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Dead-ended autologous connective tissue chambers in peripheral nerve repair – early observations

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The effects of the repair of nerve gap injuries are still unsatisfactory, despite the great progress in microsurgery. Until now, there is no effective method to induce the regeneration of the transected peripheral nerve when its distal stump is missing. The aim of this work was to examine whether the implantation of dead-ended connective tissue chambers can promote the outgrowth of injured peripheral neurites. This method differs from all previous nerve guides because it totally eliminates the distal part of the nerve and restricts the influence of surrounding tissues. We have also tried to establish whether some neurotrophic factors can be applied by means of these chambers. The results of this work show that dead-ended autologous connective tissue chambers can be a useful tool in peripheral nerve injuries treatment, even when the distal part of the nerve is missing.

Keywords: connective tissue chamber, peripheral nerve, regeneration, BDNF

The repair of the nerve gap injuries is still a serious problem, as well clinical as experimental. The method that provides the best conditions for the nerve regeneration is the primary suturing of the stumps of the injured nerve (6, 19). In peripheral nerve injuries, gaps are often too long or the distal part of the nerve is damaged so severely that it cannot be sutured to the proximal stump. In these cases the gap has to be bridged. Different techniques and materials are used to join distal and peripheral segments of the transected nerve and to bridge the gap between them (14, 19). Bridging of the gap with a fragment of an autologous nerve is often used, but it requires sacrifice of a healthy tissue

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and causes an extra mutilation of a patient. The size of the gap also limits the chances of successful regeneration. In rats, the distance of 10 mm is commonly accepted as a maximal distance covered by regrowing fibers. Until now, the presence of distal part as well as the length of the gap determine the success or failure of peripheral nerve regeneration in adult mammals (9, 22). The aim of the present paper was to ascertain whether implantation of dead-ended connective tissue chambers (deCC) in the absence of distal segment, might be an effective treatment of peripheral nerve injury. Contrary to other works, where the chambers were joining proximal and distal stumps of injured nerve (3, 4, 9, 22, 23), this technique allows to examine the ability of neurite outgrowth (a) in the absence of the distal part of the nerve and (b) with limited influence of surrounding tissues. The next step was to find out whether neurotrophic substances can be successfully applied to the injured nerve using deCC. In previous works, we have reported a way to apply the peripheral nerve extracts into the central nervous system by means of deCC (1, 7). Now we modified this method to be appropriate for peripheral nerve treatment.

Materials and Methods

The study was performed on 37 male Wistar C rats, weighing 210–230 g obtained from Medical University Animal Farm (Katowice, Poland). They were randomly assigned to one of the two equal groups and because three animals were lost during surgical procedures, the number of animals was 34 (n=17 in each group). The experimental protocol was approved by the Ethics Committee of the Medical University of Silesia. During the whole experiment rats were housed in individual cages on a natural day/light cycle and had free access to standard rodent laboratory food and tap water. All surgical procedures were performed under intraperitoneal chloral hydrate (420 mg/kg b.w., Fluka, Switzerland) anesthesia.

Two sterile 20-mm long pieces of silicone tube (Silastic, USA) of 2-mm outer diameter were implanted under the skin on the back. After 3 weeks, the tubes with the surrounding tissues were dissected and excess of connective as well as fat tissue was removed so that to obtain a thin continuous layer covering the silicone tube like a sleeve. Both ends of the tubes surrounded by connective tissue layer were cut off (one end at 45° angle that facilitates the proximal nerve stump introduction into the tube; the other end at 90°) (Fig. 1A). The connective tissue was then carefully slid from both sides to the midpart of the tube (Fig. 1B). This preparation was performed in cold sterile phosphate-buffered saline (PBS).

