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

Doctoral (PhD) Thesis

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PÉCS

2015

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LIST OF ABBREVIATIONS

(in alphabetical order)

ACD: Atopic contact dermatitis

AITC: Allyl-isothiocyanate (the active ingredient of mustard oil)

ANOVA: Analysis of variance

cAMP: Cyclic adenosine-monophosphate

CFA: Complete Freund's adjuvant

CGRP: Calcitonin gene-related peptide

CNS: Central nervous system

DRG: Dorsal root ganglion

FLS: Fibroblast like synoviocyte

FPRL1: Formyl peptide receptor-like 1

GALP: Galanin-like peptide

GalR1-3: Galanin Receptor 1-3

GPCR: G-protein coupled receptor

HE: Hematoxylin-eosin

ICG: Indocyanine green

IL: Interleukin

i.p.: Intraperitoneal

i.v.: Intravenous

LPS: Lipopolysaccharide

MMP: Matrix metalloproteinase

MPO: Myeloperoxidase

NK1-3: Neurokinin receptors (for the genes see Tacr1-4)

NKA: Neurokinin A

NKB: Neurokinin B

PACAP: Pituitary adenylate cyclase-activating polypeptide

PAC_1: The specific receptor of PACAP

PAG: Periaqueductal gray matter

PBS: Phosphate buffered saline

PNS: Peripheral nervous system

RA: Rheumatoid arthritis

RBC: Red blood cell

ROI: Region of interest

ROS: Reactive oxygen species

RTX: Resiniferatoxin

SP: Substance P

Tac1-4: Tachykinin-encoding genes

Tacr1: Tachykinin receptor gene 1

TNF: Tumor necrosis factor

TRPA1: Transient receptor potential ankyrin 1 receptor

TRPV1-4: Transient receptor potential vanilloid 1-4 receptor

$VPAC_{1-2}$: The shared receptors of PACAP and VIP

VIP: Vasoactive intestinal polypeptide

1. OVERALL BACKGROUND AND SIGNIFICANCE OF OUR WORK

1.1. The early pioneers of capsaicin research

Historically, the afferent peripheral nerves were regarded primarily as the transmitters of nociceptive and tactile stimuli from the periphery into the central nervous system (CNS). They were clearly separated from the efferent nerves held responsible for the regulation of e.g. motor and vegetative functions. However, apart from these functionally distinct groups, there is a unique subtype: capsaicin sensitive peptidergic afferents, which display both afferent and efferent functions. They can independently release peptidergic mediators in response to endogenous ligands or noxious stimuli without the involvement of higher neural complexes (Szolcsányi 2004). These secondary messengers then exert diverse effects on other afferents, immune cell subsets, and blood vessels.

I would like to briefly recapitulate some aspects of capsaicinergic afferents, and the rich history of capsaicin-research, which predated neuropeptide research by more than a century. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the pungent agent present in abundance in the *Capsicum* plant genus. Due to its well-known effect it has been a reliable part of the apothecary ingredients for centuries (*Tinctura Capsici*), and was used mainly in painful or inflammatory conditions, both topically and ingested. Endre Hőgyes was among the earliest pioneers who studied capsaicin from a pharmacological viewpoint in the 1870s, and concluded that its pungency derives from its excitatory effects on sensory nerves (Hőgyes 1878). This important discovery was followed by nearly a century of negligence, as the next important step was only taken in the 1960s. The physiologist Miklós Jancsó recognized that capsaicin is much more than just a pungent nerve irritant, and upon repeated administration it elicits sensory desensitization and consequent lasting analgesia. Through further tedious work it was deciphered that not all afferents, but only a distinct subset is targeted by capsaicin-desensitization, which can be also excited by noxious heat (Jancsó 1960). Upon the early death of Prof. Jancsó, János Szolcsányi continued to unravel these mechanisms, and described that electrical stimulation of the

sensory nerve branches elicits vasodilation, hyperpermeability, and consequent plasma protein extravasation in the innervated skin area, and more importantly this effect can be blocked by capsaicin pretreatment. This implied that these afferents topically release some kind of proinflammatory mediators (Jancsó *et al.* 1967). It was found more than a decade later that capsaicin evokes the release of the neuropeptide Substance P (SP) from primary sensory afferents (Jessell *et al.* 1978), which facilitated further research on the field. During the following decades our understanding improved vastly about the physiology of capsaicin-sensitive afferents, and novel mediators and receptors were discovered at an unprecedented pace.

Before moving on to the discussion of later proceedings, we have to emphasize however that the main focus of these experiments was not on capsaicin, as it was only employed as a selective excitatory compound to interrogate the functional importance of these afferents. As we will see later peptidergic nerves can be excited by a plethora of exogenous and endogenous compounds, some of which activate different pathways than capsaicin. Thus, the term capsaicin-sensitive afferent is used here only in a historical context, referring to a particular subset of peptidergic nocicetive terminals which possess characteristic functions.

1.2. The discovery of the endogenous receptor of capsaicin

The primary question (and a source of heated debate) remained for decades whether capsaicin has a distinct endogenous target, or if it exerts its effect via unspecific influence on the cellular homestasis (as late as the early 1990s those, who attributed its effects to the altered membrane fluidity and the formation of artificial membrane „pores” by the elongated amphiphilic molecule formed a majority). János Szolcsányi already envisioned in the 1970s, that capsaicin may be an agonist on a receptor (Szolcsányi 1975, 1976). Later it was proved, that the compound opens a cation channel in the membrane of sensory neurons (Bevan *et al.* 1990). An important milestone came in 1997, as the receptor named Transient Receptor Potential Vanilloid 1 (TRPV1) was identified (Caterina *et al.* 1997). The receptor is phylogenetically highly conserved, as human TRPV1 demonstrates 92% homology to the structure of

the rat receptor (Hayes *et al.* 2000; McIntyre *et al.* 2001). TRPV1 receptor is a membrane-bound nonselective cation channel, which serves as a polimodal sensor for various physicochemical stimuli, such as painful heat (>43ºC), or high proton concentration (pH <6) (Tominaga *et al.* 1998) (**Fig. 1**).

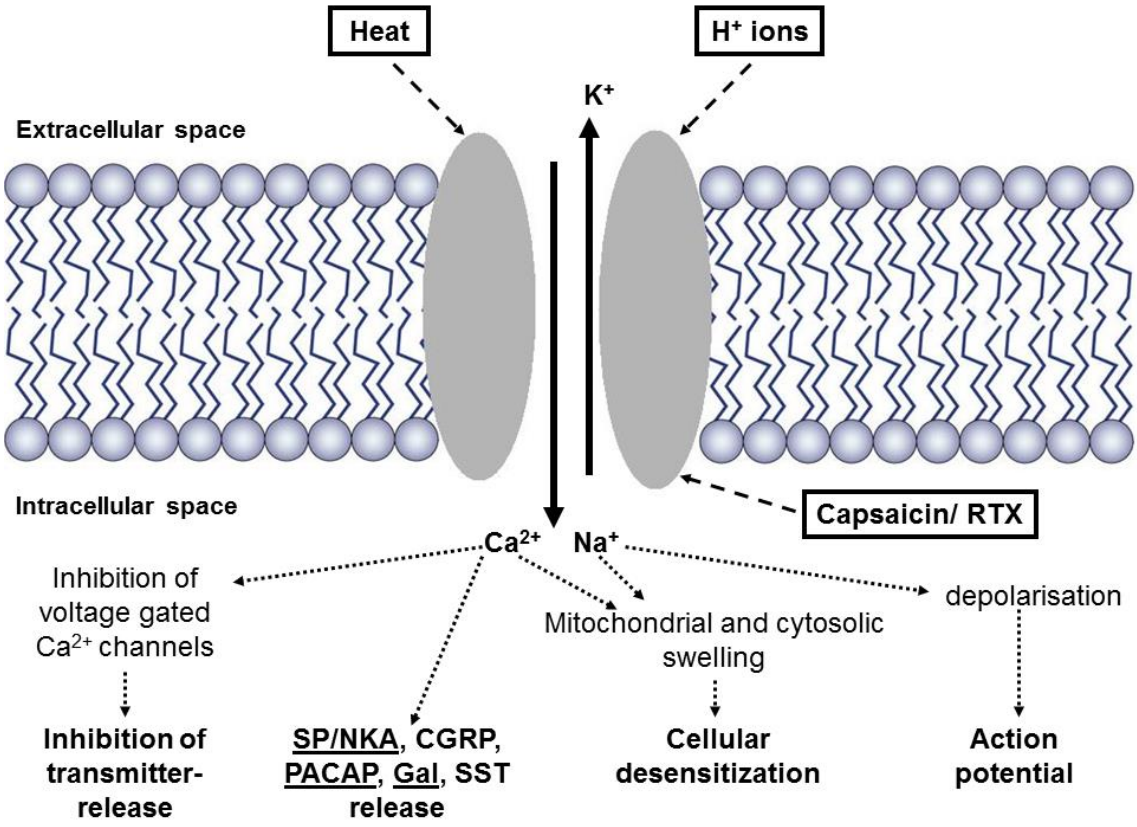

Fig. 1. Endo- and exogenous activators of the TRPV1 channel, and the effects of the receptor activation on cellular level (CGRP-Calcitonin gene-related peptide, PACAP-Pituitary adenylate cyclase-activating polypeptide, Gal-galanin, SST-somatostatin).

Besides capsaicin, other vanilloid compounds, such as resiniferatoxin (RTX), piperin (derived from black pepper), zingeron (from ginger), or eugenol (from clove) can also activate TRPV1. Bacterial lipopolysaccharide (LPS) indirectly sensititizes TRPV1 and potentiates capsaicin-evoked ion currents (Diogenes *et al.* 2011). Additionally, it also has endogenous agonists like N-arachidonoil-ethanol-amin (a.k.a anandamide) (Caterina *et al.* 1997), 12-hydroxy-eikosatetraenic acid (12-HPETE) and N-arachidonoil-dopamine (NADA). Both bradykinin and prostaglandins, which are pleiotropic and almost omnipresent proinflammatory and pronociceptive mediators are capable of indirectly sensitizing TRPV1 (Szallasi *et al.* 1999; Chuang *et al.* 2001, Shin *et al.* 2002). TRPV1 receptors can be found in the dorsal root ganglia (DRG), trigeminal

ganglia, sensory neurons, and thinly myelinated Aδ and C-fibers as well (Caterina *et al.* 1997; Tominaga *et al.* 1998). Therefore TRPV1 plays a central role in thermal hyperalgesia, highlighted by the impaired thermonociception of TRPV1 gene-deficient mice (Caterina *et al.* 2000). However, other investigations also revealed that in models of chronic pain the hyperalgesia is greater in TRPV1 knockout mice, while anti-inflammatory neuropeptide levels are diminished. This suggests that chronically TRPV1 mediates important anti-inflammatory and analgesic effects (Bölcskei *et al.* 2005). While originally it was thought to be expressed exclusively on neural cells, growing evidence supports that TRPV1 is present on various non-neural cells, including keratinocytes (Denda *et al.* 2001), mast cells (Stander *et al.* 2004), lymphocytes (Saunders *et al.* 2007, Bertin *et al.* 2014), and vascular smooth muscle cells (Kark et al. 2008).

The activation of the receptor results in Na^+ and Ca^{2+} influx, followed by K^+ efflux. Especially the Ca^{2+} inflow is important, as it elicits the release of sensory neuropeptides from the nerve terminal. If an ultrapotent agonist (e.g. capsaicin or RTX) is present, this can lead to a prolonged cation imbalance resulting in the swelling of both the mitochondria and the cytosol, and consequently decreased cellular viability (Szallasi *et al.* 1999, Vylicky *et al.* 2008, Anand *et al.* 2011). This exerts a lasting effect on the functions of the affected cells, and this is also what explains the chronic desensitization elicited by high-dosage capsaicin or RTX pretreatment (Szolcsányi 1977). It was also verified recently, that this desensitization does not affect non-neural TRPV1 expression, thus potential extraneural effects of the ion channel remain undisturbed (Kun *et al.* 2012). Since desensitization selectively defunctionalizes TRPV1-expressing sensory terminals while leaving all the other sensory modalities intact, it has proved to be a valuable tool to investigate the function of capsaicin sensitive sensory nerves (Bevan *et al.* 1990; Szolcsányi 1993; Helyes *et al.* 2003). Furthermore, as proven by the recent approval of the 8% capsaicin-containing transdermal patch (Qutenza®) for postherpetic and HIV-associated neuropathic pain, this selective desensitization also represents a pharmacotherapeutical approach of clinical relevance (Anand *et al.* 2011).

1.3. The triple function of capsaicin sensitive sensory terminals

TRPV1/TRPA1-expressing afferents possess three distinct functions acting as 1) sensory afferents, 2) local and 3) systemic efferents (**Fig. 2**). The afferent function represents their role in nociceptive transmission from the periphery to the CNS resulting in pain perception. However, upon excitation these terminals also release numerous proinflammatory peptide mediators resulting in neurogenic inflammation which constitutes their local efferent function (Szolcsányi 1984a,b; Maggi *et al.* 1988). In contrast, some of the other mediators released either simultaneously or later (e.g. somatostatin) exert systemic anti-inflammatory and analgesic effects, thus representing the systemic efferent functions (Szolcsányi *et al.* 1987; Szolcsányi *et al.* 1988, Helyes *et al.* 2000, 2004, Bölcskei *et al.* 2005), which has also been termed as sensocrine function (Szolcsányi *et al.* 2004).

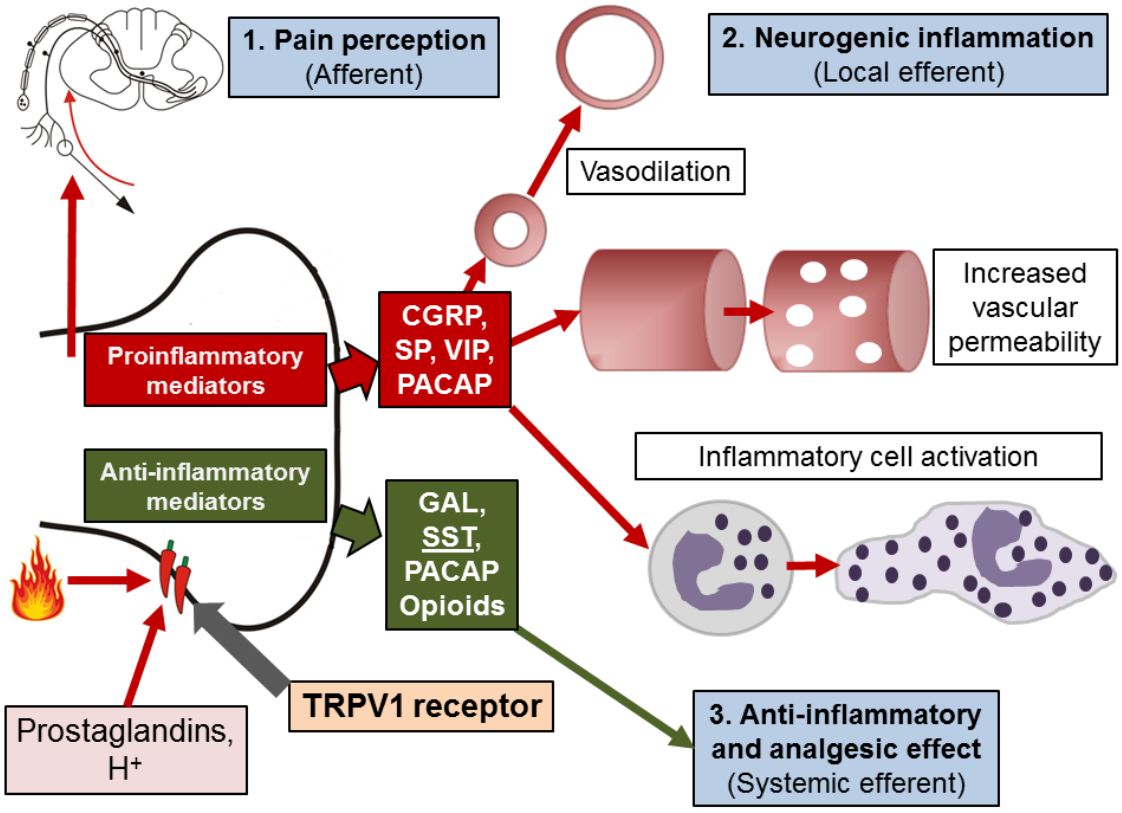

Fig. 2. The three distinct effects of the activation of capsaicin-sensitive sensory nerve endings

1.4. Other TRP receptors involved in pain and inflammation

The discovery of the TRPV1 receptor and its manifold important effects lead to a search for similar receptors conveying the effects of other exogenous irritants and physicochemical painful stimuli. As a result by now almost 30 different TRP-superfamily receptors are known. However, according to our current knowledge not all of these are related to pain or inflammation. Most of these receptors are nonselective membrane cation channels (Nilius *et al.* 2011). Here we only discuss the most important receptors other than TRPV1.

Within the TRPV family TRPV2-TRPV4 ion channels are all heat-activatable similarly to TRPV1, but they are also activated by endogenous (e.g. growth factors) and exogenous ligands (e.g. camphor on TRPV3) and mechanical stress (mainly TRPV2 and TRPV4) (Nilius *et al.* 2007). However their physiological relevance remains poorly understood.

Among the TRPM (melastatin) family the TRPM8 receptor received by far the most attention, as it is not only activated by noxious cold, but it is also the receptor responsible for the cold sensation elicited by menthol or icilin (McKemy *et al.* 2002, Peier *et al.* 2002). Some other TRPM ion channels are sensitive to noxious heat, mechanical stress, or even free radicals (Nilius *et al.* 2007).

The currenty lone member of the TRPA (ankyrin) family is the TRPA1 receptor (Jordt *et al.* 2004) for which there is a constantly growing interest due to its interesting properties. It was repeatedly claimed to be a sensor of noxious cold (Story *et al.* 2003), however knockout mouse studies reported unaltered cold sensation (Bautista *et al.* 2006). Nevertheless, TRPA1 is not the sole presumed endogenous cold sensor, thus these findings not necessarily contradict its involvement in the phenomenon (Reid 2005). TRPA1 has been shown to be the receptor responsible for the pungency of allyl-isothiocyanate (AITC) (found in mustard oil), allicin (the active ingredient of garlic) (Bautista *et al.* 2005), and cinnamaldehyde (present in cinnamon). Chemical irritants such as acrolein (the acting component of tear gas), hydrogen-sulfide (Andersson *et al.* 2012) and psychoactive compounds such as Delta(9)-tetrahydrocannabinol (found in marijuana) also activate TRPA1. It is also sensitized by endogenous inflammatory mediators such as bradykinin (Bandell *et al.* 2004, Jordt *et al.* 2004, Bautista *et al.* 2006) similarly to TRPV1. Based on these findings TRPA1

activation along TRPV1 has been shown to play a central role in nociceptive signaling, neuropathic and inflammatory pain (McMahon *et al.* 2006, Dai *et al.* 2007, Macpherson *et al.* 2007, McNamara *et al.* 2007, Koivisto *et al.* 2012), moreover it is also involved in the neuropeptide release culminating in neurogenic inflammation (Trevisani *et al.* 2007), suggesting TRPA1 as a key integrator of neuro-immune interactions during inflammation (Caceres *et al.* 2009). These results are further corroborated by the profound vascular effects of TRPA1 activation, resulting in vessel relaxation and increased blood flow (Pozsgai *et al.* 2010). TRPA1 is regarded as an at least equally important element of neurogenic vasodilation by eliciting the release of vasoactive neuropeptides (Earley 2012). A recent investigation unraveled that neurogenic inflammation induced by the bacterial products such as LPS is also mediated at least partially via TRPA1 present on nociceptive sensory neurons (Meseguer *et al.* 2014).

1.5. An overview of the released peptide mediators

Sensory neuropeptides are released form the capsaicin-sensitive primary afferents following activation of these terminals by stimuli of diverse origin (inflammation, nerve injury etc.) (Brain 1997). The most prominent member of this group is SP, the first of these mediators that became known to mankind in 1931 (Hökfelt 2001). Mainly in the 1970-90s an impressive number of other neuropeptides were described, and novel, less widely expressed members of this group are still continuously identified (Mirabeau *et al.* 2007). Among the first known neuropeptides were the SP-related other mediators, such as Neurokinin A (NKA) and Neurokinin B (NKB) (Regoli *et al.* 1994), which altogether formed the group of tachykinins. Other important sensory neuropeptides are pituitary adenylate-cyclase activating polypeptide (PACAP) (Miyata *et al.* 1989), vasoactive intestinal polypeptide (VIP) (Said *et al.* 1970), calcitonin gene-related peptide (CGRP) (Rosenfeld *et al.* 1983), somatostatin (Krulich *et al.* 1968), endogenous opioids (Hughes *et al.* 1975), and galanin (Tatemoto *et al.* 1983). These peptide mediators exert diverse effects. 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 these messengers were traditionally classified as

proinflammatory/pronociceptive, such as SP, or CGRP, whereas others were held responsible for alleviating and balancing the neurogenic response by their antiedema and antinociceptive properties (e.g. somatostatin or galanin).

Besides its profound role in peripheral nociceptive transmission, the neurogenic component has been implicated in immune-mediated disease conditions such as rheumatoid arthritis (RA) relatively early (Levine *et al.* 1985, Bozic *et al.* 1996, Brogden *et al.* 2005). Later and recent results corroborated that the release of these messengers from the afferents constitutes an integral part of numerous inflammatory diseases. This phenomenon can contribute significantly to the formation of the inflammatory microenvironment by eliciting vasodilation, vascular permeability increase, mast cell degranulation, and leukocyte egress. The above outlined process has been collectively termed neurogenic inflammation (Holzer *et al.* 1998, Steinman 2004, Keeble *et al.* 2004). Site-specific local production of these nerve-driven mediators predates the later pathologic changes, and predetermines the areas affected by the subsequently developing disease condition (Binstadt *et al.* 2006). Thus the neurogenic component 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 nerve endings by RTX leads to diminished pain perception, and surprisingly increased inflammation severity in the adjuvant-induced arthritis model, 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.

Our work focused on traditionally proinflammatory/pronociceptive mediators (SP/NKA, and their receptor), novel members with less clearly defined overall role (PACAP), and largely uncharacterized receptors of known anti-inflammatory mediators (GalR3) shedding light on their pathways and receptors as well. Our primary research tools were gene-deficient mouse strains, which provide a valuable tool to investigate the aforementioned effects.

1.5.1. PACAP - implications in pain and inflammation

PACAP is a prominent member of the VIP/secretin/glucagon peptide family showing 68% homology with VIP. Its name mirrors its initially discovered role, a hypothalamic peptide mediator stimulating adenylate cyclase in pituitary cells (Miyata *et al.* 1989). However its importance covers a much broader spectrum, which was not expected by the early investigators. PACAP is expressed in a 27 and 38 aminoacid-containing isoforms (PACAP-27 and -38 respectively). PACAP-38 is the predominantly expressed form in both humans and other mammals.

PACAP is expressed ubiquitously in the nervous system including most brain regions, the spinal dorsal horn, and peripheral nerve terminals (Vaudry *et al.* 2009). Additionally PACAP has been located in almost all kinds of non-neural tissues including endocrine glands (Arimura 1992), blood vessels, and immune cells (Delgado *et al.* 2003). It regulates nociceptive transmission in a complex manner: it is anti-hyperalgesic on the periphery, while being pronociceptive upon central administration. Therefore, PACAP has been suggested to play a crucial role in central sensitization and the induction of chronic pain (Vaudry *et al.* 2009, Ohsawa *et al.* 2002, Mabuchi *et al.* 2004, Missig *et al.* 2014). 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 (Delgado *et al.* 2003, Vaudry *et al.* 2009).

Traditionally three G-protein-coupled receptors (GPCR) were considered to be the targets of PACAP (**Fig. 3**). 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). Recently however PACAP-27 has been proved to be able to activate the Formyl Peptide Receptor-Like 1 (FPRL1) as well, which is an unrelated rhodopsin-like GPCR (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 (Ekblad 1999, Zhou *et al.* 2002, Somogyvári-Vígh & Reglődi 2004, Davis-Traber *et al.* 2008, Vaudry *et al.* 2009). PACAP receptors are also widely distributed in the immune system: PAC_1 receptor is expressed on macrophages and monocytes, but not on lymphocytes

(Pozo *et al.* 1997). $VPAC_1$ was found on both T and B 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 phagocytic leukocytes, but to a lesser extent also on lymphocytes (Kim *et al.* 2006).

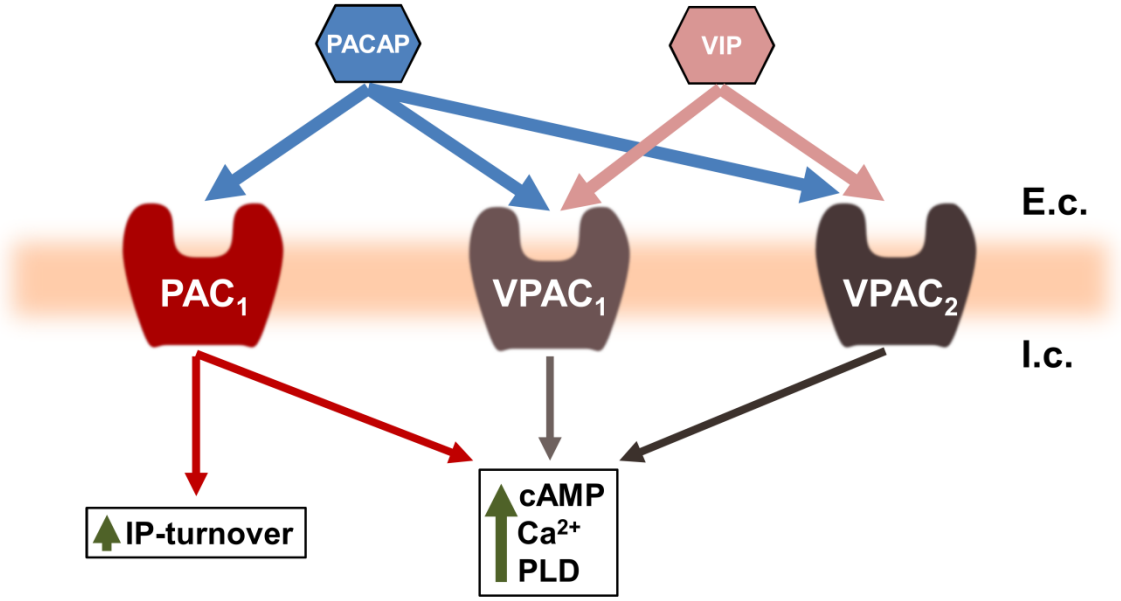

Fig. 3. The canonical endogenous receptorial targets of PACAP, its interactions with VIP-ergic signaling, and the downstream signal transduction, demonstrating a considerable overlap between the intracelullar effects of PAC_1 and $VPAC_{1/2}$ receptor activation (IP- Inositol trisphosphate, cAMP - Cyclic adenosine monophosphate, PLD- Phospholipase D).

1.5.2. The functional importance of the tacyhkinin family

Tachykinins represent a diverse family of neuropeptides released from the capsaicin-sensitive sensory nerve endings, and are considered as an important milestone of peptide research, as they were the first group of neuropeptides to be discovered owing to their abundant and ubiquitous expression. (Hökfelt 2001). The pioneering investigators were unable to isolate these messengers lacking adequate technology, however they could already demonstrate their widespread presence in the CNS, together with their prominent vasodepressor effect upon systemic administration (Euler *et al.*

1931). Due to its ability to elicit rapid contraction in smooth muscle, the presumed active ingredient of this crude extract was termed „tachykinin”. At that time hardly any peptide mediators were known, and even the idea of chemical neurotransmission was a matter of great controversy – as a consequence decades passed until the next step could be taken, by the identification (Chang *et al.* 1970), structural description (Chang *et al.* 1971) and synthesis (Tregear *et al.* 1971) of SP. Through the following decades other similar neuropeptides, (e.g. NKA, NKB) were identified along with their receptorial targets, thus the term tachykinin-family was coined (Kangawa *et al.* 1983, Folkers *et al.* 1984, Regoli *et al.* 1994).

All tachykinins are relatively short peptides, their average length is 10-11 amino acids. By this time three tachykinin encoding genes have been cloned (Tac1, Tac3, and Tac4). However due to post-translational modifications the actual diversity of the produced mature peptides is larger (Maggi 1995, Page 2005). The first identified Tac1 gene encodes primarily SP and NKA along with the less important neurokinin K and neuropeptide gamma. The Tac3 gene encodes NKB, whereas the Tac4 gene produces hemokinins, the most recently identified members of the tachykinin family (Page 2004). SP and NKA are produced in sensory nerves, and various regions of the brain (Pinto *et al.* 2004) The expression of the Tac1 gene is not restricted to neural structures, being actively expressed in immune and vascular endothelial cells as well (Linnik *et al.* 1989, Weinstock *et al.* 2004, Pinto *et al.* 2004, Douglas *et al.* 2011). SP and NKA elicit diverse effects under both normal and pathophysiological conditions. They induce neurogenic vasodilation and plasma protein extravasation, and trigger smooth muscle contraction. (Maggi 1995, Brain *et al.* 1997, Keeble *et al.* 2004). SP and NKA also stimulate lymphocyte proliferation, cytokine production, mast cell degranulation, T cell chemotaxis, and neutrophil accumulation (Grant 2002, De Swert *et al.* 2006). They facilitate the release of histamine and serotonine through mast cell activation, which consequently increases the neuropeptide-release from the sensory nerve terminals through positive feedback mechanisms (Holzer 1991, Szallasi *et al.* 1999). They are also involved in the „wind up” phenomenon occurring during central sensitization (De Felipe *et al.* 1998).

