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Inhibition of calpains fails to improve regeneration through a peripheral nerve conduit

Thomas Hausner[a,b,c], Letizia Marvaldi[d], Gábor Márton[e], Krisztián Pajer[e], Rudolf Hopf[a], Robert Schmidhammer[a,f], Barbara Hausott[d], Heinz Redl[a], Antal Nógrádi[a,e,*], Lars Klimaschewski[d,*]

[a] *Austrian Cluster of Tissue Regeneration and Ludwig Boltzmann Institute for Experimental and Clinical Traumatology at the Research Centre for Traumatology of the Austrian Workers' Compensation Board (AUVA), Donaueschingenstr. 13, 1200 Vienna, Austria*
[b] *Department of Trauma Surgery and Sports Traumatology, Paracelsus Medical University, Müllner Hauptstr. 48-50, 5020 Salzburg, Austria*
[c] *Department of Surgery, State Hospital Hainburg, Hofmeisterstr. 70, 2410 Hainburg, Austria*
[d] *Division of Neuroanatomy, Department of Anatomy, Histology and Embryology, Innsbruck Medical University, Muellerstrasse 59, 6020 Innsbruck, Austria*
[e] *Department of Anatomy, Histology and Embryology, Faculty of Medicine, University of Szeged, Hungary*
[f] *Vienna Private Clinic, Pelikangasse 15, 1090 Vienna, Austria*

HIGHLIGHTS

- Calpain inhibitor leupeptin locally applied to transected sciatic nerve of rats.
- Axon number and myelination not significantly increased 3 months after lesion.
- No difference in behavioral tests after nerve regeneration.

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ABSTRACT

Intramuscular injection of the calpain inhibitor leupeptin promotes peripheral nerve regeneration in primates (Badalamente et al., 1989 [13]), and direct positive effects of leupeptin on axon outgrowth were observed *in vitro* (Hausott et al., 2012 [12]). In this study, we applied leupeptin (2 mg/ml) directly to collagen-filled nerve conduits in the rat sciatic nerve transection model. Analysis of myelinated axons and retrogradely labeled motoneurons as well as functional 'CatWalk' video analysis did not reveal significant differences between vehicle controls and leupeptin treated animals. Therefore, leupeptin does not improve nerve regeneration *via* protease inhibition in regrowing axons or in surrounding Schwann cells following a single application to a peripheral nerve conduit suggesting indirect effects on motor endplate integrity if applied systemically.

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1. Introduction

Damage to peripheral nerves often results in abortive or inadequate regeneration due to inappropriate or missing connections between the injured nerve stumps. Various factors are required for successful regeneration in the peripheral nervous system, including the re-establishment of continuity of the peripheral nerve pathways, the fast coaptation of nerve stump endings, and the ability of axons, microglial and Schwann cells to react to signals that initiate regeneration [1,2]. Moreover, the closely regulated system of secreted proteases and protease inhibitors modifies the extracellular matrix and, thereby, allows axons to regenerate along newly assembled glial scaffolds which guide them to their targets [3].

Endogenous and pharmacological inhibitors of proteases have long been known to be potent modulators of neurite outgrowth. Among these inhibitors, the small peptide leupeptin (N-acetyl-L-leu-L-leu-arginal) inhibits the activity of calcium-activated neutral proteases (CANPs or calpains), and serin proteases such as thrombin as well as proteasomal trypsin-like activities [4]. Calpains are enzymes with high affinity for cytoskeletal proteins such as neurofilaments [5]. They have also been shown to cleave α-spectrin, collapsin response mediator protein-2, and voltage-gated sodium channels [6].

* Corresponding authors.
E-mail addresses: nogradi.antal@med.u-szeged.hu (A. Nógrádi), lars.klimaschewski@i-med.ac.at (L. Klimaschewski).