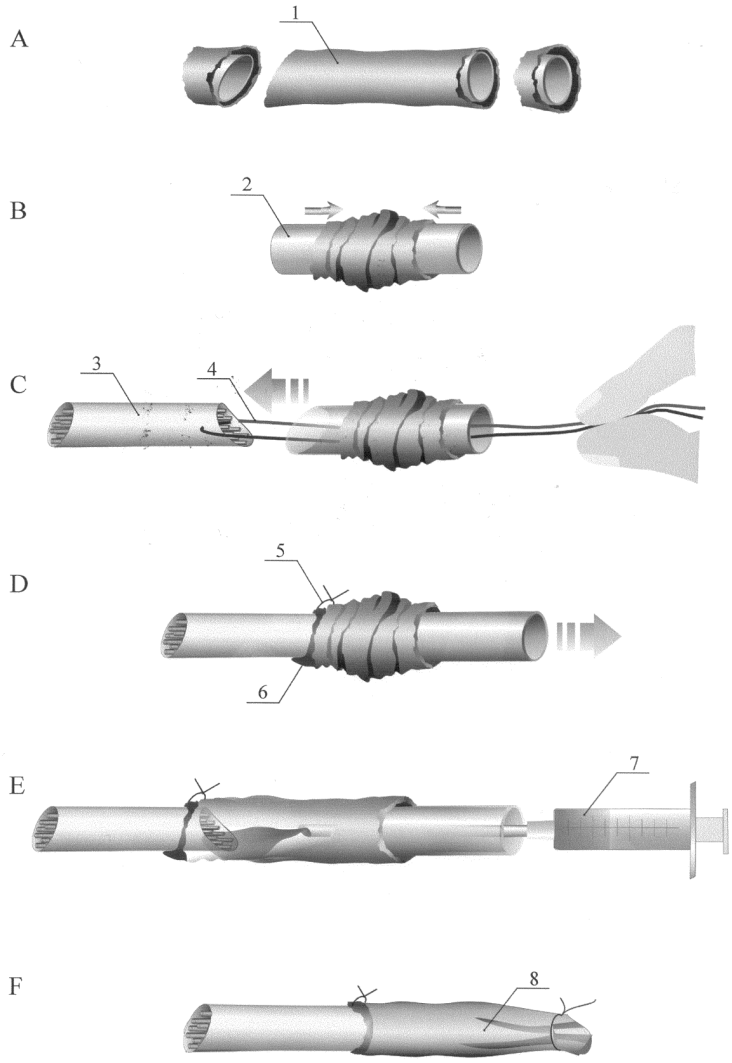


Fig. 1. The schematic presentation of consecutive steps of the surgery procedures. A, B, C, D, E and F are given according to their appearance in the text. 1 – connective tissue chamber, 2 – silicone tube, 3 – proximal stump of the transected sciatic nerve, 4 – surgical thread, 5 – suture between connective tissue chamber and the proximal stump of the nerve, 6 – tissue glue sealing the place of connective tissue chamber and the proximal stump of the nerve connection, 7 – Hamilton microsyringe, 8 – final form of dead-ended connective tissue chamber

Animals were fixed in the prone position, with legs in 30° abduction. After shaving the skin, a 30 mm incision just over the femoral bone was made. The next steps of the surgery were performed under the stereoscopic microscope (SMZ-10, Nikon, Japan). The right sciatic nerve was exposed by blunt muscle fiber preparation and then totally transected with sharp microscissors (Chifa, Poland) at the hip joint level. The peripheral fragment of the nerve was removed, till the trifurcation point. The 2 mm tip of proximal nerve stump was introduced into the transected (at 45°) end of the connective tissue chamber (deCC) (Fig. 1C*)*. The deCC was sutured to the epineurium with a single suture (8/0, Ethicon) and sealed with two-component tissue glue Tissucol Kit (Immuno AG, Austria). The tube was drawn out, like a piston, till the very end of the chamber (Fig. 1D). Subsequently, a needle of the Hamilton microsyringe was introduced into the chamber through the free end of the tube for filling (Fig. 1E). In the first group the deCCs were filled with a mixture of 20 µl of fibrinogen (3 mg/ml; Sigma, USA), 20 µl of BDNF solution (25 µg/ml; Sigma, USA) and 5 µl of thrombin (500 U.I./ml; Biomed, Poland). In the second group we used a mixture of 20 µl of fibrinogen, 20 µl PBS (that was a solvent of BDNF in the 1st group) and 5 µl of thrombin. After the chamber content consistency changing from liquid to jelly-like (~3 min), the tube was removed. The free end of the chamber was tied up with a thread (8/0, Ethicon) to preserve the deCC content from the influences of external environment (Fig. 1F). The chamber was placed in the anatomical position of the removed distal segment of the sciatic nerve. The wound was sutured in layers (4/0, Ethicon). No drugs were administered.

