The tachykinin hemokinin-1 as a mediator in neuronal- and immune functions in mouse models of pain, arthritis, and dermatitis

Doctoral (PhD) Thesis

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Pécs, 2021

1. Introduction

1.1. Pain, inflammation, and the immune system

Pain is one of the most common complaints for people seeking medical help(1). While in most cases the underlying problem can be resolved, about 10-20% of pain becomes chronic(2). The currently used main groups of analgesic drugs (nonsteroidal anti-inflammatory drugs: NSAIDs, opioids and adjuvant analgesics) are not effective in every case, and long-term administration can lead to a variety of side-effects(3). Many forms of chronic pain begin with (autoimmune) inflammation; and damage to the nervous system potentially resulting in chronic pain can lead to local inflammation as well(4, 5). Nociceptors transmit the pain stimulus from the periphery to the spinal dorsal horn, where descending pathways from the brain modulate the sensation. Sensitization of nociceptors in pathological conditions can occur through both peripheral and central mechanisms. A subtype of the nociceptors is the capsaicin sensitive nerve ending which expresses the Transient Receptor Potential Vanilloid-1 (TRPV1) ion channel on its surface(6). These have a local efferent function called neurogenic inflammation when inflammatory neuropeptides are released from the nerve endings(7). Crosstalk between the nervous- and immune systems is substantial, as nociceptors release neuropeptides that regulate immune cells(8); microglia as well as other immune cells influence the developing brain(9), and lymphatic vessels have been discovered in the meninges(10, 11). Exploring these interactions and the molecules playing a role in them could advance treatment of painful pathological conditions.

1.2. Hemokinin-1, the newest member of the tachykinin family

Tachykinins represent a classical neuropeptide family, their members include substance P (SP), neurokinin-A (NKA) and neurokinin-B (NKB). Their molecular targets are G-protein coupled receptors: tachykinin neurokinin 1, 2, and 3 (NK_1-, NK_2- and NK_3) receptors. Every tachykinin can bind to each receptor, but with different affinities: SP shows preference to NK_1 receptor, NKA to NK_2 receptor and NKB to NK_3 receptor. They are encoded by the tachykinin precursor genes: preprotachykinin-A gene (*Tac1*) encodes both SP and NKA by alternative splicing, preprotachykinin-B gene (*Tac3*) encodes NKB. Tachykinins were known primarily as central nervous system (CNS) peptides, with SP and its NK_1 receptor playing a crucial role in the wind up mechanism in the spinal cord contributing to chronic pain. Efforts have been made to develop an

NK_1 receptor antagonist as a new type of analgesic drug, however, these had no effect in human pain conditions and have been repurposed as antiemetics(12). In later years, their roles in peripheral tissues have been discovered as well, and in 2000, a dominantly peripheral tachykinin, hemokinin-1 (HK-1) was found.

HK-1 is an undecapeptide encoded by the preprotachykinin-C gene (*Tac4*) and shows a remarkable resemblance to SP with 6 out of 11 matching amino acids in mice. The *TAC4* gene in humans encodes a variety of peptides: human (h)HK-1, a truncated hHK-1 and endokinin A, B, C and D. Though HK-1 is a full agonist on all three tachykinin receptors, it has the highest affinity to the NK_1 receptor allowing a diverse biological effect due to the different isoforms and signal transduction mechanisms of NK_1 receptor. Furthermore, an unidentified molecular target was suggested as HK-1-deficient and NK_1 receptor-deficient mice do not behave the same in certain disease models. Mas related G protein coupled receptors (Mrgpr) were proposed as a potential target. Older NK_1 receptor antagonists like aprepitant can inhibit both NK_1 receptor and Mrgpr in mice, but not in humans, whereas novel dual action antagonists can inhibit human MRGPR as well(13). Since these receptors are found on primary sensory neurons and mast cells and play a role in nociception(14), this observation is a possible explanation for the ineffectiveness of NK_1 receptor antagonists as analgesics in humans despite being effective in rodents. HK-1 contributes to the development of asthma in murine models through NK_1 receptor on mast cells, while in human mast cells it acted through MRGPRX2 receptor, not NK_1 receptor(15), making MRGPRs a likely candidate for the missing target molecule of HK-1.

As opposed to the other tachykinins, HK-1 was suggested to play an important role in the periphery rather than the nervous system. Interestingly, HK-1 was scarcely found in most regions of the brain, SP being the predominant tachykinin everywhere, except for the cerebellum. Here HK-1, and not SP showed a more remarkable expression(16). As its name suggests, HK-1 is the predominant tachykinin in the bone marrow as it is expressed in both myeloid and lymphoid precursor cells(17, 18). Its preferential expression in such peripheral tissues can be explained by the regulatory mechanism in its promoter region, as well as NFκB promoting increased *Tac4* transcription in T cell lines(19). HK-1 can enhance the maturing of B cells in the transition to antigen developing cells(20), activate the mitogen-activated protein kinase (MAPK) pathway and

act as a co-stimulator in mature B cells(21). HK-1 (and SP) can be found in the synapse between T cells and dendritic cells as NK_1 receptor interacts with the T cell receptor to promote T cell maturation and survival(22). Through the NK_1 receptor HK-1 could also upregulate IL17A and IFNγ production in CD4+ T cells and contributes to the development of T helper (Th) 17 cells. HK-1 enhanced IL1β, IL6, TNF-α, and IL23 production as well(23).

Since its discovery, some roles of HK-1 in the CNS have also been revealed: evidence points to the nociceptive effect of HK-1 (with some notable exceptions), as well as its interactions with well-established components of the pain pathway like opioids and glutamate(24)(25). Beyond this, HK-1 can potentiate the effects of TRPV1 activation, suppress TRP melastatin 8 (TRPM8) mediated cold-induced nociception, but has no influence on the effects of TRP ankyrin 1 (TRPA1) channels(26). An increased amount of HK-1 was detected in the blood of patients with fibromyalgia(27), a pathological condition with heightened pain sensitivity due to alterations in the central and peripheral pain pathways(28). It has been suggested that a brain-specific N-terminal acetylation of HK-1 can increase its potency in the CNS(29).