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Direct administration of leupeptin to the sciatic nerve results in increased glial proliferation, but demyelination of axons and axon sprouting are observed, too [7–9]. Furthermore, this tripeptide promotes neurite outgrowth in neonatal and in adult sensory neuron culture [10–12]. Leupeptin also improves morphological regeneration and functional recovery *in vivo* following a median nerve lesion if injected into target muscles combined with repeated systemic intramuscular administration over 6 months in primates after median nerve injury [13]. However, these studies did not provide an unequivocal answer to the clinically relevant question whether local inhibition of calpains at the lesion site is able to improve axonal regeneration. Therefore, the aim of this study was to analyze possible pro-regenerative effects of leupeptin locally applied to a nerve conduit bridging the gap between the endings of a transected sciatic nerve in rats.

2. Materials and methods

2.1. Animals

Experiments were carried out on male Sprague–Dawley rats weighing 300–350 g (Animal Research Laboratories, Austria). The animals were anesthetized by intraperitoneal administration of a combination of ketamine hydrochloride plus xylazine (ketamine hydrochloride: 90 mg/kg body weight; xylazine: 5 mg/kg). Adequate care was taken in all cases to minimize the levels of pain and discomfort during and after the operation, and the experimental protocol was approved in advance by the Animal Protocol Review Board of the City Government of Vienna (No.: MA58-1020/2008/7). All procedures were carried out in full accordance with the Helsinki Declaration on Animal Rights and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

In experimental and control animals ($n = 10$ for each group) the right sciatic nerve was exposed through a mid-thigh incision and repaired by inserting the transected ends of the nerve into a 8 mm silicone conduit filled with a collagen solution (10 μg/ml type I rat tail collagen, Sigma–Aldrich) containing leupeptin (2 mg/ml, Sigma–Aldrich) or vehicle as a control. The distance between the stumps was adjusted to 6 mm. The internal diameter of the tube measured 1.5 mm. In another group of animals a 10 mm conduit with a gap size of 8 mm was used ($n = 5$ for each group). The nerve stumps were fixed without tension by suturing each end into the open ends of the conduit by using 8-0 epineurial sutures (Ethilon 8-0/BV-2, Ethicon-Johnson & Johnson) under an operating microscope (Leica M651). The wound was closed and animals were housed in normal cages (two animals/cage). In control animals ($n = 10$), the same procedure was performed but the conduits were filled with collagen only. The concentration of leupeptin used here was in the same range as in other *in vivo* studies [7,8], and biological activity was confirmed by measuring neurite outgrowth and receptor tyrosine kinase fluorescence changes in cultured adult sensory neurons as described before [12].

2.2. Functional analysis

Functional analysis of the locomotor pattern was performed weekly through the use of the CatWalk automated gait analysis system (Noldus) starting 4 weeks after surgery. At every time point, three successful runs produced by each animal were recorded and the results of these were averaged. The following parameters were assessed: footprint intensity (the maximum pressure exerted by one paw, expressed in arbitrary units, a.u.), footprint area (the mean area of each footprint of the affected hind limb, in mm^2), stance duration (the duration of the stance phase of the hind limb, in s), swing duration (the duration of the swing phase of the hind limbs, in s) and swing speed (the speed of the swing phase, in cm/s).

2.3. Electrophysiological analysis

At the end of the survival period, electrophysiological analysis (NeuroMax-XLTEK) was carried out during the terminal operations in all animals to assess the extent of reinnervation in the various groups. Stimulation electrodes were placed 2 mm proximal and 2 mm distal to the graft for calculation of the nerve conduction velocity. A needle electrode was placed as a recording electrode into the tibialis anterior muscle, and the sciatic nerve was stimulated for 0.05 ms first proximally and then distally to the graft in order to achieve the supramaximal stimulation amplitude. The compound action potential, the normalized amplitude and the nerve conduction velocity were determined. All measurements were carried out at a body temperature between 38 and 39 °C.

2.4. Retrograde labeling and tissue preparation

After completing the electrophysiological recordings, the common peroneal nerve on the operated side of animals in both groups was cut at the level of the tensor fasciae latae muscle and Fast Blue crystals (Illing) were applied to the proximal stump. The stump was then thoroughly covered with two layers of 1 mm thick Spongostan sheets to prevent diffusion of the tracer. Five days were allowed for retrograde transport of the dye, then the animals were re-anesthetized and perfused transcardially with ice-cold 0.9% heparinized saline solution followed by 4% phosphate-buffered paraformaldehyde (pH 7.4). The lumbar spinal cord was carefully removed, postfixed in the same fixative overnight and cryo-protected in a 30% sucrose solution at 4 °C until further use. The conduits containing the regenerated nerve were explanted and postfixed in 2.5% phosphate-buffered glutaraldehyde for 24 h.

