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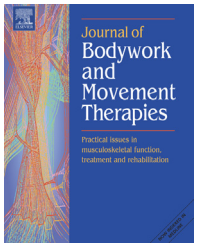

FASCIA SCIENCE AND CLINICAL APPLICATIONS: PROCEEDINGS NOTE: 4TH INTERNATIONAL FASCIA RESEARCH CONGRESS. WASHINGTON DC, SEPTEMBER 2015

Evidence for the existence of nociceptors in rat thoracolumbar fascia

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Summary Recently, the existence of nociceptive fibers in fascia tissue has attracted much interest. Fascia can be a source of pain in several disorders such as fasciitis and non-specific low back pain. However, little is known about the properties of fascia nociceptors and possible changes of the fascia innervation by nociceptors under pathological circumstances.

In this histologic study, the density of presumably nociceptive fibers and free nerve endings was determined in the three layers of the rat TLF: inner layer (IL, covering the multifidus muscle), middle layer (ML) and outer layer (OL). As markers for nociceptive fibers, antibodies to the neuropeptides CGRP and SP as well as to the transient receptor potential vanilloid 1 (TRPV1) were used. As a pathological state, inflammation of the TLF was induced with injection of complete Freund's adjuvant. The density of CGRP- and SP-positive fibers was significantly increased in the inner and outer layer of the inflamed fascia. In the thick middle layer, no inflammation-induced change occurred.

In additional experiments, a neurogenic inflammation was induced in the fascia by electrical stimulation of dorsal roots. In these experiments, plasma extravasation was visible in the TLF, which is clear functional evidence for the existence of fascia nociceptors. The presence of nociceptors in the TLF and the increased density of presumably nociceptive fibers under chronic painful circumstances may explain the pain from a pathologically altered fascia. The fascia nociceptors probably contribute also to the pain in non-specific low back pain.

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Introduction

The biomechanical properties of fascia tissue have been studied by many scientific groups (Vleeming et al., 1995; Langevin et al., 2011; Schleip et al., 2012; Corey et al., 2012). Recently, the existence of nociceptive fibers has become an important issue (Tesarz et al., 2011; Taguchi et al., 2013). Fascia can be a source of pain in fasciitis and probably also in non-specific low back pain (Langevin and Sherman, 2007). Experiments on human volunteers showed that noxious stimulation of the thoracolumbar fascia (TLF) evokes pain. Moreover, injections of pain-producing agents into the fascia have been shown to be more painful than the same injections into skin or muscle (Gibson et al., 2009; Deising et al., 2012; Schilder et al., 2014).

Not only the TLF but also other fasciae possess nociceptors (Taguchi et al., 2013). The nociceptive nature of the nerve endings was identified with immunohistochemical (Tesarz et al., 2011) or electrophysiological techniques (Taguchi et al., 2013).

In this article, the studied structure was the posterior lamina of the TLF, which covers the genuine back muscles (erector spinae muscle).

The article has two aims, namely 1. to describe inflammation-induced changes in the TLF innervation, and 2. to present immunohistochemical and functional data on the properties of fascia nociceptors. Generally, pain from fascia can be due not only to higher discharges in nociceptors but also to an increase in the innervation density by nociceptive endings.

Methods

All data were obtained from adult male Sprague—Dawley rats. The experiments were carried out in accordance with the German law on the protection of animals and the ethical proposals of the International Association for the Study of Pain (Zimmermann, 1983).

Immunhistochemistry

The experiments with fascia inflammation were carried out on deeply anesthetized rats (Ketamine 100 mg/kg i.p. and Xylazine 7.5 mg/kg i.p.; Essex Pharma, Germany and Alverat, Germany, respectively). To induce an experimental fasciitis, 50 μl complete Freund's adjuvant (CFA, Difco Lab., USA) were injected into the TLF in five animals (CFA group; cf. Hoheisel and Mense, 2015). Five naïve rats served as a non-injected control (control group). The experimenter was blinded to the experimental groups.

The CFA injection was made 3 mm lateral to the spinous processes L4 and L5. The cannula was inserted horizontally into the TLF under control of a dissecting microscope. The immunohistochemical evaluation was carried out 12 days after the CFA injection.

In histologic sections the inflamed fascia showed marked leukocyte infiltrations that were largely restricted to the fascia (middle and inner layer). Only minor infiltrations were seen in the multifidus (MF) muscle underlying the fascia.

