Chapter 1

Nociceptive nerve fibers in the sacroiliac joint in humans.

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Abstract

Background and Objectives: A positive response to sacroiliac joint intra-articular infiltration with local anesthetics is used to confirm sacroiliac joint pain. However, current anatomical and histological knowledge concerning the anatomy of pain perception within the sacroiliac joint intra- and peri-articular structures is insufficient to explain the efficacy of this infiltration, because of the use of unspecific histochemical visualization techniques.

Methods: In this study, immunohistochemistry for calcitonin gene-related peptide (CGRP) and substance P was used to trace nociceptive fibers and receptors in the anterior and interosseous sacroiliac ligaments obtained from 5 human cadavers without history of sacroiliac joint pain.

Results: Microscopic analysis of stained slides showed presence of CGRP and substance P immunoreactive fibers. Thick, wavy, formed bundles were observed in dense and loose connective tissue, whereas single, beaded nerve fibers, occasionally ramified, were observed more frequently in the dense connective tissue and next to blood vessels. Based on their morphologic features, these immunoreactive structures were classified as receptors type IV. Additionally, receptors type II were found in anterior and interosseous ligaments, which contained CGRP or substance P immunoreactive free nerve endings.

Conclusions: We conclude that the presence of CGRP and substance P immunoreactive fibers in the normal anterior capsular ligament and interosseous ligament provides a morphological and physiological base for pain signals originating from these ligaments. Therefore, diagnostic infiltration techniques for sacroiliac joint pain should consider extra- as well as intra-articular approaches.
Introduction

The sacroiliac joint (SI joint) is considered to be one of the possible causes of low back pain. When depending on patient history and the clinical examination, SI joint pain is difficult to diagnose and differentiate from other sources of low back pain. 1,2 The International Association for the Study of Pain (IASP) proposes three criteria which should be fulfilled in order to recognize the SI joint as a source of the low back pain (see tab.1). 3 Although the IASP does not recommend any specific infiltration technique, the most applied is the injection of a local anesthetic under the fluoroscopic guidance into the space surrounded by the joint capsule, i.e. the synovial part of the SI joint. 1,4-6 A prerequisite for the effectiveness of this infiltration, is reaching pain-signaling structures. Extrapolating from general anatomical knowledge 7,8, one could assume nociceptors can be found in intra-articular structures such as the ventral capsule, as well as peri-articular structures such as the anterior, posterior and interosseous ligaments, subchondral bone of the ilium and sacrum (see fig.1). In fact, the presence of myelinated and unmyelinated nerve fibers was shown in dorsal ligaments of SI joint 9,10 and in the ventral capsule of the SI joint in human. 11 Although with gold chloride staining techniques used in these studies it is possible to show the morphological characteristics of nerve structures 10,12, this technique is not suitable to distinguish between different types of neurotransmitters and neuropeptides, and is therefore not sufficient for functional discrimination of labeled nerve fibers. Currently, immunohistochemistry technique is the most sensitive and most specific way to visualize the distribution and localization of sensory nerves that are related to pain perception. Using this immunohistochemistry method, Calcitonin Gene-Related Peptide (CGRP) immunoreactive fibers were recently detected in the SI joint capsule and under the SI joint cartilage in rats. 13

Another point of consideration is that during the intra-articular infiltration of the SI joint, extra-articular leakage of local anesthetics to neighboring nerves may occur 6,14, which may have a positive influence on pain reduction 6, but reduces the diagnostic specificity of the intra-articular infiltration. In addition, peri-articular infiltration of deep interosseous ligaments of the SI joint has been used as a prognostic tool with a positive result. 15 Consequently, these two techniques: the intra-articular and the peri-articular infiltration may both be effective in patients with SI joint pain. However, as the
precise sources of the SI joint pain remain unclear, the effectiveness of both infiltration approaches is difficult to explain.

1. Pain is present in the region of the sacroiliac joint.
2. Stressing the sacroiliac joint by clinical tests that are selective for the joint reproduces the patient’s pain,
   Or
3. Selectively infiltrating the putatively symptomatic joint with local anesthetic completely relieves the patient of the pain.

