

Immunohistochemical Localization of Calcitonin Gene-Related Peptide and Substance P in the Rat Knee Cartilage at Birth

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Summary

Substance P (SP) and calcitonin gene-related peptide (CGRP) have been found in the perichondrium and within the cartilage canals. It is still unknown whether they exert a direct effect on chondrocytes during joint development. We processed 28 knees of newborn Wistar rats in 7 different fashions to perform histology and immunohistochemistry studies. Positive immunoreactivity against CGRP and SP was found in the inner aspect of the perichondrium in a close contact with chondrocytes. The presence of CGRP and SP indicates the presence of nerves fibers, and precedes the development of cartilage canals. Nerve fibers may play a role in the development of synovial joints before and during the presence of cartilage canals. The presence of CGRP and SP in the cartilage at birth may be involved in the early postnatal maturation of synovial joints. It remains to be determined whether autonomic innervation is later involved in age-related degenerative joint disease.

Key words

Cartilage development • Peripheral nervous system

Introduction

The relationship between musculo-skeletal system and peripheral nervous system had been studied more than century ago with histologic techniques (Gros 1846, Pansini 1891). With the improvement of immunohistochemical techniques, neuronal occurrence and distribution were investigated (Bjurholm *et al.* 1989). Indeed, there is increasing evidence that, in addition to conventional afferent and efferent functions, peripheal

nervous fibers are also involved in several other, not yet completely clarified, tasks.

Autonomic innervation seems to be involved in afferent sensory input for stretch and pain (Schaible and Grubb 1993). It may also cooperate with the immune system (Bjurholm *et al.* 1994), play a local effector role for immune (Berczi *et al.* 1996) and inflammatory responses (Niissalo *et al.* 2002), and in the modulation of hemodynamics and vascular permeability (Funk *et al.* 1995). Previous studies revealed the presence of

neuropeptides in regions with high osteogenesis (Bjurholm *et al.* 1988), during fracture healing (Hukkanen *et al.* 1993) and healing of soft tissues (Ackermann *et al.* 2002), and at the interface membrane of aseptic loose hip prosthesis (Ahmed *et al.* 1998), where they influence cellular proliferation, metabolism and differentiation (Bernard and Shih 1990, Konttinen *et al.* 1996), and growth factor production (Vignery and McCarthy 1996).

In the young rat, two areas participate in the formation of the mature epiphyses: the periosteal surface or collar center, and the secondary or epiphyseal centers. The primary center of ossification forms the diaphysis and the metaphysis. The cartilage canals are fundamental in the development of the vascular system of the secondary center of the cartilaginous epiphysis. They appear in the rat from the 5th postnatal day before the appearance of secondary or epiphyseal center, crossing the epiphyseal hyaline cartilage (Hedberg *et al.* 1995). The connective tissue of the cartilage canals contains several cell types: chondroclasts, osteoprogenitor cells, fibroblasts and macrophages, polymorphic cells, multivacuolated cells, perivascular cells. The chondrocytes around the canals undergo hypertrophy before the development of the secondary ossification center (Delgado-Baeza *et al.* 1991). Cartilage canals are not a complete extension of the perichondrium, and an "invasion" process underlies their formation (Delgado-Baeza *et al.* 1991).

Previous studies evidenced free nerve endings immunoreactive for the neuropeptides substance P (SP) and calcitonin gene related peptide (CGRP) within the cartilage canals (Edoff *et al.* 2000, Hara-Irie *et al.* 1996). The relationship between these neuropeptides and the cartilage canals has been studied in different species (Strange-Vognsen *et al.* 1997, Strange-Vognsen and Laursen 1997). Although postnatal denervation induces skeletal growth retardation, it is still unknown whether there is a direct function of SP and CGRP on chondrocytes during cartilage development. The presence of neuropeptides during the development of the cartilaginous epiphyses of the knee joint has not been widely investigated (Hedberg *et al.* 1995, Schwab and Funk 1998, Edoff *et al.* 2000).

