

**Involvement and relationship of transient receptor potential ankyrin 1 and
vanilloid 1 receptors and semicarbazide-sensitive amine oxidase
in mouse models of arthritis and pain**

PhD thesis



Ádám István Horváth MD

Pharmacology and Pharmaceutical Sciences Doctoral School

Neuropharmacology Program

Head of Doctoral School and Program Leader: Erika Pintér MD, PhD, DSc

Supervisor: Zsuzsanna Helyes MD, PhD, DSc

University of Pécs, Medical School

Department of Pharmacology and Pharmacotherapy

Pécs

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INTRODUCTION

1. Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) receptors

TRPA1 and TRPV1 are similar ligand-gated non-selective cation channels expressed on primary sensory afferents of dorsal root and trigeminal ganglia [1,2], as well as non-neuronal cells [3]. They can be activated by a variety of chemical irritants, endogenous inflammatory mediators and physical stimuli due to their polymodal sensory function. TRPA1 is stimulated by spicy chemicals found in the human diet (e.g. allyl-isothiocyanate, allicin, cinnamaldehyde, menthol), reactive oxygen species (ROS), SSAO products (e.g. formaldehyde, methylglyoxal and hydrogen-peroxide) and cold temperature ($<17^{\circ}\text{C}$), while TRPV1 by plant-derived vanilloids (e.g. capsaicin, resiniferatoxin, piperine, zingerone, eugenol), protons ($\text{pH}<6.0$), arachidonic acid metabolites (e.g. anandamide, 12-hydroperoxyeicosatetraenoic acid: 12-HPETE, N-oleoyl dopamine) and noxious heat ($>43^{\circ}\text{C}$) [3,4]. Furthermore several proinflammatory mediators (eg. bradykinin, prostaglandins, proteases, serotonin) are able to sensitize these receptors acting on their own receptor [5,6].

The special subpopulation of polymodal nociceptors called peptidergic, capsaicine-sensitive nerves are composed of sensory nerve terminals expressing TRPA1 and TRPV1 receptors. These nerves account for 40-50% of small diameter A δ - and C-fiber and possess three different function [7]. The classical afferent function is the sensation and pain transmission, the local efferent function is the mediation of neurogenic inflammation through the release of proinflammatory neuropeptides (e.g. calcitonine gene-related peptide: CGRP, substance P: SP, neurokinin A and B: NKA, NKB), while anti-inflammatory and antinociceptive neuropeptides (e.g. somatostatin) reach the circulation and exert systemic inhibitory efferent functions [8,9]. Neurogenic inflammation plays an important role in the pathogenesis of several inflammatory diseases, like rheumatoid arthritis, asthma bronchiale, psoriasis, ekczema, contact dermatitis, migraine or inflammatory bowel diseases, but currently we do not have drugs, which are able to inhibit this component of the mentioned disease [10]. Therefore there are pressing need to understand their patomechanisms and identify novel therapeutic targets like TRPA1 and TRPV1 receptors, which can be served as target of new anti-inflammatory and antinociceptive drugs.

2. Role of semicarbazide-sensitive amine oxidase (SSAO) in inflammation and pain, its relationship with TRPA1 and TRPV1 receptors

Semicarbazide-sensitive amine oxidase (SSAO), also known as vascular adhesion protein-1 (VAP-1), is a copper containing enzyme which catalyzes oxidative deamination of primary amines, resulting in the production of corresponding aldehydes, hydrogen peroxide and ammonia [11]. SSAO can be found in a circulating form in the plasma and as a membrane-bound form widely expressed in most tissues and organs, with particularly high levels in endothelial, vascular smooth muscle and adipose cells [12]. Besides the deamination of endogenous (e.g. methylamine, aminoacetone) and exogenous (e.g. allylamine) amines, it is involved in leukocyte trafficking and angiogenesis [13,14]. Significantly elevated plasma SSAO level was observed in several inflammatory-associated diseases, such as inflammatory liver, muscle and eye diseases, diabetes, congestive heart failure, atherosclerosis, stroke, severe obesity, chronic kidney disease, psoriasis, multiple sclerosis and Alzheimer's disease raising the possibility to use it as a biomarker in these conditions [15]. Several study using small-molecule SSAO inhibitors or anti-SSAO antibodies confirm that they can also have a therapeutic value due to anti-inflammatory and anti-angiogenic effects [16-20]. Furthermore our group addressed first their potential analgesic effect based on the decreased production of highly reactive SSAO-products (formaldehyde, methylglyoxal, hydrogen peroxide) stimulating TRPA1 receptor.

