell Transplant **11**:241-250.

Hartzell HC, Fambrough DM. 1972. Acetylcholine receptors. Distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. J Gen Physiol 60: 248-262.

Kern H, Boncompagni S, Rossini K, et al. 2004; Long-term denervation in humans causes degeneration of both contractile and excitation-contraction coupling apparatus that can be reversed by functional electrical stimulation (FES): a role for myofiber regeneration? J Neuropath Exp Neurol **63**: 919-931

Kummer TT, Misgeld T, Lichtman JW, *et al.* 2004. Nerve-independent formation of a topologically complex postsynaptic apparatus. J Cell Biol 164: 1077-1087.

Liberson WT, Holmquest HJ, Scot D, *et al.* 1961; Functional electrotherapy: stimulation of the peroneal nerve synchronized with the swing phase of the gait of hemiplegic patients. Arch Phys Med Rehabil **42**: 101-105.

Marques MJ, Conchello JA, Lichtman JW. 2000. From plaque to pretzel: fold formation and acetylcholine receptor loss at the developing neuromuscular junction. J Neurosci 20: 3663-3675.

McComas AJ. 1995. Motor unit estimation: anxieties and achievements. Muscle Nerve 18: 369-79.

Merrell GA, Barrie KA, Katz DL, *et al.* 2001; Results of nerve transfer techniques for restoration of shoulder and elbow function in the context of a meta-analysis of the English literature. J Hand Surg Am **26**: 303-314.

Millesi H. 1977; Surgical management of brachial plexus injuries. J Hand Surg Am **2**: 367-378.

Minzly J, Mizrahi J, Isakov E, *et al.* 1993; Computer-controlled portable stimulator for paraplegic patients. J Biomed Eng **15:** 333-338.

Nagano A, Tsuyama N, Ochiai N, *et al.* 1989; Direct nerve crossing with the intercostal nerve to treat avulsion injuries of the brachial plexus. J Hand Surg Am **14:** 980-985.

Okada S, Ishii K, Yamane J, Iwanami A, *et al.* 2005; In vivo imaging of engrafted neural

stem cells: its application in evaluating the optimal timing of transplantation for spinal cord injury. FASEB J **19**: 1839-1841.

Okano H. 2002; Stem cell biology of the central nervous system. J Neurosci Res **69:** 698-707.

Schwartz SD, Hubschman JP, Heilwell G, *et al.* 2012; Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet **379**:713-20.

Shi L, Butt B, Ip FC, *et al.* 2010 Ephexin1 is required for structural matu- ration and neurotransmission at the neuromuscular junction. Neuron 65: 204-216.

Steinbach JH. 1981. Neuromuscular junctions and alpha-bungarotoxin- binding sites in denervated and contralateral cat skeletal muscles. J Physiol 313:513-528.

Sternberger LA, Sternberger NH. 1983; Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc Natl Acad Sci U S A **80**, 6126-6130.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, *et al.* 1998. Embryonic stem cell lines derived from human blastocysts. Science **282**: 1145-1147.

Trounson A, Thakar RG, Lomax G, *et al.* 2011. Clinical trials for stem cell therapies. BMC Med **9**: 52.

Yohn DC, Miles GB, Rafuse VF, *et al.* 2008. Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. J Neurosci 28: 12409-12428.

Yu P, Matloub HS, Sanger JR, *et al.* 2001. Gait analysis in rats with peripheral nerve injury. Muscle Nerve **24**, 231-239.

Figure legends

Figure 1. Flow chart depicting experimental steps

Figure 2. Results from muscle anatomy and electrophysiological analyses.

The latency (A) and amplitude (B) of compound muscle action potentials (CMAP) recorded in the lateral gastrocnemius muscle, the number (C) and cross sectional area (D) of myelinated axons in the tibial nerve, and the weight (E) and muscle fiber cross-sectional area of the gastrocnemius muscle (F) of rats that received cell transplantation surgery (black bars), control surgery (grey bars) or no surgery (naïve group, white bars). Error bars represent standard deviation. Asterisks indicate $p<0.05$.

