

MRI-Based Evaluation Improves the Validity of Rodent Nerve Regeneration Studies

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Rodent models are commonly used in preclinical studies on bioengineered conduits to induce nerve regeneration, but they often fail to predict human outcomes accurately. It is suspected that conventional evaluation methods, such as evoked electromyography and motor function assessments, are prone to errors and biases, which can lead to misleading conclusions. In this study, the limitations of conventional evaluation methods are highlighted by using muscle MRI to characterize pathophysiological changes in a rodent nerve injury model. Then, an alternative MRI-based evaluation method is proposed that quantified muscle cross-sectional area and T2 signal intensity, offering a non-invasive, repeatable evaluation with minimized errors and bias. Comparative analysis demonstrated the superior validity of the MRI-based evaluations. These findings suggest that MRI evaluation can enhance the validity of preclinical studies for developing nerve guide conduits and improve translational relevance to clinical outcomes.

grafts, artificial conduits are less effective in clinical practice^[6] and their use is limited to short defects in uncritical nerves.^[7] These discrepancies between rodents and humans are considered inevitable because of the strong regenerative capacity of rodents.^[2–5] The question remains: Do rodent nerves easily regenerate?

We doubt the validity of evaluations in rodent studies because of a lack of demonstrative evidence. Many conventional evaluation methods are based on logical evidence. They have been established deductively and applied without demonstrative validation. Marshall et al. highlighted the risk of using unvalidated evaluations. They reported that unvalidated evaluations were $\approx 40\%$ more likely to conclude that interventions were effective than validated ones.^[8,9]

1. Introduction

For more than 60 years, researchers have been developing artificial conduits that can be implanted in the nerve defects caused by trauma or tumor resection to induce regeneration.^[1,2] However, considering various materials and micro-structures have yielded promising results in basic research, the clinical breakthroughs achieved have been disappointing.^[3] This is due to the low translational relevance of rodent studies to clinical outcomes, although rodents are the most commonly used in preclinical research.^[2–5] While several rodent studies have reported that artificial conduits induce regeneration in nerve defects as well as autologous nerve

The purpose of this study was to clarify the limitations of conventional methods for evaluating rodent nerve regeneration and to propose a new evaluation method accompanied by demonstrative validation. Here, we characterized the pathophysiology of the common rat nerve injury model using magnetic resonance imaging (MRI), which is an increasingly used approach in neurological investigation,^[10] to identify the problems associated with conventional evaluation methods. In addition, we developed a novel nerve regeneration evaluation method that quantifies muscle MR images and validated this method using demonstrative evidence.

2. Results

2.1. Limitation of the Evoked Electromyography

We tested the accuracy of the evoked electromyography in reflecting nerve status. While evoked electromyography is widely used, certain studies have questioned its validity because the results obtained by this approach were inconsistent with those of other evaluations.^[11,12] The technique of evoked electromyography in clinical testing has been applied to rodent studies.^[7,13] To evaluate the rodent sciatic nerve, researchers typically stimulate the sciatic nerve and record the corresponding muscle electrical activity, the compound muscle action potential (CMAP), in the intrinsic foot or gastrocnemius muscles. We transected the sciatic nerve during a typical electromyography examination and observed changes in the CMAP before and after the transection (Figure 1).

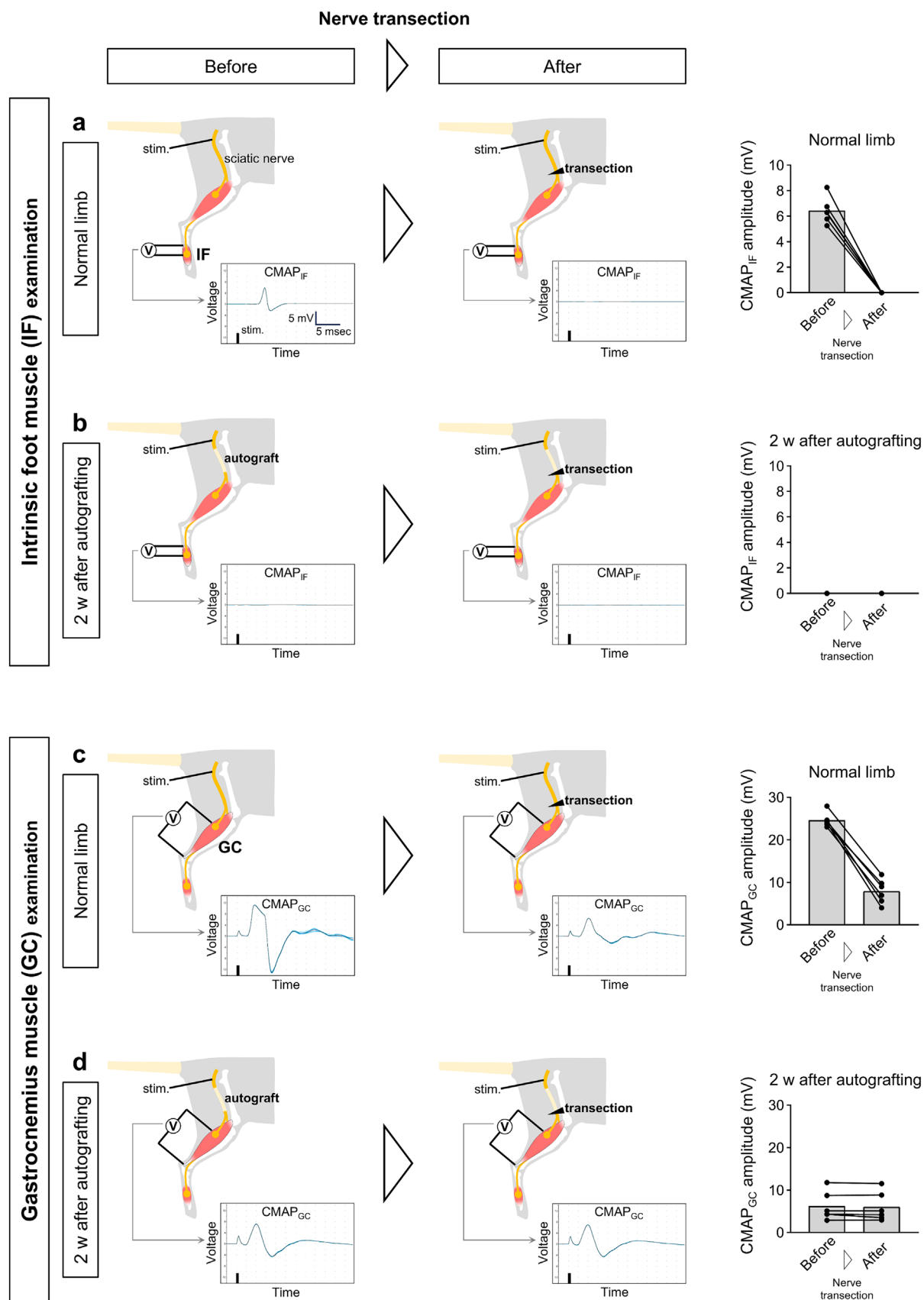
In normal limbs, the CMAP of the intrinsic foot muscles (CMAP_{IF}) disappeared after nerve transection (Figure 1a), as ex-

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pected. CMAP_{IF} was not present two weeks after autografting, even before transection (Figure 1b), indicating that the sciatic nerve did not regain conductivity. In contrast, the CMAP of the gastrocnemius muscles (CMAP_{GC}) in normal limbs did not disappear, and 23%–42% remained even after nerve transection (Figure 1c). A comparable CMAP_{GC} was detected 2 w after autografting, which was not changed by nerve transection (Figure 1d). These experiments demonstrated that CMAP_{GC} contains sciatic nerve-independent components that could lead to overestimation of sciatic nerve conductivity. Two weeks after autografting, electrical activity independent of the sciatic nerve could be mistaken as a sign of sciatic nerve regeneration.