Several small molecule, primarily hydrazine and allylamine derivate SSAO inhibitors have been developed for potential therapeutic purposes in the last 15 years, but their experimental and clinical uses have been hampered by the lack of selectivity or unfavourable physicochemical properties [21]. Our group developed and patented a novel oxime compound, SzV-1287 (3-(4,5-dipheyl-1,3-oxazol-2-yl) propanal oxime) formed from the oxime analogue of the cyclooxygenase (COX) inhibitor oxaprozin [22,23]. SzV-1287 is an innovative metabolism-activated multi-targeting drug, acting by directly inhibiting SSAO activity and simultaneously COX function through its active metabolite and independently of its main action blocking TRPA1 and TRPV1 receptors expressed on primary sensory neurons [24,25]. Latter result confirmed its potential analgesic effect, which has never been proved regarding SSAO inhibitors.

3. Joint inflammation, its treatment options and difficulties

The group of inflammatory joint diseases (arthritis) is one of the largest group of rheumatology disorders affecting approximately 130-150 thousand patients in Hungary [26]. It can be divided for infectious and non-infectious diseases. Among the latter rheumatoid arthritis (RA) should be highlighted due to its high prevalence and severity. RA is an idiopathic, chronic, progressive systemic autoimmune syndrome hallmarked by polyarthritis and joint destruction. It affects almost 1% of the population worldwide, 0.3-0.5% in Hungary, it occurs three times more often in women and starts most commonly in fourth-fifth decade [27,28]. It reduces significantly not only the life quality, but it shortens also the life expectancy with 3-10 years, therefore it is considered a significant public health problem.

Symptomatic and disease-modifying drugs are distinguished in the pharmacological management of RA. Drugs relieving the symptoms are the steroidal and non-steroidal anti-inflammatory drugs (NSAID). Disease-modifying antirheumatic drugs (DMARD) can be divided for synthetic and biological compounds, and glucocorticoids offer also disease-modifying effects [29]. All mentioned drugs are often ineffective or their long-term use induces severe adverse effects [30-32]. Although currently used biological therapies, mainly the tumor necrosis factor (TNF)- α blockers can slow down the progression of the structural damage, joint pain frequently persists and reduces physical activity and life quality. To develop novel pharmacotherapy, intensive research needs to explore the pathophysiological mechanisms of arthritis-related pain. The dense innervation of the joints by capsaicin-sensitive sensory nerves and the involvement of neurogenic inflammation in arthritis are well-known, but there is significantly less information about the role of peptidergic afferents and sensory-immune/sensory-vascular interactions in this condition. Due to their localization and function TRPA1 and TRPV1 receptors and SSAO can be promising targets for novel anti-inflammatory and analgesic drugs.

4. Mechanisms of neuropathic pain, therapeutical challenges

The neuropathic pain represents all painful conditions arising due to damage or disease of the somatosensory system [33]. Its exact prevalence is unknown, it can affect approximately 7-8% of the population in Western-Europe [34]. Based on the anatomical localization and the etiology of the damage peripheral traumatic neuropathies, polyneuropathies (caused by e.g. metabolic disorders, inflammation, tumors and toxic agents), central (e.g. poststroke pain) and other complex pain syndromes (e.g. complex regional pain syndromes) are differentiated. Since its pathomechanism is not fully elucidated and we do not possess targeted therapy, its management

means huge challenge for the doctors. Due to the ineffectiveness and limited efficacy of conventional drugs adjuvant analgesics (certain antiepileptics, tricyclic antidepressants, local anaesthetics and capsaicin patch) can be used in first line of its pharmacological treatment [35]. However approximately in the half of the patients there can not be reached adequate effect with these drugs, while the responder patients can expect severe long-term side effects. Due to its unresolved therapy it is important to understand its precise mechanism involved peripheral and central sensitization and discover novel therapeutical targets like TRPA1 receptor mediating also neuropathic pain and SSAO producing its endogenous agonists. We investigated their roles in the mouse models of peripheral traumatic neuropathy and chronic arthritis associated with neuropathic components in the late phase.