Nociceptive fibers were visualized with antibodies to calcitonin gene-related peptide (CGRP) and to substance P (SP; Danielson et al., 2006). SP-containing fibers are assumed to be nociceptive (Lawson et al., 1997). The same applies to many of the CGRP-positive fibers (Levine et al., 1993). Moreover, the nociceptive nature of free nerve endings in the fascia was tested with antibodies to the transient receptor potential receptor subtype V1 (TRPV1), one of the main receptor molecules in the membrane of nociceptors (Caterina et al., 1997).

Visualization of calcitonin gene-related peptide (CGRP)
Primary antiserum: rabbit anti-CGRP (Bachem), 1:4000 in PBS, 48 h. Secondary antiserum: biotinylated anti-rabbit IgG (Vector), 1:200, 60 min.

Visualization of substance P (SP)
Primary antiserum: rabbit anti-SP (Chemicon) 1:1000 in PBS, 24 h. Secondary antiserum: biotinylated anti-rabbit IgG (Vector) 1:200, 60 min.

Visualization of transient receptor potential vanilloid 1 (TRPV1)
Primary antiserum: rabbit anti- TRPV1 (Alomone Labs) 1:500 in PBS, 24 h. Secondary antiserum: Cy3-anti-rabbit IgG (Dianova GmbH) 1:500, 60 min.

The histologic staining techniques visualized fibers of passage and nerve endings alike. All nerve endings had the appearance of free nerve endings. A free nerve ending consists of several unmyelinated terminal axons that exhibit axonal expansions (so-called varicosities; Stacey, 1969). The decisive criterion for a free nerve ending was the presence of more than 3 axonal expansions.

Twelve days after induction of the inflammation, the animals were euthanized with an overdose of thiopental sodium i.p. (Trapanal®, Altana Pharma, Germany), and transcardially perfused with a fixative. A piece of TLF containing the site of the CFA injection together with the surrounding tissue was removed close to the spinous processes and snap frozen. Serial cryostat cross sections were made at a thickness of 40 μm and processed for immunohistochemistry.

The quantitative evaluation of immunopositive nerve fibers was carried out on sections in which the fibers were stained with the antibody-avidin-biotin complex method using 3,3-diaminobenzidine tetrahydrochloride as a chromogen. Only TRPV1-positive fibers were visualized with fluorescence staining. To quantify the innervation density, the length of the stained fibers in the tissue sections was determined using an imaging software (analySIS B, Soft imaging System, Olympus Company).

In the medial part of the fascia, three layers can be distinguished in the rat (outer layer underneath the subcutaneous tissue; middle layer with thick collagen fiber bundles orientated obliquely to the axis of the spine, and a thin inner layer of loose connective tissue between the TLF and the underlying MF (Tesarz et al., 2011)). In each layer, the length of the immunopositive fibers was measured, and for each layer the fiber length per 1000 μm^2 area was calculated. Comparisons between the groups were made

using the U-test of Mann and Whitney. A probability level of less than 5% (two-tailed) was regarded significant.

Neurogenic plasma extravasation

In additional experiments on four deeply anesthetized naïve rats (100 mg/kg thiopental sodium i.p. (Trapanal®, Altana Pharma, Germany, followed by 10–20 mg/kg each hour i.v.)), we attempted to elicit a neurogenic inflammation in the fascia. The inflammation was induced by electrically stimulating the dorsal roots L3-L6 at an intensity supramaximal for unmyelinated fibers (10 Hz, 50 V, 15–20 min). With this technique, we sent action potentials into the nerve endings in the fascia (cf. Holzer, 1998). Since the action potentials propagated in afferent fibers towards the periphery, they are called antidromic (i.e. running against their normal direction of conduction). These action potentials release the neuropeptides stored in the free endings. Specifically SP and CGRP are known to cause plasma extravasation by increasing the permeability of capillaries close to the endings. Prior to dorsal root stimulation, the rats were injected i.v. with a large volume of Evans blue (50 mg/kg dissolved in tyrode). In those regions of the fascia where plasma extravasation had occurred, the dye left the circulation and stained the fascia tissue. A neurogenic inflammation is a clear indication of the presence of nociceptors in the fascia.

