THE ROLE OF SENSORY NEUROPEPTIDES IN MOUSE MODELS OF NEUROPATHY AND IMMUNE ARTHRITIS

PhD Thesis

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INTRODUCTION

The significance of peptidergic nerves and TRP receptors in pain and inflammation

Capsaicin has mainly been used in painful or inflammatory conditions for centuries, both topically and ingested. In the 1960s it was discovered to selectively desensitize a sensory neuronal subpopulation (Jancsó 1960), and these neurons are therefore called „capsaicin-sensitive afferents". János Szolcsányi later described that stimulation of these nerves elicits vasodilation and hyperpermeability, which can be blocked by capsaicin pretreatment (Jancsó *et al.* 1967). The receptor responsible for this effect was identified in 1997 and was named Transient Receptor Potential Vanilloid 1 (TRPV1; Caterina *et al.* 1997). TRPV1 is a non-selective cation channel, which serves as a polimodal sensor for various physicochemical stimuli. Besides capsaicin, other vanilloids, such as resiniferatoxin (RTX), can also activate TRPV1. Additionally, it also has endogenous agonists like anandamide, while both bradykinin and prostaglandins are capable of indirectly sensitizing TRPV1 (Szallasi *et al.* 1999). TRPV1 receptors can be found in the dorsal root ganglia, sensory neurons, and thinly myelinated Aδ and C-fibers as well (Caterina *et al.* 1997; Tominaga *et al.* 1998). It plays a central role in hyperalgesia, but chronically it also mediates important anti-inflammatory and analgesic effects (Bölcskei *et al.* 2005). While originally it was thought to be expressed exclusively on neural cells, growing body of evidence supports that TRPV1 is functionally important in various non-neural tissues as well.

Capsaicin-sensitive afferents possess three distinct functions acting as 1) sensory afferents, 2) local and 3) systemic efferents (**Fig. 1.**). The afferent function represents their role in nociception. Meanwhile, proinflammatory peptide mediators are released from the activated peripheral terminals, thereby triggering a neurogenic inflammation (local efferent function; Maggi *et al.* 1988). However, the simultaneously released inibitory neuropeptides exert anti-inflammatory and analgesic effects both locally and in distant parts of the body through the systemic circulation (systemic efferent or „sensocrine" function; Szolcsányi *et al.* 2004). The discovery of the TRPV1 receptor lead to a search for similar receptors conveying the effects of other exogenous irritants and physicochemical painful stimuli. Almost 30 different TRP-superfamily receptors have been identified so far, among which the TRPA1 (Transient Receptor Potential Ankyrin 1) receptor seems to be particularly important for nerve-driven inflammation. It is a sensor for noxious cold, pungent spices like allyl-isothiocyanate (AITC) (found in mustard oil), and it is sensitized by inflammatory mediators such as bradykinin,

similarly to TRPV1. Based on these findings TRPA1 has also been shown to play a central role in nociceptive signaling, neuropathic and inflammatory pain, as well as peripheral neuropeptide release (Nilius *et al.* 2011). Therefore, it was suggested to be a key integrator of neuro-vascular-immune interactions during inflammation (Caceres *et al.* 2009, Pozsgai *et al.* 2010).

