Optimal conditions for graft survival and reinnervation of denervated muscles after embryonic motoneuron transplantation into peripheral nerves undergoing Wallerian degeneration

Running title

Embryonic motoneuron transplantation into peripheral nerves

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Abstract

Motoneuron transplantation into peripheral nerves undergoing Wallerian degeneration may have applications in treating diseases causing muscle paralysis. We investigated whether functional reinnervation of denervated muscle could be achieved by early or delayed transplantation after denervation. Adult rats were assigned to six groups with increasing denervation periods (0, 1, 4, 8, 12, and 24 weeks) before inoculation with culture medium containing (transplantation group) or lacking (surgical control group) dissociated embryonic motoneurons into the peroneal nerve. Electrophysiological and tissue analyses were performed 3 months after transplantation. Reinnervation of denervated muscles significantly increased relative muscle weight in the transplantation group compared with the surgical control group for denervation periods of 1 week (0.042% ± 0.0031% versus 0.032% ± 0.0020%, respectively; $p = 0.009$), 4 weeks (0.044% ± 0.0069% versus 0.026% ± 0.0045%, respectively; $p = 0.0023$), and 8 weeks (0.044% ± 0.0029% versus 0.026% ± 0.0008%, respectively; $p = 0.0023$). The ratios of reinnervated muscle contractile forces to naïve muscle in the 0-, 1-, 4-, 8-, and 12-week transplantation groups were 3.79%, 18.99%, 8.05%, 6.30%, and 5.80%, respectively, indicating that these forces were sufficient for walking. The optimal implantation time for transplantation of motoneurons into the peripheral nerve was 1 week after nerve

transection. However, the neurons transplanted 24 weeks after denervation survived and regenerated axons. These results indicated that there is time for preparing cells for transplantation in regenerative medicine and suggested that our method may be useful for paralysed muscles that are not expected to recover with current treatment.

1 Introduction

Lower motoneuron diseases, such as spinal anterior horn lesions, traumatic avulsion of spinal roots, and amyotrophic lateral sclerosis, cause motoneuron death, resulting in skeletal muscle denervation, loss of spontaneous muscle control, and atrophy of muscle fibres. Muscle atrophy progresses due to long-term denervation, causing irreversible degeneration (Kern *et al.*, 2004). Early reinnervation is required to prevent skeletal muscle atrophy; however, it is not easy to reconstruct complex neural networks in the central nervous system (CNS).

Stem cell transplantation in regenerative medicine may be a useful strategy for replacing damaged components of the CNS and has been used experimentally in various disease models, such as spinal cord injury (McDonald *et al*., 1999; Sharp *et al*., 2010). However, reconstructing the CNS's complex structures requires a large number of transplanted cells, which can lead to the risk of tumorigenesis. Besides, therapeutic transplantation in spinal cord injury is reportedly better after the inflammatory phase and before glial scar formation; thus, the optimal time window is narrow (Okano, 2010). The peripheral nervous system (PNS), on the other hand, is considered an ideal target for neuronal replacement therapy because of the simplicity of the neural network and the wide window of therapeutic opportunity (Merrell *et al*., 2001).

One experimental approach to rescue denervated skeletal muscles from atrophy is the transplantation of embryonic motoneurons into a peripheral nerve for muscle reinnervation (Erb *et al*., 1993; Thomas *et al*., 2000; Yohn *et al*., 2008). Our previous studies applied this transplantation strategy coupled with computer-programmed functional electrical stimulation (FES), demonstrating that functional muscle activity could be restored, even without a neural connection between the CNS and muscle (Kurimoto *et al*., 2016; Kato *et al*., 2015). Moreover, another previous study showed that axon regeneration and muscle reinnervation were improved when embryonic motoneurons were transplanted into peripheral nerves 1 week after denervation compared with that immediately after denervation (Grumbles *et al*., 2002). However, no studies have clarified whether functional reinnervation can be achieved with further delays in transplantation. In the clinical setting, it is challenging to perform transplantation 1 week after nerve transection; instead, intervention for reconstruction is required in chronic phases.

In peripheral nerve injury, dedifferentiated Schwann cells play a central role in axonal regeneration by producing various neurotrophic factors and forming scaffolds for axonal growth (Arthur-Farraj *et al*., 2012). However, as the time from injury elapses, Schwann cells gradually undergo atrophy, and their ability to support nerve regeneration

is reduced (Ronchi *et al.*, 2017; Sulaiman and Gordon, 2000, 2009; Walsh *et al*., 2010). In addition to Schwann cell atrophy, both muscle fibres and end plates undergo slow but steady degeneration over time, leading to impaired functional recovery. The mechanisms through which these changes in the endoneurial environment of the peripheral nerves affect nerve regeneration in transplantation strategy have not been verified.

Accordingly, in this study, we aimed to determine whether transplanted motoneurons could survive in chronically denervated peripheral nerves, regenerate nerves, and prevent muscle atrophy. Achieving good functional recovery with delayed transplantation into the peripheral nerve would suggest that there may be time to prepare cells for transplantation after denervation. Our findings provided insights into the use of neuron transplantation in the peripheral nerve for treating currently intractable diseases.

2 Materials and Methods

2.1 Animals

All experimental protocols and animal maintenance procedures used in this study were approved by the Animal Ethics Research Committee of Nagoya University and conducted in compliance with the Law for the Humane Treatment and Management of Animals and the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related

Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

The experimental protocol consisted of three steps: 1) muscle denervation, 2) cell transplantation, and 3) evaluation of muscle reinnervation.

In the first step, 36 adult (8-week-old) Fischer 344 rats (Japan SLC Inc., Shizuoka, Japan) were prepared. Both sciatic nerves were transected at the midthigh to denervate the muscle in each animal. The left hindlimb was designated as the transplantation side, and the right was designated as the surgical control side. Rats were randomly assigned to six experimental groups representing increasing periods of denervation time before cell transplantation (0, 1, 4, 8, 12, and 24 weeks; n = 6 each).

In the second step, embryonic ventral spinal cord cells were harvested from 14-day-old embryos of six pregnant Fischer 344 rats. Medium containing dissociated embryonic ventral spinal cord cells was injected into the distal stump of the peroneal nerves. On the surgical control side, medium alone was injected. In the 0-week group, neurotomy and transplantation were performed simultaneously.

In the third step, five rats from each experimental group were sacrificed 3 months after transplantation for comparative analysis. The remaining rats were sacrificed 1 year after transplantation to confirm the survival of the transplanted motoneurons.

Physiological data from the transplantation and surgical control rats were compared with those from naïve control rats (n = 5) that underwent the same procedures without denervation or transplantation (Figure 1-A). The peripheral nerve transplanted with motoneurons was more swollen than that transplanted with medium only, making it easy to recognize the transplanted side at the time of evaluation. Therefore, data were not directly collected by the researcher who was blinded to the experimental groups, except for the wet muscle weight measurement.

2.2 Surgical procedure

All surgical procedures were performed with a surgical microscope under 2% isoflurane anaesthesia. The bilateral sciatic nerves were completely transected at the midthigh and divided into the tibial nerve and peroneal nerve. The peroneal nerve was transected 30 mm proximal to the entry into the tibialis anterior muscles. The transected nerves were ligated at both ends. The proximal stump was buried in the gluteus muscle to prevent axonal regeneration. A gap of 10 mm or more was formed between the transected nerve's proximal and distal ends. In the naïve group, no surgery was performed.