Results

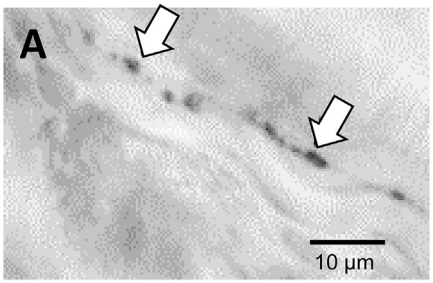
General observations

In sections of both intact and inflamed fascia, the stained nerve fibers appeared either as densely stained fibers of passage without recognizable nerve endings, or free nerve endings that looked like strings of beads with numerous varicosities (Fig. 1A). The latter were assumed to be nociceptive endings if they were stained with antibodies to SP. Most CGRP-positive fibers are likewise nociceptive, but some are known to be mechanoreceptive.

The transient receptor potential vanilloid 1 (TRPV1) is a transmembrane ion channel that can be opened by tissue acidosis (pH below 5.9), high temperatures (above 42 °C), and capsaicin which is responsible for the hot taste of chili peppers (Caterina et al., 1997). It is typical of nociceptive endings and is assumed to be one of the key receptor molecules in the pain pathway.

In the figures, the term "fiber length" includes both fibers of passage and endings. In the middle layer of the fascia, the fiber density was lowest independent of the fiber type studied.

Surprisingly, we did not find any encapsulated receptors (e.g. Ruffini or Pacinian corpuscles) in our material. We looked for these receptors not only with PGP 9.5, a universal marker for all nervous structures, but also with haematoxylin-eosin staining. Since we had no problems finding the tiny free nerve endings, it is improbable that we overlooked the relatively large encapsulated receptors. Apparently, in the medial TLF large mechanoreceptors do not exist (see Discussion).



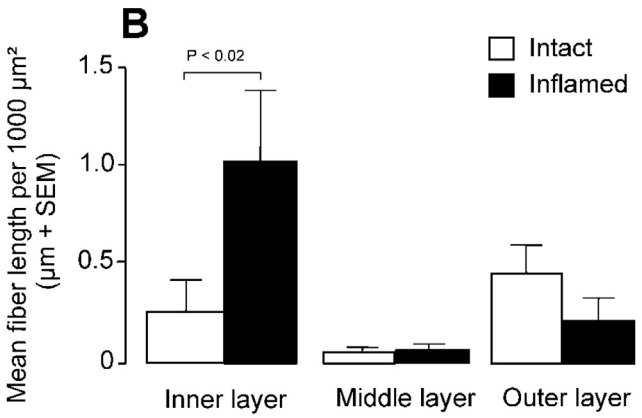


Figure 1 Calcitonin gene-related peptide-positive fibers. **A**) Terminal axon in the outer layer of the TLF. Open arrows mark varicosities. **B**) Quantitative evaluation of the fiber length in the three layers of the fascia. P, statistical difference between intact and inflamed (modified after Hoheisel et al., 2015).

Presumably nociceptive fibers

CGRP-positive fibers

CGRP-immunopositive fibers were the greatest single fiber population found. They were particularly numerous in the outer and inner layer. In the inflamed TLF, the fiber length increased significantly in the inner layer ($P < 0.02$; Fig. 1B) whereas no significant changes occurred in the middle and outer layer.

SP-positive fibers

In the intact, non-inflamed TLF, SP-containing nerve fibers existed exclusively in the outer layer (Fig. 2), but the length of these structures was very short. In the inner and middle layer not a single SP fiber was present. After induction of the inflammation, SP-positive structures were also found in the inner layer. The middle layer remained free from SP fibers also in the inflamed TLF. In contrast to the CGRP fibers – which showed a decreased innervation density in the outer layer in the inflamed fascia -, SP fibers were increased in this layer ($P = 0.054$; Fig. 2). When the length of all SP-positive in the three layers was evaluated together, the increase in the inflamed fascia was significant ($P < 0.02$).

TRPV1-positive fibers

The TRPV1-positive fibers in the TLF looked different from the free nerve endings containing CGRP or SP in that the

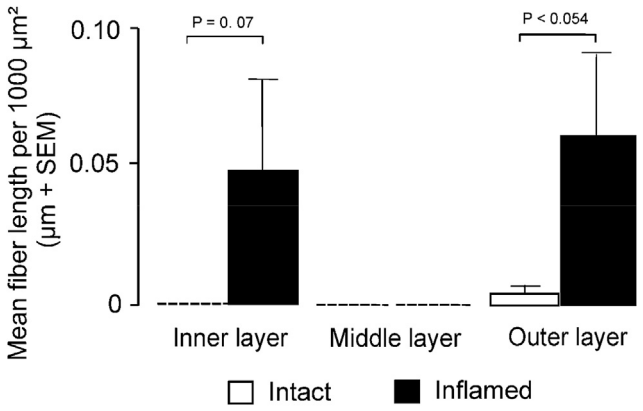


Figure 2 Substance P-positive fibers. Quantitative evaluation of the fiber length in the three layers of the fascia. All terms and numbers as in Fig. 1 (modified after Hoheisel et al., 2015).

entire terminal axon was stained and no axonal varicosities were visible. Fig. 3 shows two endings that exhibited a particularly high concentration of TRPV1 receptors in their membranes.