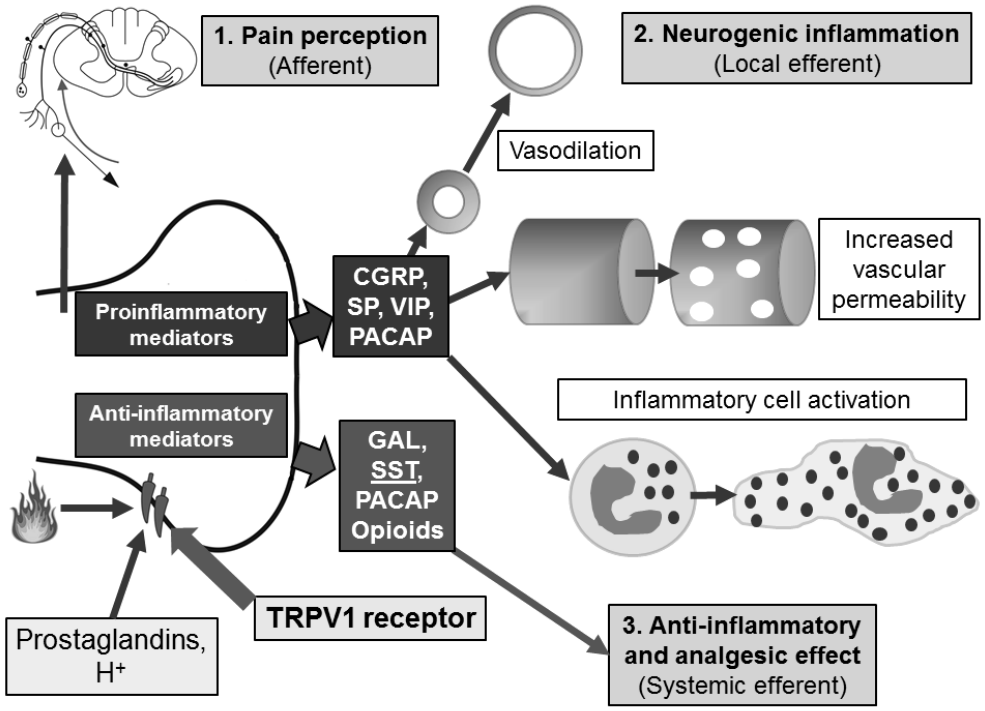

Fig.1. The three distinct effects of the activation of capsaicin-sensitive sensory nerve endings.

Peptide mediators in peripheral pain and inflammation

Sensory neuropeptides are released from the activated capsaicin-sensitive primary afferents during inflammation and nerve injury (Brain 1997). Substance P (SP) and other related peptides, such as Neurokinin A (NKA) and Neurokinin B (NKB) were among the first identified tachykinins. Other important sensory neuropeptides are pituitary adenylate-cyclase activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), somatostatin, endogenous opioids, and galanin. They regulate the vascular tone and inflammatory cell activity, influence pain behavior and altogether promote a systemic response in order to ameliorate the effects of potentially damaging stimuli. Some of them were traditionally classified as proinflammatory/pronociceptive, such as SP, or CGRP, whereas others alleviate and balance the neurogenic response (e.g. somatostatin or galanin). Besides its profound role in nociceptive transmission, the neurogenic component has been implicated in immune-mediated diseases, such as rheumatoid arthritis (RA)

relatively early (Levine *et al.* 1985). Later it has been proven that this phenomenon significantly contributes to the formation of the inflammatory microenvironment by eliciting vasodilation, vascular permeability increase, mast cell degranulation and leukocyte egress, collectively termed neurogenic inflammation (Keeble *et al.* 2004). This has been suggested as a pivotal factor in autoimmune inflammatory conditions (Stangenberg *et al.* 2014).

We have previously observed, that selective desensitization of peptidergic sensory nerves by RTX leads to diminished pain perception, and surprisingly increased inflammation severity in experimental arthritis, asserting a protective role to the TRPV1-expressing sensory terminals (Helyes *et al.* 2004). Thus, our aim was to further characterize, and preferentially attribute these effects to specific mediators released by peptidergic sensory afferents, using translational models of peripheral neuropathy and inflammatory conditions.