1.3. Arthritis

Rheumatoid arthritis (RA) is the most common autoimmune disorder of the joints characterized by chronic inflammation and severe pain. Although the inflammation can be effectively controlled by NSAIDs, steroids, disease-modifying antirheumatic drugs (DMARDs) and biologic agents(30), pain is often resistant to these drugs(31) making pain management an unmet medical need. The joints are densely innervated by capsaicin-sensitive peptidergic sensory nerves(32) expressing, among others, the TRPV1 and TRPA1 ion channels activated by a broad range of inflammatory mediators(8). Investigations have been initiated to reveal sensitization processes that convert inflammatory to central pain, contributing to persistent arthritic pain(33). Exploration of these pathophysiological processes is hindered by the fact that no single animal model can mimic every aspect of RA, conclusions drawn from a single model might not necessarily apply to the human disease(34). Studying the role of endogenous molecules of the sensory-vascular-immune interactions is essential to identify key mediators and potential novel drug targets.

1.4. Neuropathic pain

Pain can originate from several sources like trauma, inflammation, cancer, metabolic diseases, but the most debilitating and therapeutically challenging form is neuropathic pain, which is a typical symptom of diabetes, some genetic diseases and nerve injury(35). Neuropathy can be central (injury to the spinal cord or brain) or peripheral nerve damage(36). Gliosis can be an accompanying mechanism, both microglia and astrocytes can become activated at the site of nerve damage, or in the spinal cord where the central branch of the neuron ends(37). NGF can be highlighted as a critical factor in nerve development, and regeneration, as well as a transmitter of pain, based on this information it can play a protective role while also contributing to pain.

In the pharmacological treatment of neuropathy NSAIDs are ineffective, and opioids have only limited use. The adjuvant analgesics currently in use (tricyclic antidepressants, antiepileptics) have common and severe side effects(38), and in many cases the presently available pharmacotherapy is not satisfactory, there are several therapy-resistant patients(39). Therefore, neuropathic pain is still an unmet medical need; understanding the underlying mechanisms and finding new key molecules is essential in determining new therapeutic targets.

1.5. T-cell mediated skin pathologies: allergic contact dermatitis and psoriasis

Allergic contact dermatitis is a type IV delayed hypersensitivity reaction caused by a contact allergen in 2 main phases. In the sensitization phase a hapten-protein conjugate will be taken up by the Langerhans cells (LC) or dermal dendritic cells d(DC), transported to the local lymph nodes and presented to T-cells(40). The elicitation phase happens after the second encounter with the allergen, activating the T-lymphocytes and leading to the development of symptoms. This pathological immune response is modulated by the nervous system as well, TRPA1 on sensory neurons of the skin and NK_1 receptor playing a role in the integration of immune and neuronal mechanisms(41, 42). Therapy consists of avoiding the allergen, topical steroids and antihistamines(43), so exploring new therapeutic targets would be important for this condition.

Psoriasis is an autoimmune disease mediated by Th1-cells leading to characteristic skin lesions(44). Dermal dendritic cells stimulate the migration of autoimmune Th17 and Tc17 cells into the epidermis, where they produce IL17 and IL22 cytokines; and neutrophil infiltration

mediated by IL17 can lead to pustular psoriasis(45). DCs in the skin are in close contact with sensory nerve endings and inhibiting a subset of nerves can ameliorate the symptoms(46). SP and NKA and their receptors have been reported in psoriatic biopsy samples in immune cells, and NKA in nerve endings as well(47). There are several options for therapy, like local and systemic treatment and non-pharmacological approaches like light therapy(48), but in some patients therapy is still not satisfactory, so exploring new therapeutic targets would be important.

2. Aims

1. Determining the role of HK-1 in acute and chronic models of arthritis in relation to nervous system alterations.
2. Analyzing the effect of HK-1 on primary sensory neuronal cultures.
3. Investigating the role of HK-1 in acute pain conditions and peripheral traumatic mononeuropathy.
4. Examining the role of HK-1 in T-cell mediated autoimmune models as allergic contact dermatitis and psoriasis.

3. Materials and Methods

3.1. Experimental animals

Experiments were carried out on male or female *Tac1* (*Tac1*$^{-/-}$), *Tac4* (*Tac4*$^{-/-}$) gene-deficient, NK_1 receptor knockout (*Tacr1*$^{-/-}$) mice, and C57Bl/6 wild types (WT) (8–12 weeks, 20–30 g). Animals were bred and kept in the conventional animal house of the Department of Pharmacology and Pharmacotherapy under standardized conditions. Anesthesia was performed by intraperitoneal (i.p.) administration of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively). The investigator was always blinded to the treatments and the genotypes of the mice. All experiments were carried out according to regulations after the approval of the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments (BA 02/2000-9/2011), (BA 02/2000–2/2012), (BAI/35/55-76/2017).

3.2. Experimental models

3.2.1. Arthritis

Arthritis was examined with 3 different methods, one chronic and two acute models. Chronic serum transfer arthritis was induced by administering serum of K/BxN strain mice on day 0 and day 3 of the experiment. Measurements were performed in the following 3 weeks. Control animals were treated with non-arthritogenic BxN serum. Acute mast cell tryptase (MCT) arthritis was evoked by topically administering MCT to the knee joint under anesthesia, measurements were performed in the following 6 hours. In the third experiment, complete Freund's adjuvant (CFA) was injected into the right mouse knee under anesthesia, measurements were performed in the next 24 hours.

3.2.2. *In vitro* primary sensory neuron experiments

Trigeminal ganglion cultures were prepared from neonatal NMRI mice. Intracellular free calcium concentration was measured with fluorescent indicator fure-2 AM under different conditions. The activating effect of HK-1 was measured in the presence of different receptor antagonists, gene deleted cells, and Ca^{2+} free media. The desensitizing effect of capsaicin was examined in the presence of HK-1 and SP.