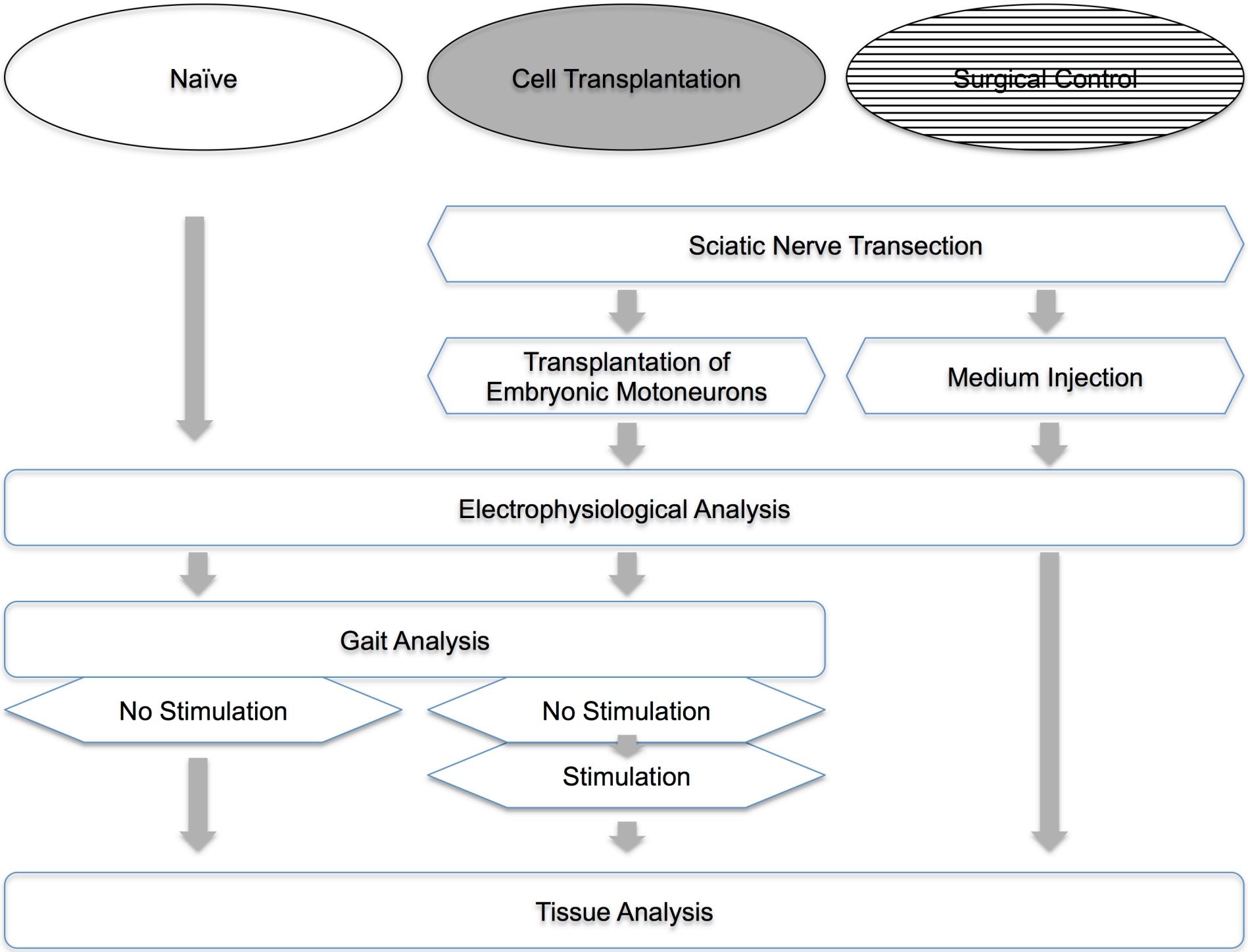
Figure 3. Photographs illustrating the quantification of ankle motion.

Reproduction of ankle motion was confirmed by alternate stimulation of the peroneal (A) and tibial nerves (B). Measurements of ankle angle during gait without (C) and with (D) functional electrical stimulation. The ankle angle was measured from the line connecting the knee and ankle joints, and the line connecting the ankle joint and the metatarsal head.

Figure 4. Ankle angle duration gait. Ankle angle at the mid-swing point of gait with and without electrical stimulation in the cell transplantation group and in naïve control group. Error bars represent standard deviation. Asterisk indicates $p<0.05$.

Figure 5. **Myelinated axons in the tibial nerve**. Microscopic images of tibial nerve sections stained with toluidine blue from naïve control (a), cell transplantation (b) and surgical control (c) groups. There were no myelinated axons in the tibial nerves of the surgical control group. Myelinated axons were present in the tibial nerves of the cell transplantation group.