2.2. Limitation of the Toe Motor Function Evaluation

The static sciatic nerve function index (SSI)^[14,15] is a common method for evaluating the recovery of motor function after sciatic nerve impairment. The SSI quantifies the toe spread function based on videos of the rat plantar pedis on a transparent floor (Figure 2b). The index is calculated from the distance between the toe tips and ranges between ≈ -100 and 0, with a higher value indicating that motor function is recovering.^[14,15] This evaluation method has the advantage of being noninvasive, and time-series data can be acquired through repeated evaluations. We prepared four groups of rats with right sciatic nerve defects repaired with different grafts (Figure 2a) and observed subsequent time-series recoveries with SSI: 1) The Auto group received autografts. 2) The Filled Conduit group received conduits containing collagen sponge. 3) The Hollow Conduit group received only the hollow conduits. 4) The defects were left unrepaired in the Defect group.

In the Auto group, the SSI began recovering 6 weeks postoperatively but regressed after 15 weeks postoperatively (Figure 3b). In addition, the number of SSI-available rats decreased over time (Figure 3c). These were caused by flexion contractures: moderate contractures shortened the distance between the toe tips and made the SSI lower than competence (Figure 3a, Auto-1), and severe contractures made the toe tips invisible and made the SSI unavailable (Figure 3a, Auto-2). In the Filled and Hollow Conduit groups, the SSI remained around -100 , indicating complete paralysis throughout the period with almost no recovery (Figure 3b). These results indicate that the SSI was not valid for long observation periods owing to flexion contractures and was not sensitive enough to detect slight recovery in the Filled and Hollow Conduit groups.

2.3. Pathophysiology of the Rat Sciatic Nerve Injury Model Based on Muscle MRI

To clarify which muscles are paralyzed and which motor deficits appear in the rat sciatic nerve injury model, we conducted an MRI scan of a rat with sciatic nerve transection. In denervated muscles, extracellular fluid increases due to enhanced muscle

cell membrane permeability^[16] and impaired Na⁺-K⁺-ATPase function.^[17] Therefore, MRI highlights denervated muscles with increased T2 signals^[18] and provides in situ 3D visualization of each muscle.

Figure 4c is an MRIs of a rat four weeks after right sciatic nerve transection, when the denervated muscles had sufficiently increased T2 signal intensity to contrast well with normal muscle but were not yet completely atrophied. The muscle T2 hyperintensity was not due to non-neural factors (e.g., traumatic response or ischemia) because the hyperintensity was not observed in the lateral thigh muscles (Figure 4c slice location 38), the most directly invaded by the operation, and because the operation did not damage any blood vessels that fed the muscles.

In the lower leg on the operated side, not all muscles were denervated, and the posterolateral and anteromedial muscles were intact (Figure 4c, asterisk and dagger). The graph showing the distribution of intact and denervated muscles (Figure 4d) revealed that the proximal half of the lower leg was dominated by intact muscles. Matching the 3D reconstructed MRI (Figure 4e) with the dissection findings (Figure 4f) revealed that the posterolateral intact muscles (asterisk) were the lateral hamstrings (biceps femoris), and the anteromedial ones (dagger) were the medial hamstrings (semitendinosus, semimembranosus, and caudo-femoris). To understand this result, the difference between the human and rat hamstrings should be noted (Figure 4g). The human hamstrings are attached to the proximal portion of the lower leg bone, whereas rat hamstrings extend in a fan shape over the lower leg muscles.^[19] The neurological status of each muscle is shown in Figure 4h.

These findings explain why, in evoked electromyography, the CMAP_{GC} contained a sciatic nerve-independent component (Figure 1c,d), even though the gastrocnemius muscle was innervated exclusively by the sciatic nerve.^[19] The gastrocnemius muscle was denervated by sciatic nerve injury but was covered by intact hamstrings (Figure 4h). Therefore, the CMAP_{GC} is confounded by the electrical activity of the hamstrings. In theory, CMAP_{GC} is more useful than CMAP_{IF} because the gastrocnemius muscle produces a higher CMAP amplitude owing to its thicker body and is reinnervated earlier owing to its proximal location. However, in practice, CMAP_{IF} allows a more valid evaluation, because the intrinsic foot muscles are distant from the hamstrings, and CMAP_{IF} is hardly confounded by the electrical activity of the intact hamstrings.

2.4. A Novel MRI Evaluation Method

We developed an MRI-based evaluation method that measures muscle cross-sectional area and T2 signal intensity. The cross-sectional area of the muscle is considered to reflect the degree of nerve regeneration,^[20] given that denervation causes progressive atrophy and reinnervation gradually improves atrophy. Similarly, the T2 signal intensity of the muscle is considered to reflect the degree of nerve regeneration because numerous basic and clinical

Figure 1. CMAP changes before and after sciatic nerve transection. a) CMAP_{IF} in normal limbs. b) CMAP_{IF} in limbs two weeks after autografting. c) CMAP_{GC} in normal limbs. d) CMAP_{GC} in limbs two weeks after autografting. CMAP waveforms of a representative rat. Stim., stimulation; circled V, voltmeter comprising the electromyograph system. The graph shows the CMAP amplitudes of six rats. The dots connected by the line show the changes in each rat, and the bars show the mean.

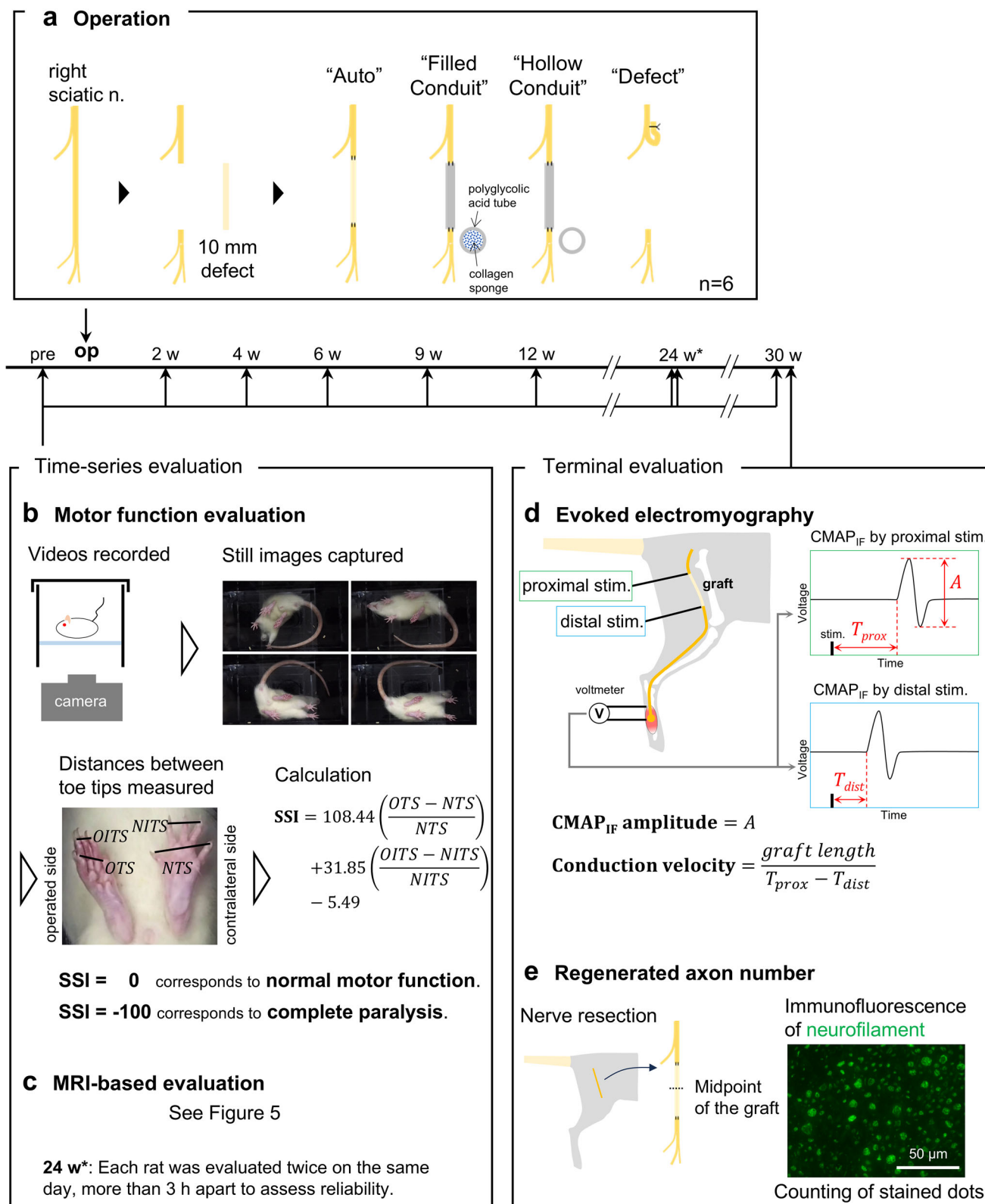


Figure 2. Overview of the operation and evaluation. a) Four groups of rats undergoing different operations were prepared for time-series recovery evaluation and to assess the correlation between different evaluation methods. b, c) Noninvasive methods were applied for the time-series evaluation. The time points were at 2, 4, 6, 9, 12, 15, 18, 21, 24, 27, and 30 weeks postoperatively. d, e) Invasive methods were performed at the termination of the study.