3.2.3. Acute and neuropathic pain

Somatic nocifensive behavior was elicited by giving a subcutaneous 2.5% formalin injection into the right hind paw. Nocifensive behavior (shaking, licking, and lifting the paw) was evaluated in 2 phases in the following 45 minutes. Acute visceral nocifensive reaction was examined by giving an i.p. injection of 0.6% acetic acid and counting the writhing movements in the next 30 minutes. Acute neurogenic inflammation was induced by giving 0.03 μg/ml intraplantar injection of resiniferatoxin (RTX) in the hind paw, pain thresholds were measured in the following 24 hours. Neuropathic pain was evoked by partial sciatic nerve ligation (PSL). Pain thresholds and motor coordination were measured in the following week, then spinal cord and brain were examined with immunohistological staining, and *Tac4* mRNA was measured in the spinal cord and DRG.

3.2.4. Autoimmune dermatitis

Allergic contact dermatitis was evoked by 2% oxazolone administration. Measurements were performed in the 3 days following ear treatment, and tissue samples were collected at every timepoint. Contralateral ear was treated with ethanol as control. Psoriasiform dermatitis was induced by applying imiquimod containing Aldara cream every day to the dorsal skin, measurements were performed for 4 days. Tissue samples were taken 24 and 96 hours after initial treatment. Contralateral dorsal skin was treated with vaseline as control.

3.3. Experimental methods

3.3.1. Measuring pain

The mechanonociceptive threshold was measured with the dynamic plantar aesthesiometer (DPA; Ugo Basile 37000) before (to determine baseline nociceptive threshold) and after treatment. Heat threshold was determined with the hot plate device (IITC Life Sciences). Cold tolerance was given as the latency to paw-withdrawal from 0° C water. Paw edema was quantified using plethysmometer (Ugo Basile 7140). In these measurements the post-treatment values are shown as the percentage of threshold-decrease of the individual mouse compared to its baseline thresholds.

3.3.2. Determining disease severity

Bodyweight was measured as a parameter of general well-being; weight loss was given as the percentage of lost weight compared to pretreatment control values. To assess joint function the grid test was performed where the grasping ability needed to perform the task correlates with the joint function. Arthritis severity was scored using a semiquantitative visual scale.

3.3.3. Measuring motor coordination

Motor coordination was examined with the accelerating rotarod (Ugo Basile, Comerio, Italy).

3.3.4. Measuring ear and skin thickness

Ear and skin thickness were measured with an engineer's micrometer (Moore and Wright, Sheffield, England).

3.3.5. *In vivo* imaging

MPO-activity and plasma leakage were measured with IVIS Lumina II instrument (PerkinElmer) under ketamine-xylazine anaesthesia. Skin perfusion was measured with laser speckle device.

3.3.6. Histology staining and evaluation

Joints were stained with safranin to evaluate fibroblast proliferation; leukocyte invasion; thickness of synovium and collagen deposition giving each parameter a 0–3 score depending on severity. Immunohistochemical staining was used in central nervous system tissues. We visualized Iba1 protein to count microglia cells, GFAP to count astrocytes and FosB protein to evaluate the chronic stress related activation of neurons. Immunopositive cells were quantified in pain-related brain regions with microscope (Nikon Microphot FXA) and Inform software (Massachusetts, USA). The locations of the investigated areas were determined based on the Paxinos and Franklin brain atlas. *Tac4* mRNA in the skin was visualized using the RNAscope *in situ* hybridization method.

3.3.7. Molecular biology essays of tissue samples

L3-L5 lumbar spinal cord and the respective DRGs were obtained from WT mice 6 days after treatment. Tissue samples were snap-frozen on dry ice, then RNA was extracted and *Tac4* mRNA was quantified in reference to the glucuronidase beta (*Gusb*(49)) reference gene. ELISA was applied to determine NGF expression at the protein level and results were calculated as pg/g tissue homogenate. Cytokine levels in ear and skin samples were quantified using the cytokine luminex immunoassay.

3.4. Statistical analysis

The treatments were not randomized within cages to prevent control animals from harming the treated animals. Results are expressed as the means ± SEM of n = 4–16 mice per group in case of in vivo functional tests. Data obtained in these experiments were analyzed as described in figure legends with GraphPad Prism 8 software. In all cases $p < 0.05$ was accepted as statistically significant. The data of the immunohistological staining, due to the larger number of groups, was analyzed with factorial ANOVA and Tukey's post hoc test with Statistica software (TIBCO Inc., Palo Alto, USA). In all cases $p < 0.05$ was accepted as statistically significant.

4. Results

4.1. Arthritis

4.1.1. K/BxN arthritis

4.1.1.1. HK-1, but not NK_1 receptor, plays a role in arthritis related early and late mechanical hyperalgesia, paw edema and heat threshold decrease

The greatest decrease in mechanonociceptive threshold developed on day 11 in WT and day 13 in *Tac4*$^{-/-}$ group and resolved in both groups by day 21. *Tac4*$^{-/-}$ group had a significantly milder threshold decrease throughout the experiment, including the last phase of arthritis on days 14-21. Paw volume measured by plethysmometry reached its peak on day 8 in WT and *Tac4*$^{-/-}$ alike, and spontaneously resolved by day 14 in both groups. The paw volume of *Tac4*$^{-/-}$ animals was significantly lower in the first 8 days of the experiment. Mechanical hyperalgesia, heat threshold and paw edema did not show a difference in *Tacr1*$^{-/-}$ mice compared to WT. There was no difference in cold tolerance, time spent on grid, change in bodyweight and arthritis severity score in *Tac4*$^{-/-}$ or *Tacr1*$^{-/-}$ mice compared to WT animals.