Figure 6. Neuron survival and the formation of neuromuscular junctions in the tibial nerve and anterior tibialis muscle. Microscopic images of tibial nerve sections stained with SMI-32 (A) and choline acetyltransferase (B), and a microscopic image of anterior tibialis muscle sections stained with antibodies against neurofilament H and α-bungarotoxin (C) from the cell transplantation group. Asterisks indicate SMI-32 staining of mature motoneurons. Arrow indicates axon and arrowhead indicates clustering of acetylcholine receptors.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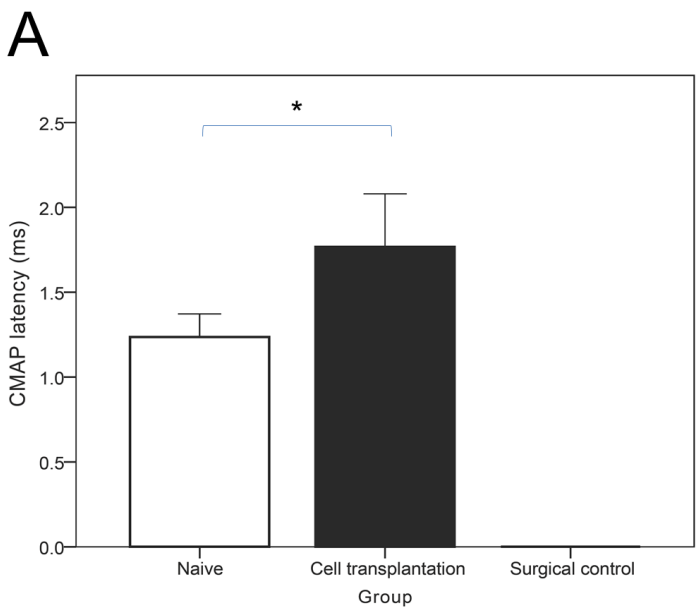