SP and CGRP synthesized in dorsal root ganglion are transported peripherally and stored in large electrondense vesicles, the accumulation of which constitutes varicosal enlargements of axon. In addition, these neuropeptides are found in unmyelinated (C type)

and small myelinated (A- δ type) primary sensory neurons (nociceptive fibers) (Niissalo *et al.* 2002). This arrangement enables their release upon stimulation very close to their targets (Niissalo *et al.* 2002). Together with the labile chemical nature of neuropeptides, their short half-life clearly depicts the need to confine the effects of these potent mediators and modulators exactly to the right moment (Niissalo *et al.* 2002).

Substance P is part of the superfamily of tachinins. SP exerts a trophic influence on neuronal tissue. The release of SP can lead to vasodilatation, plasma extravasion and leukocyte recruitment, with a clear proinflammatory action which continues through the release of collagenase, prostaglandin E₂, interleukin 1, TNF α , and oxygen metabolites (Niissalo *et al.* 2002). Peripheral nervous fibers containing SP have been identified in the synovial membrane, in ligaments, in the menisci, the subchondral bone of human or rat and in some feline and horse joints (McDougall *et al.* 1997). Recent studies showed neurofilaments in arthritic cartilage and in osteophytes. This suggests a role of this neuropeptide in pain transmission and during degenerative and reparative processes occurring in arthritic joints (Fortier and Nixon 1997).

Calcitonin gene-related peptide (CGRP) is a peptide of 37 aminoacids produced by tissue specific alternative processing of the primary RNA transcripts of the calcitonin gene. Two isoforms (CGRP-I, CGRP-II) exist deriving from the respective genetic shapes of the identified human Calc-I and Calc-II; they differ just by three aminoacids. Two types of receptors CGRP1 and CGRP2, distinct from calcitonin C receptor, are known, coupled with a G_s-protein linked to the adenylate cyclase enzyme, which allows the production of cytoplasmic cAMP and the activation of the kinase A (Konttinen *et al.* 1996, Wimalawansa 1996).

Nervous fibers containing CGRP have been identified in spinal motoneurons (Kashihara *et al.* 1989), in the motor plates of voluntary muscles (Konttinen *et al.* 1996), in the bone marrow (Konttinen *et al.* 1996), close to the epiphyseal plates in contact with osteoblasts (Edoff *et al.* 2000), in the periosteum (Konttinen *et al.* 1996), around the vessels in Volkman's channels and the Havers's channels (Konttinen *et al.* 1996), in the synovial membrane (Funk *et al.* 1995), in ligaments (Konttinen *et al.* 1996), and in subchondral bone of human joints (Konttinen *et al.* 1996). CGRP also seems to be involved in tendon and fracture healing (Konttinen *et al.* 1996; Ackermann *et al.* 2002).

CGRP can inhibit edema formation, and has been described as one of the most potent endogenous anti-inflammatory agents able to inhibit serotonin, leukotriene and mast cell mediated extravasion (Wimalawansa 1996). Substance P and calcitonin gene-related peptide have been identified in the perichondrium and in periarticular neurons (Salo *et al.* 2002). Neuronal involvement may play an important role in the development of degenerative joint disease, consequent to progressive age-related loss of joint innervation. Autonomic nerve fibres may also actively participate in bone repair (Madsen *et al.* 2000).

We report the results of a histological and immunohistochemical study aiming to ascertain whether these two neuropeptides were present in the epiphysis of rat knee at birth.

Methods

All procedures were performed after local Animal Experiment Committee approval had been granted.

Tissue preparation

Newborn (four hours of life) Wistar rats (n=14) weighing 40-50 g were euthanized with an overdose of chloroform. After removal of the skin and of the superficial soft tissues, the 28 knees joints were cut out and processed in 7 different fashions (4 knees each) (Table 1). Fixation was performed with two different solutions, namely paraformaldehyde 4 % and Zamboni's fixative (4 % paraformaldehyde in 0.2 M Söresen

phosphate buffer pH 7.3, containing 0.2 % picric acid) for six hours.