2.5. Morphological analysis

Remnants of fixative were carefully washed out from the nerve, and the tissue was next immersed in 1% OsO_4 (Agar Scientific) for 1 h, dehydrated in a graded ethanol series and in propylene oxide and then embedded in Durcupan (Fluka). Semithin sections (0.4 μm) were cut from the middle of the graft on a Leica Ultracut-R ultramicrotome and stained according to Rüdeberg (1967). Morphometric analysis was performed in a blind manner. Randomly selected semithin section were used to assess the total cross-sectional area of the whole nerve, the total fiber number, the circle-fitting diameter of the fiber, the axon myelin thickness and the g-ratio through the use of MetaMorph® (Visitron).

To determine the number of retrogradely labeled motoneurons supplying the common peroneal nerve, serial 25 μm thick cryostat sections were cut from the lumbar segments L3–L5. The sections were mounted on gelatinized slides and examined by an Olympus BX50 fluorescence microscope. To avoid double counting of the same neuron present in two consecutive sections, the retrogradely labeled neurons were mapped with the aid of an Olympus drawing tube and their locations were compared to those of labeled neurons in the previous section. All sections from the L3–L5 motoneuron pool were analyzed.

2.6. Statistical analysis

The statistical analysis was carried out with Graph Pad Prism statistical software. Groups were compared by use of ANOVA, followed by Tukey's *post hoc* test. Functional evaluations were analyzed

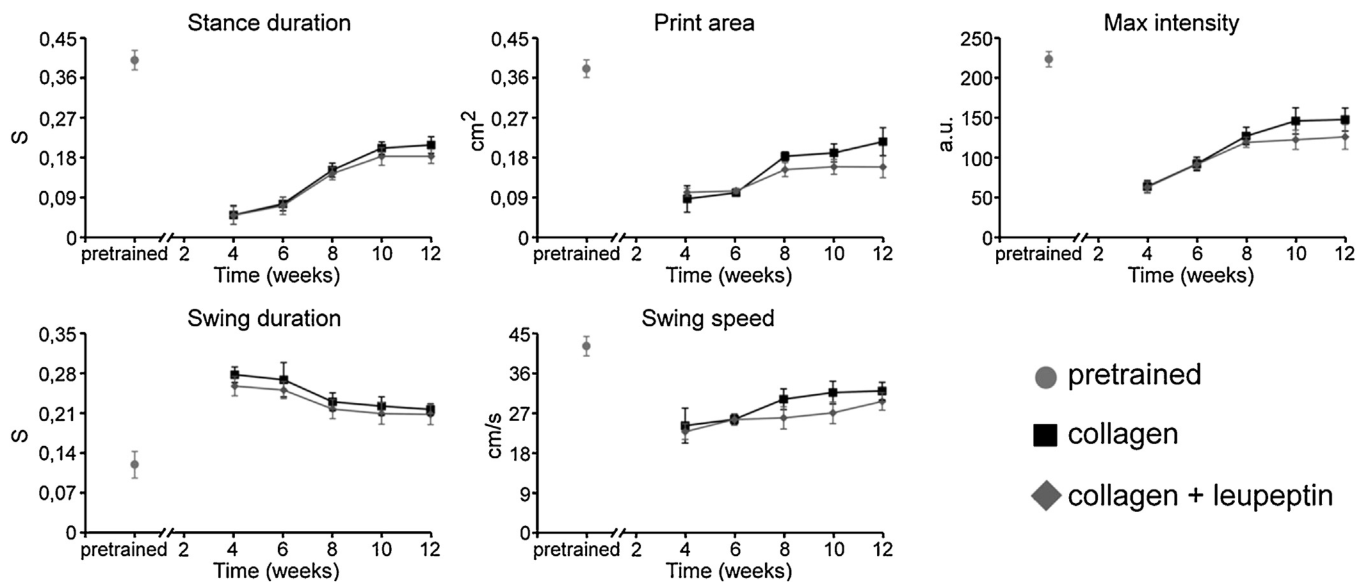

Fig. 1. CatWalk automated gait analysis data 4–12 weeks postoperatively. No significant differences were observed in various parameters indicating parallel restoration of the hind limb motor function in both experimental groups. Averaged values of pretraining are shown in the left part of each panel. Values are expressed as means ± S.E.M.