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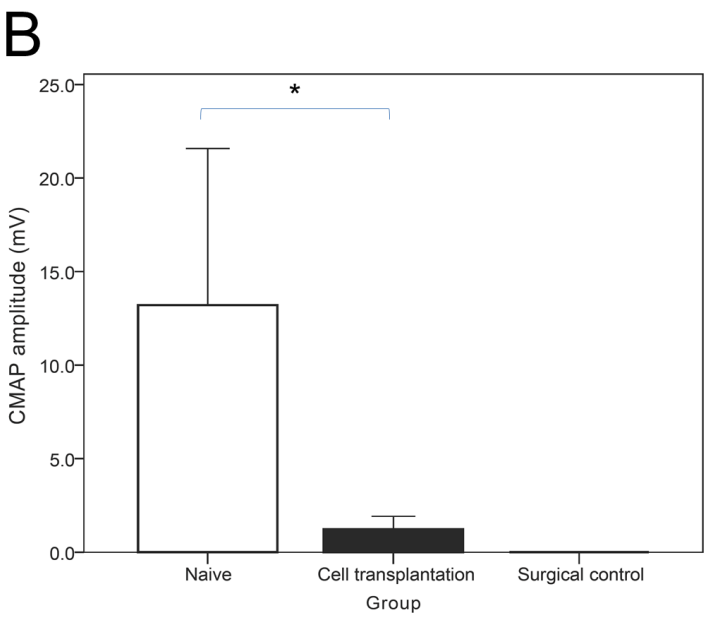
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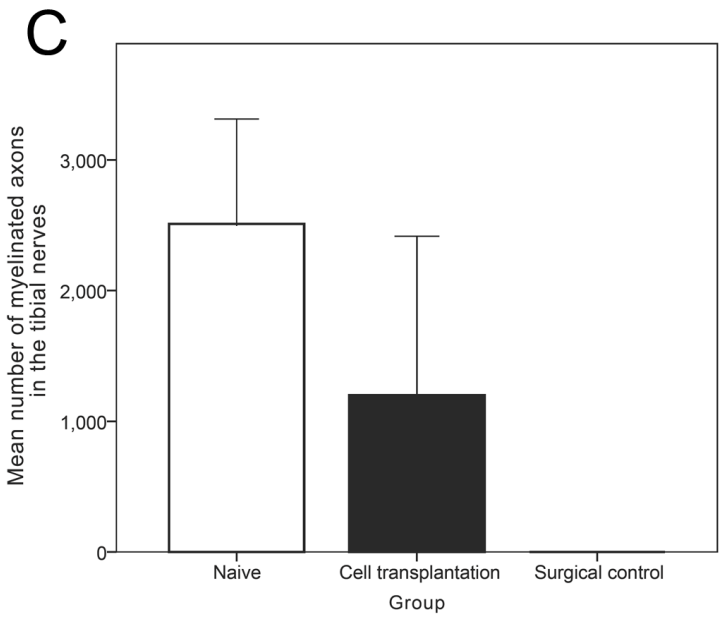
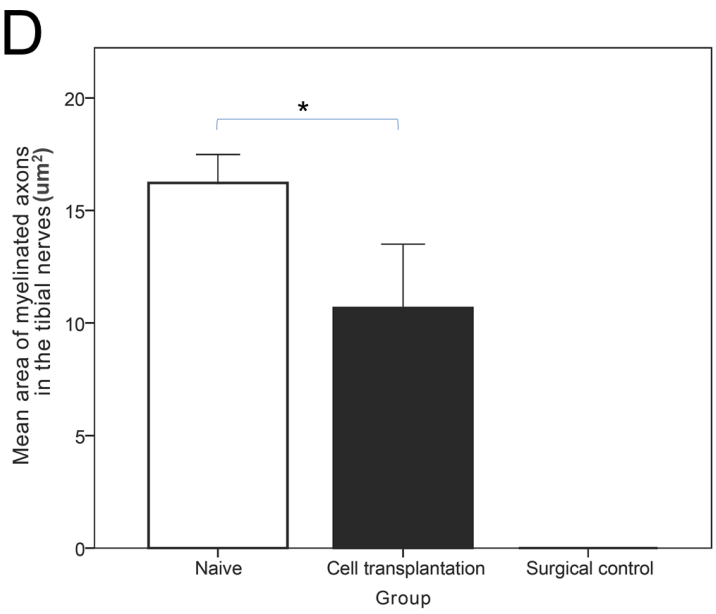
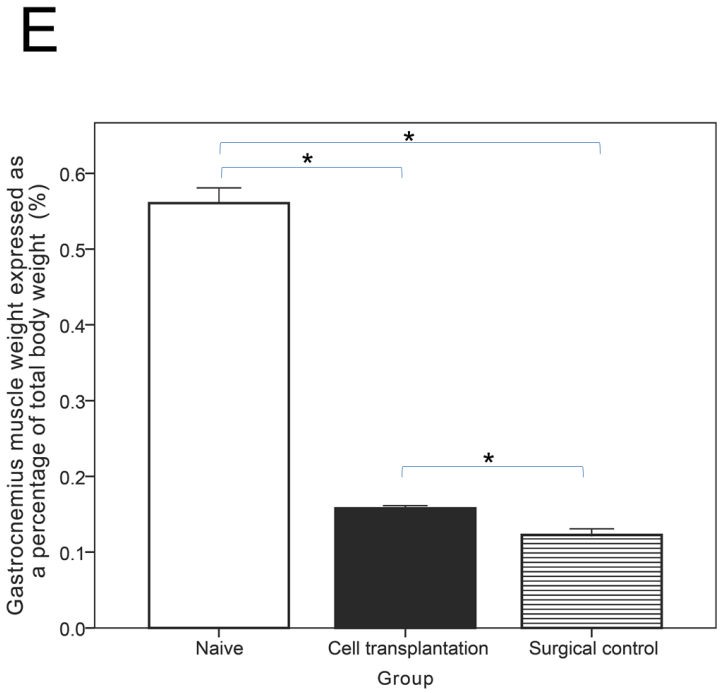
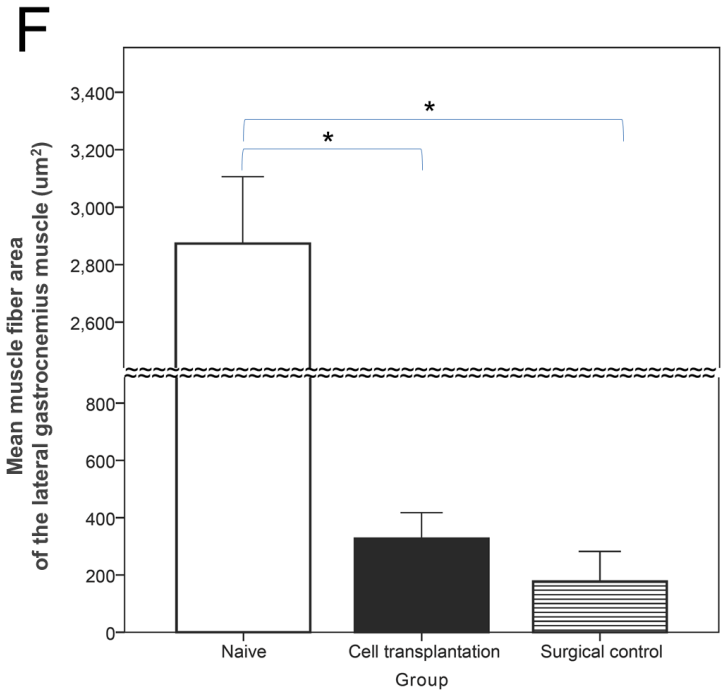
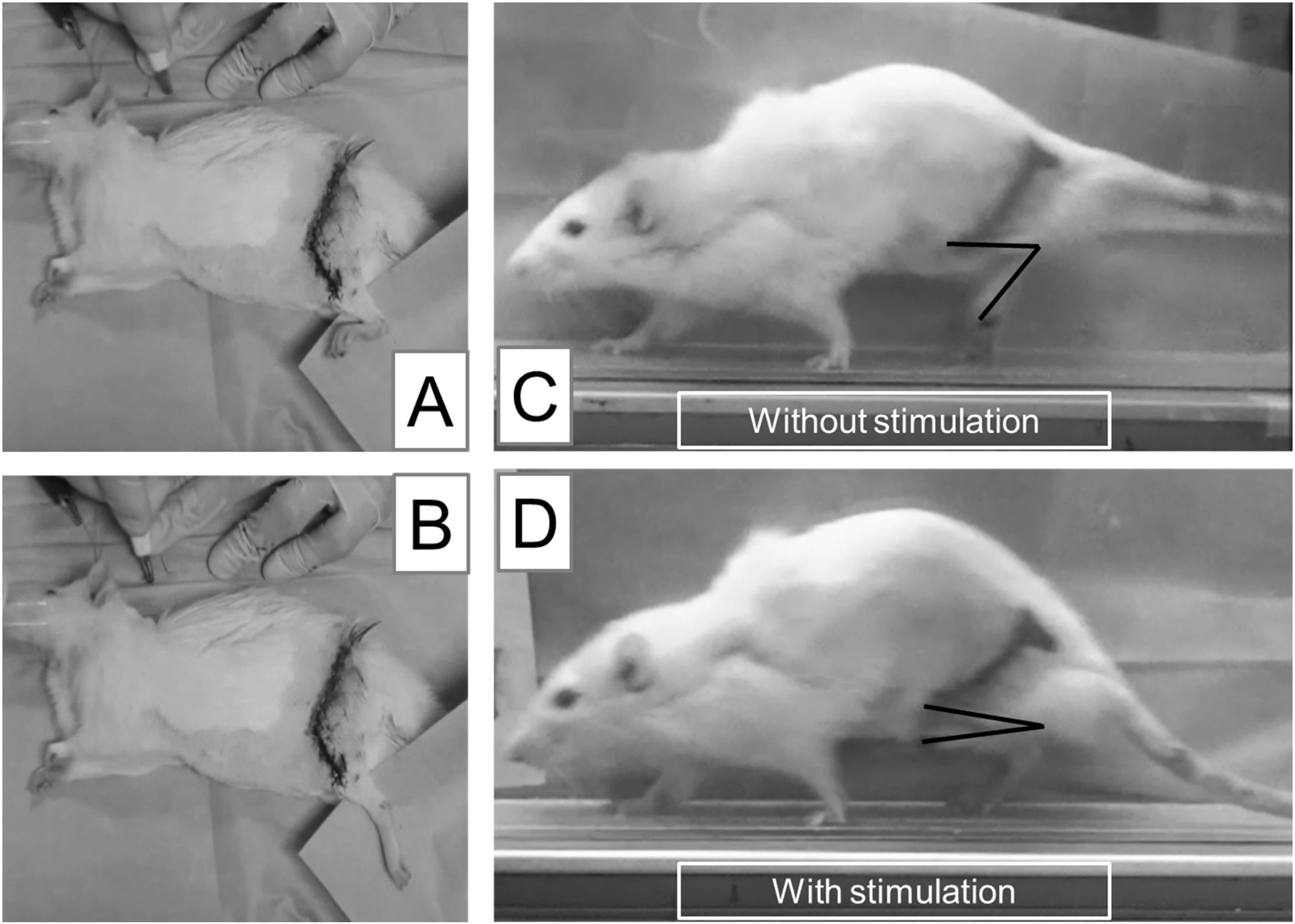
Surgical control
Group