Three substantially different tachykinin receptors have been identified: the Neurokinin 1, 2, and 3 (NK1; NK2; NK3, respectively), which are Gs/Gq-protein rhodopsin-like GPCRs (Maggi 1995, Douglas *et al.* 2011). 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 (Hastrup *et al.* 1996, Lecci *et al.* 2003, Maggi *et al.* 1997). However hemokinins are also potent agonists on the NK1 receptor (Kurtz *et al.* 2002, Page 2004). The tachykinin encoding genes, the active mediators, and the receptors are summarized in **Fig. 4**.

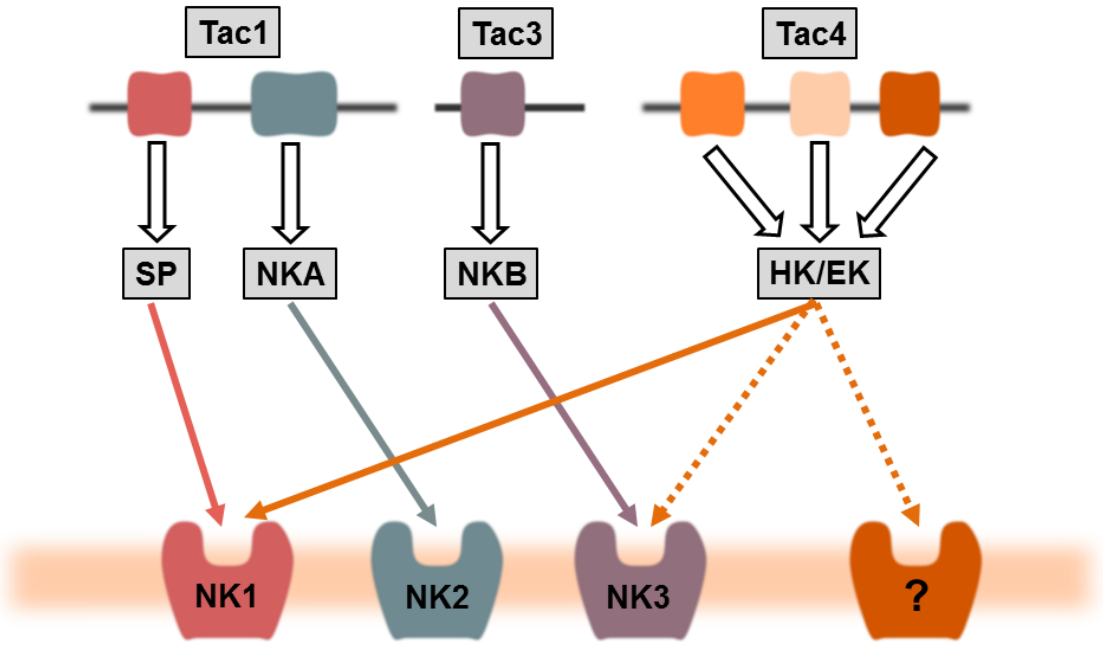

Fig. 4. Schematic representation of the tachykinin-encoding genes, and the receptor-specificity of the mediators. Dashed lines indicate the not entirely proven receptor-ligand interactions. Note: Some authors suggest that hemokinins and endokinins may also have a so far unknown receptorial target (indicated by a question mark). (HK: hemokinin, EK: endokinin)

The NK1 receptor demonstrates ubiquitous expression, and can be detected in nearly all tissues including most internal organs, however, the highest expression levels were reported in the CNS (Tsuchida *et al.* 1990, Pinto *et al.* 2004). Particulary strong NK1 expression was observed in the locus coeruleus and ventral striatum, whereas its presence is moderate in most of the cortex, the hippocampus, and the amygdaloid nuclei (Caberlotto *et al.* 2003). It is also expressed in monocytes and macrophages (Ho *et al.* 1997).

1.5.3. The Galanin Receptor 3 – an emerging mediator in peripheral peptidergic signaling

Galanin is a sensory neuropeptide with a length of 30 (29 in rodents) amino acids, that is expressed ubiquitously in both the central and peripheral nervous system (PNS). Moreover, it was corroborated as a sensory neuropeptide that is upregulated following nerve damage (Chang *et al.* 1985, Skofitsch *et al.* 1985, Hökfelt *et al.* 1987). Later studies revealed the existence of other closely related peptide mediators such as galanin-like peptide (GALP) (Ohtaki *et al.* 1999), and its splice variant alarin (Santic *et al.* 2006), which altogether form the galanin peptide family. The role of galanin has been described in a wide range of physiologic processes, such as nociception (Liu *et al.* 2002), inflammation (Schmidhuber *et al.* 2007), and it also has implications in tumor biology (Lang *et al.* 2015). Galanin gene-deficient mice have a reduced number of sensory neurons, and exhibit elevated sensitivity towards mechanical and thermal stimuli, whereas galanin-overexpressing mice demonstrate increased thermonociceptive threshold (Holmes *et al.* 2000, Blakeman *et al.* 2001, Holmes *et al.* 2003).

Galanin, GALP, and alarin (and other suggested endogenous agonists – discussed later) mediate their effects through three different GPCRs (termed GalR1-3), showing distinct tissue expression pattern (Lang *et al.* 2007). 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). Alarin, however, appears to mediate its effects through other uncharacterized receptors (Santic *et al.* 2007).

Both GalR1 and GalR2 are present in abundance throughout the entire CNS whereas GalR3 expression in the brain is limited (olfactory cortex, hippocampus, hypothalamus), and it is entirely absent in the spinal cord and DRG. (Kolakowski *et al.* 1998, Mennicken *et al.* 2002, Liu *et al.* 2002). Conversely GALR1 and GALR2 are widely expressed in the DRG (Landry *et al.* 2005), thus, it was hypothesised that these two receptors mediate galaninergic effects in nociceptive transmission. It was theorised that galanin might have a dual role in nociceptive signaling. It has pro- or antinociceptive effects depending on the pain condition, as the activation of GalR1 on dorsal horn

neurons is antinociceptive, while presynaptic GalR2 stimulation on primary afferents is pronociceptive (Liu *et al.* 2002). In non-neural tissues mainly GalR2 and GalR3 are expressed (Santic *et al.* 2007). According to the latest results GalR2 but not GalR3 is expressed in both human and murine neutrophils, whereas galanin and GalR3 are only expressed in the latter (Locker *et al.* 2014).

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, and the antiedema effect of galanin can be blocked by selective GalR3 antagonism (Schmidhuber *et al.* 2009), the interest for this receptor is growing. As recent findings indicate the presence of other endogenous GalR3 agonists surpassing the potency of galanin (Kim *et al.* 2014) this trend will presumably continoue in the future. Besides inflammation, GalR3 has been also implicated in several CNS disorders, such as depression and anxiety, as GalR3 gene-deficient mice show an increased anxiety-like behavior, suggesting GalR3-mediated effects may be anxiolytic (Brunner *et al.* 2014).

2. PRIMARY 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 peripheral and central pain conditions as well. 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. We employed global gene-deficient mouse strains for this purpose.

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. We aimed to reassess their role in this phenomenon using knockout mice and a translational model of RA.

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. 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.

3. EXPERIMENTAL MODELS AND INVESTIGATIONAL METHODS

3.1 Animals

All 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 and provided with standard rodent chow and water *ad libitum* under 12 h light-dark cycles. The original breeding pairs of the wildtype mice were purchased from Charles-River Ltd. (Budapest, Hungary). The experiments were conducted using male PACAP, Tac1, Tacr1 and GalR3 gene-deficient mice and their respective wildtype counterparts (Note: Tacr1 is the name of the NK1 receptor gene). Experiments were conducted preferentially on 10-14 week old male mice, but for the increasing temperature hot-plate test female mice were also incorporated into the studies (Note: this method often yields unreliable results with male mice, as the testicles of the subjects can get in contact with the heated surface of the plate, leading to early nocifensive reactions, and potentially biased results).

The generation of $PACAP^{-/-}$ mice on the outbred CD1 background (*Osaka University, Japan*) has been previously described in details (Hashimoto *et al.* 2001). Briefly: the heterozygous mice ($PACAP^{+/-}$) were backcrossed for 10 generations with the CD1 strain. After genotyping the offsprings of the first generation of the $PACAP^{+/-}$ breeding pairs, mice were bred on as wildtype ($PACAP^{+/+}$) and homozygous knockout ($PACAP^{-/-}$) lines in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs. Offsprings within the first three generations were used for the experiments to minimize genetic variations. As PACAP-27 and -38 are the biologically active products of the same exon $PACAP^{-/-}$ mice completely lack both.

The 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 as described previously (Helyes *et al.* 2011). As the homozygous knockouts were extensively backcrossed (8-10

generations) into the C57Bl/6 lineage, those animals served as wildtype controls.

Experiments addressing the function of GalR3 were conducted using gene-deficient ($GalR3^{-/-}$) mice and their wildtype, age-matched ($GalR3^{+/+}$) counterparts. $GalR3^{-/-}$ (LEXKO-230) mice were obtained from the European Mouse Mutant Archive. The strain was maintained on a C57Bl/6 inbred background, and was bred heterozygously as received.

3.2 Research ethics

All experimental procedures 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) and complied with the recommendations of the International Association for the Study of Pain. The studies were approved by the Ethics Committee on Animal Research of the University of Pécs according to the Ethical Codex of Animal Experiments and licenses were given (license No.: BA 02/2000-9-2011, BA 02/2000-2/2012).

3.3. Partial nerve ligation model of traumatic mononeuropathy

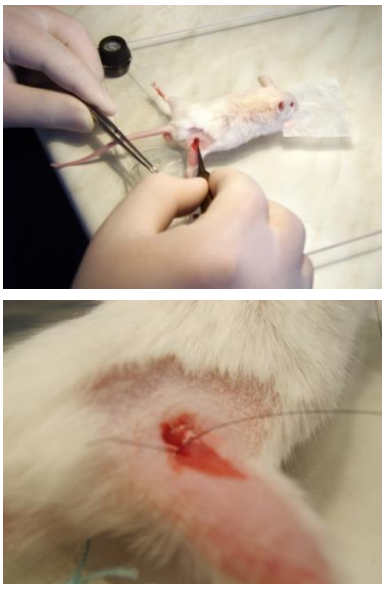

Fig. 5. Partial sciatic nerve ligation

Animals were anesthetized with ketamine-xylazine (100:5mg/kg i.p.) and topically shaved prior to the operation. The right common sciatic nerve was unilaterally exposed from a dorsal incision on the thigh, and one-third was tightly ligated using an atraumatic siliconised silk suture (Ehicone 10-0) (**Fig. 5.**). The wound was closed and the operation was followed by a 7-day interval, during which the animals were not examined to avoid interference with wound healing (Seltzer *et al.* 1990, Malmberg & Basbaum 1998). Based on our earlier investigations, this chronic model of traumatic mononeuropathy is both

reliable and highly reproducible, thus it serves as an excellent proof-of-concept setup for the evaluation of the effect of endo- or exogenous substances on nociception (Sándor *et al.* 2009, Sándor *et al.* 2010).

3.4 K/BxN serum-transfer model of autoimmune arthritis

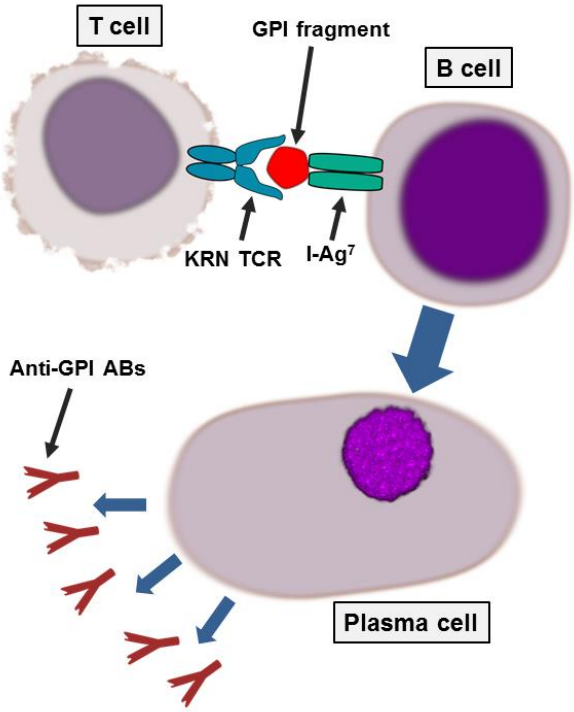

Fig. 6. The molecular mechanism of K/BxN arthritis, triggered by the recognition of self-antigen by the transgenic TCR.

The K/BxN spontaneously arthritic mouse strain was generated by a serendipitous discovery (by researchers aiming to develop a diabetes model) in 1996, as a T cell receptor (TCR) transgenic mouse line (KRN-C57Bl/6) was crossed with autoimmunity-prone non-obese diabetic (NOD) strain (Kouskoff *et al.* 1996). The resulting offsprings all develop a progressive polyarthritis, resembling numerous aspects of RA in men. The underlying cause of the arthritis model is that the transgenic KRN-TCR recognizes the self-antigen glucose-6-phosphate isomerase (GPI) peptide, presented by the NOD-derived A^{g7} major histocompatibility complex (MHC) class II molecule. Thus, the transgenic KRN T cells facilitate the formation of anti-GPI B cells, ultimately eliciting massive anti-GPI production (the primary autoantibody in the model) by plasma cells (**Fig. 6.**) (Matsumoto *et al.* 1999). This results in a joint inflammation that involves both lymphoid and myeloid immune cells. GPI is an ubiquitously expressed cytosolic enzyme, whose main function is the catalysis of the interconversion of D-glucose 6-phosphate and D-fructose 6-phosphate, which is a key element of glycolysis and gluconeogenesis. However it has important extracellular roles as well, exhibiting cytokine and growth factor-like

activities (Ditzel 2004). Besides anti-GPI the disease formation also depends heavily on the complement system (especially C_5)

The K/BxN mouse displays several common features of RA, including neutrophil infiltration, synoviocyte proliferation, cartilage and bone reorganization, polyclonal activation of B cells, hypergammaglobulinemia, and production of autoantibodies (Ditzel 2004). Nonetheless, K/BxN mice do not express the rheumatoid factor – a hallmark feature of RA. Consequent studies revealed that transfer of serum or purified immunoglobulins from the K/BxN mice elicits a robust, albeit transient polyarthritis (resolving typically <14 days after a single bolus injection) in a number of recipient strains as well (Korganow *et al.* 1999)

The blood of transgene positive (K/BxN) and negative (B/xN) littermates was collected by retroorbital bleeding (Jakus *et al.* 2010). Sera were pooled separately and stored at -80°C until use. The arthritis was induced by i.p. injecton of either arthritogenic (K/BxN) or control (BxN) serum. The typical administered dosage of the K/BxN serum varies greatly in the literature, but most commonly it is 100-300 µl/bolus injection, though occassionally lower or higher dosages have been successfully used. Therefore, the the amount of serum administered to the outbred CD1-based strains was 150 µl for the functional tests to minimize the risk of gait and weight-bearing abnormalities caused by severe arthritis which would render nociceptive tests difficult at best. For the *in vivo* imaging and histology the dose was set to 300 µl to induce a severe, uniform joint inflammation with massive cellular infiltration. As bone structural changes take an even longer time to develop, to the animals undergoing micro-CT examination two additional boosting injections (150 µl) were administered on the 10^{th} and 20^{th} day to maintain the severity of the inflammation, and potentially mimic the later, degenerative phase of arthritis.

$Tac1^{-/-}$, $Tacr1^{-/-}$, and $GalR3^{-/-}$ mice uniformly received 300 µl of serum in a single dosage, as we have observed a somewhat lower incidence and considerably slower arthritis onset in the inbred C57Bl/6-based mouse strains using the lower dosage.

3.5. The oxazolone-induced model of atopic contact dermatitis

Fig. 7. Oxazolone challenge

Repeated cutaneous challenge by the small molecule oxazolone induces a condition akin to atopic contact dermatitis (ACD) in mice. Oxazolone acts as an allergenic hapten, leading to disproportionally larger immune responses upon repated exposure to the sensitizing agent, owing to delayed type hypersensitivity. Thus, it elicits a primarily cytotoxic and T helper type-1 (Th1) cell mediated inflammation, with the consequent activation of mast cells and recruitment of neutrophils and mononuclear cells (Petersen *et al.* 2006). The model relies heavily on TNF-alpha, IL-1, and IL-6, but not on IL-4 (Traidl *et al.* 1999).

The animals were sensitized on two consecutive days by applying 50 µl of 2 w/v% oxazolone dissolved in 96% ethanol onto the depilated abdominal skin. 6 days after the second treatment 30 µl of the same solution was smeared on both sides of the the right ear, whereas on the left ear an identical amount of vehicle was applied to serve as control (**Fig. 7.**) (Bánvölgyi *et al.* 2005).

3.6. Evaluation of mechanonociception (esthesiometry)

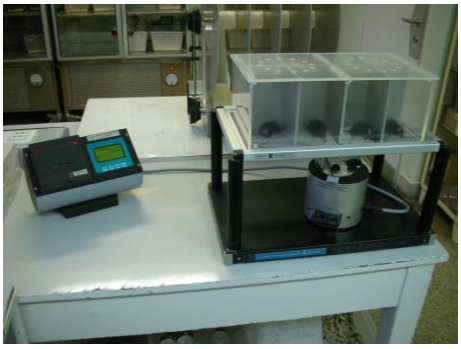

Fig. 8. Dynamic plantar esthesiometer

The mechanonociceptive threshold was measured on the plantar surface of the hindlimb with a dynamic plantar esthesiometer (Ugo Basile 37400, Comerio, Italy) (**Fig. 8.**). In the instrument the subjects were placed into an individual compartment on a wire mesh grid in which they can freely move. Each measurement was preceeded by a roughly 10 min acclimation period to decrease the initial exploratory behavior. The nociceptive threshold was measured by stimulator unit with the aid of an angled mirror. The blunt metal filament was positioned under the hindpaw and the instrument exerted an increasing upward force at a preset rate (5 sec) until

either the nocifensive reaction (paw withdrawal) was elicited, or until the upper threshold value (10 grams) was attained. The nociceptive threshold was measured three times for each paw, and the means were used for further analysis. One conditioning and 2 control measurements were performed prior to disease induction to clearly establish control thresholds and to let the subjects habituate the experimental conditions.

3.7. Assessing motor coordination (rota-rod test)

Fig. 9. Accelerating rota-rod

Motor functions were studied using an accelerating Rota-Rod device (Ugo Basile 7750, Comerio, Italy). This instrument consists of a constantly faster rotating drum, which is divided into four separate compartments, each for a single animal (**Fig. 9.**). Three control measurements preceeded disease induction as the learning proccess itself forms an important readout of this technique. Motor performance was expressed as the duration spent on the wheel in seconds (Sándor *et al.* 2007). Each subject was measured 5 times and the means were used for further evaluation.

3.8. Imaging the cutaneous blood flow (laser Doppler flowmetry)

Fig. 10. Laser Doppler imager

Microcirculation in the plantar skin of the hindpaw was measured by laser Doppler imaging (Perimed PIM II, Stockholm, Sweden) (**Fig. 10.**). The mechanism of this device is based on the Doppler-principle. It emits a monochromatic laser beam, which is reflected by the moving red blood cells (RBC) within the skin

vasculature. As the reflected light passes through Doppler-shift depending on the velocity and number of RBCs, imaging this phenomenon allows the determination of blood flow in the superficial capillaries. As most of the light is absorbed in the superficial tissues, the perfusion of the larger blood vessels does not interfere with the measurement. The mean blood perfusion was quantified in arbitrary units (flux), by automatically counting the number of RBCs in the tissue volume of interest and multiplying it by their mean velocity. Mice were anesthetized with urethane (2.4 g/kg i.p), their body temperature was maintained at 37-38°C with a controlled heating pad. First, 3-4 control images of the plantar surfaces of both the operated and the intact hindpaws were taken to establish a solid baseline blood flow. Then 30-30 µl of freshly prepared 5% mustard oil was smeared on the hindlimbs. Mustard oil contains AITC which, as we discussed earlier is a TRPA1 receptor agonist (Jordt *et al.* 2004, Bautista *et al.* 2006). Therefore topical application of this compound elicits the release of sensory neuropeptides from the intact nerve endings, leading to a neurogenic hyperemic and vasodilatory response in the innervated area. The plantar microcirculation was measured for 60 minutes after AITC application. Altogether an average of 30 images were recorded for each animal including the initial control measurements. All knockout and wildtype mice were measured within a timeframe of 48 hours to minimize the possible environmental interferences.

3.9. Evaluation of thermonociceptive threshold (hot plate)

Fig. 11. Increasing temperature hot plate

Thermonociceptive threshold was determined using an increasing temperature hot plate (IITC Life Sciences, Woodland Hills, CA, USA) (**Fig. 11.**) in which the animal is able to move unrestrained, on a temperature-controlled surface. The contact area of the device was heated from 30°C until the animal either exhibited nocifensive responses (lifting, shaking or licking either hindpaw) or the preset non-damaging temperature maximum (53°C) was attained (Sándor *et al.* 2009).

3.10. Semiquantitative evaluation of disease severity (clinical scoring)

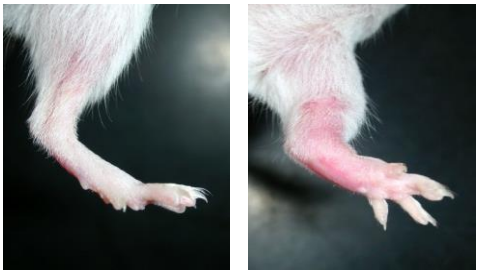

Fig. 12. Intact (Score: ~0.5) and arthritic (Score: ~9) hind limbs

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 (**Fig. 12.**). A score of ≤0.5 represents an intact, healthy limb, whereas ~8-10 marks a confluent, extreme inflammation with accompanying gait abnomalities. Two classical signs of inflammation were measured in order to determine the clinical score: edema and hyperemia (Jakus *et al.* 2010).

3.11. Assessing hindlimb edema (plethysmometry)

Fig. 13. Paw volume measurement

The hindpaw volume increase was evaluated by plethysmometry (Ugo Basile, Comerio, Italy), reporting the paw volumes in ml (**Fig. 13**). Disease induction was preceeded by at least two control measurements, from which the values were averaged to serve as baseline (Helyes *et al.* 2004).

3.12. Determination of joint dysfunction (wire grid grip test)

Fig. 14. Wire grid grip test

To assess the possible joint dysfunction, mice were placed on a horizontal wire mesh grid which was gently turned over, and maintained in this position at a height of 20-30 cm for 20/30 seconds or until the animal fell (**Fig. 14.**). This is a proven, simple but

high-throughput method to evaluate grasping inability in the K/BxN serum-transfer arthritis model (Jakus *et al.* 2010).

3.13. *In vivo* fluorescence imaging of vascular leakage

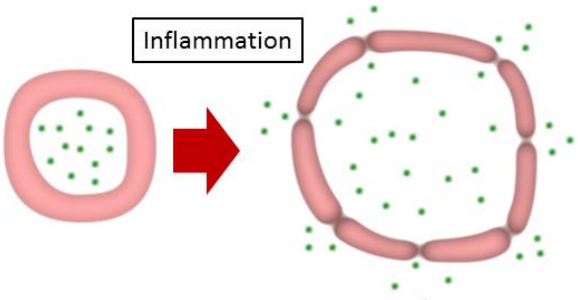

Fig. 15. The principle of the imaging technique: The fluorescently labelled micelles are unable to exit the healthy vasculature, whereas they are capable of passing through the „leaky" vessels of inflamed tissues, resulting in the local accumulation of fluorophore.

Indocyanine green (ICG) is an FDA-approved fluorescent cyanine near-infrared dye, that upon iv. injection rapidly binds to plasma proteins (mainly albumin) and remains in the healthy vasculature until excreted by the liver. Its plasma-half life is approximately 2-4 minutes when administered as a freely dissolved dye. ICG proved to be a suitable dye to image inflammatory hypervascularisation and capillary leakage in arthritis (Meier *et al.* 2010). It has been used clinically as a contrast agent and tracer for decades, however it has recently been introduced to rheumatology as well, in order to detect and evaluate rheumatoid arthritis using novel open-air clinical fluorescence imagers (Werner *et al.* 2012). To overcome its rapid clearance and stability problems in aqeous solutions (ICG formes aggregates leading to decreased fluorescence, and it easily precipitates in saline or other physiologic buffer solutions), nonionic emulsifiers have been successfully employed to encapsulate and stabilise ICG in micelles (average diameter ~12 nm) *in vitro* (Kirchherr *et al.* 2009) and to increase its plasma elimination half-life (Kim *et al.* 2010) (**Fig. 15.**). Therefore we dissolved ICG (0.5 mg/kg) in a 5 w/v% solution of non-ionic surfactant Kolliphor HS 15 (Sigma-

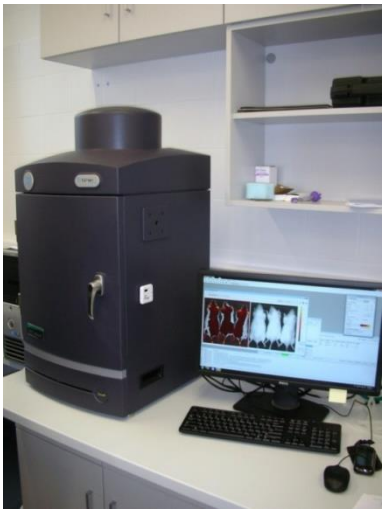

Fig. 16. IVIS Lumina II optical *in vivo* imager

Aldrich) made with sterile deionised water (Kirchherr *et al.* 2009). This formula was injected i.v. to anesthetized mice which were thereafter imaged with IVIS Lumina II optical imager (Perkin-Elmer, Waltham, MA, USA) (**Fig. 16.**). During some later studies we employed IR-676 instead of ICG, a more suitable fluorophore which offers better quantum yield and stability, and has a narrow emission peak, enabling lower dosage ($2x10^{-7}$ mol/kg) and more sensitive detection. Imaging parameters were the following: auto acquisition time, F/stop=1, Binning=2. Excitation/emission filters were 745/800 nm for ICG and 645/700 nm for IR-676. Data were analyzed using the Living Image® software (Perkin-Elmer). Standardized regions of interests (ROIs) were drawn around the ankle joints. A calibrated unit of the fluorescence, the total radiant efficiency ([photons/second/cm^2/ steradian]/[μW/cm^2]) originating from the ROIs was used for further analysis.

3.14. Investigation of neutrophil myeloperoxidase-derived free radical production *in vivo*

Luminol (3-Aminophthalic hydrazide) is a chemiluminescent compound, which can be utilised to detect the activity of the myeloperoxidase (MPO) enzyme *in vivo*. The chemiluminescence reaction occurs mainly in the presence of H_2O_2, which is primarily produced by the phagosomal MPO of activated neutrophils during the respiratory burst (**Fig. 17.**) Thus, it is an indirect but highly selective tool to investigate MPO-activity in a noninvasive manner (Chen *et al.* 2004, Gross *et al.* 2009). Luminol sodium salt (Sigma-Aldrich Ltd.) was dissolved in 1xPBS into a stock solution (20 mg/ml). Mice received luminol ip. in a dosage of 150 mg/kg. Bioluminescence imaging was performed 10 minutes postinjection with the IVIS Lumina II (Acquisition time: 60s, F/stop=1, Binning=8). ROIs of identical size were applied around the tibiotarsal joints, and luminescence was expressed as total radiance (photons/second/cm^2/steradian).

3.15. Evaluation of macrophage-derived superoxide production *in vivo*

Lucigenin (bis-N-methylacridinium nitrate) is a well-known chemiluminescent compound used in free radical detection *in vitro*. It was recently established, that *in vivo* it mainly reacts with the extracellular superoxide, produced primarily by the NADPH-oxidase enzyme of active macrophages in inflammation (**Fig. 17.**) (Tseng *et al.* 2012). Lucigenin was dissolved in saline (2.5 mg/ml) and mice were injected ip. (dosage: 25 mg/kg). Imaging was performed 10 minutes later as described earlier and was also evaluated in a similar manner.