4.1.1.2. HK-1 decreases MPO-activity in K/BxN serum-transfer arthritis

MPO-activity showed a significant increase after 2 days in *Tac4*$^{-/-}$ mice, while in WT mice it became significant compared to its control after 6 days. Both groups had an increased rate in plasma extravasation, but no effect of the gene-deletion could be observed.

4.1.1.3. HK-1 increases histopathological arthritis severity

Based on the semiquantitative scoring with a maximum of 9 points *Tac4*$^{-/-}$ mice had a significantly lower score compared to WT mice.

4.1.1.4. Tac4 mRNA expression in DRG and spinal cord

Tac4 mRNA expression could be seen on day 6 in the L4-6 DRGs of intact, BxN- and K/BxN serum treated mice with a non-significant decrease of expression in the K/BxN treated arthritic group. We could not detect *Tac4* mRNA in the lumbar spinal cord samples.

4.1.2. HK-1 contributes to pain and edema in MCT-induced acute monoarthritis

Mechanical hyperalgesia developed in WT mice 2 hours after MCT administration, while knee edema developed on the 4th hour. Both parameters were significantly less severe in *Tac4*$^{-/-}$ mice.

Increase in blood flow was detectable in the first 40 min after treatment but showed no significant difference between the groups.

4.1.3. HK-1, but not NK_1 receptor, mediates mechanical hyperalgesia and knee edema, but decreases MPO activity in CFA-induced subacute knee inflammation

Mechanical hyperalgesia and knee edema were detectable 2, 6 and 24 h after the CFA administration. *Tac4*$^{-/-}$ mice had a significantly milder mechanical hyperalgesia at every time point and less severe knee edema at 24 h. *Tac4*$^{-/-}$ mice showed a significant increase in MPO-activity 24 h after CFA administration. Changes in mechanonociceptive threshold and knee volume did not show significant difference to WT mice in NK_1 receptor-deficient mice.

4.2. In vitro primary sensory neuron experiments

4.2.1. HK-1 directly activates primary sensory neurons

HK-1 applied to a culture of primary sensory neurons in 1 µM concentration caused Ca^{2+}-influx in 26.39 ± 4.5%, whereas 500 nM HK-1 and 500 and 1 µM SP had no effect. The NK_1 receptor antagonist CP99994 did not influence the HK-1 response, this was similar in neurons of NK_1 receptor gene-deleted mice. The G-protein coupled receptor (GPCR) blocker pertussis toxin (PTX) influenced neither the ratio of responding neurons, nor the extent of the response to HK-1; neither did the TRPV1 antagonist AMG8910, nor the TRPA1 antagonist HC 030031. No Ca^{2+}- signal was detected in Ca^{2+} free ECS.

4.2.2. HK-1 and SP can counteract the desensitization caused by repeated capsaicin administration

The first application of 330 nM capsaicin induced transient Ca^{2+}-accumulation which gradually decreased in response to the second capsaicin stimulus due to TRPV1 desensitization. If 500 nm HK-1 or SP was administered after the second capsaicin stimulus, the desensitization was diminished as shown by the third and fourth capsaicin-evoked responses.

4.3. Neuropathy and acute pain

4.3.1. Acute pain

4.3.1.1. HK-1, SP/NKA and NK_1 receptor contribute to acetic acid evoked visceral pain

The number of writhing movements was significantly less in *Tac4*$^{-/-}$ mice in the 2nd observation period, and in *Tac1*$^{-/-}$ and *Tacr1*$^{-/-}$ mice in the 2nd and 3rd observation periods

4.3.1.2. SP/NKA contribute to formalin-induced nocifensive behavior

Somatic nocifensive behavior was lower in *Tac1*$^{-/-}$ mice compared to WTs in the second phase but did not differ during the experiment in *Tacr1*$^{-/-}$ and *Tac4*$^{-/-}$mice.

4.3.1.3. HK-1, SP/NKA and NK_1 receptor contribute to RTX-induced heat- and mechanical hyperalgesia

Heat and mechanical hyperalgesia were both alleviated in *Tac4*$^{-/-}$, *Tac1*$^{-/-}$ and *Tacr1*$^{-/-}$ mice. The most substantial differences were seen in the decrease of mechanonociceptive threshold in *Tac4*$^{-/-}$ mice, and in the heat threshold of *Tac1*$^{-/-}$ mice, the latter remaining at baseline level despite the RTX treatment.

4.3.2. Neuropathy

4.3.2.1. Worsened motor coordination in the absence of HK-1

Motor coordination did not worsen in WT or *Tac4*$^{-/-}$ mice due to PSL operation, but *Tac4*$^{-/-}$ mice performed worse which reached statistical significance on day 10.

4.3.2.2. Milder neuropathic mechanical hyperalgesia and cold tolerance decrease in the absence of HK-1

In the *Tac4*$^{-/-}$ group neuropathic mechanical hyperalgesia was significantly smaller throughout the whole experiment, decrease in cold tolerance was significantly milder as well.

4.3.2.3. Increased peripheral NGF-level during neuropathy in the absence of HK-1

The NGF concentration of the paw homogenates was significantly lower in intact *Tac4*$^{-/-}$ mice compared to WTs. Under neuropathic condition, 7 days after PSL, NGF level was not altered in the WT group but showed an almost 2-fold elevation in the *Tac4*$^{-/-}$ one.

4.3.2.4. Number of microglia and astrocytes decreased in the lamina I-II of operated mice in the absence of HK-1

In the spinal dorsal horn, in response to PSL, microglia density significantly increased ipsilaterally in WT mice but not in the *Tac4*$^{-/-}$ mice. Astrocyte numbers decreased in the *Tac4*$^{-/-}$ mice on both sides under neuropathic conditions. The chronic neuronal activation marker FosB-immunopositivity did not show any changes in relation to genotype, PSL or side. Astrocyte numbers were smaller in the PAG of intact *Tac4*$^{-/-}$ mice. There were no other observed differences in the PAG, amygdala and somatosensory cortex.

