Neuronal plasticity in relation to nociception and healing of rat achilles tendon

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Abstract

Nerve regeneration and the occurrence of three neuropeptides; i.e. substance P (SP), calcitonin gene related peptide (CGRP) and galanin (GAL), were studied during healing of tendon rupture in the rat by semi-quantitative immunohistochemistry. The neuronal findings were related to nociception as assessed by hindpaw withdrawal latencies at thermal and mechanical tests. Experimental rupture of rat Achilles tendon—normally devoid of nerves—elicited extensive nerve ingrowth into the rupture site in the early phase of healing followed by almost complete fiber disappearance (weeks 12–16). The ingrowth of SP and CGRP positive fibers, seen already at weeks 1–2, was associated with increased nociception. Subsequently, the occurrence of GAL positive fibers at weeks 4–6 was associated with decreased nociception. An even stronger relationship to nociception during healing was observed when the rate of change in neuropeptide expression instead of the expression in absolute terms was considered, according to the “cascade” formula of SP' + CGRP' - GAL'.

It may prove that the observed temporal occurrence of different neuropeptides reflects a role of the peripheral nervous system in regulating synchronously nociception and healing.

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Keywords: Neuropeptides; Pain measurement; Regeneration; Peripheral nervous system; Tendon rupture

Introduction

The role of the peripheral nervous system in tissue healing has only recently been recognized [5,12,23,26]. Sensory neuropeptides have been found to exert trophic effects in different tissues in addition to their nociceptive and proinflammatory actions [15,27,29]. Substance P (SP) and calcitonin gene related peptide (CGRP), representing the sensory system, participate in the regulation of fibroblast and synoviocyte proliferation and of angiogenesis [6,12,22,36]. They have also been implicated in the synthesis/release of cytokines and growth factors [7,21]. In nociception, it is well documented that CGRP potentiates the effects of SP [31,32]. Galanin (GAL), also occurring in primary afferents, has been shown to mitigate nociception and inflammation [10, 13,33].

As for the musculoskeletal system, little is known about the occurrence and role of neuropeptides. In a recent immunohistochemical study of rat Achilles tendon SP, CGRP and GAL positive fibers were identified in the surrounding tissues, but, notably, the proper tendon was devoid of nerve fibers [2]. However, in a subsequent study of ruptured Achilles tendons extensive new nerve fiber ingrowth into the proper tendon was noted in the early healing phase [1]. In the present study of neuronal plasticity in response to injury, the occurrence of SP, CGRP and GAL positive nerves was analysed over time in relation to nociception and healing of ruptured Achilles tendon.

Materials and methods

The study included 52 male Sprague–Dawley rats (180–200 g), housed 5/cage at 21 °C in a 12-h light/dark cycle with water and pellets ad lib according to the Karolinska Institute protocol. The experiments were performed with the approval of the Stockholm North Animal Ethical committee.
The study included two groups of rats, i.e. one for semi-quantitative immunohistochemistry (40 rats) and one for nociceptive tests (12 rats). Five rats in the immunohistochemical group were used as non-ruptured controls.

Surgery

Forty-seven Sprague-Dawley rats were anaesthetized by intraperitoneal (i.p.) injection of sodium pentobarbitone (60 mg/kg bw). A 3-cm midline incision was made over the right Achilles tendon, and the Achilles and plantaris tendons were dissected from the surrounding fascia. The Achilles tendon was ruptured, using a blunt instrument to tear the fibers apart, but leaving the plantaris tendon intact. The tendon was ruptured in the midpart, i.e. 0.75 cm from both the calcaneal insertion and the musculotendinous junction. The skin was closed with 5/0 non-resorbable, Polyamid 6 suture (Ethicon® II, Ethicon, Sommerville, NJ, USA). The animals were allowed to move freely post-operatively.