The demineralizing solution used was EDTA-cacodylate: 40.0 g ethylene-diamine-tetraacetic acid (EDTA; Titriplex II), 24.2 g. sodium cacodylate, and 15.0 g sodium hydroxide dissolved in 1000 ml of distilled water and pH adjusted to 7.3. Demineralization took place over an average of 12 days (11-13 days) at 4 °C under continuous stirring and with daily changes of the solution. The end point of demineralization was identified by trying to insert an insulin syringe needle through the tibial cortex. When the needle could be inserted in the tibia without any resistance, demineralization was stopped. Specimens were also radiographed to confirm that demineralization was complete. The specimens were washed in a Söresen phosphate buffer 0.1M (pH 7.2-7.4) and subsequently washed in Söresen phosphate buffer 0.1 M containing 15 % sucrose.

After immersion in Tissue-Tec, 12 of the specimens were frozen in methylbutan (-70 °C) and 16 were dehydrated and embedded in paraffin (Table 1). The 12 knees frozen in methyl-butan were sectioned at a thickness of 15 µ on a Leitz 1720 cryostat at -20 °C and mounted on SuperFrost/Plus glass slides. These sections were used for immunohistochemistry.

The 16 knees embedded in paraffin were sectioned perpendicularly to the transverse axis of the joint at a thickness of 6 µ. These sections were used for histology and for immunohistochemistry. They were then mounted on poly-L-lysine-coated slides at room temperature. The section selected for immunohistochemistry were stained with Alcian blue-PAS-hematoxylin.

Table 1. Methodologies of tissue preparation

Fixation		Demineralizing Solution		Inclusion	
Paraformal-dehyde 4 %	Zamboni's fixative	EDTA-Cacodylate	Paraffin	Frozen	
+	-	+	+	-	
+	-	+	-	+	
-	+	+	+	-	
+	-	-	+	-	
+	-	-	-	+	
-	+	-	+	-	
-	-	-	-	+	

+ = Positive; - = Negative

Immunohistochemical study

The serial sections were processed according to the indirect immunohistochemical procedure of Coons (1958). The sections were pre-incubated with TRIS-buffered saline (TBS-buffer) containing 1 % bovine serum albumin, 10 % normal serum and 0.5 % Triton X-100. PBS (0.01 mol/l at pH 7.3 phosphate-buffered saline) was used to rinse the sections. Immunostaining consisted of an overnight incubation in a wet chamber at room temperature with the primary antibody (Table 2), and of a second incubation with cyanine (Cy3)-conjugated secondary antiserum at room temperature for

1 hour. Protein gene product (PGP) (Berczi *et al.* 1996), a neural protein, was used to detect peripheric nervous fibers. As a negative control, two sections of each specimen were treated in the same manner but without the primary antibody. The slides were mounted with glycerine-PBS and examined with a fluorescence microscope (Axioplan 2 Zeiss) by the main author.

The location of the neuropeptides studied was determined by comparing each immunohistochemical section with the adjacent slice stained with Alcian blue-PAS-hematoxylin.

Table 2. Characterization of antibodies used.

Antigen	Dilution	Species	Producer	Coupling
Primary Antibody				
<i>Calcitonin gene-related peptide (CGRP)</i>	1:200	Rabbit	Peninsula, San Carlos, CA, USA	
<i>Substance P (SP)</i>	1:5000	Neat rabbit	Chemicon International, Temecula, CA, USA	
<i>Protein gene product (PGP)</i>	1:4000	Rabbit	Chemicon International, Temecula, CA, USA	
Secondary Antibody				
<i>Anti-rabbit IgG</i>	1:200	Rabbit	Chemicon International, Temecula, CA, USA	Cy3

Results

Antibodies against PGP, CGRP and SP gave a positive immunoreaction responses in the rat epiphysis at birth (Fig. 1). We obtained a positive immunoreaction in each of the 7 tissue preparations used in this study. The quality of the immunofluorescence was better in the specimens treated with paraformaldehyde 4 %, with or without the demineralizing solution, and frozen.

The epiphysis of the rat knee were composed of a massive cap of hyaline cartilage, rich of clonal cells, covered by perichondrium, rich of mesenchymal cells, which show the classical arrangement in three layers (Delgado-Baeza *et al.* 1991). The chondral tissue at this time is stained strongly with Alcian blue (Fig. 2).

Many nerves fibers immunoreacting to PGP, CGRP and SP were found in the inner aspect of the perichondrium in close contact with the chondrocytes at the interface between the epiphysis and the perichondrium. No nerve fibers were found within the center of the epiphysis.