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<th>Table 1. International Association for the Study of Pain; Diagnostic Criteria for Sacroiliac Joint Pain</th>
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Substance P and CGRP neuropeptides are recognized mediators of pain transmission and have been found in unmyelinated and myelinated nerve fibers. 16,17 These neuropeptides are released in the dorsal horn of a spinal cord as a reaction to various noxious stimuli from peripheral tissue. 16,17 Recent findings, showing the immunoreactivity for substance P in the posterior sacroiliac ligaments adjacent to the posterior superior iliac spine 18, call attention to SI joint ligaments in the generation of pain attributed to the SI joint. Since the presence of nociceptive structures in both the anterior capsular ligament and the interosseous ligament of SI joint has not yet been reported, the aim of the present study was to determine the distribution of sensory nerves in these ligaments, and by that, possibly provide new insights into the pathogenesis of SI joint pain. For this purpose, immunohistochemistry for substance P and CGRP will be applied. In addition, to obtain an overall picture of the presence of neurofilaments in the ligamentous tissue, antisera against tyrosine-hydroxylase (TH) and non-phosphorylated neurofilament (SMI-32) will be used. The TH is a rate-limiting enzyme in catecholamine biosynthesis and therefore helpful to demonstrate the presence of catecholaminergic neurons. 19 Finally, SMI-32 antibodies stain only selected neurofilaments and leave other tissue uncreative; therefore this staining method is useful for the differentiation between neural and non-neural structures. 20
Fig 1. (A) Illustration of the cross section of the sacroiliac joint. The black rectangle is magnified in (B). (1) Sacral bone, (2) iliac bone, (3) cartilaginous portion of the sacroiliac joint, (4) interosseous sacroiliac ligament, and (5) posterior sacroiliac ligament. The double-pointed arrow indicates the anterior capsular ligament, and cartilaginous facets of sacrum (triangle) and ilium (circle) are indicated.

Materials and Methods

Cadavers. The tissue used in this study was obtained from five human cadavers (one fresh and four embalmed), donated to the department of Anatomy of the VU University Medical Center between 2005-2006. The sample consisted of 3 male and 2 female cadavers with an age range 61-91. Characteristics of the cadavers are presented in table 2.

<table>
<thead>
<tr>
<th>Cadaver No.</th>
<th>Gender</th>
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<th>Musculoskeletal Disorders in history</th>
<th>Cause of death (diagnosis)</th>
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<tr>
<td>2</td>
<td>M</td>
<td>91</td>
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<td>5</td>
<td>M</td>
<td>90</td>
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<td></td>
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<td>Congestive heart failure</td>
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Table 2. Characteristics of Cadavers
During the preparation, skin, subcutaneous tissue, muscles and vessels were first dissected ventrally. The lumbosacral trunk, formed by the ventral rami of the spinal nerves L4 and L5 was then exposed, and when gently elevated at the level of pelvic brim, it showed small branches going down to the anterior sacroiliac ligaments (Fig.1). This anteroinferior capsular thickening \(^{21}\) is formed by the anterior ligament and the capsule, which cannot be separated during the gross dissection. \(^{22}\) Therefore, this entire part of the anterior sacroiliac ligament, extending from approximately 2-3 cm cranial to 2-3 cm caudal to the linea terminalis, was used for this study. Subsequently, the dorsal part of SI joints was exposed and the posterior sacroiliac ligament dissected. Then samples approximately 2-2.5cm long, 1.5-2cm wide and 3-8mm thick were taken from the interosseous sacroiliac ligaments. Finally, small samples of the ligamentous tissue adjacent to the bone and cartilage were collected from the ventral part of SI joints at the level of the pelvic brim. Findings have been categorized according to the free nerve endings nomenclature. \(^{23}\) All cadaveric tissue was handled in accordance with regulation of the VU university medical center in Amsterdam concerning the use of human material.
**Tissue preparation.** Dissected ligamentous tissues were post-fixed in 4 % buffered formalin and embedded in 10% gelatin with 30% sucrose in phosphate buffer pH 7.4 (PB) up to 5 days. Tissue blocks which contained bone were, after being treated with formalin, decalcified in Kristensen`s solution (13g sodium formate, 100ml formic acid 98-100%, 400ml AD). After completed decalcification, samples were rinsed over 24 hours with PB and embedded in 10% gelatin with 30% sucrose in PB. Subsequently, gelatin tissue blocks were fixed in 4% paraformaldehyde in PB for 24 hours. Finally, they were cut 40μm thick on a freezing microtome, placed in vials containing 30% sucrose in PB and stored in the freezer at -20°C.