2.3 Cell preparation and transplantation

Ventral spinal cord cells were obtained from 14-day-old embryos. Pregnancy Fischer 344 rats were anaesthetised with 2% isoflurane, and their embryos were collected from the uterus. The ventral spinal cords were resected from the foetuses under a surgical microscope and cut into small pieces in ice-cold Hank's balanced salt solution (Life Technologies Japan, Tokyo, Japan). Embryonic ventral spinal cord cells were dissociated using a papain-containing dissociation solution (cat. No. MB-X0801; Sumitomo Bakelite Co., Tokyo, Japan) and suspended in culture medium (cat. No. MB-X9501; Sumitomo Bakelite Co.). In a previous study, we demonstrated that motoneurons account for 83% of the cultured cells when incubated for 16 hours under these conditions (Kato *et al*., 2015). The ventral spinal cord cells were cultured for 7 days, and the existence of neurons and astrocytes was confirmed. The cells for transplantation contained neural progenitor cells with the potential to differentiate into either neural or glial lineages (Nakano *et al*., 2018).

Recipient rats were anaesthetised, and 1×10^6 embryonic ventral spinal cord cells suspended in 10 μL medium were injected into the distal stumps of the peroneal nerves on the transplantation side, using a Hamilton syringe with a 30-G needle. Medium without neurons was injected into the surgical control side. During injection, nerve swelling, which was the sign that the medium was injected intraneurally, was confirmed

visually under a microscope. The injection site was 20 mm proximal to the entry into the tibialis anterior muscles.

To evaluate the pretransplant environment, following the peroneal nerves being harvested 5 mm from the distal stumps right before transplantation in all transplantation groups, the distal stumps ligated again. The harvested nerves were immersed in 4% paraformaldehyde and sucrose, frozen, and embedded.

2.4 Electrophysiological analysis

2.4.1 Evoked electromyography

Electrophysiological analyses were performed in each group of rats 3 months after cell transplantation. After anaesthesia was induced with isoflurane, the peripheral nerve stump was exposed, and a bipolar stimulating electrode (cat. no. UM2-5050; Nihon Kohden, Tokyo, Japan) was placed around the nodular part of the nerve where the motoneurons were transplanted. Electrical stimulation (supramaximal stimulation, duration: 100 ms, frequency: 60 Hz, square-wave pulse) was performed using an isolator (cat. no. SS-201 J; Nihon Kohden) connected to an electrical stimulator. After ankle movement was confirmed, the compound muscle action potential (CMAP) of the tibialis anterior muscle was measured using a standard nerve evoked potential recording system (Neuropack S1,

cat. no. MEB -9404; Nihon Kohden). Using two stainless steel monopole recording electrodes (cat. no. H 537 A; Nihon Kohden), one was inserted into the centre of the anterior tibial muscle belly, and the other was inserted into the distal tendon. The insertion site of the electrode was adjusted to obtain a stable waveform. Latency was calculated as the time from the onset of stimulation to the first peak of the maximal CMAP, and the amplitude was measured as its peak value.

2.4.2 Muscle contraction force measurement

CMAP was measured by evoked electromyography, followed by muscle contraction force measurement of the tibialis anterior muscles in each group. The femoral condyle was exposed, taking care not to damage the peroneal nerve, and a 1.6-mm drill hole was created. A 1.5-mm Kirschner wire was inserted into the drill hole, and the hindlimbs were immobilised on a custom-made rat immobilisation platform. To further stabilise the feet, the feet were ligated with 2-0 silk and secured firmly to the rat immobilisation platform. The tibialis anterior muscle was fully exposed at the musculotendinous junction. The distal tendon was detached, ligated with 4-0 nylon, and secured to an arm connected to the transducer. The rat immobilisation platform was filled with Krebs-Ringer's solution warmed in a 37 °C thermostatic bath with continuous perfusion to ensure constant

immersion of the exposed tibialis anterior muscle (Figure 1-B).

First, the transplanted part of the peroneal nerve was stimulated with single 0.1-ms-duration pulses at 30-s intervals to fine-tune the transducer position and determine the optimal muscle length for isometric force. The voltage of stimulation intensities was increased up to 10 V using 0.1-V or 1.0-V steps. When these stimulation intensities evoked no twitch force, these were increased up to 100 V. If no force was evoked even at high stimulation intensities, these muscles were considered denervated. Next, twitch stimulation (stimulus intensity: 10 V) was performed eight times using a single digital stimulator (LE 12 I06VI; BioResearch Center, Aichi, Japan), and the muscle contraction force obtained by each stimulation was measured; the average value was calculated. Tetanus stimulation at 40, 80, 100, and 120 Hz (stimulus intensity: 10 V, duration: 300 ms) was then performed to record the maximal contractile force at the frequency of transition from incomplete to complete ankylosis. Finally, stimulation was applied every second for 2 min (stimulus intensity: 10 V, duration: 300 ms, frequency: 60 Hz) to assess muscle fatigue resistance. The muscle fatigue index was calculated by dividing the maximum muscle contraction force value at the end of stimulation by the same value at the start of stimulation. The same procedures were performed on both the transplantation and surgical control sides.

2.5 Animal sacrifice

Three months after transplantation, five rats from each group were sacrificed for tissue analysis. Under isoflurane anaesthesia, the tibialis anterior muscle was harvested and measured (wet weight/body weight). The left ventricle of the rats was perfused with 50 mL of 0.9% saline, followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

The common peroneal nerve was harvested for immunohistochemical and histochemical analyses and was divided into the proximal half, which contained the transplanted portion and the remaining distal half. The distal half was further divided into two parts; one was prefixed with 4% glutaraldehyde in cacodylate buffer and then embedded in Epon, and the other was frozen with isopentane, which was cooled with liquid nitrogen after sucrose fixation. The proximal half of the common peroneal nerve was embedded in Epon for electron microscopic evaluation and was frozen for immunohistochemical analysis. One additional rat was sacrificed 1 year after transplantation, and the nodule of the common peroneal nerve was harvested and frozen.

2.6 Tissue analysis

2.6.1 Evaluation of regenerated myelinated axons

The distal portion of the Epon-embedded common peroneal nerve was cut into 1-μm-thick cross-sections with a glass knife and stained with toluidine blue (Sigma-Aldrich, Germany) for light microscopy. The number of regenerated myelinated axons was counted.

2.6.2 Evaluation of the neuromuscular junction

To evaluate the structure of the neuromuscular junction and morphology of the end plates, extensor digitorum longus muscles containing the neuromuscular junction were harvested, stained with a FluoroPan neuronal marker conjugated with Alexa Fluor 488 (1:100; Millipore, Billerica, MA, USA), an antibody cocktail containing four antibodies (anti-NeuN, anti-microtubule-associated protein 2, anti-β3-tubulin, and anti-neurofilament H antibodies) and with Alexa Fluor 594-conjugated α-bungarotoxin (1:300; Molecular Probes, Eugene, OR, USA), and observed using confocal microscopy (A1Rsi; Nikon, Tokyo, Japan). To assess the morphology of the neuromuscular junction, the classifications shown in a previous paper were used (Marques *et al*., 2000). End plates were categorised as pretzel (a mature form with a web-like pattern containing multiple perforations) and plaque (immature and small in size without perforation) (Kummer *et*

al., 2004).

2.6.3 Evaluation of the denervated nerve environment before transplantation

The frozen common peroneal nerve harvested before transplantation was cut into 20-μm-thick cross-sections. Staining was performed with Alexa Fluor 488-conjugated anti-β3-tubulin antibodies (1:400; BioLegend, San Diego, CA, USA), anti-S100 antibodies (1:200; DAKO, Carpinteria, CA, USA), and Hoechst (1:1,000; Dojindo, Kumamoto, Japan) and observed by fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan) or confocal microscopy (A1Rsi; Nikon).