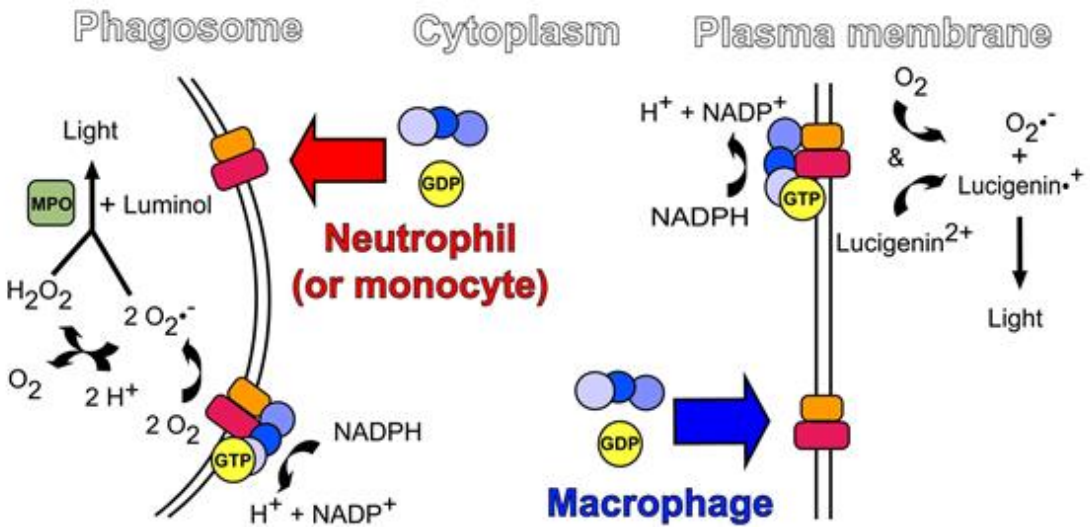

Fig. 17. Overview of the mechanisms of bioluminescence-generation of luminol and lucigenin in inflammation. Luminol penetrates the plasma membrane and enters into intracellular organelles, whereas lucigenin is not membrane permeable. Therefore, the main source of luminol bioluminescence is generated by reaction with phagosomal MPO-derived free radicals of activated neutrophils, while lucigenin reacts with extracellular superoxide generated by the membrane-bound NADPH-oxidase of activated macrophages. Thus, the two compound are suitable to image the acute (neutrohil-dominated) and chronic (macrophage-dominated) phase of ROS production respectively *(Source: Tseng et al. 2012)*

3.16. *In vivo* micro-computed tomography (micro-CT) analysis of bone reorganization

Fig. 18. Micro-computer tomograph

Micro-CT imaging was performed using the same mice at every time-point to minimize inter-individual differences. The right ankles were repeatedly scanned using the same settings and 17.5 μm voxel size by a SkyScan 1176 micro-CT (**Fig. 18.**) (Bruker, Kontich, Belgium). After the reconstruction of the scans, the bone structure alterations were analyzed by the CT Analyser® software. ROIs of predefined standard size 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 (μm^3) and bone surface (μm^2) were calculated and expressed as % of the standardized total volume of the ROI (% bone volume and surface density).

3.17. Evaluation of ear edema

Fig. 19. Measurement of ear thickness

Ear thickness was measured with a microcaliper (Moore and Wright, Sheffield, England) on the pinna of the ear, leaving the rim of the pinna free from the caliper jaws. Measurements were taken from the same relative position before and after oxazolone-treatment (**Fig. 19.**). Data were expressed as % increase of ear thickness compared to pre-challenge controls.

3.18. Histology and evaluation of joint inflammation

PACAP$^{+/+}$ and PACAP$^{-/-}$ mice were sacrificed on day 4 and 28 to investigate acute and chronic alterations, GalR3$^{+/+}$ and GalR3$^{-/-}$ mice were euthanized on the 14th day. Animals were deeply anesthetised (100 mg/kg sodium-pentobarbital i.p.) and transcardially perfused with 4% buffered paraformaldehyde. The ankle joints were further fixed in the same buffer, decalcified, dehydrated, and embedded in paraffin. Sections were made (3-4 µm) and stained with safranine O (a staining protocol exceptionally good for cartilage, bone, and fibroblasts). Slides were semiquantitatively evaluated by a pathologist in a blinded fashion. Synovial cell proliferation and mononuclear cell infiltration were scored (0-normal state, 3-maximal severity) (Helyes *et al.* 2004).

The ear samples of GalR3$^{+/+}$ and GalR3$^{-/-}$ mice were taken upon completion of the *in vivo* experimental readouts, 48 hours post oxazolone-challenge, processed in a similar manner, and stained with hematoxylin-eosin (HE) (Bánvölgyi *et al.* 2005).

3.19. Statistical analysis

Statistical evaluation was performed by the GrapPad Prism® software package. All data were expressed as means with standard errors of means (SEM). The detailed statistical methods are indicated along the tentative results of each experiment. At least $p<0.05$ were considered statistically significant.

CHAPTER 1

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

4.1. INTRODUCTION

4.1.1. Significance of peripheral neuropathies

In general neuropathic pain represents all painful conditions arising due to damage of the somatosensory system (Jensen *et al.* 2011). Pain management in neuropathic disorders is difficult, yet often encountered task for clinical specialists. According to current estimations neuropathic pain can affect as much as 7-8% of the population in Western-Europe (Treede *et al.* 2008, Bouhassira *et al.* 2008). Middle-aged and elderly people are especially susceptible due to the increased prevalence of numerous disease groups capable of eliciting neuropathy in this age group (e.g. diabetes, malignancies, cerebrovascular disorders among many). The therapy of this disease group however is far from being solved, as the background and mechanism of neuropathic disorders varies greatly, and also because even the most potent opioid analgesics have only a limited use due to their inherent risks (abuse, tolerance etc.) in these chronic disorders.

The two main aspects in the classification of neuropathic pain are 1) the anatomical localization of the lesion, and 2) the etiology of the damage. By these two considerations these conditions can be subdivided into four broad categories: 1) Painful peripheral neuropathies (mostly of traumatic origin), 2) polineuropathies (caused by e.g. metabolic disorders, tumors, drugs and other noxious agents), 3) central pain syndromes (arising secondarily upon CNS damage), and 4) other miscellaneous complex pain syndromes (e.g. complex regional pain syndromes). The present work focuses on the first group: namely the peripheral neuropathies induced by direct nerve injury.

A damage to the nervous system has two potential consequences: 1) loss of the somatosensory functions, and 2) abnormal (extra) perceptions. Thus, traditionally the symptoms of neuropathies are categorized into negative (loss of function), and positive (abnormal extra function) symptoms (Komoly *et al.* 2010).

Negative symptoms are for example the loss of various sensory modalities (tactile, sensory etc.), hypesthesia (decreased pain perception), loss of vibration sensing. The most common positive signs are paresthesia (spontaneous, uncomfortable, but not definitely painful sensation), dysesthesia (spontaneous, painful sensation, „pins and needles", burning feeling), or hyperalgesia (decreased pain threshold), allodynia (painful sensation caused by normally non-painful stimuli e.g. mild tactile or thermal stimuli) (**Fig. 20.**).

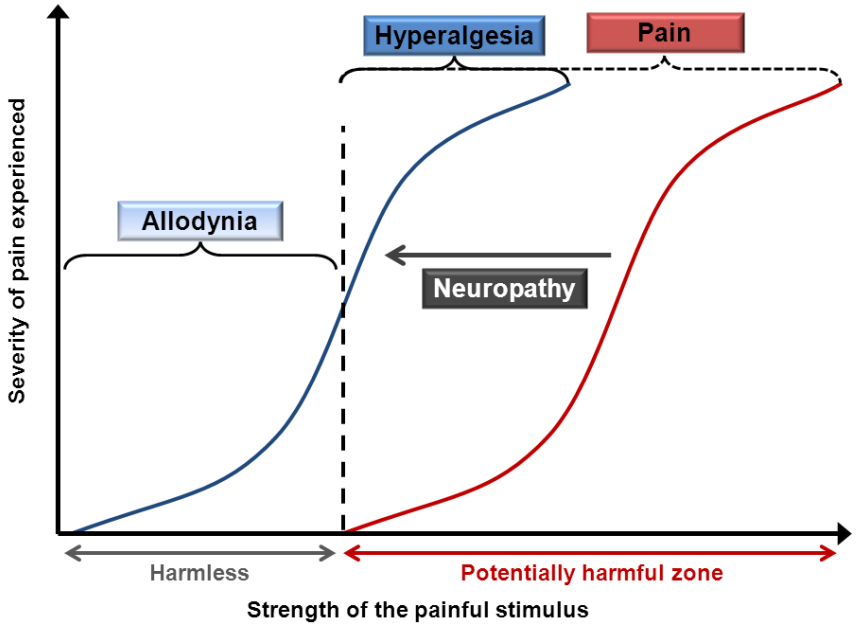

Fig. 20. The types of neuropathic pain on the basis of their intensity and causing factor. Allodynia represents pain elicited by normally non-painful stimuli, while hyperalgesia constitutes increased sensitivity towards painful phenomena. Especially the latter can be accurately modelled in mice. *(Based on Cervero et al. 1996)*

One of the main problems in the management of neuropathic pain is, that during their development, irrespectively of the original disease and the localisation of the primordial injury, secondary maladaptive changes occur at all levels of the nervous system. This means, that the originally localized peripheral nerve trauma leads to lasting pathological changes, ultimately resulting in adverse

CNS reorganization and functional alterations (Todd 2010). Thus, the originally confined disease gradually evolves into a multi-level disorder of the nervous system drastically reducing the chance of succesful pain management. This vicious cycle of the pathomechanism of neuropathies has been illustrated in **Fig. 21.**

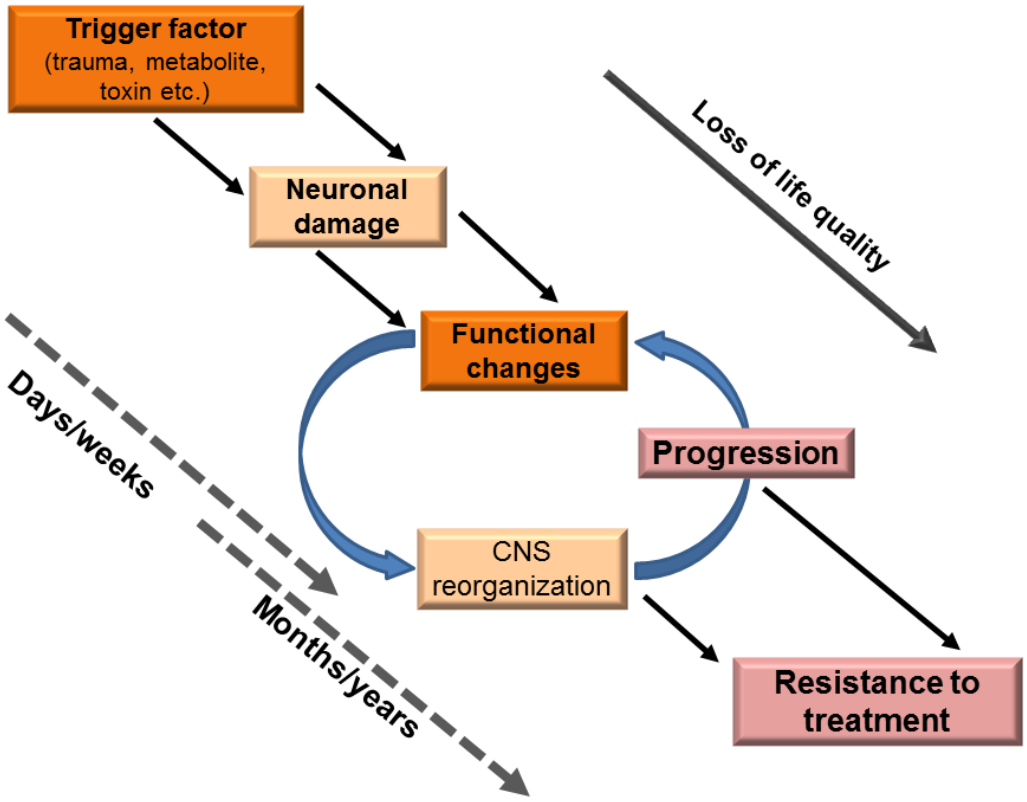

Fig. 21. Schematic representation of the key mechanisms involved in the development of neuropathic pain, and their role in the process. Note that the initial damage gives rise to secondary maladaptive changes initiating a self-propelling vicious cycle.

PACAP and tachykinins, such as SP or NKA released from the capsaicin-sensitive peptidergic sensory nerve endings were shown to be involved in both acute and chronic pain conditions (Dickinson *et al.* 1999, Cahill *et al.* 2002, Shimizu *et al.* 2004, Davis-Traber *et al.* 2008, Sandor *et al.* 2009, Schytz *et al.* 2010, Tatsushima *et al.* 2011), however their overall role in chronic neuropathic pain has not been elucidated.

4.1.2. PACAP in nociception and neuropathy

It was observed early, that PACAP shows increased expression in the superficial layer of the spinal dorsal horn and in capsaicin-sensitive primary sensory neurons, moreover it demonstrates elevated expression in the spinal cord following peripheral nerve injury, suggesting it as a potential mediator of nociception (Moller *et al.* 1993, Zhang *et al.* 1996, Zhang *et al.* 1997, Zhang *et al.* 1998, Mulder *et al.* 1999, Petterson *et al.* 2004). However, the results of early *in vivo* studies turned out to be contradictory (Sakashita *et al.* 2001). PACAP upon intrathecal administration showed an early analgesic effect, followed by prolonged algesia in the formalin-induced acute pain model (Shimizu *et al.* 2004). Centrally administered PACAP also demonstrated marked pronociceptive effects in several other models: it increased thermal hyperalgesia and potentiated nociceptive transmission to the spinal dorsal horn (Ohsawa *et al.* 2002). It also facilitated spinal nociceptive flexor reflexes (Xu *et al.* 1996, Sakashita *et al.* 2001) and induced hyperalgesia (Narita *et al.* 1996). We have demonstrated that PACAP modulates pain-signaling in a pleiotropic manner. Following peripheral administration, it induces antinociceptive, antihyperalgesic and antiallodynic effects in both acute somatic and visceral pain models. In contrast, it does not influence mechanical hyperalgesia in the traumatic mononeuropathy model, but induces sensitization of knee joint primary afferents (Sándor *et al.* 2009). Moreover, PACAP inhibits the release of several pronociceptive and proinflammatory sensory neuropeptides (CGRP, SP and other tachykinins) from peptidergic sensory nerve endings, and also diminishes acute neurogenic and non-neurogenic inflammatory processes (Németh *et al.* 2006, Helyes *et al.* 2007, Elekes *et al.* 2011). Based on our observations, we suggested that PACAP might have a dual role in nociception. Thermal hyperalgesia is increased in PACAP knockout mice, whereas somatic and visceral nocifensive reactions, and neuropathic mechanical hyperalgesia influenced by nociceptive signal transduction within the CNS are decreased. Thus, PACAP can have both inhibitory and excitatory roles depending on the site of action and the predominant mechanism in the different pain models investigated, providing an explanation for the observed superficially conflicting effects (Sándor *et al.* 2010).

4.1.3 Tac1 gene-derived tachykinins in the somatosensory system and nociception

The involvement of the Tac1 gene-derived tachykinins in several models of chronic inflammation and pain has been postulated by many (Garrett *et al.* 1992, Brain 1997, Harrison *et al.* 2001, Keeble *et al.* 2004). Early observations demonstrated that SP expression is greater in the dorsal than in the ventral horn, suggesting that SP is a sensory neurotransmitter (Lembeck *et al.* 1982). As later studies demonstrated that SP is also present in the smaller, unmyelinated sensory fibers, it became associated with nociceptive transmission (Salt *et al.* 1983).

Both SP and NKA are synthesized in the DRG, and they regulate nociceptive information at the first sensory synapse in the spinal cord (De Koninck *et al.* 1991, Vedder *et al.* 1991). SP has been proposed to be an important mediator of the „wind up" phenomenon occurring during central sensitization in the spinal dorsal horn – which is absent in NK1 knockout mice (De Felipe *et al.* 1998). SP, NKA, and NK1 receptor were all suggested to be involved in inflammatory and neuropathic pain syndromes, such as traumatic mononeuropathies (Goff *et al.* 1998, Cahill *et al.* 2002, Gonzalez *et al.* 2002), streptozotocin-induced diabetes (Coudoré *et al.* 2000), and paclitaxel-induced peripheral neuropathy (Tatsushima *et al.* 2011). However, despite these widespread effects it was also demonstrated that SP is not necessarily involved in the development of neuropathic hyperalgesia (Keeble *et al.* 2004).

4.1.4. Specific aims of our study

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. We

have tested the animals for mechanical hyperalgesia, motor coordination deficits, and alterations of vascular responsiveness towards AITC, a specific irritant on the peptidergic sensory terminals.

4.2. RESULTS

4.2.1. Mechanical hyperalgesia

The initial control mechanonociceptive thresholds of the gene-deficient mice mice were similar to the wildtypes, the sole exception being Tacr1$^{-/-}$ animals where a slightly increased threshold was observed (**Fig. 22.**).

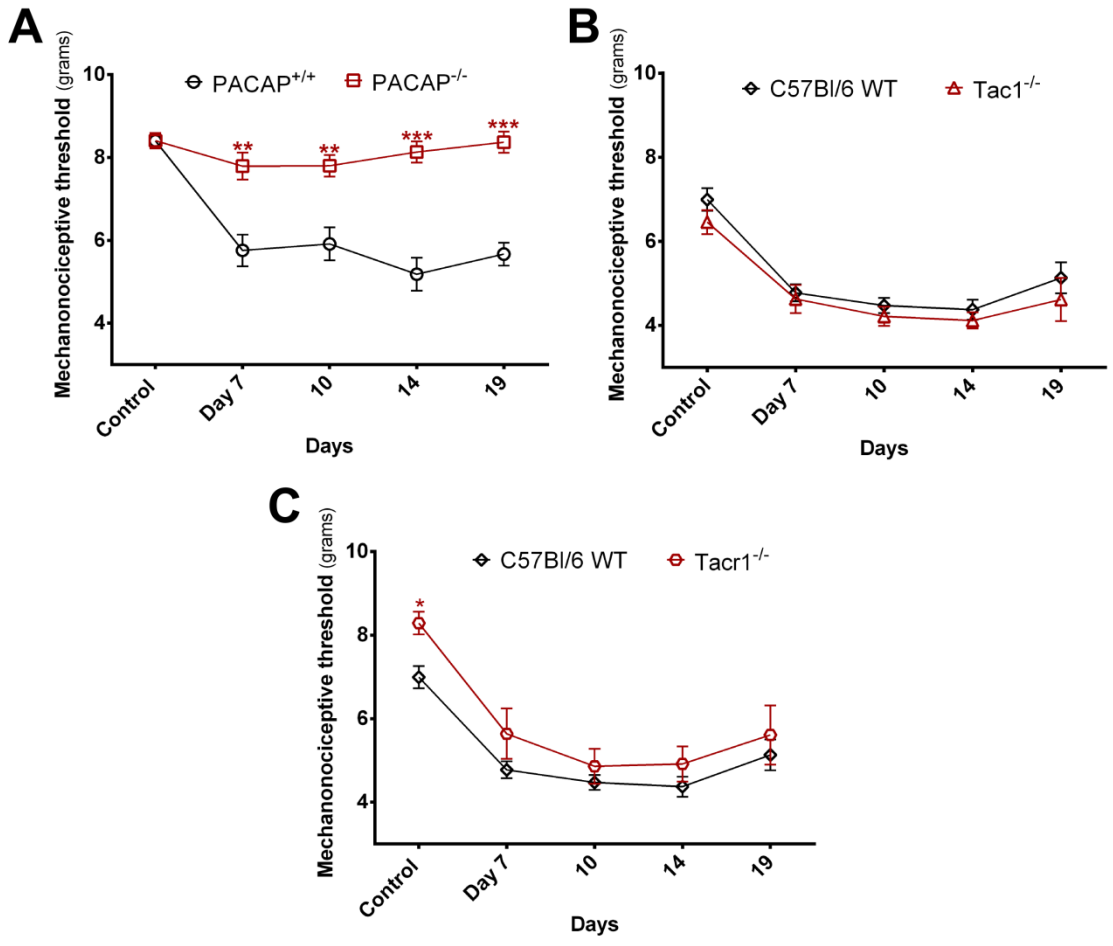

Fig. 22. Change in mechanonociceptive threshold of the hindpaw after partial sciatic nerve ligation. **(A)** The results of PACAP$^{-/-}$ mice compared to their wildtypes. **(B)** The mechanonociceptive threshold of Tac1$^{-/-}$ and **(C)** Tacr1$^{-/-}$ mice compared to C57Bl/6 wildtypes (WT). (repeated measures ANOVA + Bonferroni's post-test; n=10-12 (PACAP$^{+/+}$ and PACAP$^{-/-}$) n=6-8 (C57Bl/6, Tac1$^{-/-}$, Tacr1$^{-/-}$); *p<0.05 **p<0.01, ***p<0.001 vs respective wildtype)

Tight ligation of one-third of the sciatic nerve induced a significant, about 30-40% decrease of the threshold of the affected hindpaw in wildtype animals between the 7th and 19th postoperative days. In comparison, neuropathic mechanical hyperalgesia was negligible in the PACAP$^{-/-}$ group, the nociceptive threshold remained similar to the initial control values throughout the total duration of the experiment (**Fig. 22./A**).

In Tac1$^{-/-}$ and Tacr1$^{-/-}$ 35-45% mechanical hyperalgesia developed in response to the nerve ligation, which was very similar in all the groups. SP/NKA or NK1 receptor deficiency did not influence the mechanonociceptive threshold during the whole study (**Fig. 22./B-C**).

4.2.2. 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 (**Fig. 23./A-B**).

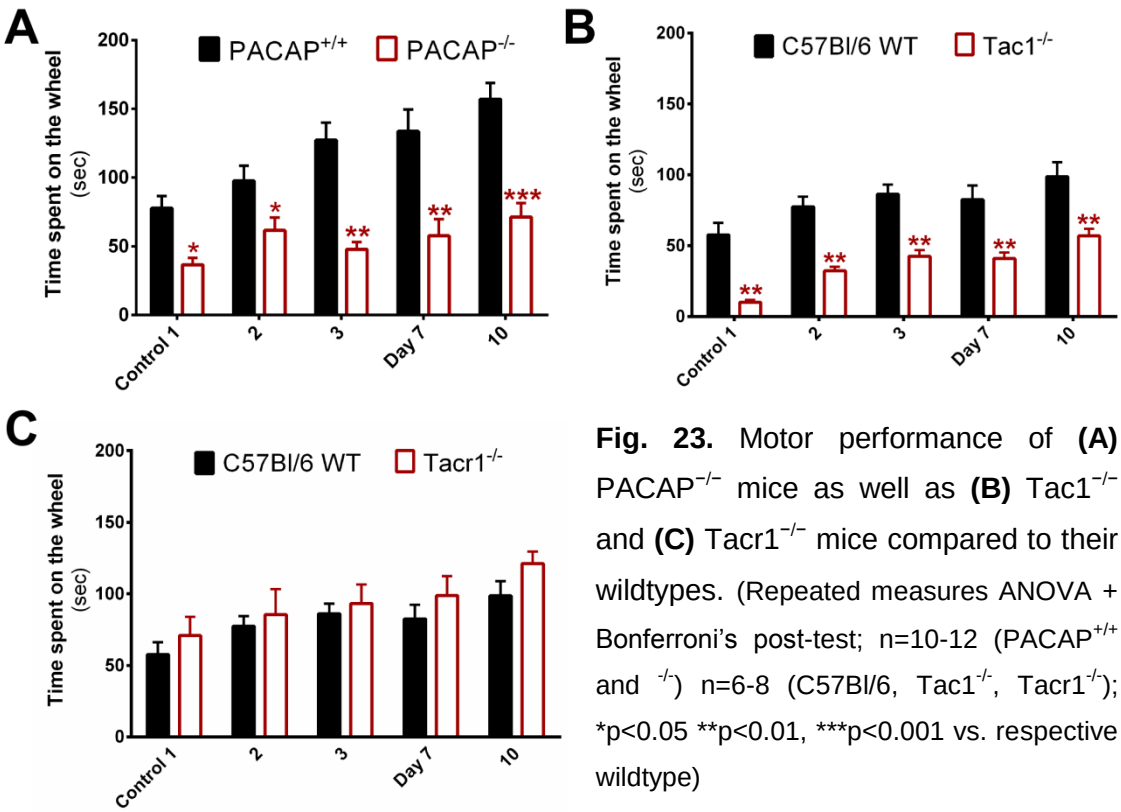

Fig. 23. Motor performance of **(A)** PACAP$^{-/-}$ mice as well as **(B)** Tac1$^{-/-}$ and **(C)** Tacr1$^{-/-}$ mice compared to their wildtypes. (Repeated measures ANOVA + Bonferroni's post-test; n=10-12 (PACAP$^{+/+}$ and $^{-/-}$) n=6-8 (C57Bl/6, Tac1$^{-/-}$, Tacr1$^{-/-}$); *p<0.05 **p<0.01, ***p<0.001 vs. respective wildtype)

In contrast, deletion of the NK1 tachykinin receptor (Tacr1$^{-/-}$) did not influence motor coordination (**Fig. 23./C**). The partial sciatic nerve ligation did not affect performance in any of the groups. During the control preoperative and even the

postoperative periods a continuous learning process was observed in wildtype, PACAP, and Tac1 gene-deleted groups, but not in Tacr1$^{-/-}$ animals. However, impaired basal motor coordination in PACAP$^{-/-}$ and Tac1$^{-/-}$ mice was also observed after the operation (**Fig. 23./A-B**).

4.2.3. Cutaneous blood flow of the hindpaw and neurogenic vasodilation

In both wildtype (PACAP$^{+/+}$ and C57Bl/6) and all gene-deficient (PACAP$^{-/-}$, Tac1$^{-/-}$, Tacr1$^{-/-}$) groups, the basal microcirculation in the plantar skin was somewhat diminished on the operated limb, however the difference was not statistically significant. The basal perfusion was significantly higher in inbred C57Bl/6 and Tac1$^{-/-}$, but not in Tacr1$^{-/-}$ mice when compared to the PACAP$^{+/+}$ and PACAP$^{-/-}$ animals on outbred CD1 background. Topical application of 5% mustard oil (AITC) induced significantly reduced neurogenic vasodilatory response in C57Bl/6-based groups compared to the CD1-based ones (15-20% vs. 50-60%). This suggests a potential strain difference in the regulation of microcirculation (**Fig. 24.**).

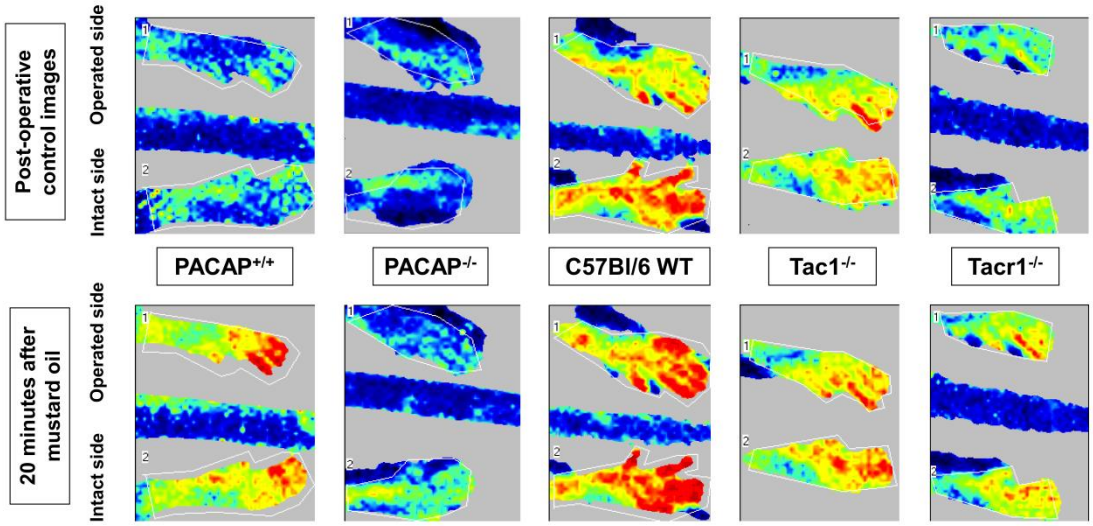

Fig. 24. Representative laser Doppler images of the plantar skin of the operated (right/upper in the figure) and the intact (left/lower in the figure) hindpaws before and 20 min after topical application of 5% mustard oil (AITC). Blue color represents low perfusion areas, green and yellow refer to higher perfusion and red shows the highest microcirculation.