Immunohistochemistry

Forty rats were anaesthetized by injection of sodium pentobarbitone (60 mg/kg i.p.) before euthanasia in groups of five after 1, 2, 4, 6, 8, 12 and 16 weeks, while the control animals were euthanized at week 0. In vitro perfusion and cryosectioning was followed by immunostaining (all described in detail elsewhere [1]) using primary antiserum for protein gene product 9.5 (PGP), a general marker of nerve fibers (1:10,000, Ultraclone, Cambridge, UK), SP and CGRP (1:10,000, Peninsula Laboratories, USA) and GAL (1:5,000, Peninsula Laboratories, USA). The following controls were used to demonstrate specificity of the staining: (a) preadsorption of the primary antiserum with excess of homologous antigen (50 μg/ml SP, CGRP, GAL, Peninsula laboratories, USA) and GAL (1:5000, Peninsula Laboratories, USA) for 12 h at room temperature; (b) omission of either the primary antiserum, the secondary antibody or the secondary biotinylated antibody. A Nikon epifluorescence microscope (Eclipse E800 Yokohama, Japan) was used to analyse the sections.

Semi-quantitative analysis

This has been described in detail in a previous study [1].

Rate of change in neuropeptide occurrence

The analyses considered both the absolute values and the rate of change (slope) in neuropeptide occurrence between different time points [13]. As for the latter, both changes for individual neuropeptides and combinations of neuropeptides were assessed by a new approach to obtain an estimate of the net physiological effect. Given the well-known fact that CGRP potentiates the nociceptive effect of SP [31,32], the rates of change in occurrence of the respective neuropeptides were combined to get an estimate of the total pronociceptive effect. Furthermore, the known counteracting (antinociceptive effect) of GAL [9,13,33] was considered by subtracting the rate of change in GAL occurrence from that of SP plus CGRP, giving an estimate of a net nociceptive effect. This estimate was subsequently related to the results of nociceptive tests.

Nociception

In 12 male Sprague-Dawley rats, hindpaw withdrawal latencies (HWLs) at thermal and mechanical stimulation were measured each week up to 8 weeks postrupture. Briefly, the entire plantar surface of the rat hindpaw was placed on a hot plate, which was maintained at a temperature of 52 °C (51.8–52.4 °C) [30,34,35]. The time to hindpaw withdrawal was measured in second (s) and referred to as the HWL to thermal stimulation. The Randall-Selitto Test (Ugo Basile, Type 7200, E800 Yokohama, Japan) was used to assess the HWL to mechanical stimulation. A wedge-shaped pusher at a loading rate of 30 g/s was applied to the dorsal surface of the hindpaw. The latency required to initiate the withdrawal response was assessed and expressed in seconds. The average values obtained before Achilles tendon rupture were considered the basal HWL in both tests. All rats were accustomed to the test procedures for 5 days to minimize stress at the actual experiments. An upper limit of 15 s of stimulus in both tests was set to avoid tissue damage.

Statistics

Differences in thermal and mechanical hyperalgesia between the left and right side were analysed using Student's t-test for paired observations. Differences in time courses for the hyperalgesia variables were determined by ANOVA for repeated measurements. As the distributions of the neuropeptides were positively skewed (the subjects were clustered in the lower end), we used a non-parametric approach. Thus, Mann–Whitney's U test was used to analyse differences between weeks. All statistical tests were two-tailed and a significance level of 0.05 was applied.

Results

Immunohistochemistry including semi-quantitative analysis

Nerve fibers immunoreactive to SP, CGRP and GAL were consistently identified in the tissues surrounding the Achilles tendon, i.e. the paratenon and the loose connective tissue at week 0 (controls), whereas the proper tendon tissue was devoid of neuronal immunoreactivity (Fig. 1A–C). In the rupture groups there was new nerve ingrowth during the inflammatory and early proliferative phase (weeks 1–2) of healing, where SP and CGRP occurred mostly in the proper tendinous tissue (Fig. 2A, B, D). There were clear differences in temporal occurrence of SP and CGRP compared to GAL (Fig. 3). GAL firstly emerged in the end of the proliferative phase (weeks 4–6), and was expressed in the "border zone" between the proper tendinous tissue and the surrounding loose connective tissue (Fig. 2C). This was followed by nerve fiber withdrawal (weeks 6–12) from the proper tendinous tissue, and the neuropeptides were again mostly found in the surrounding connective tissue (Fig. 4A–D). A neuronal marker, PGP, confirmed the neuronal occurrence of the staining.

