INVESTIGATION OF THE ROLE OF SENSORY-IMMUNE INTERACTIONS IN RODENT MODELS OF ARTHRITIS

PhD thesis

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INTRODUCTION

1. Prevalence of chronic arthritis, current therapeutical strategies

Rheumatoid arthritis (RA) is a severe autoimmune disease affecting not only the joints of the hands and legs, but also other organs. The cause and the precise pathomechanism is still unknown. Around 70.000-80.000 people suffer from the disease in Hungary (female/male ratio: 3). RA is a chronic disease characterized by the progressive destruction and deformation of the joints leading to persistent pain, movement disability and decreased life quality (Jones et al., 2003; Harris, 2005; Kourilovitch et al., 2014). It is a great public health problem due to its high incidence and prevalence, unsatisfactory therapeutic outcomes and unfavorable life expectancy.

The management of RA is composed of symptomatic and disease-modifying antirheumatic drugs (DMARDs). Drugs relieving the symptoms are the steroidal and non-steroidal anti-inflammatory agents, but their use is limited by the severe adverse effects. RA treatment should start with the DMARDs, which do not only attenuate the symptoms, but also slow down the progression of the structural damage. Furthermore, they also cause several side effects. The most important therapeutic tools are TNFα or B cell action inhibitors, but their most common adverse effect is the increased risk of infections (especially tuberculosis).

2. Capsaicin-sensitive sensory nerves, neurogenic inflammation

Approximately half of the primary sensory neurones belong to the capsaicin-sensitive subpopulation, which expresses the Transient Receptor Potential Vanilloid 1 (TRPV1) receptor. TRPV1 is a ligand-gated non-selective cation channel, its activation causes Na^{+}- and Ca^{2+} influx to the cells resulting in pain sensation and sensory neuropeptid-release. These receptors can be activated by physical, as well as chemical stimuli, both from the intra- and extracellular spaces (high temperature, protons, vanilloids – e.g.: resiniferatoxin). Furthermore there are several transmitters, acting on their own receptors that sensitize the TRPV1 receptor and increase the responsiveness of the nerve endings. These transmitters (bradykinins, prostaglandins, proteases, serotonin) show a significantly high concentration under inflammatory conditions and are crucial participants of the inflammatory processes.

Besides the classical afferent function of these nerves mediating sensory input to the central nervous system and generating pain, these fibres possess local and systemic efferent functions too. Their activation by antigens or non-immunological provocation triggers sensory neuropeptide (Substance-P: SP and calcitonin gene-related peptide) release. This process is neurogenic inflammation, which is the local efferent function of the sensory nerve-endings

(Jancsó et al., 1967; Holzer, 1988). This phenomenon plays an important role in the pathogenesis of several inflammatory diseases, like RA, asthma, allergic rhinitis, konjunctivitis and dermatitis, migraine or inflammatory bowel diseases (Pintér et al., 2014). From the same fibres somatostatin and opioid peptides are released, reach the systemic circulation and act in distant parts of the body, exerting antiinflammatory and antinociceptive functions (Szolcsányi, 1998). Somatostatin acts at its five G_i protein-coupled receptors (sst_1-sst_5). Capsaicin-sensitive peptidergic sensory nerves densely innervate the joint capsule and the synovium, their pathophysiological relevance is beyond doubt, since increased proinflammatory and decreased antiinflammatory neuropeptide levels have been demonstrated in the serum and/or synovial fluid of RA patients.

3. Protease-activated receptors

The family of protease-activated receptors (PARs) consists of four members: three receptors for thrombin (PAR-1, PAR-3 and PAR-4) being involved in mainly coagulation processes and one for trypsin/mast cell tryptase (PAR-2). They are all heptahelical G-protein-coupled receptors activated by proteolytic cleavage through several proteases (Macfarlane et al., 2001; Hollenberg and Compton, 2002). Furthermore, it is well-known, that serine proteases play an important role in several inflammatory processes (Kanke et al., 2005) and pain (Vergnolle et al., 2001; Coelho et al., 2003). PAR-2 has drawn great attention due to its different localization and function compared to the other three PARs. It is expressed in the joints (Ferrell et al., 2003; Nakano et al., 2007), and also on capsaicin-sensitive peripheral sensory nerves (Steinhoff et al., 2000). Recently, the involvement of capsaicin-sensitive fibres in PAR-2 activation-induced inflammatory reactions and nociception has been suggested by the markedly reduced responses observed after capsaicin desensitization or with TRPV1 receptor antagonists (Paszcuk et al., 2008). PAR-2 stimulation in the murine knee promotes synovial hyperaemia and oedema (Ferrell et al., 2003) indicating a role for PARs in inflammatory joint diseases.