For spinal motoneurons retrograde labeling, the modified method of Wang-Bennett and Coker was used (21). Four weeks following implantation, in 9 rats from each group a 2-mm long distal fragment of the chamber end was cut off and this freshly opened tip was introduced into the polyethylene tube (Tomel, Poland, 3-mm o.d.) and the junction place was sealed with a tissue glue. Subsequently, the microcrystal of Dil (1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine Chlorate, Sigma, USA) with 30 µL of its solvent DMOS (Dimethyl Sulfoxide, Sigma, USA) was administered through the tube to the deCC end. To prevent the dye leaking, the free outlet of the tube was sealed with the white vaseline plug (Hesco-Lek, Poland) which was then fixed with a piece of spongostan (Polfa, Poland).

Forty-eight hours after operations animals were perfused transcardially with PBS (200 ml), followed by a cold fixative containing 4% formaldehyde (FA) in PBS (500 ml). The L2-L5 segments of the spinal cord were immediately dissected, postfixed in the FA for 1 h at 4 °C and placed in 15% sucrose/PBS overnight. Serial 20 µm thick transverse sections were cut by means of a cryostat microtome (Cryotome 620 Anglia Scientific, England) and mounted on gelatin-coated slides. Air-dried sections were dehydrated and coverslipped with DPX. They were examined under a fluorescence microscope (Labophot-2 Nikon, Japan) at the wavelength of 550 nm and photographed. Cell profiles in the ventral horn with distinct nucleus contour were counted in every second section (5 of ten consecutive sections taken at the L3-L4 level).

Four rats from each group were also perfused transcardially, first with PBS (200 ml), and then with cold fixative containing a 2.5% glutaraldehyde in 0.1 M

cacodylate buffer (500 ml). The mid-parts (2-mm long) of the chambers were removed and immersed in the same solution for about 1 h. After post-fixation with 1% OsO4 in 0.1 M cacodylate buffer, the specimens were dehydrated and embedded in Epon resin. Semithin sections (1 μm) were cut (Ultramicrotome, Reichert-Jung, Germany) and stained with the toluidine blue, then mounted and coverslipped. The sections were examined in the light microscope (Labophot-2, Nikon, Japan) and photographed. The myelinated nerve fibers were counted in the same area (five vision fields – one central and four peripheral) in each examined slide.

Additionally, three rats from each group were perfused transcardially with PBS (200 ml), and then with cold FA (500 ml). Transection sites were re-exposed and the whole implanted chambers along with the short fragments of the sciatic nerves proximal to the junction were carefully dissected, cryoprotected and embedded in TissueTek. Ten μm thick longitudinal cryostat sections were mounted onto slides (Menzel-Gleser, Germany). The sections were treated with rabbit polyclonal antibody against growth associated protein-43 (GAP-43; Chemicon, USA). Secondary goat anti-rabbit IgG antibodies conjugated with Alexa 568 (Molecular Probes, USA) were used. Coverslipped sections were examined and photographed under a confocal laser scanning microscope (Fluoview, Olympus, Japan) at the wavelength of 568 nm and saved. We measured the distance from the nerve-chamber junction to the place where most of distal GAP-43 positive fiber endings were present.