Substance P and calcitonin gene related peptide

One week after rupture, i.e. during the inflammatory phase, an increased number of SP/CGRP-positive nerve fibers were observed in both the connective tissue around the rupture and in the tendon tissue proper (Table 1). The increased SP/CGRP-immunoreactivity in the connective tissue, at week 1, was predominantly found in blood vessel walls surrounded by inflammatory cells. The immunoreactivity in the proper tendinous tissue, which normally is SP/CGRP negative, firstly occurred in free sprouting nerve fibers at the musculotendinous junctions and in the connective tissue close to the rupture site. These nerve fibers progressively invaded the rupture site. During weeks 2–6, i.e. the proliferative phase, the sprouting SP/CGRP-positive fibers in the proper tendinous tissue at the rupture site exhibited their peak occurrence (Fig. 2A, B, D; Table 1). These nerve
Fig. 1. (A)–(C) Normal tendon tissue devoid of nerves. Overview micrographs at week 0 (before rupture) of longitudinal sections through the Achilles tendon built up by putting together computerized images of smaller micrographs stained with H and E (A) and immunofluorescence (B, C). Incubation with antisera to GAL (B) and SP (C). Arrows denote varicosities and nerve terminals. The predominant immunoreactivity is seen in the paratenon and surrounding loose connective tissue, not in the main substance of the tendon (B, C). Nerve fibers immunoreactive to GAL (A) are present in small amounts in the paratenon and the loose connective tissue as free nerve endings. The SP-positive fibers (C) are observed to be more abundant than GAL. The SP fibers are also located in the paratenon and loose connective tissue both perivascularly and as free nerve fibers.

$t$ = tendon tissue; $pt$ = paratenon; $v$ = blood vessel; $lct$ = loose connective tissue. Bar = 250 μm.
Fig. 2. (A)–(D) Nerve ingrowth into the rupture site. Overview micrographs, at week 4 (GAL at week 6) post rupture, of longitudinal sections through the Achilles tendon from computerized images of smaller micrographs stained with H and E (A) and immunofluorescence (B–D). Incubation with antisera to CGRP (B), GAL (C) and SP (D). Arrows denote varicosities and nerve terminals. The predominant immunoreactivity is seen in the proper healing tendinous tissue where CGRP- and SP-immunoreactivities (B, D) both occur in the same sprouting free nerve fibers. Closer analysis of SP shows immunoreactivity (D) both around newly formed vessels and in free nerve endings. Nerve fibers immunoreactive to GAL (C) are present in high amounts in the tendinous tissue and paratenon as both free nerve endings and around vessels. t = tendon tissue; pt = paratenon; v = blood vessel; lct = loose connective tissue. Bar = 250 μm.
fibers were evenly distributed between vascular and non-vascular structures. Some of the vascular fibers were localized in the walls of newly formed blood vessels. During this phase immunoreactivity to SP/CGRP in the surrounding connective tissue decreased. At the end of the proliferative phase and over the following 10 weeks, the SP/CGRP-immunoreactive nerve fibers almost disappeared from the proper tendon tissue, and returned to the normal (preoperative) level in the redeveloping paratenon and surrounding loose connective tissue (Fig. 4A–C).

Galanin

The temporal occurrence of the sensory modulating neuropeptide GAL clearly differed from that of the sensory neuropeptides SP/CGRP (Table 2). Immunoreactivity to GAL was almost undetectable during the first 4 weeks postrupture. However, at the end of the proliferative phase, weeks 4–6, GAL-positive nerve fibers emerged as free nerve endings (Fig. 2C). Some fibers were perivascular in newly formed blood vessels located in the “border zone” between the paratenon and proper tendon tissue. Subsequently, from weeks 6 to 16, GAL-immunoreactivity returned to normal (Fig. 4D; Table 2).