with the Mann–Whitney U test. All data in this study are given as means ± standard error (S.E.M.).

3. Results

3.1. Functional observations

Detailed analysis of hindlimb function through the use of the automated gait analysis system CatWalk indicated moderate return of function of hindlimb muscles throughout the 12 week observation period in both experimental groups. Parameters indicating return of hindlimb function began to improve 6 weeks after injury and reached levels of typically over 50% of the pretraining values (Fig. 1). Animals with conduits containing leupeptin revealed slightly limited recovery in some of the parameters such as print area and maximum intensity, without showing statistically significant differences relative to the control animals (collagen only).

3.2. Morphological observations

Twelve weeks after axotomy, conduits in both experimental groups contained a regenerated segment of axon bundles connecting the 6 mm gap between the proximal and distal nerve stumps (Fig. 2A). The cross sectional area at the middle of the regenerate was compared. The area of control regenerates (collagen-filled tubes) was slightly, but not significantly greater relative to the leupeptin-treated regenerates (0.24 ± 0.02 *vs.* $0.20 \pm 0.01\ mm^2$, Fig. 2B). Retrograde tracing by applying the fluorescent tracer Fast Blue to the common peroneal nerve revealed considerable numbers of reinnervating motoneurons (Fig. 2C) in the control and leupeptin-treated groups (587 ± 116 *vs.* 569 ± 66, Fig. 2D) without significant differences between the two groups.

The numbers of myelinated axons were also determined in the regenerates (Figs. 3A and B), and these data correlated with the motoneuron counts. Leupeptin-treated regenerates had fewer myelinated axons as compared with control animals (7389 ± 853 *vs.* 5774 ± 848, Fig. 3C) indicating a non-significant trend toward reduced axon numbers in the presence of leupeptin. Morphometric analysis demonstrated no differences in myelin thickness (Fig. 3D) or axon diameter between control and leupeptin-treated regenerates (Fig. 3E). However, the g-ratio (the ratio of the inner axonal diameter relative to the outer diameter) was slightly, but significantly increased (Fig. 3F) suggesting a minor negative effect of leupeptin on remyelination of axons as reported before [8]. The trend toward smaller regenerates supports this assumption, too.

To exclude the possibility that leupeptin may promote nerve regeneration in a more severe injury paradigm the lesion experiment and morphological analysis of sciatic nerves was repeated applying a 10 mm conduit with a gap size of 8 mm. Following this approach no significant differences in number or myelination of regenerated axons were observed either (data not shown).

3.3. Electrophysiological observations

Electrophysiological recordings were made from the tibialis anterior muscle 3 months after surgery. Stimulating electrodes were placed either proximal or distal to the nerve graft, and the conduction velocity within the grafted nerve segment was calculated. At survival time of 3 months, considerable amplitude (19.7 ± 3.8 *vs.* 17.6 ± 6.4 mV) and compound nerve action potential area values (CNAP, 31.4 ± 5.7 *vs.* 27.4 ± 9.8 mV ms) were observed in the control *vs.* leupeptin-treated animals (Fig. 4). These values did not differ significantly between the two experimental groups. More striking but still non-significant differences were detected in nerve conduction velocity values between the leupeptin-treated and the control animals (40.2 ± 3.3 m/s *vs.* 30.4 ± 6.7 m/s).

4. Discussion

The results of the present study suggest that a single application of leupeptin into an artificial nerve conduit placed between transected nerve stumps does not promote nerve regeneration and re-innervation of target muscles. We even observed a trend toward reduction in number of regenerating motoneurons and in number of myelinated axons within the conduit as well as in myelin thickness (without reaching statistical significance at $p < 0.05$).