2.6.4 Morphological evaluation of the nodule from the transplantation site

Frozen-embedded nodules of the peroneal nerve were cut into 20-μm-thick cross-sections and longitudinal sections; stained with Alexa Fluor 488-conjugated anti-β3-tubulin antibodies (1:400; BioLegend), anti-glial fibrillary acidic protein (GFAP) antibodies [G-A-5] (Cy3; 1:400; Abcam, Cambridge, UK), and Hoechst (1:1,000; Dojindo); and observed with fluorescence microscopy (BZ -9000; Keyence) or confocal microscopy (A1Rsi; Nikon).

The Epon-embedded nodules were cut into 80-nm-thick ultrathin sections, and

the morphology of neurons, glia, and axons was observed under a transmission electron microscope (JEM1200 EX; JEOL Ltd., Tokyo, Japan).

2.7 Statistics

Analysis of variance with Tukey or Games-Howell post-hoc comparisons or Student's t test were used to compare outcome measures across groups, as appropriate. All statistical analyses were performed using the Statistical Package for Social Science version 22.0 software (IBM, Armonk, NY, USA). Results with *p*-values of less than 0.05 were considered statistically significant. All data are described as the means ± standard errors (SEs).

3 Results

3.1 Electromyography findings

3.1.1 Evoked electromyography

The mean latencies of CMAP in reinnervated tibialis anterior muscles in the naïve, 0-week, 1-week, 4-week, 8-week, and 12-week groups were 1.58 ± 0.12, 1.99 ± 0.59, 2.47 ± 0.96, 2.27 ± 0.28, 3.00 ± 1.17, and 3.08 ± 0.93 ms, respectively. The mean amplitudes of CMAP in these groups were 18.11 ± 8.29, 2.03 ± 1.74, 1.54 ± 1.5, 1.05 ± 0.83, 0.83 ±

0.16, and 0.71 ± 0.51 mV, respectively. CMAP was not evoked in the 24-week group.

In all denervation period groups, CMAP was not evoked in the surgical control group. The latency tended to be prolonged as the denervation period increased; however, no significant differences were observed between groups. The naïve group showed a larger amplitude compared with any of the transplantation groups ($p < 0.001$). Among the transplantation groups, the amplitudes tended to decrease with increasing denervation period; however, there were no significant differences between groups as the denervation period increased (Figure 2A, B).

3.1.2 Muscle contraction force measurement

The mean muscle contractile forces of twitch stimulation (stimulus intensity: 10 V) in the naïve, 0-week, 1-week, 4-week, 8-week, and 12-week groups were 37 ± 4.5, 3.5 ± 0.19, 7.0 ± 2.3, 2.8 ± 0.51, 5.4 ± 0.01, and 3.1 ± 0.75 cN, respectively. The naïve group showed larger values than any of the transplantation groups ($p < 0.001$). The mean muscle contractile forces of tetanus stimulation (stimulus intensity: 10 V, duration: 300 ms, frequency: 120 Hz) in the naïve, 0-week, 1-week, 4-week, 8-week, and 12-week groups were 157 ± 5.4, 6.0 ± 0.75, 30 ± 19, 13 ± 5.7, 9.9 ± 5.1, and 9.1 ± 4.8 cN, respectively. The naïve group showed larger values than any of the transplantation groups (naïve versus

0-week: $p = 0.003$, naïve versus 1-week: $p = 0.001$, naïve versus 4-week: $p = 0.002$, naïve versus 8-week: $p = 0.004$, naïve versus 12-week: $p = 0.004$). The ratios of contractile force compared with the naïve group were 3.79%, 18.99%, 8.05%, 6.30%, and 5.80% for the 0-, 1-, 4-, 8-, and 12-week groups, respectively, and fatigue indices were 16.2 ± 4.52, 52.7 ± 24.65, 57.3 ± 19.19, 48.3 ± 4.39, 39.4 ± 36.37, and 30.4 ± 25.64 in the naïve, 0-week, 1-week, 4-week, 8-week, and 12-week groups, respectively. Although reinnervated muscles were more resistant to fatigue than naïve muscles, as the denervation period increased, the resistance to fatigue decreased (naïve versus 0-week: $p = 0.58$, naïve versus 1-week: $p = 0.043$, naïve versus 4-week: $p = 0.002$, naïve versus 8-week: $p = 0.91$, naïve versus 12-week: $p = 0.94$; Figure 2C–E).

In all denervation period groups, no forces were evoked in the surgical control side muscles even when stimulated at 100-V. These data suggested that these muscles were denervated, as expected.

3.2 Tissue analysis

3.2.1 Effects of preventing muscle atrophy

The wet muscle weights of the tibialis anterior (as a percentage of body weight [%BW]) in the transplantation group / surgical control group for 0-, 1-, 4-, 8-, 12-, and 24-week

groups were 0.048%BW ± 0.0017%BW / 0.042%BW ± 0.0031%BW, 0.042%BW ± 0.0031%BW / 0.032%BW ± 0.0020%BW (p = 0.009), 0.044%BW ± 0.0069%BW / 0.026%BW ± 0.0045%BW (p = 0.0023), 0.044%BW ± 0.0029%BW / 0.026%BW ± 0.0008%BW (p = 0.0023), 0.037%BW ± 0.0038%BW / 0.027%BW ± 0.0041%BW, and 0.032%BW ± 0.0023%BW / 0.027%BW ± 0.0017%BW, respectively. In the surgical control group, wet muscle weight decreased as the denervation period increased, but then levelled off when the denervation period was 4 weeks or more. There were significant differences in the 1-, 4-, and 8-week transplantation and surgical control groups (Figure 3A).

3.2.2 Evaluation of regenerated myelinated axons

No myelinated axons were found in the common peroneal nerve of the surgical control group. The numbers of myelinated axons in the 0-, 1-, 4-, 8-, 12-, and 24-week transplantation groups were 636 ± 388, 1251 ± 623, 514 ± 321, 236 ± 130, 191 ± 122, and 127 ± 123, respectively. The 0-, 4-, 8-, 12-, and 24-week groups had significantly fewer myelinated axons than the naïve group (2252 ± 719). The 8-, 12-, and 24-week groups had significantly fewer myelinated axons than the 1-week group (Figure 3B).

3.2.3 Evaluation of neuromuscular junctions

Neuromuscular junctions in the reinnervated extensor digitorum longus muscles were evaluated by fluorescence immunohistochemistry. Regenerative axons labelled with the FluoroPan neuronal marker reached the neuromuscular junction region where α-bungarotoxin-labelled acetylcholine receptor clusters were located. Additionally, in the cluster classification of acetylcholine receptors, the pretzel phenotype was predominant in the 0-, 1-, and 4-week groups, whereas the plaque phenotype was predominant in the 8-, 12-, and 24-week groups (Figure 3C).

3.2.4 Evaluation of denervated nerve environment before transplantation

The common peroneal nerve harvested before transplantation in each group was compared immunohistochemically. In the 0-week group, a typical peripheral nerve structure was observed, in which β3-tubulin-positive axons were surrounded by S100-positive Schwann cells. In the 1-week group, axonal structures were disrupted, and the staining of β3-tubulin-positive axons was reduced. S100-positive Schwann cells proliferated markedly, reflecting Wallerian degeneration. In the 4- and 8-week groups, S100 staining was decreased, reflecting the decrease in Schwann cell number. Neither β3-tubulin nor S100 staining was observed in the 12- and 24-week groups (Figure 4).

