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

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## Abstract

Intramuscular injection of the calpain inhibitor leupeptin promotes peripheral nerve regeneration in primates (*Badalamente et al., 1989*). Recently, we observed direct positive effects of leupeptin on axon outgrowth of adult sensory neurons overexpressing growth factor receptors *in vitro* (*Hausott et al., 2012*). 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 local application to a peripheral nerve conduit suggesting indirect effects on motor endplate integrity if applied systemically.

## Introduction

Damage to peripheral nerves often results in abortive or inadequate regeneration due to inappropriate or missing connections between the injured nerve stumps. There are a number of factors required for successful regeneration in the PNS, 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 (*Raivich and Makwana, 2007; Klimaschewski et al., 2013*). 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 to guide them to their targets (*Monard, 1988*).

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) strongly inhibits the activity of Calcium-activated neutral proteases (CANPs or calpains), but serin proteases such as thrombin and proteasomal trypsin-like activities as well (*Tsubuki et al., 1996*). Calpains, the enzymes with high affinity for cytoskeletal proteins such as neurofilaments (*Croall and DeMartino, 1991*), have been shown to cleave  $\alpha$ -spectrin, collapsin response mediator protein-2, and voltage-gated sodium channels (*Schoch et al., 2013*).

Direct administration of leupeptin to the sciatic nerve results in increased glial proliferation, but demyelination of axons and axon sprouting are observed, too (*Moreno et al., 1996; Alvarez et al., 1995; Alvarez et al., 1992*). Furthermore, this tripeptide promotes neurite outgrowth in neonatal and in adult sensory neuron culture (*Hawkins and Seeds, 1986; Hausott et al., 2008; Hausott et al., 2012*). 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 (*Badalamente et al., 1989*). However, these studies did not provide an unequivocal answer 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 this common peripheral nerve injury model.

## **Material & Methods**

### *Animals*

Experiments were carried out on 20 male Sprague-Dawley rats weighing 300-350 g (Animal Research Laboratories, Himberg, Austria) and lasted for a period of 3 months. The animals were anaesthetized 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 (publication NIH 86-23, revised 1985).

Two experimental groups were set up. In the first group of animals (n = 10) 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, Austria) containing leupeptin (2 mg/ml). The distance between the stumps was adjusted to 6 mm, and the internal diameter of the tube measured 1.5 mm. 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, Brussels, Belgium) under an operating microscope (Leica M651, Leica Microsystems, Vienna, Austria). The wound was closed and animals were housed in normal cages (2 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 (*Moreno et al., 1996; Alvarez et al., 1995*), and biological activity was confirmed by measuring axon outgrowth of sensory neurons in culture as described before (*Hausott et al., 2008*).

### *Functional analysis*

Functional analysis of the locomotor patterns was performed weekly through the use of the CatWalk automated gait analysis system (Noldus Ltd., The Netherlands) 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<sup>2</sup>), 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).

#### *Electrophysiological analysis*

At the end of the survival period, electrophysiological analysis (NeuroMax-XLTEK, Oakville, ON, Canada) 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 normalised amplitude and the nerve conduction velocity were determined. All measurements were carried out at a body temperature between 38 and 39 °C.

#### *Retrograde labelling 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 GmbH, Breuberg, Germany) 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-anaesthetized 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.

#### *Morphological analysis*

Remnants of fixative were carefully washed out from the nerve, and the tissue was next immersed in 1% OsO<sub>4</sub> (Agar Scientific, Stansted, UK, in PBS) for 1 h, dehydrated in a graded ethanol series and in propylene oxide and then embedded in Durcupan (Fluka, Switzerland). Semithin sections (0.4 µm)

were cut from the middle of the graft on a Leica Ultracut-R ultramicrotome and stained according to R deberg (1967). Morphometric analysis was performed in a blind manner. Randomly selected semithin sections 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®.

To determine the number of retrogradely labelled 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 (Olympus Ltd, Tokyo, Japan,). To avoid double counting of the same neuron present in two consecutive sections, the retrogradely labelled neurons were mapped with the aid of an Olympus drawing tube and their locations were compared to those of labelled neurons in the previous section. All sections from the L3-L5 motoneuron pool were analyzed.

#### *Statistical analysis*

The statistical analysis was carried out with Graph Pad Prism statistical software (Graph Pad Software Inc., San Diego, CA, USA). Groups were compared by use of ANOVA, followed by Tukey's *post hoc* test. Functional evaluations were compared with the Mann-Whitney U test. All data in this study are given as means ± standard error (S.E.M.).