AIMS

Alleviating neuropathic pain is one of the greatest challenge in the everyday doctoral practice, because the currently used painkillers are usually ineffective or cause severe adverse effects during the long-term use. There is an urging need to develop effective compounds with new mechanism of action, which do not possess severe side effects. Therefore we investigated the mechanisms of pain processing, role of the neuro-immune interactions, promising therapeutical targets, effects and mechanism of a novel drug candidate in mouse models of arthritis and traumatic nerve injury.

Our primary aims were the following:

1. To investigate the role of TRPA1 and TRPV1 receptors in acute and chronic arthritis and pain models using gene-deficient mice.
2. To examine the involvement of SSAO in serum transfer- and adjuvant-induced chronic arthritis mouse models using small molecule SSAO inhibitors.
3. To analyze the relationship of TRPA1, TRPV1 and SSAO in the mouse models of acute chemocifensive pain, serum transfer-induced chronic arthritis and traumatic nerve injury using gene-deficient mice and small molecule SSAO inhibitors.

EXPERIMENTAL MODELS AND INVESTIGATIONAL TECHNIQUES

1. Animals

Experiments were carried out on 8-15-week-old male TRPV1- (TRPV1^{-/-}) and TRPA1-deficient mice (TRPA1^{-/-}) and their wildtype (C57BL/6J and TRPA1^{+/+}) counterparts weighing 20-30 g. The effects of the SSAO inhibitor compounds in the different models were tested on C57BL/6J mice weighing 20-30 g and CD1 mice weighing 30-40 g of the same age and sex. The breeding pairs of homozygous TRPV1^{-/-} mice were purchased from The Jackson Laboratory and bred as homozygotes. Since the original breeding pairs were already backcrossed to the C57BL/6J mice, this background strain was used as wild type (WT) controls. Homozygous TRPA1^{-/-} and TRPA1^{+/+} mice were generated from TRPA1^{+/-} heterozygous mice generously donated by Pierangelo Geppetti (University of Florence). The genotype of the animals was determined by polymerase chain reaction (PCR). All mice were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pécs in standard (160x137x330 mm sized) cages with 5-10 mice/cage density under a 12-hour light/dark cycle at 24-25 °C, provided with standard mouse chow and water *ad libitum*.

2. Ethics statement

All experiments were performed according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments and the 40/2013. (II.14.) Hungarian Government regulation on the protection of animals used for scientific purposes complied with the directive 2010/63/EU of the European Parliament and Council and the recommendations of the International Association for the Study of Pain (IASP). All procedures were approved by the Ethics Committee on Animal Research of University of Pécs according to the Ethical Codex of Animal Experiments (licence No.: BA 02/2000-3/2011 and BA 02/2000-2/2012).

3. Experimental models

3.1. CFA-induced chronic arthritis model

The chronic joint inflammation was induced by intraplantar injection of complete Freund's adjuvant (CFA, killed Mycobacterium suspended in paraffin oil, 1 mg/ml; Sigma Aldrich) into the right hindpaw and subcutaneously (s.c.) into the tail root (20-20 µl in CD1, 50-50 µl in TRPA1^{+/+} és TRPA1^{-/-} mice). An additional s.c. injection was administered on the following

day into the tail root in order to potentiate the systemic effects [36]. Decrease of mechanonociceptive and termonociceptive threshold, paw swelling, cold sensitivity, plasma leakage and neutrophil myeloperoxidase (MPO) activity were assessed *in vivo* during the 21-day experimental period.

3.2. K/BxN serum transfer-induced arthritis model

Transient polyarthritis was induced by a single intraperitoneal (i.p.) injection of arthritogenic K/BxN serum in CD1 mice (300 µl on day 0) and by its repeated injection in C57BL/6J, TRPV1^{-/-}, TRPA1^{+/+} and TRPA1^{-/-} mice (150 µl on the days 0 and 3). Control animals received non-arthritogenic BxN serum following the same protocols [37,38]. Mechanonociceptive threshold, paw volume, clinical arthritis severity score, joint function, neutrophil MPO activity and plasmaprotein extravasation were measured repeatedly *in vivo* during the 14- and the 21-day experimental period.

3.3. Traumatic mononeuropathy model

Under ketamine/xylazine anesthesia (Calypsol/Sedaxylan, 100/5 mg/kg i.p.) the nerve injury was evoked by the partial ligation of 1/3 of the right common sciatic nerve in C57BL/6J, TRPV1^{-/-}, TRPA1^{+/+} and TRPA1^{-/-} mice (Seltzer operation) [39,40]. The mechanonociceptive thresholds were determined on the 7th postoperative day.

