Novel experimental surgical strategy to prevent traumatic neuroma formation by combining a 3D-printed Y-tube with an autograft

Anne Bolleboom, BSc,1,2 Godard C. W. de Ruiter, MD, PhD, J. Henk Coert, MD, PhD, Bastiaan Tuk, Jan C. Holstege, MD, PhD, and Johan W. van Neck, MD, PhD

Departments of 1Neuroscience, and 2Plastic and Reconstructive Surgery, Erasmus University Medical Center, Rotterdam; 3Department of Neurosurgery, Medical Center Haaglanden, The Hague; and 4Department of Plastic and Reconstructive Surgery, Utrecht University Medical Center, Utrecht, The Netherlands

OBJECTIVE Traumatic neuromas may develop after nerve injury at the proximal nerve stump, which can lead to neuropathic pain. These neuromas are often resistant to therapy, and excision of the neuroma frequently leads to recurrence. In this study, the authors present a novel surgical strategy to prevent neuroma formation based on the principle of centro-central anastomosis (CCA), but rather than directly connecting the nerve ends to an autograft, they created a loop using a 3D-printed polyethylene Y-shaped conduit with an autograft in the distal outlets.

METHODS The 3D-printed Y-tube with autograft was investigated in a model of rat sciatic nerve transection in which the Y-tube was placed on the proximal sciatic nerve stump and a peroneal graft was placed between the distal outlets of the Y-tube to form a closed loop. This model was compared with a CCA model, in which a loop was created between the proximal tibial and peroneal nerves with a peroneal autograft. Additional control groups consisted of the closed Y-tube and the extended-arm Y-tube. Results were analyzed at 12 weeks of survival using nerve morphometry for the occurrence of neuroma formation and axonal regeneration in plastic semi-thin sections.

RESULTS Among the different surgical groups, the Y-tube with interposed autograft was the only model that did not result in neuroma formation at 12 weeks of survival. In addition, a 13% reduction in the number of myelinated axons regenerating through the interposed autograft was observed in the Y-tube with autograft model. In the CCA model, the authors also observed a decrease of 17% in the number of myelinated axons, but neuroma formation was present in this model. The closed Y-tube resulted in minimal nerve regeneration inside the tube together with extensive neuroma formation before the entrance of the tube. The extended-arm Y-tube model clearly showed that the majority of the regenerating axons merged into the Y-tube arm, which was connected to the autograft, leaving the extended plastic arm almost empty.

CONCLUSIONS This pilot study shows that our novel 3D-printed Y-tube model with interposed autograft prevents neuroma formation, making this a promising surgical tool for the management of traumatic neuromas.

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KEY WORDS neuroma; sciatic nerve; nerve regeneration; axon; rat; 3D printing; peripheral nerve
A neuroma often leads to a transient relief of pain, but the problems often return within a few months because of the regenerative capacity of the peripheral nervous system. As a consequence, surgery often does not lead to sufficient pain relief in the long term, while pharmaceutical pain treatment is notoriously ineffective. Therefore, neuromas remain a clinical problem and urgently require a more effective approach.

The primary aim of each therapeutic approach after nerve injury is to restore the function of the nerve by connecting the proximal nerve stump to the distal nerve. If a nerve defect cannot be directly restored without tension to the nerve ends, the current gold standard for repair is interposition of an autologous nerve graft to bridge the defect. However, the distal nerve stump is not always available for reconstruction, for example, after amputation or in cases of small sensory nerve branches. As an alternative for different covering and capping methods of the proximal nerve stump, Slooff and Samii introduced a novel clinical technique in the late 1970s, the centro-central anastomosis (CCA). In this CCA method, each pair of fascicles of the proximal nerve is reconnected to this nerve—or, in cases of multiple severed nerves, with each other—to prevent the occurrence of neuromas. This method was later investigated experimentally. Gil-Salú et al. created an anastomosis between 2 proximal nerve ends with the interposition of an autograft. Recently, there has been a renewed interest in the CCA method by Boroumand et al., who used this model in a clinical setting in patients who underwent lower-limb amputation. Both experimental and clinical studies have suggested that the CCA method is effective in reducing the size of terminal neuromas and in minimizing neuroma-related pain.