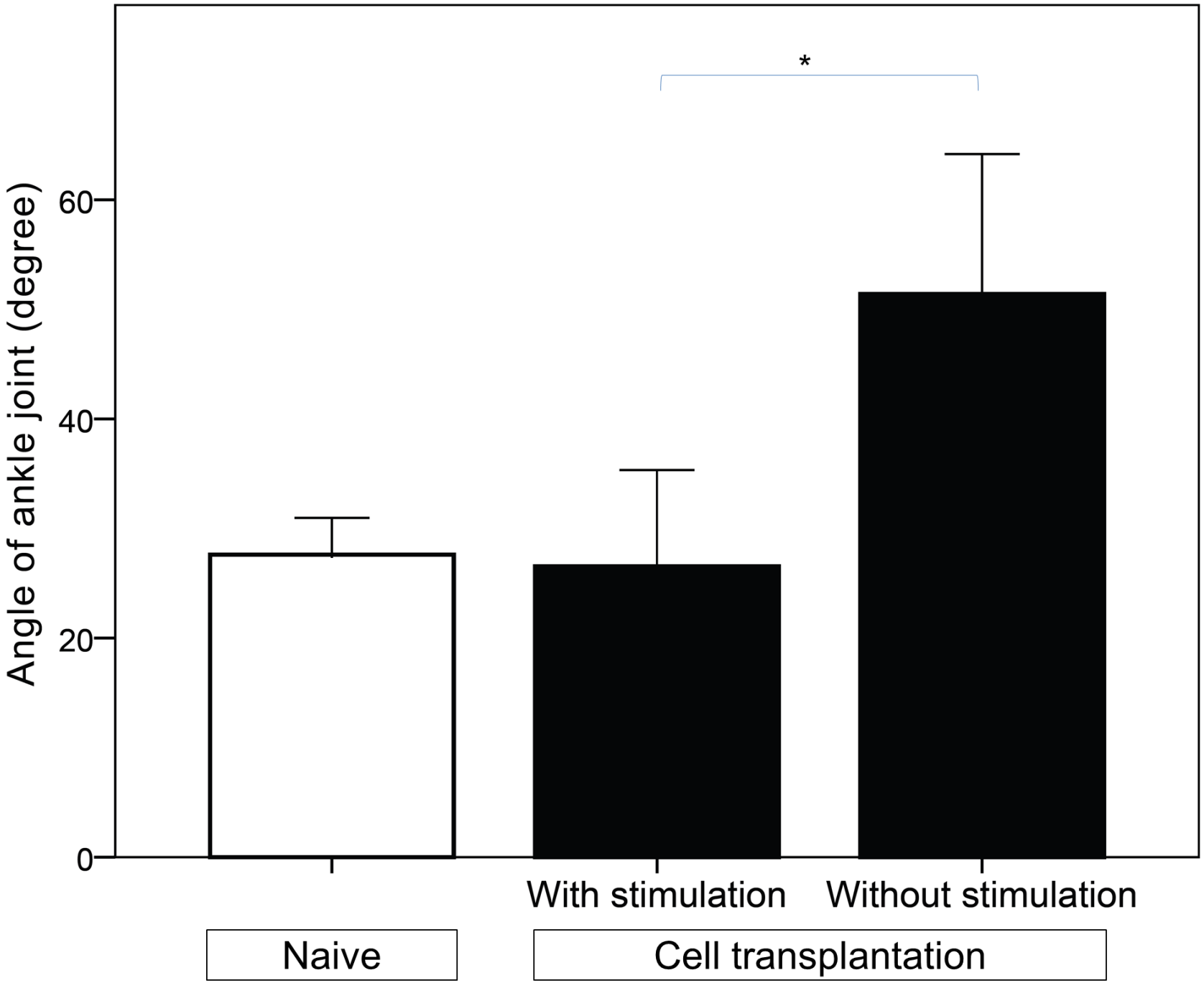

C
Mean number of myelinated axons in the tibial nerves
3,000
2,000
1,000
0
Naive
Cell transplantation
Surgical control
Group

