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

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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.

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].

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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