The mechanisms by which the CCA method reduces neuroma formation are not known. One theory is that the CCA method reduces neuromas by guiding regenerating axons into an autograft, protected from surrounding tissues, where they grow into the remaining empty endoneurial tubes of the graft. If an autograft is not interposed and the terminal stumps of the fascicles are sutured directly end to end, it is suspected that the axons of each fascicle could not penetrate into the opposite fascicle because the endoneurial tubes are filled, and thus the axons would penetrate into neighboring tissue where they can form a neuroma. Experimental studies have demonstrated that axonal regeneration in the CCA model is not haphazard but rather arranged, and that the number of regenerating axons in the CCA model is reduced.

In the present study, we developed a novel surgical model to prevent the formation of neuromas by creating a 3D-printed Y-tube that can be customized to exactly fit the proximal injured nerve end. In this model, the upper arms of the Y-tube were connected by a peroneal autograft, while the single arm was connected to the proximal sciatic nerve stump. With the aid of this Y-tube with autograft, we intended to create a closed loop to induce regrowth of injured axons into the Y-tube and thereafter through the graft, which we hypothesized would reduce the possibility of an irregular arrangement of regenerating axons and thereby prevent neuroma formation. An advantage of this Y-tube with autograft is that it is applicable to single-nerve injuries, with a relatively straightforward surgical procedure. The CCA method was used as a control in our experiments. Here, we show that the use of a Y-tube with autograft is an effective approach in preventing neuromas, which also reduces the number of regenerating axons and is easily applicable in nerve surgery.

Methods
Preparation of the 3D-Printed Nerve Conduits

In this study, 3 types of customized nerve conduits were used: the open Y-tube, the closed Y-tube, and the extended-arm Y-tube (Fig. 1). Conduits were made of polyethylene-like material (Endur RGD450, Stratasys) using layer-by-layer 3D printing performed on a commercially available 3D printer (Objet 30 Pro, Stratasys). During fabrication, support material (FullCure 705, Stratasys) was used to prevent any changes in the dimensions of the conduits and was removed preoperatively. To fabricate conduits with anatomically accurate dimensions, the diameters of the sciatic and peroneal nerves in a 180-g Lewis rat were measured (0.8 mm and 0.6 mm, respectively) and used as tissue templates to design the conduits. To prevent

![Image of different types of customized 3D-engineered conduits: the open Y-tube (A), the closed Y-tube (B), and the extended-arm Y-tube (C). The dimensions presented are millimeters. Ø = diameter.](image-url)
nerve compression due to swelling of the nerve, the inner diameters of the conduits (1.0 mm for the common arm and 0.8 mm for the smaller arms) were made slightly larger than the diameters of the donor nerves. With the aid of CAD software (Solidworks, CAD2M), the conduits were designed and 3D printed. All conduits were Y-shaped and consist of 1 common arm (inner diameter 1.0 mm, outer diameter 1.2 mm) and 2 smaller arms (inner diameter 0.8 mm, outer diameter 1.0 mm) to ensure a perfect fit for the donor nerves. The open Y-tube is 4 mm long (Fig. 1A). The closed Y-tube is 8 mm long and includes a plastic cap that fits the 2 smaller arms of the tubes, thereby creating a continuous loop (Fig. 1B). The extended-arm Y-tube consists of a short arm and a 5-mm extended arm (Fig. 1C). Two small holes were made in all tubes (0.15 mm wide and located approximately 1 mm from the tube ending; Fig. 1A) at all insertion sides, which were used to facilitate pulling the nerve into the conduit and to fix the nerve inside the conduit.

Experimental Animals

Thirteen adult female Lewis rats (160–200 g, Charles River) were used. The rats were housed in pairs with ad libitum access to food and water. All experiments were performed in accordance with the European guidelines for the care and use of laboratory animals and approved by the Dutch Ethical Committee on Animal Welfare. The animals were monitored daily for signs of stress, discomfort, or autotomy.