Basal perfusion in the plantar skin of either the operated or intact paws of PACAP$^{+/+}$ and PACAP$^{-/-}$ mice did not differ significantly. In response to activation of the capsaicin-sensitive sensory nerve terminals by topical application of 5% mustard oil microcirculation increased by 50-60% in both groups on both limbs after 5-10 minutes. This neurogenic vasodilatory response persisted in wildtype mice during the next 60 minutes of the experiment, whereas in PACAP$^{-/-}$ animals it started to wear off after 10-15 minutes. Significantly lower perfusion was detected in the PACAP$^{-/-}$ group from the 30th minute onwards (**Fig. 25.**)

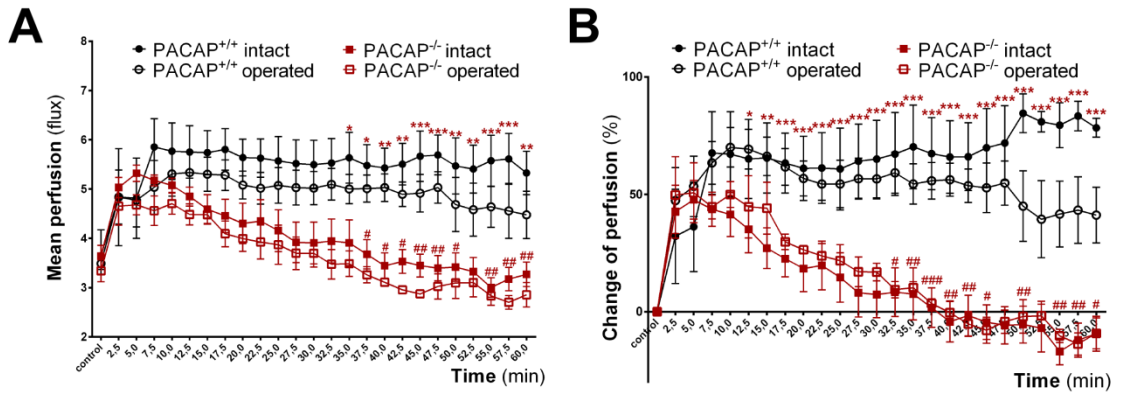

Fig. 25. Mustard oil-induced vasodilation compared to the respective initial reference images in the plantar skin of the operated and intact paws of PACAP$^{-/-}$ mice compared to their wildtypes **(A)** mean flux, **(B)** percentage). (n=5-6; two-way ANOVA + Bonferroni's post-test; *p<0.05 **p<0.01, ***p<0.001 vs. the respective wildtype group, #p<0.05, ##p<0.01, ###p<0.001 vs. the respective self-control).

In the Tac1$^{-/-}$ and Tacr1$^{-/-}$ groups the basal cutaneous microcirculation was significantly lower on both limbs compared to C57Bl/6 wildtypes (**Fig. 26./A-B**). In response to mustard oil smearing, the perfusion increased steadily by 15-20% on both limbs of the wildtype mice. In contrast, in cases of SP/NKA and NK1 receptor deficiency there was an initial transient decrease on the intact side, but after the first 10 minutes, the blood perfusion did not differ significantly from the results of the C57Bl/6 wildtype animals for the rest of the experiment (**Fig. 26./C-D**).

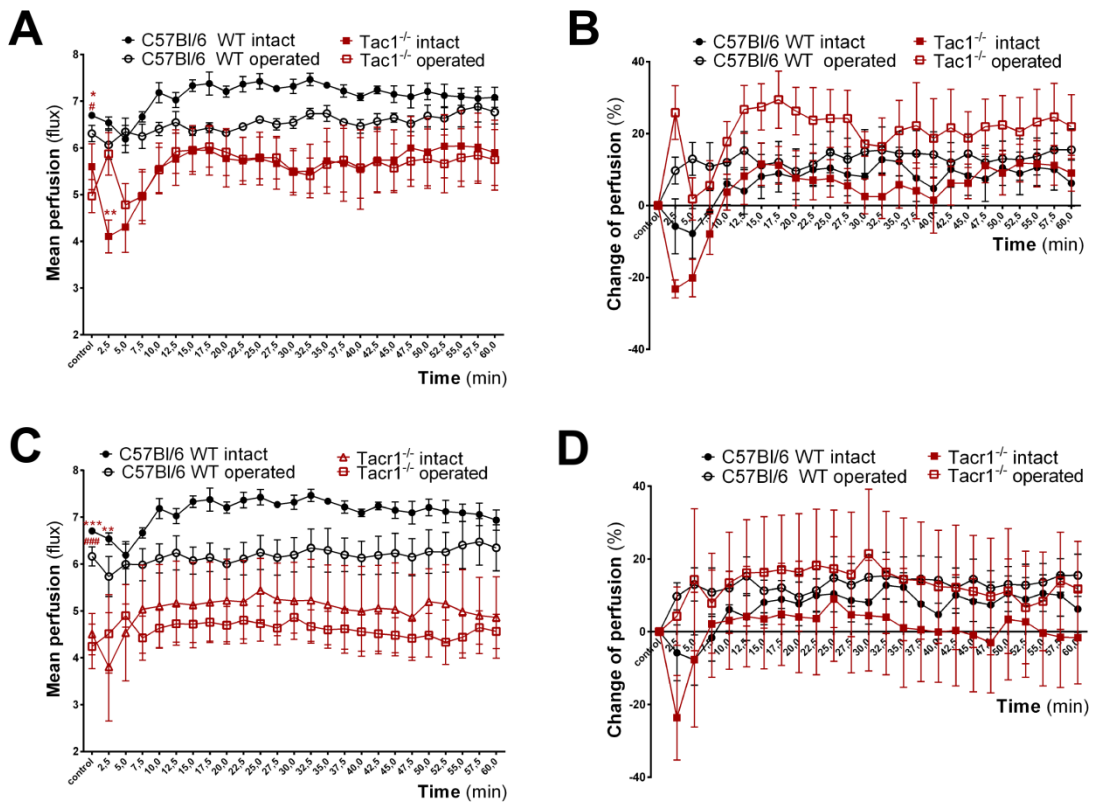

Fig. 26. Vasodilatory response induced by 5% mustard oil on the plantar skin of the operated and intact paws of the **(A)-(B)** Tac1$^{-/-}$ and **(C)-(D)** Tacr1$^{-/-}$ mice in comparison with their wildtypes. (n=5-6; two-way ANOVA + Bonferroni's post-test) *p<0.05 **p<0.01, ***p<0.001 vs. the respective wildtype group, #p<0.05 ###p<0.001 vs. the respective self-control)

4.3 DISCUSSION

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.

The observed significant mechanical hyperalgesia, but normal motor performance and cutaneous microcirculation following the sciatic nerve ligation

broadens our knowledge about this neuropathy model. Although the sciatic nerve contains sensory, motor and autonomic fibers, the traumatic mononeuropathy arising following partial ligation is affecting exclusively the sensory functions. However, it does not interfere with either the motor performance or the vascular regulatory functions. Nevertheless this claim should be further validated by comparing these results to the effect of sham-operation (e.g. by exposing but not ligating the nerve).

We found remarkable strain differences between the mice based on the inbred C57Bl/6 and outbred CD1 strain in the examined sensory, motor and vascular parameters. This observation is not surprising, since numerous publications described such findings, and reviews extensively discuss the strain differences affecting both normal physiological parameters and disease susceptibility (Mogil *et al.* 1999, Smith *et al.* 2004). The observed differences highlight the importance of using the appropriate wildtypes when interpreting data of gene-deficient mice.

4.3.1. PACAP is a pronociceptive mediator of neuropathic mechanical hyperalgesia

Recent data showing that PACAP has a hyperalgesic and pro-nociceptive role in a variety of pain models are in agreement with the present findings (Davis-Taber *et al.* 2008, Sándor *et al.* 2010). However we and others have also observed earlier that peripherally administered PACAP exerts marked anti-nociceptive effects, presumably due to its direct neuropeptide release-blocking effect on sensory nerve terminals (Sándor *et al.* 2009). Nevertheless, the overall effect of PACAP within the CNS is clearly pronociceptive. Upon intra-amygdalar injection PACAP elicits a PAC_1-mediated hyperalgesia, which, taken together with immunohistochemical studies, suggests its involvement in the development of chronic pain via its effect on sensory fiber projections from the lateral parabrachial nucleus along the spino-parabrachioamygdaloid tract (Missig *et al.* 2014). Others also demonstrated that PACAP may play a key role in spinal sensitization, and therefore, in the development of neuropathic pain (Dickinson *et al.* 1999, Mabuchi *et al.* 2004) through PAC_1 receptor activation in the dorsal root ganglia (Davis-Taber *et al.* 2008, Jongsma *et al.* 2000, Jongsma

et al. 2001). Furthermore, PACAP has a pivotal pronociceptive function in animal models of migraine and also in human migraneurs (Markovics *et al.* 2012, Schytz *et al.* 2010b, Tuka *et al.* 2012). Others suggested that the peripheral nociceptive responses elicited by intradermally administered PACAP are mainly mediated through VPAC receptors (Schytz *et al.* 2010a). Therefore, further investigations are required in order to identify the targets responsible for the hyperalgesic actions of PACAP. The Janus-faced involvement of PACAP in nociception has been summarized in **Fig. 27.**

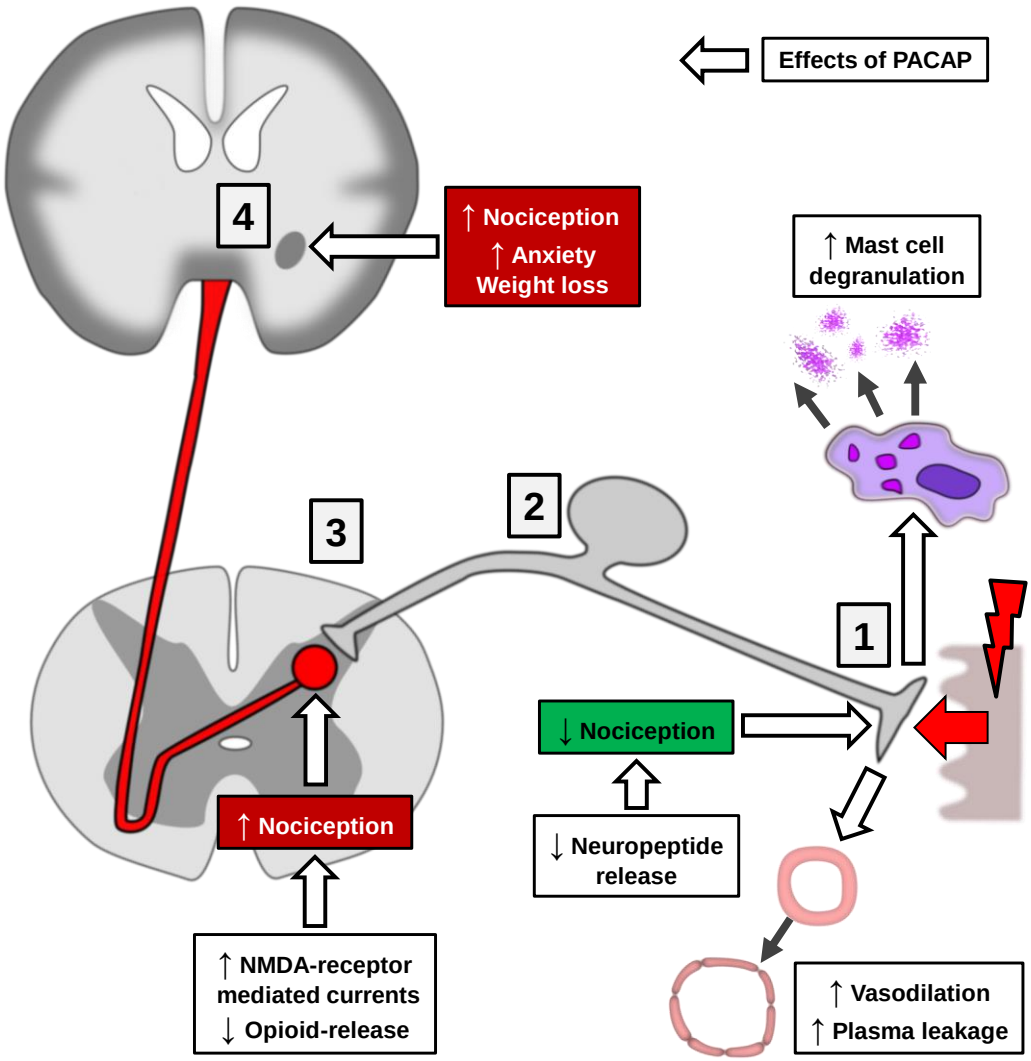

Fig. 27. Our current understanding of the impact of PACAP in nociception and related phenomena on the periphery and in the central nervous systems (1. Sensory nerve terminal, 2. Dorsal root ganglion, 3. Spinal dorsal horn, 4. Amygdala)

4.3.2. Tac1 gene-encoded tachykinins and the NK1 receptor are not involved in nociceptive transmission in the model

While the increased expression of the Tac1 gene-encoded tachykinins in the DRG under neuropathic conditions has been established (Wallin *et al.* 2002), our results indicate that neither SP/NKA nor the NK1 receptor play an important role in the development of neuropathic hyperalgesia. Studying the analgesic mechanisms of the action of topical capsaicin application in neuropathies, it was found that the increased expression of SP has only marginal impact, and capsaicin exerts its therapeutically beneficial effects via different pathways (Anand *et al.* 2011). Additional results also indicate that SP/NKA upregulation is not a crucial factor in the development of neuropathies: while SP was found to be involved in the development of paclitaxel-induced neuropathy, oxaliplatin treatment leads to a similar condition without influencing the SP/NKA levels. (Tatsushima *et al.* 2011). Additionally, in the intraplantar formalin-induced pain model NK1 receptor antagonism reduced pain behavior but had a limited effect on CNS c-Fos (marker of neuronal activation) increase. It reduced c-Fos levels in some areas (e.g. prefrontal cortex, PAG), but not in other localisations (e.g. thalamic nuclei, central amygdaloid nucleus) (Baulmann *et al.* 2000).

Our results about the role of Tac1 gene-derived tachykinins contradict the predominantly positive outcomes from late 1980s to the early 2000s. Blocking SP activity by NK1 antagonism was once heralded as a promising therapautic approach not only in pain management, but also in inflammatory conditions (see later). As a consequence, large-scale research was initiated to develop small molecule NK1 receptor antagonists. However these drug candidates (despite their promising performance in the preclinical stage) demonstrated minimal effect in clinical trials, that appeared to be completely enigmatic (Urban *et al.* 2000, Hill 2000).

This incosistency is still open to interpretation. However it may stem at least partially from the fragmentary understanding of the complex and overlapping tachykininergic pathways. We have noted that hemokinins encoded by the Tac4 gene were not discovered until the early 2000s (Page 2004). Thus, all NK1 antagonists were developed as simple competitive antagonists of SP, assuming

that SP is the only endogenous tachykinin mediator of pathophysiological relevance in the respective disease conditions. As a result hemo/endokinins can potentially bypass the missing SP-mediated effects via NK1 or other receptors. This phenomenon may explain why painful disease conditions can develop undisturbedly in mice, deficient for the Tac1/Tacr1 genes. It also implies that these rescue mechanisms might mitigate the theorized effects of SP-antagonists. This is further corroborated by the facts that the NK1 receptor has two isoforms demonstrating different expression pattern (Caberlotto *et al.* 2003), and it even possesses two distinct binding sites 1) classical ones, which exclusively bind SP, and „septide sensitive" sites which can also be activated by other members of the tachykinin-system (Maggi 1995).

4.3.3 PACAP is involved in motor coordination

PACAP gene deficient mice demonstrated a reduced motor performance both before and after the induction of neuropathy. Our findings are supported by previous reports implicating PACAP as an endogenous regulator of motor coordination (Marquez *et al.* 2009), however its exact mechanism and site of action within the CNS have not been elucidated.

4.3.4. SP/NKA but not the NK1 receptor plays a role in normal motor functions

The motor performance of Tac1 gene deficient animals was significantly diminished compared to their wildtype controls under both normal and neuropathic conditions. The coordination of the NK1 receptor gene-deficient mice was comparable to the controls. The above results indicate that SP/NKA may also act as mediator of motor coordination under physiological conditions. However, the lack of difference in the NK1 knockouts suggests, that this effect is possibly not NK1 receptor-mediated. There is a limited amount of evidence available about the role of Tac1 gene-derived tachykinins in motor functions. These sporadic results obtained in markedly different models (less complex species e.g. *Drosophila*) also suggest a regulatory function (Kahsai *et al.* 2010). SP has been suggested to exert a protective role in a preclinical model of

amyotrophic lateral sclerosis, which is caused by motor neuron degeneration, supporting its role in motor coordination (Caioli *et al.* 2011). SP and the NK1 receptor activation was also linked to the development of Parkinson's disease, in which the loss of motor coordination is the characteristic symptom. Elevated SP expression in this model accelerated disease progression, while NK1 receptor antagonist treatment alleviated the motor performance deficit (Thornton *et al.* 2012).

4.3.5. PACAP is a mediator of neurogenic vasodilation

Besides neuropathic hyperalgesia, PACAP is also involved in the development of neurogenic vasodilation, particularly in its long-term maintenance phase. This was higlighted by the observed effect of AITC, which induced a remarkable initial vasodilation in PACAP deficient mice, which however rapidly subsided. PACAP is a well-known and potent vasodilator (Warren *et al.* 1992), that also increases the permeability of microvessels (Svensjö *et al.* 2009, 2012), an effect which is mediated primarily via the VPAC receptors (Schytz *et al.* 2010a). Subsequently it is suggested, PACAP contributes to the later, stable phase of neurogenic vasodilation. Our results imply that other vasoactive sensory neuropeptides released from the stimulated sensory nerve terminals (e.g. CGRP) are likely to be responsible for the initial phase of this response, since it was preserved in PACAP knockout mice.

4.3.6. SP/NKA and the NK1 receptor influence basal vascular tone, but not AITC-induced neurogenic vasodilation

As the basal blood flow was significantly lower in both Tac1 and Tacr1 gene-deficient mice, SP through NK1 receptor activation is likely to play an important role in the regulation of the vascular tone under normal conditions. In contrast, they exert no visible influence on the neurogenic vasodilatory response. We ought to note that the main vascular effect of SP is not vasodilation, but venular permeability increase, which is however not measured by laser Doppler flowmetry (Holzer 1998). In addition, CGRP is considered to

be the most potent sensory neuropeptide mediator of vasodilation (Lin *et al.* 2007).

It was hypothesised that NK1 receptor activation ultimately increases the production of nitric oxide and other local vasodilatory substances (Wong *et al.* 2006). It must be taken into account, that SP is not the sole endogenous ligand of NK1. Thus, other mediators (e.g. hemokinins) may be capable of bypassing the role of lacking SP. Moreover, it is established, that tachykinins can also induce vasoconstriction, and their net effect is heavily influenced by the local differences of the endothelium (Walsh *et al.* 2006). These findings are supported by the fact that the neurogenic vasodilation elicited by AITC is not affected by NK1-antagonism (Pozsgai *et al.* 2010), suggesting that tachykinins may not consititute a vital element of nerve-driven vascular responses.

4.3.7. Conclusions

1. PACAP is pronociceptive in peripheral traumatic neuropathy.
2. PACAP and SP/NKA are both involved in motor coordination.
3. PACAP is a key mediator of neurogenic vasodilation.
4. SP/NKA and the NK1 receptor have a crucial role in the maintenance of normal vascular tone.

The main findings have been recapitulated in **Table 1.**

	PACAP	**SP/NKA**	**NK1**
Neuropathic mechanical hyperalgesia	↑	-	-
Motor coordination	involved	-	-
TRPA1-driven neurogenic vasodilation	↑	-	-
Basal blood flow	-	↑	↑

Table 1. The effect of PACAP, SP/NKA, and the NK1 receptor in the mononeuropathy model according to our results.

CHAPTER 2

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

5.1. INTRODUCTION

5.1.1. The global burden of rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory condition, being one of the most prevalent autoimmune diseases in the developed world, affecting roughly 0.5-1% of the population with marked geographical differences (Firestein *et al.* 2003, Aletaha *et al.* 2009). It is characterized by progressive inflammation of the joints, functional loss (joint stiffness), and an overall greatly reduced quality of life. Its management advanced considerably due to the introduction of biological therapeutics (primarily monoclonal antibodies), however pain management in this chronic disease can still pose a challenge to the clinician as potent analgesics within reach can easily induce addiction/tolerance (not to mention side effects), whereas most over the counter analgesics provide inadequate relief (besides having their own untoward side-effects during chronic administration). RA is a multifactorial disease, both genetical and environmental factors playing a role in its induction, however the ultimate triggering factor of the disease is still missing (Smolen *et al.* 2007).

5.1.2. The mechanism of rheumatoid arthritis - insights from preclinical and clinical observations

„CAPSICUM ANNUM: …it has lately been given with success in the advanced stages of acute rheumatism; in various diseases attended with cold feet, it has been recommended to wear socks dusted with Cayenne Pepper."

(J.A. Paris, Pharmacologia, Collins and Hannay, 1831, London)

Experimental evidences point towards a biphasic development of RA. In the initial phase of the disease genetic and environmental factors elicit the

proliferation of autoreactive T cells. In the second „effector" stage these T cells trigger joint inflammation, cartilage and bone damage via the activation of myeloid cells and synovial fibroblasts. The main connection between these two phases is a massive autoantibody and cytokine production (e.g. TNF and ILs) and activation of complement pathways (Jakus *et al.* 2010). This theory was corroborated by the fact that B cell depletion ameliorates RA (Edwards *et al.* 2004, Edwards *et al.* 2006), and by observing the potential of administered autoantibodies to elicit arthritis in preclinical models, such as in the K/BxN serum transfer model (Korganow *et al.*1999).

In the last decades several models of RA have been described which gradually improved by the time. Among these the K/BxN serum transfer model proved to be particularly suitable for studying the onset, and early inflammatory phase of the disease. It results in an immune-mediated polyarthritis, which can be induced in a number of native or transgenic mouse strain, unlike some other popular models (e.g. the collagen or proteoglycan induced arthritis) which work properly in a few or only a single strain (Asquith *et al.* 2009). As we have discussed earlier, its mechanism of action involves complement activation, mast cell degranulation, an neutrophil infiltration.

Immunological aspects predominantly involved in the inflammatory components of RA have been extensively studied. Although the importance of neurogenic inflammation in its pathophysiological mechanisms has already been suggested in the 1980s (Levine *et al.* 1985a,b), the mechanisms of chronic pain and sensitization, as well as the complexity of neuro-vascular and neuro-immune interactions received much less attention in this disease. The first mechanistic insights were predated, by early, crude objections made by simply observing the interesting but profound influence of nervous system diseases on RA. As early as the 1930s it was noted that stroke-sufferers who later develop RA present their symptoms in an unusual asymmetric way, as only the neurologically unaffected limbs display the signs of arthritis. This phenomenon was later noted in PNS disorders as well, such as in hemiplegia caused by e.g. polio or syphilis, and traumatic nerve dissection (see **Fig. 28.**) (Jacqueline *et al.* 1953, Thompson *et al.* 1962, Bland *et al.* 1968, Kane *et al.* 2005, Kim *et al.* 2012, Stangenberg *et al.* 2014).

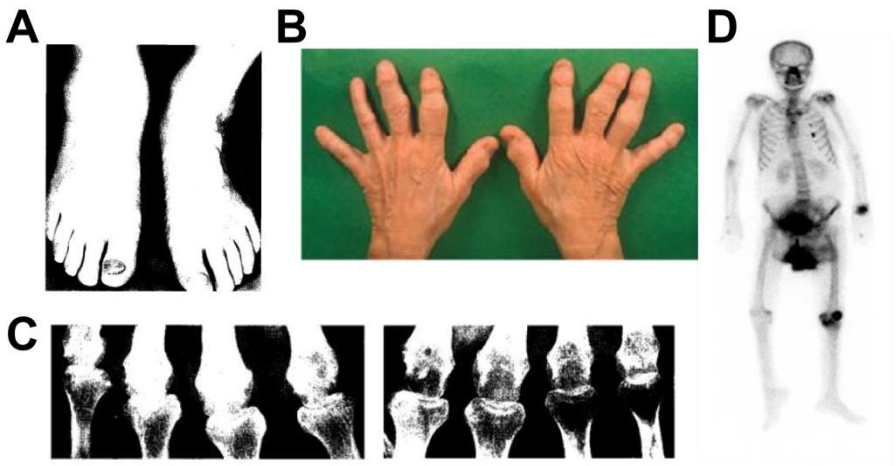

Fig. 28. Examples of the critical influence of nervous system lesion on the course of inflammatory arthritis. **(A)** The left ankle of a patient is unaffected by arthritic edema. The patient was partially hemiplegic due to an earlier attack or neurosyphilis *(Source: Thompson et al. 1962)*. **(B)** All fingers of a patient are affected by arthritis with the sole exception of the 4th on the left hand, which was deafferented by an earlier injury *(Source: Kane et al. 2005)*. **(C)** Radiography of the metacarpophalangeal joints of a patient hemiplegic due to a massive stroke sustained earlier. Arthritic bone thinning only affects the functioning hand (right) *(Source: Thompson et al. 1962)* **(D)** Bone scintigraphy scan of a patient suffering from an acute RA shub that evolved more than twenty years after a cerebral hemorrhage rendered her hemiplegic on the right side. In the wrist and knee joints of the paralysed side no signs of contrast enhancement can be observed *(Source: Kim et al. 2012)*

It is well known, that joints are densely innervated by both sympathetic and sensory nerve fibers, and that nociceptors are present throughout the joint capsule. Inflammation induces a sensitization of these elements, leading to hyperalgesia and allodynia (McDougall 2006b). Preclinically it was first observed in the adjuvant-induced joint inflammation that peripheral sympathectomy or neonatal systemic capsaicin administration (which defunctionalizes small-diamater afferents) reduces arthritis severity, whereas unilateral dorsal rhizotomy results in increased joint disease in the deafferented limb (Levine *et al.* 1986). According to the latest results a similar effect is observed in the K/BxN serum-transfer model. It has been known, that the effector phase of this model is predated by an early disease-initiating phase characterized by a rapid vascular permeability increase in and around the joints affected by the disease (Binstadt *et al.* 2006). So far no particular mediator, including immune cell subsets or various messengers (e.g. vasoactive amines

such as serotonine) could be labelled as the main culprit responsible for this important step during disease formation. It was also observed, that unilateral chronic total denervation completely abolished this early vascular response, almost completely preventing arthritis in the affected limb (Stangenberg *et al.* 2014). We have to note however that the time elapsed after the denervation proved to be of key importance, as acute denervation conversely increased arthritis severity. TRPV1 gene deficiency had no effect on the disease course, or on the impact of denervation. Similarly, administration of agents blocking cathecholaminergic or cholinergic signal transmission in the PNS resulted in no visible differences.

The possible bidirectional interaction of the proinflammatory cytokines and the nervous system has been suggested by many. The reason seems to be that large proportion of DRG neurons express TNF receptors. Moreover, intraarticularly administered TNF-alpha blockers (infliximab, etanercept) had profound effects on TRPV1 expression in the DRG besides alleviating hyperalgesia by decreasing C-fiber responsiveness (Schaible *et al.* 2010). A decade ago our group observed that RTX-desensitization or somatostatin antagonism increases edema severity, mechanical hyperalgesia, and joint damage assessed by histology in the complete Freund's adjuvant (CFA)-induced paw inflammation model of the rat. These results implied that peptidergic afferents, contribute significantly to the alleviation of joint inflammation by releasing anti-inflammatory mediators (incl. somatostatin) (Helyes *et al.* 2004). Recently, we could recapitulate these findings and provided a deeper insight into their mechanism in the K/BxN model, which provided an even more convincing evidence, that 1) the model is heavily influenced by nerve-driven mediators, and 2) these afferent qualities play a remarkable role in arthritis, and present an endogenous regulatory mechanism that can bidirectionally influence disease development. The main findings of these studies have been summarized in **Table 2**. These results neccesitated further investigation on the level of the released mediators, which ultimately culminated in the experiments discussed herein.

Model	**CFA**	**K/BxN serum transfer**
Species	rat	mouse
Effect of RTX desensitization		
Mechanical hyperalgesia	↑	↓(late phase)
Edema	↑	
Joint damage		
Somatostatin level	↓(plasma)	↓(joint)
Thermal hyperalgesia	not evaluated	absent in the model
MPO activity		↑
MMP-activity		
Bone reorganization		↑?*

Table 2. The summary of our earlier experiments implicating the important role of capsaicin sensitive peptidergic afferents in arthritis. *The effect on bone reorganization could not be decided clearly by the acute experiment, thus, further surveys using chronic models (e.g. repeated serum transfer or proteoglycan-induced-arthritis) might be warranted.

5.1.3. PACAP in inflammation and arthritis

Earlier results by our group provided evidence, that peripherally administered PACAP is anti-nociceptive in several acute pain models, but induces the sensitization of the knee joint primary afferents (Sándor *et al.* 2009). It has been postulated that PACAP activates $VPAC_1$/$VPAC_2$ receptors located on nociceptive nerve terminals within the articular capsule, thus increasing joint hypersensitivity similarly to VIP (McDougall *et al.* 2006b). PACAP also exerts a potent vasodilatory effect through the PAC_1 receptor and facilitates plasma leakage, edema formation and leukocyte egress (Warren *et al.* 1992, Svensjö *et al.* 2012). PACAP induces mast cell degranulation and histamine release, thereby contributing to edema formation (Schmidt-Choudhury *et al.* 1999). In

human polymorphonuclear granulocytes it also enhances respiratory burst, elastase, lactoferrin, and matrix metalloproteinase (MMP-9) -release (Harfi *et al.* 2004). Interestingly, it stimulates the activity of resting macrophages, whereas it inhibits activated cells (Delgado *et al.* 2003). PACAP also increases the expression of several neutrophil activation markers, such as CD11b, CD63 and CD66b, suggesting its role in the induction of neutrophil-activity during inflammation (Kinhult *et al.* 2002). Additionally, the shorter isoform PACAP-27 specifically induces neutrophil-chemotaxis through FPRL1 receptor activation and induces phagocyte-activation and Cd11b upregulation (Kim *et al.* 2006). PACAP gene-deficiency resulted in increased proinflammatory cytokine and chemokine production, but decreased anti-inflammatory cytokine synthesis in a mouse model of chronic autoimmune encephalomyelitis (Tan *et al.* 2009).

