Neuropeptides and neurotrophins in arthritis – Studies on the human and mouse knee joint

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Umeå 2008

ur Göteborgs Handels- och Sjöfartstidning, Torgny Segerstedt, 9 oktober 1940

"Den mänskliga personlighetens betydelse är den grundtanke, i vilken liberalismens hela politiska och kulturella åskådning till sist bottnar."

ur Tidskriften Forum, Torgny Segerstedt, 1914

Till min familj
LIST OF PUBLICATIONS

This Thesis is based on the following papers, which are referred to in the text by their roman numerals


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ABSTRACT

Neuropeptides, such as substance P (SP) and bombesin/gastrin-releasing peptide (BN/GRP), and neurotrophins are involved in neuro-immunomodulatory processes and have marked trophic, growth-promoting and inflammation-modulating properties. The impact of these modulators in rheumatoid arthritis (RA) is, however, unclear. An involvement of the innervation, including the peptidergic innervation, is frequently proposed as an important factor for arthritic disease. Many patients with RA, but not all, benefit from treatment with anti-TNF medications.

The studies presented here aimed to investigate the roles of neuropeptides, with an emphasis on BN/GRP and SP, and neurotrophins, especially with attention to brain-derived neurotrophic factor (BDNF), in human and murine knee joint tissue. The expression patterns of these substances and their receptors in synovial tissue from patients with either RA or osteoarthritis (OA) were studied in parallel with the levels of these factors in blood and synovial fluid from patients with RA and from healthy controls. Correlation studies were also performed comparing the levels of neuropeptides with those of pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6)]. Furthermore, the impact of anti-TNF treatment on the levels of BDNF in blood was investigated. In a murine model of RA, the expression of these substances on articular chondrocytes along with their expression in synovial tissue was investigated.

The expression of BN/GRP in human synovial tissue was confined to fibroblast-like and mononuclear-like cells whereas SP was detected in nerve-related structures. Receptors for these neuropeptides (GRP-R and NK-1R) were frequently present in blood vessel walls, and on fibroblast-like and mononuclear-like cells. The expression of BDNF and its receptors, p75 neurotrophin receptor and TrkB, was mainly confined to nerve structures. The levels of SP, and particularly those of BN/GRP, in synovial fluid and peripheral blood correlated with the levels of pro-inflammatory cytokines. There were clearly more correlations between SP-BN/GRP and inflammatory parameters than between BDNF and these factors. Plasma levels of BDNF were decreased following anti-TNF-treatment. In the joints of the murine model, there was a marked expression of neurotrophins, neurotrophin receptors and NK-1R/GRP-R in the articular chondrocytes. The expression was down-regulated in the arthritic animals. A neurotrophin system was found to develop in the inflammatory infiltrates of the synovium in the arthritic mice.

The results presented suggest that there is a local, and not nerve-related, supply of BN/GRP in the human synovial tissue. Furthermore, BN/GRP and SP have marked effects in the synovial tissue of patients with RA, i.e., there were abundant receptor expressions, and these neuropeptides are, together with cytokines, likely to be involved in the neuro-immunomodulation that occurs in arthritis. The observations do on the whole suggest that the neuropeptides, rather than BDNF, are related to inflammatory processes in the human knee joint. A new effect of anti-TNF treatment; i.e., lowering plasma levels of BDNF, was observed. Severe arthritis, as in the murine model, lead to a decrease in the levels of neurotrophin, and neurotrophin and neuropeptide receptor expressions in the articular cartilage. This fact might be a drawback for the function of the chondrocytes. Certain differences between the expression patterns in the synovial tissue of the murine model and those of human arthritic synovial tissue were noted. It is obvious that local productions in the synovial tissue, nerve-related supply in this tissue and productions in chondrocytes to different extents occur for the investigated substances.
ABBREVIATIONS

ACh  Acetylcholine
ACR  American College of Rheumatism
AP   Alkaline phosphatase
BDNF Brain-derived neurotrophic factor
BN/GRP Bombesin/gastrin-releasing peptide
BSA  Bovine serum albumin
CGRP Calcitonin gene-related peptide
ChAT Choline acetyltransferase
CIA  Collagen-induced arthritis
CNS  Central nervous system
CRP  C-reactive protein
DAB  Diaminobenzidine
DAS  Disease activity score
ESR  Erythrocyte sedimentation rate
FITC Fluorescein isothiocyanate
FLS  Fibroblast-like synoviocytes
GRP-R Gastrin-releasing peptide receptor
htx  Hematoxylin
IL-  Interleukin
IHC  Immunohistochemistry
ISH  In situ hybridisation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-LI</td>
<td>-like immunoreactions</td>
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<tr>
<td>LIA</td>
<td>Local injection-induced arthritis</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
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<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NK-1R</td>
<td>Neurokinin-1 receptor</td>
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<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>NSAAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<td>NT-</td>
<td>Neurotrophin-</td>
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<td>OA</td>
<td>Osteoarthritis</td>
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<tr>
<td>PAP</td>
<td>Peroxidase-anti-peroxidase</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
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<td>RF</td>
<td>Rheumatoid factor</td>
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<td>SP</td>
<td>Substance P</td>
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<tr>
<td>TNF-alpha</td>
<td>Tumour necrosis factor-alpha</td>
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<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
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<tr>
<td>trk</td>
<td>Tyrosine receptor kinase</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

## INTRODUCTION

<table>
<thead>
<tr>
<th>Rationale</th>
<th>Neuropeptides</th>
<th>Neuropeptides</th>
<th>Neurotrophins and their receptors</th>
<th>Rheumatoid arthritis (RA)</th>
<th>Animal models of rheumatoid arthritis</th>
<th>Osteoarthritis</th>
<th>The normal and inflamed human knee joint</th>
<th>Tumour necrosis factor-alpha and other cytokines</th>
<th>Anti-TNF treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>11</td>
<td>11</td>
<td>14</td>
<td>15</td>
<td>17</td>
<td>18</td>
<td>18</td>
<td>21</td>
<td>22</td>
</tr>
</tbody>
</table>

## MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Patient material</th>
<th>Rheumatoid arthritis patients</th>
<th>Osteoarthritis patients</th>
<th>Mouse material and induction of arthritis</th>
<th>Sampling</th>
<th>Immunohistochemistry (IHC)</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>24</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>26</td>
<td>30</td>
</tr>
</tbody>
</table>

### Sampling

- Biopsies from human knee joint
- Mouse tissue samples
- Blood and synovial fluid sampling

### Immunohistochemistry (IHC)

- Pre-treatment procedures
- Immunofluorescence
- Peroxidase anti-peroxidase (PAP) staining
- Primary antibodies
- Control stainings

### In-situ hybridisation

### ELISA

- Tissue homogenisation for ELISA
Neuropeptides and neurotrophins in arthritis

ELISA procedures ...........................................................................................................30
Statistics ..........................................................................................................................31
Ethical considerations .................................................................................................32
RESULTS AND DISCUSSION .........................................................................................33
Methodological considerations ..................................................................................33
Human studies ..............................................................................................................34
Morphological aspects (Paper I, II) .............................................................................34
Neuropeptide expression in human knee joint synovial tissue (Paper I) ..................34
  SP and NK-1R expression .........................................................................................34
  BN/GRP and GRP-R expression ..............................................................................36
BDNF and neurotrophin receptor expression in knee joint synovial tissue (Paper II) .36
Neuropeptides and the neurotrophin BDNF in blood and synovial fluid and in relation to inflammatory parameters and pro-inflammatory cytokines (Paper II, III) ..............................................................................................37
Impact of anti-TNF treatment on the levels of BDNF in peripheral blood (Paper II) ..............................................................38
Studies on a mouse model of arthritis .......................................................................39
Morphological aspects (Paper IV, V) ...........................................................................39
Neuropeptides and their receptors in articular chondrocytes and synovial tissue (Paper IV) .................................................................39
  Expression patterns ................................................................................................39
  Interpretations of the findings ..................................................................................40
Neurotrophins and their receptors in articular chondrocytes and synovial tissue (Paper V) .................................................................41
  Expression patterns ................................................................................................41
  Interpretations of the findings ..................................................................................41
CONCLUSIONS ..............................................................................................................43
Human synovial tissue, synovial fluid and blood .......................................................43
  The neuropeptides SP and BN/GRP and associated receptors.........................43
  The neurotrophin BDNF and its associated receptors ....................................43
  Overall comparisons between SP- BN/GRP and BDNF ................................44
Mouse studies on experimental arthritis ....................................................................44
A FURTHER CORRELATION, STUDIES IN PARALLEL AND FUTURE STUDIES ..........46
SVENSK SAMMANFATTNING ......................................................................................48
Inledning ......................................................................................................................48
Syften ..........................................................................................................................49
Material och metod ...................................................................................................49
  Material ....................................................................................................................49
  Metoder ...................................................................................................................50
Resultat ...............................................................................50
Sammanfattning/diskussion ..................................................51
FUNDING ..............................................................................53
TACK TILL... ........................................................................54
REFERENCES .........................................................................56
INTRODUCTION

Rationale

In this study, the importance of neuropeptides and neurotrophins in the knee joint tissues is examined. For a schematic overview of the knee joint, see Figure 1. Parallel focus is applied to pro-inflammatory cytokines, in particular tumour necrosis factor-alpha (TNF-alpha). The examinations include studies on the normal knee joint and that in rheumatoid arthritis (RA), comparisons being also made with that in osteoarthritis (OA). The normal and arthritic knee joint of mice from an experimental model of RA is also examined.

Figure 1. Schematic overview of the normal human knee joint

Neuropeptides

General aspects

Neuropeptides are small molecules of amino acid chains of varying length. The production of neuropeptides in nerves occurs in the cell body from which they are transported to the varicosities and eventually released upon stimulation. Classically it was considered that neuropeptides were only secreted by sensory and autonomic neurons innervating peripheral tissues such as gastrointestinal and joint
tissues. However, they are also produced by endocrine cells, e.g., cells in endocrine tumours [Reubi and Waser, 2003; Zhou et al., 2003]. Furthermore, it has more recently been found that neuropeptides can be produced locally by various other cells, including inflammatory cells [Jonsson et al., 2005]. Neuropeptides can affect the production of cytokines such as TNF-alpha and, thus, modulate joint inflammation (for reviews see [Keeble and Brain, 2004; Ganea et al., 2006]). For example, substance P (SP) increases the production of TNF-alpha whereas vasoactive intestinal peptide (VIP) has the opposite effect. Details of the correlation patterns between neuropeptides/neurotrophins and cytokines in RA are, however, not clearly understood. This hampers understanding whether medications targeting the neuropeptides could be useful in RA, as has been discussed by several groups [Foey et al., 2003; Keeble and Brain, 2004].

**Substance P and the neurokinin-1 receptor**

Substance P is an 11 amino acid peptide produced after post-translational modification of preprotachykinin A. It is mainly produced in sensory neurons but also cells in endocrine tumours, inflammatory cells [Jonsson et al., 2005] and other cell types such as corneal epithelial cells and keratinocytes [Watanabe et al., 2002]. Whether there is a local SP production in synovial tissue remains unknown. SP produced in sensory nerves can function as potent mediator of pain, vessel regulation and inflammatory reactions (see below).

The distribution of SP-innervation in the synovium and the role of SP in RA has been investigated for more than 20 years. SP-containing sensory neurons are present in rat knee synovium [Iwasaki et al., 1995], as well as the synovium from patients with RA [Gronblad et al., 1988], and the synovium of OA patients [Witonski et al., 2005]. However, different studies have shown that nerve-related SP expression can be either up- or down-regulated, or even depleted, in arthritic synovia (for review see [Keeble and Brain, 2004]). In studies using the synovium from the rat ankle joint an increase in SP-positive structures was observed after induction of arthritis [Ahmed et al., 1995a]. Intra-articular injection of SP leads to worsening of the severity of arthritic symptoms and induces endothelial cell proliferation [Levine et al., 1984; Seegers et al., 2004]. It has also been shown that SP can induce angiogenesis in the synovial tissue of the rat knee joint [Seegers et al., 2003] and that SP, to a certain extent, has mitogenic effects on rabbit intervertebral disc cells in vitro [Ashton and Eisenstein, 1996]. In RA per se, it has been shown that SP can stimulate the proliferation of rheumatoid synoviocytes and exert pro-inflammatory effects via increasing TNF-alpha, IL-1 and IL-6 production [Lotz et al., 1987; Cuesta et al., 2002; Pennefather et al., 2004]. Decreased, unaltered, but in most cases elevated levels of SP in knee joint synovial fluid has been observed in several studies of RA (e.g., [Marshall et al., 1990; Larsson et al., 1991; Matucci-Cerinic et al., 1991; Hernanz et al., 1993; Matucci-Cerinic et al., 1993; Cerinic et al., 1998; Westermark et al., 2001]).