Surgery

All surgical procedures were standardized and performed by the same extensively trained surgical team, consisting of a surgeon and a technical assistant, to avoid variability with regard to the surgical techniques. Under isoflurane inhalation anesthesia (3%) and by using standard aseptic microsurgical techniques with an operating microscope (OP-MI 6-SD, Carl Zeiss), the sciatic nerve of the right hindlimb of the rat was exposed through a longitudinal incision down the right thigh from where the 3 branches of the sciatic nerve, i.e., the tibial, peroneal, and sural nerves, were exposed. The animals were randomly assigned to one of 4 experimental groups: the Y-tube with autograft (n = 4), the CCA (n = 3), the closed Y-tube (n = 3), and the extended-arm Y-tube (n = 3).

Experimental Groups

Y-Tube With Autograft

After sharply transecting the sciatic nerve 3 mm before its trifurcation, the proximal stump of the injured sciatic nerve was inserted into the single arm of the Y-tube. A 10-mm-long nerve graft was taken from the distal peroneal nerve; each side was inserted into the other tubes and secured with 1 suture in an upper arm of the Y-tube to form a loop (Fig. 2A).

CCA

After exposing the sciatic nerve and its trifurcation, the tibial and peroneal nerves were sharply transected 3 mm distal from the trifurcation. A 10-mm-long nerve graft was taken from the distal peroneal nerve; each side was inserted into the other tubes and secured with 1 suture in an upper arm of the Y-tube to form a loop (Fig. 2B).

Closed Y-Tube

After a sharp transection of the sciatic nerve, the injured proximal nerve stump was inserted in the single arm of the closed Y-tube (Fig. 2C) and single sutured to the conduit.

Extended-Arm Y-Tube

After sharp transection of the sciatic nerve, the proximal nerve stump was inserted in the lower short arm of the Y-tube. A 5-mm-long nerve graft was taken from the distal peroneal nerve and single sutured to the upper short arm of the conduit, leaving the distal part of the graft in open connection with the environment (Fig. 2D). The upper extended arm was left in open connection with the surrounding tissue.
In all groups, a gap of at least 10 mm was maintained between the transection site of the proximal nerve stump and the distal denervated nerve stumps to avoid spontaneous regeneration to the distal nerve stump. The excised nerve stumps were inserted into the conduits to a depth of 1–1.5 mm and fixed to the conduits using a single epineural 10-0 monofilament nylon suture (Ethicon) to prevent the nerve stumps from shifting and escaping the tube after closing the surgery site. All muscle wound beds and skin incisions were closed using 4-0 Vicryl sutures (Ethicon). Immediately postoperatively and at 24 hours, all rats received intramuscular buprenorphine (0.05–0.1 mg/kg, Temgesic).

Nerve Morphometry

Twelve weeks after surgery, the animals were killed by administering an overdose of sodium pentobarbital (100 mg/kg). Peripheral nerve segments, including the surgical conduits, were carefully dissected from the rat’s hindlimb, and the conduits were removed from the nerves. Proximal nerve segments were transected 3 mm proximal to the conduit. The excised nerve segments were processed for resin embedding and paraffin embedding. For paraffin embedding, tissue samples were kept in formalin 10% solution for 24 hours and stored at 4°C. The tissue samples were embedded in paraffin and mounted on slides. Five-micrometer transverse nerve sections were cut and stained for 1 hour in Picro Sirius Red (Direct Red 80, Sigma) solution (0.1% solution of Sirius Red F3BA in saturated aqueous picric acid, pH 2). For resin embedding, tissue segments were postfixed in 2.5% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C, followed by overnight fixation using 1% osmium tetroxide. The next day, the tissue was dehydrated in graded ethanol followed by propylene oxide (PO) twice for 30 minutes, PO/Araldite (dilution 1:1; Huntsman Advanced Materials) for 90 minutes, PO/Araldite (1:3) for 90 minutes, and pure Araldite overnight. Subsequently, the nerves were embedded in Araldite and polymerized at 60°C for 72 hours. Semi-thin cross sections (0.5 μm) were cut with the aid of a microtome (Ultracut, Leica) using a diamond knife, 1 mm proximal from the conduit or the suture lines and at every following 2–3 mm of the nerve and stained with 1% paraphenylenediamine (PPD) for 2 minutes at 60°C.