4. Tachykinins, tachykinin receptors

Mammalian tachykinins are 10-12-aminoacid-long peptides sharing the hydrophobic C-terminal region. SP and neurokinin A (NKA), encoded by the preprotachykinin A (Tac1) gene, are expressed predominantly in capsaicin-sensitive primary sensory neurones of the dorsal root ganglia and act as an excitatory transmitter (Otsuka és Yoshioka, 1993), although transient expression in response to challenge is seen in a variety of non-neuronal cells. Neurokinin B (NKB), derived from the preprotachykinin B (Tac3) gene, is found

predominantly in the central nervous system (Lundberg, 1996). The newest member of the tachykinin gene family is the preprotachykinin C (Tac4) gene discovered in 2000 (Zhang et al., 2000). Tac4 encodes hemokinin-1 (HK-1) in mice and its equivalent peptides, endokinin A, B, C and D (EKA-D) and human HK-1 in humans (Steinhoff et al., 2014). It can be found predominantly in immune cells, but also in various brain regions, but the pattern of its peripheral and central expression is significantly different from that of SP (Page et al., 2003, Duffy et al., 2003). Three G-protein-coupled mammalian receptors, tachykinin NK1, 2 and 3 have been identified, to which tachykinins have different affinities. SP binds predominantly to the NK1 tachykinin receptor localized mainly on neuronal, endothelial and immune cells. NKA shows the greatest affinity to NK2 receptors, while NKB to NK3 receptors in the brain. HK-1 is very similar to SP regarding its structure and pharmacology, it has similar receptor binding and preference for NK-1. However, some data raised the possibility of a new tachykinin receptor, because the results can not be interpret with the actions on the presently known 3 receptors (Lau et al., 2001; Page et al., 2003). Investigation of the role of tachykinins in chronic joint inflammatory processes is reasonable because the magority of the proinflammatory sensory neuropeptide released during neurogenic inflammation belong to this group. As a result of the activation of capsaicin-sensitive nerve-terminals, the released Tac1 gene-encoded SP and NKA and their NK1 and 2 receptors, are responsible for activation of inflammatory cells (lymphocytes, mast cells), vasodilation and pain transmittion, which effect are well-documenteted in the literature (Longmore et al., 1997). Furthermore, their role in some inflammatory disease models has also been proven (Helyes et al., 2004; 2010). The importance of these neuropeptides can be explained by the fact that in the serum and synovial tissue of RA patients (Anichini et al., 1997; Larsson et al., 1991), as well as in animal models of arhritis (Bileviciute et al., 1993) eleveted levels of SP could be detected. Both the remarkable expression of HK-1 on different inflammatory cells and the NK1 receptor preference strongly suggest that the Tac4-encoded tachykinins play an inportant role in the mediation of inflammatory processes and neuro-immune interactions.

AIMS

There is still no drug available that is able to influence the neurogenic inflammatory factor, sensory-immune interactions, and the late pain component of RA. There is a great need for more effective drugs with less side-effects with special emphasis on the neuropathy-like chronic persistent pain. Therefore, in our experiments we aimed to reveal the complex pathophysiological mechanisms in these processes, identify key mediators and potential therapeutical targets for novel drug development.

The general objectives of our work were:

I. To investigate the role of capsaicin-sensitive peptidergic nerves in the serum-transfer mouse model of RA with integrative approaches.
II. To study the involvement of the TRPV1 channel in PAR2 activation-induced rodent arthritis.
III. To analyse the role of tachykinins in the adjuvant-induced chronic arthritis model of the mouse.

MATERIALS AND METHODS

1. Experimental animals

We performed experiments on male and female C57Bl/6J, wild type mice (WT; 10-12-week-old; 25-30 g), and male Wistar rats (250-300 g). The original breeding pairs were purchased from Charles-River Ltd. (Hungary).

In PAR2- and CFA-induced arthritis models we used Trpv1 gene-deficient ($Trpv1^{-/-}$; Jackson Laboratories, USA), Tac1 ($Tac1^{-/-}$), Tac4 ($Tac4^{-/-}$) and NK1 receptor gene-deficient ($Tacr1^{-/-}$) as well as double gene-deficient ($Tac1^{-/-}$/$Tac4^{-/-}$) animals. $Tac1^{-/-}$ and $Tacr1^{-/-}$ mice were generated at the University of Liverpool (Zimmer et al., 1998). $Tac4^{-/-}$ and $Tac1^{-/-}$/$Tac4^{-/-}$ mice were obtained from the University of Toronto (Berger et al., 2010).

Animals were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs at 24–25 ˚C under a 12-h light-dark cycle were used in all studies. Standard mouse chow and water were provided *ad libitum*.

2. Experimental models

2.1. K/BxN serum-transfer arthritis

Chronic arthritis of male and female C57Bl/6 mice was induced by intraperitoneal (i.p.) injection of K/BxN serum on the days 0. and 3. Control groups of intact animals were treated with BxN (not arthritogenic/control) serum following the same protocol. Measurements were performed during 2 weeks.

2.2. PAR2 activation induced arthritis

The PAR2-activating peptide, was injected s.c. into the plantar surface of the paw or into the knee joint of mice and rats under isoflurane anaesthesia, which evokes SP and CGRP release from the capsaicin-sensitive sensory nerve endings and result in inflammation. Measurements were performed at certain time points after the injections throughout the 6-h experimental period.