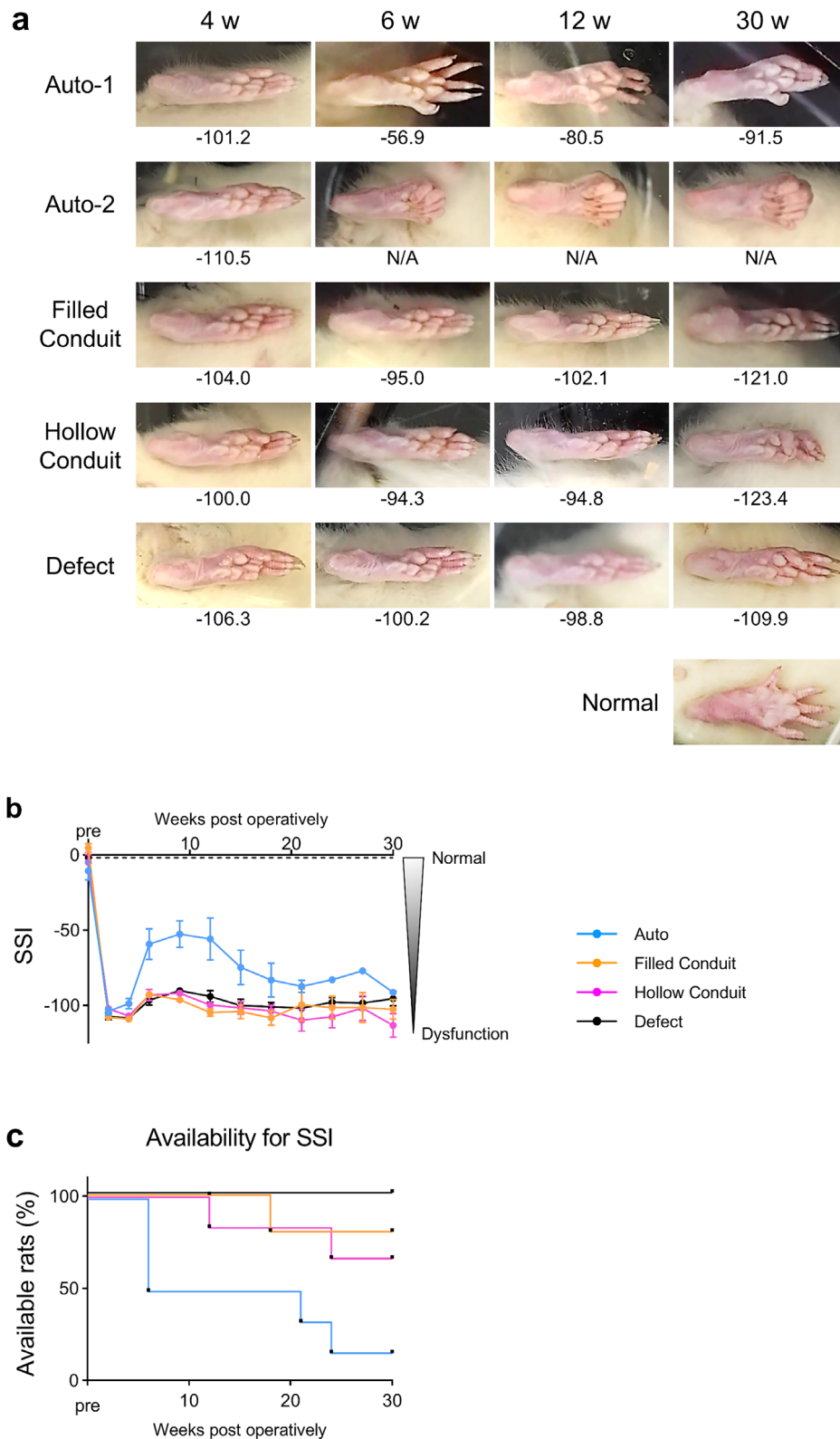
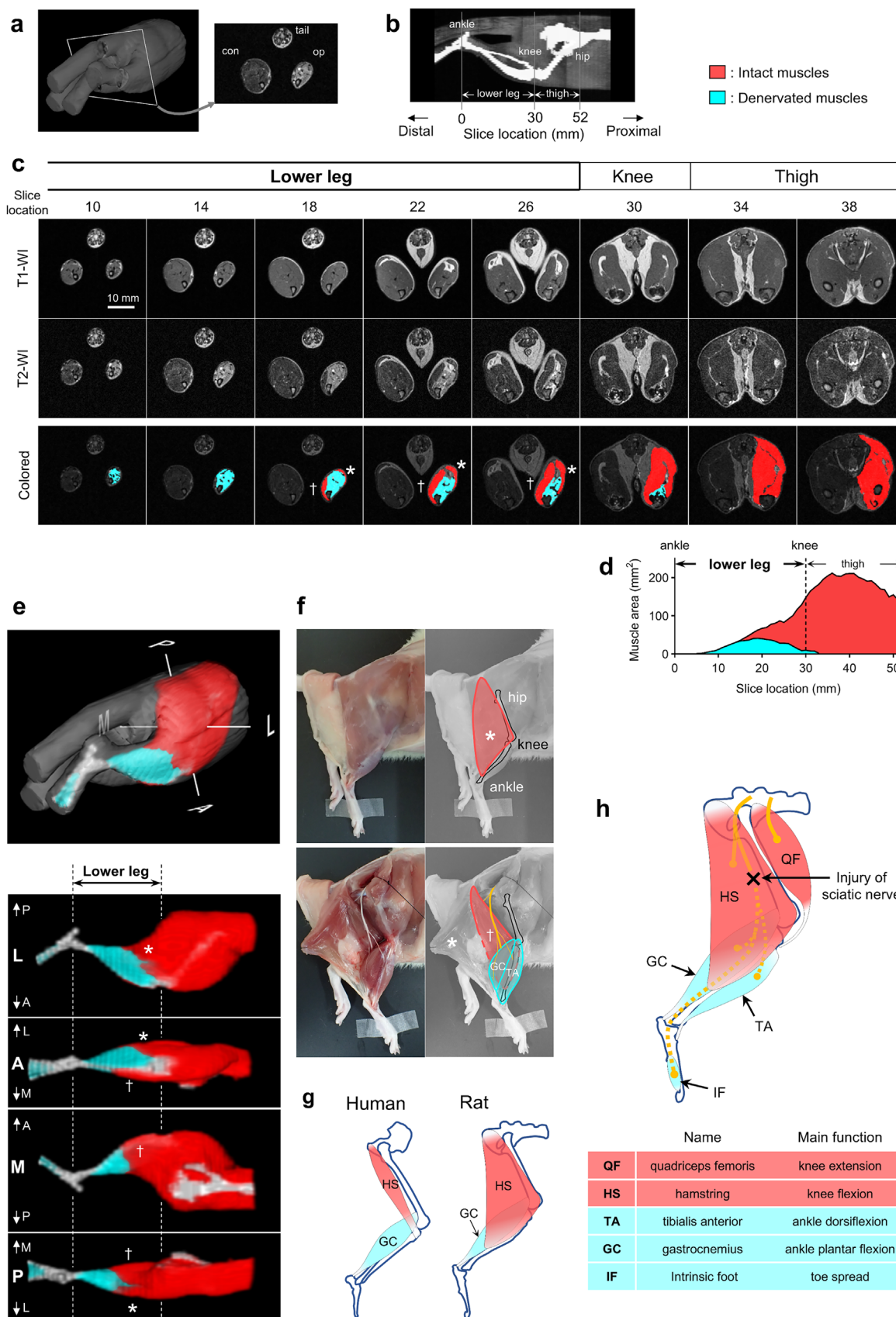