4.4. Skin disease models

4.4.1. Oxazolone-induced allergic contact dermatitis

4.4.1.1. Decreased ear swelling in the absence of HK-1

The most pronounced ear swelling developed 24 hours after administering oxazolone solution to the ear in both WT and *Tac4*$^{-/-}$ mice. At this timepoint *Tac4*$^{-/-}$ mice had significantly milder ear swelling compared to WTs. Contralateral ear treated with solvent did not show significant increase in thickness compared to control values.

4.4.1.2. MPO-activity or plasma leakage did not change in the absence of HK-1

MPO-activity and plasma leakage increased significantly 24 hours after oxazolone treatment in both WT and *Tac4*$^{-/-}$ mice, but there was no significant difference between WT and *Tac4*$^{-/-}$ groups.

4.4.1.3. Increased IFNγ and IL4 in the absence of HK-1, Tac4 mRNA located in hair follicles

IFNγ- and IL4-level increased 24 hours after treatment with a significantly higher amount in *Tac4*$^{-/-}$ mice. TNFα-level began to elevate after 24 hours and reached its peak after 72 h with no observed difference in the absence of HK-1. IL2, and IL5 became elevated in both WT and *Tac4*$^{-/-}$ oxazolone treated groups 24 h after ear treatment and remained elevated until the end of the experiment with no significant difference between WT and *Tac4*$^{-/-}$ groups.

Upregulation of *Tac4* mRNA could not be seen after treatment. In all samples *Tac4* specific signal was detected in the hair follicle.

4.4.2. Aldara-induced psoriasiform dermatitis

4.4.2.1. Milder skin swelling and blood flow at 96h in the absence of HK-1

Thickening of skin was already detectable 24 h after first treatment. Though the results of both *Tac4*$^{-/-}$ and *Tacr1*$^{-/-}$ mice were below the WT's curve, it only reached statistical significance at the 96 h timepoint in *Tac4*$^{-/-}$ mice. *Tac4*$^{-/-}$ mice had significantly lower levels of detectable blood perfusion at 96 h.

4.4.2.2. Tac4 mRNA located in hair follicle

Accumulation of *Tac4* mRNA could not be seen after treatment. In all samples *Tac4* specific signal was detected only in the hair follicle. There was no significant difference in IL1β or TNFα levels in the absence of HK-1 or NK_1 receptor.

5. Discussion

We showed that HK-1 is an important mediator of central- and peripheral sensitization during joint inflammation, which was independent of NK_1 receptor in the K/BxN and CFA models. HK-1 also contributes to edema formation, histopathological processes and prevents MPO increase. In our experiments we saw that edema (which correlates to inflammatory responses) resolve 14 days after serum administration, while mechanical hyperalgesia persisted for another week indicating central sensitization independent of inflammation on the periphery. HK-1 contributes to both the early, dominantly peripheral pain as well as the late, central sensitization related pain, while NK_1 receptor did not contribute to arthritis related pain. Edema formation and pathohistological score were also milder in the absence of HK-1, this reinforces that HK-1 has a role in the peripheral inflammatory reaction. On day 2 and 6 of K/BxN arthritis the absence of HK-1 did not affect dye extravasation in the fluorescent imaging study. Vascular leakage is not the only component of edema formation, so it cannot exclusively explain the differences in paw swelling.

Despite the detrimental effect of HK-1 in joint inflammation so far, MPO increase occurred earlier (on day 2) in the absence of HK-1. MPO is produced by neutrophils and macrophages and is known as a mediator of tissue damage and inflammation. Some studies have found that in certain

circumstances, when elevated in the early phase of the cascade, MPO can prevent inflammation(50), but the mechanism is not well understood.

Acute CFA-induced arthritis(51) is initiated by macrophages and based on our findings HK-1 contributed to both pain and knee swelling, while NK_1 receptor did not. MCT is a local mediator of inflammation and elicits its effects through sensory neurons(52). HK-1 played a role in related mechanical hyperalgesia and edema, but not in increasing blood flow, possibly through the NK_1 receptor(52). Based on these findings we can see compelling evidence that HK-1 mediates its effects in arthritis not only through inflammatory cells, but also through the sensory neurons, in certain models independently of the NK_1 receptor.

In our *in vitro* studies on cultured primary sensory HK-1 had an effect independently of NK_1 receptor and PTX-sensitive receptors, which could not develop in Ca^{2+}-free media. This suggests an ion channel-coupled receptorial mechanism, but not mediated by TRPV1 or TRPA1, as their inhibitors did not influence the HK-1-induced Ca^{2+} influx response. Repeated administration of capsaicin to primary sensory neurons causes a decreasing intensity in Ca^{2+} influx as the TRPV1 receptors become desensitized from the repeated stimulus(53). Both HK-1 and SP were able to neutralize this effect and elicit the full Ca^{2+}-influx response in the neurons. The neuronal activating ability of HK-1 has been shown in cholinergic hippocampal neurons, where it acted post-synaptically in a tetrodotoxin-resistant manner, however this response was not dependent on Ca^{2+} from the extracellular space(54).

When evoking acute neurogenic inflammation in the foot with the administration of the TRPV1 agonist RTX we saw that the decrease of heat threshold and mechanical hyperalgesia were ameliorated in the absence of HK-1, NK_1 receptor and SP/NKA. This reinforces our in vitro findings where the presence of HK-1 and SP maintained the TRPV1 agonist sensitivity of the sensory neurons.

During the release of inflammatory mediators in the 2^{nd} phase of formalin-induced nocifension only SP/NKA played a role, HK-1 and NK_1 receptor did not. None of the peptides or NK_1 receptor were involved in the 1^{st} phase direct activation of sensory neurons in this model. SP/NKA and NK_1

receptor were the dominant mediators of visceral pain, HK-1 only had a weaker effect in the 2nd phase of the activation of visceral nociceptors.