One chamber (in one animal) in each group was subjected to histochemical analysis for acetylcholinesterase (AChE), according to Hedreen et al. to visualize motor nerve endings inside the chamber (5). Briefly, rats were perfused transcardially with 0.1 M phosphate buffer (PB) pH 7.2 (200 ml), and subsequently with cold fixative containing 4% formaldehyde in PB (500 ml). The deCC were dissected, and placed in 30% sucrose/PB overnight. The 10 μm thick longitudinal sections were cut using a cryostat microtome (Cryotome 620 Anglia Scientific, England) and then mounted on SuperFrost+ slides (Menzel-Glaeser, Germany). Incubation was performed for 15 min at room temperature in medium containing acetylthiocholine iodide, sodium acetate, sodium citrate, cupric sulfate and potassium ferricyanide. After rinsing in acetate buffer, sections were treated with 1% ammonium sulfide solution followed by rinsing in 0.1 M sodium nitrate and treating with 0.1% silver nitrate. Finally rinsed sections were dehydrated and coverslipped. They were analyzed in the light microscope (Labophot – 2 Nikon, Japan) and photographed.

All counts were made by two independent observers unaware of the analyzed group.

Statistical analysis

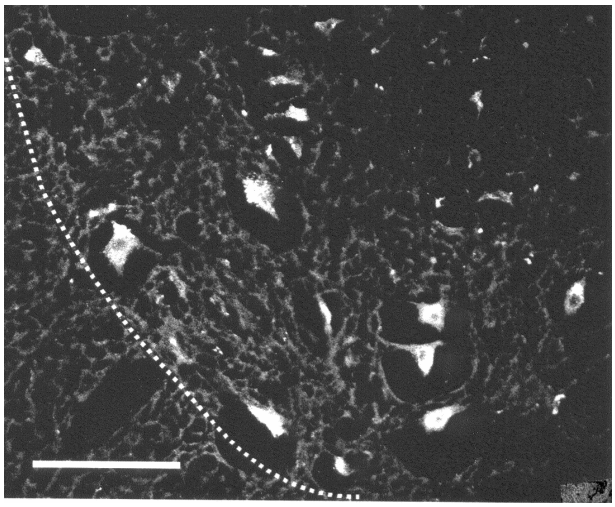
Statistical analysis was done using one-way analysis of variance (ANOVA) and non-parametric Kruskal-Wallis test. The Tukey test was then used as *post hoc* test. Statistical significance was set at $p<0.05$. All data are expressed as means ± SD.

Results

The number of failures in our study was relatively low (less than 10%). No wound inflammation symptoms were observed.

The number of Dil-labeled motoneurons in individual groups was different. In rats treated with BDNF it was significantly higher (65.5±12.4) than in the B group (37.7±11.9) (Fig. 2A).