2.3. CFA induced arthritis

Arthritis was induced by intraplantar injection of of Complete Freund's Adjuvant (CFA, killed Mycobacteria suspended in paraffin oil, 1 mg/ml; Sigma, St. Louis, MO) into the right hind paw and s.c. into the root of the tail. An additional s.c. injection was given on the following day into the tail in order to potentiate the systemic effects. Measuments were performed throughout the 21-day experimental period.

3. Pharmacological tools

3.1. RTX desensitization

Pretreatment with the ultrapotent TRPV1 agonist resiniferatoxin (RTX, Sigma-Aldrich; 30, 70, 100 μg/kg s.c. on 3 consecutive days) leads to long-lasting defunctionalization of capsaicin-sensitive nerves (desensitization) (Szolcsányi et al., 1990). Two weeks later the success of the pretreatment was verified by the lack of eye-wiping after capsaicin drops (50 μl, 0.1%).

3.2. PAR2 activation

The PAR2-activating peptide, SLIGRL-NH_2 (or in the control group the inactive LRGILS-NH_2) was injected s.c. into the plantar surface of the right paw (100 μg in 50 μl) or into the right knee joint of mice (100 μg in 50 μl) and rats (100 μg in 100 μl) under isoflurane anaesthesia.

In one group of rats, pre-treatment with the selective TRPV1 receptor antagonist SB366791 (500 lg/kg i.p.) was performed 15 min before intraarticular SLIGRL-NH_2 injection and before each measurement.

4. Experimental methods

4.1. Measurement of touch sensitivity of the paw

Touch sensitivity of the plantar surface of the paw was determined by dynamic plantar aesthesiometry (Ugo Basile 37400, Comerio, Italy). This device is a modified, electronic von Frey technique, which is used to assess mechanonociception. In case of mice mechanical hyperalgesia was expressed as percentage of control mechanonociceptive threshold compared to the initial values. Since this mechanical stimulus is basically non painful in rats, but slightly painful in mice, the drop of the threshold is considered to be mechanical allodynia in rats, but hyperalgesia in mice.

4.2. Measurement of the mechanonociceptive threshold of the rat paw

The mechanonociceptive threshold of the rat hindpaw was measured with the Randall-Sellitto test (Ugo Basile Analgesimeter 7210, Comerio, Italy) and hyperalgesia was expressed in percentage compared to the initial, pre-injection control values.

4.3. Measurement of spontaneous weight distribution

Spontaneous weight bearing on the two hindlimbs was determined by incapacitance tester (Linton Instrumentation, Norfolk, England). The percent weight distributed onto the right (treated) hindlimb was calculated and expressed in percentage before and after treatment.

4.4. Measurement of thermal hyperalgesia

The thermonociceptive threshold of the paw was determined on increasing temperature hot plate (IITC Life Sciences, Woodland Hills, CA, USA) by nocifensive reactions (lifting, licking, shaking) or reaching the maximum value (53°C). Thermal hyperalgesia was expressed in °C drop of thermonociceptive threshold compared to the control values.

4.5. Measurement of the paw volume

Paw volume was measured by plethysmometry (Ugo Basile Plethysmometer 7140, Comerio, Italy). Change in paw volume (oedema) was expressed in percentage compared to the initial values.

4.6. Measurement of knee diameter in mice

The anterio-posterior and medio-lateral the diameter of the knee joint was measured with a digital micrometer (Mitutoyo, Japan). Change in knee diameter was expressed in percentage compared to the initial values.

4.7. Assessment of arthritis severity

Arthritic changes were semiquantitatively scored using a grading scale of 0 to 10 by evaluating edema and hyperemia.

4.8. Assessment of joint function (grid test)

An easy and reproducible method to determine grasping ability correlating with joint function. Mice were placed on a horizontal wire-grid, then it was turned over and the latency to fall was determined.

4.9. In vivo bioluminescence imaging of myeloperoxidase-activity

Luminol bioluminescence (BLI; 5-amino-2,3-dihydro-1,4-phthalazine-dione) correlates with neutrophil myeloperoxidase activity in arthritis in vivo, which was detected with IVIS Lumina II (PerkinElmer, Waltham, USA). Identical Regions of Interests (ROIs) were applied around the ankles and luminescence was expressed as total radiance (total photon flux/s).

4.10. In vivo fluorescence imaging of matrix-metalloproteinase activity

Matrix-metalloproteinase (MMP) activity was assessed using MMPSense680 (PerkinElmer), an activatable fluorescent imaging probe for MMP-2, -3, -9 and -13. Measurements were performed with the FMT 2000 fluorescence molecular tomography system (PerkinElmer). Three-dimensional reconstructions of the ankles were made, isocontour ROIs were applied, and MMP was expressed as pmol fluorophore.

4.11. In vivo micro-computed tomography (micro-CT) analysis of bone structure

The right tibiotarsal joints were scanned by SkyScan 1176 in vivo micro-CT (Bruker, Kontich, Belgium). Changes of bone structure were evaluated by CT Analyser® software. Standard size ROIs were applied around the periarticular tibia and fibula regions, and around the tibiotarsal and tarsometatarsal joints. Bone volume (μm3) was quantified and expressed as a percentage of the total ROI volume.