Discussion

This study shows the presence of CGRP and SP in the rat knee epiphysis at birth. The inner layer of the perichondrium is richly innervated by peripheral nervous fibers. These fibers were often found at the interface with the cartilage, in a close contact with the outer layers of epiphyseal chondrocytes, actively producing cartilaginous matrix and arranged in clonal grouping.

The external layer of the perichondrium is richly vascularized (Delgado-Baeza *et al.* 1991). Therefore, it seems that there is a close relationship between the fibers that we identified and the perichondrial vessels. In the rat knee joint, cartilage canals are detected around the fifth postnatal day (Delgado-Baeza *et al.* 1991), while CGRP and SP are detected at the L3-L4 cord level around the eighth postnatal day (Kontinen *et al.* 1996). Secondary ossification centers appear around the 10th postnatal day (Hedberg *et al.* 1995). Our study shows that, in the rat knee, CGRP and SP nerves fibers precede the development of the cartilage canals. These nerve fibers

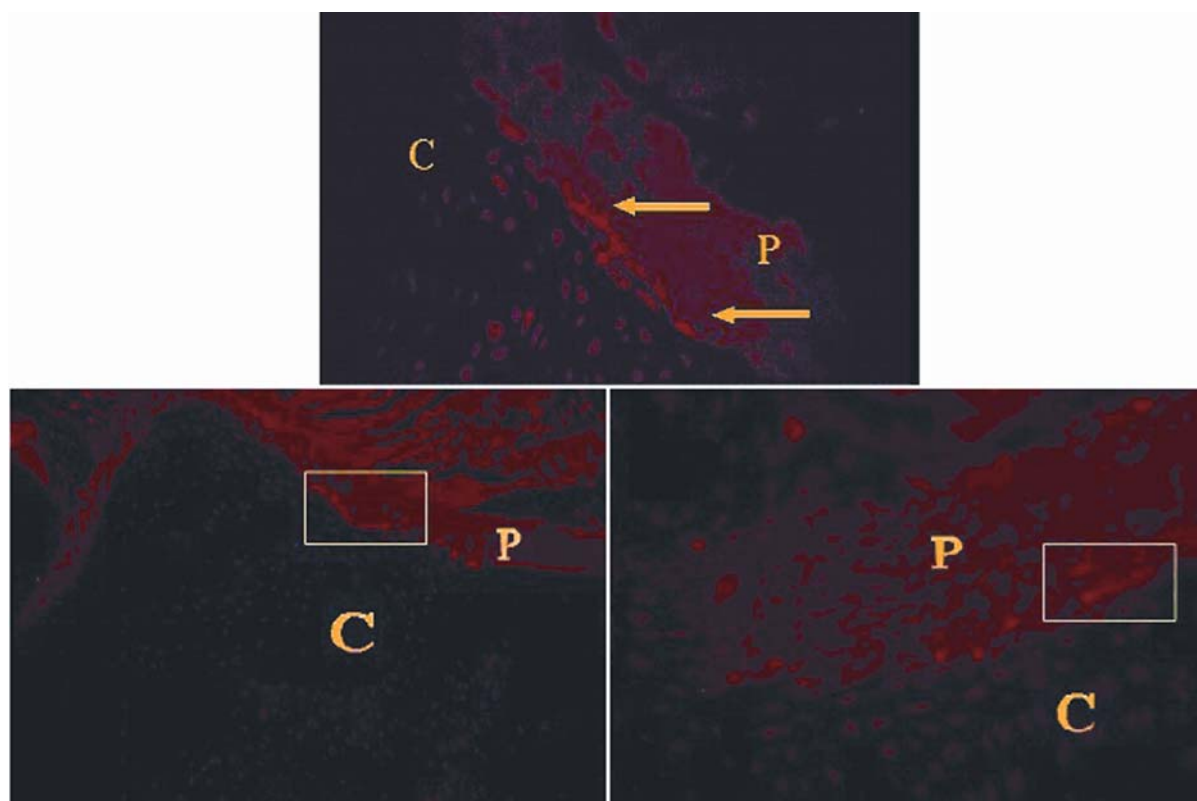