As a corollary, existing data suggest a surprisingly widespread and complex effect of PACAP on immune functions, which may be attributed to rapid changes of receptor expression profile during inflammation, as inflammatory mediators are known to upragulate some of its receptors (like FPRL1 or $VPAC_1$), thus causing diverging downstream effects (El Zein *et al.* 2008).

The effect of PACAP on bone physiology is an emerging research area. Currently only a handful of articles are available on this topic. The possible role of PACAP in bone metabolism has been first suggested more than a decade ago on the basis of the presence of its receptors in bone tissue: osteoclasts express the PAC_1 receptor, whereas bone marrow cultures express $VPAC_1$, $VPAC_2$ and PAC_1 (Ransjö *et al.* 2000). $VPAC_2$ is also expressed in osteoblastic cell lines. Both PACAP and VIP block osteoblast differentiation by inhibiting alcalic phosphatase production. They also enhance bone resorption by stimulating IL-6 production and thereby osteoclast-activity (Persson *et al.* 2005, Nagata *et al.* 2009). PACAP inhibits osteocalcin synthesis in osteoblasts and decreases bone formation (Kanno *et al.* 2005). It has a considerably greater binding affinity and elicits more potent activation at $VPAC_2$ receptors on osteblastic cells than VIP, underlining its importance in bone proliferation (Lundberg *et al.* 2001). It has recently been demonstrated, that both PACAP and VIP increase the RANKL (receptor activator of nuclear factor kappa-B ligand)/osteoprotegerin ratio through the activation of $VPAC_2$ osteoblasts, which in turn promotes osteoclastogenesis (Persson *et al.* 2011).

The possible involvement of PACAP in cartilage homeostasis is even less well understood. The first article dedicated to the role of PACAP in chondroprotection has only been published recently, indicating a beneficial effect of this peptide *in vitro* primarily by increasing calcineurin levels (Juhász *et al.* 2014).

5.1.4. The significance of tachykinins in arthritis

Due to their long history as key proinflammatory peptide mediators, SP and other tacyhkinins were implicated much earlier in inflammatory arthritis (Lotz *et al.* 1987, Garrett *et al.* 1992, Brain 1997, Keeble *et al.* 2004). This was based on clinical observations such as reporting significantly higher levels of SP in the serum but not in the synovial fluid of RA patients regardless of disease duration, severity, or other factors (Larsson *et al.* 1991, Anichini *et al.* 1997). SP has also been found to stimulate enzyme production (PGE_2, collagenase) and proliferation of synoviocytes from RA patients (Lotz *et al.* 1987). A later study corroborated, that SP increases cytokine-induced vascular cell adhesion molecule-1 (VCAM-1) expression via the NK1 receptor on cultured RA synoviocytes (Lambert *et al.* 1998), suggesting SP might facilitate inflammatory cell infiltration, cell proliferation, and joint damage. Thus, NK1 antagonism by the mid-1990s appeared to be a clinically relevant, tangible approach to alleviate joint disease in RA patients.

It is well known, that the joints and surrounding blood vessels are both innervated by SP-positive nerve fibers (Iwasaki *et al.* 1995). It has also been noted that SP facilitates mast-cell degranulation and leukocyte-transmigration from venules, which are important steps during the development of arthritis (Suzuki *et al.* 1995). Moreover, NK1 receptor antagonists have been shown to weakly constrict synovial blood vessels in non-arthritic joints and that this vasoconstrictor effect can be abolished by capsaicin-induced desensitization. The authors therefore proposed that continuous, „basal" release of SP/NKA from sensory afferents might provide a physiologic influence opposing the vasoconstrictor tone (Ferrel *et al.* 1997). It was also noted that SP is a potent inducer of joint angiogenesis, and that this effect can be abolished by selective NK1 antagonism (Seegers *et al.* 2003). Thus, it was hypothesised that SP might

also contribute to joint inflammation by influencing the vascular phase (Keeble *et al.* 2004). In the adjuvant arthritis model an early and transient increase of Tac1-expression was observed in the DRG (Garrett *et al.* 1995, Bulling *et al.* 2001), while expression in the joint synovium only became elevated in the chronic phase. However, this not necessarily mirrors an upregulation of SP which is produced by further post-translational modifications. Others reported that SP levels in the spinal dorsal horn are initially depleted in inflammatory arthritis, followed by an increase in the chronic stage (Sluka *et al.* 1993). Contradictorily, other authors described a reduction in SP-expressing nerves within the synovium using the same model, especially in the tissues heavily infiltrated by immune cells, suggesting that neuropeptide-releasing afferents may be destroyed during the course of the disease (Konttinen *et al.* 1992). In experimental monoarthritis both SP and NKA level increased in the synovial fluid, however this effect was also observed in the contralateral sham-treated joint (Bileviciute *et al.* 1993). In addition to the above discussed alterations of SP-expression during arthritis, a marked downregulation of NK1 receptors was demonstrated as well (Kar *et al.* 1994). In summary, these findings confirmed the presence and disease-specific up/downregulation of these mediators, but they provided little insight about their actual roles and effects.

The available *in vivo* results apparently contradict the expectations based on the *in vitro* results: in the adjuvant arthritis model mechanical hyperalgesia develops identically in NK1 receptor knockout mice (De Felipe *et al.* 1998). Interestingly, recent results by our group described a slightly decreased mechanical hyperalgesia in NK1 but not in SP/NKA-deficient mice, whereas paw swelling or cytokine-levels were unaltered. These results implied that NK1 contributes to the development of inflammatory pain, but this effect is mediated by endogenous agonists other than SP (Borbély *et al.* 2013). Earlier results pointed toward the same direction as well, as intraarticularly administered NK1 receptor antagonist reduced inflammatory joint pain, but only if it was injected topically and not systemically and prior to arthritis induction. This raised the possibility that NK1 agonism might contribute to the induction, but not to the maintenance of arthritic pain (Hong *et al.* 2002). In an other study spinally or systemically administered NK1 antagonists had no effect on mechanonociception, but decreased thermal hyperalgesia (Sluka *et al.* 1997).

5.1.5. Specific aim of the experiments

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 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.

5.2. RESULTS

5.2.1. Arthritic mechanical and thermal hyperalgesia

The control mechano- and thermonociceptive thresholds of wildtype and PACAP$^{-/-}$ mice were similar. A significant 20-25% mechanical hyperalgesia developed by day 5 after arthritis induction in wildtypes, which gradually vanished by day 9. This phenomenon was absent in either the arthritic PACAP gene-deficient mice, or sham-treated controls (**Fig. 29./A**). Arthritis did not result in a significant change of the thermonociceptive thresholds in any of the observed groups (**Fig. 29./B**). Thermal hyperalgesia was found to be absent in all later experiments using Tac1 and NK1 gene-deficient mice as well, thus it was determined that thermal hyperalgesia does not constitute a general feature of this model.

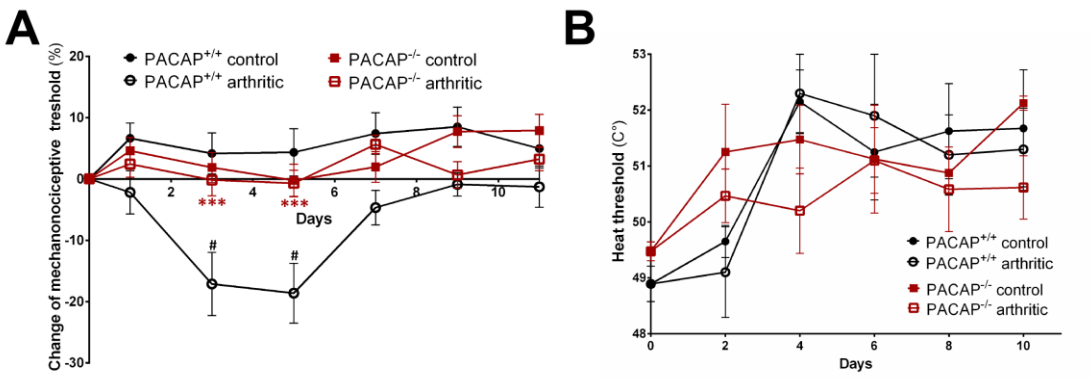

Fig. 29. Evaluation of nociceptive changes in PACAP$^{-/-}$ mice and the respective wildtype controls. **(A)** Mechanonociceptive threshold measured on the hindpaw (n=8-12/group). **(B)** Thermonociceptive threshold on the increasing temperature hot plate in °C (n=4-6/group; repeated measures ANOVA + Tukey's post-test; ***p<0.001 vs. the respective wildtype group, $^{\#}$p<0.05 vs. the respective self-control).

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, peak values representing a threshold drop of 40-45% (**Fig. 30.**)

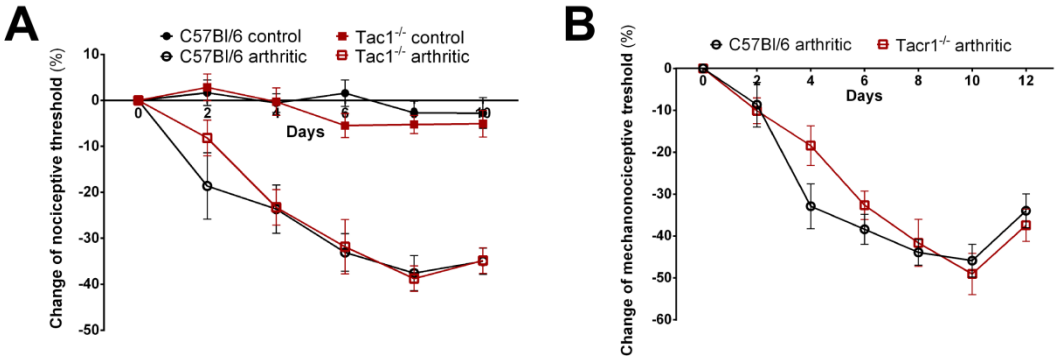

Fig. 30. Mechanonociceptive alterations of **(A)** Tac1$^{-/-}$ arthritic mice and C57Bl/6 wildtypes, **(B)** Tacr1$^{-/-}$ animals. (n=5-8/group).

5.2.2. Hindlimb edema and disease severity

Remarkable hindpaw edema developed in wildtypes after the induction of arthritis by 150 µl arthritogenic serum, which peaked on day 3 (40%) and gradually decreased thereafter. The hindpaw edema was markedly reduced in PACAP$^{-/-}$ mice (peak value: ~20%). The edema was not only significantly

smaller, but the kinetics was also slower, as it reached its maximum on day 5 (**Fig. 31./A**). Semiquantitative clinical scoring of hindlimb edema and hyperemia yielded a comparable outcome, peak values observed on day 3-4 in wildtypes and on day 5 in PACAP$^{-/-}$ ones. PACAP$^{-/-}$ mice had overall significantly lower arthritis severity scores (**Fig. 31./B**).

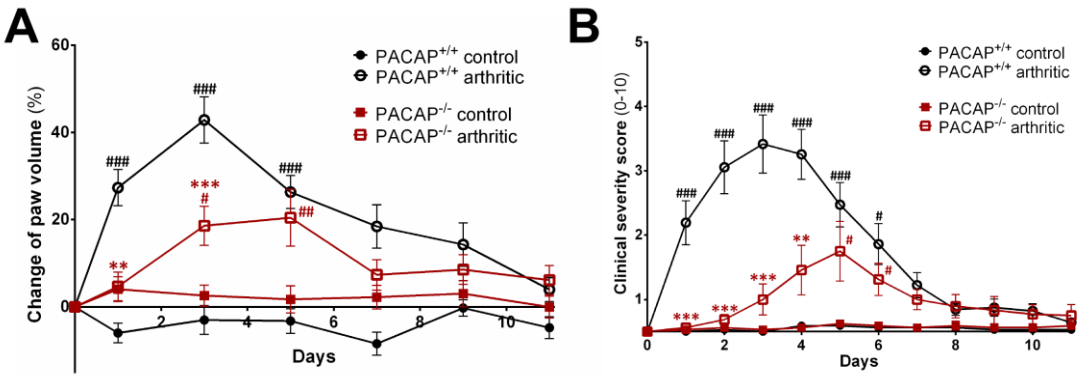

Fig. 31. Investigation of edema and clinical disease severity in PACAP$^{-/-}$ arthritic mice and their respective wildtypes. **(A)** Plethysmometric determination of the hindpaw volumes. **(B)** Clinical scores of the disease severity. (n=8-12/group; repeated measures ANOVA + Tukey's post-test; **p<0.01 , ***p<0.001 vs. the respective wildtype group, $^{\#}$p<0.05 $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. the respective self-control)

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 (**Fig. 32.**). In the experiment involving Tacr1 knockout mice a different pooled K/BxN serum stock was used, thus somewhat slower disease kinetics was observed, though mice reached overall the similar degree of arthritis severity.

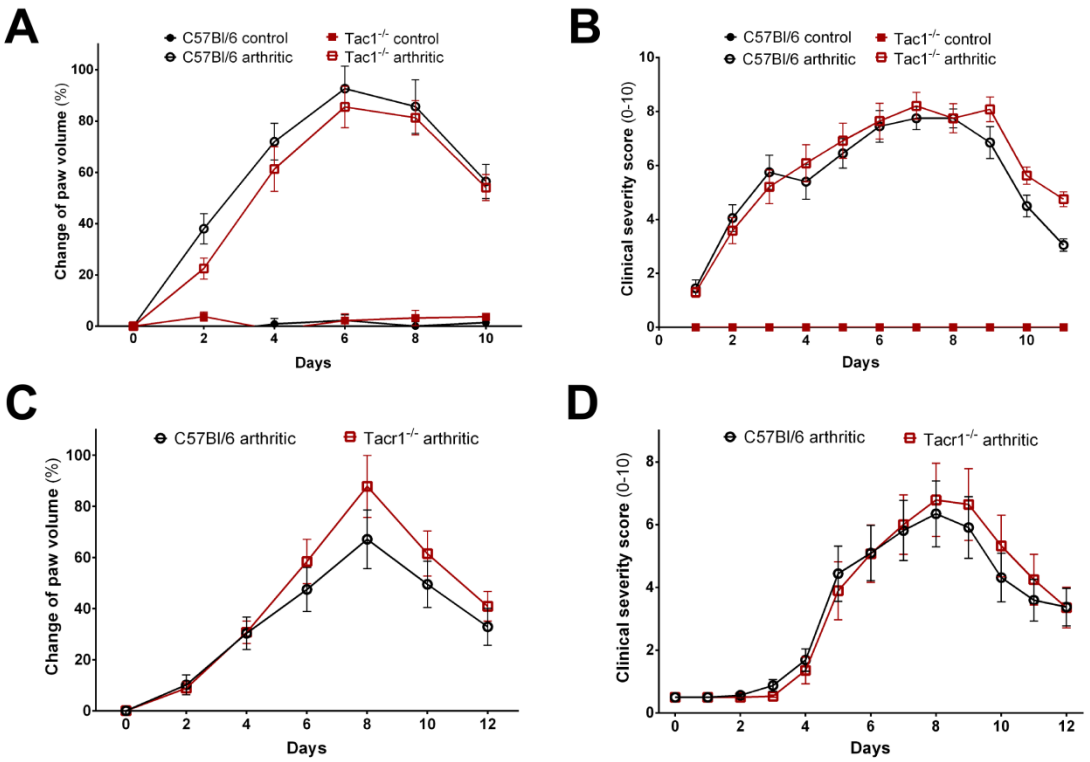

Fig. 32. Hindpaw edema and clinical disease severity in **(A)-(B)** Tac1$^{-/-}$ arthritic mice and C57Bl/6 wildtypes, **(C)-(D)** Tacr1$^{-/-}$ animals. (n=5-8/group)

5.2.3. Microvascular plasma leakage in the inflamed hindlimbs

The pre-treatment ICG fluorescence intensity was similarly low in the ankle joints of the two groups, demonstrating negligible extravasation of the dye formula. Two days after the induction of arthritis the accumulation and fluorescence of ICG increased remarkably in the ankle joints of wildtypes both immediately after injection and 1 hour later, indicating hyperemia and plasma leakage (80% increase at 5 minutes, 170% at 1 hour post-injection compared to initial control data). In contrast, in PACAP-deficient animals, the rise was significantly less pronounced (25% increase at 5 minutes, 65% after 1 hour) (**Fig. 33.**). By day 5 ICG-fluorescence increased even further in both wildtypes (120% at 5 minutes, 320% after 1 hour) and PACAP$^{-/-}$ mice (60%, 130% respectively) (**Fig. 34.**).

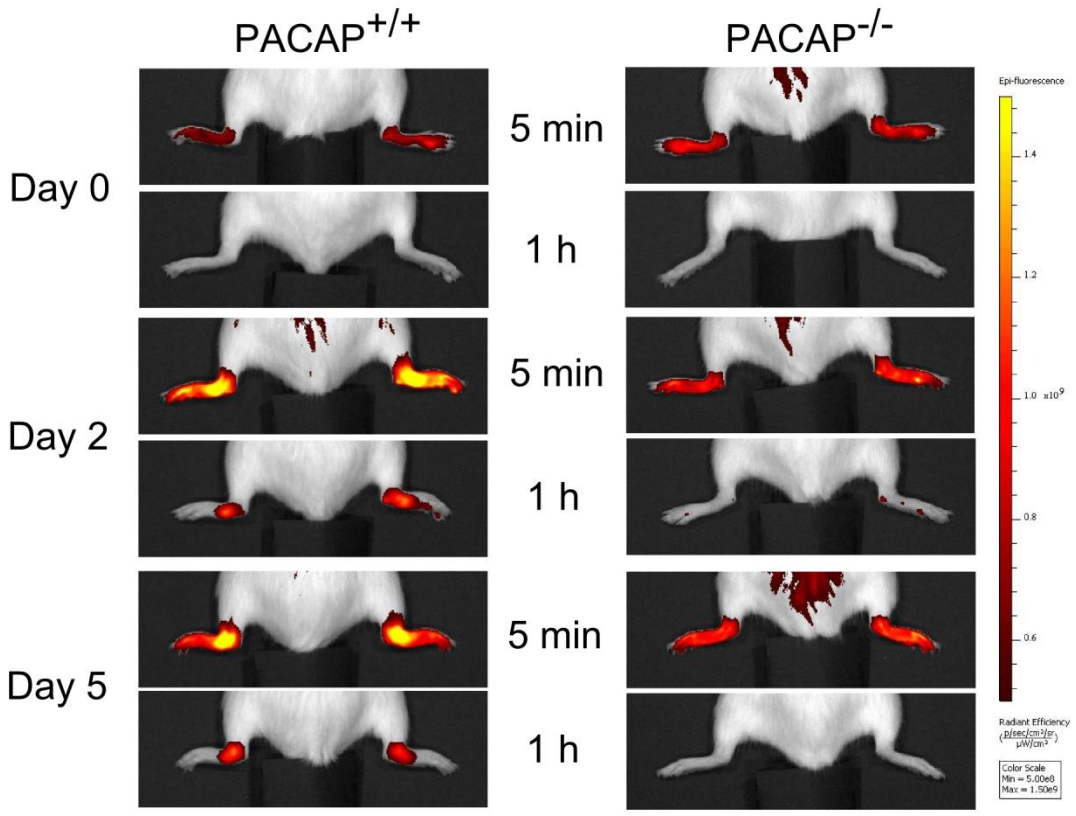

Fig. 33. Representative images of ICG in the ankle joints taken 5 and 60 minutes post-injection.

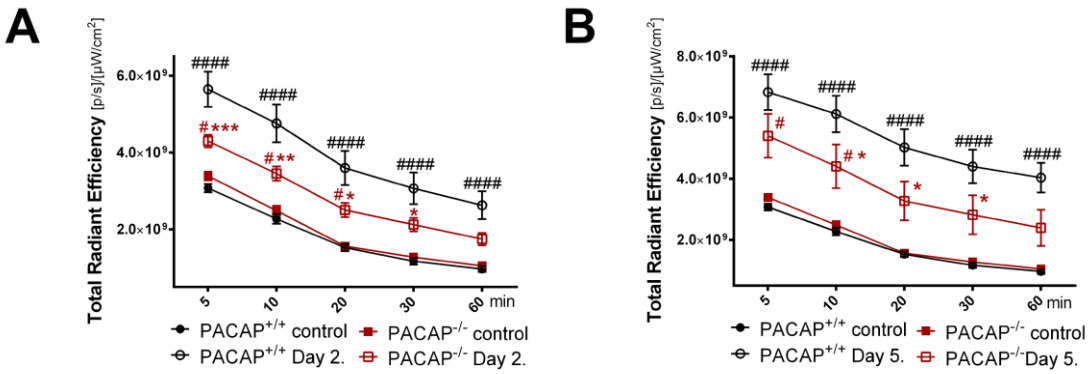

Fig. 34. ICG-fluorescence intensity in the ankle joint in the intact state, 2 and 5 days after the induction of arthritis. (n=5-6/group; two-way ANOVA + Tukey's post-test; *p<0.05, **p<0.01 , ***p<0.001 vs. the respective wildtype group, $^{\#}$p<0.05, $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001 vs. the respective self-control)

5.2.4. Alteration of grasping ability and motor coordination

The horizontal wire grid grip-test revealed an abrupt decrease of grasping ability in wildtypes, by day 4, as only 30% could stay on the grid for 20 s. In contrast, 75-80% of the PACAP$^{-/-}$ animals could stay on the grid during this period (**Fig. 35./A**). Motor performance on the Rota-Rod gradually improved in

all groups during the experiment demonstrating learning, being significant in the PACAP$^{+/+}$ but not in the gene-deficient group (**Fig. 35./B**). Therefore it was concluded that the Rota-Rod test is inadequate to measure functional incapacitance in this model thus we abandoned this method in the later experiments.

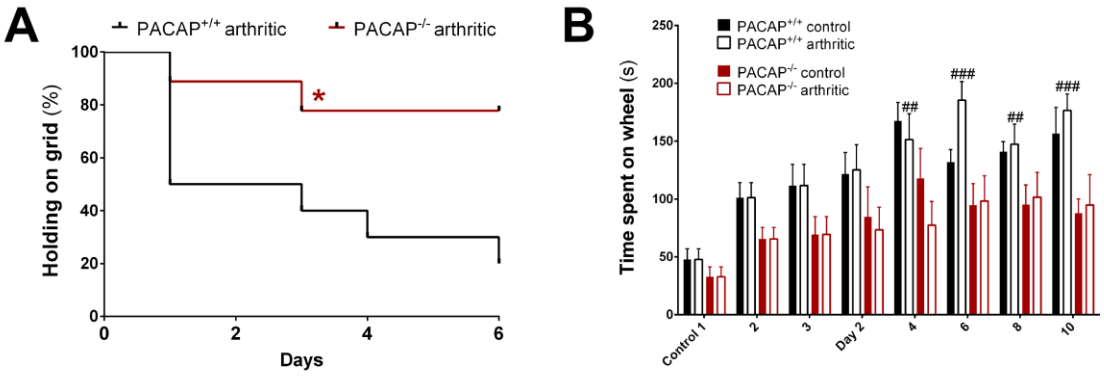

Fig. 35. (A) Kaplan-Meier curve of wire-grid holding ability of PACAP$^{-/-}$ and wildtype arthritic mice for 20s (n=8-12/group; logrank test). Animals were habituated by 3 control-sessions prior to the induction of arthritis. **(B)** Motor performance on the accelerating Rota-Rod during the 3 consecutive control measurements, and following the induction of arthritis. (n=4-6/group; *p<0.05 vs. the respective wildtype group, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, vs. the respective self-control)

The performance of Tac1 and Tacr1 gene-deficient mice proved to be indifferent from their wildtype controls, loss of grasping ability was complete by day 6 owing to the higher dosage of the K/BxN serum administered (**Fig. 36.**)

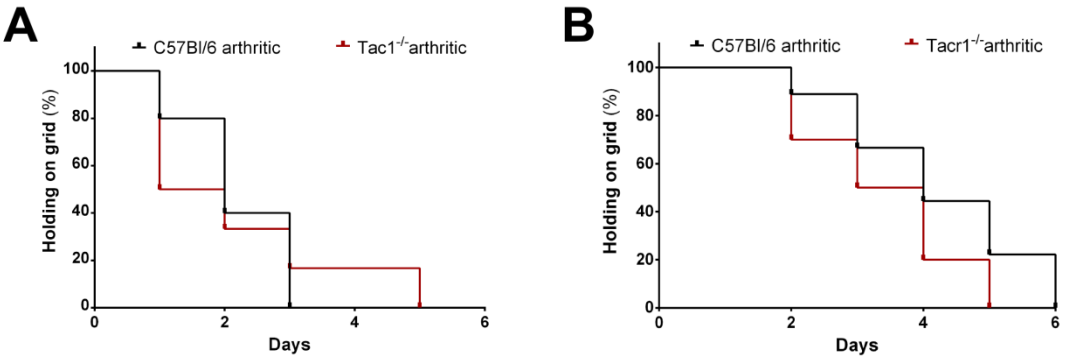

Fig. 36. Kaplan-Meier curve of the grasping ability of **(A)** Tac1$^{-/-}$ and **(B)** Tacr1$^{-/-}$ mice compared to C57Bl/6 wildtypes. (n=5-8)

5.2.5. Neutrophil-derived MPO-activity and hydrogen peroxyde generation

Bioluminescence imaging of luminol 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. The initially diffuse activity in the hindpaws rapidly declined and concentrated in the tibiotarsal joints by day 4. In contrast, in PACAP$^{-/-}$ mice early MPO-activity was significantly smaller, but by day 4 it became significantly greater in the ankles (**Fig. 37.**).

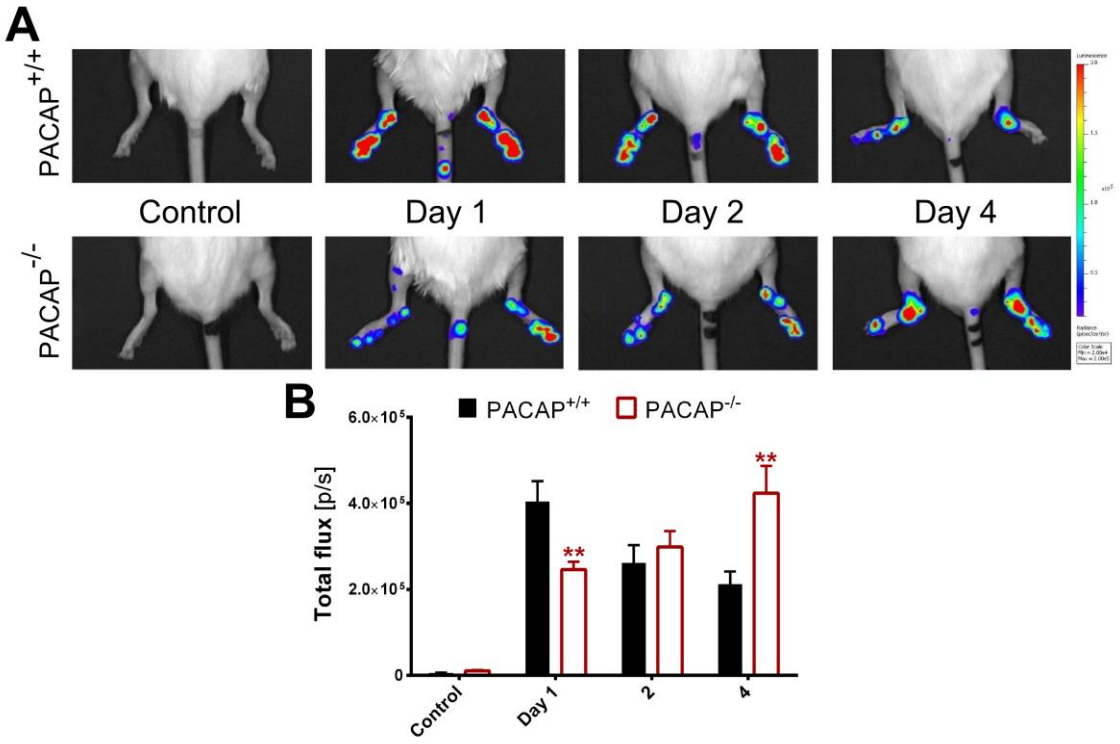

Fig. 37. Bioluminescence imaging of neutrophil-derived MPO-activity in PACAP$^{-/-}$ mice and their wildtype controls. **(A)** Representative images taken 10 min after ip. injection of luminol and **(B)** quantification of luminescence in the diseased joints. (n=4-6/group; Student's t-test; **p<0.001 vs. the respective wildtype group).

In the experiment involving NK1 gene-deficient mice and their C57Bl/6 wildtype counterparts the arthritis onset was delayed mirroring the functional results, however there was no difference between the two groups in neither the early, nor the late phase (**Fig. 38.**).