The main receptor for SP is the neurokinin-1 receptor (NK-1R), a protein of 407 amino acids belonging to the family of G protein-coupled receptors. It consists of seven hydrophobic transmembrane domains with three extracellular and
intracellular loops, an extracellular amino-terminus and an intracellular carboxy-terminus (for review see [Pennefather et al., 2004]).

SP can stimulate synoviocytes ex vivo to release prostaglandin E2 and collagenase [Lotz et al., 1987]. In that study it was shown that these effects were blocked by a NK-1R antagonist. NK-1R is present on human synovial fibroblasts [Sakai et al., 1998], on cells of the blood vessel walls of the synovium [Sakai et al., 1998] and in blood vessel walls and the counterpart of fibroblasts, the tenocytes, in human tendons [Forsgren et al., 2005]. The expression of the NK-1R gene in rheumatoid synoviocytes correlates with levels of c-reactive protein (CRP) and the radiographic grade of joint destruction [Sakai et al., 1998]. Furthermore it has been reported that human cartilage cells express both SP and NK-1R [Millward-Sadler et al., 2003].

Further information on the importance of SP and on NK-1R expression patterns in RA is of interest as blocking the effects of SP via use of NK-1R antagonists is suggested to be a potential therapeutic possibility in RA [Keeble and Brain, 2004]. Since a down-regulation of SP-containing nerve fibres in arthritis may occur (cf. above), it is of apparent interest to clarify whether a local production of SP exists in the synovial tissue.

**Bombesin/gastrin-releasing peptide (BN/GRP) and the GRP-receptor**

Bombesin/gastrin-releasing peptide (BN/GRP) is a tetradecapeptide which was first discovered in the skin of the frog Bombina bombina [Anastasi et al., 1971]. The BN/GRP peptide family is known to have potent effects in multiple systems in mammals, e.g., related to thermo-regulation and satiety [Anastasi et al., 1971; Pert et al., 1980; Okuma et al., 1995]. The peptide is known to be implicated as a growth factor in different tumours and to function as a paracrine hormone [Cuttitta et al., 1985; Carney et al., 1987; Saurin et al., 1999]. There is rather limited information regarding involvement of BN/GRP in inflammatory diseases. However, it has been observed that BN/GRP can have chemotactic properties for monocytes and to be mitogenic for fibroblasts in the airway system [Aguayo et al., 1990]. It has been suggested that BN/GRP has a marked role in airways [Aguayo et al., 1990; Lemaire et al., 1991; Ashou et al., 2006; Dal-Pizzol et al., 2006]. Furthermore, BN/GRP has been shown to have healing effects on cutaneous wounds and to show anti-ulcerogenic effects on intestinal damage [Gulluoglu et al., 1999; Yamaguchi et al., 2002].

There are several receptors for BN/GRP, i.e., BB1, BB2 (GRP-R) and BB3. GRP-R is the most widely distributed of these receptors in the peripheral tissues (for review, see [Jensen et al., 2008]). It consists of 384 amino acids and is expressed, among others, on many different tumours, cells of the central nervous system, smooth muscle cells and pancreatic acinar cells [Jensen et al., 2008]. Targeting GRP-R is suggested to be of importance in the treatment of several cancers, such as lung cancer and endocrine tumours [Moody et al., 2003; Zhou et al., 2003]. In a model of septic shock, the levels of pro-inflammatory cytokines were decreased following blockage of the GRP-R [Dal-Pizzol et al., 2006].
BN/GRP was detected in studies on knee joint synovial fluid, in which it was shown that levels of BN/GRP were increased in patients with RA [Westermark et al., 2001]. According to Iannone & Lapadula (1998), BN/GRP-like peptides are expressed by cultured human chondrocytes [Iannone and Lapadula, 1998]. It is completely unknown whether BN/GRP and its receptor, are present in the synovial innervation in the synovium of patients with RA. Furthermore, the source of the BN/GRP detected in knee joint synovial fluid is unknown [Westermark et al., 2001]. As with SP, it is also not known as yet whether or not there is a local production of BN/GRP in the human synovial tissue. Information on the occurrence of possible correlations between this neuropeptide and cytokines is also relevant for the understanding of the inflammatory process in RA.

Other neuropeptides
There are several other neuropeptides of interest in inflammatory diseases. Vasoactive intestinal peptide (VIP) has, apart from effects on the vasculature and on various cell types such as bone cells [Lundberg and Lerner, 2002], anti-inflammatory properties. This peptide can thus inhibit the production of pro-inflammatory cytokines by macrophages [Martinez et al., 1998; Delgado et al., 1999]. It has also been shown to have beneficial effects on arthritis symptoms in studies on the collagen-induced arthritis (CIA) model [Delgado et al., 2001]. Patients with RA have higher levels of VIP in their synovial fluid than do OA patients [Hernanz et al., 1993].

Calcitonin gene-related peptide (CGRP) is a neuropeptide known to have potent effects on vasodilation and in neurogenic inflammation [Brain et al., 1985; Gamse et al., 1987]. It is released by sensory neurons and is considered to have pro-inflammatory effects (for a review, see [Brain, 1997]). It has, however, also been shown to reduce the production of IL-6 and matrix metalloproteinase-2 (MMP-2) from whole blood cells [Hernanz et al., 2003]. There is an increase in proportion of CGRP-immunoreactive dorsal root ganglion cells in response to inflammation of the rat ankle [Hanesch et al., 1993].

Neuropeptide Y (NPY) is another neuropeptide that may be involved in joint diseases. It is mainly released from the nerve fibres of the sympathetic nervous system [Lundberg et al., 1982] and has vasoconstrictive effects [Lundberg et al., 1985]. Levels of both NPY and CGRP in synovial fluid from the knee joint of patients with RA have been found to be elevated compared with those for healthy control subjects [Larsson et al., 1991].

Neurotrophins and their receptors
The family of neurotrophins consists of four members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4). The different neurotrophins bind to different high affinity receptors, but the same low affinity receptor, namely neurotrophin receptor p75 [Martin-Zanca et al., 1989; Chapman and Kuntz, 1995]. The high affinity receptors, i.e., the tyrosine kinase (trk) receptors (TrkA, TrkB and TrkC), have different affinities for the
neurotrophins. Thus, TrkA binds NGF, whilst NT-4 and BDNF bind to TrkB and NT-3 binds to TrkC [Martin-Zanca et al., 1989]. The neurotrophins are expressed in the nervous system, in inflammatory cells and several other cell types such as epithelial cells and skeletal muscle cells [Nockher and Renz, 2005].

It is well-established that neurotrophins play important roles in the differentiation, survival and proliferation of cells in the nervous system. They possess trophic and growth-promoting effects on neurons [Levi-Montalcini, 1987; Linnarsson et al., 2000]. Primarily NGF, but also BDNF, have been implicated in inflammatory processes including in autoimmune diseases [Halliday et al., 1998; Weidler et al., 2005]. NGF possesses a capacity to attract leucocytes to the site of inflammation and it is upregulated in inflammatory diseases, e.g., pancreatitis [Gee et al., 1983; Friess et al., 1999]. Neurotrophins are also suggested to be implicated in the pathogenesis of inflammatory pain and elevated levels of NGF in the periphery is thought to be a major contributor to this state [Woolf et al., 1994]. Nevertheless, neurotrophins may also have healing effects [Aloe, 2004; Muangman et al., 2004].

It has been shown that NGF is found in high levels in the synovial fluid from patients with RA, that macrophages and fibroblast-like synoviocytes (FLS) express BDNF and that BDNF can be detected in human synovial fluid [Halliday et al., 1998; Rihl et al., 2005; Weidler et al., 2005]. It has been suggested that NGF can modulate joint inflammation [Manni et al., 2003], possibly, in part at least, by modulating release of neurotrophines from sensory neurons [Bowles et al., 2004; Muangman et al., 2004]. Nevertheless, the involvement of neurotrophins in arthritic processes is largely unknown, with the impact of BDNF, on the whole, being clearly less investigated than NGF. Consequently, it is unknown whether or not there is an expression of BDNF in synovial innervation and in the articular chondrocytes. However, there is interesting information concerning effects of anti-TNF treatment on BDNF levels. In a preliminary study it was thus shown that there was a tendency for the levels of BDNF in plasma to decrease following anti-TNF treatment [del Porto et al., 2006]. An interesting background factor in this respect is the finding that TNF-alpha can enhance BDNF secretion by peripheral blood monocytes [Schulte-Herbruggen et al., 2005].

Rheumatoid arthritis (RA)

Rheumatoid arthritis is an inflammatory autoimmune joint disease that affects approximately 1% of the human population. It mainly affects the synovium, the cartilage and subchondral bone but the disease has systemic effects with increased comorbidity and mortality, particularly due to cardiovascular disease [Wallberg-Jonsson et al., 1997]. There are well-developed criteria for the characterisation of RA in the clinical practice, i.e., the so-called ACR criteria [Arnett et al., 1988]. Briefly, these are as follows: (1) morning stiffness, (2) arthritis of 3 or more joint areas, (3) arthritis of the hand joints, (4) symmetrical arthritis, (5) the presence of rheumatoid nodules, (6) detectable serum rheumatoid factor (RF), and (7) radiographic changes. At least 4 out of 7 of these criterias must be fulfilled, with
criteria 1 to 4 being present for at least 6 weeks, in order to diagnose RA. Recently assay protocols for an antibody, anti cyclic citrullinated peptide/protein antibody, with higher specificity and at equal sensitivity than RF, have been developed, increasing diagnostic ability for RA [Rantapaa-Dahlqvist, 2005].

The exact mechanisms of disease initiation and propagation are still essentially unknown. However, research on the aetiology of RA has been, and still is, extensive and it is now well-established that genetic factors, both human leucocyte antigen (HLA) and non-HLA genes, are important [Gregersen, 1999; Bowes and Barton, 2008]. Nevertheless, RA is a multifactorial disease in which environmental and life-style factors, e.g., obesity and smoking, are potentially relevant [Symmons, 2003].

The disease is characterised by joint inflammation, i.e. synovitis (inflammation in the synovium) together with hyperplasia (thickening of the synovial membrane) leading to pannus formation (see further below). Cartilage and bone destruction gradually appear, in the end leading to deformity of the inflamed joints (Figure 2), which, concerning the knee joint, eventually may lead to prosthesis operations.

No curative treatment is available but disease modifying anti-rheumatic drugs (DMARDs) (e.g., methotrexate, sulphasalasine, leflunomide, injectable gold, etc.) can reduce disease activity when used efficiently. Treatment has recently been improved by the use of drugs targeting TNF [Emery, 2006]. For evaluation of disease status, several clinical parameters are analysed. Laboratory analyses of both CRP and erythrocyte sedimentation rate (ESR) are valuable, and easily performed, tools for estimating the degree of inflammation. To evaluate response to therapy of the disease activity over time, disease activity scores (DAS) are calculated. Such scores are based on the number of tender and swollen joints, patients global assessment (visual analogue scale, VAS) and ESR. DAS has been validated in different studies and are currently used with a 28 joint count (DAS28 score) [Prevoo et al., 1995].

Figure 2. Human hands affected by rheumatoid arthritis, photograhpic picture and radiograhpic picture.
Animal models of rheumatoid arthritis

Several different animal models of arthritis have been used in order to elucidate early events in joint pathology and to study effects of potential anti-arthritic agents. Commonly used models are adjuvant arthritis, antigen-induced arthritis and collagen II-induced arthritis (CIA) models. One of the most commonly used model for studies of arthritis resembling human RA is the CIA model [Kannan et al., 2005]. This model shares several clinical and histological features of human RA such as infiltration of neutrophils and macrophages, formation of pannus and destruction of cartilage and bone. This type of arthritis was first described in 1977 by Trentham and colleagues [Trentham et al., 1977]. CIA is induced after intradermal injection of collagen II, together with complete Freund’s adjuvant, at the base of the tail. For the present studies a new model, developed in Umeå and referred to as local injection-induced arthritis (LIA), which also uses collagen II as antigen but the arthritis induction is complemented with an intra-articular injection on day 21, was used. It shares most features of the CIA model, but it also includes an innate immune response triggered by the local injection of collagen II [Li et al., 2005]. (Figure 3, 4).

Figure 3. Knee joint section showing articular chondrocytes (below) from a healthy mouse.

Figure 4. Arthritic knee joint from a mouse where you can see how the pannus (below) is destroying the cartilage.
Osteoarthritis

Osteoarthritis (OA) of the knee joint is a common disease affecting both men and women. The prevalence increases with age. OA can be primary or secondary to trauma, surgery, infection or other disease processes. OA of the knee joint is diagnosed using Altman’s diagnostic criteria [Altman et al., 1986]. The disease is caused by an imbalance in cartilage metabolism, i.e., levels of synthesis vs destruction, which leads to degradation of cartilage. There is also inflammation in the synovium and damage to the subchondral bone. The processes of OA are very complex and the aetiology is not well defined. Many different mediators and cell types are involved including cytokines, growth factors, matrix metalloproteinases (MMPs) and chondrodegradative enzymes (for a review see [Moskowitz et al., 2004]). When the disease is very pronounced, a knee joint prosthesis operation may have to be performed. The knee joint involvement in OA is graded according to Ahlback’s criteria [Ahlback, 1968].