In the PSL-induced neuropathic pain model HK-1 contributed to both mechanical hyperalgesia and decrease in cold threshold. This is unique to HK-1 among the tachykinins, as neither SP/NKA nor NK_1 receptor played a role in PSL-induced pain(55). The level of NGF did not change in WT animals after PSL, in *Tac4*$^{-/-}$ animals it began from a significantly lower level and increased when neuropathy developed. While NGF is best known for contributing to pain, it is also an important trophic factor of neurons which could contribute to the regeneration of damaged nerves.

HK-1 contributed to the increase of microglia cells after operation in the lamina I-II of the spinal dorsal horn. HK-1 seems to be an important mediator of microglia activation: the production of HK-1 is upregulated when microglia(56) become activated; and blocking microglia activation with minocyclin decreases HK-1 production, as well as pain related behavior in rats(57).

The PSL operation did not influence motor coordination, HK-1-deficient mice had worse motor coordination than WT mice. This correlates with previous reports of a paradoxically high HK-1 expression in the cerebellum(16). The cerebellum has the lowest NK_1 receptor expression among different regions of the brain(58). With this finding we provided the first functional data in correlation with the high HK-1 expression in the cerebellum.

The results of our experiments showed that HK-1 only has a minor role in oxazolone-induced allergic contact dermatitis, decreasing ear edema only at the 24h timepoint. At this timepoint the inflammatory cytokines IFNγ and IL4 increased despite the milder reaction in the ear. Though IL4 is known as a promoter of Th2-cell differentiation and allergic reaction(59), it can also down-regulate the inflammation in contact dermatitis which may explain the alleviated skin swelling at this timepoint(60). Furthermore, neutralization of IL4 leads to the increased production of inflammatory mediators as IFN-γ, IL2, IL12 p40, and IL1β. In a previous study the absence of NK_1 receptor showed a more robust and persistent alleviation of symptoms of allergic contact dermatitis(22, 42). NK_1 receptor produced by the T-cells has a pivotal role in survival and developement of Th1 then Th17 type cells(22). Resting and stimulated T cells can produce both

HK-1 and SP, so based on our findings, the NK_1 receptor seems to be the crucial component of the process, and the presence of one of its agonists (SP) was enough to elicit its effects in T cells.

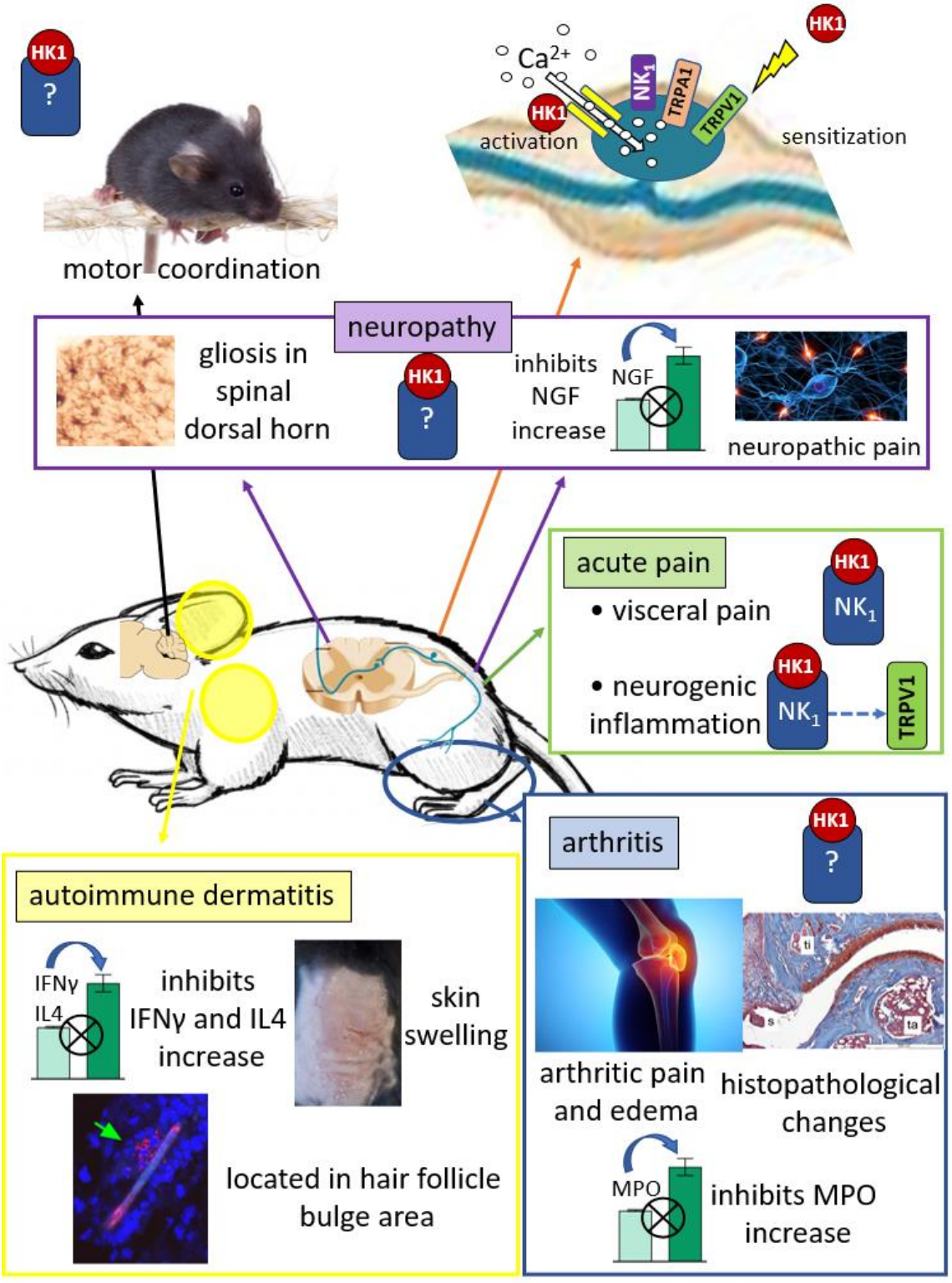
While increase in skin thickness was markedly milder in both *Tac4*$^{-/-}$ and *Tacr1*$^{-/-}$ mice, this only reached statistical significance in case of *Tac4*$^{-/-}$ mice at the last measured timepoint. There was no difference in blood flow between WTs and KOs, which correlates with previous findings. The significant decrease in blood flow at 96 hours in case of *Tac4*$^{-/-}$ mice can be contributed to the more intensive scaling of the skin in these animals, as the scaling skin influenced the laser speckle device's ability to detect the moving blood cells in the layers below. The process of T cell activation in psoriasis also includes Th17 cells, it is more dependent on IL23 than IL2. TCR signaling pathways are fully functional in NK_1 receptor-deficient cells, and T-cell apoptosis in NK_1 receptor-deficient cells could be prevented with IL2 supplementation in a study examining the mechanism of allergic contact dermatitis(22). A study of skin biopsies of patients with plaque psoriasis with pruritus concluded that not just NK_1, but NK_2 receptor were upregulated in the lesions as well as NKA positive immune cells and nerve fibers(47). Probably due to the complexity of the tachykinin system psoriasis was able to develop in mice even in the absence of HK-1 or NK_1 receptor. As in the ear hair follicles, *Tac4* mRNA was found in the bulge area of the hair follicles of the dorsal skin as well. Though it does not seem to play a role in dermatitis either, this was the first study to precisely locate HK-1 in the skin. The bulge is the area of the hair follicle at the insertion of the arrector pili muscle, it contains epidermal stem cells (61)(62). The expression of HK-1 in this bulge area proposes exciting new research prospects.