The rationale of this study was based on previous findings that leupeptin strongly promotes axon outgrowth if applied directly to growth factor stimulated primary adult sensory neuron cultures [10–12]. These cultures are nearly devoid of Schwann cells,

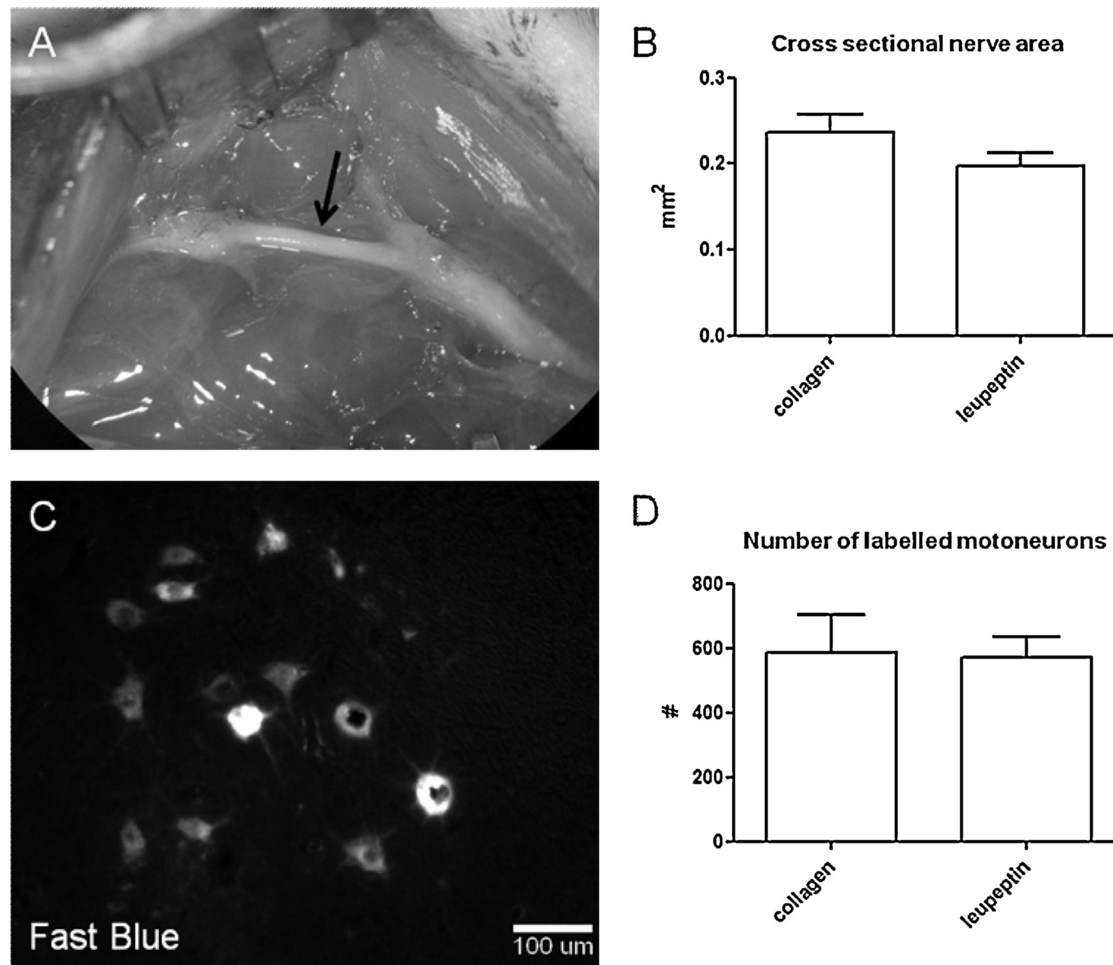

Fig. 2. Axonal regeneration in control (collagen only) and leupeptin-treated peripheral nerve conduits 3 months after surgery. (A) The panel shows the macroscopic view of the established conduit (arrow) populated by regenerated axons in a control animal. (B) The cross sectional area of the conduit was non-significantly smaller in leupeptin-treated animals than in the controls. (C and D) Numerous retrogradely labeled motoneurons were found in the ventral horn of the L4–L5 spinal segments after labeling with the fluorescent tracer Fast Blue from the common peroneal nerve (C). No significant difference was observed between the experimental groups in the number of retrogradely labeled motoneurons (D).