**Immunohistochemistry for substance P and CGRP.** Defrosted free-floating sections were first rinsed with 50 mM Tris-buffered saline pH 7.6 (TBS)(Merck, Sigma). Endogenous tissue peroxidase activity was reduced by treating the sections with 1% hydrogen peroxide (Sigma) solution in TBS at room temperature (RT) for 15 min and rinsed with TBS. Then, the sections were treated over 20 min at RT with 5% normal goat serum (DAKO Cytomation code no. X0907) blocking solution in TBS-tx (triton X-100). Thereafter, the solution was carefully decanted and the sections were allowed to react up to 24 hours at 4°C, with the primary antisera against either substance P (Chemicon International Inc., Temecula, catalog no. AB1566) or CGRP (Chemicon International Inc., Temecula, catalog no. AB5920), diluted 1:2000 and 1:1000 respectively. The sections were then rinsed and reacted with biotinylated goat anti-rabbit IgG (DAKO Cytomation, Denmark, code no. E0432) diluted at 1:200, for 1 hour at RT. After washing in TBS, the sites of antibody binding were visualized using the avidin-biotin peroxidase method (ABC Standard kit, Vectastain, Vector Labs) diluted at 1:200, for 1 hour at RT. The sections were then rinsed with TRIS-HCl buffer pH 7.6 and finally, treated with 3,3'-Diaminobenzidine (Sigma) until desired color intensity. Finally, sections were mounted on slides, counterstained with Nissl-thionin stain, cover-slipped with Entellan and observed in the light microscope.

**Immunohistochemistry for tyrosine-hydroxylase (TH) and non-phosphorylated neurofilaments (SMI-32).** The sections for TH and SMI-32 immunohistochemistry were, before incubation with primary antibody, steamed at 80°C in preheated buffers: TBS pH 9.0 and Citrate Buffer pH 6 respectively, for 25 min for antigen retrieval. The sections
were left for 1 hour in the solutions to cool down, rinsed with TBS and treated for 20 min at RT with 5% normal horse serum blocking solution (Nordic, NHoS) in TBS-tx. The serum was tapped off and sections were incubated overnight in primary antisera: mouse anti-TH (Incstar Catalog No. 1510-22941), diluted 1:2000 and mouse anti-SMI-32 (Sternberger Monoclonal Inc), diluted 1:600. The next day the sections were rinsed and incubated with biotinylated Anti-Mouse IgG, made in horse (Vector Labs, Catalog No. BA-2000) diluted at 1:200, for 1 hour at RT. After rinsing with TBS, the sections were further reacted with the ABC kit according to the above-mentioned staining technique for substance P and CGRP. The differences between staining protocols are summarized in table 3.

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<thead>
<tr>
<th>Immunohistochemical protocols</th>
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<tr>
<td>Protocol steps</td>
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<tr>
<td>Antigen retrieval</td>
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<tr>
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<tr>
<td>Blocking solution</td>
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<tr>
<td>Incubation with 1st antibody</td>
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<tr>
<td>Incubation with 2nd antibody</td>
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**Table 3.** Differences Between Avidin-Biotin Method Immunohistochemistry Protocols for TH, SMI-32, SP, and CGRP Abbreviations: CGRP, calcitonin gene-related peptide; SMI-32, nonphosphorylated neurofilaments; SP, substance P; TH, tyrosine hydroxylase.
Control tissue. Control staining was run in order to test the protocols and the specificity of the antibodies. For positive control, sections of a cervical spinal cord have been used. For negative control, 2-3 sections of the series were stained without the primary antiserum.

Results

Technical notes. In total, 1219 sections of approximately 1,5 cm x 1,5 cm x 40 μm in size were stained: 445 sections with substance P, 415 with CGRP, 123 with SMI-32 and 167 with TH antiserum. The microscopic evaluation was performed by KS and PVJMH. The anterior capsular sacroiliac ligament was easier to stain than the interosseous ligament, particularly because it contained considerably less background and was more resistant to the high temperature, needed for antigen retrieval during TH and SMI-32 stainings. The frontal sections appeared to better for staining of anterior capsular ligament, whereas sagittal oblique sections were better suited for staining of the interosseous ligament. The expression of immunoreactive structures was more intense in the fresh sections. However, these sections were more difficult to judge because of the strong background staining.

![Fig 3. Photomicrograph of a nerve fiber bundle in the anterior capsular ligament. Substance P staining. Scale bar: 100μm.](image)

Substance P immunoreactivity. The SP antiserum revealed nerve tissue in the anterior sacroiliac ligament and ligamentous tissue adjacent to cartilage in 3,4% of the sections.
Both, the loose and dense connective tissue contained SP positive structures. As shown in Figure 2, immunoreactive bundles were wavy-formed and due to their considerable thickness 122-142μm, they were easy to screen with the lowest power lens (4x magnification). Solitary immunoreactive fibers were very difficult to identify, however, an adjustment to the fine focus (40x) of the microscope showed the presence of beaded fibers, enclosed in the positive stained bundles. These fibers were 0.8-1μm thick, and therefore classified as receptors type IV a.