PACAP - implications in pain and inflammation

PACAP is a prominent member of the VIP/secretin/glucagon peptide family expressed in a 27 and 38 aminoacid-containing isoforms (PACAP-27 and -38 respectively). PACAP-38 is the predominant form in mammals. It is expressed ubiquitously in the nervous system and various non-neural tissues (blood vessels, immune cells). It is anti-hyperalgesic on the periphery, while pronociceptive in the CNS. Therefore, PACAP has been suggested to play a crucial role in central sensitization and the induction of chronic pain (Vaudry *et al.* 2009). The role of PACAP in inflammation and immunoregulation has been investigated to a lesser extent, however it was suggested to be an important endogenous immunomodulator. Traditionally three G-Protein-Coupled Receptors (GPCR) were considered to be the targets of PACAP. On one of these (PAC_1) PACAP is the sole endogenous agonist, whereas its potency on the $VPAC_1$ and $VPAC_2$ receptors is similar to that of VIP (Laburthe *et al.* 2007). PACAP-27 has been proved to be able to also activate the Formyl Peptide Receptor-Like 1 (FPRL1) as well (Kim *et al.* 2006). The PAC_1 receptor is expressed mainly on neural and smooth muscle cells, whereas the $VPAC_1$/$VPAC_2$ receptors are localized primarily on the DRG, sensory nerve terminals, and inflammatory cells (Vaudry *et al.* 2009). PACAP receptors are also widely distributed in the immune system: PAC_1 receptor is expressed on macrophages and monocytes. $VPAC_1$ was found on lymphocytes, macrophages, and monocytes, whereas the $VPAC_2$ receptor is only expressed on stimulated lymphocytes and macrophages (Delgado *et al.* 2003). The FPRL1 receptor is expressed on leukocytes and lymphocytes (Kim *et al.* 2006).

The functional importance of tacyhkinins

Three tachykinin encoding genes have been cloned up to now (Tac1, Tac3, and Tac4). However, due to post-translational modifications the diversity of the peptides is larger. The first identified Tac1 gene encodes primarily SP and NKA, the Tac3 gene encodes NKB, whereas the Tac4 gene produces hemokinins, the most recently discovered members of the tachykinin family (Page 2005). SP and NKA are expressed in both neural, vascular and immune cells. They elicit diverse effects, such as neurogenic vasodilation and plasma protein extravasation, visceral smooth muscle contraction (Keeble *et al.* 2004), stimulation of lymphocyte proliferation, cytokine production, T cell chemotaxis and neutrophil accumulation. They facilitate the release of histamine and serotonine through mast cell activation, which consequently increases the neuropeptide-release from the sensory nerves through positive feedback (Szallasi *et al.* 1999). Three G-protein coupled tachykinin receptors have been identified: Neurokinin 1, 2, and 3 (NK1; NK2; NK3, respectively; Maggi 1995). Although all tachykinins activate all receptors, SP is considered to be the main endogenous agonist on the NK1, NKA on the NK2, and NKB on the NK3 receptor. However hemokinins are also potent agonists on the NK1 receptor. The NK1 receptor demonstrates ubiquitous expression, however, the highest expression levels were reported in the CNS (Pinto *et al.* 2004).

Galanin Receptor 3 – an emerging target in neurogenic inflammation

Galanin is a sensory neuropeptide that is expressed ubiquitously in the nervous system. Later studies revealed the existence of other closely related peptide mediators such as Galanin-Like Peptide (GALP), and its splice variant alarin, which altogether form the galanin peptide family (Lang *et al.* 2015). The role of galanin has been described in a wide range of physiologic processes, such as nociception, inflammation. Galanin, GALP, and alarin mediate their effects through three different GPCRs (termed GalR1-3). Galanin was found to be a potent agonist on GalR1 and GalR2 but not on GalR3, while GALP has high affinity only towards GalR3 and GalR2 (Webling *et al.* 2012). Both GalR1 and GalR2 are present in abundance throughout the CNS whereas GalR3 expression in the brain is limited. Thus, it was hypothesised that these two receptors mediate galaninergic effects in nociceptive transmission. In non-neural tissues mainly GalR2 and GalR3 are expressed. Due to its limited expression in the CNS, GalR3 received less attention than the other receptors. However, since it is the predominant galaninergic receptor on the periphery the interest for this receptor is growing, especially as recent findings also indicate the presence of other endogenous GalR3 agonists surpassing the potency of galanin (Kim *et al.* 2014).

EXPERIMENTAL MODELS AND METHODS

Animals

The experiments were done on 10-14 week-old male PACAP, Tac1, Tacr1 and GalR3 gene-deficient mice and their respective wildtype counterparts. For the increasing temperature hot-plate test female mice were also used. $PACAP^{-/-}$ mice were generated on the outbred CD1 background (*Osaka University, Japan*). SP and NKA deficient ($Tac1^{-/-}$) (*University of Liverpool, UK*) and NK1 receptor gene-deleted ($Tacr1^{-/-}$) (*University of Bonn, Germany*) mice were generated on the inbred C57Bl/6 background (Helyes *et al.* 2010). $GalR3^{-/-}$ (LEXKO-230) mice were obtained from the European Mouse Mutant Archive.