4.12. Histological processing and assessment of joint inflammation

Ankle joints excised on day 14 were fixed, decalcified and dehydrated, embedded in paraffin, sectioned (3-5 μm) and stained with hematoxylin-eosin or Safranin O. Histopathological changes were scored by a pathologist blinded from the study on the basis of certain inflammatory parameters to create composite arthritis scores.

4.13. Determination of inflammatory cytokin concentrations and somatostatin-like immunoreactivity (SOM-LI) in tissue homogenates

The tibiotarsal joints were homogenized, centrifuged, and the supernatants were collected for cytokine concentration with ELISA or SOM-LI determination with a specific and sensitive radioimmunoassay (RIA).

4.14. Ethics Statement

Experiments were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal

Experiments (243/1988), complied with the recommendations of IASP, and approved by the Ethics Committee on Animal Research of University of Pécs (licence: BA 02/2000-2/2012).

4.15. Statistical analysis

Hyperalgesia, allodynia, spontaneous pain, edema and weight loss and joint function were evaluated by repeated measures two-way analysis of variance (ANOVA) + Bonferroni's modified t-test, semiquantitative clinical and composite histopathological scores by non-parametric Kruskal-Wallis test + Dunn's post-test, micro-CT results by two-way ANOVA + Dunett and Tukey post-tests to evaluate the time-dependent self-control changes and the different groups, respectively. Bioluminescence and fluorescence imaging, as well as somatostatin-LI and cytokin concentrations were analyzed by Student's t-test for unpaired comparisons. In all cases $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Capsaicin-sensitive sensory nerves exert complex regulatory functions in the serum-transfer mouse model of autoimmune arthritis

Results

1. Increased joint edema after desensitization of capsaicin-sensitive sensory nerves

In non-pretreated arthritic mice an approximately 45% edema developed in both males and females, which was maintained till the end (day 11) of the experiment. In RTX-desensitized arthritic animals this swelling was significantly higher in both genders during the whole study with a maximum of 90-95%. Similarly, arthritis scores reached a maximum of 7 in mice without pretreatment and 9 in RTX-pretreated animals showing that the significant increase in paw volume was visible on all limbs between days 2 and 7-8 in male female mice, respectively.

2. Attenuated late mechanical hyperalgesia in RTX-desensitized mice

Mechanical hyperalgesia in non-pretreated arthritic mice reached an approximately 25-30% after 5 days, which further increased to 45% by days 10 in both male and female mice. Significant reduction of mechanical hyperalgesia was measured in RTX-desensitized animals from day 10. Despite the development of mechanical hyperalgesia, the noxious heat threshold was not influenced by the arthritis. However, the thermonociceptive threshold of RTX-pretreated animals was significantly higher compared to mice without pretreatment between days 1 and 5.

3. Similar weight loss and impaired joint function in non-desensitized and RTX-desensitized mice

Arthritis resulted in weight loss until day 7 in both groups, after that a slight increase could be detected, but it did not reached the initial values till the end of the experiment. Similarly, a significant decrease of time spent on the grid was measured both in the non-pretreated and RTX-pretreated groups and it retured to the control values to day 14.

4. Greater neutrophil-activity in desensitized mice in the acute arthritis phase

Luminol-BLI revealed a remarkable increase in neutrophil-derived MPO activity in the arthritic ankle joints of both groups, being significantly higher in RTX-pretreated mice in the early phase (day 2). This difference ceased during the later phase by day 6.

5. Increased MMP activity in RTX-pretreated animals

Fluorescent molecular tomography revealed that a considerable increase in MMP activity occurred in the inflamed ankle joints of arthritic animals similarly on days 5 and 8, but no signal could be detected in intact, non-inflamed mice. MMP activity significantly enhanced after functional impairment of the capsaicin-sensitive afferents on day 5 when the differences in swelling and arthritis severity scores of the two groups were the greatest.

6. Altered inflammation-induced structural changes in the bone of RTX-pretreated female mice

The basal bone mass (BV/TV) was lower in the female group as compared to age-matched males. RTX-desensitization alone did not induce any change in the bone mass in male animals, it evoked a moderate, but significant decrease in females. Self-control quantitative analysis of the bone structure revealed significant increase in the ankle joint of non-pretreated both males and females already on day 7 due to pathological new bone formation. Meanwhile, in RTX-pretreated arthritic females bone mass gradually and significantly increased reaching a remarkable, 20% gain by day 14 compared to the initial control values of the same animals.

7. More severe arthritic histopathological alterations after RTX pretreatment

There was no histopathological difference between the intact joints of untreated and RTX-pretreated animals. In non-pretreated arthritic mice characteristic chronic arthritic changes developed by day 14. In desensitized arthritic animals these changes were more pronounced with significantly greater synovial swelling, higher number of fibroblasts and more collagen. Semiquantitative scoring of these parameters showed remarkable worsening effect of RTX pretreatment on these characteristic histopathological features in both sexes.