Induction of a neurogenic inflammation as a test for a nociceptive function

The results presented so far included **histologic** evidence for a nociceptive nature of the free nerve endings, specifically for the SP- and TRPV1-positive fibers. To obtain **functional** data on a nociceptive nature, we attempted to induce a neurogenic inflammation in the TLF. A neurogenic inflammation is a common clinical disorder; it occurs also in patients with a compression of spinal nerves.

In all four experiments a clear neurogenic plasma extravasation was visible in form of dark patches of Evans blue in the fascia. In those areas, where the antidromic action potentials had set free SP and CGRP, the dye left the circulation and stained the fascia tissue. Fig. 4B shows the result of one experiment. The finding that a neurogenic inflammation can be elicited in the fascia, is a clear

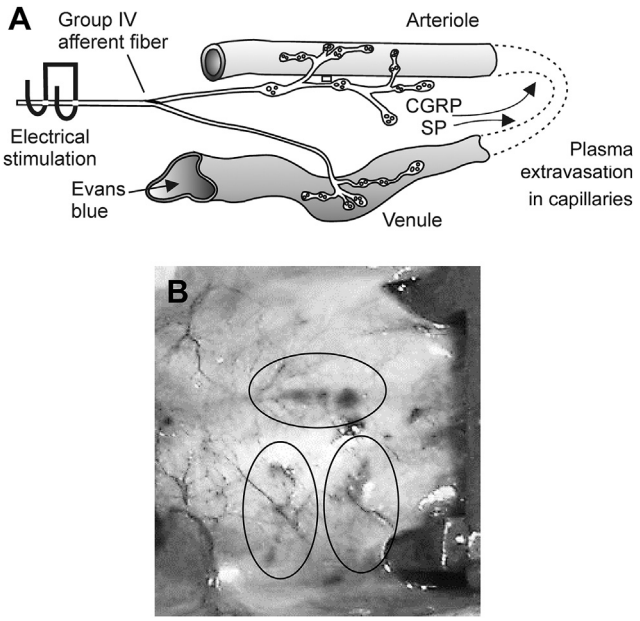


Figure 4 Functional test for the existence of nociceptive fibers in the TLF. A) Scheme of the experimental set-up for inducing a neurogenic inflammation in the fascia. For details, see Methods. **B**) Dorsal view of the exposed caudal thoracolumbar fascia. Along the midline and in the lateral fascia several dark patches are visible (marked with ellipses). The dark patches are areas of plasma extravasation. These patches are signs of a neurogenic inflammation and indicate the existences of fascia nociceptors.

functional evidence for the existence of nociceptive fibers in the TLF.

Discussion

An experimental fascia inflammation was chosen for our study, because the inflammation mimics several painful conditions in which the TLF may become a source pain. These conditions are fasciitis (Malghem et al., 2013; Lebeaux and Sène, 2012) and mechanical overload leading

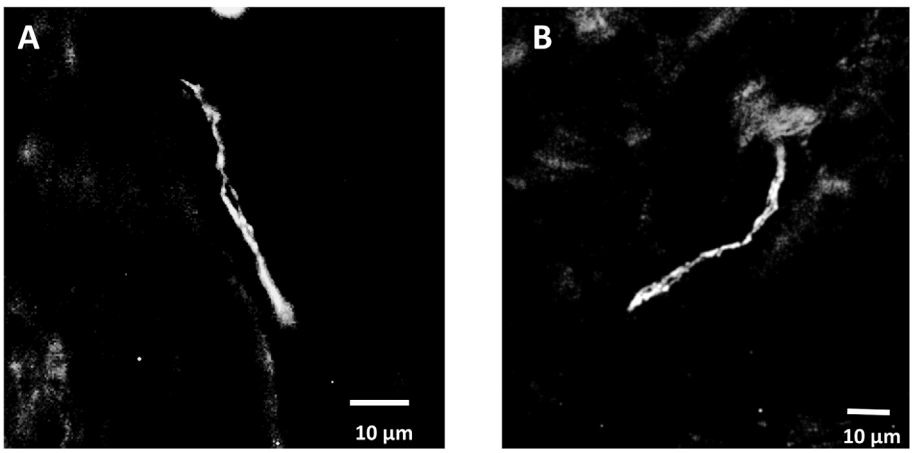


Figure 3 TRPV1-positive fibers. A, B) Two fibers showing a particularly high density of TRPV1 receptor molecules in their membranes.

to microlesions in the fascia which are repaired by an inflammatory process (Solomonow, 2012).