3.2.5 Morphological evaluation of the nodules formed in the transplantation group

Nodules of the peroneal nerve formed by the transplantation were evaluated by fluorescent immunostaining. βIII-tubulin-positive neurons and GFAP-positive astrocytes were seen in the transplantation group of all denervation periods. In the 1-week group with a short denervation period, β3-tubulin-positive neurons were buried in GFAP-positive astrocytes. Central glia differentiated from the transplanted cells and appeared to be involved in the survival of neurons. In contrast, in the 12-week group with a long denervation period, the GFAP-positive astrocytes decreased, and isolated neurons were confirmed. Neither βIII-tubulin-positive neurons nor GFAP-positive astrocytes were seen in surgical control and naïve peripheral nerve tissues. The number of surviving neurons decreased as the denervation period increased, but the neurons were still alive 1 year after transplantation in each transplantation group (Figure 5).

3.2.6 Electron microscopic evaluation

In the 12-week group, nodules of the peroneal nerve were evaluated using electron microscopy. In addition to transplanted neurons, Schwann cells with basement membrane and glia cells without basement membrane were observed. There were two types of

myelinated axons, one wrapped with a thick myelin sheath and the other wrapped with a thin myelin sheath. The thick myelin had a basement membrane, whereas the thin myelin did not, suggesting that both PNS and CNS cells were involved in the myelination of the regenerated axon. These structures were absent in surgical control tissues (Figure 6).

4 Discussion

This study examined whether transplanted motoneurons could survive in chronically denervated peripheral nerves and prevent denervated muscle degeneration. Comparing changes in wet muscle weight between the transplantation and surgical control groups showed that even if transplantation was performed 8 weeks after denervation, it was still effective in preventing muscle atrophy. The reinnervated muscle strengths decreased as time after denervation increased. Ploeg *et al.* (1984) noted that the muscle strength equivalent to the MMT3 level is different for each muscle: 24% for the hip abductor, 13.7% for the quadriceps, 2% for the biceps, and less than 1% for the supinator versus maximum contractile force. In another study, lower extremity muscle strength required for locomotion was about 5% of maximal muscle strength in rodents and 5% in humans (Jung *et al*., 2009; Klein *et al*., 2010). Accordingly, the contraction force of the reinnervated muscles obtained in the current study corresponded to the muscle strength

sufficient for walking, even when transplantation was 12 weeks after denervation. Notably, the reinnervated muscles were resistant to muscle fatigue, consistent with previous reports showing that muscles reinnervated by embryonic motoneurons were predominantly type I and IIA, and that their contractile properties included fatigue resistance (Thomas, 2003; Botterman *et al*., 1985; Kernell *et al*., 1983). Furthermore, our results confirmed that the neurons transplanted into peripheral nerves 24 weeks after denervation survived and regenerated axons.

Our approach provides a source of motoneurons in the peripheral nerve to reinnervate denervated muscles that cannot be regenerated because of lower motoneuron disease. The number of peripheral motoneurons needed to innervate the muscle is much smaller than the number of neurons and glia cells used in CNS regenerative medicine. Previous studies that estimated the number of motor units per muscle using the motor unit estimation method (MUNE) found that the mean MUNE values of the thenar, biceps brachii, extensor digitorum brevis, tibialis anterior, and adductor muscles were 230, 109, 143, 256, and 224, respectively (McComas, 1995). The number of motor axons required to innervate each muscle ranges from several hundred to several 1000, and the number of motoneurons required for nerve transplantation to peripheral nerves is much less than 10,000; this smaller number of cells required for transplantation can reduce the risk of

tumour formation.

Schwann cells are essential for peripheral nerve regeneration. Nerve injury triggers myelin and non-myelin Schwann cell conversion to a repair-specialised cell phenotype. Distal to damage, these repair Schwann cells provide the necessary signals and spatial cues for axonal regeneration and target reinnervation. (Jessen *et al.*, 2016; Michalski *et al*., 2008; Chen *et al*., 2007; Jonsson *et al.*, 2013). However, this change has a time window. If the regenerated axons fail to regenerate within a limited time, the transformed Schwann cell advantages will be lost (Gordon *et al*., 2011; Zhang *et al*., 2017). Gordon *et al*. (2014) have shown that at 3 months after nerve transection, Schwann cells in the distal nerve stump atrophied, and the expression levels of neurotrophic factors significantly decreased. This chronic atrophy of the distal nerve stumps is the primary factor that results in poor axonal regeneration and subsequently poor functional recovery after peripheral nerve injuries (Xu *et al*., 2010; Sulaiman and Gordon, 2000, 2009).

Regarding functional regeneration of denervated muscle in this study, both the number of regenerated axons and muscle contractile forces were the best outcomes in groups in which transplantation was performed at 1 week after denervation, this period was considered to be the optimal timing for transplantation. This result indicates that pre-degeneration of the nerve before cell transplantation provides the following advantages,

Schwann cell activation and secretion of neurotrophic factors, leading to neuronal survival and axonal regeneration. However, no studies have examined how long this favourable environment for transplantation may last. Our results showed that transplanted neurons in the peripheral nerve denervated for 24 weeks survived and regenerated axon. However, functional recovery was obtained only when transplantation was performed within 12 weeks after denervation. It is thought to be due to a decrease in the number of transformed Schwann cells to support the transplanted motoneurons and degeneration of the distal peripheral nerve stumps and postsynaptic structures.

Past studies have reported that FK 506, transforming growth factor β, and neurotrophic factors help prevent Schwann cells from atrophying over time and maintains their ability to support axonal regeneration (Sulaiman and Gordon, 2009; Hoke *et al*., 2002). Another approach to compensate for reduced Schwann cells is direct muscle neurotisation combined with motoneuron transplantation. Nakano *et al*. (2018) reported that a new neuromuscular junction was formed by implanting a fresh free nerve graft into a denervated muscle and transplanting embryonic motoneurons to reinnervate the denervated muscle. This not only provides fresh Schwann cells but also allows flexibility in the time and site of transplantation, using it as a free nerve graft. Combining these techniques may improve the acceptance of motoneuron transplantation and reinnervation

for long-term denervated muscles, thereby leading to further functional improvement.

In cell transplantation therapy, the detailed conditions in which transplanted motoneurons survive and act on axonal regeneration and reinnervation are unclear. Many embryonic motoneurons die within hours to days of transplantation if certain conditions are not met (Onifer *et al.*, 1997). Harper *et al.* (2004) transplanted motoneuron-associated embryonic stem cells into the spinal cord of adult rats with motor neuron damage and found that 25% of the transplanted motor neurons survived up to 3 months after transplantation. However, embryonic stem cell-derived axonal growth was inhibited by myelin, graft-derived axons did not reach skeletal muscle targets, and there was no electrophysiological or functional recovery. Based on this study, Deshpande *et al.* (2006) transplanted myelin inhibitors and administered a glial cell-derived neurotrophic factor into peripheral nerves, resulting in the formation of host/transplant neuromuscular junctions and mediated partial recovery from paralysis. However, in humans it may take more than a year for axons starting from the spinal cord to reach distal skeletal muscles. During this time, irreversible degenerative changes in the denervated muscle progress, making it difficult for the axons to reinnervate functionally. One experimental approach to rescue denervated muscles from atrophy is to transplant cells into peripheral nerves to shorten the distance and time required for the axon to regenerate and reach the target

muscles. To improve the conditions in the peripheral nerve, Grumbles *et al.* (2009) reported that the addition of neurotrophic factors to embryonic motoneurons transplant increased the survival of neurons and the functional recovery of reinnervated muscle.