8. RTX desensitization decreases arthritis-induced elevation of somatostatin-LI in the tissue homogenates

Somatostatin-LI significantly increased to 75.54±3.07 fmol/g wet tissue in the arthritic paws of non-pretreated mice compared to their intact controls (25.19±1.53 fmol/g wet tissue), while in RTX-desensitized arthritic animals its inflammation-induced elevation was significantly smaller, from 28.43±1.19 to 62.39±2.58 fmol/g wet tissue.

Discussion

We provided here the first evidence that capsaicin-sensitive peptidergic sensory nerves play an important and complex regulatory role in a primarily autoimmune arthritis model of the mouse. Although there was no difference between male and female mice in any inflammatory parameters, our unique finding obtained by quantification of the self-control micro-CT scans is that there was in fact a decreased bone mass in females compared to age-matched males. Furthermore, in females inactivation of the capsaicin-sensitive afferents resulted in basically decreased bone volume, but the arthritis-induced pathological bone formation was more severe. These results are supported by recent animal (Cai et al., 2014) and human (Vanderschueren et al., 2014) data. Since unlike in female mice, in RTX-pretreated males we could not detect a significantly increased pathological bone formation as compared to the non-pretreated animals, it can be suggested that the androgens might have a protective role on the bones particularly under inflammatory conditions. It is clear that sex steroids are important influencing factors in osteoclast/osteocyte/chondrocyte functions (Vanderschueren et al., 2014). However, the precise mechanisms of peptidergic sensory nerve activation and bone turnover regulation are still unclear, because there are very few data about the importance of afferents/TRP channels in chondrocyte-osteoclast-osteoblast functions. It is well-known that in the early stage of the inflammatory reaction in this model, as well as in human rheumatoid arthritis, the activation of neutrophils is a predominant component (Beevart et al., 2010) and MPO is the major constituent of neutrophil azurophilic granules. Similarly to what we found in this arthritis model, elevated MPO-levels in RTX-desensitized mice were previously detected in LPS-induced acute airway inflammation (Elekes et al., 2007). Our MMP results are also in good correlation with sporadic earlier evidence demonstrating that TRPV1 receptor activation results in decreased MMP-9-secretion (Tauber et al., 2012). We found that somatostatin level was increased in the arthritic joints, but significantly decreased after RTX-pretreatment. Therefore, its inflammation-evoked elevation is likely to be derived from the capsaicin-sensitive fibers, and be involved the protective actions of these nerves. Intriguingly, in contrast to our earlier findings in RTX-pretreated rats,

the increased inflammation was not accompanied by a proportionally enhanced mechanical hyperalgesia. In the late phase, when inflammatory signs were attenuated, but mechanical hyperalgesia was still present, it was even significantly milder in RTX-pretreated mice. Compared to the greater severity of inflammation, mechanical hyperalgesia is clearly smaller in the RTX-pretreated group. Therefore, capsaicin-sensitive nerves might participate in arthritic mechanical hyperalgesia during the whole process, but the difference was only manifest when the degree of inflammation was equal in both groups. Furthermore, recent data showed that in the chronic phase of the K/BxN arthritis, when swelling and hyperemia disappeared, neuropathic pain developed (Christianson et al., 2010). In heat and mechanical hyperalgesia TRPV1 channels are the most important participants (Sousa-Valente et al., 2014). In agreement with these results we detected a significantly attenuated mechanical hyperalgesia in RTX-pretreated animals only in the late phase (from day 10). This strongly suggests that TRPV1 channels play a pivotal role in pain mediation, as described in neuropathy (Brito et al., 2014) and arthritis models (Fernandes et al., 2011). We found a significant difference in the RTX-pretreated group during the first week, which is probably due to the well-known heat threshold increasing effect of RTX (Almási et al., 2003), but arthritis did not alter thermosensitivity. The activation of capsaicin-sensitive peptidergic sensory nerves inhibits the characteristic arthritis symptoms (edema, inflammatory cell activation and functions) at least partially through somatostatin release, but despite this potent anti-inflammatory role, they mediate the later pain response. It can be concluded that neuropeptide-containing sensory nerves exert a complex regulatory function in inflammatory conditions, and the overall effect of their activation depend on the tissues and the pathophysiological mechanisms of the disease.

Involvement of transient receptor potential vanilloid 1 receptors in protease-activated receptor 2-induced joint inflammation and nociception

Results

1. Role of the TRPV1 receptor in intraarticular SLIGRL-NH_2-induced pain behaviour in rats

Injection of the PAR2 receptor activating peptide, SLIGRL-NH_2 into the knee joint induced 12–17% mechanical allodynia, which was markedly reduced by i.p. pre-treatment with the TRPV1 receptor antagonist. The drop in the mechanonociceptive threshold of the paw was much greater (about 35%) in response to injection of SLIGRL-NH_2, and was abolished by

TRPV1 receptor antagonist pre-treatment. In addition, PAR2 activation in the knee joint caused spontaneous pain, which was significantly diminished by TRPV1 receptor antagonism 3, 4 and 5 h after SLIGRL-NH_2 administration. The inactive peptide had no effect on either mechanonociception or spontaneous weight bearing.