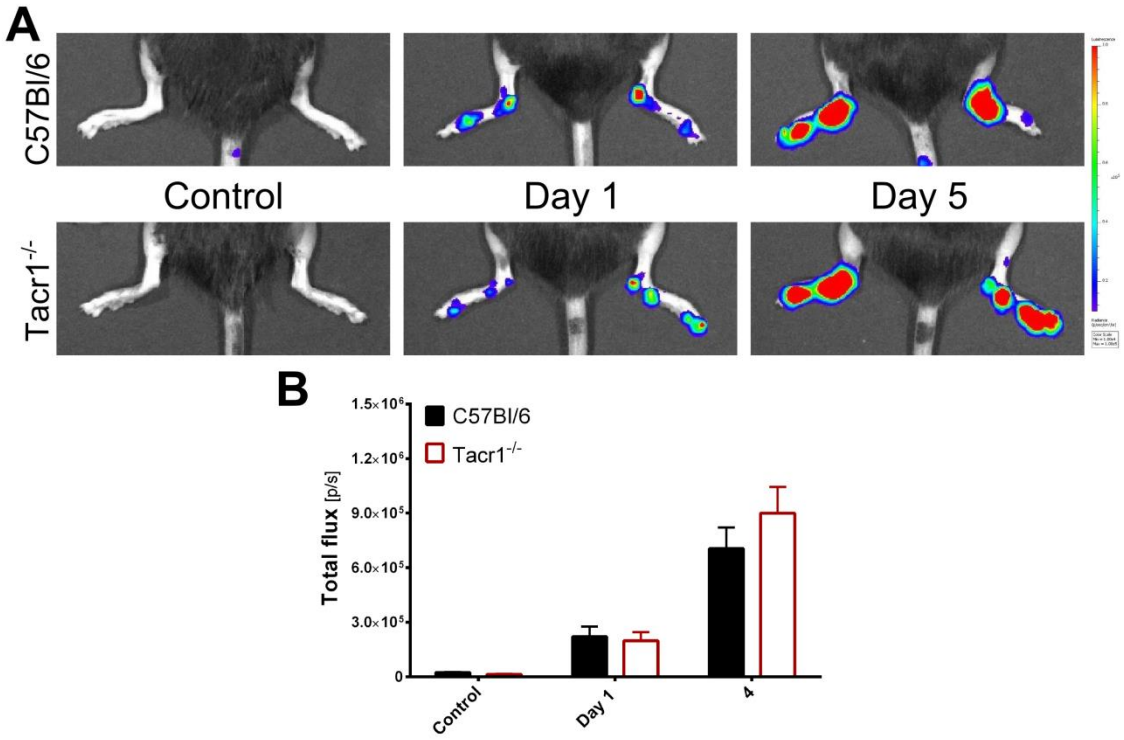

Fig. 38. Bioluminescence imaging of neutrophil-derived MPO activity in Tacr1$^{-/-}$ gene-deficient mice and wildtypes. **(A)** Representative bioluminescence images and **(B)** quantification of luminescence in the diseased joints.

5.2.6. Macrophage-derived superoxide production

In PACAP$^{+/+}$ mice, lucigenin-based bioluminescence imaging indicating extracellular superoxide production increased steadily reaching its maximum on day 6. Its elevation was slower compared to that of luminol highlighting the differences between these markers. Superoxide generation in PACAP$^{-/-}$ mice remained similar to the initial controls, being significantly lower than in wildtypes (**Fig. 39.**).

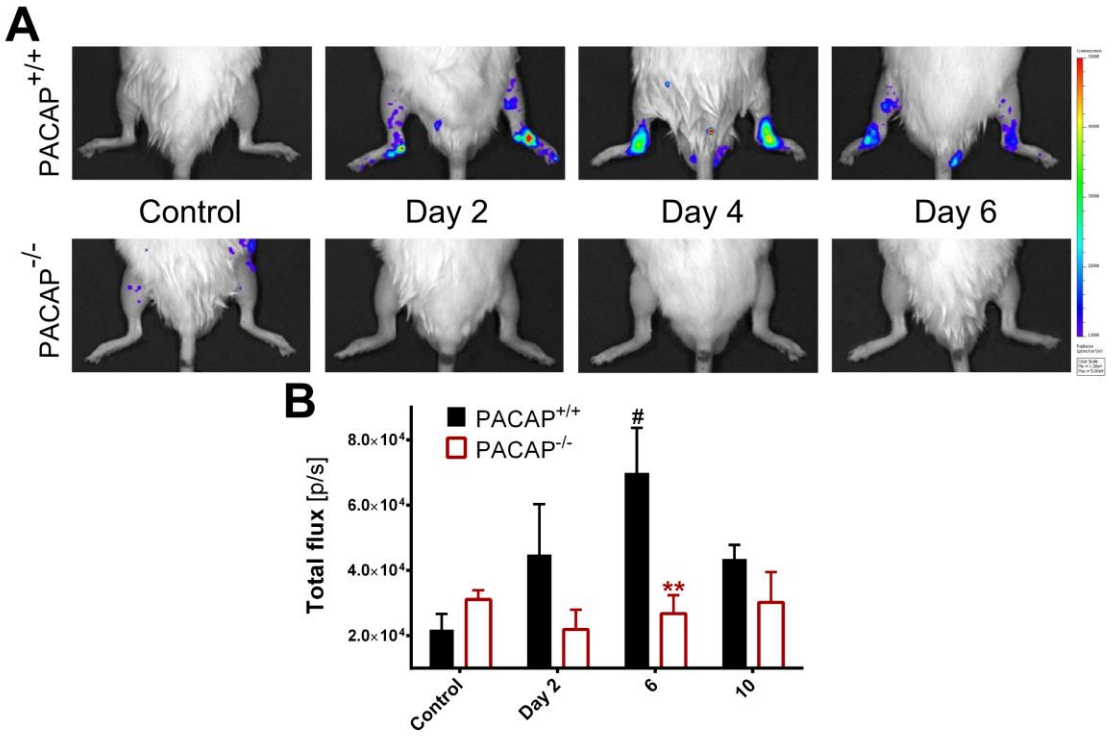

Fig. 39. Bioluminescence imaging of macrophage-derived superoxide activity. **(A)** Representative images and **(B)** quantitative evaluation of the total luminescence in the ankle joints. (n=4-6/group; Student's t-test; **p<0.01 vs. the respective wildtype group, #p<0.05 vs. respecive internal control)

5.2.7. 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 in both the talocrural and distal periarticular regions of the tibia and fibula. Their bone surface density expressed as Bone Surface/Total Volume ratio (BS/TV) was similar to the age-matched wildtypes. Arthritis did not remarkably alter the bone structure in neither PACAP$^{+/+}$ nor PACAP$^{-/-}$ mice in the region of the ankle joint (**Fig. 40-41.**)

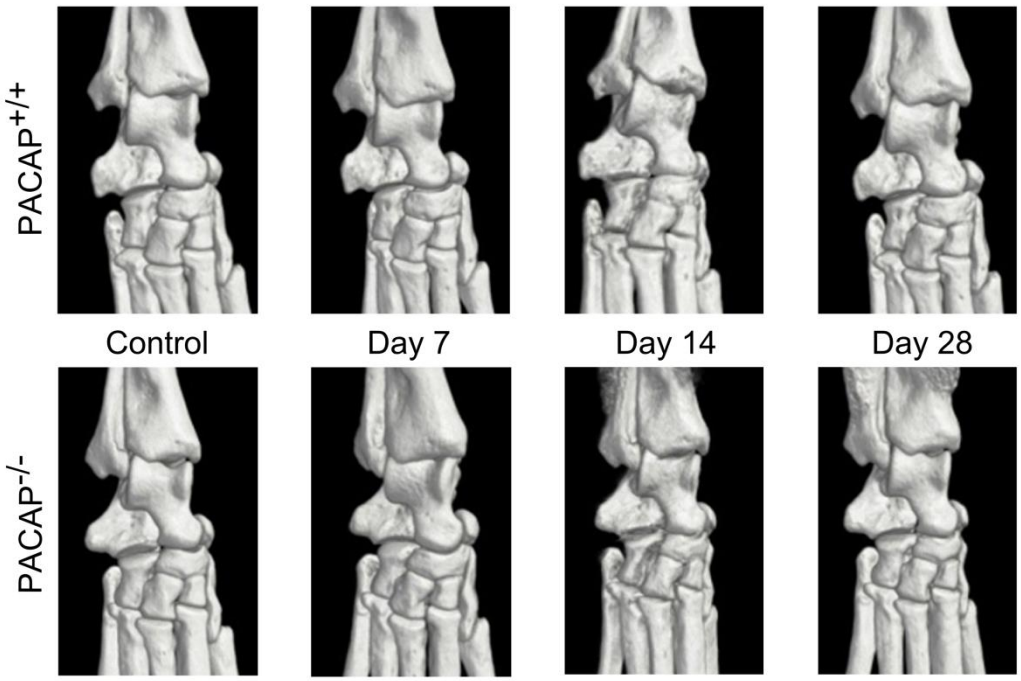

Fig. 40. Representative 3D micro-CT reconstruction of the tibiotarsal joints.

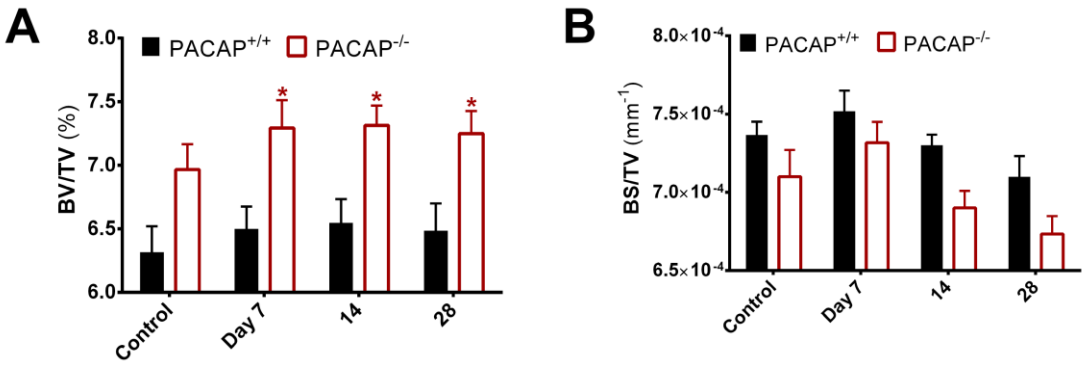

Fig. 41. Change of **(A)** Bone Volume/Total Volume (BV/TV) ratio and **(B)** bone surface density (BS/TV) throughout the study in the vicinity of the tibiotarsal joint. (n=6/group; two-way ANOVA+ Tukey's post-hoc test; *p<0.05 vs. respective wildtype)

In contrast, in PACAP$^{-/-}$ mice it induced extensive, progressive osteophyte formation in the periarticular region of the tibia and fibula by the 14th day (**Fig. 42.**). These bone spurs turned into compact, dense bone by day 28 leading to a prominent, significant increase of bone mass, even reaching a 70% extra bone in some subjects compared to the initial controls (**Fig. 43.**).

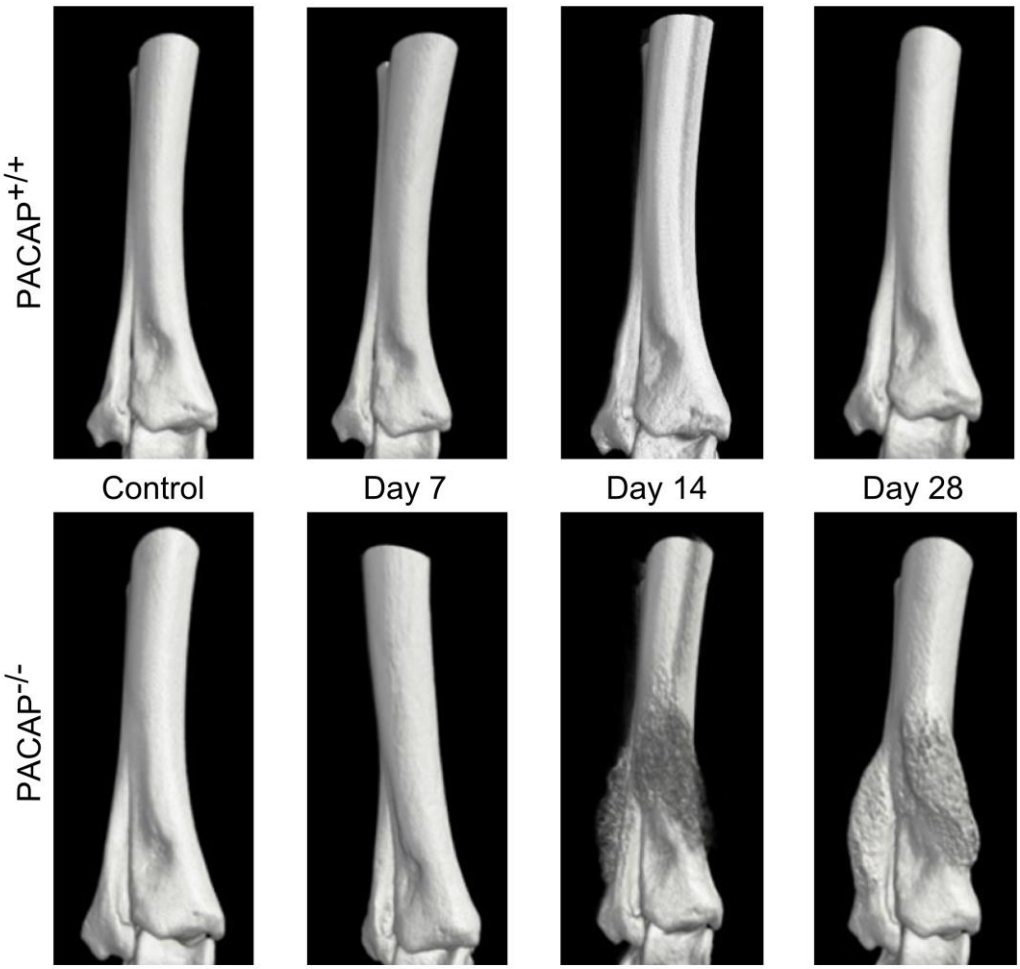

Fig. 42. Representative 3D micro-CT reconstructions of the periarticular region of the tibia and fibula.

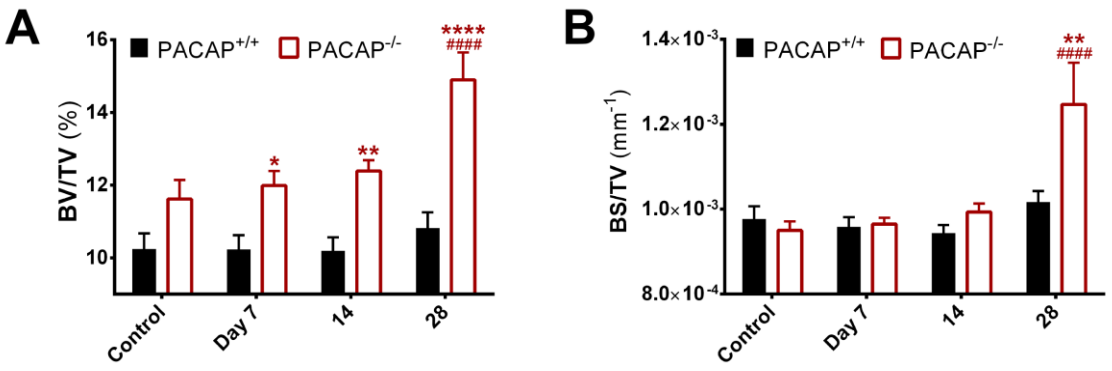

Fig. 43. Change of **(A)** Bone Volume/Total Volume (BV/TV) ratio and **(B)** bone surface density (BS/TV) throughout the study in the the periarticular region of the tibia. (n=6/group; two-way ANOVA + Tukey's post-hoc test; *p<0.05, **p<0.01, ****p<0.0001 vs. respective wildtype, ####p<0.0001 vs. respective self-control)

5.2.8. Histopathologic alterations in the ankle joints

Analysis of the joints revealed no difference between control serum-treated PACAP$^{+/+}$ and PACAP$^{-/-}$ mice, the tibiotarsal joint (**Fig. 44.**), the synovium (**Fig. 45.**), and the periarticular connective tissue (**Fig. 46.**) all appeared to be normal. 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 the PACAP$^{-/-}$ mice (**Fig. 47.**).

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 (**Fig. 44.**). The previously infiltrated synovial lining and connective tissue showed fibroblastic transformation and prominent collagen-deposition indicating the chronic stage of the inflammation (stained blue; **Fig. 46.**).

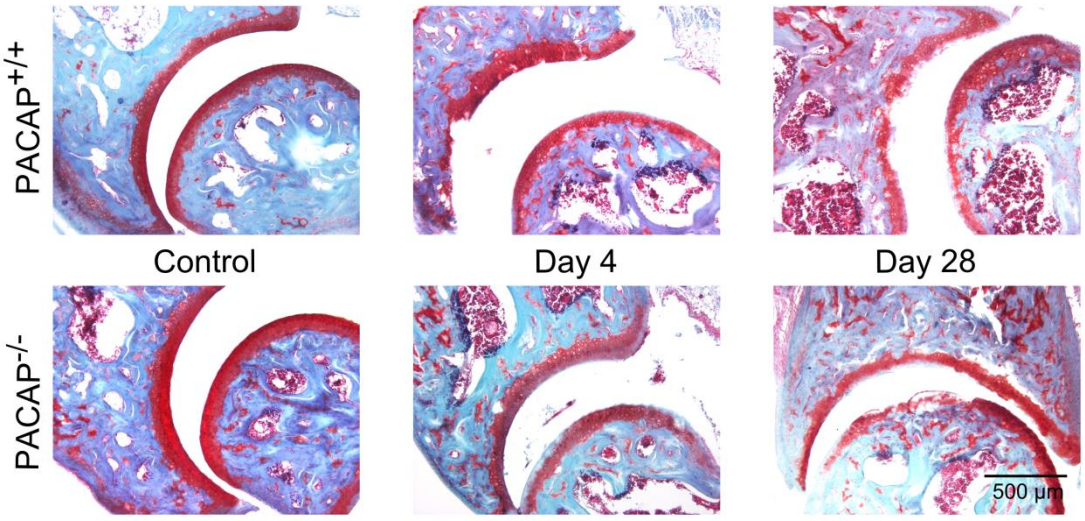

Fig. 44. Representative images of the tibiotarsal joints, showing the cartilage lining.
(Magnification: 40x, Scale bar: 500 µm, Safranine O staining)

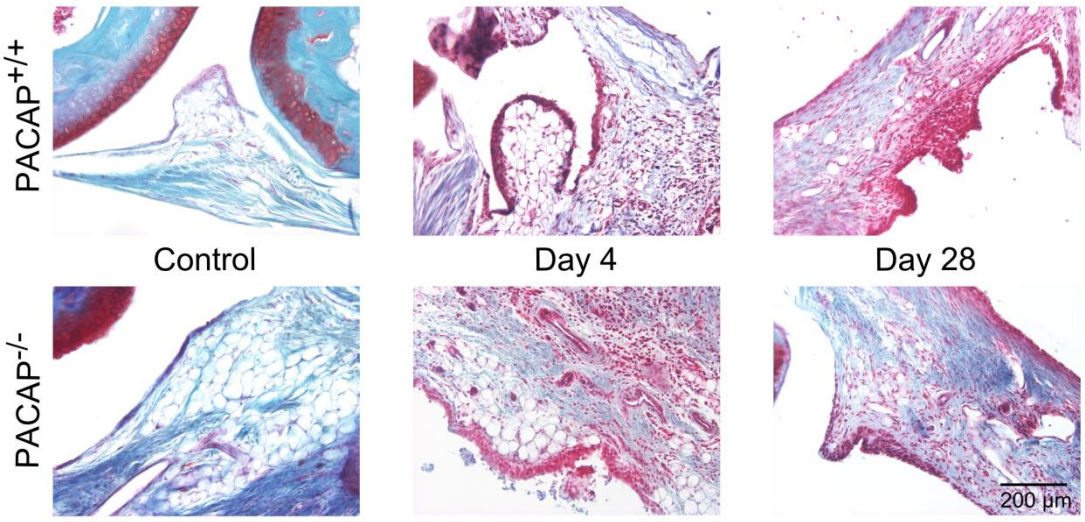

Fig. 45. Microphotographs of the synovium of the ankle joint.(Magnification: 100x, Scale bar: 200 µm Safranine O staining)

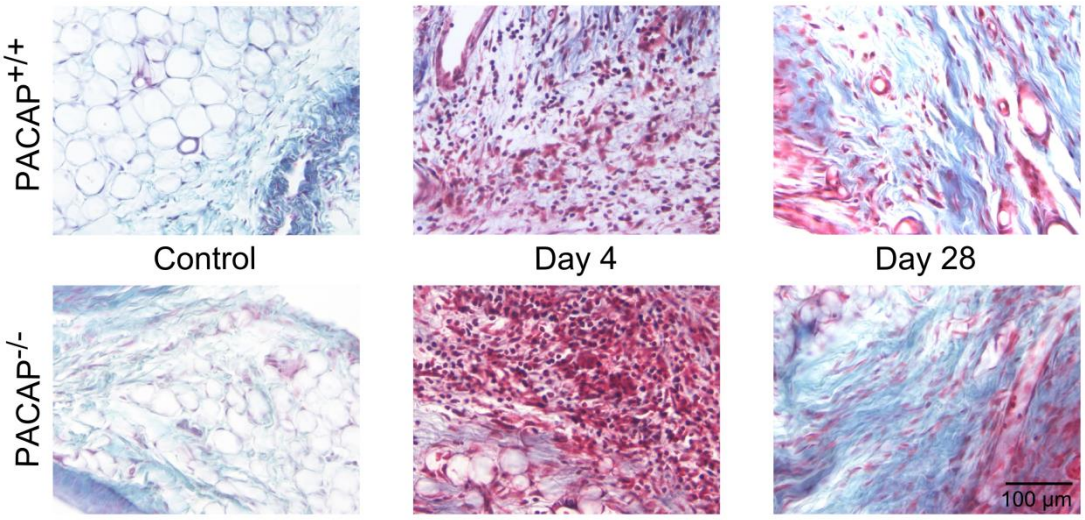

Fig. 46. Representative images of the periarticular soft tissue (Magnification: 200x, Scale bar: 100 µm, Safranine O staining)

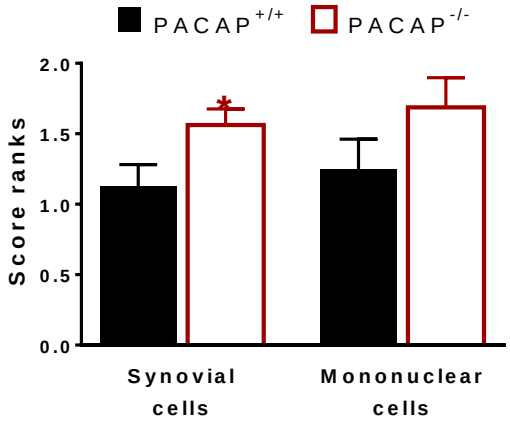

Fig. 47. Semiquantitative histopathologic scores of synovial hyperplasia and mononuclear cell infiltration on day 4. (n=4-6/group; Mann-Whitney U-test; *p<0.05 vs. respective wildtype)

The pathological spurs in PACAP$^{-/-}$ mice demonstrated in the micro-CT scans were also identified by their irregularity, distinct staining and remarkable vascularization in the histological slides (**Fig. 48.**). This phenomenon was absent in wildtype arthritic mice.

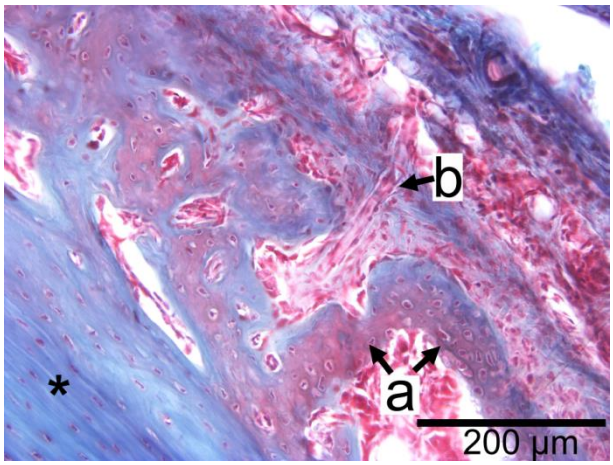

Fig. 48. Representative image of a bone spur observed in the PACAP$^{-/-}$ animals by the 28th day. The tibia is marked by an asterisk (*****), the arrows show **a)** irregular, cell-rich new bone, and **b)** a vessel entering into the affected area. (Magnification 200x, Scale bar: 200 µm Safranine O staining)

5.3. DISCUSSION

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 deficiency decreases vasodilation, plasma leakage, inflammatory cell accumulation, hyperalgesia, functional loss and ROS generation, while facilitating 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.

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 *in vivo* functional and optical imaging techniques, as well as self-controlled experimental paradigm that help to identify key pathophysiological mechanisms in inflammatory and degenerative joint diseases.

5.3.1. PACAP promotes arthritic edema formation by increasing vasodilation and microvascular permeability

We have observed a dramatic reduction of edema and disease severity in PACAP$^{-/-}$ mice, suggesting released PACAP from sensory nerve terminals contributes to the formation of the inflammatory microenvironment. These results are in agreement with our laser Doppler flowmetry findings upon AITC-challenge.

The potent edema-forming and acute inflammatory action of PACAP may be explained by its well-established vasoactive effect (Warren *et al.* 1992, Schytz *et al.* 2009, Schytz *et al.* 2010a, Svensjö *et al.* 2012). The *in vivo* imaging provided further validation, as arthritic gene-deficient mice displayed decreased vascular leakiness. On a receptorial level both PAC_1 and $VPAC_{1/2}$ are likely to be involved in downstream mediation of the vascular effects of PACAP: VPAC-driven pathways interact with the nitric oxide synthesis in eliciting vasodilation (Kellogg *et al.* 2012) and the PAC_1 receptor is of key importance for plasma leakage induction (Svensjö *et al.* 2009, Svensjö *et al.* 2012).

5.3.2. PACAP is a potent mediator of arthritic mechanical hyperalgesia

The absence of arthritic hyperalgesia in PACAP gene-deficient mice correlates with previous results of our and other groups demonstrating that PACAP has a pronociceptive role in diverse pain conditions, where sensitization mainly occurs in the spinal dorsal horn (Ohsawa *et al.* 2002, Mabuchi *et al.* 2004, Sándor *et al.* 2009, Schytz *et al.* 2010a, Sándor *et al.* 2010). A special emphasis must be placed on the PAC_1 receptor activation within the CNS, as intrathecal administration of the relatively selective PAC_1 receptor antagonist PACAP6-38 diminished hyperalgesia in a rat osteoarthritis model (Davis-Traber *et al.* 2008).

5.3.3. PACAP aggravates the loss of joint function

The almost normal grasping ability of arthritic PACAP knockout mice is likely to be due to milder joint swelling and pain. We did not find disease-related alterations of motor coordination on the Rota-Rod in our experiments. This reiterates our earlier results obtained using the peripheral neuropathy model (Chapter 1). The contradictorily retained motor performance during an apparently ongoing painful condition is likely to be attributed to the fact, that motor coordination is primarily a CNS process, and the inflammation of the small joints or a peripheral neuropathy is unable to influence this parameter. Moreover, the K/BxN model initiates an inflammatory arthritis, but poorly mimics the joint dysfunction (stiffness) characteristic of the chronic arthritis. Indeed, others relying on degenerative joint disease models found the Rota-Rod test to be useful for disease evaluation (Allen *et al.* 2009).

5.3.4. PACAP exerts a biphasic effect on neutrophil activity, and facilitates macrophage ROS-production

We found that the neutrophil-derived MPO-activity in PACAP$^{-/-}$ mice followed a biphasic pattern. In the early phase it was lower than in wildtypes, which is in correlation with the functional results, but virtually contradicts the traditional view about the inhibitory effects of PACAP on immune cells (Delgado *et al.* 2003). In the later, subacute phase of the arthritis the MPO-activity of knockouts became greater than their wildtype controls. The possible explanations for the observed phenomenon are 1) increased migration of immune cells into the inflamed tissue during the initial phase of the disease, mainly due to the PACAP-driven capillary permeability-increase mediated through PAC_1 receptor activation (Warren *et al.* 1992, Schmidt-Choudhury *et al.* 1999, Svensjö *et al.* 2009), and 2) the key importance of PACAP-27 (and possibly -38) to facilitate neutrophil chemotaxis and migration during the onset of the inflammation (Kim *et al.* 2006). Thus, we can conclude, that the proinflammatory effects of PACAP lead to a more rapid formation of the inflammatory microenvironment (Kinhult *et al.* 2002, Harfi *et al.* 2004). In comparison, the increased neutrophil-derived MPO-activity in the later phase

suggests that PACAP inhibits neutrophil activity by suppressing proinflammatory cytokine production and increasing the expression of anti-inflammatory mediators (Delgado *et al.* 2003, Tan *et al.* 2009). This is in agreement with previous results demonstrating that exogenous administration of PACAP reduces disease severity and inflammatory enzyme production in the collagen-induced arthritis model (Abad *et al.* 2001). However, in the collagen-induced arthritis T and B cell responses are essential. In contrast, the K/BxN serum-transfer arthritis depends mainly on myeloid lineages and develops similarly even if T and B cells are completely absent (since the autoantibodies are produced exogenously) (Jakus *et al.* 2010). The significantly smaller extracellular superoxide production and NADPH-oxidase activity in the PACAP gene-deficient mice suggests, that PACAP might stimulate macrophage-activity in arthritis thereby having a proinflammatory effect on phagocytes.