![Fig 4. Photomicrograph showing a mechanoreceptor type II (A) in the anterior sacroiliac ligament with the enclosed substance P positive nerve fiber (B). V, blood vessel. Scale bars: 100μm.](image)

Furthermore, receptors classified as mechanoreceptors type II, were found in the anterior sacroiliac ligament. These mechanoreceptors contained SP-ir fibers as shown in figure 3. In addition to the presence of substance P in nerve fibers, immunoreactivity for substance P was seen in the loose connective tissue next to the blood vessels, and the wall of arteries and veins stained light brown, whereas small capillaries stained “entirely” dark brown. Finally, cell membranes of adipose cells stained positive.

**Calcitonin Gene Related-Peptide immunoreactivity.** CGRP-ir nerve fibers were found in 6% of the sections in the anterior and interosseous sacroiliac ligament, and in the ligamentous tissue attached to the bone. CGRP-ir nerve fibers were more frequent in the dense connective tissue compared to SP-ir fibers and were also seen in perivascular tissue. Positive nerve bundles were 25-125μm thick and solitary immunoreactive fibers
were 1,2-4.4 μm thick and were classified as Type IV α receptors. Single axons were ramified, with irregular bulging and beads and less waved than SP-ir fibers (see Fig. 4). Moreover, receptors Type II, labeled with the CGRP in the interosseous ligament contained positive nerve fibers. In addition, non-nervous tissue, like adipose tissue and blood vessels stained positive, as was the case with substance P. Finally, CGRP-ir nerve fibers were detected in the tunica adventitia of some blood vessels (see Fig. 5).

**Fig 5.** Calcitonin gene-related peptide-positive nerve fibers indicated by arrowheads in the (A, B) anterior sacroiliac ligament, and (C) dense connective tissue adjacent to iliac bone. (D) Cross-section through an immunoreactive nerve fiber bundle in the interosseous ligament and a few solitary calcitonin gene-related peptide-positive nerve fibers (arrowheads). Scale bar: 100 μm.

*Tyrosine hydroxylase and SMI-32 immunoreactive structures.* The free-floating sections appeared to be very delicate and fragile after the antigen retrieval process. Although
almost all sections showed some damage, the staining was usually very good, showing positively stained structures in 2.4% of the sections. Perivascular nerve bundles in the loose connective tissue of anterior sacroiliac ligament were well labeled. (See Fig. 6) Bundles stained with TH were 65-120μm thick and those stained with SMI-32 40-100μm thick. No solitary fibers were detected. Blood vessels stained positive TH but negative with SMI-32 antisera. In the interosseous ligament background staining was so robust, that no positive nerve fibers could be differentiated.

Fig 6. (A) Blood vessel in the loose connective tissue adjacent to the iliac bone. The black rectangle is magnified in (B) in which the calcitonin gene-related peptide positive nerve fiber (arrowheads) is visualized in the tunica adventitia. V, blood vessel; F, adipose tissue. Scale bar: (A) 100μm, and (B) 50μm.

Control tissue staining. The spinal cord sections stained positive in all series. Substance P and CGRP immunoreactivity was found in the in lamina I and II of the dorsal horn. TH-ir fibers were labeled in the total area of spinal cord, whereas non-phosphorylated neurofilaments stained predominantly in the ventral horn. (See schema 1 and figure.7) In control sections, stained with omission of the primary antibody there was no immunoreactivity detected.
**Fig 7.** (A, B) Nonphosphorylated neurofilament-32 positive nerve bundles in the loose connective tissue of the anterior sacroiliac ligament. (C, D) Tyrosine hydroxylase positive perivascular nerve fiber bundles in the anterior sacroiliac ligament. V, blood vessel. Scale bars: 100μm.

**Fig 8.** This figure illustrates the anatomy of the cervical part of a spinal cord. Photomicrographs of marked sections are shown in Figure 9 as follows: dorsal horn (A, B), lateral horn (C), and anterior horn (D).
Fig 9. Photomicrographs of the spinal cord. Dorsal horn labeled with (A) substance P and (B) calcitonin generelated peptide, (C) axons positive for nonphosphorylated neurofilaments-32 in the ventral horn, and (D) tyrosine hydroxylase-positive nerve fibers in the lateral horn. Scale bar: 100μm.