Figure 3. Time series evaluation by SSI. a) Photographs of the plantar pedis of representative individuals in each group. The numbers below are the calculated SSI values; N/A, not available for the SSI. b) SSI changes in the four groups. Data are expressed as the mean $\pm$ standard error ($n = 6$). c) The availability for SSI over time is described by Kaplan–Meier curves. Loss of measurement because of flexion contracture was regarded as an event, and accidental death of the rat was regarded as censoring. None of the rats whose SSI was unavailable were returned.



cal studies have shown that a high T2 signal in denervated muscles is restored with reinnervation.^[18,20,21]

To minimize the influence of examiner skill and arbitrariness, we designed a simple and unambiguous evaluation protocol (Figure 5a). The hind limbs were set symmetrically. The axial T1-weighted images and T2-weighted images of the entire lower leg were scanned at 2 mm intervals. Afterward, a slice just distal to the end of the tibial crest, which is a distinct landmark in axial images, was selected for analysis. This slice corresponded to the middle level of the lower leg, and preliminary experiments demonstrated that it was free of hamstrings and the most appropriate for analysis (Figure S1, Supporting Information). While our method did not require the precise positioning skills or efforts of an MRI machine operator, the position of the selected slice might theoretically deviate up to 2 mm from the exact level of the distal end of the tibial crest. However, we confirmed that slices from the end of the tibial crest to 6 mm distal to it were available for the subsequent analysis (Figure S1a, Supporting Information).

Image analysis was automated using a macro in Fiji,^[22] an image analysis software, to eliminate examiner bias and errors. Figure 5b shows the macro mechanism. First, the macro detects muscle regions from T1-weighted images. Because T1-weighted images have higher contrast than T2-weighted images and the T1 signal intensity of the muscle is hardly affected by the state of the nerve, muscle regions can be detected accurately from T1-weighted images by the following process: Detect regions of *whole* lower leg cross-section by binarizing and filling the holes (i), detect *fat* by picking up pixels brighter than surrounding pixels (ii), detect *bone and tendon* by picking up pixels darker than the surrounding pixels (iii), and then subtract the *fat, bone, and tendon* from the *whole* region and the *muscle* region remains (iv). Second, the macro overlays the detected *muscle* regions on the T2-weighted image (v). Finally, the macro measures the area and average T2 signal intensity of the muscle region of each side (vi). The full code of the macro is provided in the Note S1 (Supporting Information).

The operated/contralateral ratios of the muscle cross-sectional area (A_{op}/A_{con}) and T2 signal intensity (I_{op}/I_{con}) were used as nerve regeneration indices.

2.5. Reliability of the MRI Evaluation Method

Reliability, the reproducibility of measurement, is a prerequisite for validity assessment because the upper limit of validity is regulated by reliability.^[9,23] The intraclass correlation coefficient (ICC)

and standard error of measurement calculated using the test-retest method are common indicators of reliability.^[9,23,24] To assess the reliability of the MRI evaluation, each rat in the four groups was tested twice on the same day, 24 weeks postoperatively, to assess the agreement between the two measurements (Figure 2).

In both A_{op}/A_{con} and I_{op}/I_{con} , all individuals in the four groups were plotted near the line $x = y$ (Figure 6a), suggesting that both A_{op}/A_{con} and I_{op}/I_{con} had sufficient reliability. Because ICC and standard error of measurement were only available for populations normally distributed and not for mixed populations of different groups,^[23,24] these values were calculated within each group (Figure 6b). The Filled and Hollow Conduit groups showed high ICCs for both A_{op}/A_{con} and I_{op}/I_{con} , indicating high reliability. The Auto and Defect groups showed low ICCs, but these groups were not suitable for ICC assessment because the individuals in each group clustered in small ranges in the test-retest plots (Figure 6a), and the ICCs were calculated as low values. The measurement error of A_{op}/A_{con} was expected to be within 0.1, and the measurement error of I_{op}/I_{con} was expected to be within 0.2, according to the 95% confidence interval of the standard error of measurement (Figure 6c). These results demonstrate that the MRI evaluation is reliable enough to be worthy of validity assessment.

2.6. Validity of the MRI Evaluation Method

We tested the validity of the MRI evaluation by comparing the time-series recovery of the four groups of rats in the same manner as for the SSI (Figure 2, time-series evaluation). In terms of A_{op}/A_{con} (Figure 7b), the Auto group began recovering 6 weeks postoperatively, followed by steady improvement. The Filled and Hollow Conduit groups recovered more slowly and weakly than the Auto group. The Defect group did not recover during the study period. These recovery patterns in the four groups held true in terms of I_{op}/I_{con} (Figure 7c). These results demonstrate that both A_{op}/A_{con} and I_{op}/I_{con} are valid indicators of nerve regeneration. The vigorous nerve regeneration provided by the autologous nerve graft was reflected in A_{op}/A_{con} and I_{op}/I_{con} as the Auto group's rapid recovery; paradoxical regression, as seen in the SSI, was not observed. A_{op}/A_{con} and I_{op}/I_{con} detected the slight regeneration in the Filled and Hollow Conduit groups, which was not detectable using SSI. The absence of recovery in the Defect group indicated that A_{op}/A_{con} and I_{op}/I_{con} did not contain a sciatic nerve-independent component, unlike CMAP_{GC}.

Figure 4. MRI findings in a rat four weeks after right sciatic nerve transection. a) Orientation of the cross-sectional view. The upper side of the image is posterior, the lower side is anterior, the right side is operated upon (op), and the left side is contralateral (con). b) Definition of slice location. The numbers indicate the distance in millimeters from the level of the ankle joint. In this rat, 30 corresponded to the knee joint and 52 to the hip joint. c) Scanned images. T1-WI, T1-weighted images; T2-WI, T2-weighted images. The colored images highlight the intact muscles in red and the denervated muscles in blue. Asterisks indicate intact muscles in the posterolateral portion of the lower leg, and daggers indicate intact muscles in the anteromedial portion of the lower leg. d) Graph of the relationship between slice location and intact/denervated muscle sectional area. e) 3D reconstruction of the colored images. The bones were highlighted in white as anatomical landmarks. L, lateral; A, anterior; M, medial; P, posterior. Asterisks and daggers correspond to those in Figure 4c. f) Dissection findings in a retired rat. In the upper photograph, the skin was removed. The lateral hamstring is the muscle that covers the posterolateral aspect of the lower leg (asterisk). In the lower photograph, the lateral hamstrings are detached and retracted. The medial hamstring is the muscle that covers the anteromedial aspect of the lower leg (dagger). Yellow line, sciatic nerve and branches; GC, gastrocnemius and adjacent muscles; TA, tibialis anterior and adjacent muscles. g) Anatomical differences between the human and rat hamstrings. HS, hamstrings; GC, gastrocnemius. h) Illustration of the neurological status of each muscle.