A



B

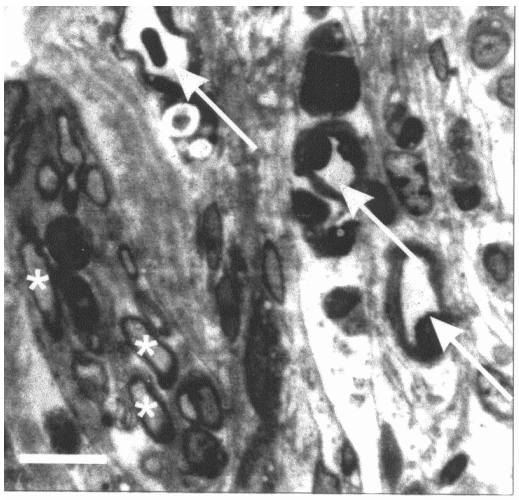


Fig. 2A and B

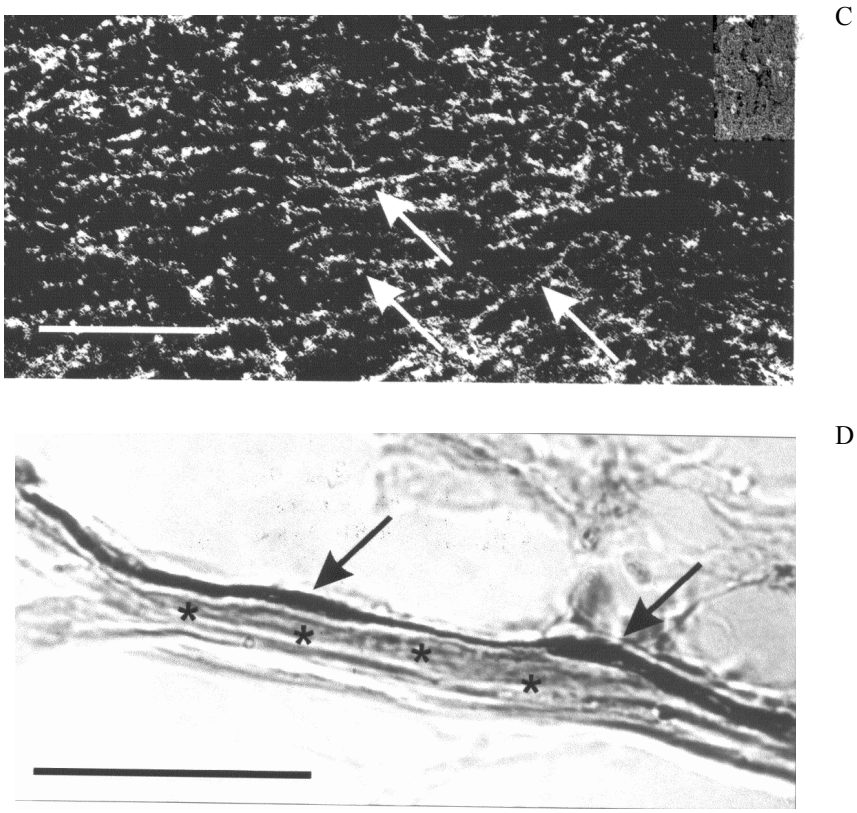


Fig. 2. Photos of slides from B group, where connective tissue chamber (deCC) filled with fibrin was implanted into transected sciatic nerve. A – DiI labeled motoneurons of L3-L4 spinal cord segments; dashed line marks the anterior horn of spinal cord; bar 100 μm; B – myelinated fibers (asterisks) and vessels (arrows) growing in the deCC, center of the vision field; bar 10 μm; C – GAP-43-positive nerve endings inside deCC; bar 50 μm; D – AChE - positive fibers (arrows) growing along fibrin network (asterisks) inside the implanted deCC; bar 10 μm

The number of myelinated nerve fibers present in the mid-part of deCC, was significantly higher in the BDNF group (557.2±147.7) than in the B group (151.7±113.1). Although the analysis of the chamber vascularization was not the aim of the paper we have found numerous blood vessels growing into them (Fig. 2B).

The distances covered by GAP-43-positive nerve endings were in the BDNF group (9.0±1.3) significantly higher than in the B group (5.7±1.1) (Fig. 2C).

The AChE reaction revealed parallelly arranged regenerating motor nerve fibers inside all examined chambers (Fig. 2D).

Discussion

We have described the method of preparation and implantation of deCC filled with neuroactive substances into the injured peripheral nerve. The method seems to be advantageous for we observed neurite outgrowth in all groups examined.