Discussion
The present study aimed to determine the distribution of sensory and sympathetic nerves in the anterior capsular ligament and the interosseous ligament of SI joint, and to provide new insights concerning the origin of SI joint pain from ligaments adjacent to the synovial part of SI joint. This study shows the presence of substance P, CGRP, TH and SMI-32 in ligaments bordering the intra-articular cavity of the SI joint. Substance P immunoreactive nerve bundles were revealed in the anterior capsular sacroiliac ligament, whereas solitary fibers were labeled in ligaments, attached to the bone and cartilage. Furthermore, receptors type II, which were found in the anterior capsular ligament, contained single substance P positive axons. CGRP immunoreactive structures,
predominantly single nerve fibers, were found in the anterior and interosseous sacroiliac ligaments as well as in the receptors type II. SMI-32 and TH positive bundles were detected in the anterior sacroiliac ligament, indicating the overall presence of neurofilaments as well as presence of sympathetic nerves. Walls of blood vessels and adipose tissue showed some immunoreactivity for substance P and CGRP and TH antisera.

The immunohistochemistry combines anatomical, histological and chemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. Consequently, compared to previous histological studies reporting the presence of myelinated and unmyelinated axons in SI joint ligaments 9-11, physiological discrimination of labeled structures could be established in the present study with an immunostaining technique. Furthermore, the morphology of substance P and CGRP positive fibers shown in the present study, particularly their thickness (0.8 to 4.4 μm), indicates their probable nociceptive character. However, without application of any specific myelin staining, the discrimination of 'myelinated' and 'unmyelinated' fibers is not possible, therefore these terms were avoided in our report, and the labeled fibers were categorized as receptors type IV. 23,26,27 Additionally, our results complete the finding of substance P in posterior sacroiliac ligaments as described by Fortin and co-workers 18, as we found substance P and CGRP positive fibers in the ligaments bordering the SI joint intra-articular space.

We succeeded in visualizing catecholaminergic filaments in the anterior sacroiliac ligament. Localization of TH-positive nerve bundles nearby the blood vessels may provide an indication of sympathetic regulation of vasomotor activities. We failed in visualization of TH and SMI-32 immunoreactive structures in the interosseous ligament. The immunostaining protocol with these antisera requires heat induced antigen retrieval. A great deal of the interosseous ligament consists of the adipose tissue and blood vessels 28,29, which, we assume, caused extensive tissue destruction during the antigen retrieval procedure. Because immunoreactivity to TH and SMI-32 was not our principal goal, we did not attempt to apply other forms of antigen retrieval.
A limitation of the present study is that the samples were obtained from specimens older than 60 years, of which the density of myelinated and unmyelinated fibers may be decreased. Furthermore, the specimens history with regard to SI joint pain was lacking. This could be debatable, as our goal was to determine the presence of nociceptors in the SI joint, and to provide new information about the pain origin from this joint. Histological studies of degenerative discs and painful facet joints reported occasional presence of nociceptive neuropeptides in “healthy” control samples, compared to increased amount in the affected one. Therefore, the results of our study could be an underestimation of the actual density of nociceptive structures in painful SI joints.

According to the literature, SI joint painful pathology involves not only intra-articular causes (such as arthritis), but also peri-articular causes (for instance ligamentous injury, enthesopathy, fractures and myofascial pain). Although this infiltration is still the most commonly used diagnostic technique in patients whose complaints presumably originate from SI joint, the evidence for the specificity and validity of diagnostic sacroiliac joint intra-articular injections is moderate, and the evidence for therapeutic intra-articular sacroiliac joint injections is limited. Even though pain reduction after this infiltration may be an important diagnostic parameter, little is known about the background of the pathology and the observed pain complaints. Therefore, the response to this infiltration is difficult to explain. The current study supports the theory that nociceptive signals may originate from the SI joint, particularly from the anterior capsular ligament and the interosseous ligament. In addition, based on results of the present study and previous reports about the innervation of the SI joint, the effectiveness of intra-articular infiltration of SI joint could be explained by leakage of the anesthetics to the SI joint ligaments adjacent to the intra-articular space. In conclusion, our data give new details considering the nociceptive innervation of the SI joint, and therefore suggest that (part of) the pain associated with the SI joint may originate from peri-articular ligaments. Finally, these results suggest that the infiltration techniques for diagnosing of SI joint pain should be extended and comprise both, intra-articular and peri-articular structures.
Acknowledgments

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Reference List