2. Inflammatory cytokine concentrations in the rat joint

The concentration of TNFα in the rat joints were around the detection limit of the ELISA assay both in LRGILS-NH_2 and SLIGRL-NH_2-injected knees 6 h after the injection. The level of IL-1β significantly increased in response to intraarticular PAR2 activation (792 ± 32.6 pg/g wet tissue in the SLIGRL-NH_2-injected knees compared to the 687 ± 48.4 pg/g wet tissue measured in the LRGILS-NH_2-treated joints). However, IL-1β levels were not significantly altered by SB366791 pre-treatment (709 ± 45.5 pg/g wet tissue).

3. Role of the TRPV1 receptor in intraarticular SLIGRL-NH_2-induced pain behavior and inflammatory cytokine release in mice

Injection of SLIGRL-NH_2 into the knee joint of wildtype mice induced about a 15% decrease of the mechanonociceptive threshold. In the TRPV1 gene-deficient group, this secondary hyperalgesia was almost absent at 3 and 4 h. WT mice demonstrated decreased ipsilateral hindlimb weight bearing in response to intraarticular injection of SLIGRL-NH_2 but it was significantly reduced in TRPV1 gene-deficient mice at 5 and 6 h. PAR2 receptor activation did not elevate joint TNFα concentration, which was at the detection limit of the ELISA assay both in LRGILS-NH_2 and SLIGRL-NH_2-injected knees. Intraarticular administration of SLIGRL-NH_2 caused a significant increase in joint IL-1β levels in both WT and TRPV1$^{-/-}$ mice, there was no difference between these groups.

4. No effect of intraarticular SLIGRL-NH_2 on knee diameter

Moderate oedema formation was detected as a result of PAR2 activation, which remained unchanged during the whole experimental period. Similar changes were observed in TRPV1$^{-/-}$ mice, significant differences could not be determined between any groups.

5. Involvement of the TRPV1 receptor in intraplantar SLIGRL-NH_2-induced tissue oedema in mice

In WT animals paw volume increased by about 22–26% and in the TRPV1$^{-/-}$ animals by 10%, so it was markedly smaller than in wildtypes throughout the whole 6 h-period.

6. TRPV1 have no effect on intraplantar SLIGRL-NH_2-induced pain behavior and cytokine concentrations in mice

Moderate primary hyperalgesia and change in spontaneous weight distribution induced by intraplantar injection of SLIGRL-NH_2 was detected in WT mice, and there were no

significant difference between the gene-deleted and WT groups. Intraplantar injection of SLIGRL-NH_2 did not induce increased TNFα production in the paw. IL-1β, however, significantly increased in response to PAR2 activation, but it was not altered by genetic deletion of the TRPV1 receptor.

Discussion

The present results provide the first evidence that injection of the selective PAR2-activating peptide, SLIGRL-NH_2 into the knee joint results in the development of a secondary mechanical hyperalgesia/allodynia in the paw as well as impaired weight distribution in both rats and mice. The effects of articular PAR2 stimulation was examined by Ferrell et al. (2003) who described swelling, hyperaemia and histological changes as well-defined signs of the development of an inflammatory reaction. We, on the other hand, did not find any change in knee joint diameter in response to PAR2-activating peptide. Our experiments with rats pre-treated with the selective TRPV1 receptor antagonist SB366791, as well as studies with TRPV1 receptor gene-deleted mice revealed, that the secondary hyperalgesia/allodynia and the decreased spontaneous weight distribution on the affected side are predominantly mediated via TRPV1 receptor activation. While the PAR2 activation-evoked changes were abolished by the TRPV1 antagonist in rats, these were significantly, but not completely inhibited by the genetic deletion of this ion channel in mice. This difference can be explained by a species difference, but compensatory mechanisms counteracting the lack of this receptor are also possible in knockout mice. Furthermore, activation of TRPV1 channel is likely to be a little faster in rats than in mice. Activation of PAR2 receptors on the synovial cells, fibrocytes, inflammatory and immune cells, as well as sensory nerves results in the release of several proinflammatory mediators, which in turn activate/sensitize the TRPV1 channels. Intraplantar administration of SLIGRL-NH_2 in mice produced paw edema through a TRPV1-mediated mechanism, which is most likely to be the result of SP and CGRP release from the activated sensory nerve terminals. In contrast, this ion channel was not involved in the development of PAR2-evoked primary hyperalgesia or impaired weight distribution. It has been suggested by previous studies performed on TRPV1 receptor gene-deleted mice that thermal and mechanical hyperalgesia evoked by SLIGRLNH_2-injection into the paw was absent or significantly smaller in the TRPV1 knockout group (Amadesi et al., 2004; Dai et al., 2004) but we could not prove this observation. Primary and secondary hyperalgesia develop by fundamentally different mechanisms. While primary hyperalgesia, especially to thermal stimuli, is predominantly mediated by peripheral sensitization of primary nociceptive nerve terminals, secondary hyperalgesia is attributed to altered processing in the central nervous