Fig. 1. Immunofluorescence micrograph of a sagittal section of the rat knee epiphyses at birth, incubated with antiserum to protein gene product (PGP), calcitonin gene-related peptide (CGRP), substance P (SP). **Above:** Immunoreactive nerve fibers (arrows) in the inner aspect of the perichondrium (P) projecting in the hyaline cartilage (C) in close contact with chondrocytes, in the femoral condyle, incubated with antiserum to protein gene product (PGP). Original magnification X 40. **Left:** Immunoreactive nerve fibers in the inner aspect of the perichondrium in the tibial epiphysis, incubated with antiserum to calcitonin gene related peptide (CGRP). Original magnification X 40. **Right:** Immunoreactive nerve fibers in the inner aspect of the perichondrium in the tibial epiphysis, incubated with antiserum to substance P (SP). Original magnification X 40.

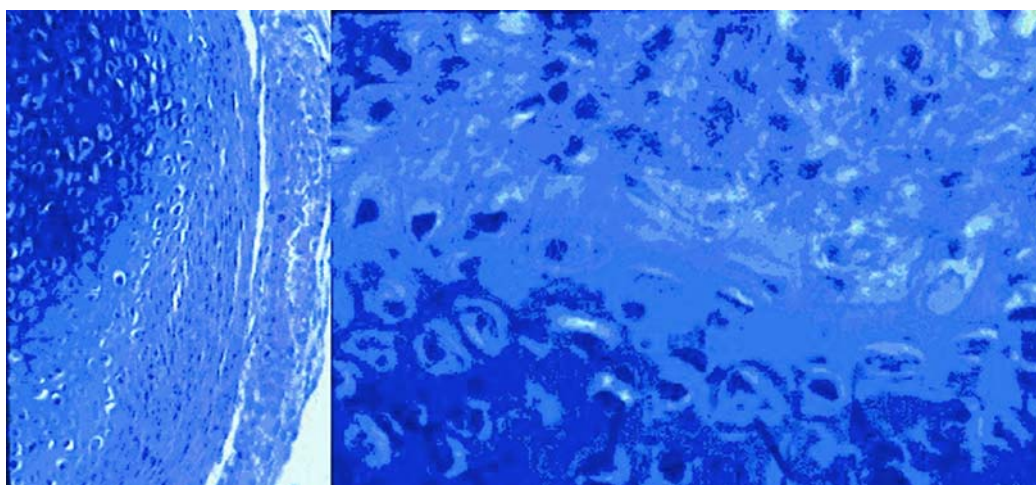


Fig. 2. Micrograph of a sagittal sections of the rat distal femur epiphysis at birth, stained with Alcian blue-PAS-hematoxylin. **Left:** The different gradient of the Alcian blue while the cells become cartilaginous. Original magnification X 60. **Right:** The close contact between perichondrium and articular cartilage. Original magnification X 100.