In the case of neural progenitor cell transplantation, the direction of differentiation is determined by the host environment (Cao *et al.*, 2002). Su *et al*. (2009) transplanted neural progenitor cells from the spinal cord into the damaged anterior horn of the spinal cord, most of which differentiated into astrocytes. Nevertheless, they concluded that neurotrophic factors produced by neural progenitor cells acted effectively and favoured the survival and axogenesis of damaged motoneurons.

Embryonic day 14 ventral spinal cells are a mixture of motoneurons and neural progenitor cells. These cells transplanted into the peripheral nerve, survived, regenerated axons, and reinnervated the target muscle. However, we have not yet determined how these additional cells other than motoneurons affected the reinnervation process. It is possible that glial cells derived from neural progenitor cells after transplantation affected motoneuron survival. However, the immunohistochemical evaluation showed that some transplanted motoneurons could survive even in an environment where glia cells were scarce. This finding suggests the existence of additional support for the survival of transplanted motoneurons in the Schwann canal, which is currently under investigation.

Further investigation of this molecular mechanism may lead to identifying key factors involved in improving functional recovery in this cell transplantation therapy.

Our results showed that the number of regenerated axons was significantly higher in the 1-week group, but CMAP amplitude was not significantly larger compared to the other groups. This result leads us to speculate that neurolysis during electrical stimulation may have an adverse effect on the transplanted sites, to where motor neurons extend their axons. Alternatively, some of the regenerated axons may not have functioned as motor nerves. It is necessary to investigate the relationship between the number of functional motor neurons reinnervating denervated muscles and tetanic forces in future studies.

One of the limitations of this study is that functional evaluations, such as gait analysis or rat sciatic function index, were not performed. This was because, it was difficult to evaluate functional parameters unless electrical stimulation synchronized with the gait cycle was applied, the peripheral nerves were completely transected from CNS to investigate the effect of cell transplantation. Another limitation is that we have not confirmed that all motor units are derived from transplanted motoneurons. However, there was no anatomic continuity between the proximal and distal sciatic nerves in the

surgical control and the cell transplantation groups at the time of transplantation and tissue analysis. It was considered that transplanted cells did not work as a factor to cross-link the nerve stump or to induce the axon from the proximal side. Our results support the view that implanted neurons were a source of the myelinated axons in the cell transplantation group. And, we have already histologically confirmed the reinnervation of muscles by axons extended from the transplanted embryonic motoneurons by retrograde labelling in 1-week group (see Supplementary Figure). Moreover, rats underwent bilateral sciatic nerve transection to reduce the number of sacrificed animals, the left side was used for transplantation and right side as a surgical control, raising concern about the remote effect of surgery and transplantation. Nevertheless, there was no axonal regeneration in the surgical control groups, so it was considered that the cells implanted into the transplantation side did not affect the reinnervation of denervated muscles on the surgical control side.

In conclusion, we found that transplanted embryonic motoneurons survived, regenerated axons, and reinnervated target muscles, even in long-term denervated peripheral nerves. In terms of functional outcomes, the optimal time for transplantation was 1 week after neurotomy. The effect of preventing muscle atrophy was achieved until the delay of transplantation was 8 weeks after nerve transection. We also showed that

reinnervated muscles generated sufficient muscle contractile forces for walking, even after delayed transplantation. These results indicated an optimal time window for the preparation of cells for transplantation in regenerative medicine approaches. Overall, our method may serve as a reconstruction approach for paralysed muscles that are not expected to recover with current treatment.

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Conflict of interest

The authors have declared that there is no conflict of interest.

Author contributions

H.S. designed the study, executed the experiments, interpreted the data, and drafted the manuscript. K.T. and S.S. executed the experiments. H.H. conceived and supervised the

study, interpreted the data. S. K. critically reviewed the manuscript, and performed the literature review. All authors approved the final version of the manuscript.

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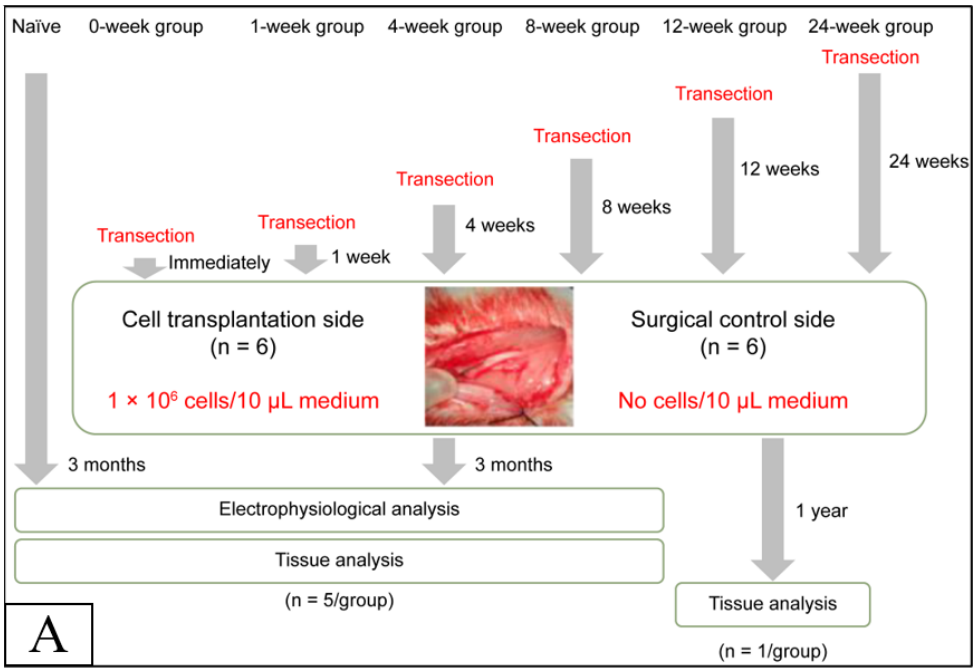
Figure



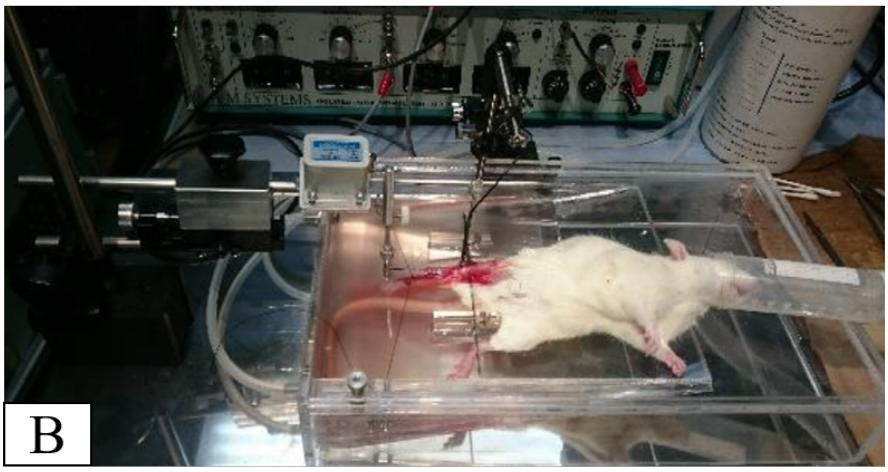


Figure 1

Caption: Flow chart showing the experimental steps (A) and Image of the custom-made rat immobilization platform for muscle contractile force measurement (B). The rat hindlimbs were immobilized on a rat immobilization platform and were constantly immersed in Ringer's solution maintained at 37 °C using a thermostatic bath. The distal tendon of the tibialis anterior muscle was detached, ligated with 4-0 Nylon, and secured to an arm connected to the transducer.