The DiI labeled motoneurons were counted in L3-L4 segments of the spinal cord because the cell bodies of the neurons sending their axons to the sciatic nerve are located in these segments. The regenerating ends of the transected axons picked up the fluorescent dye and transported it to the cell bodies. The highest number of labeled motoneurons in the BDNF group did not excess 90. It is known that number of rat's motoneurons of spinal segments L3 to L4 is about 300 (16). This suggests that only a part of adult motoneurons population has a tendency to regenerate their fibers on such a long distance. This finding is in agreement with Swett et al. who have shown that most motoneurons of the adult rats survive injury but only some of them reinnervate their original targets (17). According to Suzuki et al., sensory neurons regenerate their axons more dominantly than motoneurons in the early stages of the regenerating process following peripheral nerve transection (18). This may be due to inherent motor and sensory differences as well as the distance from injury site to somata of the motor and sensory neurons, respectively. For the sciatic nerve, the sensory neurons are located at the S4-S5 level, and the motoneurons are 2 cm rostral from them (10).

Each deCC contained fibrin which is known to be essential for the scaffold formation that enables the fibers to grow (23). It is commonly accepted that some components of connective tissue as well as extracellular matrix such as collagen, laminin and fibronectin also possess similar properities (3, 4, 22, 23). The mechanism by which these substances influence regeneration is not clear, but it has been shown to affect both neurite outgrowth and migration of glial cells (4, 14).

Schwann cells provide basal lamina components as well as trophic factors that promote axonal elongation (15). We used BDNF as a neurotrophic factor because its action on injured motoneurons is well documented. It has been previously shown that BDNF protect adult spinal motoneurons from retrograde degeneration following ventral root avulsion and reduces cell death and tissue necrosis after spinal cord hemisection (11, 12, 20). Moreover, combination of ventral root reconstruction by means of peripheral nerve graft or embryonic spinal cord transplants and BDNF treatment may be an especially promising method to support of motoneuron survival and regeneration their axons (19).

We conclude that the method described above offers a useful delivery system supporting the regrowth of injured peripheral neurites and enabling an application of active substances. Our results break the dogma that the presence of the distal part of injured nerve is indispensable for the regeneration of peripheral nerves (2, 9, 13, 22). The distal part of the nerve can be replaced with deCC filled with substances able to support neurite outgrowth. Moreover, this delivery system provides the possibility of testing the putative neuroactive substances when the influence of neighboring environment is limited.

We presume that deCC may have clinical implications in temporary nerve injury treatment when a delay in the proper reconstruction occurs. In states of emergency, the connective tissue for the chamber formation can be obtained from different sources of the human body (e.g. fascia, epimysium or epineurium) (19).