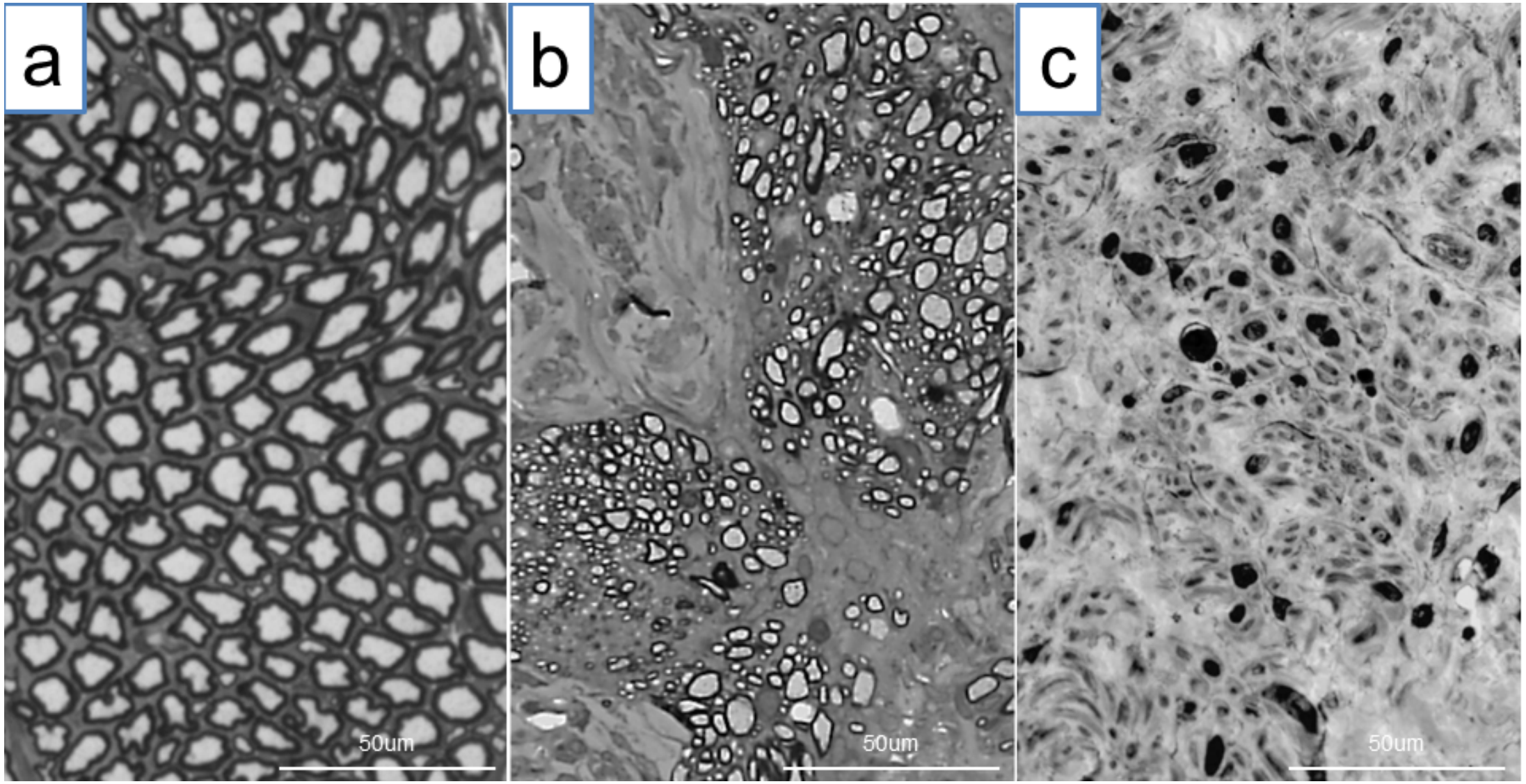

D
Mean area of myelinated axons in the tibial nerves (um²)
20
15
10
5
0
*
Naive
Cell transplantation
Surgical control
Group