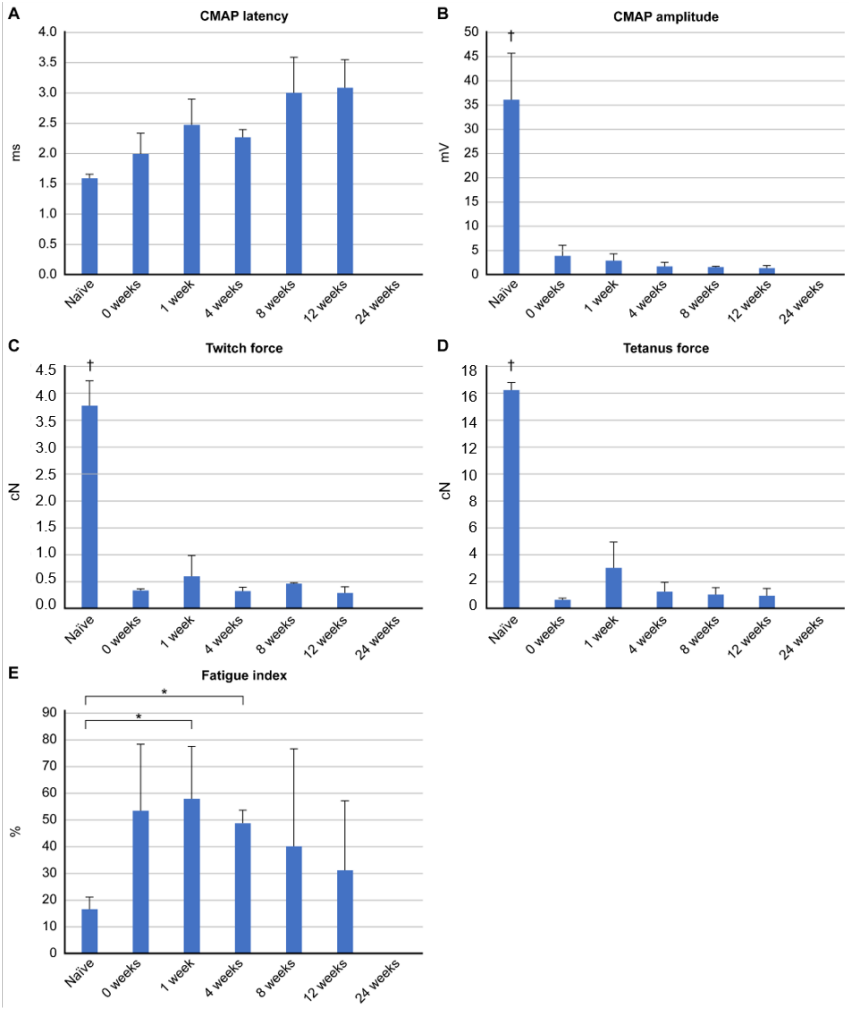


Figure 2

Caption: Results from electrophysiological analyses. The latency (A) and amplitude (B) of compound muscle action potentials (CMAPs) recorded in the tibialis anterior muscle. The twitch force (C), tetanus force (D), and fatigue index (E) of the muscle contractile force measurement in the tibialis anterior muscle. Error bars represent SEs. †$p < 0.001$ versus all transplantation groups, *$p < 0.05$ between groups.

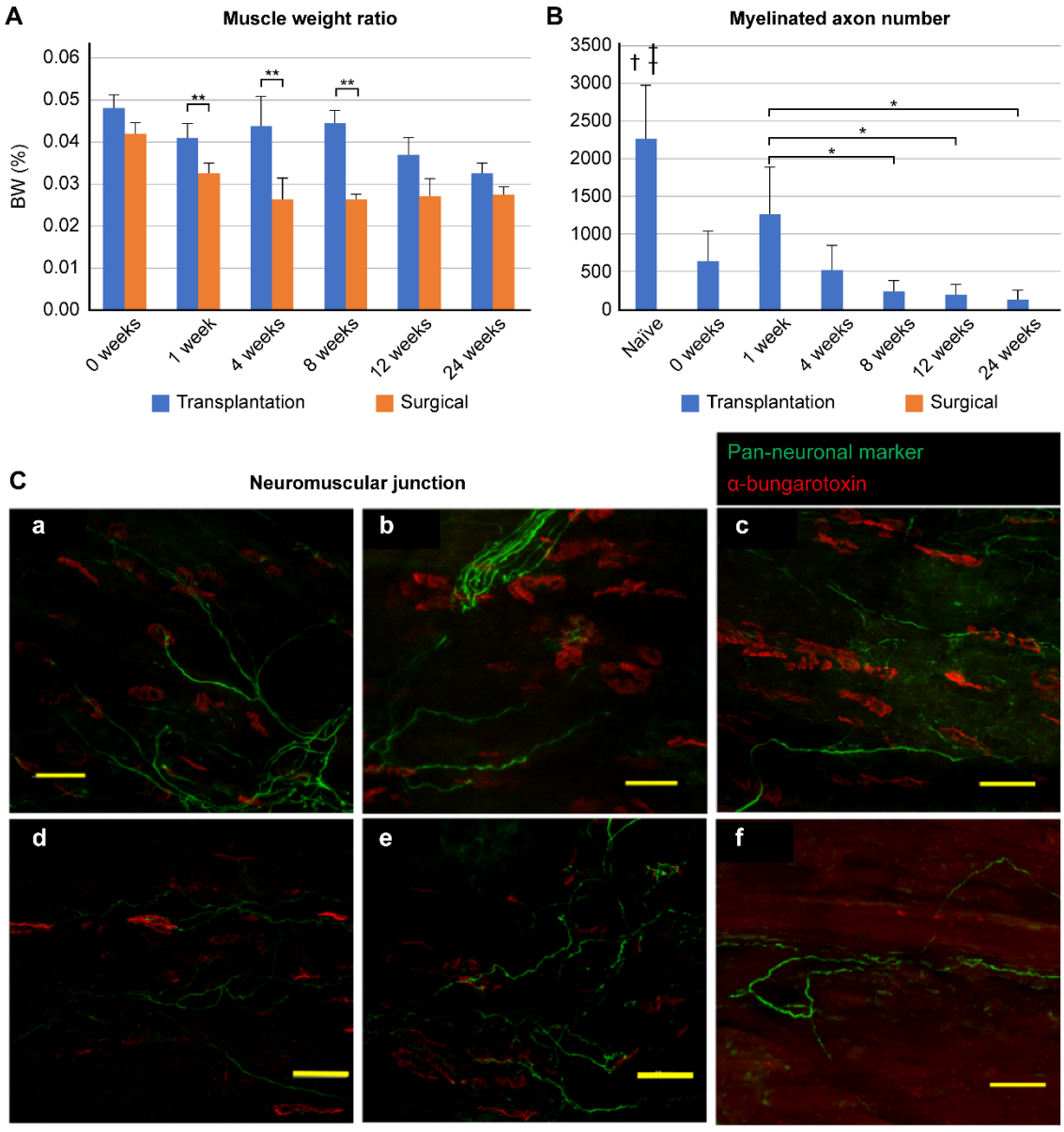


Figure 3

Caption: Regenerated axon and reinnervated muscle analyses. Tibialis anterior muscle wet weight ratios (A), myelinated axon numbers (B), and neuromuscular junction formation (C) in the transplantation groups. 0-week group (a), 1-week group (b), 4-week group (c), 8-week group (d), 12-week group (e), 24-week group (f). Error bars represent SEs. †$p < 0.05$ versus 0- and 4-week groups; ‡$p < 0.001$ versus 8-, 12-, and 24-week groups; *$p < 0.05$ across groups; **$p < 0.01$ across groups. Scale bar = 50 μm.