however. Considering the effects of leupeptin on Schwann cell proliferation and myelin disruption in sciatic nerve branches with intact axons [8], the absence of effects on axon regeneration in the sciatic nerve bridging model may have been caused by this inhibitory effect of leupeptin on axon remyelination. It has to be pointed out, however, that a *g*-ratio of 0.6 as observed here is still in the regular range of sciatic nerve axons (healthy or regenerated).

Methodological differences to the study by Alvarez et al. [8] may explain why myelin thickness only slightly decreased and the g-ratio correspondingly increased following a single application of leupeptin at the time of nerve transection as performed here. Alvarez et al. carried out repeated subperineurial injections or continuous infusions of leupeptin (*via* a mini-osmotic pump) into the intact tibial nerve which may have caused more prominent effects on non-neuronal cells. The inhibitory effects of leupeptin on myelination could be successfully avoided by repeated intramuscular injections resulting in increased numbers of axons distal to the lesion site which was accompanied by improved motor and sensory conduction velocities and reduced muscle atrophy [13]. Since leupeptin has been shown to maintain neuromuscular contacts if directly applied to the muscle following nerve crush at birth [14], it appears likely that the primary site of action in the primate study [13] was within the denervated muscles and not within the regenerating nerve.

However, chemoattractive effects of muscle-derived factors on regenerating axons may also underlie the improved morphological outcome and enhanced conduction velocities, because intact myofibers are expected to release a plethora of trophic factors after denervation [15]. The median nerve lesion in Badalamente's study was relatively close to the denervated muscles, which further supports this hypothesis.

The main pharmacological targets of leupeptin are calcium-activated neutral proteases (calpains). Calpain activation plays a key role not only in traumatic nerve injuries or stroke lesions, but also in neurodegenerative diseases such as Alzheimer's and Parkinson's as well as in amyotrophic lateral sclerosis [16]. Calcium influx, as observed after nerve injury, leads to calpain activation, resulting in the cleavage of a variety of cellular substrates in neuronal and non-neuronal cells. Considering the results of this study, the effects of local calpain inhibition within a nerve conduit appear to be minor and mainly involve Schwann cells that migrate into the conduit, but not axons. In contrast, the effects on preventing muscle denervation atrophy appear to be more significant and may warrant intramuscular injection of leupeptin as adjunctive treatment for peripheral nerve repair.