Quantitative Analysis

Microscopic images of the PPD-stained cross sections were digitalized using a digital slide scanner (Nanozoomer 2.0 series system, Hamamatsu), and examined in the Hamamatsu NPDI file format. Low-magnification images were captured for a manual measurement of the cross-sectional area of the regenerated tissue using ImageJ and digital microscope NDPI software. At 100× magnification, randomly selected fields of the regenerated nerve—which, taken together, represented at least 10% of the total tissue area—were manually counted for the number of myelinated axons by an observer who was blinded to the experimental groups. Beforehand, and on statistical analysis, it was determined that additional images captured beyond 10% of the total tissue area did not significantly alter the mean number of axons. Thus, the density and the total number of axons per nerve were calculated.

The presence or absence of a neuroma was macroscopically and microscopically determined by an investigator blinded to the experimental groups. Macroscopic observations were based on the presence of a bulb before the entrance of the conduits and the presence of excessive scar and connective tissue. The microscopic presence or absence of a neuroma was determined on histological evaluation of multiple PPD-stained cross sections of each transected nerve by looking at the following criteria: disorganized, with regenerating axon sprouts; erosion of the perineurium and epineurium; loss of funicularchitecture; and the presence of intraneural fibrosis.

Statistical Analysis

Data are expressed as means ± SEM. The 2-tailed independent Student t-test was used for statistical analysis between 2 groups and the paired-samples t-test for statistical analysis in 1 group for the data, which was normally distributed, using IBM SPSS software (version 21.0, IBM). One-way ANOVA was used for comparison among multiple groups. The post hoc Tukey test was applied when there was a significant difference; p < 0.05 was considered statistically significant.

Results

The different experimental conduits were successfully fabricated using the 3D printing technique, implanted into the hind paw of the rats, and adjusted to the severed nerves. Neuroma formation and nerve regeneration efficiency through the models was evaluated for the 4 experimental groups (Y-tube with autograft, CCA, closed Y-tube, and extended-arm Y-tube) by macroscopic observations and histomorphometry. No complications occurred during the surgical procedures. During the survival period, all rats exhibited motor deficiencies consistent with a lesion of the sciatic nerve. No overt signs of autotomy were observed. Table 1 shows the number of myelinated axons in the different experimental models.

Y-Tube With Autograft

Macroscopic Observations

Examination of the surgical area 12 weeks after surgery showed neither bulbous tissue nor excessive fibrous or adhesive tissue surrounding the surgery site (Fig. 3A). The epineurium appeared intact without signs of inflammation.

Neuroma Formation

The lack of signs of neuroma formation at the macroscopic level was confirmed by the microscopic observations at the different sites in and around the Y-tube corresponding to the locations illustrated in Fig. 4A. The epineurium was present and not disrupted at any location (Fig. 3B–D). Connective tissue was observed surrounding the intact epineurium (Fig. 3B).

Microscopy of Nerve Regeneration

Different nerve sections were microscopically ana-
lyzed for the number of myelinated axons at positions a–g (Fig. 4A). Abundant nerve regeneration was observed in the Y-tube and the graft (Fig. 4C). Bundles of regenerating axons, from the proximal part of the Y-tube (27,800 ± 1,400), split into 2 bundles of approximately uniform axonal density (14,000 ± 725 and 13,300 ± 835 for positions d and e, respectively; Fig. 4B). Both bundles entered the autograft at their respective side with a significant decrease in the number of axons inside the graft, when comparing positions d to f and e to g (p = 0.03 and p = 0.004, respectively, Student t-test), with a 10.1% ± 1.2% and 16.3% ± 4.0% decrease in myelinated axons, respectively.