Partial nerve ligation model of traumatic mononeuropathy

Animals were anesthetized and one-third of the the right common sciatic nerve was unilaterally tightly ligated using an atraumatic suture. (Seltzer *et al.* 1990). Based on our earlier results, this model is both reliable and highly reproducible.

K/BxN serum-transfer model of autoimmune arthritis

The genetically engineered K/BxN mouse strain displays spontaneous polyarthritis characterized by the production of autoantibodies and other inflammatory mediators. Transfer of serum from the K/BxN mice elicits a robust, albeit transient polyarthritis (dominated by neutrophils and macrophages) (Korganow *et al.* 1999). The arthritis was induced by i.p. injecton of the arthritogenic (K/BxN) or control (BxN) serum.

The oxazolone-induced model of atopic contact dermatitis

Repeated cutaneous challenge with the allergenic hapten oxazolone induces an atopic contact dermatitis (ACD)-like delayed type hypersensitivity reaction in mice. This is a primarily Th1 lymphocyte-mediated inflammation with a consequent activation of mast cells and recruitment of neutrophils and mononuclear cells (Petersen *et al.* 2006). Mice were sensitized on two consecutive days by oxazolone smearing on the abdominal skin, and elicitation was performed 6 days later on the ear (Bánvölgyi *et al.* 2005).

Evaluation of mechano- and thermonociception

The mechanonociceptive threshold was measured on the plantar surface of the hindlimb with a dynamic plantar esthesiometer. Thermonociceptive threshold was also determined on the hindlimb using an increasing temperature hot plate.

Assessing motor coordination and grasping ability

Motor functions were studied using an accelerating Rota-Rod device. Arthritic joint dysfunction was also evaluated by the horizontal wire mesh grid test.

Imaging the cutaneous blood flow (laser Doppler scanning)

Microcirculation in the plantar skin of the hindpaw was measured by laser Doppler imaging. Mice were anesthetized, their body temperature was maintained with a controlled heating pad. First, control images of the plantar surfaces of both the operated and the intact hindpaws were taken to establish a solid baseline blood flow. Then freshly prepared AITC (mustard oil) was smeared on the hindlimbs in order to elicit a TRPA1-driven vasodilatory response. The plantar microcirculation was measured for ~60 minutes after AITC application.

Evaluation of arthritis severity and hindpaw edema

The severity of the arthritis including the hyperemia and paw edema were determined daily by the means of semiquantitative clinical scoring of both hindlimbs between 0 and 10 The hindpaw volume increase was evaluated by plethysmometry.

In vivo fluorescence imaging of vascular leakage

We dissolved the fluorescent marker indocyanine-green (ICG) using the non-ionic surfactant Kolliphor HS 15 into a micellar solution (Kirchherr *et al.* 2009), which limits the blood clearance of the dye to sites affected by increased vascular permeability. This formula was injected i.v. to anesthetized mice which were thereafter imaged with an IVIS Lumina II optical imager. During some later studies we employed IR-676 instead of ICG, a more suitable fluorophore enabling lower dosage and more sensitive detection. Standardized regions of interests (ROIs) were drawn around the ankle joints, and the fluorescence intensity was quantified within these regions.

Investigation of free radical production *in vivo*

Luminol is a chemiluminescent compound, which can be utilised to detect the activity of the myeloperoxidase (MPO) enzyme *in vivo*. The reaction occurs mainly in the presence of H_2O_2, which is primarily produced by the phagosomal MPO of activated neutrophils. Thus, it is an indirect, but highly selective tool to investigate MPO-activity in a noninvasive manner (Gross *et al.* 2009). Mice received luminol i.p. and were imaged 10 min later with the IVIS

Lumina II. ROIs of identical size were applied around the joints, and luminescence was quantified.

Lucigenin is also a chemiluminescent compound that reacts with the extracellular superoxide produced primarily by the NADPH-oxidase enzyme of active macrophages in inflammation (Tseng *et al.* 2012). Lucigenin solution was injected i.p., and bioluminescence imaging was performed as described earlier and was also evaluated in a similar manner.