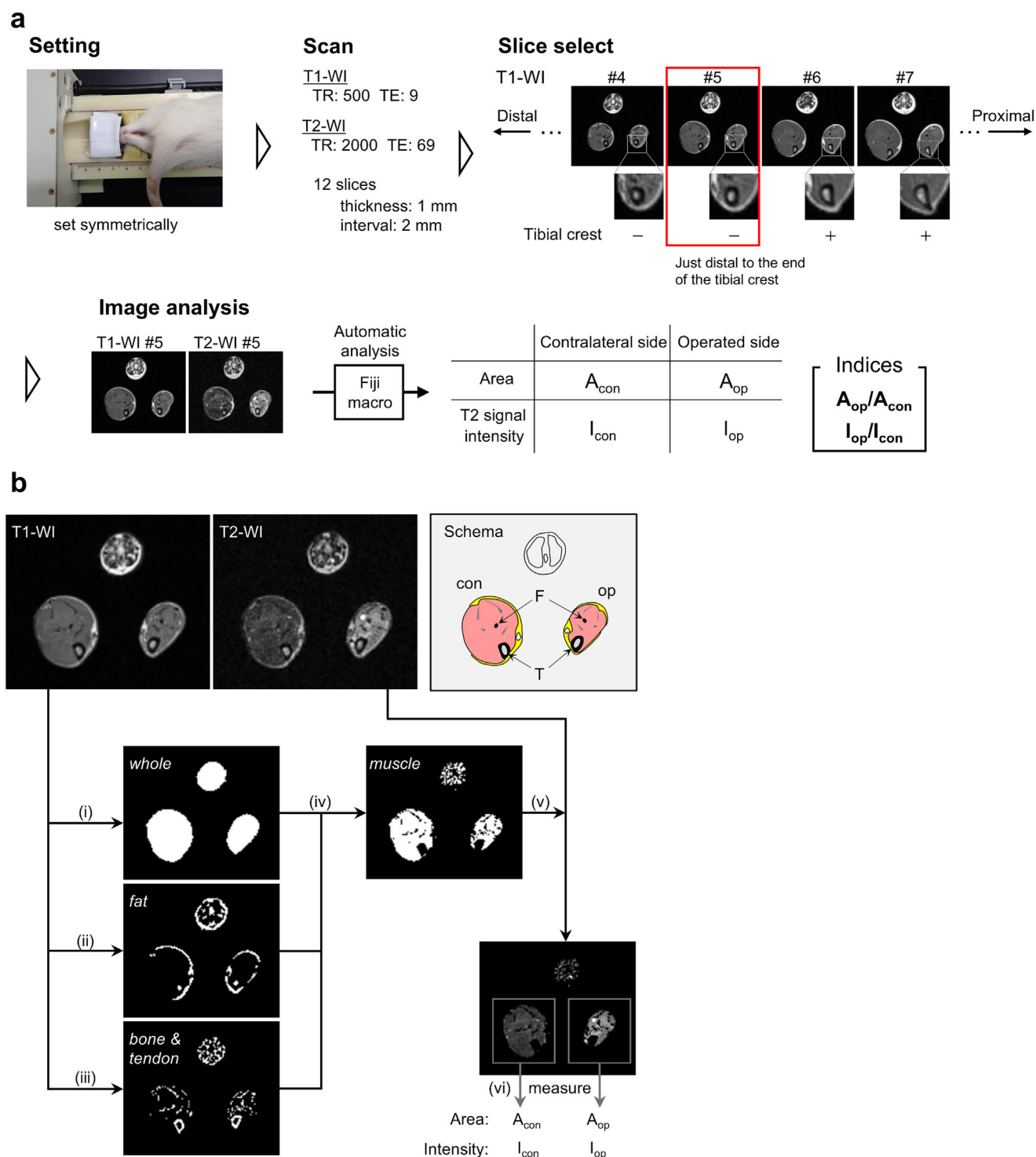


Figure 5. Novel MRI-based evaluation method. a) Overview of the protocol. b) Mechanism of the macro for automated image analysis. In the schema, red areas indicate muscle, yellow areas indicate fat, black areas indicate bones (T, tibia; F, fibula), and gray areas indicate tendons.

All rats were available for the MRI evaluation throughout the study period (Figure 7d). There were no scan failures, except for the death of one rat in the Filled Conduit group owing to an accident caused by failure of anesthesia 12 weeks postoper-

atively. We visually confirmed the availability of the automated image analysis; the Fiji macro accurately identified the muscle regions to be measured in the images of all rats at all time points.

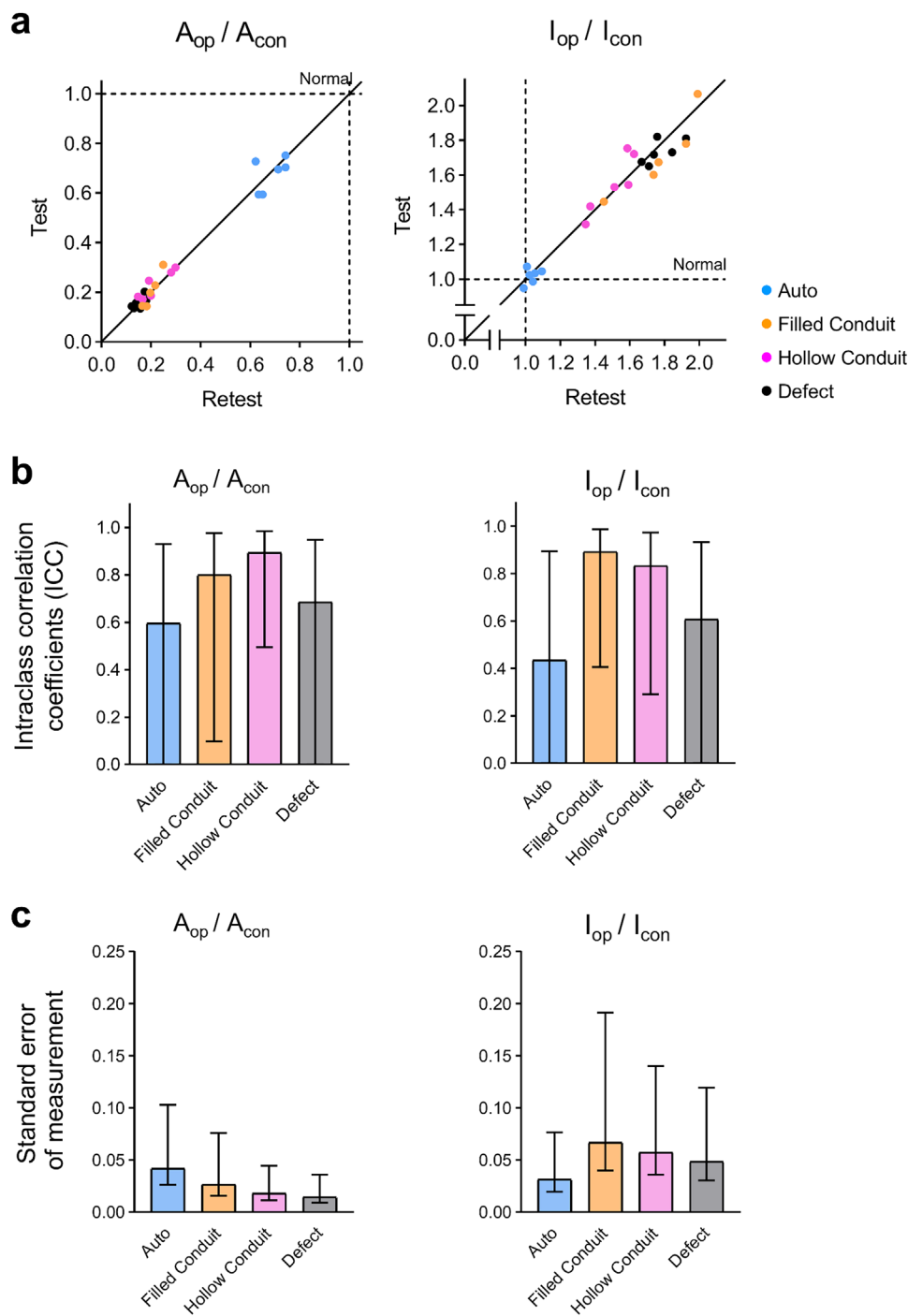


Figure 6. Reliability of MRI-based evaluation. a) Plot of the test-retest method. b) Intraclass correlation coefficients (ICC). c) Standard error of measurement. The colored and error bars indicate estimates and 95% confidence intervals, respectively.