CCA

Macroscopic Observations

Examination of the surgical area 12 weeks after surgery showed excessive adhesion of the nerves to the surrounding tissues and abundant scar tissue covering the surgical area. Bulbous vascularized tissue covered the epineural stitches at the positions where the proximal nerves were sutured to the interposed autograft (Fig. 3A).

Neuroma Formation

The macroscopic observations suggesting neuroma formation surrounding the suture lines were confirmed by microscopic observations. Random axons that did not enter the autograft and penetrated the surrounding connective tissue were observed especially surrounding the suture lines of the coaptation sites with intraneural fibrosis and disruption of the epineurium (Fig. 3B–D). Connective tissue was observed both intraneural and surrounding the epineurium, as shown in Fig. 3B.

Microscopy of Nerve Regeneration

Different nerve sections were microscopically analyzed at the locations illustrated in Fig. 5A. Sections of the proximal peroneal and tibial nerves, corresponding to positions a and b, respectively, in Fig. 5B and C, showed an axonal morphology and number of axons that reflected a noninjured situation (7829 ± 534 and 3216 ± 534, respectively). The number of axons increased after entering the autograft corresponding to positions c and d (13,410 ± 625 and 13,213 ± 652, respectively; Fig. 5B). A significant decrease in the number of axons was found in the middle of the autograft in positions e and f (11,398 ± 795 and 10,839 ± 405, respectively) when compared with positions c and d (13,410 ± 625 and 13,213 ± 652 respectively; p = 0.036 left and p = 0.027 right), with a 13.7% ± 2.7% and 19.2% ± 3.2% decrease, respectively.

Closed Y-Tube

Macroscopic Observations

Examination of the surgical area 12 weeks after surgery showed excessive connective tissue surrounding the surgical area. Bulbous, vascularized tissue surrounded the proximal nerve stump before the entrance of the Y-tube (Fig. 3A). No nerve tissue was observed in the connecting cap of the conduit. The total nerve matrix that grew through the 2 upper arms of the tube was smaller than the nerve matrix that grew through the proximal part of the conduit (Fig. 3A).
Neuroma Formation

The macroscopic observations suggesting neuroma formation were confirmed by microscopic observations, which showed randomly oriented regenerating axons before the Y-tube entrance (Fig. 3C and D), with loss of the epineurium and axons penetrating the surrounding connective tissue and the presence of intraneural fibrosis and connective tissue (Fig. 3B).

Microscopy of Nerve Regeneration

Different nerve sections were microscopically analyzed at the locations illustrated in Fig. 6A. Extensive nerve re-
generation was seen in the proximal part of the Y-tube corresponding to section a in Fig. 6C (14,919 ± 1439). Significantly fewer axons were found after the bifurcation of the Y-tube when comparing positions b (1300 ± 823) and c (1162 ± 802) to position a (p = 0.005 [b vs a] and p = 0.004 [c vs a], respectively; Fig. 6B), which is a total decrease of 90.5% of myelinated axons compared with the proximal arm. No axons were observed through the connecting cap of the Y-tube.

Extended-Arm Y-Tube
Macroscopic Observations
Examination of the surgical area 12 weeks after surgery showed vascularized bulbous tissue at the distal end of the sciatic nerve before the entrance of the Y-tube (Fig. 3A), and connective tissue surrounding the surgical area. In all rats, nerve matrix was extending beyond the adjusted autograft, making the graft appear longer than the 5-mm graft connected to the tube during surgery. In 2 rats, no clear endpoint of the nerve matrix extending beyond the adjusted autograft was identified. A clear difference in size was found in the diameter of the regenerated nerve tissue of position c compared with d (Fig. 3A).