3.4. Carrageenan-induced acute inflammation model

Acute paw inflammation was evoked by intraplantar (i.pl.) injection of 50 µl 3% carrageenan solution (Sigma Aldrich) in TRPA1^{+/+} and TRPA1^{-/-} mice. Contralateral paws injected with same volume of saline (0.9% NaCl) served as control [41]. Mechanonociceptive and termonociceptive thresholds, paw volume and cold sensitivity were measured throughout the 24-hour experimental period.

3.5. CFA-induced acute inflammation model

Acute knee joint inflammation was induced by 20 µl intra-articularly (i.a.) injected CFA (Sigma Aldrich) in TRPA1^{+/+} and TRPA1^{-/-} mice under ketamine/xylazine anesthesia (Calypsol/Sedaxylan, 100/5 mg/kg i.p.) Contralateral knee joint injected with same volume of saline (0.9% NaCl) served as control [42]. Measurements of mechanonociceptive thresholds and knee diameters were carried out over a period of 24 hours.

3.6. Acute somatic chemonocifensive behavior and hyperalgesia models

3.6.1. TRPV1 activation-induced acute thermal and mechanical hyperalgesia model

The ultrapotent selective TRPV1 agonist RTX (Sigma Aldrich) was injected i.pl. into the right hindpaws to induce direct activation of the TRPV1-expressing capsaicin-sensitive peptidergic sensory nerves in C57BL/6J mice. After applying a single dose (20 µl, 0.03 µg/ml, 10 minutes

before the first measurement), the noxious heat thresholds were repeatedly determined at 5, 10, 15 and 20 min, while mechanonociceptive testing was performed at 2, 4, 6 and 24 h [43].

3.6.2. TRPA1 activation-induced acute nocifensive behavior and mechanical hyperalgesia model

The TRPA1 agonist formalin (20 μ l, 2.5%) was administered i.pl. into the right hindpaw to evoke direct activation of the TRPA1-expressing peptidergic sensory nerves in C57BL/6J mice. The duration of paw lickings was measured in two phases (0-5 min and 20-45 min), while the mechanonociceptive threshold was determined at 2 and 4 h [41].

4. Pharmacological tools

4.1. SzV-1287 and LJP-1207 treatment

In CFA- and K/BxN serum transfer-induced arthritis models SzV-1287 and the reference SSAO inhibitor LJP-1207 (N'(2-fenyl-allyl)-hydrazine hydrochloride) were administered daily in 20 mg/kg dose i.p., in traumatic mononeuropathy model SzV-1287 in a single 2, 5, 10, and 20 mg/kg dose i.p. as 20 min pretreatment, daily in 20 mg/kg dose i.p., LJP-1207 in a single 20 mg/kg dose as 20 min pretreatment. In acute neurogenic inflammation models the compounds were injected i.p. in 20 mg/kg dose 20 min before RTX- and formalin injection. LJP-1207 was dissolved in distilled water, while SzV-1287 at the beginning in a vehicle containing 2% Tween (polyethylene-glycol-sorbitan-monooleate), 2% ethanol and 96% distilled water, later in a vehicle containing 20% Kolliphor HS 15 (polyethylene-glycol-15-hydroxisterate) and 80% distilled water. 2 mg/ml solutions were prepared always immediately before use. Vehicle-treated mice served as controls.

4.2. Oxaprozin and diclofenac treatment

In traumatic mononeuropathy model oxaprozin, the active metabolite of SzV-1287 and diclofenac, a non-steroidal anti-inflammatory drug served as reference compound. Similar to SzV-1287 they were administered i.p. in a single 20 mg/kg dose as a 20 min pretreatment in C57BL/6J mice.

5. Investigational techniques

5.1. Measurement of mechanonociceptive thresholds

Mechanonociceptive thresholds of the hindpaw was evaluated using dynamic plantar esthesiometry (DPA, Ugo Basile 37400). Decrease of mechanonociceptive thresholds (mechanical hyperalgesia) was expressed as a percentage decrease compared to the baseline mechanonociceptive threshold values [36].