The normal and inflamed human knee joint

Synovial tissue and its cells

The synovial membrane (synovium) in normal, non-diseased, joints is thin being lined by only a few layers of cells. In the deep parts, it contains tissue constituted of loose connective tissue, fibrous tissue or adipose tissue. Collagen, particularly of type I and III, fibronectin and proteoglycans are present in the matrix of the synovium. The main function of the synovium is production of the synovial fluid. Another important function for the normal synovium is to remove debris from the joint space. The cells in normal synovial tissue are predominantly phagocytic cells generated from monocytes (tissue specific macrophages), and fibroblasts. The synovium in patients with RA is hypertrophic and contains numerous mononuclear cells. Pannus tissue, the destructive tissue in RA, is a hypertrophic and inflammatory synovial tissue localised at the junction of the synovial lining and cartilage and bone (Figure 5). Macrophages and FLS are the predominant cell types in the inflamed synovium (for review see [Tak and Bresnihan, 2000]). The macrophages are of great importance for the inflammation in RA being numerous in the inflamed synovial membrane and at the cartilage-pannus junction [Mulherin et al., 1996; Kinne et al., 2007]. The macrophages are pro-inflammatory and are major contributors to the joint destruction via secretion of cytokines and MMPs [Kinne et al., 2007].
The FLS are, together with osteoclasts, involved in the processes of bone erosion, and the destruction of cartilage and bone (for reviews see [Goldring and Gravallese, 2000; Abeles and Pillinger, 2006]). They function in a direct manner by secreting MMPs and indirectly by secreting cytokines leading to recruitment of many other cells such as monocytes, lymphocytes, neutrophils and mast cells [Abeles and Pillinger, 2006]. Lymphocytes, neutrophils and mast cells also participate in the process(es) of joint destruction in RA [Tak and Bresnihan, 2000]. The role of lymphocytes in the synovium has been studied extensively such that it is well-established that both T- and B-lymphocytes play central roles in the pathogenesis of RA (for reviews see [Bugatti et al., 2007; Lundy et al., 2007]). B-lymphocytes are important, not least for autoantibody production, whilst T-lymphocytes contribute to inflammation and tissue destruction [Bugatti et al., 2007; Lundy et al., 2007].

Fine venules and capillaries occur beneath the lining cells of the synovium. These superficial vessels have a fenestrated endothelium via which fluid transudates in order to contribute to the joint fluid. Larger vessels are present in the deep parts of the synovium. Angiogenesis is crucial for the formation of the pannus in RA. Hence, the ingrowth of arterioles and venules in the deep parts of the synovium is obvious. In this context, it is known that vascular endothelial growth factor (VEGF) plays an important role for the angiogenesis in RA synovium (for review see [Malemud, 2007]). There are also several other factors which are of potential interest in the neo-angiogenesis in RA, e.g., growth hormone and insulin-like growth factor-1 (IGF-1) [Malemud, 2007].
**Joint innervation**

From early animal studies, it has been observed that knee joints are supplied with sensory and sympathetic nerve endings [Samuel, 1952; Skoglund, 1956; Heppelmann, 1997]. The cat knee joint has been an important model for studies into the innervation of the joint in both normal and inflamed joint tissue [Heppelmann, 1997]. The innervation pattern consists of group I – IV sensory afferents and sympathetic fibres (for review see [Heppelmann, 1997]). Peptide-containing sensory [Saito and Koshino, 2000] as well as sympathetic [Miller et al., 2000] nerve fibres have been found in the human knee joint synovium. The presence of SP in the sensory innervation in animal joints has been frequently documented (cf above).

The main function of sensory nerves in joints is to detect and transmit mechanical information to the central nervous system. The process of pain in arthritis is, to a large extent, unknown although much effort has been made in understanding this process [McDougall, 2006]. Nevertheless, early studies showed that sensory denervation with capsaicin attenuated inflammation and nociception in arthritic rats [Cruwys et al., 1995].

Joint innervation is of interest as the nervous system generally is assumed to be involved in the development of arthritis. For example, it is known that the paralytic limb of hemiplegic patients with RA is spared from the inflammatory process [Thompson and Bywaters, 1962; Glick, 1967]. The midline symmetry and the involvement of the richly innervated peripheral joints in RA also suggest that the innervation may be of importance in the pathogenesis of RA [Konttinen et al., 2006]. However, interruption of sensory nerve supply to joints cannot fully prevent the development of arthritis [Ahmed et al., 1995b]. Neuro-immune pathways are, on the whole, suggested to be of importance for the modulation of arthritic processes [Kane et al., 2005]. It has been suggested that the imbalance between sympathetic and sensory innervation in the arthritic joint may be of importance for the joint inflammation [Weidler et al., 2005]. Interesting observations are also the findings in studies on rat knee joints that monoarthritis leads to bilateral changes in neuropeptide levels in the synovial fluid [Bileviciute et al., 1993] and bilateral SP and CGRP changes in the spinal cord [Mapp et al., 1993].

**Synovial joint fluid**

The synovial joint fluid consists of hyaluronic acid produced by the lining cells and fluid from superficial capillaries and venules, together with low molecular weight proteins. A normal synovial fluid has a high viscosity and is difficult to aspirate. However, a synovial fluid sample from a patient with an inflammatory arthritis, e.g., RA, has a lower viscosity and contains numerous polymorphnuclear cells. Inflammatory markers and nerve signal substances can also be detected in the synovial fluid from patients with RA or OA.

**Articular cartilage**

Hyaline cartilage is found on the joint surfaces of the bones forming the knee joint. It functions as the self-lubricating, low-friction gliding and load-distributing joint...
surface. This articular cartilage, as well as other types of cartilage, contains two types of cells – chondrocytes and chondroblasts. Furthermore, articular cartilage is made up of a fibrillar meshwork of collagen II fibres and proteoglycans. Water is, nevertheless, a major component of cartilage. Electron microscopy studies has revealed four different zones of the articular cartilage in the human knee joint. The chondrocytes have secretory capacitites, not least including production of collagen and chondromucoprotein. The articular cartilage obtains nutrients from the synovial fluid.

The level of proliferation of chondrocytes in healthy individuals is limited, as is the level of penetration of other cell types from the joint cavity into the articular cartilage (for review see [Otero and Goldring, 2007]). The destruction of cartilage in arthritis occurs in the junction between the pannus and cartilage [Kobayashi and Ziff, 1975; Woolley et al., 1977]. Both FLS and macrophages can attach to the cartilage and initiate the destruction by secreting proteinases [Edwards, 2000]. The chondrocytes respond to different mediators by alteration in their metabolism and can start to produce and secrete pro-inflammatory factors such as nitric oxide and prostaglandin E (PGE) [Otero and Goldring, 2007]. Osteoclasts do also contribute to the destruction of cartilage [Bromley and Woolley, 1984; Gravallese et al., 1998].

**Tumour necrosis factor-alpha and other cytokines**

Pro-inflammatory cytokines such as TNF-alpha, interleukin-1 (IL-1β), IL-6, IL-15 among others are of great importance in the pathogenesis and inflammatory processes in RA. The immunology of RA synovial inflammation actually involves numerous pro-inflammatory cytokines and extensive research in this area has been made during the past two decades. TNF-alpha, a crucial cytokine in the inflammatory process in RA, is measurable in the synovial fluid and serum from patients with RA [Saxne et al., 1988; Brennan et al., 1992; Cope et al., 1992; Feldmann and Maini, 2003]. It is mainly produced by monocytes and macrophages, but also by other cells such as lymphocytes and fibroblasts [Chu et al., 1991]. It is a potent inducer of other important cytokines such as IL-1β, IL-6 and also MMPs. TNF-alpha is found in elevated levels in synovial fluid and is highly expressed in the pannus from patients with RA [Saxne et al., 1988; Chu et al., 1991; Brennan et al., 1992]. There are two cellular receptors for TNF-alpha, TNFα and TNFβ, which also exist as soluble forms in circulating blood and synovial fluid following cleavage of the extracellular portions [Cope et al., 1992].

IL-6, produced by T-cells, monocytes, macrophages and FLS, is also of importance for the RA synovial inflammation [Van Snick, 1990]. The main functions of IL-6 are promoting development of B-cells into plasma cells, induction of CRP, formation of osteoclasts and proliferation of FLS [Van Snick, 1990]. The levels of IL-6 in synovial fluid and sera of patients with RA are elevated [Houssiau et al., 1988]. The concentration of IL-6 in sera has been found to correlate with disease activity and treatment response [Dasgupta et al., 1992; Watson et al., 1992]. Antagonists of IL-6 are currently being evaluated in patients with RA.
IL-1β is mainly produced by monocytes and macrophages, but also by B- and T-cells [Koch et al., 1995]. It is an important pro-inflammatory cytokine which can induce secretion of TNF-alpha and chemokines from chondrocytes and fibroblasts [Arend and Dayer, 1990]. IL-1β can also stimulate release of MMPs from fibroblasts and chondrocytes. Taken together these findings show the importance of IL-1β in joint damage [MacNaul et al., 1990; Shingu et al., 1993].

**Anti-TNF treatment**

The discovery of TNF-alpha as a crucial mediator of inflammation in RA led to a revolution in the therapy of this disease and other autoimmune diseases [Maini et al., 1998; Feldmann and Maini, 2003]. Currently there are three main different TNF-alpha blocking agents in clinical use: infliximab, adalimumab and etanercept. The two first are anti-TNF-alpha monoclonal antibodies whereas etanercept is a fusion protein consisting of the extracellular domain of the p75 TNF receptor. All of these medications efficiently neutralise the effect of TNF-alpha, thereby dramatically dampening the inflammation in the joint. The exact mechanisms are, as yet, not fully understood. However, many results indicate that the joint inflammation is more or less abolished following anti-TNF treatment (for a review see [Valesini et al., 2007]). For example, CRP, serum amyloid A protein and haptoglobin are reduced as are several pro-inflammatory cytokines, e.g., IL-6 and IL-1β [Charles et al., 1999]. Anti-TNF treatment also leads to reduced angiogenesis as seen by lower levels of VEGF [Paleolog et al., 1998]. Cell recruitment is also altered upon anti-TNF treatment, leading to fewer leucocytes in the joints [Taylor et al., 2000], an observation that may possibly be explained by the increased frequency of apoptotic macrophages [Catrina et al., 2005]. As with many therapies, there are problems with the anti-TNF treatments such as variability in efficacy and the formation of antibodies against the anti-TNF medications [Valesini et al., 2007]. Furthermore, the costs of these treatments are rather high, reaching about 15 000 US Dollar per patient. It is recommended that administration of infliximab (the anti-TNF factor used in this study) should be in combination with methotrexate [Emery, 2006].
AIMS

The overall aim of this study was to obtain a better knowledge on the importance of the neuropeptides SP and BN/GRP and neurotrophins in arthritis. The specific aims were:

• To study the distribution patterns of the neuropeptides SP and BN/GRP, the neurotrophins NGF and BDNF, with particular emphasis on BDNF, and the receptors for all these substances in knee joint tissues from both man and laboratory animal (mouse).

• To include studies on the patterns seen in marked inflammation and reorganisation of the synovium in RA and in marked joint destruction in experimental arthritis.

• To evaluate the expression patterns of the above described neuropeptides/neurotrophins in synovial innervation vs. in local cells in the synovium.

• To investigate the relationships between SP, BN/GRP, other neuropeptides, the neurotrophin BDNF and inflammatory parameters and pro-inflammatory cytokines in patients with RA.

• To examine the levels of neuropeptides/BDNF in the synovial fluid and serum from arthritic and healthy humans.

• To elucidate the impact of anti-TNF treatment on the levels of a substance, namely BDNF, that, according to known facts, may possibly be influenced in peripheral blood from patients with RA.
MATERIALS AND METHODS

Patient material

For an overview of the RA and OA patient materials see Table 1 indicating into which studies the different patient materials were included. Healthy control subjects were included in the studies where ELISA analyses on synovial fluid and blood were performed.