Mechanical sensitivity

Compared to baseline (week 0), mechanical sensitivity on the injured side exhibited a significant increase (decreased HWL) at weeks 2, 4, 6 and 7 postrupture (Fig. 5A). On the contralateral side there was no change during the whole study period.

Thermal sensitivity

Compared to baseline (week 0), thermal sensitivity on the injured side exhibited a significant increase weeks 1–4, followed by a decrease at week 5 postrupture (Fig. 5B). On the contralateral side thermal sensitivity was increased significantly only during week 7.

Neuropeptide occurrence in relation to nociception

When comparing data from the two experimental groups, i.e. neuropeptide occurrence in one and nociception in the other, we observed: (1) the increased SP/CGRP occurrence during weeks 1–4, temporally coincided with increased thermal sensitivity, (2) the increased GAL occurrence during weeks 4–6, temporally coincided with decreased thermal sensitivity, and (3) also with increased mechanical sensitivity.

The relationship between neuropeptide occurrence and nociception over time became more evident when analysing the rates of change (slope) in the occurrence of the different neuropeptides from one time point to another instead of the absolute values (Fig. 6A). Thus, thermal sensitivity was closely related to the rate of change in SP and CGRP occurrence during weeks 1–4 and inversely related to the rate of change in GAL occurrence during weeks 4–8 (Fig. 6A and B). Mechanical sensitivity was not related to the rate of change in SP/CGRP occurrence, but was clearly related to the rate of change in GAL up to week 6 (Fig. 6C). At week 8, however, the rate of change in GAL occurrence was decreased without a concomitant decrease in mechanical sensitivity (Fig. 6C).

When considering the combined rates of change in occurrence of all three neuropeptides, according to \( SP + CGRP - GAL \), there was a close relationship to thermal nociception over the whole experimental period (Fig. 6B). Thus, the combined rates of change appeared to provide a better correlate to thermal nociception than the individual rates.

Discussion

This study demonstrates that the healing process of ruptured tendons is associated with new nerve ingrowth and a specific temporal pattern of neuropeptide occurrence. Notably, the rate of change in peripheral neuropeptide occurrence, rather than the occurrence in absolute terms at each time point, is related to nociceptive thresholds. The results suggest that neuropeptides occur in an orchestrated pattern during tendon healing, which presumably reflects a regulatory role in both nociception and tissue repair.

Our experimental model based on a tissue devoid of nerves under normal conditions appears highly appropriate for studies of neuronal plasticity after injury. As compared to other peripheral tissues, the model provides a clearer picture of the neuronal response to tissue injury and the role of the peripheral nervous system in
Fig. 4. (A)–(D) Nerve fiber withdrawal. Overview micrographs, at week 16 postrupture, of longitudinal sections through the Achilles tendon built up by putting together computerized images of smaller micrographs stained with H and E (A) and immunofluorescence (B–D). Incubation with antisera to CGRP (B), SP (C) and GAL (D). Arrows denote varicosities and nerve terminals. The predominant immunoreactivity is seen in the paratenon and surrounding loose connective tissue (B–D). The CGRP- and SP-immunoreactivities (B, C) occur predominantly in the loose connective tissue. Closer analysis of SP shows immunoreactivity (C) in the loose connective tissue both around vessels and in free nerve endings. Nerve fibers immunoreactive to GAL (D) are sparsely found in the loose connective tissue and paratenon both as free nerve endings and around vessels. t = tendon tissue; pt = paratenon; v = blood vessel; lct = loose connective tissue. Bar = 250 μm.
tissue regeneration. The observation of SP and CGRP immunoreactive nerves emerging already at week 1 after rupture would seem to comply with a role in regulating inflammation and nociception. The ensuing steady state peak during weeks 2-6 may reflect a role in cell proliferation, i.e. tissue repair. The subsequent disappearance of SP and CGRP from the rupture site appears to coincide with the completion of the regenerative process. The observed biphasic curve of SP-CGRP occurrence is in accordance with other reports [14,18,20] including that of Gronblad et al., who demonstrated increased SP and CGRP occurrence at week 4 followed by a decrease at 14 weeks after ligament rupture [11].