References

1. Barski J, Lewin-Kowalik J, Krause M, Górka D, Larysz-Brysz M, Gołka B, Święch-Sabuda E: Autologous connective tissue bars as vehicles for the administration of growth-influencing substances to the brain. Brain Res. Brain Res. Prot. 1, 27–32 (1997)
2. Brushart TM, Mesulam MM: Alteration in connections between muscle and anterior horn motoneurons after peripheral nerve repair. Science 208, 603–605 (1980)
3. Chamberlain LJ, Yannas IV, Hsu HP, Strichartz G, Spector M: Collagen-GAG substrate enhances the quality of nerve regeneration through collagen tubes up to level of autograft. Exp. Neurol. 154, 315–329 (1998)
4. Chen YS, Hsieh CL, Tsai CC, Chen TH, Cheng WC, Hu CL, Yao CH: Peripheral nerve regeneration using silicone rubber chambers filled with collagen, laminin and fibronectin. Biomaterials 21, 1541–1547 (2000)
5. Hedreen JC, Bacon SJ, Price DL: A modified histochemical technique to visualize acetylcholinesterase axons. J. Histochem. Cytochem. 33, 134–140 (1985)
6. Jaremczyk C: Results of surgical treatment for traumatic injury of upper extremity peripheral nerves in own material. Chir. Narzadow Ruchu Ortop. Pol. 60, 77–80 (1995)
7. Lewin-Kowalik J, Górka D, Larysz-Brysz M, Gołka B, Świech-Sabuda E, Małecka-Tendera E, Krause M: Purified extracts from short time predegenerated rat's sciatic nerves promote the regrowth of injured hippocampal neurites. Acta Physiol. Hung. 85, 325–334 (1997–98)
8. Lundborg G, Hansson HA: Nerve regeneration through preformed pseudosynovial tubes. A preliminary model of a new experimental model for studying the regeneration and reorganization capacity of peripheral nerve tissue. J. Hand. Surg. Am. 5, 35–38 (1980)
9. Lundborg G, Dahlin LB, Danielsen N, Grelberman RH, Longo FM, Powell HC, Varon S: Nerve regeneration in silicon chambers; influence of gap length and of distal stump components. Exp. Neurol. 76, 361–375 (1982)
10. Macias M, Lehman C, Sanger J, Riley D: Myelinated sensory and alpha motor axon regeneration in peripheral nerve neuromas. Muscle and Nerve 21, 1748–1758 (1998)
11. Novikova L, Novikov L, Kellerth JO: Brain-derived neurotrophic factor reduces necrotic zone and supports survival after spinal cord hemisection in adult rats. Neurosci. Lett. 220, 203–206 (1996)
12. Novikov L, Novikova L, Kellerth JO: Brain-derived neurotrophic factor promotes axonal regeneration and long-term survival of adult rat spinal motoneurons *in vivo*. Neuroscience 79, 765–774 (1997)
13. Politis MJ, Ederle K, Spencer PS: Tropism in nerve regeneration in vivo. Attraction of regenerating axons by diffusible factors derived from cells in distal nerve stumps of transected peripheral nerves. Brain Res. 253, 1–12 (1982)
14. Rosen JM, Kaplan E, Jewett D (1980): Suture and sutureless methods of repairing experimental nerve injuries. In: Nerve Repair and Regeneration, Its clinical and experimental basis, eds. Jewett, D and McCarroll, HR Jr. C.V. Mosby, St. Louis, pp. 235–243.
15. Stoll G, Muller WH: Nerve injury, axonal degeneration and neural regeneration: basic insight. Brain Pathol. 9, 313–325 (1999)
16. Swett JE, Wikholm RP, Blanks RH, Swett AL, Conley LC: Motoneurons of the rat sciatic nerve. Exp. Neurol. 93, 227–252 (1986)
17. Swett JE, Hong CZ, Miller PG: All peroneal motoneurons of the rat survival crush injury buy some fail to reinnervate their original targets. J. Comp. Neurol. 304, 234–252 (1991)

18. Suzuki G, Ochi M, Uchio Y, Matsuura Y: Sensory neurons regenerate more dominantly than motoneurons during the initial stage of the regenerating process after peripheral axotomy. Neuroreport 9, 3487–3492 (1998)
19. Terzis JK, Sun DD, Thanos PK: Historical and basic science review: past, present, and future of nerve repair. J. Reconstr. Microsurg. 13, 215–225 (1997)
20. Vejsada R, Tseng JL, Lindsay RM, Acheson A, Aebischer P, Kato C: Synergistic but transient rescue effects of BDNF and GDNF on axotomized neonatal motoneurons. Neuroscience 84, 129–139 (1998)
21. Wang-Bennett LT, Coker NJ: Analysis of axonal regeneration through the silicon regeneration chamber: a retrograde tracing study in the rabbit facial nerve. Exp. Neurol. 107, 222–229 (1990)
22. Williams LR, Powell HC, Lundborg G, Varon S: Competence of nerve tissue as distal insert promoting nerve regeneration in a silicone chamber. Brain Res. 293, 201–211 (1984)
23. Zhao Q, Dahlin LB, Kanje M, Lundborg G: Repair of the transected rat sciatic nerve: matrix formation within implanted silicone tubes. Restor. Neurol. Neurosci. 5, 197–204 (1993)