Our results provided evidence for the complex regulatory roles of HK-1 in pathologies related to sensory-immune interactions, whether in immune mechanism-triggered pain like arthritis, or nerve damage-induced neuroinflammation (gliosis). Its presence in the stem cell rich bulge region is a particularly valuable novel information. The discovery of murine MrgprB2 and human MRGPRX2 receptors as novel targets of HK-1, as well as the dual antagonists of both NK_1 receptor and MRGPRX2 has reopened the possibility of novel analgesics targeting the tachykinin system.

8 Summary: conclusions from the novel results

The tachykinin HK-1 mediates

- arthritis-related pain and inflammation in acute and chronic models, including late central sensitization independently of NK_1 receptor activation, while it prevents neutrophil MPO activity increase;
- TRPV1 activation-induced acute neurogenic inflammation, somatic and visceral nocifensive behaviors via the NK_1 receptor;
- primary sensory neuronal activation in a pertussis toxin-insensitive, NK_1 receptor-independent, but extracellular Ca^{2+}-dependent manner, as well as the prevention of capsaicin-induced desensitization;
- neuropathic pain and related neuroinflammation (gliosis) in the lamina I-II of the spinal dorsal horn independently of the NK_1 receptor, while it prevents peripheral NGF increase;
- motor coordination independently of NK_1-receptor, which is supported by the high HK-1 and low NK_1 receptor expressions in the cerebellum;
- skin swelling in psoriasis and allergic contact dermatitis models, while it prevents IFNγ and IL4 level increase in allergic contact dermatitis. The HK-1 encoding *Tac4* mRNA is present in the bulge area of the hair follicles of the mouse skin.


HK1
?
motor coordination
Ca2+
NK1
TRPA1
TRPV1
HK1
activation
sensitization
HK1
neuropathy
gliosis in spinal dorsal horn
HK1
?
inhibits NGF increase
NGF
neuropathic pain
acute pain
• visceral pain
HK1
NK1
• neurogenic inflammation
HK1
NK1
TRPV1
arthritis
HK1
?
arthritic pain and edema
histopathological changes
MPO
inhibits MPO increase
autoimmune dermatitis
IFNγ
IL4
inhibits IFNγ and IL4 increase
skin swelling
located in hair follicle bulge area

6. References

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9 List of publications

9.1 Publications related to the thesis:

Ágnes Hunyady*, Zsófia Hajna*, Tímea Gubányi, Bálint Scheich, Ágnes Kemény, Balázs Gaszner, Éva Borbély, Zsuzsanna Helyes; Hemokinin-1 is an important mediator of pain in mouse models of neuropathic and inflammatory mechanisms. Brain Res Bull. 2019 Apr;147:165-173. PMID: 30664920 DOI: 10.1016/j.brainresbull.2019.01.015

Éva Borbély*, **Ágnes Hunyady***, Krisztina Pohóczky, Maja Payrits, Bálint Botz, Attila Mócsai, Alexandra Berger, Éva Szőke, Zsuzsanna Helyes; Hemokinin-1 as a Mediator of Arthritis-Related Pain via Direct Activation of Primary Sensory Neurons. Front Pharmacol. 2021 Jan 13;11:594479. PMID: 33519457 PMCID: PMC7839295 DOI: 10.3389/fphar.2020.594479

9.2 Publications not related to the thesis:

B Scheich, P Vincze, É Szőke, É Borbély, **Á Hunyady**, J Szolcsányi, Á Dénes, Zs Környei, B Gaszner, Zs Helyes; Chronic stress-induced mechanical hyperalgesia is controlled by capsaicin-sensitive neurones in the mouse. Eur J Pain. 2017 Sep;21(8):1417-1431. PMID: 28444833 DOI: 10.1002/ejp.1043

Éva Borbély, Maja Payrits, **Ágnes Hunyady**, Gréta Mező, Erika Pintér; Important regulatory function of transient receptor potential ankyrin 1 receptors in age-related learning and memory alterations of mice. Geroscience. 2019 Oct;41(5):643-654. PMID: 31327098 PMCID: PMC6885083 DOI: 10.1007/s11357-019-00083-1