It is woth noting that the maximal inflammatory cell activity was seen on the 1th day as shown by luminol-bioluminescence. It is surprising, since this is a very early phase of the disease, when functional changes are minimal. This highlights that it is necessary to investigate the early period to elucidate mechanisms preceeding edema formation and functional loss.

5.3.5. PACAP is a positive regulator of pathologic bone neoformation

The effects of PACAP on bone/cartilage metabolism and turnover has not been thoroughly investigated *in vivo* under either normal or arthritic conditions. Therefore, our study provides substantial new evidence about PACAP's crucial role in bone pathophysiology. We revealed this peptide is a key regulator of bone turnover, particularly in inflammation. There are few, exclusively *in vitro* data describing the ability of PACAP to inhibit osteoblast precursors (Persson *et al.* 2005, Nagata *et al.* 2009) and to diminish osteoclastogenesis (Persson *et al.* 2011). Taken together these findings and our results both suggest that PACAP plays a crucial role in chondro- and osteogenesis by regulating bone formation and inhibiting pathological osteophyte growth. Osteophyte-formation in the late phase of the experiment indicates, that this model has translational relevance regarding the degenerative

complications of RA if maintained for a prolonged period by repeated K/BxN serum challenges.

5.3.6. PACAP moderates synovial cell hyperplasia

Synovial hyperplasia is a characteristic and widely-investigated feature of RA. It arises due to the formation of disinhibited fibroblast-like synoviocytes (FLS), that express NFκ-B and secrete several inflammatory cytokines and adhesion molecules (IL-6, CCL-2, VCAM-1, ICAM-1). Consequently, there is an influx and accumulation of inflammatory cells, that produce cytokines, chemokines and proteases (MMPs, cathepsins etc.) leading to cartilage and bone destruction. It was recently demonstrated, that in the K/BxN serum-transfer model FLS develop and behave similarly to human RA (Hardy *et al.* 2013). This, taken together with the increased synovial hyperplasia observed in knockouts, suggests that PACAP might decrease FLS-formation having a protective effect in chronic arthritis. This is also supported by the pathological bone neoformation in $PACAP^{-/-}$ mice, which can be at least partially attributed to disinhibited FLS-derived mediators.

5.3.7. Tac1 gene-derived tachykinins and the NK1 receptor are not paramount to the K/BxN serum-transfer arthritis

Our results indicate, that neither SP/NKA, nor the NK1 receptor deficiency impacts the development or severity of this arthritis model. This is in good agreement with our earlier negative results discussed extensively in the previous chapter. As we have elaborated earlier, a significant evidence pointed towards the marked involvement of SP and the NK1 receptor in arthritis (Lotz *et al.* 1987, Garrett *et al.* 1992, Brain 1997, Keeble *et al.* 2004). This was based on both clinical reports showing elevated serum SP levels in RA patients (Anichini *et al.* 1997), but also on experimental results demonstrating the presence of SP-positive nerves around the joints (Iwasaki *et al.* 1995), which taken together with its known vasoactive, proinflammatory and pronociceptive effects suggested SP as a key mediator of edema formation and arthritic pain (Sluka *et al.* 1993,

Suzuki *et al.* 1995, Ferrel *et al.* 1997, Bulling *et al.* 2001, Seegers *et al.* 2003, Keeble *et al.* 2004).

However, functional investigations revealed a less pronounced effect, as adjuvant-arthritis and concomitant hyperalgesia develops similarly in the absence of the NK1 receptor (De Felipe *et al.* 1998). Also in the adjuvant arthritis locally administered NK1 antagonists produced only a partial analgesia (Uematsu *et al.* 2011). It was found however that Tac1 (and also Tac4) gene expression changes only slightly in the DRG and spinal cord of mice suffering from autoimmune arthritis, while NK1 antagonists were not effective in relieving the mechanical hyperalgesia, although they slightly attenuated joint swelling (Makino *et al.* 2012). Our group has also described a modest decrease of arthritic hyperalgesia in NK1 but not in Tac1 knockout mice in the adjuvant arthritis model, with unaltered disease severity (Borbély *et al.* 2013). Results obtained with NK1 antagonists also proved that these compounds only have a modest influence on arthritic mechanical hyperalgesia (Sluka *et al.* 1997, Hong *et al.* 2002), and that NK1 antagonism does not affect TRPA1-mediated neurogenic vascular responses (Pozsgai *et al.* 2010) Taken together these results, we conclude that experimenters relying on simple inflammation models usually found a convincing effect of SP/NKA and the NK1 receptor, whereas those who used translational disease-mimicking models reported a less remarkable effects, similary to our results. Most importantly the authors of a recently published paper also found an unaltered disease course in Tac1 knockout mice using the same K/BxN serum transfer experimental paradigm as ours (Stangenberg *et al.* 2014).

5.3.8. Conclusions

1. PACAP affects arthritis development in a complex manner. It increases hyperemia, plasma leakage and edema (most likely due to the lack of the potent vasodilating effect of PACAP).
2. It also aggravates functional impairment, pain and sensitization.
3. PACAP increases early neutrophil-accumulation by facilitating their extravasation from the vessels, but it diminishes their function in the later phase.

4. PACAP promotes macrophage-activity and ROS production, but limits inflammation-induced pathological bone neoformation.
5. Mice deficient in SP/NKA or the NK1 receptor develop the same degree of inflammation 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.

The main findings of the study have been summarized in **Table 3.**

	PACAP	**SP/NKA**	**NK1**
Mechanical hyperalgesia	↑	-	
Thermal hyperalgesia	Absent in the model		
Joint functional loss	↑	-	
Synovial hyperplasia	↓	-	
Neutrophil activity	↓ (in the late phase)	N.E.	-
Plasma leakage	↑	N.E.	
Macrophage activity	↑		
Bone reorganization	↓		

Table 3. The effect of PACAP, SP/NKA, and the NK1 receptor in the K/BxN serum transfer arthritis model according to our results. (N.E.= not examined)

CHAPTER 3

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

6.1. INTRODUCTION

6.1.1. Implications of the Galanin receptor 3 in inflammation

It was previously demonstrated that galanin has anti-inflammatory, and primarily antiedema effects in rodent inflammation models (Lang *et al.* 2011). However, contradictorily galanin knockout mice exhibit an absent neurogenic inflammatory response, and diminished neutrophil recruitment into the disease-ridden tissues (Schmidhuber *et al.* 2008). GalR3 was found to be a promising candidate among the presumed mediators of the galaninergic antiedema effect on a receptorial level, as treatment with GalR3 antagonist small molecule SNAP 37889 dose-dependently abolished the antiedema effect of galanin in the murine skin (Schmidhuber *et al.* 2009). However, recent *in vitro* results raised concerns about the selectivity of this compound, and also found it to exhibit potent cytotoxicity towards a variety of cells of the immune and nervous system (Koller *et al.* under review).

The latest developments in neuropeptide research also casted an entirely different perspective onto GalR3 and its functions, as it was found that spexin, a recently identified novel neuropeptide is a substantially more potent agonist on GalR3 than galanin itself. Spexin also acts as an agonist on GalR2 but not on GalR1. Thus, it was hypothesised that spexin might be the primary endogenous ligand for GalR3. (Kim *et al.* 2014). In **Fig. 49.** we aimed to outline this unexpected complexity of galaninergic pathways. Spexin is a highly conserved octadecapeptide predicted and identified by computational methods, that is expressed both within the CNS and on the periphery (Mirabeu *et al.* 2007, Sonmez *et al.* 2009). More detailed examinations revealed widespread presence of spexin (skin, respiratory, digestive, urinary, and gonadal organs, endocrine glands, and several brain regions) (Porzionato *et al.* 2010, Rucinski

et al. 2010). Its physiological importance is largely uncharted, the few papers published to date all demonstrating pleiotropic effects of this peptide. It was found to be antinociceptive and vasodepressive upon central administration while it also causes decreased caloric intake and weight loss (Toll *et al.* 2012, Walewski *et al.* 2014). Due to their evolutionary homology kisspeptins (KISS), galanin (GAL) and spexin (SPX) has been collectively proposed to be members of the same peptide superfamily (SPX/GAL/KISS) (Kim *et al.* 2014).

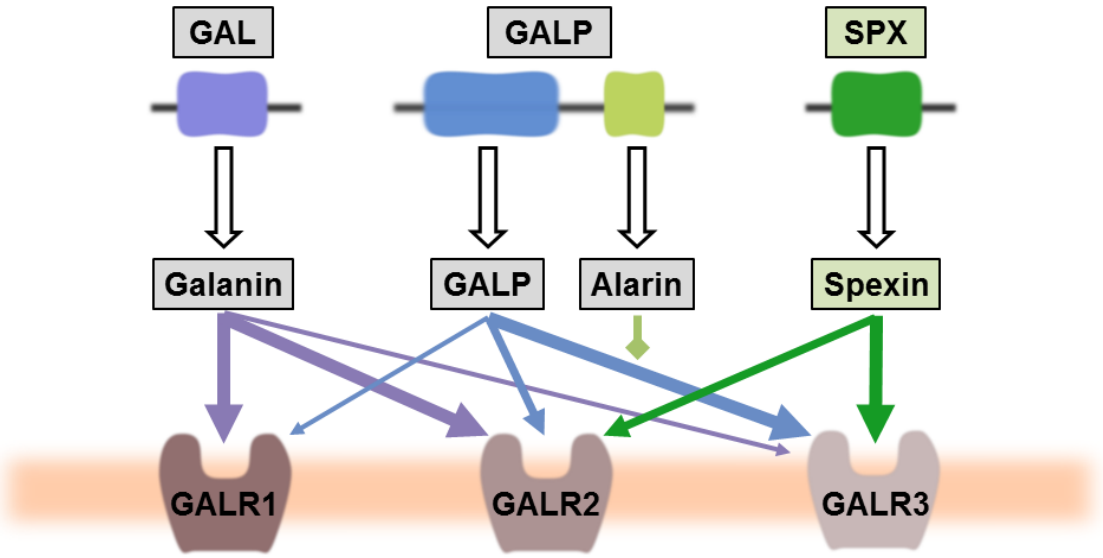

Fig. 49. The canonical and novel endogenous agonists on the galanin receptors according to our current knowledge. The potency of the endogenous ligands is indicated by the size of the arrows. The blunt arrowhead displayed along alarin indicates, that the receptorial target of that peptide is currently unknown, despite its marked biological effects.

Due to the aforementioned physiologic roles of GalR3 we hypothesised it as a potential contributor to various inflammatory conditions, and tested this idea on two markedly different autoimmune disease models resembling RA and ACD respectively. The importance, and relevance of neuropeptidergic mechanisms in RA has been elaborated in Chapter 2. Thus, here we only discuss the characteristics of ACD.

6.1.2. Mechanism of atopic contact dermatitis and potential involvement of neurogenic mediators

ACD is a disease of the skin characterized by chronic pruritis (itching), and inflammation (Leung *et al.* 2003, Harskamp *et al.* 2013). The condition usually develops in a young age, but it can also emerge in adulthood. Its prevalence is constantly increasing in the industrialized world (15-30% among children and 2-10% among adults) (Bieber 2008), for which the so-called „hygiene hypothesis" is the most widely known explanation. This theory attributes the observed phenomenon to the decreased exposure to microbial antigens in the early life (Harskamp *et al.* 2013). The hallmark feature of ACD is a localised T cell activation within the skin, initiated by either air- or foodborne antigens, haptens, and autoantigens. ACD is dominated by T-helper type-2 (Th2) cell response within the skin, in the acute phase, whereas in the chronic stage the T-helper type-1 (Th1) cells are predominant. Both type of cells release a range of ILs and IFN-gamma, thereby aggravating the inflammation (Bieber 2008). The disease is potentiated by impaired functioning of the epithelial barrier (most commonly due to mutations of the filaggrin gene), which consequently increases the susceptibility to bacterial colonisation and allergen exposition.

Since local inflammatory mediators potently sensitize the TRP receptors of the peptidergic nerve fibers in the skin (Shim *et al.* 2007), these receptors and the mediators released upon their activation were suggested to be able to influence the course of the disease. Indeed it was demonstrated that TRP channels (primarily TRPA1) play a pivotal role in itch induced by non-histaminergic pruritogens (Wilson *et al.* 2011). Mice deficient in TRPA1 but not in TRPV1 receptor or animals treated with TRPA1 antagonist show diminished inflammation following oxazolone-challenge, presumably because this receptor is directly activated by the hapten (Liu *et al.* 2013). Both galanin and its receptors show marked expression in the human skin (Kofler *et al.* 2004) and both galanin and GALP were able to inhibit neurogenic edema formation (Schmidhuber *et al.* 2007). Moreover, galanin immunoreactivity was found to be increased in mice following oxazolone-treatment, suggesting its involvement in contact dermatitis (El-Nour *et al.* 2004).

6.1.3. Aims of the study

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 model of RA, and the oxazolone-model of ACD for this purpose, and induced these models in GalR3 knockout mice. Galanin itself has been implicated as an endogenous regulatory messenger in inflammatory arthritis, but the receptorial pathways have not been identified (Calzá *et al.* 2000, Qinyang *et al.* 2004), whilst its importance in the edema formation in dermal microvasculature has been previously demonstrated (Schmidhuber *et al.* 2009).

6.2. RESULTS

6.2.1. Hindpaw edema and arthritis severity

Clinical severity scoring end plethysmometry both indicated an accelerated disease induction in GalR3$^{-/-}$ mice as both paw volumes (**Fig. 50./A**) and disease severity scores (**Fig. 50./B**) were significantly greater in knockouts until the 6th day following arthritis induction. However wildtype mice reached only slightly lower peak values.

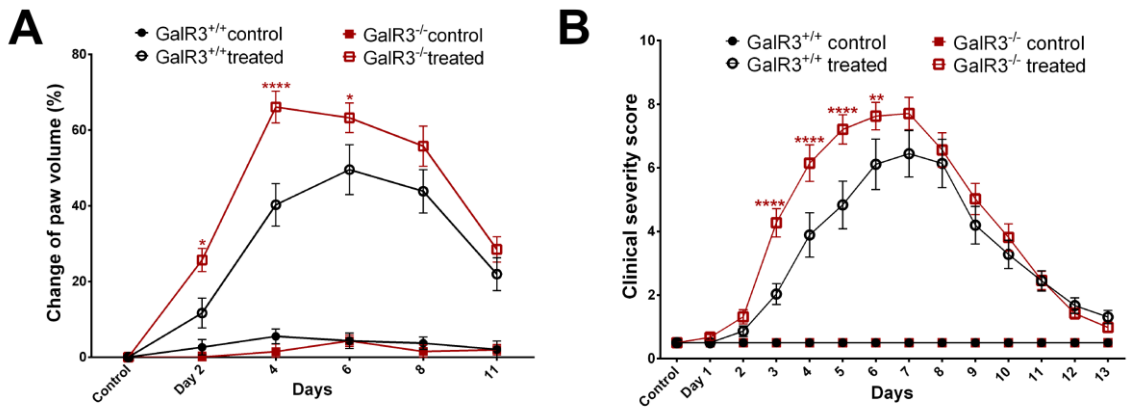

Fig. 50. (A) The change of hindlimb volume and **(B)** disease severity in the model, compared to the pretreatment controls. (Two-way repeated measures ANOVA + Tukey's post-test; Controls: n=6-7 Arthritic groups: n=9-12; *p<0.05, **p<0.01, ****p<0.0001 vs. respective wildtype)

6.2.2. Arthritic mechanical hyperalgesia and change of joint function

A considerable and similar mechanonociceptive threshold drop was observed in both groups (40-50%) with no observable differences between wildtypes and knockouts (**Fig. 51./A**). Joint function of the mice decreased steadily to the extent that by the 6th day almost all animals became unable to maintain their position on the grid for the observation period (**Fig. 51./B**). There were no remarkable differences between the two groups.

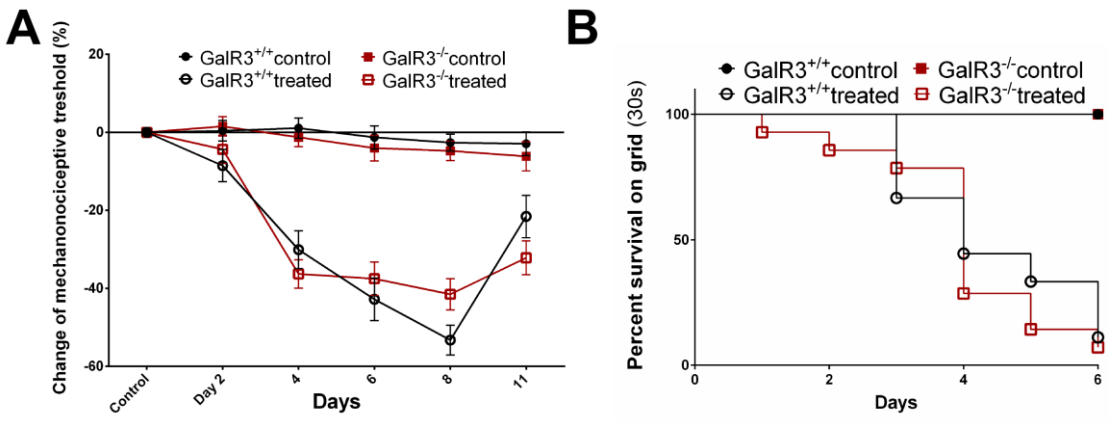

Fig. 51. (A) The change of mechanonociceptive threshold and **(B)** grasping ability. (Two-way repeated measures ANOVA + Tukey's post-test and logrank test; Controls: n=6-7 Arthritic groups: n=9-12)

6.2.3. Microvascular plasma leakage 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 (**Fig. 52.**).

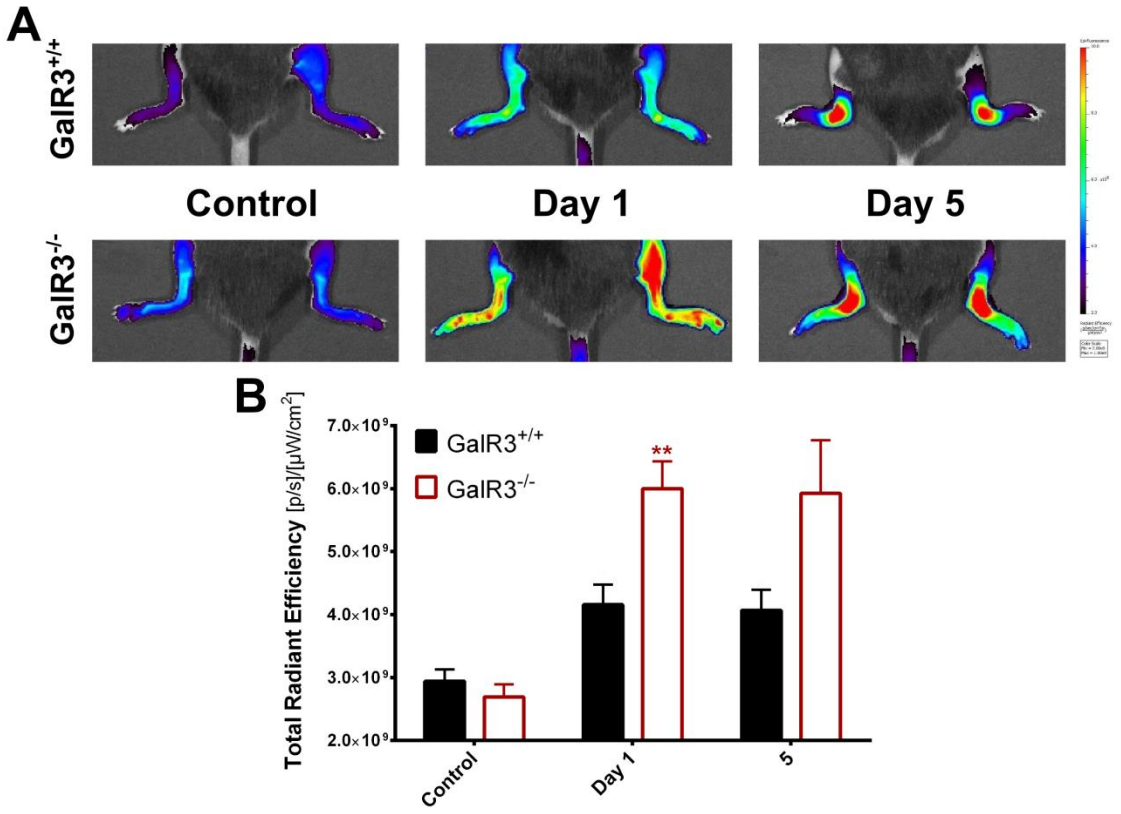

Fig. 52. Change of plasma extravasation measured by i.v. administered micellar ICG. **(A)** Representative fluorescence images. **(B)** Quantification of normalized fluorescence intensities in the hind paws. (Student's t-test; n=5-6; **p<0.01, vs. respective wildtype,)

6.2.4. Neutrophil-derived MPO-activity in arthritis

The MPO-activity of activated neutrophils peaked in both groups on day 1 in the hyperacute phase of the disease, with no significant difference between the study groups. The MPO-activity decreased considerably by the 5th day (**Fig. 53.**).

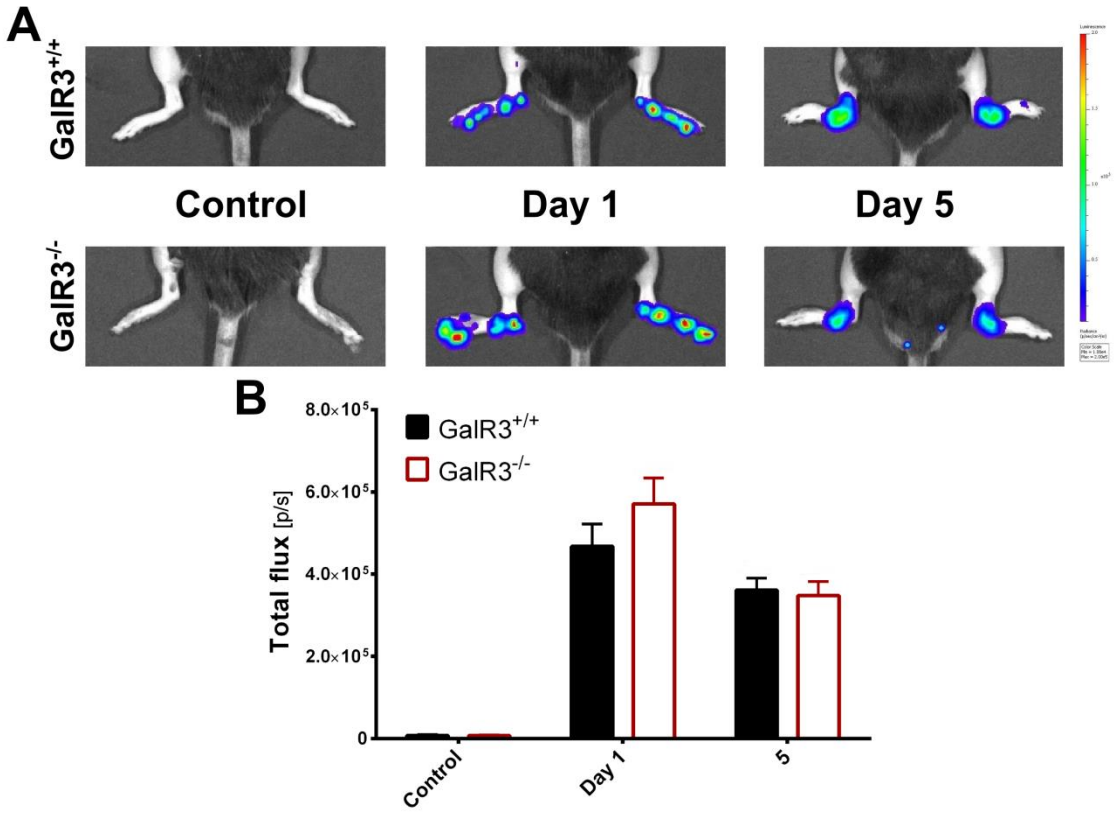

Fig. 53. Neutrophil-derived myeloperoxidase activity in the disease ankle joints. **(A)** Representative bioluminescence images. **(B)** Quantification of normalized luminescence intensities in the hind paws. (Student's t-test; Controls: n=6-7 Arthritic groups: n=9-12)

6.2.5. 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 (**Fig. 54-55.**)

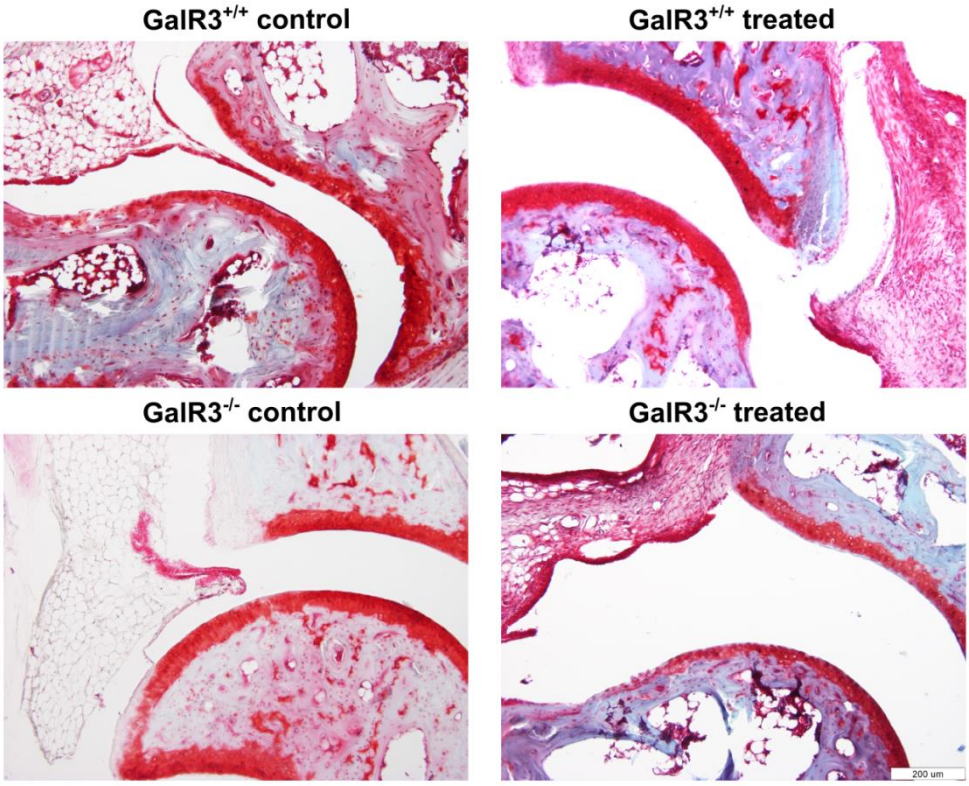

Fig. 54. Representative microphotographs of the joint samples taken on the 14th day of the arthritis. (Magnification: 100x, Scale bar: 200 μm, Safranin O staining)

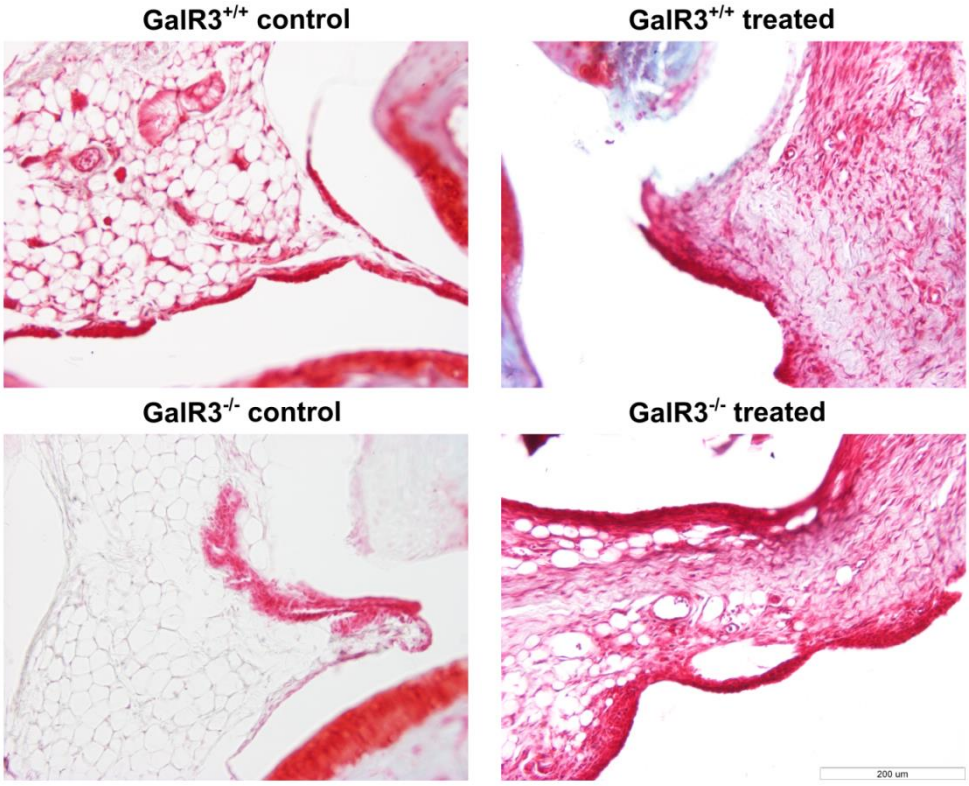

Fig. 55. Higher magnification images showing the alterations of the synovial lining and the underlying soft tissues. (Magnification: 200x, Scale bar: 200 μm, Safranin O staining)

6.2.6. 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 (**Fig. 56.**).