system (Treede and Magerl, 2000). The basic mechanisms of secondary hyperalgesia may consist of modulation of neural transmission in the spinal cord and in higher neural centres (thalamus, somatosensory cortex). Several mechanisms are triggered in the dorsal horn by noxious stimuli, which lead to enhanced synaptic activation (central sensitization). However, intraarticular PAR2 activation induced a weaker secondary hyperalgesia than the primary response after direct intraplantar administration of the agonist. There might be more constitutive endopeptidase activity in the synovial fluid, which limits the PAR2 induced responses. Besides the localization of the PAR2 on sensory nerve endings, in the joints they have also been described on other specific cell types such as synovial cells, immune- and inflammatory cells including mast cells. Proinflammatory mediators released from these specific cells in the joints might be involved in activation/marked sensitization of the TRPV1 receptors (Kelso et al., 2006). Although PAR2 activation cannot generate action potentials itself, it has the ability to functionally interact with other receptors and ion channels (e.g. TRPV4; Grant et al., 2007) to induce depolarization in the spinal cord (Seeliger et al., 2003; Dai et al., 2004). Based on our results, it can be inferred that the activation of joint sensory nerve terminals themselves by the PAR2 agonist is not TRPV1 receptor-mediated, however, TRPV1 does appear to be involved in PAR2-mediated nociceptive processing involving higher pain centres.

Role of tachykinin 1 and 4 gene-derived neuropeptides and the tachykinin NK1 receptor in adjuvant-induced chronic arthritis of the mouse

Results

1. Role of tachykinins in adjuvant-induced inflammatory mechanical hyperalgesia.

In WT mice an approximately 40% decrease of the mechanonociceptive threshold developed 4 days after adjuvant injection, which was significantly reduced in the Tac4 and Tacr1 gene-deleted groups starting on day 11 of the experiment. In contrast, no significant difference in pain thresholds was detected in either $Tac1^{-/-}$ or $Tac1^{-/-}/Tac4^{-/-}$ mice.

2. Tachykinins are not involved in adjuvant-induced oedema.

In control animals, the volume of the CFA-injected paws increased to about 90% within 4 days post adjuvant injection, reaching a maximal swelling 11 days after the induction of inflammation. No significant differences were observed in any knockout strains.

3. Role of tachykinins in adjuvant-induced arthritic histopathological alterations.

There was no difference between the intact joint structures of C57Bl/6 and any gene-deleted mice. Meanwhile, joints of adjuvant-injected WT mice were damaged by expanding synovial pannus. Widening of the synovial cavity, synovial hyperplasia and its infiltration with inflammatory cells, as well as cartilage destruction and minimal bone erosion were apparent. Histopathological alterations seen in arthritic joints of the WT mice were not altered in $Tac1^{-/-}$ and $Tacr1^{-/-}$ mice, while they were significantly reduced in the $Tac4^{-/-}$ and $Tac1^{-/-}/Tac4^{-/-}$ groups.

4. Role of tachykinins in adjuvant-induced IL-1β production in the joints.

The IL-1β concentration in the intact tibiotarsal joint homogenates of all mouse groups was below the detection limit of the ELISA technique. Adjuvant administration induced an approximately 9000 pg/g production of this inflammatory cytokine in WT control mice. In accordance with the histopathological scoring, IL-1β production was significantly lower in the joints of $Tac4^{-/-}$ and $Tac1^{-/-}/Tac4^{-/-}$ double knockout mice compared to WT controls.

Discussion

The present study provides the first evidence that HK-1 increases inflammatory pain in the chronic phase of CFA-induced and plays a predominant role in the development of inflammatory morphological alterations and inflammatory cytokine production arthritis. HK-1 is expressed in the peripheral and central nervous system and has a remarkable selectivity and potency for the NK1 receptors. For decades, SP was the only tachykinin known to be detected by anti-SP antibodies, and a positive readout in a SP radioimmunassay was interpreted as SP immunoreactivity. Since hemokinins and endokinins exhibit structural homology, this consequently results in immunological crossreactivity with anti-SP antibodies. Thus, to date SP and HK-1 cannot be differentiated by radioimmunoassay. It has therefore been suggested, that in several experimental layouts the measured SP-like immunoreactivity reflects both SP and HK-1 contents. Nevertheless, HK-1 might have different binding sites on the NK1 receptors, distinct receptor activation mechanisms and signal transduction pathways compared to SP. Furthermore, Endo and colleagues (2006) have raised the possibility of a presently unidentified proper receptor related to HK-1 on the basis of several actions of hemokinins different from that of SP. Concerning chronic autoimmune/inflammatory diseases in human, a recent publication has suggested that HK-1 may be involved in the pathophysiology of inflammatory bowel diseases, such as ulcerative colitis (Liu et al., 2011), but no data are available on its role in arthritic diseases. However, promising approaches highlight the importance of B cell targeting in arthritis therapy (Chen et al., 2012; Popa et al., 2007). Since