At week 1 (inflammatory phase), the increased immunoreactivity to SP and CGRP was predominantly found in the connective tissue adjacent to the rupture site, mostly located in blood vessel walls surrounded by inflammatory cells, which complies with a proinflammatory role of sensory neuropeptides. SP release has been shown to enhance vasopermeability [25] and recruitment of leukocytes [4,8,24]. During weeks 2-6 (proliferative/regenerative phase), the occurrence of SP and CGRP peaked in the rupture site of the proper tendinous tissue. SP and CGRP were partly observed in free nerve endings among fibroblasts in the tendinous tissue, which may reflect a stimulatory role in proliferation as has been demonstrated in cultured fibroblasts [22,36]. SP and CGRP are also known to stimulate proliferation of endothelial cells [12,22,37]. Our observation of free sprouting SP and CGRP fibers around newly formed blood vessels in the rupture site would seem to comply with a role in angiogenesis [37] in addition to vasoregulation [6]. A role for SP/CGRP in healing is corroborated by studies of their depletion, which show impaired wound healing [17,28].

Between weeks 4 and 6, corresponding to the transition from the regenerative to the remodeling phase, there was a dramatic increase in the occurrence of GAL, seen both as free nerve endings and around vessels in a "border zone" enveloping the healing tendon. GAL has been shown to inhibit the proinflammatory and nociceptive effects of SP [9,13,16,33]. The observed inverse

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Table 1
Significance of differences between levels of CGRP, above the diagonal, and SP, below the diagonal, from week 0 (baseline) to week 16, as analysed by two-tailed Mann-Whitney’s U-test

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 6</th>
<th>Week 8</th>
<th>Week 12</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>-</td>
<td>-2.61**</td>
<td>-2.61**</td>
<td>-2.45*</td>
<td>-2.24*</td>
<td>-2.61**</td>
<td>-2.45*</td>
</tr>
<tr>
<td>Week 1</td>
<td>-2.61**</td>
<td>-2.19*</td>
<td>-2.45*</td>
<td>-2.61**</td>
<td>-1.94</td>
<td>-0.52</td>
<td>1.23</td>
</tr>
<tr>
<td>Week 2</td>
<td>-2.61**</td>
<td>0.10</td>
<td>0.00</td>
<td>-0.45</td>
<td>-2.19*</td>
<td>-1.96*</td>
<td>-1.96*</td>
</tr>
<tr>
<td>Week 4</td>
<td>-2.61**</td>
<td>-1.57</td>
<td>-1.78</td>
<td>-1.77</td>
<td>-2.45*</td>
<td>-2.31*</td>
<td>-2.02*</td>
</tr>
<tr>
<td>Week 6</td>
<td>-2.24*</td>
<td>-1.04</td>
<td>-1.64</td>
<td>-0.45</td>
<td>-2.45*</td>
<td>-2.12*</td>
<td>-1.06</td>
</tr>
<tr>
<td>Week 8</td>
<td>-2.24*</td>
<td>-0.52</td>
<td>-0.10</td>
<td>2.19*</td>
<td>-1.94</td>
<td>-0.49</td>
<td>-0.49</td>
</tr>
<tr>
<td>Week 12</td>
<td>-2.61**</td>
<td>-1.36</td>
<td>-1.57</td>
<td>-2.40*</td>
<td>2.24*</td>
<td>-1.98*</td>
<td>-0.58</td>
</tr>
<tr>
<td>Week 16</td>
<td>1.64</td>
<td>-1.64</td>
<td>-1.64</td>
<td>-2.24*</td>
<td>-1.96*</td>
<td>-1.34</td>
<td>-0.75</td>
</tr>
</tbody>
</table>

All differences are calculated as the earlier week minus the later, i.e. an increase in the level is represented by a minus sign.

*p < 0.05, **p < 0.01.

* n = 5 for both CGRP and SP.

* n = 4 for CGRP and n = 5 for SP.

* n = 3 for both CGRP and SP.

* n = 4 for CGRP and n = 3 for SP.

