INTRODUCTION
Research on the mechanisms regulating tendon healing has predominantly focused on mechanical rather than biological features. Over the last decade, however, accumulating data suggests that besides cytokines and vascular factors also neuronal mediators are critical components of tissue repair. A variety of neuronal mediators, so-called neuropeptides, have been identified and found to exert nociceptive, vasoactive, inflammatory and, notably, also trophic effects in several tissues. As for tendons, however, not even the normal innervation has been clarified. This applies not only to the type of nerve fibers, but also to the specific transmitters. Recently, we were able to demonstrate the sensory neuropeptides substance P and calcitonin gene related peptide (CGRP) in normal rat Achilles tendon [1].

Considering that neuropeptides have been shown to mediate trophic effects, it is quite plausible that an intact nervous system is a prerequisite for normal tendon healing. This, however, presupposes a potent and rapid nerve regenerative capability after rupture. To our knowledge, this issue has not been addressed previously in studies of tendon healing. It may prove that nerve regeneration including a complete renewal of transmitters is required for normal neovascularisation or and the proliferation/differentiation of different cell types involved in tissue repair.

In the present study, the occurrence of nerve fibers during healing of ruptured Achilles tendon was monitored up to 8 weeks post rupture. A specific marker of nerve regeneration, the B-50/growth associated protein 43 (GAP), and a marker for mature nerve fibers, the protein gene product 9.5 (PGP), were used. An attempt was also made to quantify these two neuronal markers over time. Moreover, the occurrence of two specific transmitters representing the sensory and autonomic nervous system was analysed, i.e. CGRP and neuropeptide Y (NPY).

METHODS
Operation: Twenty-five male Sprague-Dawley rats were anaesthetised and a 3-cm midline incision was made over the right Achilles tendon. The Achilles tendon was exposed and ruptured 0.5 cm proximal to its calcaneal insertion using a blunt instrument. Subsequently, the skin was sutured without any attempts to repair the tendon. The experiments were approved by the local animal ethics committee.

Immunohistochemistry: The animals were euthanized in groups of five after 1, 2, 4, 6 and 8 weeks. The Achilles tendons were dissected bilaterally, the left intact side serving as an internal control. The tissues were fixed in 4% paraformaldehyde solution containing 0.2% picric acid and cut (15 µm) on a cryostat. Staining was performed with primary antisera for PGP, GAP, CGRP and NPY according to the avidin/biotin method. After incubation with the primary antisera, the sections were incubated with biotinylated goat anti-rabbit antibodies for 40 min. The sections were then incubated for 40 minutes with fluorescein isothiocyanate (FITC)-conjugated avidin. A Nikon epifluorescence microscope (Eclipse E800,Yokohama,Japan) was used to examine the sections.

Computerized analysis: For each time-point (1,2,4,6,8 w.), two longitudinal sections of each Achilles tendon in five rats were stained for GAP and PGP. In each section, images of six microscopical fields (10x objective) from different parts of the tendon were analysed using a soft ware (Easy Analysis, Bergström Instrument) that denotes and considers all positively stained nerve fibres beyond a defined threshold of fluorescence intensity. The results were expressed as the fractional area occupied by positive fibres in relation to the total area. The mean fluorescent/total area, as determined in six microscopical fields in five rats at each time point, i.e. in 30 microscopic fields, was calculated. Statistical analysis was performed by the unpaired t-test.

RESULTS
The two neuronal markers and two neuropeptides exhibited a distinct time related expression. Thus, there were an increased number of GAP-positive nerve fibers 2 weeks after rupture. While PGP-immunoreactivity in the paratenon and connective tissue increased at week one and subsided the ensuing three weeks, PGP-positive fibres, in the tendinous area were first seen at week 2 with a peak expression at week 6. The expression of CGRP-positive fibres coincided with that of PGP, while the increase in NPY-positive fibres was seen somewhat later, i.e. at 6 weeks.

DISCUSSION
The present study suggests that healing of ruptured tendons is characterized by the appearance of nerve fibres expressing different neuronal markers and neuropeptides in an accurately orchestrated, temporal manner. Presumably, early ingrowth of new nerves in traumatized tissue provides a delivery system of neuronal mediators, e.g. sensory and autonomic neuropeptides, required for tissue repair. In fact, CGRP, a potent vasodilator, has been shown also to stimulate cell differentiation and tissue regeneration. Conversely, NPY, a well known vasoconstrictor, has been shown to inhibit fibroblast proliferation, thereby moderating the effects of CGRP. It may prove that early nerve regeneration after injury is prerequisite for normal tissue repair. Whether neuropeptides have direct or indirect effects in tissue repair has yet to be investigated. Thus, one mechanism may be the initiation of proliferation and differentiation of specific cell types. Another may be the promotion of neovascularisation and/or increased blood flow.

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REFERENCE