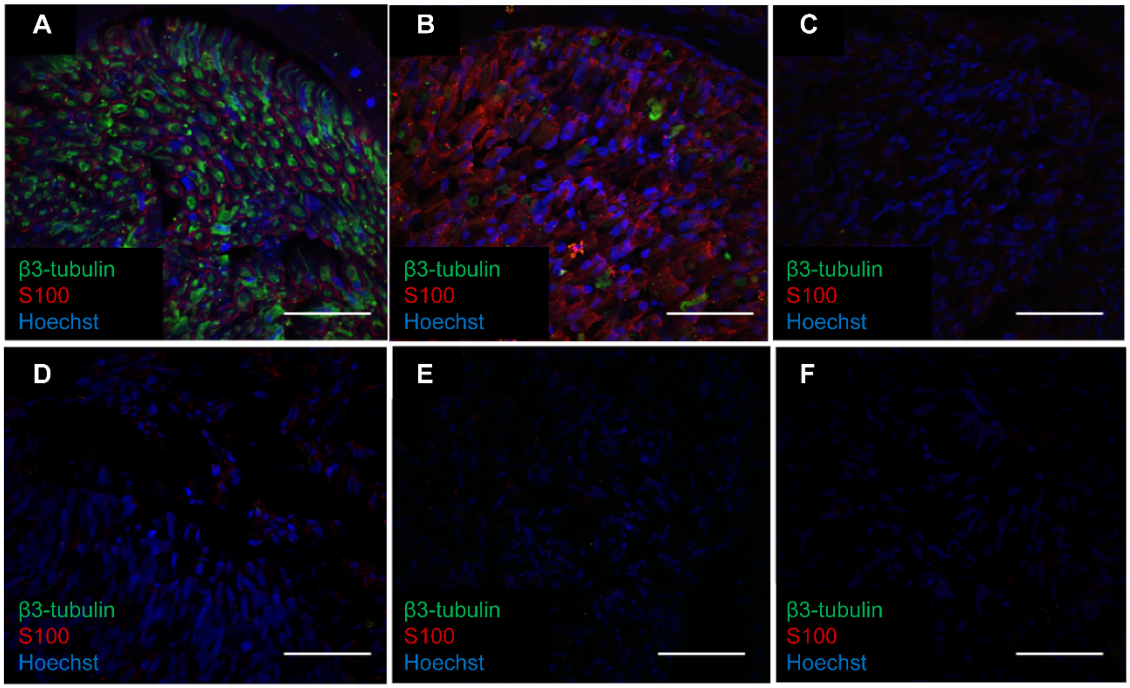


Figure 4

Caption: Evaluation of the transplant environment. Axial sections of the peroneal nerve harvested right before transplantation. 0-week group (A), 1-week group (B), 4-week group (C), 8-week group (D), 12-week group (E), 24-week group (F). Scale bar = 50 μm.

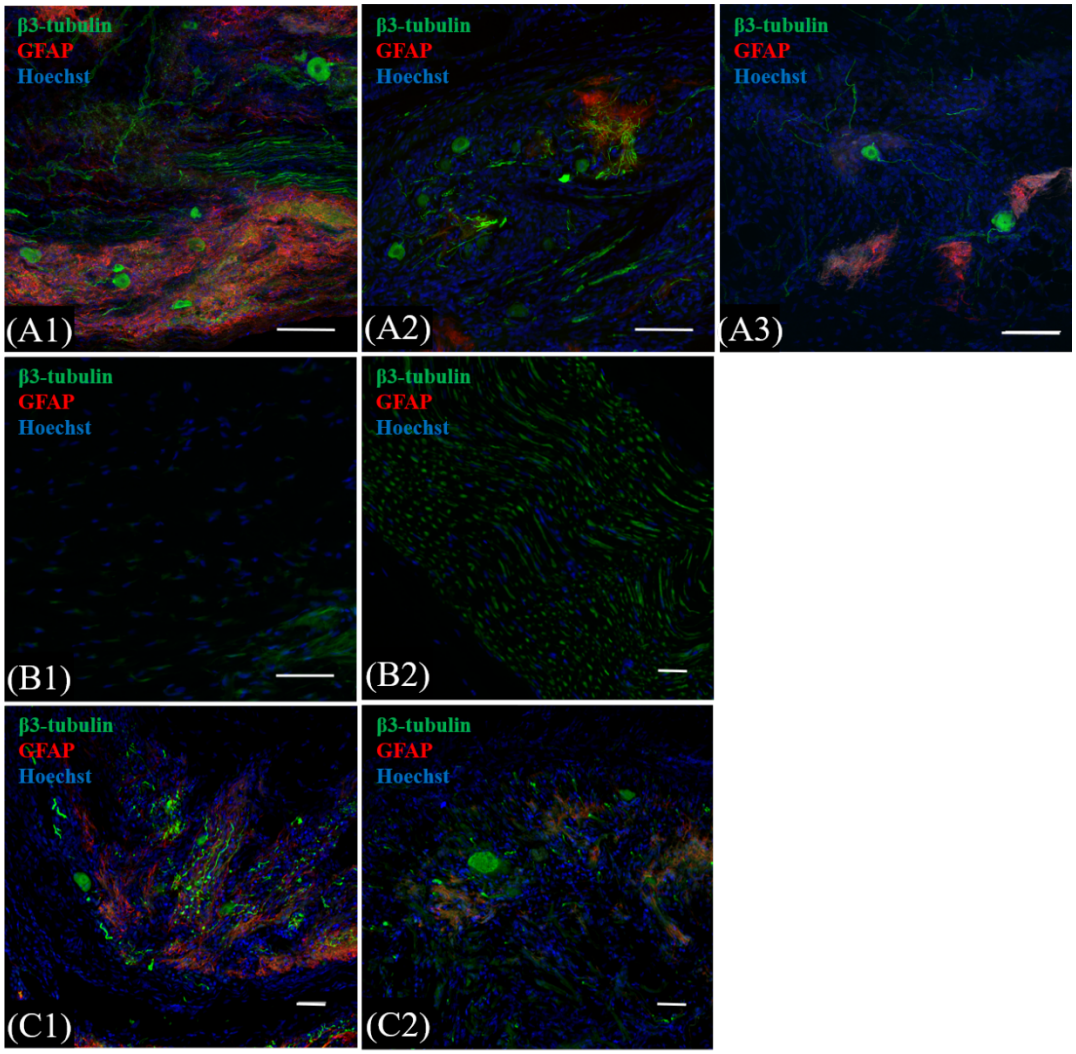


Figure 5

Caption: Morphological comparison of neuron survival. Sagittal sections of the peroneal nerve harvested at 3 months after transplantation (A1-3). 1-week (A1), 12-week (A2), and 24-week (A3) groups. Sagittal sections of the peroneal nerve (B1, 2). surgical control (B1) and naïve (B2) groups. Axial sections of the peroneal nerve harvested at 1 year after transplantation (C1, 2). 1-week (C1) and 12-week (C2) groups. Scale bar = 50 μm (A1–3, B1) or 20 μm (B2, C1,2).