2.7. Comparison of the Validity of the Conventional and the MRI Evaluation Methods Based on Correlation with the Regenerated Axon Numbers

Sprouting and extension of regenerated axons from the proximal nerve stump is a primordial phenomenon that precedes muscle reinnervation or motor function recovery. Histological

counting of the regenerated axons in harvested grafts is a common method for evaluating nerve regeneration in rodent models. In the early phase after nerve repair, not all regenerated axons in the graft reach the target muscles.^[7,13,25] However, after a sufficient period, regenerated axons that fail to reach the target muscle disappear, whereas those that successfully reinnervate muscles survive; consequently, the number of regen-

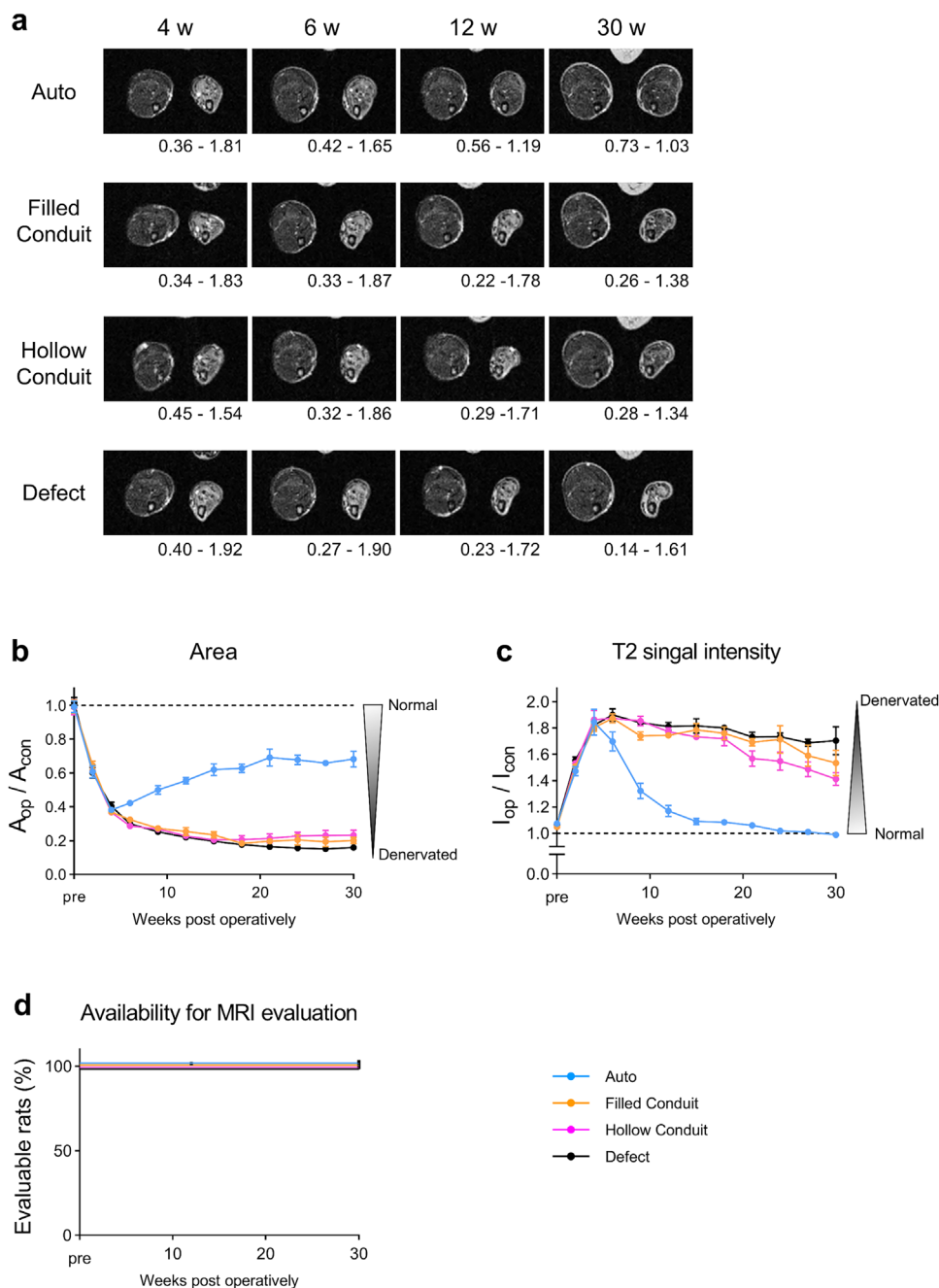


Figure 7. Time-series evaluation using the MRI-based method. a) Images of representative individuals in each group. The numbers below indicate $A_{op}/A_{con} \cdot I_{op}/I_{con}$. b) A_{op}/A_{con} changes in the four groups. c) I_{op}/I_{con} changes in the four groups. d) Availability for the MRI evaluation over time. In the Kaplan–Meier curves, the accidental death of the rat was regarded as censoring.

erated axons correlates more directly with muscle reinnervation or motor function recovery.^[13] Therefore, an evaluation method that directly correlates the number of regenerated axons can be considered valid. We counted the regenerated axons 30 weeks postoperatively and compared the conventional evaluations with MRI-based methods in terms of the correlation to the number of regenerated axons (Figure 2e). The correlation was assessed using Spearman's rank correlation coefficient (r_s).

The SSI showed a negligible correlation with the number of regenerated axons ($r_s = -0.02$; Figure 8a). This was because SSI was diminished or unavailable owing to the flexion contracture of the toes in rats with a high number of regenerated axons. This result is consistent with that shown in Figure 3.

In evoked electromyography, the amplitude of CMAP_{IF} showed a significant correlation with the number of regenerated axons ($r_s = 0.88$; Figure 8b), and the conduction velocity showed a significant but weaker correlation ($r_s = 0.83$; Figure 8c). These

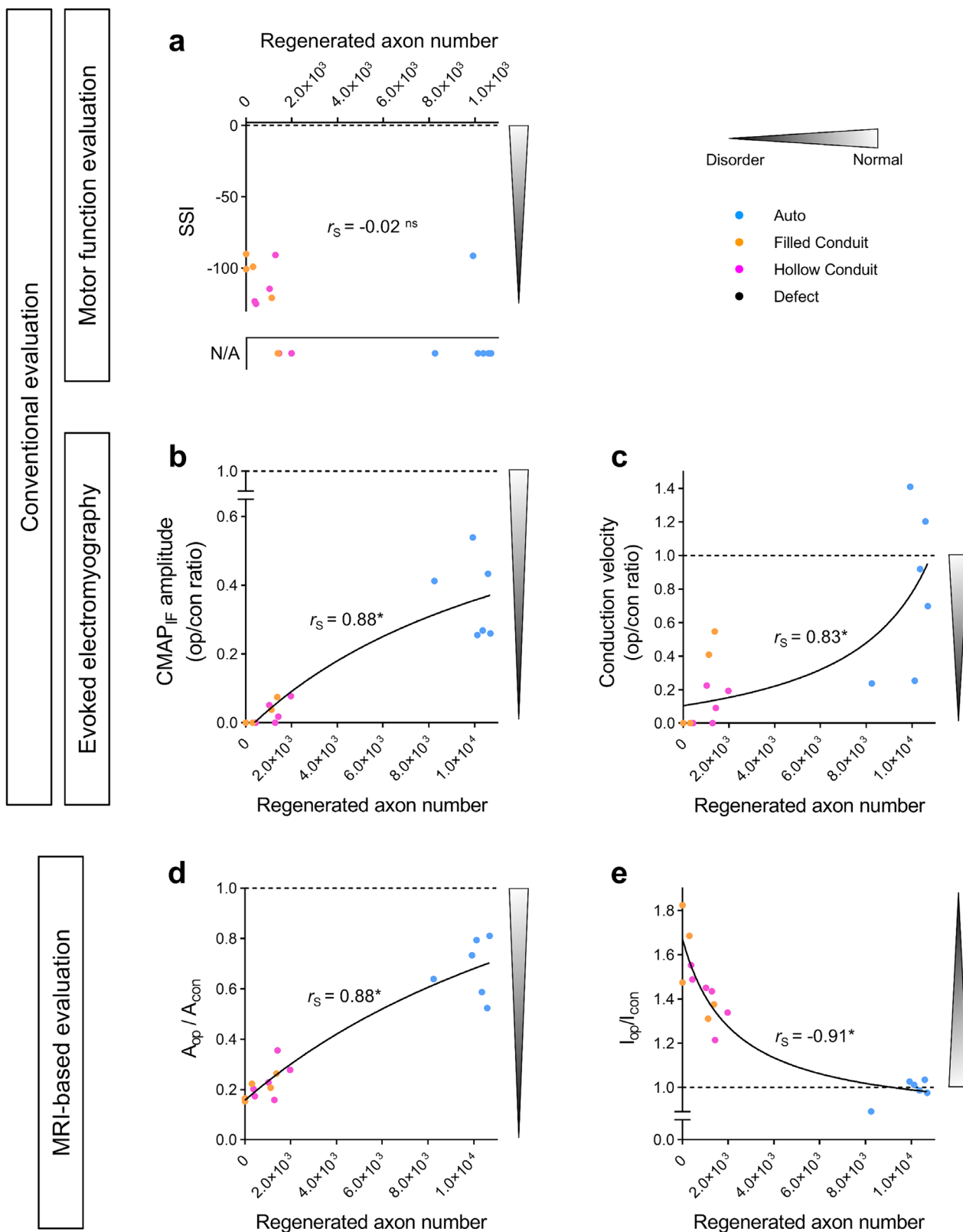


Figure 8. Correlation with the number of regenerated axons. a) Correlation between the SSI and the number of regenerated axons. b) Correlation between the CMAP amplitude and the number of regenerated axons. c) Correlation between the nerve conduction velocity and the number of regenerated axons. d) Correlation between the A_{op}/A_{con} and the number of regenerated axons. e) Correlation between the I_{op}/I_{con} and the number of regenerated axons. r_s , Spearman's rank correlation coefficient; $^*p < 0.01$. Approximating curves were fitted using the [1/1] Padé approximation.