Table 1. Demographic data and patient characteristics of this study

<table>
<thead>
<tr>
<th></th>
<th>Synovial biopsy</th>
<th>Synovial fluid</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Longstanding RA, n=11</td>
<td>OA n=16</td>
<td>Longstanding RA, n=28</td>
</tr>
<tr>
<td>Age at onset, years, mean ± SD</td>
<td>41.8±21.2</td>
<td>67.1±9.8</td>
<td>44.8±11.8</td>
</tr>
<tr>
<td>Disease duration, years, mean ± SD</td>
<td>26±14.4</td>
<td>7.3±5.6</td>
<td>13.6±7.5</td>
</tr>
<tr>
<td>ESR, mm/h, mean ± SD</td>
<td>30.6±23.5</td>
<td>14.7±9.2</td>
<td>37.7±21.3</td>
</tr>
<tr>
<td>DAS28</td>
<td>nt</td>
<td>na</td>
<td>nt</td>
</tr>
<tr>
<td>RF, ever</td>
<td>9 (82)</td>
<td>na</td>
<td>25 (89)</td>
</tr>
<tr>
<td>DMARDs</td>
<td>8 (73)</td>
<td>na</td>
<td>16 (57)</td>
</tr>
<tr>
<td>Prednisolone (≤ 7.5 mg/d)</td>
<td>5 (45)</td>
<td>na</td>
<td>11 (39)</td>
</tr>
</tbody>
</table>

nt = not tested; na = not applicable

1 anti-malarials, cyclosporin A, azathioprine, sulphasalazine, leflunomide and injectable gold

Values in brackets represent percentages (%).

Serum was also analysed from patients from whom synovial fluid was withdrawn (see Paper III).

Rheumatoid arthritis patients

All patients with RA fulfilled the criteria of the American Rheumatism Association [Arnett et al., 1988]. In Paper II and III, both patients with early RA, i.e., disease diagnosed for less than one year and patients with longstanding, established disease (i.e., a disease duration of more than one year) were included. In Paper I, only
Neuropeptides and neurotrophins in arthritis

Patients with longstanding RA were included. Non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) were prescribed for the majority of the patients with RA as is presented in Table 1. For the study on the impact of anti-TNF treatment on the levels of BDNF (paper II) the patients were those who had failed to respond to therapy with DMARDs.

Osteoarthritis patients

All OA patients fulfilled the criteria of Altman et al. (1986), and the grading system of Ahlback et al. (1968) was applied. They were all graded as Ahlback II – III. For further details of the OA patient group see Table 1.

Mouse material and induction of arthritis

Male littermates [C57BL/6 were backcrossed twice with DBA/1 (H2q)] were used for the experiments (see Table 2). All mice were maintained on a 12h light/12h dark cycle, and were fed chow and water ad libitum.

For the induction of arthritis, mice were injected intra-dermally at the base of the tail with 100 μg collagen II emulsified in 0.1 ml complete Freund’s adjuvant containing 200 mg mycobacterium strain H37RA on days 0 and 7. At the same time points additional adjuvant, i.e., heat-killed Bordetella pertussis (2 x 10⁹ organisms), was given intra-peritoneally. Local arthritis was induced at day 21 by intra-articular injection of 25 μg collagen II in 10 μl sterile 0.9% NaCl. Control joints were injected with 10 μl sterile 0.9% NaCl or were handled without any local injection.

Table 2. Number of murine joints examined for each experimental setup. All animals, except the controls, were also given systemic collagen II injection.

<table>
<thead>
<tr>
<th>Control</th>
<th>Local collagen II</th>
<th>NaCl locally</th>
<th>No local treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 8</td>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 4</td>
</tr>
</tbody>
</table>

Sampling

Biopsies from human knee joint

Synovial biopsy specimens were collected from patients with RA or OA during prosthetic knee surgery. The samples were immediately transported to the laboratory. The biopsies were cut into small pieces of tissue and either mounted directly onto cardboard with OCT medium or fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.0) at 4°C for 24h followed by washing in Tyrode’s solution (pH 7.2) containing 10% (w/v) sucrose for 24h, after which the specimens were frozen. The freezing procedure was performed by immersion in propane chilled
with liquid nitrogen. The mounted specimens were used for histological, immunohistochemical or in-situ hybridisation staining. Additionally, pieces of unfixed tissue weighing approximately 30 mg were directly frozen in liquid nitrogen prior to preparation for ELISA analysis.

**Mouse tissue samples**

Mice were sacrificed, the knee joints dissected and fixed in 4% phosphate buffered paraformaldehyde (PFA) at 4°C for 24h. Fixed joints were decalcified for 3 weeks in 10% EDTA followed by dehydration and embedding in paraffin.

**Blood and synovial fluid sampling**

Synovial fluid was aspirated from human knee joints (patients with RA and healthy control subjects) according to the method of Dixon and Emery [Dixon, 1992]. The fluid was centrifuged and the white blood cells were removed. Thereafter, the samples were frozen and stored at – 80°C until analysed. Peripheral blood (from patients with RA and healthy control subjects) was collected and aliquots of plasma were stored at -80°C.

**Sectioning**

A series of 7 µm thick sections of the human tissue specimens (fixed and unfixed), were cut using a cryostat. The sections were mounted on slides pre-coated with chrome-alum gelatin. They were then stored at -20°C until processed for either immunohistochemistry (IHC) or haematoxylin-eosin (htx-eosin) staining. For in situ hybridisation, 10 µm sections of fixed tissue specimens were cut and mounted on Super Frost Plus slides (nr. 041300: Menzel-Gläser, Braunschweig, Germany) and processed immediately. Sections then underwent post-fixation with 4% paraformaldehyde.

Sections, 8 µm thick, of the mouse tissue were cut on a vibratome and mounted on Super Frost Plus slides (nr. 041300: Menzel-Gläser). They were then dried on a warmed plate and then stored at RT until being processed for IHC or stained with htx-eosin.

**Immunohistochemistry (IHC)**

**Pre-treatment procedures**

Microwave antigen retrieval was applied for demonstration of certain of the substances. The sections were for this purpose placed in 0.01 M citrate buffer, pH 6, and placed in a microwave oven and boiled at 650 W for three 5-min cycles. After each cycle, the slides were placed in fresh buffer. After the last cycle, the slides were allowed to cool to room temperature and were then washed three times for 5 min in phosphate-buffered saline (PBS). An alternative pre-treatment used was acid potassium permanganate solution (1 vol. of 2.5% KMnO₄ and 1 vol. of...
Neuropeptides and neurotrophins in arthritis

5% H₂SO₄ in 80 vol. of distilled water, pH 2.0) for 2 min [Hansson and Forsgren, 1995]. For details of when pre-treatment procedures were used, see Papers.

**Immunofluorescence**

Immunofluorescence staining using tetramethylrhodamine isothiocyanate (TRITC) was utilised for the detection of those substances and receptors under investigation. For some of the antibodies pre-treatment was performed (see the respective papers).

The sections were incubated for 20 min in a 1% solution of Triton X-100 (Kebo Laboratories, Stockholm) in 0.01 M PBS, pH 7.2, containing 0.1% sodium azide as preservative, and thereafter rinsed three times for 5 min in PBS. The sections were then incubated in 5% normal swine serum in PBS supplemented with 0.1% bovine serum albumin (BSA) for 15 min. The sections were then incubated with the primary antibody diluted in PBS containing BSA in a humidity chamber either overnight at 4°C or 1h at 37°C. After 3 x 5 min washes in PBS, sections were further incubated in normal swine serum followed by incubation with a secondary antibody corresponding to TRITC-conjugated swine anti-rabbit IgG (Dakopatts, Denmark), diluted 1:40 in PBS containing BSA, for 30 min at 37°C. The sections were washed three times for 5 min in PBS and then mounted in Vectashield Hard Set Mounting Medium (H-1400: Vector Laboratories Inc, Burlingame, CA, USA). Examination of the stained sections was carried out using a Zeiss Axioskop 2 plus microscope equipped with an Olympus DP70 digital camera.

**Peroxidase anti-peroxidase (PAP) staining**

After the necessary pre-treatments, the sections were incubated in a 1% solution of Triton X-100 for 20 min and then rinsed in PBS and incubated in 1% H₂O₂ in H₂O for 30 min. Then the sections were rinsed once more in PBS and incubated in 5% normal swine serum (Dakopatts, Denmark) in PBS supplemented with 0.1% BSA for 15 min before incubation with the primary antibody overnight at 4°C or for 60 min at 37°C (p75) in a humidity chamber. Sections were rinsed in PBS and again incubated in 5% normal swine serum before incubation with swine anti rabbit antibody (Dakopatts, Denmark) diluted 1:100 for 30 min in a humidity chamber at room temperature. The sections were rinsed in PBS before incubation with PAP-complex (PAP-Rabbit, diluted 1:100; Dakopatts, Denmark) for 30 min in the humidity chamber at room temperature. Then the sections were rinsed in PBS and developed in diaminobenzidine (DAB) solution (3-3 diaminobenzidine tetrahydrochloride, Sigma, St. Louis, MO, USA) for 5 min. The reaction was stopped with running water for 5 min. The sections were counterstained in Mayer’s htx (Histolab, Göteborg, Sweden) for 20 seconds. Then they were dehydrated in ethanol (70%, 2x96%, 3x99.5%), and mounted in Pertex microscopy mounting medium (Histolab, Göteborg, Sweden). The sections were examined using a Zeiss Axioskop 2 plus microscope and Olympus DP70 digital camera.
**Primary antibodies**

A list of all primary antibodies utilised in these studies is presented in Table 3. For detailed descriptions of the primary antibodies, see the corresponding papers as given in the Table.

**Table 3. Overview of primary antibodies used in this study**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody raised in</th>
<th>Antibody dilution</th>
<th>Preferential tissue processing</th>
<th>Code</th>
<th>Source</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Fixed</td>
<td>8450-0004</td>
<td>Biogenesis, Poole, UK</td>
<td>I, IV</td>
</tr>
<tr>
<td>SP</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Fixed</td>
<td>S-184</td>
<td>RBI, Natick, MA</td>
<td>I</td>
</tr>
<tr>
<td>SP</td>
<td>Rat</td>
<td>1:50</td>
<td>Fixed</td>
<td>8450-0505</td>
<td>Biogenesis, Poole, UK</td>
<td>I</td>
</tr>
<tr>
<td>SP</td>
<td>Rabbit</td>
<td>1:300</td>
<td>Fixed</td>
<td>H-061-05</td>
<td>Phoenix, Belmont, CA</td>
<td>I</td>
</tr>
<tr>
<td>NK-1R</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Fixed</td>
<td>s-8305</td>
<td>Sigma, St Louis, USA</td>
<td>I, IV</td>
</tr>
<tr>
<td>NK-1R</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Unfixed</td>
<td>NB300-119</td>
<td>Novus, Littleton, CO</td>
<td>I</td>
</tr>
<tr>
<td>BN/GRP</td>
<td>Rabbit</td>
<td>1:50</td>
<td>Fixed</td>
<td>RPN.1692</td>
<td>Amersham, Sweden</td>
<td>I, IV</td>
</tr>
<tr>
<td>GRP-R</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Unfixed, Fixed</td>
<td>NLS830</td>
<td>Novus, Littleton, CO</td>
<td>I, IV</td>
</tr>
<tr>
<td>BDNF</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Fixed</td>
<td>Sc-546</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>II, V</td>
</tr>
<tr>
<td>NGF</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Fixed</td>
<td>Sc-548</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>V</td>
</tr>
<tr>
<td>p75</td>
<td>Rabbit</td>
<td>1:50-1:100</td>
<td>Fixed</td>
<td>N3908</td>
<td>Sigma, St Louis, USA</td>
<td>II, V</td>
</tr>
<tr>
<td>TrkA</td>
<td>Rabbit</td>
<td>1:50</td>
<td>Fixed</td>
<td>Sc-118</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>V</td>
</tr>
<tr>
<td>TrkB</td>
<td>Rabbit</td>
<td>1:20</td>
<td>Fixed</td>
<td>Sc-12</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>II, V</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Fixed</td>
<td>7863-0504</td>
<td>Biogenesis, Poole, UK</td>
<td>II</td>
</tr>
</tbody>
</table>
**Control stainings**

Continuous test stainings were performed in order to delineate specific immunoreactions for every antibody used in this study. This included preabsorption stainings where the antibody was preabsorbed with corresponding peptide, control stainings on other tissues (e.g. human tendon, human colon), or replacing primary antibody by PBS/BSA. For specific details regarding control stainings performed for each antibody, see separate papers as listed in Table 3.

**In-situ hybridisation**

Commercially available digoxigenin (DIG)-hyperlabelled oligonucleotide probe (ssDNA) were used in all experiments (Gene Detect, New Zealand). For specification of probes used in the experiments, see Table 4. *In-situ* hybridisation (ISH) was performed according to an established protocol [Panoskaltsis-Mortari and Bucy, 1995], using an alkaline phosphatase (AP)-labelled anti-DIG antibody for detection, with a few modifications [Danielson et al., 2007]. For details concerning the contents of solutions and buffers used, see Paper I.