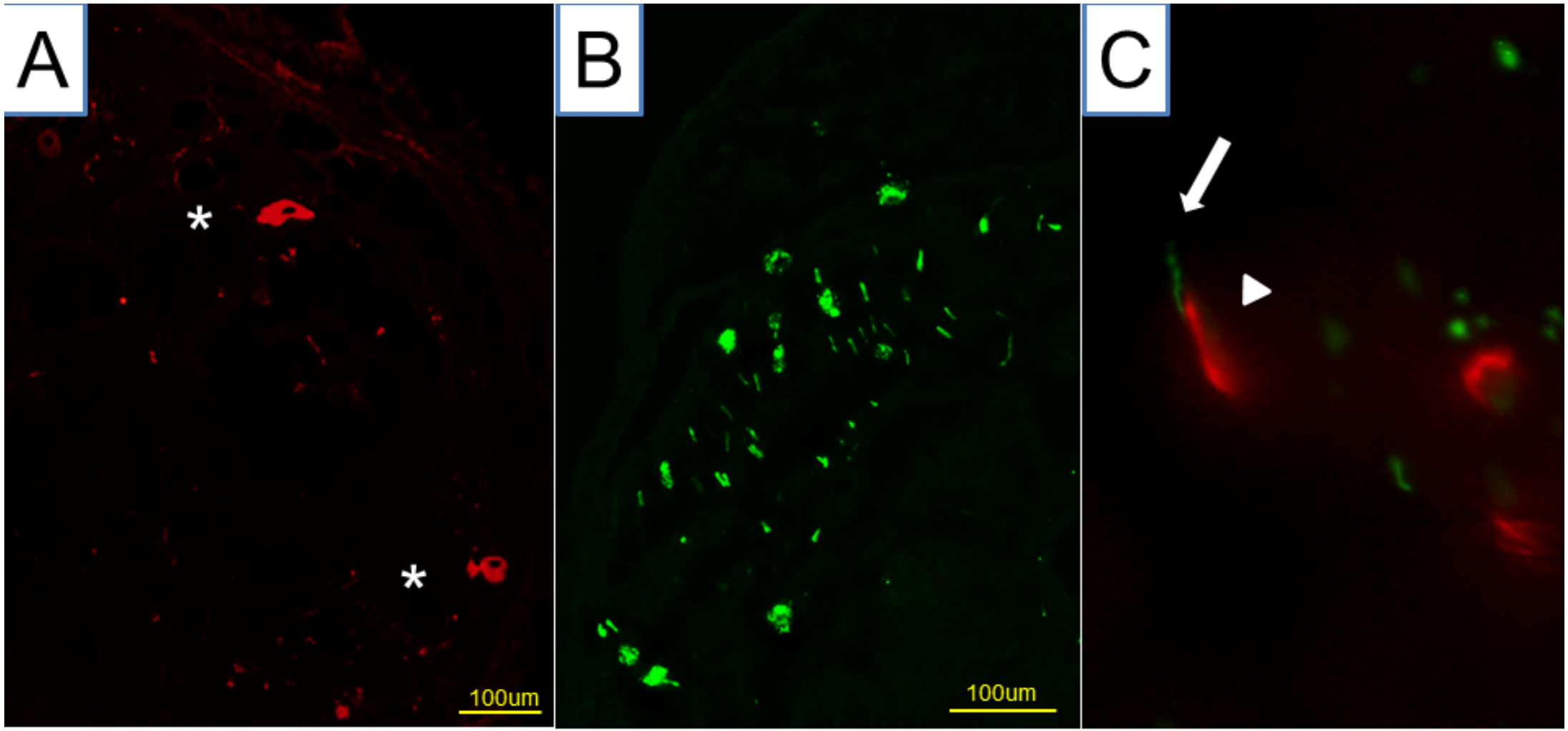

E
Gastrocnemius muscle weight expressed as a percentage of total body weight (%)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
Naive
Cell transplantation
Surgical control
Group


F
Mean muscle fiber area of the lateral gastrocnemius muscle (um²)
3,400
3,200
3,000
2,800
2,600
800
600
400
200
0
*
*
Naive
Cell transplantation
Surgical control
Group


A
B
C
Without stimulation
D
With stimulation


*
Angle of ankle joint (degree)
60
40
20
0
With stimulation
Without stimulation
Naive
Cell transplantation


a
50um
b
50um
c
50um


A
B
C
100um
100um