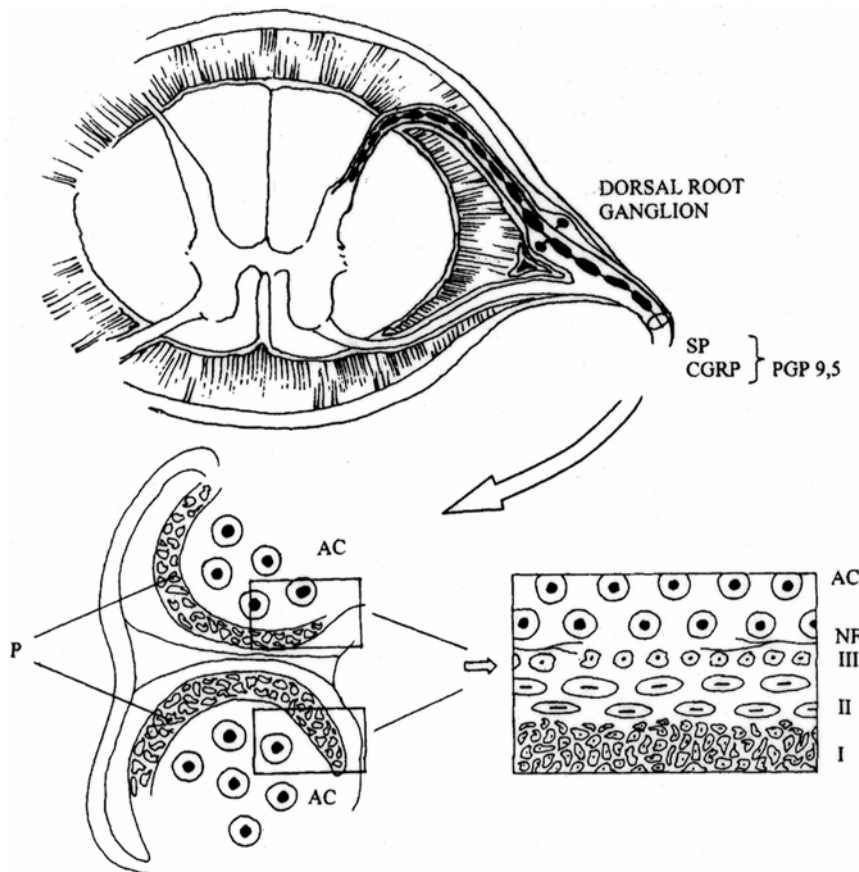


Fig. 3. Above: Schematic view of cross section of spinal cord showing the dorsal root ganglion where SP (substance P), CGRP (calcitonin gene-related protein) and PGP (protein gene product) were detected. Left: Sagittal section of a rat knee at birth, articular cartilage (AC) covered by perichondrium (P) innervated (arrow) by autonomic nerve fibers (SP-CGRP). The inserts indicate the areas of femur and tibia epiphysis whereof the immunoreactive nerve fiber has been detected. Right: The detail of a rat knee at birth showing immunoreactive nerve fibers (NF) situated between the third (III) and inner layer of P and chondrocytes (AC) coming from P as shown in Fig. 1.

therefore seem to be transient and will not be identified with the disappearance of the canals (Hedberg *et al.* 1995). The development of secondary ossification centers is consequent to the presence of cartilage canals. Nerve fibers may exert an influence on the development of synovial joints. In the secondary ossification centers, some neuropeptides play a role in angiogenesis, and exert a trophic action on different cells types within the cartilage canals. CGRP is a potent vasodilator, and, as SP, has a proinflammatory effect causing protein extravasion. It may be possible that the increase of vasopermeability of the perichondrial capillary may be the first step for the development of cartilage canals. This would be the natural consequence of CGRP found during the gestational period in a close proximity to the developing cartilaginous limb skeleton of the mouse (Bidegain *et al.* 1995).

It is still unknown whether all the cells found within the canals have surface receptors for neuropeptides, although some studies show that this is indeed the case for some of them (Vignery *et al.* 1991). The activation of the receptor for CGRP induces IGF-I on rat fetal osteoblasts (Edoff *et al.* 2000). Macrophages

have CGRP receptors (Vignery *et al.* 1991), and CGRP and SP are involved in the inflammation process (Niissalo *et al.* 2002). In concert with other investigations that suggest a neurogenic influence of neuropeptides on the physiology of bone tissue (Bjurholm *et al.* 1990), our data point towards a possible regulatory role of neuropeptides in the development of joints before and during the presence of cartilage canals.

Our investigation has several limitations, e.g. the number of animals is relatively limited. However, our results are univocal, and we believe that our findings are valid and real. Therefore, increasing the number of animals would not increase our knowledge. We are aware that our experiments were performed with only three antigens in mind. Nevertheless, the antigens that we selected are well studied and characterized, and, given the preliminary nature of our investigations, the ones which were more likely to give a positive result.

In conclusion, nerve fibers from the perichondrium are present before the appearance of the cartilage canals at birth in the rat knee epiphyses, and are closely related to chondrocytes. It is possible that the periarticular autonomic nerve fibers, through their

neuropeptides, exert a trophic effect on joint development (Fig. 3). We are still working to understand the mechanisms that regulate the physiology and metabolism of chondrocytes, but the evidence of a close contact between these and CGRP and SP and nerves fibers during the development of joints suggests further investigations, possibly using radioimmunohistochemistry, to clarify their role.

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