The sections (10 µm) were air-dried at room temperature (RT) for 30 min, and then fixed in filter-sterilised 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 60 min at RT. The slides were then washed twice with 2x saline sodium citrate (SSC) for 10 min. The sections were incubated in 0.2 M HCl for 8 min at RT to inhibit endogenous AP activity, following which the sections were acetylated by incubation of slides for 15 min at RT in a mixture of 195 ml DEPC-H₂O, 2.7 ml tiethanolamine, 0.355 ml HCl, and 0.5 ml acetic anhydride. Slides were then again rinsed in 2xSSC. An aliquot (50 - 100 ng) of the ssDNA probe (see Table 4) was added to 15 µl of hybridisation solution in a 1.5 ml microfuge tube, denatured for 5 min in 80°C and then put on ice. The probe-containing hybridisation solution was then applied to each section and then incubated at 56°C overnight.

The slides were washed twice in 2xSSC and once in STE-buffer. Subsequently, the slides were incubated in 100 µl RNase A for 30 min at 37°C, following which they were washed for 20 min at 56°C in 2xSSC, 50% formamide, set into a 56°C bath, then washed twice in 1xSSC, and twice in 0.5xSSC. The following steps included washings in buffers, incubation with AP-labelled antibody, counterstaining and mounting (for further details, see Paper II). The corresponding sense DIG-hyperlabelled ssDNA probes were used as negative controls; α-actin probe (GD5000-OP, Gene Detect, New Zealand) was used as a positive control.
Table 4. Details of probes used for in-situ hybridisation

<table>
<thead>
<tr>
<th>Probe</th>
<th>Code</th>
<th>Source</th>
<th>Dilution</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>GD1001-CS</td>
<td>GeneDetect, New Zealand</td>
<td>100 ng</td>
<td>CCGTTTGCCCATTAATCCAAAG A CTGCTGAGGCTTGGGTCTCCG</td>
</tr>
<tr>
<td>NK-1R</td>
<td>GD1001-DS</td>
<td>GeneDetect, New Zealand</td>
<td>50 ng</td>
<td>TGGACACCTTGCGTTGGGAGAGA CTTGCCGTCGGA TGCAGAGG</td>
</tr>
<tr>
<td>BN/GRP</td>
<td>GD1001-DS</td>
<td>GeneDetect, New Zealand</td>
<td>50 ng</td>
<td>CGCCCCAGTGGTTGCCGCGCGGGTA CATCTTGGTCAGCACGGTCCTCC</td>
</tr>
<tr>
<td>GRP-R</td>
<td>GD1001-DS</td>
<td>GeneDetect, New Zealand</td>
<td>100 ng</td>
<td>CGAACAGGCCCAAAACACCAGCA CTTCTTGGCAAGTCGCTTCCGGG</td>
</tr>
</tbody>
</table>

Reprinted from Paper I, dilution refers to ng in 15 µl hybridisation solution.

ELISA

Tissue homogenisation for ELISA

Tissue samples were mechanically homogenised in a 100mM TRIS-HCl buffer pH 7.0, containing 1M NaCl, 2% BSA, 4mM EDTA, 0.2% Triton X-100 (pH 7.0), 0.02% sodium azide and the following protease inhibitors: Pepstatin A (0.1µg/ml), Aprotinin (5µg/ml), Antipain (0.5µg/ml), Benzamidin (167µg/ml) and PMSF (5.2µg/ml). All protease inhibitors were purchased from Sigma, Germany. Tissue and buffer were mixed in a 1:20 ratio and homogenisation was performed on ice. Directly after homogenisation, the synovial tissue extracts were centrifuged at 13000 x g and 4°C for 15 min. Aliquots were frozen at -80°C until assayed.

ELISA procedures

The levels of the substances in synovial fluid, blood or synovial tissue were measured with commercially available kits (see Table 5 for all details). All assays were performed according to the corresponding manufacturer’s instructions. The levels in the supernatants from the homogenised tissue were normalised to the weight of tissue samples and expressed as pg/mg.
Table 5. Overview of ELISA kits used in this study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Code</th>
<th>Source</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>CYT306</td>
<td>Chemicon, CA, USA</td>
<td>II</td>
</tr>
<tr>
<td>BN/GRP</td>
<td>EK-027-07</td>
<td>Phoenix Pharmaceuticals, CA, USA</td>
<td>I, III</td>
</tr>
<tr>
<td>CGRP</td>
<td>EK-015-02</td>
<td>Phoenix Pharmaceuticals, CA, USA</td>
<td>III</td>
</tr>
<tr>
<td>IL-6</td>
<td>EH2IL6</td>
<td>Pierce-Endogen, Rockford, IL, USA</td>
<td>III</td>
</tr>
<tr>
<td>MCP-1</td>
<td>EHMCP1</td>
<td>Pierce-Endogen, Rockford, IL, USA</td>
<td>III</td>
</tr>
<tr>
<td>NPY</td>
<td>EK-049-03</td>
<td>Phoenix Pharmaceuticals, CA, USA</td>
<td>III</td>
</tr>
<tr>
<td>SP</td>
<td>EK-061-05</td>
<td>Phoenix Pharmaceuticals, CA, USA</td>
<td>I, III</td>
</tr>
<tr>
<td>sTNFRI</td>
<td>DRT100</td>
<td>R&amp;D Systems, Wiesbaden-Nordenstadt, Germany</td>
<td>III</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>EH3TNFA</td>
<td>Pierce-Endogen, Rockford, IL, USA</td>
<td>II, III</td>
</tr>
<tr>
<td>VIP</td>
<td>EK-064-16</td>
<td>Phoenix Pharmaceuticals, CA, USA</td>
<td>III</td>
</tr>
</tbody>
</table>

Statistics

The software used for statistics was SPSS 11 for Macintosh (SPSS, Chicago, Illinois, USA). The Mann-Whitney statistical method was used to compare continuous data. The Spearman rank correlation test was applied for correlation analyses. Factor analysis, explorative data analysis was performed to find patterns among measured variables. Factor loadings >0.3 were here considered. P-values, two-sided, <0.05 were considered as significant. For specification of where each method was applied see the individual papers.
Ethical considerations

Approval from the Ethics Committee at the Faculty of Medicine, Umeå University, and by the Regional Ethical Review Board in Umeå was obtained for the studies on human tissue. For the studies on mouse arthritis, approval was given by the Animal Research Committee in Umeå. All patients and controls had given their informed consent. All experiments followed the guidelines of the declaration of Helsinki.
RESULTS AND DISCUSSION

Methodological considerations

Several aspects concerning the human synovial tissue materials and the methods used in this study are open to discussion when interpreting the results. Firstly, the synovial tissue material used in Papers I and II was from patients with RA who had the disease for a long period of time (for details see Table 1). The RA biopsies are “end stage biopsies” meaning that, in some cases at least, the inflammation had declined. This fact could be observed during morphological examination of these specimens. Consequently, the degree of inflammation seen histologically varied substantially between the different RA specimens. However, in general, the degree of inflammation was significantly higher in the RA group compared with the OA group. In contrast, the murine arthritic material always showed active inflammation and was, therefore, a more acute model of arthritis.

Considering the immunohistochemical staining for neuropeptides, neurotrophins and high affinity neurotrophin receptors, extensive pre-absorption controls (first order control) were always performed (Paper I, II, IV and V). In certain previous immunohistochemical studies describing the occurrence of neuropeptide immunoreactions in non-neuronal cells pre-absorption controls were not performed. This may have lead to erroneous results. Pre-absorption of the antiserum against BN/GRP used in the present studies was of particular importance since cross-reactivity with SP has been demonstrated [Cimini et al., 1989]. Hence, the BN/GRP antiserum was pre-absorbed with both SP and BN/GRP peptide in order to clarify the specific reactions and with SP in the regular stainings (Paper I). Furthermore, second order controls were also regularly performed, i.e., the staining protocol being followed but omitting the primary antibody or replacing the primary antibody with normal serum. Staining was always evaluated using other human tissues for which reaction patterns had previously been established. Reference tissue was also included in the in situ hybridisation experiments (Paper I). All estimations of immunoreactivity performed during this study were semi-quantitative and determined independently by two observers.

The protocol for tissue homogenisation had been developed in this laboratory several years previously. A mechanical homogenisation method was used and this has, in principle, been used by another group studying synovial tissue [Rosengren et al., 2003]. Using this protocol it was, thus, possible to measure neuropeptides and neurotrophins (Paper I, II). However, it should be taken into consideration that other, newly discovered tachykinins, such as hemokinins and endokinins (cf [Kurtz et al., 2002; Page et al., 2003]) may cross-react with the anti-SP antibodies supplied in the commercial assay kit used. It has recently been shown that these tachykinins show high affinity for NK-1R [Kurtz et al., 2002; Page et al., 2003].
Human studies

Morphological aspects (Paper I, II)
As described above, the level of inflammation seen histologically varied between the synovium specimens from patients with RA. Mononuclear-like cells were, however, present in all specimens from the RA subjects and were, in many cases, present in high numbers (Figure 6). The level of inflammation was higher in the RA group than in the OA group, as defined by semi-quantitative estimations of the number of mononuclear-like cells (see Paper I). Lymphoid aggregates were present in some of the RA specimens and these were also included when assessing the level of inflammation. The number of fibroblast-like cells also varied in both patient groups and these variations were taken into consideration when assessing the degree of hypercellularity in the tissue. Nerve fascicles could be observed in specimens from both RA and OA patient groups but they were, however, only seen infrequently.

Figure 6. Histological feature of a human knee joint affected by rheumatoid arthritis. There is a massive infiltration of mononuclear cells in the synovium.

Neuropeptide expression in human knee joint synovial tissue (Paper I)
SP and NK-1R expression
Immunoreactive substance P could only be detected in nerve-related structures, i.e., in nerve fascicles and in the perivascular innervation. No SP mRNA could be detected in the specimens. With respect to NK-1R immunoexpressions, these were observed in fibroblast-like cells, in the blood vessel walls (Figure 7a) and in lymphoid aggregates. NK-1R mRNA was also detected in fibroblast-like cells, in cells of the blood vessel walls and in lymphoid aggregates. Semi-quantitative
estimation of levels of immunoreactive NK-1R revealed that the levels were higher in the RA specimens than the OA specimens. These observations show that SP is confined to synovial innervation and that no local SP production occurs in the synovium. The widespread expression of NK-1R in the human synovial tissue indicates that SP has several effects in this tissue. It is likely that SP affects the vasculature, including effect on angiogenesis [Seegers et al., 2003] and inflammatory oedema, and that it has pro-inflammatory effects [Lotz et al., 1988]. Interestingly, it has also been shown that SP can stimulate the proliferation of synovial fibroblasts [Lotz et al., 1987]. Furthermore, it is known that SP has mitogenic effects on fibroblasts [Ziche et al., 1990] and that administration of SP and neutral endopeptidase inhibitors can stimulate fibroblast proliferation, as well as angiogenesis, during Achilles tendon healing in the rat [Burssens et al., 2005].

The effects of SP in arthritic situations and the possible implications of blocking its effects has been extensively discussed [Keeble and Brain, 2004] (see also Introduction). It is not clear whether NK-1R antagonist treatment alone can ameliorate arthritic symptoms in arthritis [Hong et al., 2002]. It is more probable that combination therapy in which an SP antagonist is combined with other treatment(s) would be more effective. The possible effectiveness of combination therapy in arthritis, including another neuropeptide, VIP, has been put forward [Gomariz et al., 2006]. It has, nevertheless, been suggested that an NK-1R antagonist can be useful in minor inflammatory conditions [van der Kleij et al., 2003].

**Figure 7.** Sections of synovial tissue from patients with RA. In (a), immunoreactions for NK-1R can be seen in the wall of and in the proximity of an arteriole. In (b), immunoreactions for GRP-R can be viewed in a blood vessel wall.


**BN/GRP and GRP-R expression**

Immunoreactions for BN/GRP were observed in both mononuclear-like and fibroblast-like cells, and sometimes in the lymphoid aggregates, in the synovial tissue. BN/GRP mRNA was seen in similar structures. Conversely, no immunoreactions were found in relation to nerve fascicles or perivascular innervation. Cells positive for GRP-R were located in perivascular areas and in blood vessel walls (Figure 7b). Also mononuclear-like cells showed immunoreaction for GRP-R. GRP-R mRNA showed a similar pattern of distribution as the GRP-R immunoreactive cells. Semi-quantitatively the levels of expression of both BN/GRP and GRP-R were, in principle, greater in specimens from RA patients when compared with those from OA patients. Tissue homogenates from either RA and OA subjects contained detectable levels of BN/GRP, and there was a tendency, although not reaching statistical significance, for the levels to be higher in RA specimens compared with OA specimens.