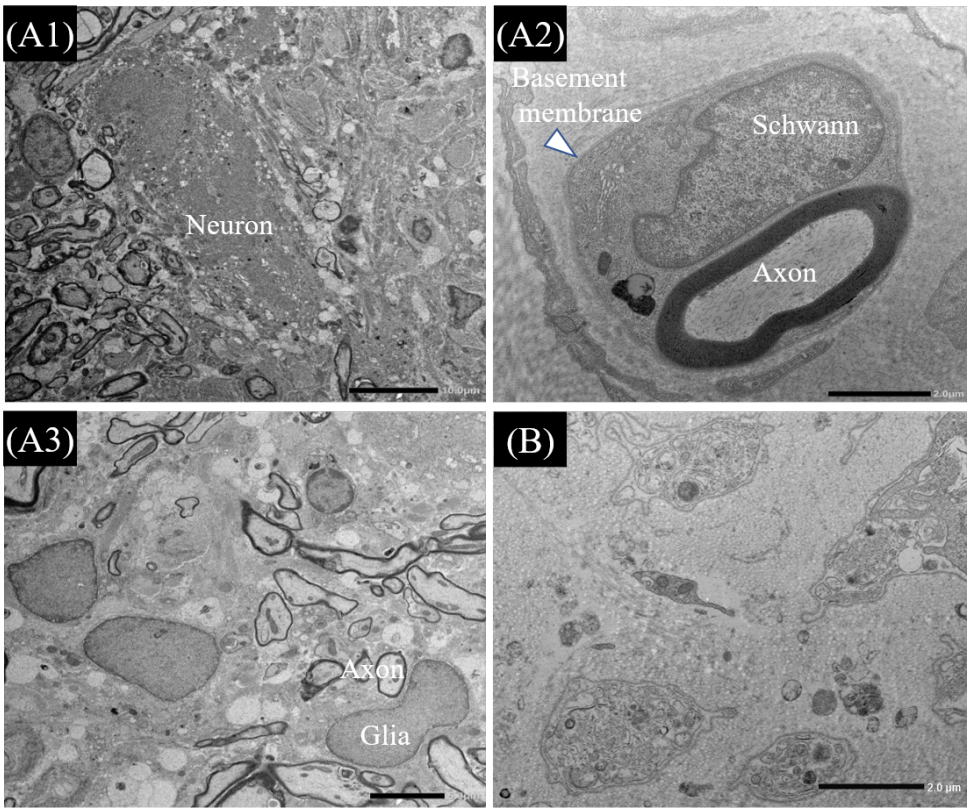
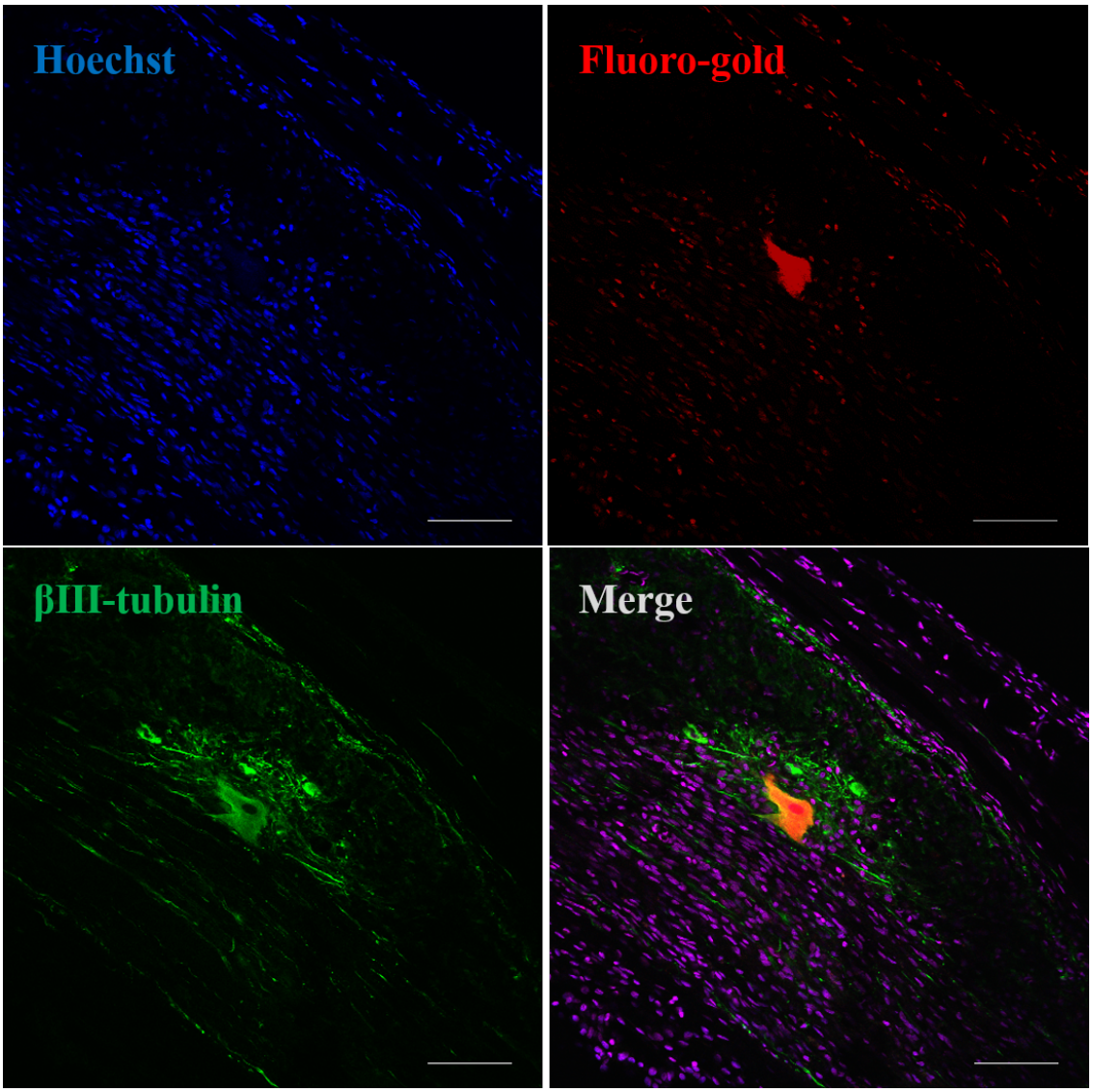


Figure 6

Caption: Electron microscopic images of transplantation (A1-3) and surgical control (B) groups. Electron microscopically confirmed neurons (A1); Schwann cells, axons with a thick myelin sheath, and basement membranes (A2); glia and axons with a thin myelin sheath (A3). Neither neurons, glia, nor axons were detected in surgical control groups (B). Scale bar = 10 μm (A1), 2 μm (A2, B), or 5 μm (A3).



Supplementary Figure.

Sagittal sections of the transplanted site of the peroneal nerve harvested at 1 week post Fluoro-Gold injection. Fluoro-Gold labeled motor neurons were observed. Merged image shows that the βIII-tubulin-positive neuron is labeled with Fluoro-Gold. Scale bar = 100 μm.