## **Results**

#### *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 (collagen only) animals.

#### *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 ( $0.24 \pm 0.02$  vs  $0.20 \pm 0.01$  mm<sup>2</sup>, 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 ( $587 \pm 116$  vs  $569 \pm 66$ , Fig. 2D) without significant differences between the two groups.

The numbers of myelinated axons were also determined in the regenerates (Figs. 3A, B), and these data correlated with the motoneuron counts. Leupeptin-treated regenerates had fewer myelinated axons as compared with control animals ( $7389 \pm 853$  vs  $5774 \pm 848$ , Fig. 3C) indicating a non-significant trend towards 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 (*Alvarez et al., 1995*). The trend towards smaller regenerates supports this assumption, too.

#### *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 ( $19.7 \pm 3.8$  vs  $17.6 \pm 6.4$  mV) and compound nerve action potential area values (CNAP,  $31.4 \pm 5.7$  vs  $27.4 \pm 9.8$  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 ( $40.2 \pm 3.3$  m/s vs  $30.4 \pm 6.7$  m/s).

#### **Discussion**

The results of this study suggest that a single application of leupeptin to an artificial nerve conduit placed between the transected nerve stumps does not promote nerve regeneration and reinnervation of target muscles. Considering the effects of locally injected leupeptin on Schwann cell proliferation and myelin disruption in sciatic nerve branches with intact axons (*Alvarez et al., 1995*), the absence of effects on axon regeneration in the sciatic nerve bridging model is surprising. Although we observed a trend towards reduction in the number of motoneurons projecting into the conduit and in the number

of myelinated axons within the conduit as well as in myelin thickness, the effects were not statistically significant at  $p < 0.05$ .

As demonstrated in a previous study (*Badalamente et al., 1989*), repeated intramuscular injections of leupeptin clearly increased the number of myelinated and non-myelinated axons distal to the lesion site at three months after nerve transection which was accompanied by improved motor and sensory conduction velocities and reduced muscle atrophy. Since leupeptin has been shown to maintain neuromuscular contacts if directly applied to the muscle following nerve crush at birth (*Harding et al., 1996*), it appears likely that the primary site of action in the primate study was within the denervated muscles and not within the regenerating nerve. Furthermore, in our study leupeptin was applied to an artificial nerve conduit and thus it could be argued that the compound may have little or no direct effect on the nerve stump distal to the conduit.

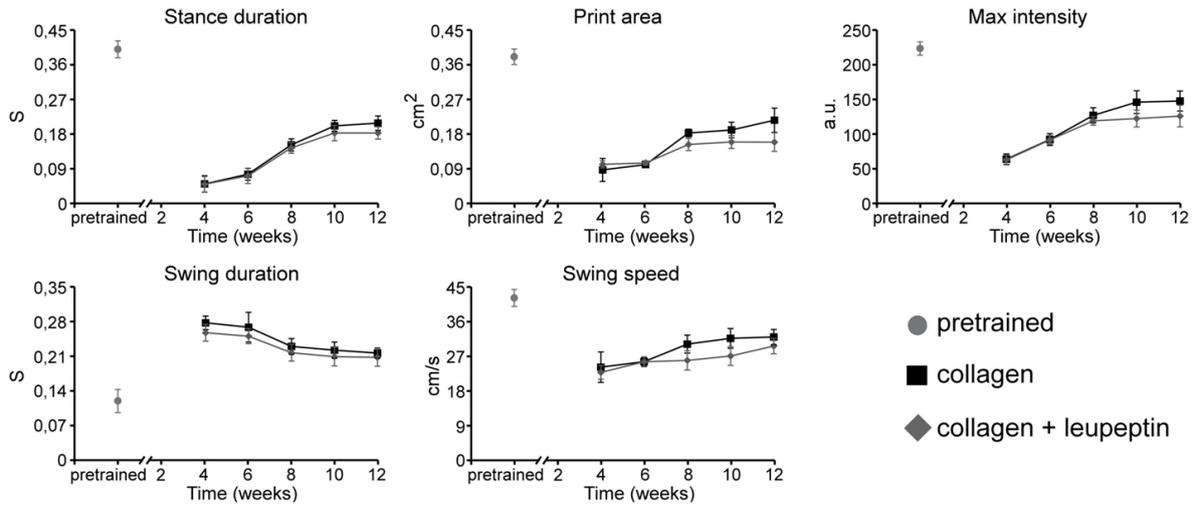
On the other hand, chemoattractive effects of muscle-derived factors on regenerating axons may underlie the improved morphological outcome and enhanced conduction velocities, because intact myofibers are expected to release a plethora of trophic factors after denervation (*Zhao et al., 2004*). The median nerve lesion in Badalamente's study was relatively close to the denervated muscles, which further supports this hypothesis.