This is the first time that BN/GRP and the GRP-R have been shown to be present in human synovial tissue. As GRP-R was found to be widely expressed in this tissue, it is likely that BN/GRP has multiple effects in joints, including in arthritic situations. The results also suggest that GRP is produced locally in the tissue. In this context, it is interesting to note that BN/GRP has been shown to have both inflammatory [Lemaire et al., 1991], healing and growth-promoting (e.g., [Gunal et al., 2002]) as well as mitogenic effects [Aguayo et al., 1990] in other tissues. Therapeutic targeting of GRP-R has been studied in certain cancers for some time (e.g., [Moody et al., 2003]) and more recently has been applied to inflammatory conditions [Dal-Pizzol et al., 2006]. Thus, targeting GRP-R in animal models of arthritis would be an interesting topic for future experimental studies. This rationale is strengthened by the fact that the levels of BN/GRP correlate with those of pro-inflammatory cytokines in synovial fluid and serum from patients with RA (see below).

**BDNF and neurotrophin receptor expression in knee joint synovial tissue (Paper II)**

The expression patterns of BDNF was concentrated on here because those of NGF in joint tissues are well described [Aloe et al., 1993; Manni et al., 2003]. Immunoreactions for BDNF and the neurotrophin receptors p75 and TrkB were observed in nerve fascicles of synovial tissue from both RA and OA patients. The reactions for BDNF, TrkB and p75 were confined to intra-fascicular nerve fibres. However, p75 immunoreactions were also detectable in the perineurium and these were more distinct than the intrafascicular ones. Immunoreactions for BDNF, p75 and TrkB were also observed in sensory corpuscles. p75-immunoreactions were found in the capsule of the corpuscles and in their interiors there were nerve fibres that were immunopositive for BDNF and TrkB. Both mononuclear-like and fibroblast-like cells showed immunoreactions for BDNF. No immunoreactions were observed for the neurotrophin receptors in these cell types, whilst p75 immunoreactions were seen in blood vessel walls.
These results are the first that show BDNF and p75 to be detectable in the innervation of the human synovial tissue. It has previously been shown that BDNF is detectable in cells of the synovium [Rihl et al., 2005; Weidler et al., 2005] and that TrkB is present in nerve fascicles of the synovial tissue [Miller et al., 2000]. The results for BDNF add new and relevant information on its possible role in synovial tissue showing that nerve-related effects of BDNF, apart from effects of BDNF produced in mononuclear-like and fibroblast-like cells [Weidler et al., 2005], should be taken into consideration. As immunoreactive neurotrophin receptor was not detected on these latter cells it appears as if the principal effects of BDNF in the tissue are nerve and blood vessel related. The nerve-related effects include those in sensory corpuscles, which are structures with a proprioceptive function having previously been found especially in ligaments and skin (e.g., [Vega et al., 1993; Hagert et al., 2004]). The present results show that the synovial tissue of the human knee joint is supplied with these structures.

**Neuropeptides and the neurotrophin BDNF in blood and synovial fluid and in relation to inflammatory parameters and pro-inflammatory cytokines (Paper II, III)**

All the measured neuropeptides (BN/GRP, CGRP, NPY, SP and VIP) could be detected in serum and synovial fluid from patients with RA. Serum levels of SP were higher in those patients with longstanding disease whereas the levels of BN/GRP were lower for those with longstanding disease when compared with patients with early disease. The levels of SP and BN/GRP in synovial fluid of healthy controls were significantly lower than those of the two patient groups. Interestingly, in synovial fluid of patients with longstanding RA, the levels of BN/GRP correlated both with the levels of IL-6 and of TNF-alpha, respectively. Furthermore, factor analysis on composition of synovial fluid showed that both SP and BN/GRP grouped together with IL-6. Additionally, SP grouped together with both MCP-1 and TNF-alpha. It was also observed that ESR grouped together with SP and BN/GRP in the factor analysis on synovial fluid. In serum, the levels of soluble TNF receptor I (sTNFRI) correlated with those of BN/GRP in patients with longstanding RA. It was also shown, in factor analysis, that both CGRP and BN/GRP grouped together with sTNFRI in serum.

Higher levels of SP were observed in the tissue homogenates from patients with RA when compared with those with OA and there was a tendency for the levels of BN/GRP to be higher in the RA specimens compared with OA samples.

The observations show that the levels of SP and BN/GRP correlate with the levels of the pro-inflammatory cytokines TNF-alpha and IL-6 in synovial fluid, and that BN/GRP correlates with sTNFRI in serum from patients with RA. Thus, both these groups of substances, i.e., cytokines and the neuropeptides SP and BN/GRP are upregulated simultaneously during RA. However, it can not, from this study, be definitely concluded whether it is the cytokines that enhance the secretion of neuropeptides or vice versa. Nevertheless, it is tempting to suggest that the increased concentrations of the neuropeptides seen in the tissue might stimulate the production of cytokines from immunocompetent cells. In this respect it has been
shown that SP can stimulate the release of IL-1β, IL-6 and TNF-alpha from human monocytes [Cuesta et al., 2002]. However, considering the effects of BN/GRP, it has actually been shown that BN/GRP lowers the levels of TNF-alpha but augments the levels of IL-6 in human whole blood cells [Hernanz et al., 1996].

Based on these findings and the general assumption that SP exhibits pro-inflammatory effects [Lotz et al., 1987], it can be suggested that SP stimulates the cytokine production from immunocompetent cells in RA. As discussed above, the role of SP in arthritic situations has been discussed by many groups (e.g., [Sakai et al., 1998; Seegers et al., 2004]). On the other hand, the role of BN/GRP seems to be more complex in inflammation. Anti-inflammatory actions and ameliorating effects of BN/GRP have actually been shown in situations involving injury and tissue damage [Gulluoglu et al., 1999; Yegen, 2003]. In contrast, it has been demonstrated that BN/GRP has chemotactic properties and modulates phagocytosis in murine peritoneal macrophages [De la Fuente et al., 1991; Del Rio and De la Fuente, 1994]. Apart from inflammation-modifying effects, one must keep in mind that both SP [Bursens et al., 2005] and BN/GRP [Yamaguchi et al., 2002] have marked trophic effects.

Only a small subset of the patients with longstanding RA showed detectable levels of BDNF in synovial fluid. Furthermore, the levels were not significantly different to those of healthy control subjects. Thus, it appears as though a disease such as RA is not related to a marked release/leakage of BDNF into the synovial fluid. The levels of BDNF in plasma from patients with RA did not correlate with either DAS28 or ESR and there were no correlations between the levels of BDNF in tissue homogenates and the level of inflammation in the tissue as judged by semi-quantitative estimations. Neither were there any correlations between the levels of BDNF in synovial fluid and ESR or white blood cell counts. These observations, and the findings concerning p75 and TrkB in the synovial tissue, suggest that, as discussed above, the main area of interest concerning BDNF is related to neuronal effects within the synovial tissue and effects on circulating and to some extent on local cells. On the other hand, it might be that NGF, which has been the subject of previous extensive studies on inflammation in joints [Aloe et al., 1993; Manni et al., 2003], is more inflammatory-related than BDNF.

**Impact of anti-TNF treatment on the levels of BDNF in peripheral blood (Paper II)**

In a preliminary study it was suggested that anti-TNF treatment did not have any distinct impact on the levels of BDNF in plasma from patients with RA [del Porto et al., 2006]. However, in that study, it could be observed that the levels of BDNF were actually decreased after anti-TNF treatment, but not with any statistical significance. Based on these earlier studies, the effects of anti-TNF treatment on the levels of BDNF in the blood were investigated. Following anti-TNF treatment, the levels of BDNF in plasma of patients with RA decreased significantly after 14 weeks of treatment. Thereafter, the levels reached a plateau with no further decrease. The levels of BDNF at baseline in the patients were significantly higher.
than those of healthy control subjects. On the other hand, unpublished results indicate that anti-TNF treatment has no impact on the levels of SP and BN/GRP in plasma from patients with RA.

Our results suggest that the inhibition of TNF-alpha by infliximab to a certain extent modulates the production of BDNF. However, since the levels of BDNF after 14 weeks of treatment were still significantly higher than those of healthy control subjects, the situation is complex.

The possible effects of anti-TNF treatment on nerve structures have been discussed in several reports [Onda et al., 2004; Murata et al., 2005], showing that injection of infliximab prevents hyperalgesia and attenuates the levels of BDNF in the dorsal root ganglion and spinal cord of the rat [Onda et al., 2004; Murata et al., 2005]. It has also been shown that BDNF is upregulated in the dorsal horn following peripheral inflammation [Garraway et al., 2003]. Hence, it is possible that anti-TNF treatment can affect the expression of BDNF in joint nerves and thereby modulating pain in RA. It is well-known that joint nerves become sensitised during inflammation [McDougall, 2006].

Studies on a mouse model of arthritis

Morphological aspects (Paper IV, V)
The healthy control animals had a normal joint morphology, i.e., without any sign of inflammation or joint destruction. In the arthritic animals, a marked inflammation and pannus formation was seen. The level of inflammatory cell invasion, however, varied between the animals. The inflammatory cells appeared as clusters and were also sometimes seen in the muscular tissue surrounding the joint. Cartilage destruction was present to a high extent in severely arthritic animals. On the whole, the most marked inflammation/destruction appeared in the animals receiving both systemic and local CII-treatment. In this study the joints were stratified into those with mild-moderate arthritis and those exhibiting severe arthritis when describing the expression patterns of the substances and receptors under investigation. For exact details on the stratification and on the morphological classifications, see Papers IV and V.

Neuropeptides and their receptors in articular chondrocytes and synovial tissue (Paper IV)
Expression patterns
The specific SP-immunoreactions that were observable were related to nerve structures. A few fine nerve fibres in the synovial tissue from both control and arthritic joints thus exhibited SP-immunoreactivity. Immunoreactions for SP were not observed for the articular chondrocytes. Noticeably, the articular chondrocytes of all specimens from control animals showed strong NK-1R immunoreactions. Immunoreactions in specimens from arthritic animals were of variable strength, many of the chondrocytes showing weak or very weak reactions. In other words,
the chondrocytes from control animals showed more pronounced NK-1R immunoreactivity.

BN/GRP immunoreactive nerve fibres were observed in the synovial tissue of the controls but particularly in association with the inflammatory infiltrates in the pannus. No BN/GRP cellular reactions were noted nor were BN/GRP immunoreactions observed in articular chondrocytes. GRP-R immunoreactions were apparent in cells present in the inflammatory infiltrates. In the case of articular chondrocytes, GRP-R showed a similar pattern to NK-1R, i.e., there were more marked reactions in joints of control than arthritic animals.

**Interpretations of the findings**

The articular chondrocytes of the human knee joint could not be examined in this study. The information obtained from studying the mouse knee joints are, therefore, complementary in showing aspects of these cells. Interestingly, it was observed that the articular chondrocytes of the control animals are markedly equipped with NK-1 and GRP receptors. Although these receptors were also seen in the arthritic joints, the level of expression was generally decreased. The observations suggest that SP and BN/GRP are important for cartilage metabolism and that the extent of this importance apparently decreases with advanced arthritis. To date very little is known regarding SP and BN/GRP effects on articular chondrocytes and on the receptor expression of these cells. However, *in vitro* studies have shown that NK-1R is indeed present on human chondrocytes [Millward-Sadler et al., 2003].

The apparent down-regulation in receptor reactions by the chondrocytes is not readily explained. One interpretation is that it is related to compensatory mechanisms related to the peptide levels in the synovial fluid. From the literature, including a publication from this laboratory [Westermark et al., 2001], it is thus known that there usually are increased levels of SP and BN/GRP in the synovial fluid from arthritic joints (cf. above). Nevertheless, it is possible that the decrease in NK-1 and GRP- receptors noted is a negative aspect for the articular cartilage. Thus, apart from the well-known effects of SP in relation to neurogenic inflammation, angiogenesis, pain sensation and inflammation, it is also known that SP can have trophic and healing effects [Brain, 1997; Ackermann et al., 2002]. Accordingly, it has been shown that SP has functional effects on cultured human chondrocytes [Millward-Sadler et al., 2003]. Furthermore, it is known that BN/GRP can have effects on ovine foetal chondrocytes [Hill and McDonald, 1992]. Likewise it is well-known that BN/GRP may have trophic and wound-healing effects [Yamaguchi et al., 2002; Yegen, 2003].

In contrast to the situation in the human knee joint, nerve fibres containing BN/GRP were detected in the mouse knee joint synovium, particularly in association with the pannus. Conversely, there was no expression of cellular BN/GRP in the synovial tissue. These findings show that there are species variations between man and mouse in terms of BN/GRP expression patterns in the knee joint synovial tissue.