The lack of Ruffini and Pacinian corpuscles in our material is surprising. In a previous study, in which specimen from the human TLF were included (Tesarz et al., 2011), likewise no encapsulated receptors were found. This is a clear difference to the work of other groups who found encapsulated mechanoreceptors in the human TLF (Yahia et al., 1992) and in the supraspinal and intraspinal ligaments (Jiang et al., 1995).

One explanation for this discrepancy is that in the cited studies the TLF specimen were taken from regions other than those used in our investigation. Probably, encapsulated proprioceptors do not exist in all types of fascia and are not equally distributed over all parts of a given fascia. Differences in the density of these receptors in different fasciae have been described (Stecco et al., 2007). We do not want to state that the medial TLF in the rat does not possess proprioceptive endings, though. In a previous electrophysiological study on unmyelinated afferents from the gastrocnemius-soleus muscle — which likewise all terminate in free nerve endings -, a considerable portion of the endings had a low mechanical threshold and could serve as proprioceptors (Hoheisel et al., 2005).

A general finding was that the inflammation-induced changes did not occur in all layers to the same extent. For instance, all fiber types had the lowest density in the middle layer, and for SP-positive fibers the density in this layer was zero. Moreover, we never found a significant inflammation-induced change in innervation density in this layer. The low density of nerve fibers in the middle layer may be due to the fact that the middle layer is composed of thick collagen bundles that probably have to transmit the mechanical forces during trunk movements. During all movements the collagen fiber bundles are likely to move against each other. Therefore, if receptive endings —particularly nociceptors — existed between the bundles the receptors will be squeezed and excited during all trunk movements.

Antibodies to CGRP and SP were used as general stain for sensory fibers (Carr and Lipkowski, 1990). As in other tissues, CGRP-positive fibers were more common than SP-positive afferents, and SP is known to coexist with CGRP in many fibers (Merighi et al., 1988). CGRP-positive fibers include both nociceptive and mechanoreceptive ones. CGRP-positive fibers showed a highly significant increase in the inner layer, but no increase in the other layers (Fig. 1).

SP-positive fibers differed from the other fiber types studied in that they were completely lacking in the middle layer and showed a marked increase in both inner and outer layer. However, when all layers were evaluated together the increase in fiber density was significant (Fig. 2). The increase in SP- and CGRP-fiber density in the inner layer of the inflamed fascia may cause tenderness or pain, e.g. when the multifidus muscle contracts and the muscle moves against the inner layer.

Through what mechanism the fiber length increases in the inflamed fascia is obscure. Possibilities are sprouting or increased branching of the fibers. In a previous study on afferents from the inflamed gastrocnemius-soleus muscle (Reinert et al., 1998), also NGF-positive and growth associated protein 43- (GAP 43-) containing fibers were increased in number. This finding is a strong indication of sprouting. Presently, we cannot tell if this mechanism also applies to the TLF.

As stated above, SP-positive fibers are generally assumed to be nociceptive (Raja et al., 1988; Lawson et al., 1997). Therefore, the increased density of these fibers in the inflamed TLF may indicate greater pain sensitivity. In this line, results from previous rat experiments on dorsal horn neurons showed that the cells were sensitized by the fascia inflammation and showed increased responsiveness (Hoheisel and Mense, 2015).

Conclusion

In the present article both histologic and functional evidence for the existence of nociceptors in the TLF are described. The inflamed TLF exhibited an increase of presumably nociceptive fibers, which may explain the pain from a pathologically altered fascia, for instance in nonspecific low back pain. Because some free nerve endings may function as proprioceptors, the lack of corpuscular proprioceptors in the TLF is no argument against a possible proprioceptive function of the fascia.

Author contribution

SM and UH conceived the study, UH designed and carried out the experiments. SM prepared the manuscript. All authors were involved in the revision of the manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

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