In vivo micro-computed tomography (micro-CT) analysis

Micro-CT imaging was performed using the same mice at every time-point to minimize inter-individual differences. The ankles were repeatedly scanned by a SkyScan 1176 micro-CT. After the reconstruction, the bone structure was analyzed. ROIs were drawn around the periarticular region of the tibia and fibula, as well as the tibiotarsal and tarsometatarsal joints based on anatomical landmarks. In the ROIs bone volume and bone surface were calculated and expressed as % of the standardized total volume of the ROI.

Evaluation of ear edema

Ear thickness was measured with a microcaliper on the pinna of the ear. Measurements were taken from the same relative position before and after oxazolone-treatment.

Histology

$PACAP^{+/+}$ and $PACAP^{-/-}$ mice were sacrificed on day 4 and 28 $GalR3^{+/+}$ and $GalR3^{-/-}$ mice on the 14^{th} day under deep anesthesia. The ankle joints were fixed in paraformaldehyde, decalcified, dehydrated and embedded in paraffin. Sections were made and stained with safranine O and semiquantitatively evaluated by a pathologist. Synovial cell proliferation and mononuclear cell infiltration were scored (0-normal state, 3-maximal severity). The ears of $GalR3^{+/+}$ and $GalR3^{-/-}$ mice were taken upon completion of the *in vivo* experimental readouts 48 hours post oxazolone-challenge and stained with hematoxylin-eosin (HE).

Statistical analysis

Statistical evaluation was performed by the GrapPad Prism® software package. All data were expressed as means±SEM. The majority of the functional results was evaluated by repeated measures or two-way ANOVA. Simple comparisons were made using unpaired t-test. The Kaplan-Meier curves were analyzed by logrank test. $P<0.05$ was considered significant.

AIMS

In the present work we examined the pathophysiological relevance of three distinct peptide mediator groups. Our aims were the following:

1. **To investigate the role of PACAP and Tac1-gene derived tachykinins, particularly SP and the NK1 receptor in a mouse model of traumatic neuropathy.** Both PACAP and SP/NKA are prominent representatives of sensory neuropeptides, and have been implicated in both peripheral and central pain conditions. PACAP has been shown to play a role in pain, while tachykinins, but most importantly the NK1 receptor were until recently also considered to be a promising target for analgesic drug candidates. Here we aimed to evaluate their effect on mechanical hyperalgesia, motor coordination, and peripheral vasoregulation under normal and neuropathic conditions.

2. **To analyze the effect of PACAP, Tac1-gene derived tachykinins, and the NK1 receptor in a murine rheumatoid arthritis model.** Since PACAP is not only a nerve-driven mediator, but it is also expressed by numerous non-neural cells, we employed a broad range of readouts to address its function in nociception, inflammation, and neurovascular interactions. The fact that this peptide has a distinct specific receptor (PAC_1) renders it particularly important in context of potential drug developmental perspectives. Tachykinins, but particularly SP were among the earliest neuropeptide candidates implicated in RA, however later results proved to be contradictory, some results supporting, while others opposing their role in nerve-driven inflammation.

3. **To examine the role of the galanin receptor 3 in the pathophysiology of inflammation.** Galanin is widely acclaimed due to its anti-inflammatory and analgesic properties. However, little is known about its downstream signaling and targets on a receptorial level. Due to its low expression in neural tissues GalR3 received particularly little attention until recently. Its profound presence in the periphery, especially around blood vessels indirectly suggested, it might be among the receptors responsible for the manifold anti-inflammatory effects of galanin-family peptides. Here we examined the role of GalR3 in inflammatory disease models for the first time, using translational murine models of RA and atopic contact dermatitis.

RESULTS AND DISCUSSION

1. The role of PACAP, Tac1 gene-derived tachykinins, and NK1 receptor in a traumatic mononeuropathy mouse model

Since PACAP and tachykinins are colocalized in peptidergic sensory nerve terminals, our main goal was to investigate their roles using the Seltzer-model (unilateral partial sciatic nerve ligation) of traumatic mononeuropathy and global PACAP, SP/NKA, and NK1 receptor knockout mouse strains. Additionally, the potential involvement of the NK1 receptor, the main target of SP, was also addressed. This was because results obtained using solely Tac1 gene-deficient animals leave completely unexplained whether the possible differences in the parameters investigated are due to the lack of SP or NKA.