Neuroma Formation
The macroscopic observations suggesting neuroma formation were confirmed in 2 rats by microscopic observations, which showed randomly oriented regenerating axons before the extended Y-tube entrance (Fig. 3C and D). In addition, microscopic observations showed signs of loss of epineurium and randomly regenerating nerve fibers around the Y-tube with connective tissue in and around the epineurium (Fig. 3B and D).
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Microscopy of Nerve Regeneration

Different nerve sections were microscopically analyzed at the locations illustrated in Fig. 7A. Nerve regeneration was observed through the entire conduit and into the autograft (Fig. 7C). The axons extending through the proximal tube, corresponding to position a (16,288 ± 1808), were split into 2 unequal bundles. Significantly more axons were present in the graft arm (7936 ± 522) when compared with the extended plastic arm (98 ± 84, p = 0.0001; Fig. 7B). No significant differences were found in the number of axons at different positions in the autograft.

Comparing the Different Experimental Groups

Neuromas

Macroscopic and microscopic results showed that, 12 weeks after surgery, no neuroma formation was observed in the Y-tube with autograft group, whereas all other groups showed randomly growing axons and loss of epineurium, indicating neuroma formation. Histologically confirmed neuromas were only observed at the position where the nerve was about to enter the Y-tube or surrounding the coaptation sites in the CCA, as shown in Fig. 3. An organized pattern of regenerating axons was observed inside the different Y-tubes.

Nerve Regeneration

A significant difference, considering the number of myelinated axons, was observed in the proximal part of the Y-tube between the following groups: the Y-tube with autograft group, the closed Y-tube group, and the extended-arm Y-tube group (1-way ANOVA; F = 22.763, p = 0.001). A Tukey post hoc test revealed that the number of axons in the proximal part of the Y-tube was significantly lower...
in the closed Y-tube (14,919 ± 1439, p = 0.001) and the extended-arm Y-tube (16,288 ± 1808, p = 0.003) compared with the Y-tube with autograft (27,802 ± 1393). There was no statistically significant difference between the number of axons in the proximal part of the closed Y-tube and the extended-arm Y-tube (p = 0.826). In the closed Y-tube, no axons were observed in the connecting tube, while many axons were observed at the same position when using an autograft in the Y-tube with autograft model (compare Fig. 6C to Fig. 5C).

Discussion
In the present study, we have developed a novel surgical strategy that prevents neuroma formation after nerve injury by using a 3D-printed Y-tube with an interposed autograft. Following nerve transection, the proximal sciatic nerve stump was inserted into the lower arm of a custom-made Y-tube, while the 2 upper arms of the Y-tube were connected by an autologous peroneal autograft to form a closed loop. At 12 weeks of survival, we found that regenerating axons had extended through the Y-tube and grown into the autograft from both sites. No signs of neuroma formation were observed in this experimental model, while the number of regenerating axons in the middle of the autograft was significantly reduced. In contrast, the other experimental models, i.e., the CCA, closed Y-tube, and extended-arm Y-tube models, all showed signs of neuroma formation.

The concept of nerve tubes has evolved from an investigation tool to a surgical device that is used in peripheral nerve injury. In recent years, both scientists and the medi-
The peripheral nerve injury may involve one or more nerves, and the injured nerve may be varied in geometry. The natural variance in patient anatomies has motivated the development of personalized treatment for peripheral nerve injury. For this purpose, 3D printing is an advancing technology in the field of surgery and enables the fabrication of personalized constructs. In this study, 3D printing with polyethylene-like material allowed us to successfully fabricate conduits with dimensions that nicely fit the sciatic and peroneal nerves in the rat. The commercially available material we used to fabricate the conduits was Endur RGD450 with Fullcure 705 as support material, which allowed us to accurately print the conduits with the desired small dimensions on our 3D printer. The small holes, which were designed at the nerve insertion sites of the tubes, made it easy to pull the transected nerves into the tubes using a single suture, which minimized damage to the epineurium and securely fixed the nerve stumps in the tubes. In addition, this ensured that a closed loop is formed in which regenerating axons are directed in a tension-free environment between the nerve ends. The simplicity and flexibility of the 3D printing technique is a clear advantage, allowing for individual-based treatment after nerve injury.