results indicate that the conduction velocity has a lower validity. This was due to the complexity of the procedure for measuring the conduction velocity, which increased the susceptibility to measurement errors. As shown in Figure 2d, the measurement of conduction velocity requires CMAP recordings at two stimulus points to calculate the difference in latency (time lag from stimulus to CMAP onset), whereas amplitude can be measured from a single CMAP recording.^[26]

In the MRI evaluation, both A_{op}/A_{con} and I_{op}/I_{con} showed strong correlations ($r_S = 0.88$ and -0.91 , respectively) with the number of regenerated axons, which suggests high validity of these indices. While A_{op}/A_{con} showed an approximately linear correlation (Figure 8d), I_{op}/I_{con} showed a strongly curved correlation (Figure 8e); I_{op}/I_{con} changed more steeply in the lower region of the regenerated axon number. This result indicates that I_{op}/I_{con} is more sensitive to slight regeneration than A_{op}/A_{con} . This was consistent with the time-series change in the Auto group (Figure 7b,c); A_{op}/A_{con} showed a constant and gradual recovery throughout the period, whereas I_{op}/I_{con} showed rapid recovery in the early phase (6–12 weeks postoperatively) when nerve regeneration was immature.

3. Discussion

This study identified problems with common conventional evaluation methods and indicated that the results could be misleading. Evoked electromyography is disturbed by the hamstrings, the anatomical structures of which in rodents and humans are different. Given the anatomy of the rodent, it is difficult to prevent evoking electrical activity in the hamstrings wherever the sciatic nerve is stimulated (Figure S2, Supporting Information). When the sciatic nerve is stimulated at the level of the lumbus, the nerve branch that goes to the hamstrings is excited (Figure S2a, Supporting Information), and when it is stimulated at the level of the thigh, the stimulating current leaks directly into the surrounding hamstrings (Figure S2b, Supporting Information). Although CMAP_{IF} is hardly confounded by the electrical activity of the hamstrings, the inherent technical complexity limits the reliability of electromyography.^[13] Particularly in rodents, the results of evoked electromyography are susceptible to random errors and examiner bias because rodents are so small that slight differences in electrode positions can affect the results.

Our MRI findings on the pathophysiology of rodent sciatic nerve injury shed light on the gait function. Gait impairment rapidly recovers in rodent models of sciatic nerve injury. Even rats with a defective sciatic nerve (the Defect group) dragged their foot behind, but within a few days, they walked almost normally, pushing off their foot against the floor. Figure 4h shows that the muscles responsible for knee extension (QF) and flexion (HS) were intact. The ankle moved with the knee owing to the dynamic tendinosis effect (Figure S3, Supporting Information). Therefore, rodents with complete sciatic nerve paralysis can move their knees and ankles and regain gait function independently of sciatic nerve regeneration, probably because of adaptation to impaired conditions.

The rapid recovery of the rodent gait function and the early appearance of CMAP_{CC} have been interpreted as an indication that the rodent nerve regenerated easily. However, we found that these symptoms were not necessarily caused by nerve regenera-

tion and that rodent nerves did not regenerate easily. According to our results, it takes 6 weeks for rat nerves to regenerate across a 10 mm gap with autografts and over 20 weeks with artificial conduits. Although rodent nerves have been criticized for regenerating too easily to be an appropriate model,^[2–5] our results suggest that they work sufficiently for a preclinical model if they are evaluated validly.

SSI, an evaluation based on toe motor function, is disrupted by flexion contracture, which has been reported to occur frequently in nerve grafting models.^[27–29] In the studies that established the SSI^[14] and its original method, SFI,^[30,31] validation was primarily performed with sciatic nerve crush models, which rarely develop flexion contracture.^[32] Even the validated methods may not be valid when applied to other models.

The first advantage of our MRI evaluation method is its non-invasiveness, which enables repeated evaluations and provides time-series data. In invasive and one-time conventional methods, it is impossible to demonstrate the reliability of the evaluation using the test-retest method. In addition, a one-time-point evaluation can overlook differences in the capacity of nerve regeneration because poorly regenerating groups catch up with the superior group at later time points.^[33] However, there is no consensus regarding the timing of evaluation. Therefore, a time-series evaluation is more convincing and sensitive than a one-time-point evaluation in detecting differences. To demonstrate this advantage, we attempted to determine whether filling nerve guide conduits with a 3D structure would increase their capacity to induce regeneration.^[34,35] but no definitive conclusions could be drawn from the time-series data. We could not determine whether filling the conduit with the collagen sponge did not improve the capacity or whether our evaluation was not sufficiently sensitive.

Another advantage of our MRI evaluation method was its technical simplicity. We designed a simple and unambiguous scanning protocol and a fully automated image analysis using computer software to minimize the influence of examiner skill, arbitrariness, and assumptions. This technical simplicity may contribute to the reliability. The protocol can be further simplified, and its reliability can be further enhanced with superior MRI equipment that can measure absolute T2 values using mapping sequences.

Finally, this study demonstrated the validity of I_{op}/I_{con} and suggested that this parameter may be more sensitive than A_{op}/A_{con} . Moreover, for models of spinal cord injury or systemic neurological disease in which both sides are impaired and the ratio of the impaired side to the normal side is unavailable, the T2 signal intensity (I) is a more promising evaluation indicator than the cross-sectional area (A). This is because bilaterally impaired models can be evaluated based on T2 signal intensity in absolute value measured by a mapping sequence or in ratio to a phantom set beside the legs, whereas evaluation based on the absolute value of the cross-sectional area of the leg muscle would make little sense because it varies between individuals and increases with age.

4. Conclusion

We demonstrated that conventional evaluation methods in a rodent sciatic nerve injury model could produce misleading results, because they show nonspecific reactions that do not depend on nerve regeneration. The proposed MRI-based evaluation method,

with demonstration of superior validity, can be applied to rodent models other than sciatic nerve injury and will improve the translational relevance to clinical results.

5. Experimental Section

Animals: All animal experiments were performed in accordance with the guidelines for animal experiments of the Ministry of Health, Labor, and Welfare of Japan and the National Cerebral and Cardiovascular Center Research Institute of Japan. The Committee on Ethics of Animal Experiments at the National Cerebral and Cardiovascular Center Research Institute approved the study protocol (approval number: 22053). In this study, female Lewis rats aged 11–12 weeks (Japan SLC, Hamamatsu, Japan) were used in all experiments except for one. The rats were randomly assigned to the experimental groups and housed them under the same conditions. The animals were regularly weighed and confirmed that none of them were extremely heavy or light. Lewis rats were well suited for evaluating toe motor function in nerve injury models because they rarely self-mutilate their numbed feet compared to other rat strains.^[36,37] Females were chosen because they showed little growth in body size after 10 weeks of age and provided consistent conditions for MRI scanning throughout the long observation period. The one exception was the dissection experiment (Figure 4f), presenting universal anatomical features of rats. Since sex, strain, or age did not matter in this experiment, a retired male Sprague–Dawley rat was used to minimize the number of sacrificed animals.

Conduit Preparation: A commercially available nerve guide conduit (Nerbridge, inner diameter of 2.0 mm, Toyobo, Osaka, Japan) was used for the Filled Conduit group. The conduit was made of braided polyglycolic acid fibers, and the lumen was filled with a collagen sponge to support the migration of cells involved in regeneration. To prepare the sample for the Hollow Conduit group, the collagen sponge was removed by incubating with 2100 U mL⁻¹ collagenase type I solution (Fujifilm Wako Pure Chemical, Osaka, Japan) at 37 °C for 4 h, followed by the deactivation of collagenase with 0.1 mM EDTA solution (Dojindo, Kumamoto, Japan) at 4 °C overnight.