Mechanical hyperalgesia

Tight ligation of one-third of the sciatic nerve induced a significant decrease of the threshold of the affected hindpaw in wildtype animals. In comparison, neuropathic mechanical hyperalgesia was negligible in the $PACAP^{-/-}$ group. SP/NKA or NK1 receptor deficiency did not influence the mechanonociceptive threshold during the whole study.

Motor coordination and performance

The basal motor performance on the accelerating Rota-Rod was significantly worse in both the $PACAP^{-/-}$ and $Tac1^{-/-}$ groups compared to respective wildtypes. In contrast, deletion of the NK1 receptor did not influence motor coordination. The partial sciatic nerve ligation did not affect performance in any of the groups.

Cutaneous blood flow of the hindpaw and neurogenic vasodilation

After AITC-challenge a significantly lower perfusion was detected in the $PACAP^{-/-}$ group from the 30^{th} minute onwards. In the $Tac1^{-/-}$ and $Tacr1^{-/-}$ groups the basal cutaneous microcirculation was significantly lower on both limbs compared to C57Bl/6 wildtypes but the response elicited by AITC was similar in knockouts

Our results provide evidence that: 1) PACAP is a crucial mediator of neuropathic hyperalgesia. 2) Under normal conditions both PACAP and Tac1 gene-derived tachykinins play an important role in motor coordination. 3) Tachykinins regulate the basal cutaneous microcirculation via NK1 receptor activation, whereas PACAP is involved in neurogenic vasodilation. 4) Partial ligation of the sciatic nerve, which is a widely used traumatic mononeuropathy model, induces purely sensory neuropathy (mechanical hyperalgesia) without affecting the motor and vascular functions. Although the sciatic nerve contains sensory, motor and autonomic fibers, partial ligation is affecting exclusively the sensory functions.

2. The role of PACAP, Tac1 gene-derived tachykinins, and NK1 receptor in a murine model of autoimmune arthritis

Based on the prior results, we decided to investigate the role of PACAP, Tac1-gene derived tachykinins, and the NK1 receptor using global gene-deficient mice in the K/BxN serum-transfer model of autoimmune arthritis, which relies at least partially on neurogenic mediators and a functioning peripheral innervation (Stangenberg *et al.* 2014). Thus, it provides a state-of-the-art workhorse model for studying the effect of neuro-immune and neuro-vascular interactions in a complex, disease-mimicking experimental setup. First we aimed to establish the overall effect of these peptidergic mediators using functional readouts. Promising candidates would be then further interrogated using more elaborate methods, placing special emphasis on the vascular phase, as this is the most important parameter influenced by neurogenic messengers.

Arthritic mechanical and thermal hyperalgesia

A significant mechanical hyperalgesia developed after arthritis induction in wildtypes, which was absent in the arthritic PACAP gene-deficient mice. Thermal hyperalgesia was found to be absent in this model of autoimmune arthritis. In the experiments involving Tac1 and Tacr1 gene-deficient mice, the arthritic mechanical hyperalgesia developed similarly to their wildtype controls, with no observable differences in the early or late phase of the disease.

Hindlimb edema and disease severity

The hindpaw volume was markedly reduced in PACAP$^{-/-}$ mice. The edema was not only significantly smaller, but the kinetics was also slower. Semiquantitative clinical scoring of edema and hyperemia yielded a comparable outcome, PACAP$^{-/-}$ mice had overall significantly lower arthritis severity scores. In contrast, neither the Tac1, nor the Tacr1 gene-deficient mice displayed any difference regarding disease severity, or hindlimb edema compared to the C57Bl/6 wildtype mice.

Microvascular plasma leakage in the inflamed hindlimbs

Two days after the induction of arthritis the accumulation of ICG increased remarkably in the ankle joints of wildtypes both immediately after injection and 1 hour later, indicating hyperemia and plasma leakage. In contrast, in PACAP-deficient animals, the rise was significantly less pronounced.