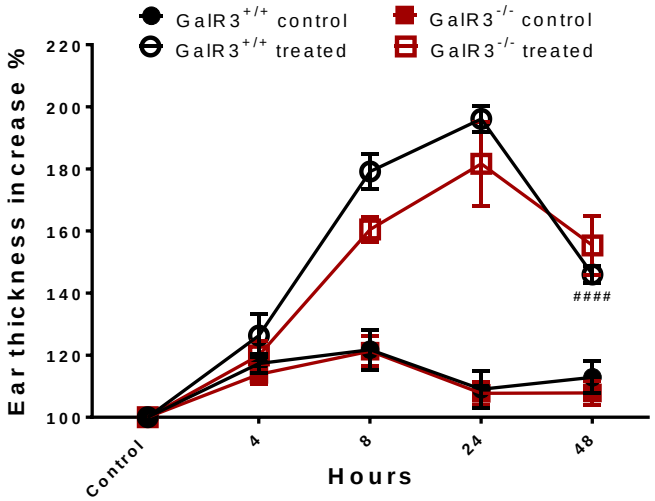

Fig. 56. Ear edema expressed by % increase of ear thickness in µm compared to pretreatment controls. (Two-way ANOVA + Tukey and Dunnett post-tests. n=7-8; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.0001$ vs. respective internal control)

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 (**Fig. 57.**).

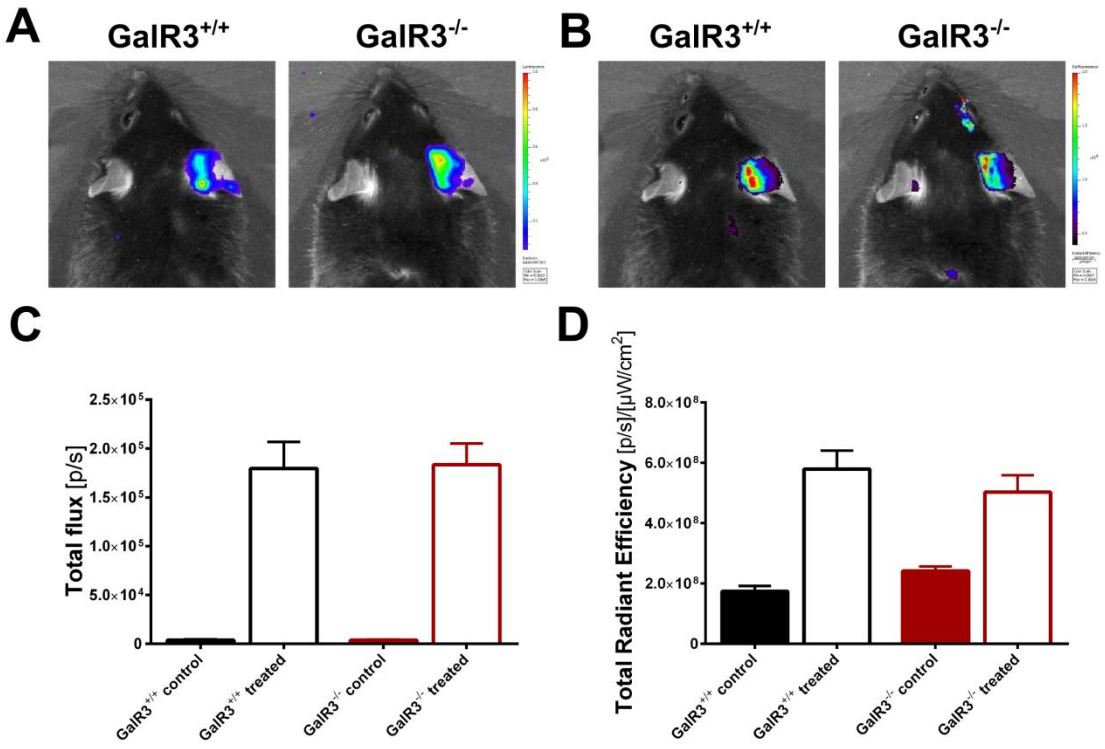

Fig. 57. MPO-activity and inflammatory vascular leakage. **(A)** Representative bioluminescence images (left ear: control, right ear: oxazolone-treated). **(B)** Representative fluorescence images. **(C)** Quantification of MPO-activity. **(D)** Quantification of fluorescence intensity. (Student's t-test; n=5-7; $^{####}$p<0.0001 vs. respective internal control)

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 (**Fig. 58.**)

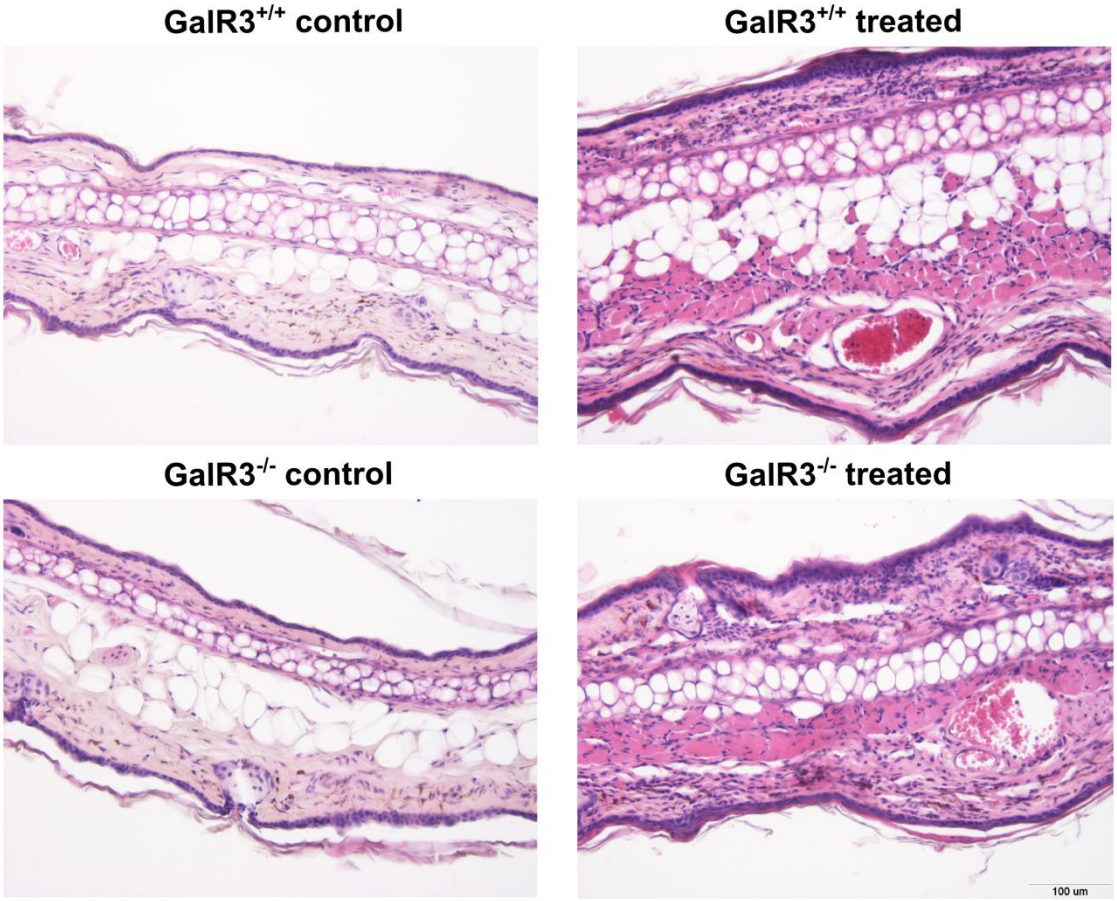

Fig. 58. Representative microphotographs of the ear samples taken 48 hours after the oxazolone-challenge. (Magnification: 200x, Scale bar: 100 μm, HE staining)

6.3. DISCUSSION

Our results suggest a modest, model-dependent involvement of GalR3 signaling in nerve-driven inflammation which affects mainly the inflammatory microvascular leakage and consequent edema formation, without any observable impact on the inflammatory cell activity.

6.3.1. GalR3-deficiency accelerates arthritic microvascular leakage and edema formation

The important regulatory effect of locally released galanin has been established long ago, and it was found to be able to diminish the histamine-induced edema formation in the skin (Jancsó *et al.* 2000). Later investigations identified that GalR2 and GalR3 but not GalR1 are expressed in the skin.

Consequently, the antiedema effect of galanin was hypothetically asserted to one or both of these receptors, which are present on perivascular neural but not on endothelial or smooth muscle tissues. (Schmidhuber *et al.* 2007).

Interestingly however, further experiments using galanin gene-deficient mice yielded contradictory results as the knockouts failed to follow the normal course of the neurogenic inflammatory response, after stimulation by either the TRPV1 agonist capsaicin or noxious heat. Moreover these mice demonstrated a deficiency in neutrophil recuitment into the injured areas (Schmidhuber *et al.* 2008). These findings were attributed at least partially to the *in utero* developmental deficits of the sensory nervous system, and not to the lack of galanin in the adult mice. In contrast GalR3 gene-deficient mice exhibit minimal phenotypic difference under normal conditions including lifespan, growth, and fertility. CNS development and hematologic parameters were also found to be normal in GalR3 knockouts, whereas plasma cholesterol and triglyceride levels were slightly elevated. Further behavioral assessment however recently revealed an anxiogenic phenotype in these knockouts (Brunner *et al.* 2014).

Other members of the galanin peptide family such as the GALP or alarin were also found to possess potent antiedema, and vasoconstrictor effects (Santic *et al.* 2007), while the direct effects of spexin, an even more potent endogenous agonist on GalR3 (Kim *et al.* 2014) has not been investigated. Interestingly a recent study found that in a mouse model of acute pancreatitis GalR3 antagonism by the selective nonpeptide SNAP 37889 ameliorated disease severity (Barreto *et al.* 2011). However SNAP was also found to be potently cytotoxic on a variety of cell types, including but not limited to myeloid lineages, moreover this effect is GalR3-independent (Koller *et al.* under review), suggesting that the surprising anti-inflammatory effect via GalR3 antagonism theorised by the authors could simply mirror the unspecific cytotoxic impact of SNAP 37889 on various immune cell subsets.

Our results suggest that GalR3 is a mediator of endogenous protective mechanisms in inflammatory arthritis initiated by neurogenic vascular responses. Previously it was demonstrated that galanin immunoreactivity increases in the DRG during experimental arthritis (Calzá *et al.* 2000). Others found a decreased galanin immunoreactivity in the sciatic nerve and macrophage-like cells, whereas it was found to be elevated in fibroblasts,

osteoblasts, and the polymorphonuclear lineage cells of the bone marrow (Qinyang *et al.* 2004). However taking into consideration the later results questioning the prevailing concepts about the endogenous GalR3 agonists, the observed effect in our model is not neccessarily galanin-mediated.

6.3.2. GalR3-deficiency has no impact on arthritic mechanical hyperalgesia, grasping ability, or neutrophil activity

We observed no difference between wildtype and GalR3$^{-/-}$ mice in these parameters, as expression of GalR3 in the CNS is much more confined than that of GalR1 or GalR2 (Mennicken *et al.* 2002, Landry *et al.* 2005). Nevertheless we could not rule out its involvement in the nociceptive signaling on the periphery *a priori*, but these findings suggest that GalR3 does not exert a remarkable effect on pain transmission.

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. Indeed, independent results published since the completion of our survey showed that GalR3 is not even expressed in mature human blood neutrophils. Contradictorily, GalR3 was found to be expressed on murine bone marrow neutrophils, though whether this is caused a species-difference, or the difference between the loci of collection (peripheral blood vs. bone marrow) remains to be addressed (Locker *et al.* 2014). Nevertheless, galanin was found to act as an immunomodulator on neutrophils increasing responsiveness towards IL-8, but the latest evidences prove that it is mediated via different pathways.

6.3.3. GalR3-deficiency does not affect disease severity, immune, and vascular functions in the oxazolone model of ACD

The disease progression and severity was similar in the wildtype and gene-deficent group. Neutrophil MPO-activity and inflammatory vascular leakage was also unaltered. This may suggest that galanin only exerts its antiedema effect through GalR3 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 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 Th, Tc 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.

6.3.4. Conclusions

1. GalR3 mediates important antiedema functions in during nerve-driven inflammation.
2. GalR3 is not involved in arthritic pain signaling and does not directly affect inflammatory cell functions.
3. Galanin or other endogenous agonists of GalR3 might be considered as endogenous protective or regulatory factors which balance and limit the extent of the vascular phase of neurogenic inflammation.

The main findings of the study are recapitulated in **Table 4.**

	K/BxN serum-transfer arthritis	**Oxazolone-induced ACD**
Edema formation	↓	-
Plasma leakage		
Hyperalgesia	-	N.A.
Neutrophil activity	-	
Histopathologic damage		

Table 4. Summary of the theorised effect of GalR3-agonism based on our results. (N.A.= not applicable)

7. NOVELTY AND PRACTICAL RELEVANCE OF OUR FINDINGS

7.1. BRIEF SUMMARY OF OUR KEY RESULTS

1. We have provided evidence that PACAP, but not SP/NKA or the NK1 receptor is involved in pain signaling during peripheral traumatic mononeuropathy. PACAP and tachykinins are involved in motor coordination, furthermore, PACAP is also an important mediator of the TRPA1-mediated neurogenic vasodilation.
2. PACAP exerts manifold influence on inflammatory arthritis: It increases nociception, disease severity, and the vascular phase of the inflammation. Conversely, its effect on the cellular phase seems to be more ambigous. It diminishes the activity of neutrophils while increasing macrophage-derived ROS-production. Chronically PACAP exerts a beneficial effect on bone remodeling and synovial damage. Surprisingly, SP/NKA or the NK1 receptor are not paramount to our arthritis model.
3. Peripheral GalR3 activation provides a peptidergic antiedema mechanism which should be counted among the endogenous regulatory pathways during neurogenic inflammation. Its direct effect on inflammatory cells, or its involvement in nociception is not supported by our findings.

7.2. THE RELEVANCE OF THE 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*. As we have discussed the results in the previous chapters, here I would like to focus on the consequences, relevance and future perspectives of these findings.

PACAP has a rich history as an endogenous regulator of nociceptive signaling on multiple levels (Dickinson *et al.* 1999, Vaudry *et al.* 2009), and also as neuroprotective factor (Somogyvári-Vígh *et al.* 2004, Dejda *et al.* 2005, Armstrong *et al.* 2008, Tamás *et al.* 2012). However this peptide shows a

pleiotropic effect being involved not only in pain signaling and sensitization, but also in immune and vascular functions. These functions all have biological relevance, but PACAP also possesses another valuable feature, as it has a receptor (PAC_1) on which it is the sole endogenous agonist. As we have mentioned earlier, the lack of success with tacyhkinin-antagonists may originate at least partially from the fact, that neuropeptide functions are redundant on several levels. Sensory nerve terminals (but also other cells) typically release a broad range of peptide mediators (simultaneously or sequentially) with overlapping functions, each of them being capable of activating several receptors, with converging downstream signaling. Thus, simple agonism/antagonism of any given mediator on any receptor may be easily bypassed on a functional level. Whether PACAP or any other peptide messenger can be seriously considered as a target of pharmacological interest, depends largely upon the uniqueness of their signaling pathways. However there are currently no known non-peptide compounds having affinity to any of the PACAP receptors, which considerably limits its potential for drug development at least in the near future.

One important conclusion of our study is the observed consistent and ubiquitous lack of effect of Tac1 or NK1 gene-deficiency in both the neuropathy and autoimmune arthritis models. These findings admittedly contradict numerous earlier results, and oppose the prevailing principles of the heyday of tachykinin research. However our findings are in good correlation with the later, predominantly negative results obtained during clinical trials of NK1 receptor antagonists (Hill 2000), or experiments on global knockout mice (De Felipe *et al.* 1998, Stangenberg *et al.* 2014). Being tested in various painful conditions NK1 antagonists only proved to be effective against postoperative dental pain, and even for this indication they were found to be inferior compared to ibuprofen (Dionne *et al.* 1998). Efforts were made to interpret the disappointing findings as a consequence of the poor bioavailability of these drugs in the CNS, or of the different supraspinal NK1 expression pattern in humans (Urban *et al.* 2000). However this argument is questionable, as NK1 antagonists were engineered against the human and not the rodent NK1 receptor, resulting in decreased potency on the latter (which would translate into decreased and not increased efficacy of the drug candidate in animals). In fact the drug development was

based on largely circumstantial evidences as described earlier (Hill 2000). It has to be briefly mentioned that some NK1 antagonists (after being ineffective in both pain and depression) serendipitously found an alternative, albeit limited use as antiemetics (Rupniak *et al.* 1999), which is their sole current clinical application either as monotherapy, or as the recent success of netupitant shows in combination with novel $5HT_3$-antagonists (Gralla *et al.* 2014). The current standing of NK1-antagonist research has been summarized in **Table 5.**

In conclusion, historical evidence and the overwhelming majority of the early articles (1980s-90s) related to the topic dictated that SP/NKA and their primary receptor NK1 have crucial role in pain and inflammation, but a substantial amount of evidence gathered in the last 15 years opposes this former collective „frame of mind". One enigmatic point remains however, namely the interpretation of these negative results in light of the ubiquitous expression of these mediators and their receptors, at levels unmatched by other similar neuropeptides (enabling their early discovery). As a final remark, the lack of biologically relevant effects of the potent and proven tachykinin antagonists continues to be an open question, that is definitely worthy of further investigation. In the meantime tachykinin antagonist „boom" of the 1990s and its bitter outcomes less than a decade later, remain with us as a warning sign about the perils of the translation of *in vitro* and animal research findings into the clinical phase of drug development.

Drug	**Planned application**	**Application (if approved)**	**Current state of development**
Aprepitant	analgetic antidepressant	**antiemetic**	**Approved (2003)**
Fosaprepitant (prodrug of aprepitant)	antiemetic	**antiemetic for iv. use**	**Approved (2008)**
Netupitant	antiemetic	**antiemetic**	**Approved in combination with palonosetron (2014)**
Rolapitant	antiemetic	-	In development
Vestipitant	antiemetic hypnotic anxiolytic tinnitus treatment		Yet to be approved, promising clinical results as antiemetic and hypnotic, modest anxiolytic effect, lack of effect in tinnitus.
Casopitant	antidepressant antiemetic		Promising effect in depression and postoperative nausea, further toxicity studies needed.
Orvepitant	antidepressant		In development
Ezlopitant	Irritable bowel disorder (IBD) antiemetic analgetic		Development halted.
Dapitant	migraine		tolerated but ineffective, abandoned
Lanepitant	analgetic migraine		tolerated but ineffective, abandoned

Table 5. The overview of NK1-antagonists, and their current use. Only compounds that reached clinical phase are listed. So far the antiemetic effect of these compounds in postoperative and chemotherapy-induced nausea (either alone as adjuvants of setrons) seems to be their most successful application.*(Sources: Goldstein et al. 2001, Diener et al. 2003, Tsuchiya et al. 2005., Lohr 2008., Altorjay et al. 2011, Gan et al. 2011, Roberts et al. 2011., Ratti et al. 2011, Ratti et al. 2013a,b, Gralla et al. 2014, Kranke et al. 2014, Poma et al. 2014)*

For a long time after its discovery the Galanin receptor 3 remained a largely ignored and hardly investigated target, as it demonstrates limited expression in the CNS/PNS, where the biologically most important (according to the tradition) galaninergic effects are mediated (Mennicken *et al.* 2002, Landry *et al.* 2005). However in spite of this, it has been recently found to have a considerable impact on behavior and cognitive functions (Brunner *et al.* 2014). Moreover, due to its predominance on the periphery (Schmidhuber *et al.* 2009) it emerged as the main target of galanin in non-neural tissues. Our results also corroborated that GalR3 has little if any involvement in nociceptive signaling, but that it mediates antiedema effects during neurogenic inflammation. However these results must be interpreted with great care, since as we have discussed, the latest findings suggest not galanin but spexin, a novel, largely uncharacterized peptide as the main endogenous agonist on GalR3 (Kim *et al.* 2014). Since the field of galanin research is in transition due to the novel mediators and potentially important pathways that have surfaced recently (Lang *et al.* 2015), we conclude that our findings contribute to the understanding of the anti-inflammatory effect of galanin-receptors, regardless of the nature of the actual mediator.

7.3. FUTURE PERSPECTIVES

A growing number of findings support the paramount involvement of neurogenic components and neuropeptides in pain and inflammation. Whether these results can be translated into actual clinical applications, depends however on a number of confounding factors, that needs to be solved.

An important problem stems from the divergent effect and widespread presence of these peptidergic mediators, and also from the coexistence of parallel pathways possessing similar functions. As a corollary, the simple antagonistic approach that proved to be fruitful in countless applications, seems to be more perilous in neuropeptide research. Indeed, the example of NK1 antagonists shows that blockade of single agonist-receptor interactions can result in far more modest effects, than what would be logically expected. This obstacle can only be avoided by careful selection of targets, taking into consideration the possible convergent mechanisms present on the level of mediators, receptors

(including binding sites), and downstream intracellular signal transduction. Better undestanding of the complex interplay of these mediators is necessary in order to transform the knowledge obtained by basic research into targets of solid theraputical implications as well as the development of receptor-selective agonists/antagonists to selectively modulate these effects.

Our experiments provided insight into the role of some representative neuropeptides in the pathophysiology of pain and inflammation of neurogenic origin. However the role of other, biologically important mediators is left to be addressed especially as sensory afferents are capable of releasing both beneficial (anti-inflammatory and analgesic), and largely detrimental (proinflammatory and algesic) mediators upon stimulation during disease conditions. Fine-tuning this mediator release could provide an alternate approach in variety of disease conditions. Here we briefly mention the significance of some promising mediators, which were not investigated by the present study.

Among the detrimental factors CGRP, an important pro-inflammatory mediator, and a central element of neurogenic vasodilation has been suggested as an important contributor to hyperalgesia and joint inflammation by both preclinical and clinical studies (Zhang *et al.* 2001, Terenzi *et al.* 2013). CGRP seems to be especially valuable target as it acts on a specific, distinct receptors, and as recently a number of non-peptide antagonists have been developed against it (primarily for the treatment of migraine) (Ho *et al.* 2008). Clinical phase trials with the first small molecule CGRP-antagonist (telcagepant) had to be however terminated recently, as the otherwise effective drug elicited liver enzyme increase in some study participants. Nevertheless, with novel, unrelated CGRP antagonists already in the pipeline, this problem can hopefully be eliminated, and the indication range of these drugs could be in time extended to encompass other painful and inflammatory conditions (Negro *et al.* 2012).

The potently anti-inflammatory somatostatin on the other hand, has received attention as a positive factor in pain and inflammation, possessing valuable analgesic, and anti-inflammatory effects (Helyes *et al.* 2004, Blake *et al.* 2007, Suto *et al.* 2014). However as it has five distinct receptors (termed sst1-5), and as its function appears to be astonishingly pleiotropic, its primary targets must be deciphered in order to consider it as a suitable therapeutical target. Our

earlier results have suggested, that in a number of painful and inflammatory conditions especially sst1 and sst4 receptors are involved in the mediation of these favorable effects (Markovics *et al.* 2012). Thus, receptor-selective somatostatinomimetics could also provide a prospective therapeutical approach in pain and inflammation.

As a final remark, large parts of the neurogenic involvement in the initiation of inflammation remain enigmatic. As an example have seen earlier that complete deafferentation protected limbs from arthritis in several cases (Kane *et al.* 2005, Kim *et al.* 2012), and insights obtained from preclinical models also corroborated that denervation is acutely proinflammatory, but chronically it prevents disease formation, mainly by preventing the early associated changes within the microvasculature (Stangenberg *et al.* 2014). Deafferented joints proved to be resistant not only towards the arthritogenic serum, but other related mediators such a serotonin or histamin. However no particular nerve quality could be identified as the primary causing factor, including the vegetative and motor nervous system. The authors also ruled out the importance of sensory afferents, by using TRPV1 and SP-deficent mice. However, as we have seen earlier, not only SP but also other neuropeptides are involved in inflammation, and additionally TRPV1-deficent mice are not suitable to address the overall role of capsaicin-sensitive afferents. The reason behind this is that these afferents express not only TRPV1 but a number of other receptors of similar importance and intracellular signaling. (e.g. TRPA1), that can only be eliminated by desensitization. Our earlier results showed however that systemic defunctionalization of these afferents not decreases but rather increases arthritis severity (Helyes *et al.* 2004). This contradiction is certainly worthy of further investigations, especially as exogenous activators of capsaicin sensitive afferents elicit pronounced vasodilation and microvessel permeability increase (**Fig. 59.**)

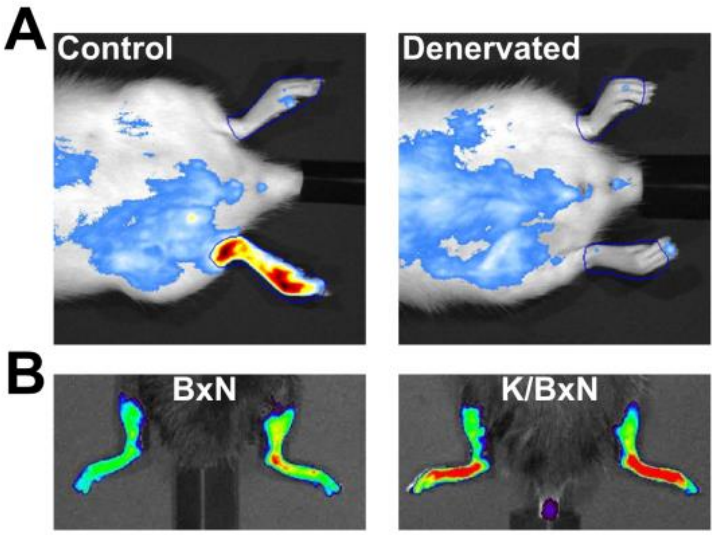

Fig. 59. (A) AITC-induced selectively TRPA1-mediated plasma extravasation on the rat hindpaw is abrogated by complete denervation *(Courtesy of Kata Bölcskei)*. **(B)** A mouse treated with arthritogenic (K/BxN) serum 30 minutes before the imaging (right) already shows an early, nerve-driven vascular permeability increase in the areas that become later affected by the arthritis, when compared to its BxN serum-treated control (left) *(The authors preliminary experiment).* Whether sensory nerve terminals or other neurogenic modalities (serotoninergic, sympathetic etc.) represent a common denominator of these phenomena remains to be understood.

As we have seen, these afferents are capable of releasing a plethora of messengers, some of which facilitate, while others mitigate disease progression. Modulating their action in a favorable direction is a challenging, yet also promising endeavor.

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. Zsuzsanna Helyes for tutoring me through all these years, and for introducing me as a student research fellow into the world of biomedical research. I am most grateful to Prof. Erika Pintér, the of the Neuropharmacology doctoral program and head of the institute for giving me the opportunity to work and study in her school. I would like to express my gratitude to Prof. János Szolcsányi, the founder of this research group and to Prof. Loránd Barthó, the former head of the institute.

I would like to also thank the members of the research group for constantly advicing and helping me through all these years regarding both the theoretical and technical parts of the research and for sharing their expertise with me, especially Kata Bölcskei, Ágnes Kemény, Éva Borbély, Valéria Tékus, Zsófia Hajna.

I also thank Dóra Ömböli, Nikolett Szentes, Anikó Perkecz, and Tamás Kiss for their valuable technical assistance in our experiments.

Many thanks go to our collaborators, Prof. Dóra Reglődi, Prof. Attila Mócsai, Prof. Barbara Kofler, and Prof. László Kollár for their important contributions, and for providing us with valuable research materials and critical insights.

I wish to thank all members of the institute for their varying but indispensable help.

Finally, I would like to thank my parents, family, and friends for supporting and encouraging me through all these years, and my fiancée for her loving care.

LIST OF PUBLICATIONS

Articles related to thesis:

Role of Pituitary Adenylate-Cyclase Activating Polypeptide and Tac1 gene derived tachykinins in sensory, motor and vascular functions under normal and neuropathic conditions. **Botz B**, Imreh A, Sándor K, Elekes K, Szolcsányi J, Reglődi D, Quinn JP, Stewart J, Zimmer A, Hashimoto H, Helyes Z. Peptides. 2013 13;43:105-112. **(IF: 2.614)**

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(*5-year impact factor)

Cumulative impact factor (without citable abstracts): 45.102

Citations: 26

Independent citations: 11

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