Leupeptin's main pharmacological targets 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 (*Camins et al., 2006*). 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.

## **Acknowledgements**

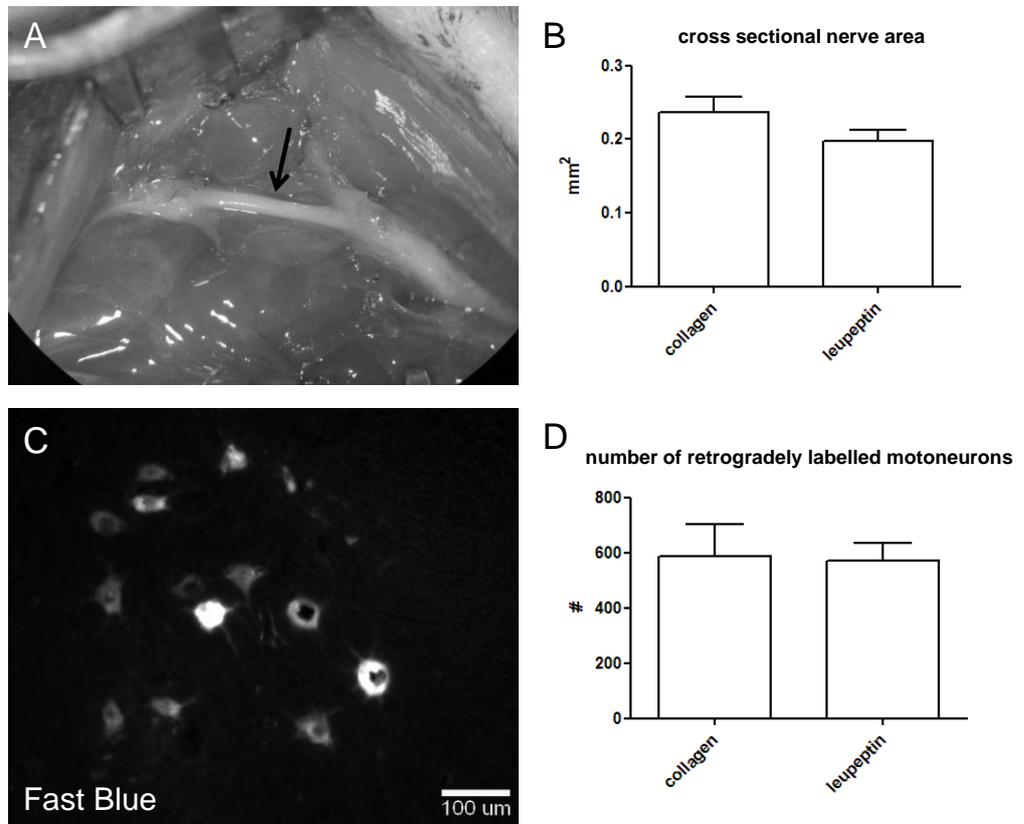
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Fig. 1



**Figure 1. CatWalk automated gait analysis data 4 to 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  $\pm$  S.E.M.