Alteration of grasping ability and motor coordination

The horizontal wire grid grip-test revealed an abrupt decrease of grasping ability in wildtypes, but not in PACAP$^{-/-}$ animals. Motor performance on the Rota-Rod gradually improved in all groups during the experiment demonstrating learning. Therefore it was concluded that the Rota-Rod test is inadequate to measure functional incapacitance in this model. The performance of Tac1 and Tacr1 gene-deficient mice proved to be indifferent from their wildtype controls.

Neutrophil-derived MPO-activity

Bioluminescence imaging showed that MPO-activity in the inflamed ankle joints peaked in the hyperacute phase of the disease, reaching its maximum on day 1 and gradually decreasing thereafter. In PACAP$^{-/-}$ mice early MPO-activity was significantly smaller, but by day 4 it became significantly greater.

Macrophage-derived superoxide production

In PACAP$^{+/+}$ mice extracellular superoxide production increased steadily upon arthritis induction. Superoxide generation in PACAP$^{-/-}$ mice remained significantly lower than in wildtypes.

Micro-CT imaging of bone structural changes

The control micro-CT-scans of intact mice revealed that PACAP$^{-/-}$ animals have different bone architecture even under normal condition. Their Bone Volume/Total Volume (BV/TV) ratio was consistently, although not significantly higher. Arthritis did not remarkably alter the bone structure in neither PACAP$^{+/+}$ nor PACAP$^{-/-}$ mice in the region of the ankle joint. In contrast, in PACAP$^{-/-}$ mice it induced extensive, progressive osteophyte formation in the periarticular region of the tibia and fibula. These bone spurs turned into compact, dense bone leading to a prominent, significant increase of bone mass.

Histopathologic alterations in the ankle joints

Four days after arthritis induction there were prominent changes in the wildtype group: 1) Irregular cartilage-bone border, 2) Enlarged synovium infiltrated with inflammatory cells 3) Massive infiltration of the periarticular connective tissue by immune cells and formation of mononuclear cell aggregates. Synovial hyperplasia, but not cellular infiltration was greater in PACAP$^{-/-}$ mice. By day 28 these acute inflammatory signs decreased, but the cartilage-bone border became more irregular and the cartilage width remarkably decreased in both groups.

The primary outcome of our study is that we provided the first evidence for a surprisingly pleiotropic effect of PACAP on various characteristics of a RA disease model. According to our results PACAP increases vasodilation, plasma leakage, inflammatory cell accumulation, hyperalgesia, functional loss and ROS generation, while abrogating late phase inflammatory cell activity, synovial proliferation and pathological bone formation. Contrarily, our results about the role of SP/NKA and the NK1 receptor do not support their involvement in this model of autoimmune arthritis, This is in good agreement with our earlier negative results discussed extensively in the previous chapter. Secondly, this model proved to be appropriate to investigate several early and late phase characteristics of RA using *in vivo* non-invasive imaging modalities. We adopted and modified structural and optical imaging techniques, as

well as self-controlled experimental paradigm that help to identify key pathophysiological mechanisms in inflammatory and degenerative joint diseases.

3. The role of Galanin receptor 3 in a mouse model of autoimmune arthritis and atopic contact dermatitis

It was previously demonstrated that galanin has anti-inflammatory, and primarily antiedema effects in rodent inflammation models (Lang *et al.* 2011). As the main galaninergic receptor on the periphery GalR3 was found to be a promising candidate among the presumed mediators of this antiedema effect on a receptorial level (Schmidhuber *et al.* 2009). On the basis of these prior results we aimed to investigate the potential involvement of GalR3 activation in mouse models of immune-mediated inflammatory diseases, placing special emphasis on the edema formation and inflammatory cell activity. We have selected the K/BxN serum transfer model of RA, and the oxazolone-model of ACD for this purpose.

Arthritis severity mechanical hyperalgesia and change of joint function

Clinical severity scoring and plethysmometry both indicated an accelerated disease induction in GalR3$^{-/-}$ mice until the 6th day following arthritis induction. However wildtype mice reached only slightly lower peak values. A considerable and similar mechanonociceptive threshold drop was observed in both groups with no observable differences between wildtypes and knockouts. Joint function of the mice decreased steadily without any differences between groups.