Thus far, there have been many attempts to prevent neuroma formation and to treat painful terminal neuromas. However, in most cases the results have been ineffective, as a neuroma often reappears together with neuroma-associated pain. In this study, we focused on inducing an environment for controlled regrowth of regenerating axons, which is supposed to reduce irregular outgrowth of the regenerating axons, thus preventing neuroma formation. Our Y-tube with autograft model was found to be very well suited in preventing neuroma formation in a single transected nerve by providing a closed and controlled pathway. The closed loop in this model suppresses the formation of communication with the surrounding tissues, where intrusion of scar tissue is minimized, but also prevents the formation of neuromas.

![Diagram](https://example.com/diagram.png)
escape of regenerating axons. Moreover, a physical barrier protects the regenerating axons from various external stimuli, such as pressure, which reduces scar tissue and also reduces pressure around the nerve. In the Y-tube with autograft, we found all the regenerating axons from the proximal nerve growing into the Y-tube and into the autograft in an organized way, without signs of neuroma formation or scar formation in or around the conduit or autograft.

An autograft likely provides a gradient of essential neurotrophic factors for guiding regenerating axons from the proximal nerve stump in the direction of the autograft and thereafter into the denervated endoneurial tubes that remain inside the autograft following Wallerian degeneration. Many studies have shown the benefits of the autograft in guiding regenerating axons; however, their possible role in neuroma prevention has not been clearly documented. Nerve autografts are immunologically inert, possess the extracellular matrix structure meant to host axons, and contain Schwann cells, which are known to secrete diffusible factors that guide axonal regeneration. In our study, the attracting influence of the autograft on regenerating axons was clearly demonstrated in the extended-arm Y-tube, where 99.4% of the regenerating axons in the Y-tube merged into the Y-tube arm connected to the autograft, leaving the extended plastic arm almost empty. Furthermore, this is also supported by our observations of the closed Y-tube, which lacked neurotrophic factors, showing the avoidance of axons to grow into the tube and, consequently, the formation of a neuroma outside the tube. Therefore, we can conclude that the autograft is important for both the guidance of regenerating axons and the moderation of their growth in the absence of a target, making the autograft an important tool in preventing neuromas.

An early attempt to prevent neuroma formation using an autograft is the CCA. The CCA method, in which 2 nerve branches are sutured to an autograft to create an anastomosis, was found to limit, but not prevent, neuroma formation by offering the regenerating axons an autograft to restore the continuity of the nerve fascicles. This result may be explained by the mismatch in diameter between the proximal nerves and the autograft and in the number of axons between the 2 proximal nerve stumps, leading to a bad fit. In our CCA model, we aimed to minimize the mismatch between the proximal nerves and the autograft by using nerves with a diameter that better aligned with the diameter of the autograft, thereby increasing the chance of all axons growing into the empty endoneurial tubes inside the autograft. The autograft in this model is approached by regenerating axons from both sides, which, in theory, should create a situation in which endoneurial channels of the autograft are occupied by regenerating axons from both sides. We found the number of regenerating axons to be significantly reduced in the middle of the autograft, making it conceivable that a form of axonal growth inhibition occurred at the position where the growth cones of the regenerating axons meet. However, in this model we still found neuromas surrounding the coaptation sides. This result can likely be explained by the inequality between the diameters of the proximal nerve stumps and the autograft and the lack of a physical barrier, which does not allow a perfect-fit connection between the nerve stumps and the autograft. Weiss proposed that open connections at the suture lines might offer opportunities for axons to regenerate outside the nerve environment and for connective tissue and vessels to penetrate along the sutures. This makes the CCA method less suitable for neuroma prevention.