The findings in the present study of both immunoreactive BN/GRP and GRP-R associated with inflammatory infiltrates suggest that BN/GRP is involved in the
joint inflammatory process. The existing published information concerning the effects of BN/GRP in inflammation in other tissues is, as discussed above, however, controversial. For example, it is reported that BN/GRP has a role in mediating lung damage [Dal-Pizzol et al., 2006] and to be chemotactic for airway monocytes [Aguayo et al., 1990] but BN/GRP also has anti-ulcerogenic and healing effects [Gunal et al., 2002; Yamaguchi et al., 2002; Yegen, 2003].

**Neurotrophins and their receptors in articular chondrocytes and synovial tissue (Paper V)**

**Expression patterns**

Cells immunoreactive for BDNF and NGF were observed in the inflammatory infiltrates, although no cellular neurotrophin reactions were observed in the synovial tissue of control animals. BDNF- and NGF-immunoreactions were also found as fine nerve varicosities in the synovial tissue. NGF immunoreactions were clearly visible in the articular chondrocytes of all subjects. However, controls showed significantly stronger reactions in these when compared with arthritic animals. Weak immunoreactions for BDNF were observed for the articular chondrocytes throughout the tissue material, the immunoreactions being stronger for the controls also in this case.

It was observed that immunoreactive p75 positive nerve fibre-like varicosities were present, predominantly in inflammatory infiltrates. Similarly, TrkB immunoreactions were detectable in cells in the inflammatory infiltrates. Receptor reactions were observed for all the three receptors (p75, TrkA and TrkB) on the articular chondrocytes. The immunoreactions for both p75 and TrkA observed were more distinct in controls compared with arthritic animals, the difference being confirmed by statistical analyses. There was also a tendency for TrkB immunoreactions to be stronger in the arthritic animals but the difference compared with normal animals was not statistically significant.

**Interpretations of the findings**

The findings in this study suggest that NGF and BDNF are involved, to some extent at least, in the inflammatory process in mouse synovial tissue. These results represent new information for murine arthritic joints. However, cellular and nerve-related BDNF immunoreactions were also noted in the synovial tissue of RA and OA patients (presented in Paper II). Similar results on cellular BDNF immunoreactions were also obtained by Weidler and collaborators in studies on inflamed synovial tissue from RA patients [Weidler et al., 2005]. Particularly notable were the findings of immunoreactive varicose strands of p75 within the inflammatory infiltrates in some of the arthritic joints (see Paper V). Similar immunoreactive strands have been reported in association with the clusters of immune cells in the pancreas of NOD mice [Persson-Sjogren et al., 2005]. It has been suggested that these strands may be related to survival and nerve growth effects, or alternately apoptosis-modifying effects (cf [Zhu et al., 2003; Persson-Sjogren et al., 2005]). Neurotrophin (i.e., BDNF and NGF) immunoreactions were
detected in nerve fibres of the synovial tissue suggesting that the neurotrophins are also related to synovial innervation.

Most surprisingly, downregulation of NGF, TrkA and p75 in the lining chondrocytes in arthritic joints was found when compared with healthy non-diseased joints. The pattern was thus the same when compared with NK-1R and GRP-R expression. It has previously, in a small study on osteoarthritic chondrocytes, been observed that the expression of NGF and TrkA became upregulated as compared with normal healthy chondrocytes [Iannone et al., 2002]. Those results stand in contrast to the results observed in the present study. There can be several possible explanations to this discrepancy. Firstly, whole joints were examined in this study and not single cells as in the previous study. Secondly, the experimental conditions used here are acute and do not represent a chronic disease such as osteoarthritis. In earlier studies on experimental arthritis it was actually shown that the chondrocytes of the articular cartilage are non-responsive towards another type of growth factor (IGF-1) leading to the inhibition of chondrocyte proteoglycan synthesis [Joosten et al., 1989].

The results suggest that neurotrophin autocrine/paracrine effects on articular chondrocytes are diminished in severe arthritis. Occurrence of neurotrophin effects on chondrocytes is, to a certain extent, supported by previous results on BDNF and NGF expression in cultured human chondrocytes [Yamashiro et al., 2001; Iannone et al., 2002]. Furthermore, it has previously been suggested that NGF may regulate chondrocyte metabolism [Iannone et al., 2002].

Collectively, it is apparent that both neuropeptides (SP and BN/GRP) and neurotrophins have trophic effect on the chondrocytes lining the synovial joint cavity. Furthermore, that there, on the whole, is a decrease in this effect in severe arthritis.

Certain differences between human and mouse synovial tissue were observed indicating that BDNF and its associated receptors are more related to the inflammatory process in the mouse synovial tissue than in that of man. In man, BDNF is nevertheless also related to the synovial innervation.
CONCLUSIONS

In conclusion, the present study shows that:

Human synovial tissue, synovial fluid and blood

The neuropeptides SP and BN/GRP and associated receptors

• A local production of BN/GRP but not SP occurs in fibroblast-like and mononuclear-like cells in human knee joint synovial tissue. On the other hand, SP, but not BN/GRP, was detected in the synovial innervation. The observations concerning both SP and BN/GRP were confined to findings at both protein and mRNA levels.

• There was a pronounced expression of GRP- and NK-1 receptors in the human synovial tissue at both the mRNA and protein level. The receptors were present on perivascular/vascular cells, and in mononuclear-like and fibroblast-like cells in the synovial tissue.

• The results indicate that both SP and BN/GRP have marked effects in the synovial tissue, including effects in relation to the inflammatory cells, the vasculature and the fibroblasts. Occurrence of growth-promoting and healing effects cannot be excluded.

• Correlations between SP and especially BN/GRP and pro-inflammatory cytokines/cytokine receptor (IL-6, TNF-alpha and sTNFRI) were noted in synovial fluid and serum from patients with RA. These observations show that both neuropeptides and cytokines in principle are upregulated in parallel in RA.

• Semi-quantitative estimations of levels of immunoreactions for BN/GRP, GRP-R and NK-1R revealed higher levels in RA specimens compared with OA. In parallel there were higher levels of inflammation and a higher degree of hypercellularity in the RA specimens. Furthermore, the levels of SP in tissue homogenates of RA specimens were higher than those of OA specimens, and there was also a tendency for higher BN/GRP levels in the RA specimens.

The neurotrophin BDNF and its associated receptors

• BDNF immunoreactions were detected in fibroblast-like and mononuclear-like cells. BDNF and the neurotrophin receptors p75 and TrkB were also observed in the synovial innervation, including in sensory corpuscles. The receptors were also located in relation to blood vessels. The presence of BDNF and p75 immunoreactions in nerve structures has not been shown
previously. It is possible that the major BDNF effects in the human synovial tissue are nerve- as well as blood vessel-related.

- No correlation was found between the levels of BDNF in blood and inflammatory parameters (ESR, DAS28). Nor were there any correlations between the levels of BDNF in tissue homogenates with the degree of inflammation in the tissue. BDNF in synovial fluid was only detected in a subset of the RA patients.

- The levels of BDNF in peripheral blood from patients with RA decrease following anti-TNF treatment. It may be that anti-TNF treatment can modulate the pain experiences by patients with RA through the modulation of the neurotrophin BDNF.

**Overall comparisons between SP- BN/GRP and BDNF**

- There were clearly more correlations between SP-BN/GRP and inflammatory parameters and pro-inflammatory cytokines than between BDNF and these factors. This suggests that the neuropeptides, rather than BDNF, are related to inflammatory processes in the human knee joint.

- Studies on the receptors suggest that there are more SP and BN/GRP effects than BDNF effects in the synovial tissue.

- BDNF and SP, but not BN/GRP, are associated with synovial innervation.

**Mouse studies on experimental arthritis**

- Classification of the degree of arthritis led to the stratification of the joints into mild/moderate arthritic and severe arthritic.

- The lining articular cartilage was severely damaged in joints exhibiting severe arthritis. Inflammatory infiltrates were seen in the synovial tissue in arthritic but not control joints.

- The articular chondrocytes of mice express both NK-1R and GRP-R and these receptors were expressed more strongly in healthy animals compared with arthritic ones. No expression of SP or BN/GRP was seen in the chondrocytes. Expression of the neurotrophins, especially NGF, and the neurotrophin receptors p75 and TrkA, was also observed on articular chondrocytes. These immunoreactions (NGF, p75 and TrkA) became downregulated following induction of arthritis. The observations suggest that SP and BN/GRP, as well as the neurotrophins, have marked trophic, and
autocrine/paracrine effects on the chondrocytes. Furthermore, there appears to be production of neurotrophins, but not SP and BN/GRP, in the articular chondrocytes. The decrease in receptor reactions in the chondrocytes in severe arthritis may be a drawback for the chondrocyte functions.

- Immunoreactions for both SP and particularly BN/GRP were seen in the synovial innervation in the arthritic joints in the mouse studies. They were predominantly noted in the proximity of inflammatory infiltrates. The results for BN/GRP in this regard are in contrast to the results of the human study (Paper I) where no nerve-related immunoreactions were observed in the synovium.

- Both NGF and BDNF were expressed in the synovial tissue of arthritic mice but not of the control animals. Reactions were seen both in cells and to some extent as nerve varicosities. TrkB could be seen in cells and p75 immunoreactions as nerve fibre-like varicose strands in the inflammatory infiltrates. The observations show that a neurotrophin system develops in the synovium in parallel with arthritis development.
A FURTHER CORRELATION, STUDIES IN PARALLEL AND FUTURE STUDIES

When going through all the details of the statistical analyses concerning Paper III it was observed that one value erroneously had not been inserted in the table on factor analysis of serum levels (Table 4). That was the value concerning BDNF. A possible explanation for the fault is that this was an isolated feature concerning BDNF during the studies which lead to Paper III, while BDNF was not considered at all in any respect in the final manuscript. The value was 0.829 on Factor 2. The fully correct table is shown below. This shows that there is a correlation between BDNF and certain other variables. However, this can be considered as an isolated observation concerning correlations with respect to BDNF. Thus, as shown in Paper II, there were no correlations between BDNF levels and TNFalpha levels in the synovial tissue nor did the levels of BDNF in synovial tissue/synovial fluid correlate with inflammatory parameters (level of infiltration of inflammatory cells in the synovium and levels of ESR or white blood cell counts in the blood). The finding does not change the interpretations drawn above concerning BDNF.

<table>
<thead>
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<th>Variable</th>
<th>Factor</th>
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</thead>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>sTNFR1</td>
<td>0.323</td>
</tr>
<tr>
<td>ESR</td>
<td></td>
</tr>
<tr>
<td>BN/GRP</td>
<td>0.324</td>
</tr>
<tr>
<td>SP</td>
<td>0.907</td>
</tr>
<tr>
<td>VIP</td>
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</tr>
<tr>
<td>CGRP</td>
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<tr>
<td>NPY</td>
<td>0.925</td>
</tr>
<tr>
<td>BDNF</td>
<td></td>
</tr>
</tbody>
</table>

One surprising finding in this study is that there is a local production of the neuropeptide BN/GRP in the synovial tissue of human knee joints. This means that instead of a BN/GRP-innervation there is a local supply of BN/GRP. The cell types expressing the peptide were mononuclear- and fibroblast-like.

In order to explore whether there is local production of signal substances of the neurotransmitter/neuromodulator type further studies have been undertaken. These have shown that the cell types of the human knee joint synovial tissue referred to above, as well as to some extent cells of blood vessel walls, express the acetylcholine-synthesising enzyme choline acetyltransferase (ChAT) [Grimsholm et al., 2008]. I.e. the cells appear to be acetylcholine (ACh)-synthesising. The findings are of interest as there is no certain proof of cholinergic innervation within human synovial tissue. These findings, and those on BN/GRP, show that non-neuronal cells should be taken into consideration to a larger extent than previously thought concerning local productions of signal substances normally detected in...
neurons. Of importance regarding ACh production is the recently presented concept of a "cholinergic anti-inflammatory pathway" [Jonsson et al., 2007; Tracey, 2007] in tissues like airways and the intestine. This means that released ACh unexpectedly has anti-inflammatory properties, e.g., decreasing the production of pro-inflammatory cytokines from inflammatory cells [Czura and Tracey, 2005]. Ongoing studies in the laboratory also suggest that catecholamines might be produced locally in the human synovial tissue. In accordance with this, noradrenaline is described to be produced by local cells in the human knee synovium in studies by Miller and collaborators [Miller et al., 2000].