Boglárka Kántás, Rita Börzsei, Éva Szőke, Péter Bánhegyi, Ádám Horváth, **Ágnes Hunyady**, Éva Borbély, Csaba Hetényi, Erika Pintér, Zsuzsanna Helyes; Novel Drug-Like Somatostatin Receptor 4 Agonists are Potential Analgesics for Neuropathic Pain. Int J Mol Sci. 2019 Dec 11;20(24):6245. PMID: 31835716 PMCID: PMC6940912 DOI: 10.3390/ijms20246245

Boglárka Kántás, Éva Szőke, Rita Börzsei, Péter Bánhegyi, Junaid Asghar, Lina Hudhud, Anita Steib, **Ágnes Hunyady**, Ádám Horváth, Angéla Kecskés, Éva Borbély, Csaba Hetényi, Gábor Pethő, Erika Pintér, Zsuzsanna Helyes; In Silico, In Vitro and In Vivo Pharmacodynamic Characterization of Novel Analgesic Drug Candidate Somatostatin SST 4 Receptor Agonists. Front Pharmacol. 2021 Jan 27;11:601887. PMID: 33815096 PMCID: PMC8015869 DOI: 10.3389/fphar.2020.601887

Cumulative impact factor of publications related to the thesis: 7.595
Impact factor of other publications: 11.772
Number of citations (MTMT): 36
Number of independent citations (MTMT): 21
Number of citations (Google Scholar): 41

10 List of conference presentations

10.1 International conferences

Hemokinin-1 mediates neuropathic pain in a mouse model of traumatic neuropathy: role in spinal glia activation and peripheral NGF production
AMSE Congress, Pécs, Hungary, 2018.10.4-6., poster
Agnes Hunyady, Eva Borbely, Balint Scheich, Agnes Kemeny, Balazs Gaszner, Zsuzsanna Helyes

Hemokinin-1 is an important mediator of arthritic and neuropathic pain in mouse models
11th FENS Forum of Neuroscience, Berlin, Germany 2018.07.7-11., poster
Agnes Hunyady, Eva Borbely, Balint Scheich, Agnes Kemeny, Balazs Gaszner, Zsuzsanna Helyes

Hemokinin-1 is an important mediator of arthritic and neuropathic pain in mouse models
14th International Medical Postgraduate Conference, Hradec Kralove, Czech Republic, 2017.11.23-24., oral presentation
Agnes Hunyady, Eva borbely, Zsuzsanna Helyes

The effect of somatostatin 4 receptor agonists in mouse models of neuropathic pain, anxiety and depression-like behavior
FENS Regional Meeting, Pécs, Hungary, 2017.09.20-23., poster
Agnes Hunyady, Eva Borbely, Boglarka Kantas, Erika Pinter, Janos Szolcsanyi, Éva Szőke, Zsuzsanna Helyes

10.2 Domestic (Hungarian) conferences

A hemokinin-1 gyulladáskeltő szerepe pszoriáziform bőrgyulladás és allergiás kontakt dermatitisz egérmodelljeiben
FAMÉ 2019 - Magyar Kísérletes és Klinikai Farmakológiai Társaság, Magyar Anatómus Társaság, Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság, Magyar Élettani Társaság közös Vándorgyűlése

Budapest, 2019.06.05-08., oral presentation, **Pharmacology section grand prize**
Hunyady Ágnes, Helyes Zsuzsanna, Kemény Ágnes, Horváth Szabina, Horváth Ádám

A hemokinin-1 szerepet játszik krónikus traumás neuropátia kialakulásában centrális és perifériás mechanizmusokkal
Idegtudomanyi Centrum PhD/TDK konferencia, Pécs, 2018.11.22-23., oral presentation,
3. place
Hunyady Ágnes, Borbély Éva, Helyes Zsuzsanna

Új típusú, szájon át adható szomatosztatin 4 receptor agonisták hatásának vizsgálata neuropátiás fájdalomra, szorongásra és depresszió-szerű viselkedésre egérmodellekben
Magyarországi Fájdalom Társaság kongresszusa, Szeged, 2018.11.9-10., poster
Hunyady Ágnes, Borbély Éva, Kántás Boglárka, Pintér Erika, Szolcsányi János, Helyes Zsuzsanna

Szomatosztatin 4 receptor agonisták vizsgálata neuropátiás fájdalom, szorongás és depresszió-szerű viselkedés egérmodelljeiben
Magyarországi Fájdalom Társaság konferenciája, Szeged, 2017.11.10-11., poster
Hunyady Ágnes, Borbély Éva, Kántás Boglárka, Pintér Erika, Szolcsányi János, Helyes Zsuzsanna

Szomatosztatin 4 receptor agonisták vizsgálata neuropátiás fájdalom, szorongás és depresszió-szerű viselkedés egérmodelljeiben
MÉT Konferenciája Debrecen 2017.06.13-16., poster
Hunyady Ágnes, Borbély Éva, Kántás Boglárka, Pintér Erika, Szolcsányi János, Helyes Zsuzsanna

11 Acknowledgements

I would like to thank my supervisors, Prof. Zsuzsanna Helyes and Dr Borbély Éva for their guidance throughout my PhD years and the student research years preceding it. I am most grateful to Prof. Erika Pintér, the head of the institute and leader of the Neuropharmacology doctoral program for the opportunity to conduct my research. I would also like to thank the members of the research group. Kata Bölcskei, Valéria Tékus for their guidance, Maja Payrits, Krisztina Pohóczky, Ádám István Horváth, Bálint Botz, Szabina Horváth, Angéla Kecskés, János Konkoly, Zsófia Hajna, Tímea Gubányi, Katinka Békefi and Nikolett Szentes for their contributions to the experimental work, Dóra Ömböli, Teréz Bagoly, Zsanett Szarka, Izabella Orbán and Anikó Perkecz for their professional technical assistance; as well as our collaborators Gaszner Balázs, Mócsai Attila for their contributions. I wish to thank all members of the Department of Pharmacology and Pharmacotherapy valuable help. Finally, I would like to thank my family and friends for the help and support they provided over the years.