In our Y-tube with autograft model, we found that all regenerating axons sprouted through the Y-tube. These regenerating axons, coming from the proximal sciatic nerve, extended through the Y-junction where it splits into 2 bundles of approximately uniform axonal density. At the point of entering the autologous nerve graft, we observed a 4% increase in the number of myelinated axons, which likely reflects axonal sprouting. In contrast, the number of axons in the middle of the autograft, where the axons from opposite directions are expected to encounter each other, was significantly reduced by about 13% compared with the lateral edges of the graft. This suggests that a form of axonal growth inhibition occurred in the middle of the autograft where the axons are supposed to encounter each other. It is conceivable that the growth cones of the regenerating axons are involved in this process, as they react to changes in the environment. In support of this view, in our extended-arm Y-tube, the number of regenerating axons extending through the graft arm did not significantly change at any position in the autologous graft, which further strengthens the idea of axonal growth inhibition in the middle of the autograft in the Y-tube with autograft. However, the mechanisms underlying the decrease in axons in the middle of the autograft of the Y-tube with autograft model remain unknown.

Our observations hold promise for clinical use as they indicate that introducing the proximal nerve end into a perfect-fit structure with an autograft may guide regenerating axons and induce axon growth inhibition, thereby preventing neuroma formation. In addition, an advantage is that the autograft can be obtained from the injured nerve itself by harvesting a piece of the proximal stump, thereby preventing the sacrifice of other nerves or the use of donor nerves with possible associated morbidity. This surgical strategy for preventing neuromas may be suitable for treatment of painful neuromas as well as fresh nerve injuries where end-to-end reconstruction is not an option (e.g., due to tissue loss or amputation).

Our aim in this work was to produce a surgical model in which neuroma formation was prevented. In this study, evaluation of neuroma formation and nerve regeneration was assessed using nerve morphometry. Other techniques, like behavioral outcomes, in which neuropathic pain behavior can be measured, were not assessed in this study. Behavioral outcomes may often be applied when continuity of the nerve is partially spared. In our model, however, we aimed to create an amputation-like situation in which the sciatic nerve was transected without the possibility of being rejoined with the distal end.

From nerve repair surgery in the clinic and from literature, it is known that the presence of rigid and semirigid nerve guidance conduits, such as an interposition graft, should be avoided, as nerve compression is reported over time. In our experimental study, we used nondegrad-
able Y-tubes as a proof of principle to assess nerve regeneration and neuroma formation in a closed-loop model. However, for clinical translation, biodegradable conduits are of interest since they can be used to prevent possible compression problems. Hu et al.\textsuperscript{10} reported biodegradable cellularized designer conduits, which degraded in 2–4 months in vivo. Computer-aided design/computer-aided manufacturing (CAD-CAM) and 3D printing warrant the delivery of nerve repair tubes that can be customized to anatomical dimensions and combined with degradable materials.\textsuperscript{10} The use of a 3D-printed biodegradable Y-tube with autograft could prevent possible nerve compression problems, thereby making this an interesting technique for the production of the Y-tube, with the potential for clinical translation in preventing and managing neuromas.

Conclusions
Our study demonstrates that the Y-tube with autograft prevents neuroma formation and significantly reduces the number of axons in the middle of the autograft, thereby outperforming the CCA and capping techniques. In our view, these findings, combined with the feasibility of this surgical model, hold promise for a novel clinical approach to both treat painful neuromas and prevent neuroma formation after nerve injury.

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Author Contributions
Conception and design: all authors. Acquisition of data: Bolleboom, Tuk. Analysis and interpretation of data: all authors. Drafting the article: van Neck, Bolleboom, de Ruiter, Coert, Holstege. Critically revising the article: van Neck, Bolleboom, de Ruiter, Coert, Holstege. Reviewed submitted version of manuscript: van Neck, Bolleboom, de Ruiter, Coert, Holstege. Approved the final version of the manuscript on behalf of all authors: van Neck. Statistical analysis: Bolleboom. Administrative/technical/material support: Bolleboom, Tuk. Study supervision: van Neck, de Ruiter, Holstege.

Correspondence
Johan W. van Neck: Erasmus MC, University Medical Center, Rotterdam, The Netherlands. jvanneck@erasmusmc.nl.