The importance of local productions of nerve signal substances in relation to that of synovial innervation should be investigated in future studies. Another important future task is to elucidate further the relationship between cytokines, including TNF-alpha, and neuropeptides/neurotrophins. New treatments ameliorating the severe damage that occurs in RA are obviously desirable. It may be that interference not only with cytokines, but also with neuropeptides or neurotrophins would be worth attention. That may include combination therapy (see also Discussion in Paper IV).
SVENSK SAMMANFATTNING

Inledning

Neuropeptider och neurotrofiner är två grupper av substanser som inte minst har neuroimmuno-modulatoriska egenskaper. De kan båda ha en påtaglig relevans för normal ledfunktion och särskilt för led som drabbats av reumatoid artrit (RA). Neuropeptider är en familj av substanser som traditionellt setts som peptider som frisätts från nervutslag ute i perifera vävnader såsom mag-tarmkanalen, leder m.fl. Det har dock visat sig att även andra typer av celler kan producera och frisätta neuropeptider, t.ex. senceller, inflammationsceller och endokrina celler [Reubi and Waser, 2003; Jonsson et al., 2005; Andersson et al., 2008].

En neuropeptid som undersökts mycket i samband med RA är substans P (SP). Den har visats sig ha pro-infammatoriska egenskaper såsom att stimulera TNF-alfa-produktion från inflammationsceller och att ge upphov till vänndsödemed. SP har också betydelse vid småftörnimmer, den kan ha trofiska effekter och den kan stimulera till blodkärlstillsynt [Seegers et al., 2003; Burssens et al., 2005]. Det har föreslagits att inhibitor mot SP’s receptor, NK-1R, skulle kunna ha terapeutiska effekter vid artrit [Keeble and Brain, 2004]. Det finns dock fortfarande många frågetecken kvar avseende SP’s betydelse för normal och artritisk led.

En annan neuropeptid, bombesin/gastrin-releasing peptide (BN/GRP), har visat sig ha sårläkande, tillväxtbärande och trofiska effekter [Aguayo et al., 1990; Gulluoglu et al., 1999; Yamaguchi et al., 2002]. Vidare har man visat att BN/GRP kan ha kemotaktiska egenskaper på monocyter samt mitogena egenskaper på fibroblaster i luftvägarna [Aguayo et al., 1990]. Det är okänt huruvida BN/GRP uttrycks localt i synovialvävnad eller om den uttrycks i nerver i denna. Vidare är det okänt hur dess receptorer uttrycks i synovialvävnad. Det är inte heller känt om BN/GRP frisätts ut till ledvätskan.

En annan familj av substanser som visat sig vara involverade i inflammatoriska processer är neurotrofinerna [Halliday et al., 1998; Weidler et al., 2005]. De mest undersökt medlemmarna i denna familj är nerve-growth factor (NGF) och brain-derived neurotrophic factor (BDNF). Det finns tecken på att NGF kan vara involverad vid ledinflammation. Bland annat har man funnit att NGF-nivåerna i ledvätska från patienter med RA är förhöjda [Halliday et al., 1998]. Kunskapen om BDNF avseende leder är väldigt begränsad, t.ex. vet man inte alls om substansen uttrycks i synovialvävnadens innerverning. Betydelsen för både neuropeptider och neurotrofiner för ledbrosket är ofullständigt beskriven.

Kronisk ledgångsreumatism, RA, är en kronisk autoimmune ledsjukdom. Kroppens leder blir inflammgerade och i slutändan bryts ledbrosket ned med svår smärta som följd. Behandlingar mot RA har blivit mer effektiva de senaste decennierna i och med att mer specifika inflammationshämmande läkemedel tagits fram, t.ex. TNF-blockerare. TNF-alfa är en viktig substans för inflammationscellers kommunikation som visats sig vara viktig för sjukdomsutvecklingen vid RA. Dock är det så att nya behandlingsstrategier behövs eftersom många patienter fortfarande...
inte svarar på de läkemedel som finns tillgängliga. En intressant aspekt är att det sker interaktionseffekter mellan cytokiner, inklusive TNF-alfa, och neuropeptider och neurotrofiner.

Eftersom det finns många frågetecken avseende betydelsen för SP, BN/GRP och neurotrofiner, särskilt BDNF, i normal ledvävnad och i artritisk sådan, har här studier över dessa substanser och deras receptorer genomförts.

**Syften**

Det övergripande målet för denna avhandling är att bättre förstå betydelsen för neuropeptiderna SP och BN/GRP och neurotrofiner (särskilt BDNF) i knäled, inklusive vid artrit. Mer specifikt är målen följande:

- Att för detta ändamål studera var (i vilka celltyper) de produceras i synovialvävnad, uttrycket för substanserna i ledbrosket, och halterna i synovialvätska och blod.

- Att studera de ovan nämnda substansernas mottagarställen, dvs. receptorer, i synovialvävnad och ledbrok.

- Att avseende dessa frågeställningar göra studier på mänsklig synovialvävnad från patienter med RA och osteoartrit samt på hel knäled från normal och artritisk mus.

- Att avseende RA studera möjliga korrelationer mellan substanserna ifråga och inflammationsparametrar alt. halterna av proinflammatoriska cytokiner i ledvätska, plasma och synovialvävnad.

- Att observera anti-TNF behandlings effekt på blodnivåerna av BDNF från patienter med RA eftersom tidigare studier visat att dessa möjligen kan påverkas av denna behandling.

**Material och metod**

**Material**

Synovialvävnadsbiopsier från knäled, blod och synovialvätska (knäled) från patienter med RA har studerats i denna avhandling (för detaljer se Tabell 1). Gällande proverna från synovialvätska var dessa från olika stadier av sjukdomen, tidig och etablerad RA. Därtöver har också synovialvävnadsbiopsier (knäled) från patienter med OA studerats. Blod från RA-patienter som startade och genomgick anti-TNF behandling har också analyserats. Avseende blod och synovialvätska analyserades också prover från friska kontroller. Vidare har hela knäleder från
möss som utvecklat artrit studerats och vilka särskilt har legat till grund för studier på ledbrocket. Parallellt studerades normala knäleder från möss.

**Metoder**

Två olika metoder har använts för att studera substansernas uttryck i vävnaden. Immunohistokemi användes för att lokalisera neuropeptider, neurotrofiner och deras receptorer (för detaljer om antikroppar, se Tabell 3). 7 μm tunna vävnadssnitt användes till immunohistokemin. Både enzymatisk immunohistokemi (peroxidasa-antiperoxidas) och immunofluorescensmetodik användes.


För att ytterligare studera peptiduttrycket i synovialvävnaden homogeniserades vävnad för att mäta i en enzymbaserad absorbansmätning s.k. ELISA. ELISA användes även för att studera halterna av aktuella substanser i synovialvätska och blod.

**Resultat**

- Lokalt uttryck för BN/GRP sågs i fibroblast-liknande och mononukleär-liknande celler i synovialvävnaden från patienter med RA och OA. Något lokalt uttryck kunde inte ses för SP. SP, men inte BN/GRP, observerades i innerveringen. Både GRP-receptorn (GRP-R) och SP’s receptor, NK-1R, detekterades i stor omfattning i synovialvätnaden.

- BDNF och dess receptorer p75 och TrkB sågs uttryckta i nerv-relaterade strukturer, inklusive sensoriska korpusklar, i synovialvätnaden hos både patienter med RA och OA. BDNF kunde i viss omfattning även ses i celler i synovialvätnaden.

- Det var högre SP-halter och det var en tendens till högre BN/GRP-halter i synovialvävdsextrakt från RA-patienter jämfört med sådant extrakt från OA-patienter. Det var övergripande sett en högre grad av BN/GRP, GRP-R och NK-1R reaktion i synovialvävnad från patienter med RA än OA.

- Vi fann korrelationer mellan neuropeptiderna SP, BN/GRP och proinflammatoriska cytokiner i synovialvätska och blod från patienter med RA. Särskilt BN/GRP korrelerade med IL-6 och TNF-alfa i synovialvätska och med löslig TNF receptor 1 i blod.
Neuropeptides and neurotrophins in arthritis

• Det var ej någon korrelation mellan BDNF-nivåer i synovialvävnaden/synovialvätskan och olika typer av inflammatoriska parametrar.

• Anti-TNF behandling ledde till minskade BDNF-halter i blod från patienter med RA. Effekten var statistiskt säkerställd efter 14 veckors behandling.

• Uttryck för neurotrofinerna NGF, BDNF och deras receptorer uppkom i synovialvävnaden i samband med att artrit etablerades i musmodellen. BN/GRP och SP sågs i nervstrukturer och GRP-R i cellstrukturer i synovialvävnaden i både artritiska och normala möss.

• Broskcellerna i musled uttryckte neurotrofiner, särskilt NGF, och neurotrofinreceptorer liksom NK-1R och GRP-R. Det skedde en nedreglering av NGF och dess receptorer (p75 och TrkA) i ledens broskeller hos artrit-drabbad mus jämfört med friska möss. Det skedde även en nedreglering av NK-1R och GRP-R uttrycken i ledens broskeller hos artrit-drabbad mus.

Sammanfattning/diskussion


Det är även tydligt att SP och BN/GRP är mer associerade till inflammationsgraden i leden än vad fallet är med BDNF. BDNF uttrycktes främst i nervstrukturerna i synovialvävnaden. Tilläggas bör att NGF i tidigare studier beskrivits ha inflammationsrelaterade egenskaper. Det är sannolikt att NGF är mer inflammations-relaterad än BDNF. Det faktum att SP- och särskilt BN/GRP-nivåerna var korrelerade med nivåerna för de pro-inflammatoriska cytokinerna visar på ett samband mellan dessa modulatorer. En möjlighet är att BN/GRP har en pådrivande effekt på cytokinsyntesen, vilket skulle vara intressant eftersom cytokiner, inte minst TNF-alfa, är kraftigt påsluga vid artrit.
Vidare visar avhandlingen att anti-TNF behandling påverkar nivåerna av BDNF i perifert blod. Detta, tillsammans med resultaten i artikel III där vi visar att BDNF uttrycks främst av nerv-liknande strukturer i leden, är en intressant infallsvinkel för ytterligare studier om BDNFs betydelse vid RA. Det går alltså inte att utesluta att effekterna av anti-TNF behandling inkluderar effekter på nervstrukturer.


Sammantaget visas att både lokala celler i synovialvävnaden, neuronen i denna och ledbroskcellerna på olika sätt kan tillhandahålla de aktuella substanserna. Detta, tillsammans med observationerna avseende receptoruttrycken för dessa, är viktigt att ha i åtanke för fortsatt förståelse av substansernas betydelse.
FUNDING

- The Faculty of Medicine, Umeå University
- Arnerska forskningsfonden
- JC Kempes Minnes Stipendiefond
- Wallenbergs stiftelse för resestipendium vid Umeå Universitet
- JC Kempes resestipendium för Umeå Universitet
- Anna Cederbergs stiftelse
TACK TILL...

Den här doktorsavhandlingen utgår huvudsakligen från avd. för Anatomi och har genomförts i samarbete med avdelningen för Reumatologi vid Norrlands universitetssjukhus samt med Institutionen för Medicinsk kemi och biofysik.

Jag skulle vilja tacka alla som har varit behjälpliga under dessa åren då jag genomfört forskningen som ligger till grund för denna avhandling. Jag skulle särskilt vilja tacka följande personer:

- Professor Sture Forsgren, min huvudhandledare, för att han har delat med sig utav den enorma kunskap han besitter inom forskningsområdet. Han har alltid tagit sig tid att hjälpa mig och förklara oavsett hur upptagen han har varit med annat. Han har brinnat verkligen för forskningen och det har gjort min doktorandtid till en mycket gedigen grund att stå på inför framtiden. Jag är Dig evigt tacksam.

- Professor Solbritt Rantapää-Dahlqvist, min bihandledare, för att hon har delat med sig av sina gedigna kunskaper inom reumatologifältet och alltid kommit med kritiska synpunkter på manuskripten. Utan dessa synpunkter hade inte denna avhandling hållit samma nivå. Stort tack också för all hjälp med statistik. Jag uppskattar de raka kommentarerna som jag fått genom åren. De har fört oss framåt.

- Laboratorieassistenten Ulla Hedlund för all hjälp genom åren. Utan henne hade inte denna avhandling kunnat genomföras rent praktiskt. Denna service har verkligen förenklat livet på anatomen för min del. Tack också för alla trevliga pratstunder och kafferaster tillsammans. De har förgyllt tillvaron.

• Thanks to Dr Brian Ellis for all the help with language corrections. Your help has been very valuable to me.

• Thanks to Dr Guo Yongzhi for a fruitful co-operation in the experimental animal studies. It was a pleasure to work with you. Thank you for all your kindness.

• Docent Tore Dalén for all biopsies and medförfattarskap och till Professor Tor Ny för medförfattarskap.


• Till slut mitt tack till de viktigaste personerna i mitt liv, min syster, Elin, och mina föräldrar, Annika och Lennart, för all er kärlek genom hela livet. Ni har varit mitt stöd och alltid stöttat mig i mina val var jag än befunnit mig i livet. Tack för att ni stått ut med all flytt fram och tillbaka de senaste åren. Utan er hade jag inte varit där jag är idag. Ni är helt underbara. Jag älskar er.
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Neuropeptides and neurotrophins in arthritis