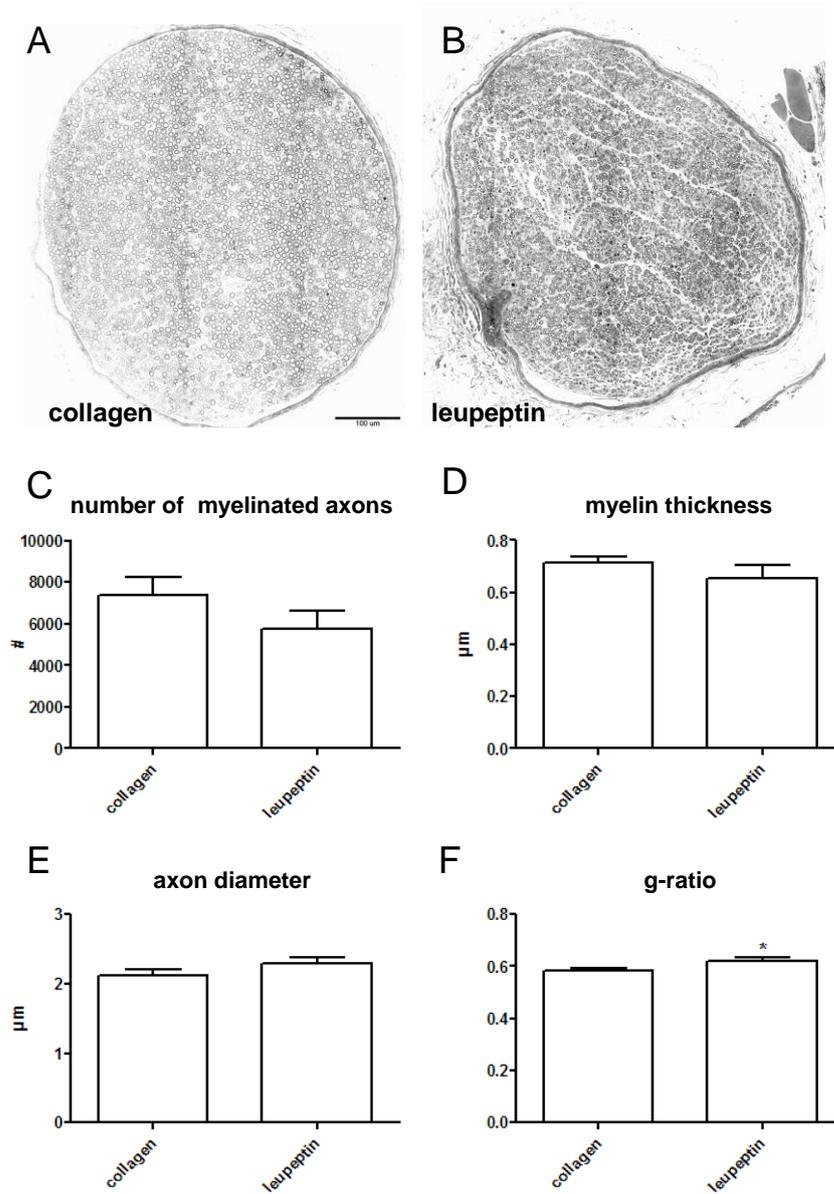
Fig. 2



**Figure 2. Axonal regeneration in control (collagen only) and leupeptin-treated peripheral nerve**

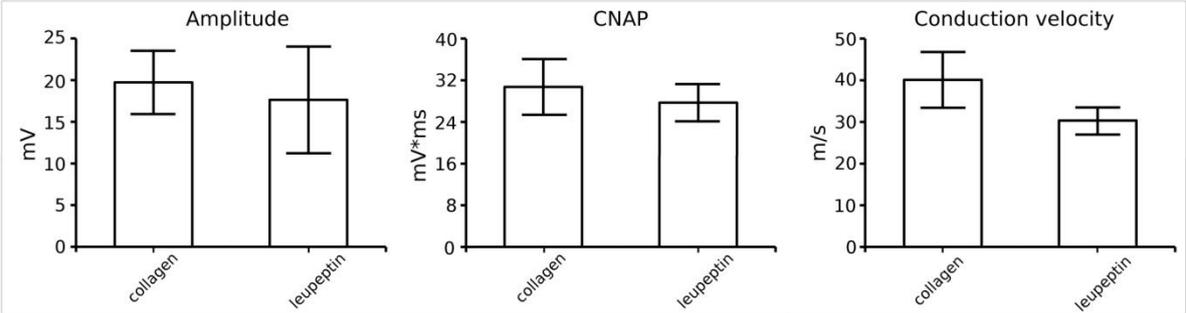
**conduits 3 months after surgery. A:** The panel shows the macroscopic view of the established conduit (arrow) populated by regenerated axons in a control animal. **B:** The cross sectional area of the conduit was non-significantly smaller in leupeptin-treated animals than in the controls. **C-D:** Numerous retrogradely labelled motoneurons were found in the ventral horn of the L4-5 spinal segments after labelling 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 labelled motoneurons (D).

Fig. 3



**Figure 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,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. 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  $\pm$  S.E.M.

Fig. 4



**Figure 4. Electrophysiology stimulation data 3 months postoperatively.** At the end of the survival period the amplitude and CNAP values 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  $\pm$  S.E.M.

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## **Legends**