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Table 2
Significance of differences between levels of GAL from week 0 (baseline) to week 16, as analysed by two-tailed Mann-Whitney’s U-test

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 6</th>
<th>Week 8</th>
<th>Week 12</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>-</td>
<td>-0.31</td>
<td>-0.94</td>
<td>-2.61**</td>
<td>-2.24*</td>
<td>-2.61**</td>
<td>-2.40*</td>
</tr>
<tr>
<td>Week 1</td>
<td>-0.73</td>
<td>-2.19*</td>
<td>-2.24*</td>
<td>-2.40*</td>
<td>-1.57</td>
<td>-0.10</td>
<td>-0.49</td>
</tr>
<tr>
<td>Week 2</td>
<td>-0.94</td>
<td>-1.34</td>
<td>-2.24*</td>
<td>-0.52</td>
<td>-1.57*</td>
<td>-1.96*</td>
<td>-1.96*</td>
</tr>
<tr>
<td>Week 4</td>
<td>-1.34</td>
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<td>-1.57*</td>
<td>-2.24*</td>
<td>-2.24*</td>
<td>-2.12*</td>
<td>-2.21*</td>
</tr>
<tr>
<td>Week 6</td>
<td>-2.24*</td>
<td>-2.40*</td>
<td>-1.36</td>
<td>-1.57*</td>
<td>-2.24*</td>
<td>-1.98*</td>
<td>-0.58</td>
</tr>
<tr>
<td>Week 8</td>
<td>-1.98*</td>
<td>-2.24*</td>
<td>-2.12*</td>
<td>-1.36</td>
<td>-2.45*</td>
<td>-1.23</td>
<td>-1.23</td>
</tr>
</tbody>
</table>

All differences are calculated as the earlier week minus the later, i.e. an increase in the level is represented by a minus sign.

*p < 0.05, **p < 0.01.

* n = 5 for GAL.

* n = 3 for GAL.

* n = 4 for GAL.
relationship over time in the occurrence of SP-CGRP on one hand and GAL on the other would seem to comply with counteracting effects in tissue repair and nociception.

Our experiments on neuropeptide occurrence and nociceptive thresholds, albeit based on two separate groups of rats, support a modulating role of GAL on SP and CGRP. Thus, nociception to thermal and mechanical stimulus was related over time to the occurrence of these partly counteracting neuropeptides. Increased occurrence of SP-CGRP weeks 1 and 2 was associated with increased thermal nociception. Increased GAL occurrence weeks 4–6 was associated with decreased thermal nociception but, notably, also with increased mechanical nociception. These features were even more pronounced when considering the rate of change (slope) in neuropeptide expression, indicating that this is a more accurate reflection of nociception than the absolute occurrence at each time point.

The strongest relationship between neuropeptide occurrence and nociception was obtained by combining the rates of change according to findings that CGRP potentiates [31,32] and GAL inhibits the nociceptive effect of SP [3,9,33]. Thus, adding the rate of change in CGRP occurrence to that of SP minus that of GAL, was found to closely match the thermal nociception over time. The approach seems to give a good estimate of the net “cascade” effect of the three neuropeptides regarding thermal nociception.
As for mechanical nociception, there was a relationship to the rate of change in GAL occurrence, but not to that of SP-CGRP. Increased rate of change in GAL occurrence was associated with increased mechanical nociception up to 6 weeks. Whether this should be attributed to the increased occurrence of GAL in the periphery or a concomitant decreased occurrence centrally has yet to be determined. Taken together, our results indicate that GAL occurrence in the periphery inhibits thermal nociception and that mechanical nociception possibly is stimulated in interaction with the CNS. Notably, it has been shown that intrathecal GAL injection inhibits mechanical, but not thermal nociception [19].

In summary, rat Achilles tendon, normally devoid of nerve fibers, offers an excellent tissue for studies of neuronal plasticity in response to injury. The observed temporal pattern of SP, CGRP and GAL suggests a combined role in regeneration and nociception, which is more accurately reflected by the rate of change in occurrence according to a cascade formula than the absolute occurrence of each neuropeptide.

Acknowledgement

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