Plasma leakage and MPO-activity in the arthritic hindlimbs

The plasma leakage measured by ICG was found to be greater in the paws of GalR3$^{-/-}$ mice on day 1 than in arthritic wildtypes, however this significant difference vanished by day 5. The MPO-activity of activated neutrophils peaked in both groups during the hyperacute phase of the disease, with no significant difference between the study groups.

Histology of arthritic ankle joints

Structural interrogation of joint samples taken for histology 14 days after K/BxN serum-challenge revealed mainly alterations characteristic of chronic arthritis. The synovial lining was thickened, and the adipocyte-rich connective tissue was replaced with a dense fibroblastic scar tissue, with a limited presence of inflammatory cells. No difference was observed in these respects between the study group, in agreement with the absent functional difference at this stage of the disease.

Ear edema, MPO-activity, plasma extravasation, and histology

A considerable and similar ear thickness increase could be observed following oxazolone-challenge in both groups, which peaked at 24 h. MPO activity and microvessel permeability also increased dramatically after 24 hours, however no difference could be observed between the wildtypes and knockouts with any of the readouts employed. The histology of the ear lobes by HE staining confirmed the considerable thickening of the connective tissues, inflammatory cell infiltration, the dilation of the ear microvasculature, and the presence of a considerable amount of exsudate in both groups between the ear cartilage and the subcutis.

Our results suggest that GalR3 is a mediator of endogenous protective mechanisms in inflammatory arthritis initiated by neurogenic vascular responses. However taking into consideration the most recent forthcomings, the observed effect in our model is not necessarily galanin-mediated (Kim *et al.* 2014). The absent difference in the neutrophil-derived MPO-activity highlights that the heightened inflammatory reaction in knockouts is caused entirely by the increased vascular leakiness, and not by GalR3-mediated effects on the polymorphonuclear cells. The absence of difference in the ACD model may suggest that the GalR3-mediated antiedema effect is only encountered if the disease model in question is triggered by neurogenic vasodilation in the early phase. In our point of view the way of the formation of the inflammatory microenvironment is a key difference between these two models, in the K/BxN serum-transfer model there is a very early neurogenic permeability increase of the microvessels around the joints (Stangenberg *et al.* 2014), which enables inflammatory cell recruitment. In contrast in the cell-mediated oxazolone-model is initiated by the locally present dendritic and T cells, with consequent permeability increase and leukocyte influx (Petersen *et al.* 2006). Thus the absence of the antiedema effect of galanin through GalR3 will presumably not have a noticeable impact on the disease course.

SUMMARY AND MAIN CONCLUSIONS

In the present study we have provided a comprehensive picture on the effects of several peptidergic mediators in pain and inflammation, where the main emphasis was placed on the disease development *in vivo*.

1. PACAP is pronociceptive in not only peripheral traumatic neuropathy but also in autoimmune arthritis.
2. PACAP and SP/NKA are both involved in motor coordination.
3. PACAP is a key mediator of neurogenic vasodilation, while SP/NKA and the NK1 receptor have a crucial role in the maintenance of normal vascular tone.
4. PACAP affects arthritis development in a complex manner. It increases hyperemia, plasma leakage and edema.
5. PACAP increases early neutrophil-accumulation by facilitating their extravasation from the vessels, but it diminishes their function in the later phase. In contrast, it promotes macrophage-activity and ROS production, but limits inflammation-induced pathological bone neoformation and synovial degeneration.
6. Mice deficient in SP/NKA or the NK1 receptor develop the same degree of arthritis undisturbedly, suggesting that the pathways represented by these mediators can be either bypassed, or that they do not constitute a pivotal mechanism in the development of the serum transfer arthritis.
7. GalR3 mediates important antiedema functions in during nerve-driven inflammation but it is not involved in arthritic pain signaling and does not directly affect inflammatory cell functions. Thus, endogenous agonists of GalR3 might be considered asprotective or regulatory factors which balance and limit the extent of the vascular phase of neurogenic inflammation.

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