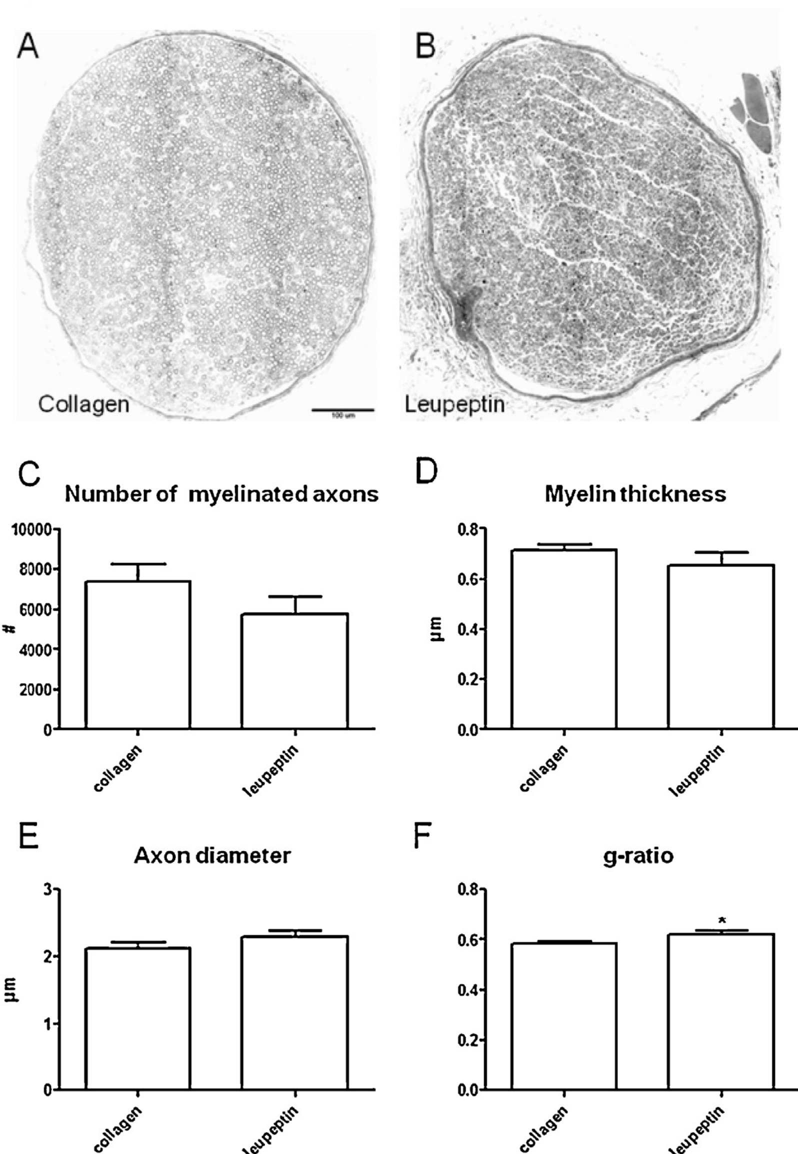

Fig. 3. Features of the regenerating axons in the peripheral nerve conduit. Photographs of semithin cross-sections from the middle of the conduit of control and leupeptin-treated animals (A and B) 3 months postoperatively. The control conduits (collagen only) contain more myelinated axons (C) with marginally thicker myelin sheaths (D), while the leupeptin-treated conduits appear to have slightly thicker axons (E), in a non-significant manner. (F) The *g*-ratio is, however, higher in leupeptin-treated conduits as compared with controls. * Significant difference between the control and leupeptin groups, $p < 0.05$, by ANOVA, computed by using Tukey's all pairwise multiple comparison procedures. Values are expressed as means ± S.E.M.

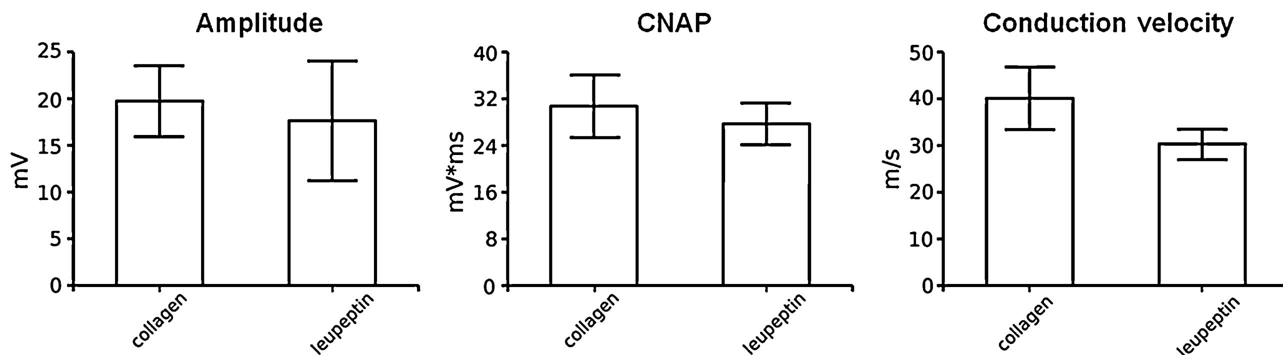

Fig. 4. Electrophysiology stimulation data 3 months postoperatively. At the end of the survival period, the amplitude and CNAP values only marginally differ, whereas the conduction velocity in the leupeptin-treated animals appear considerably lower than that in the collagen only group, without significant difference. Values are expressed as means ± S.E.M.

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