B cells are an important source of HK-1, decreased HK-1 production and release might be an explanation for the efficacy of rituximab, which induces B cell depletion. High expression-level of NK1 receptor mRNA in the synovia of RA patients, and the antinociceptive, oedema and joint destruction reducing effect of the antagonists is well-known (Lam and Ng, 2010; Uematsu et al., 2011). Adjuvant-induced inflammatory mechanical hyperalgesia was significantly and similarly reduced from the 11th day of the experiment in $Tac4^{-/-}$ and $Tacr1^{-/-}$ animals, but not in the other knockouts compared to wildtypes, suggesting that HK-1 induces hyperalgesia through NK1 receptor activation on sensory neurons. Besides peripheral mechanisms at the nerve terminals, central sensitization in the spinal cord also plays a predominant role in this process. Although we do not have a precise explanation for why the hyperalgesic action of HK-1 presumably at the NK1 receptors is not observed when SP and NKA are also missing from the system, some hypothesis can be made: a) SP exerts its hyperalgesic effect in the nociceptive pathway through the NK1 tachykinin receptor, but it is counteracted by NKA acting at NK2 receptors in the central nervous system (Tauer et al., 2012), b) HK-1 and SP act at the same receptors (NK1), but they might have different binding sites, affinities and intinsinc efficacies, as well as distinct activation mechanisms and signalling pathways. When both are removed from the system, the inhibition observed in case of the HK-1 absence, might be counteracted via intracellular molecular mechanisms.

The inflammatory histopathological changes and cytokine concentrations in the joint were also significantly decreased in Tac4 gene-deleted mice, Therefore, a different mechanism seems to mediate the inflammatory functions of HK-1, and a role for a putative HK receptor can be proposed. There are data showing interactions between the tachykinin system and cytokines in rheumatoid arthritis, like reduced substance P release and disease severity observed after the TNFα inhibitor etanercept treatment in RA patients (Origuchi et al., 2011). Although RA is known as a TNFα dominant disease, other interleukines are also involved in chronic joint destruction: IL-1β plays a role in the immune response modulation and osteoclast activation. In contrast to $Tac4^{-/-}$ animals there were no changes in the inflammatory parameters (histopathological score, cytokine concentrations) in Tacr1 knockouts. In summary, we provided the first evidence for inflammatory and nociceptive roles of HK-1 in a mouse model of chronic arthritis. However, the mechanisms of these actions are different and a putative, new tachykinin receptor mediates the inflammatory actions of HK-1.

SUMMARY OF THE NEW FINDINGS PRESENTED IN THE THESIS

1. **We provided here the first evidence that capsaicin-sensitive peptidergic sensory nerves play an important and complex regulatory role in an immune arthritis model of the mouse.** These fibers are able to attenuate inflammatory alterations, in which a mediator role for somatostatin can be suggested. Meanwhile, despite the decreased inflammation, they can induce and/or increase late mechanical hyperalgesia. Based on the present results, further investigations are needed to identify the released neuropeptides which are the most important mediators of these effects in arthritis.

2. The present results show that a PAR2 activating peptide results in the development of a remarkable pain reaction, oedema formation and IL-1β production. **PAR2-induced inflammation and pain can be reduced, certain nociceptive mechanisms abolished by pharmacological inhibition or genetic deletion of the TRPV1 receptor, but these do not affect the cytokine release.** Based on these data, it can be concluded, that in PAR2-medieted acute arthritis, TRPV1 receptors play an important role in the mediation of inflammation and secondary mechanical hyperalgesia.

3. We provided the first evidence for **inflammatory and nociceptive roles of HK-1 in a mouse model of chronic arthritis**. However, the mechanisms of these actions are different: the peripheral inflammatory effects are not NK1 receptor-mediated, but mechanical hyperalgesia involving central sensitization is dependent on NK1 activation. This observation raises the possibility of a presently not identified receptor for HK-1.

Our knowledge about the immunological aspects of RA has extensively increased in the last decade, but the regulatory role of sensory nerves and the complexity of neuro-immune interactions in this condition are still not completely understood, although it is well-known that nervous system is normally involved in immune regulation and homeostasis, and sensory-immune interactions can influence the disease development (McInnes and Schett, 2011). Our results prove that somatostatin released from the sensory nerve ending and PAR2 on the surface of nerves and immune cells, as well as HK-1 from the same cell types play an important role in the modulation and regulation of arthritis. Identification of their targets and the precise signalling pathways might open new perspective for the treatment of arthritis.

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PUBLICATIONS

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Borbély É, Botz B, Bölcskei K, Kenyér T, Kereskai L, Kiss T, Szolcsányi J, Pintér E, Csepregi JZ, Helyes Z (2015). Capsaicin-sensitive sensory nerves exert important anti-inflammatory functions in the serum-transfer mouse model of autoimmune arthritis. *Brain Behav Immun.* 45: 50-9. doi: 10.1016/j.bbi.2014.12.012. (**IF: 6.128**) (50% of the article)

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