Surgical Procedure: During surgery for nerve defects and grafting, the animals were anesthetized by inhalation of 2.5% isoflurane (Pfizer, New York, USA), and the body temperature was maintained using a heating pad. All surgical procedures were performed under a stereoscopic microscope (SZX7, Olympus, Tokyo, Japan). The right sciatic nerve was approached through the intermuscular plane between the vastus lateralis and biceps femoris muscles and exposed from the distal margin of the piriformis muscle to the popliteal fossa. Sciatic nerves were resected to a length of 10 mm (Figure 2a). For autografting, the resected segments were flipped in the opposite direction to bridge the gap with 10-0 string (Asflex, Kono Seisakusyo, Tokyo, Japan) epineural simple sutures (Auto Group). During conduit grafting, the gap was bridged with a 14-mm-long conduit, with nerve stumps placed 2 mm inside either side of the conduit. Two or three 9-0 epineural loop sutures were placed on each side (the Filled and Hollow Conduit groups). As a negative control, the defects were left unrepaired, and the proximal stump was ligated and inversely fixed with a 9-0 string to ensure that spontaneous repair was prevented (Defect group). Finally, the wounds were closed in layers; the fascia was approximated using 5-0 nylon; and the skin was approximated using 3-0 silk.

Evaluation of the Toe Motor Function: Based on Bervar's method^[14] toe spread function was evaluated using SSI (Figure 2b). The animals were placed in a dark box with a transparent bottom, and after acclimation, their plantar pedis were video-recorded. From the video, four static images were selected at different times, while the animals stood still. The following variables were measured in each image using Fiji: the distance between the first and fifth toes (operated side, OTS; contralateral side, NTS), and the distance between the second and fourth toes (operated side, OITS; contralateral side, NITS). SSI was calculated using the following equation:

$$SSI = 108.44 \left(\frac{OTS - NTS}{NTS} \right) + 31.85 \left(\frac{OITS - NITS}{NITS} \right) - 5.49 \quad (1)$$

MRI Scanning and Image Processing: Anesthesia was administered in the same manner as during surgery. A small-animal MRI system (MR-mini SA, Neomax Engineering, Takasaki, Japan) was used that can be installed in a normal laboratory and involves simple scanning procedures. The animals were secured to the coil in the prone position, with their hind limbs stretched caudally, ensuring that the knee and ankle positions were symmetrical. The MRI system was operated according to the manual to obtain T1- and T2-weighted images in multislice sequences.

Wide-ranging and fine 3D images for consideration of pathophysiology (Figure 4) were obtained as follows: the field of view (from the hip joint to the toes, 80 mm in the axial direction) was divided into four segments. Each segment was scanned at 1.0 mm slice intervals. The 3D image data of the four segments were stitched together using the Fiji plugin 3D Stitching.^[36] The 3D images shown in Figure 4e were constructed using the Fiji plugin, 3D viewer.^[37]

The images for the routine evaluation methods were obtained simply, as shown in Figure 5a.

Evoked Electromyography: Anesthesia and temperature maintenance were performed in the same manner as during the surgery. It was confirmed that the noise emitted by the heating pads was in the microvolt range and did not interfere with CMAP recordings.

On the operated side, the sciatic nerve was carefully exposed to minimize conductivity attenuation. Bipolar stimulating electrodes were placed proximal to the grafts. The recording electrodes were placed according to the belly-tendon method; the active electrode was placed on the surface of the muscle belly, and the reference electrode on the tendon.^[26] The stimulus duration was set at 0.20 ms, and the stimulation intensity was escalated to the supramaximal stimulation level, at which all axons at the electrode were excited.^[26] Ten serial stimuli were applied at 1.0-s intervals, and the CMAP amplitude and latency were measured from the average waveform. The stimulating electrodes were then displaced distal to the graft, and CMAP latency was measured again. Conduction velocity was calculated from the difference in latency between the two stimulus locations and the distance between the stimulus points.^[26] The same test was also performed on the contralateral side.

In the Defect group, evoked electromyography was unavailable because the sciatic nerve was absent.

The equipment was composed of an electronic stimulator (SEN-3401, Nihon Kohden, Tokyo, Japan), an isolator (SS-203, Nihon Kohden), a bioelectric amplifier (AB-621G, Nihon Kohden), and an analog/digital converter (Power Lab 8/30, AD Instruments, Bella Vista, Australia).

Histological Evaluation: After all other evaluations, the grafts were removed, fixed overnight in 10% formalin, and placed in 30% sucrose until cryoembedding (Tissue-Tek OCT Compound, Sakura Finetek, Torrance, CA, USA). A 10 µm frozen section at the midpoint of the graft was prepared for immunofluorescence analysis. The sections were permeabilized with methanol at 4 °C for 30 min. Antigen retrieval was performed in distilled water at 70 °C for 40 min. After washing with PBS, normal goat serum (catalog no. 005-000-001; Jackson ImmunoResearch, West Grove, USA) was added to the slides, which were then incubated at 37 °C for 1 h. The sections were incubated at 37 °C for 2 h with primary antibodies against neurofilaments (catalog no. 412551; Nichirei Bioscience, Tokyo, Japan; pre-diluted). Slides were washed with PBS and incubated at 37 °C for 45 min with Alexa Fluor 488 conjugated secondary antibodies (catalog no. A11001; Invitrogen, Waltham, USA; 1:1000).

The stained sections were photographed using a fluorescence microscope (BZ-X800, Keyence, Osaka, Japan), and whole images were obtained by stitching. The number of regenerating axons was counted using Fiji in the following way. The entire neural area of each specimen was measured. The images were magnified to 600%, and nine fields of view (45×34 µm each) were randomly selected. The axons were counted in each field of view to calculate the axon density. The total number of axons in each specimen was estimated by multiplying the entire neural area by the mean axon density.

Histological evaluations were unavailable for the Defect group due to the absence of the sciatic nerve.

Blinding Procedure: Evaluations were performed in a blinded manner to minimize examiner bias. In this study, to prevent confounding between

the evaluation methods, the primary data of each rat were relabeled based on random numbers generated using the Excel (Microsoft, Redmond, USA) random function, and the subsequent process was conducted separately later. The blinding method of concealing which group each rat belonged to by randomly assigning identification codes to the rats was inadequate because the visual impression of each rat (e.g., thick/thin operated lower leg, active/poor toe movement) might affect all evaluation methods and falsely link the results of the different evaluation methods.

In the evaluation of motor function using SSI, the video files were randomly renamed. The examiner was blinded to the individual being examined while selecting static images and taking measurements.

In the historical evaluation, all the specimens were stained in one batch and randomly relabeled. The examiner was blinded to the individual being examined while photographing sections and counting regenerated axons.

For the MRI evaluation, the files of the scanned images were randomly renamed. Slice selection and analysis were blinded.

Blinding was practically impossible in evoked electromyography. The examiner inevitably knows whether the graft was an autograft or a conduit because the examiner had to expose it to stimulate it directly.

Statistical Analysis: The reliability analysis (i.e., calculation of ICC and standard error of measurement) was performed in R4.1.2 (CRAN) based on the formulae described by Shrout^[23] and Stratford^[24], respectively. Correlation with the regenerated axon numbers was assessed in Prism 8 (GraphPad Software, California, USA) using r_s , because samples were not normally distributed. p -values < 0.01 were considered statistically significant. In Figure 8, the curves were fitted using [1/1] Padé approximation because it allowed for fitting curves accurately regardless of the underlying mechanism. In this section, the underlying mechanism of the correlations remains unexplained, and the purpose of curve fitting was not to fit a theoretically correct function but to visually show the characteristics of the correlation (i.e., whether it was almost linear or strongly curved). Therefore, [1/1] Padé approximation was suitable for achieving this purpose.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

T.Y. and N.M. conceived the study. T.Y., A.M., and K.O. designed the experiments. K.O. performed the experiments and data analysis and developed the MRI-based evaluation method, with T.Y. and A.M. as supervisors. K.O. prepared the manuscript, and all authors reviewed and edited the manuscript.

Data Availability Statement

All data generated during this study are available upon request.

Keywords

electromyography, motor function evaluation, MRI-based evaluation, nerve regeneration, rodent models

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