5.2. Measurement of paw volume

Paw volume was measured using plethysmometry (Ugile Basile Plethysmometer 7140). Paw edema was expressed as a percentage change compared to the baseline paw volume [36].

5.3. Measurement of knee diameter

The anteroposterior and mediolateral diameter of the knee joints were measured with a digital micrometer (Mitutoyo Corporation). Knee joint swelling was expressed as a percentage change compared to the baseline knee diameter [44].

5.4. Measurement of thermonociceptive thresholds

The thermonociceptive threshold of the paw was determined on increasing temperature hot-plate (IITC Life Sciences). Thermal hyperalgesia was expressed in °C drop of thermonociceptive threshold compared to the control values [43].

5.5. Measurement of cold sensitivity of the paw

The cold sensitivity was determined by the withdrawal latency after immersing the hindpaw in 0 °C icy water. It was expressed as withdrawal latency decrease compared to the control values [45].

5.6. Evaluation of arthritis severity

The classical signs of the inflammation, edema and hyperemia on both hindlimbs, were semiquantitatively scored daily using a scale of 0 to 10 in the K/BxN serum-transfer arthritis model [37].

5.7. Assessment of joint function (grid test)

The grasping ability correlating with joint function was determined daily using the grid test in the K/BxN serum-transfer arthritis model. Mice were placed on a horizontal wire grid, then it was turned over and the latency to fall was determined. The grid was maintained in horizontal position for a maximum of 20 seconds. The joint function impairment was expressed in sec drop of time spent on the grid compared to the control values [37].

5.8. Measurement of *in vivo* neutrophil MPO activity and plasmaprotein extravasation

Activity of neutrophil MPO playing important role in the pathomechanism of arthritis was determined by luminescence, plasmaprotein extravasation by fluorescence *in vivo* imaging using IVIS Lumina II (PerkinElmer) [46].

5.9. Histology

In CFA-induced arthritis model TRPA1^{+/+} and TRPA1^{-/-}, and CD1 mice were euthanized using sodium pentobarbital (Euthanimal 100 mg/kg) on day 10 and 21, respectively, to remove their tibiotarsal joints for histological processing. The sections were stained with haematoxylin and eosin, then arthritic changes were scored using a scale of 0 to 3 according to predetermined

parameters. The scores for each of criteria were accumulated to generate a composite arthritis score ranging between 0 and 9 [36].

5.10. Measurement of inflammatory cytokine concentrations in the tibiotarsal joint homogenates and plasma

Under sodium pentobarbital anesthesia (Euthanimal 100 mg/kg) blood samples of CD1 mice were taken by cardiac puncture and tibiotarsal joints were excised on the 4th day after CFA- or K/BxN serum administration for measurement of inflammatory cytokine concentrations. Following the centrifugation of blood samples and homogenization of joints interleukin (IL)-1 β , IL-6, keratinocyte chemoattractant (KC), macrophage inflammatory protein-2 (MIP-2) and TNF- α concentrations were determined with Luminex Multiplex Immunoassay using customized Milliplex Mouse Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore) [47]. The results were expressed based on tissue weight in pg/mg in case of joint homogenates and in pg/ml in case of plasma.

5.11. Cell culturing and metachromatic staining

Chondrifying primary micromass cell cultures were treated with SzV-1287 and LJP-1207 in 10 μ M final concentrations continuously from day 6 or from day 8. Their vehicles served as controls. On day 10 the amount of metachromatic cartilage matrix stained with 0.1% dimethyl methylene blue was assessed with microplate reader (Chameleon, Hidex Ltd.) following 0.1% toluidine blue (TB) staining [48].

5.12. Statistical analysis

Statistical analysis was performed by repeated measures two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test in cases of mechanical hyperalgesia (except in traumatic mononeuropathy model), paw and knee joint edema, thermal hyperalgesia, cold sensitivity, clinical scoring and grid test results, while one-way ANOVA followed by Bonferroni's multiple comparison test in cases of neuropathic hyperalgesia, the *in vivo* optical imaging, cytokine concentrations and histopathological scoring data. The TB data and the formalin-induced nocifensive behavior were analyzed by unpaired Student's t-test. The significance level was set at $p < 0.05$.

RESULTS

1. Role of TRPA1 and TRPV1 receptors in the mouse models of arthritis and pain

1.1 CFA-induced mechanical hyperalgesia and paw edema are attenuated in TRPA1-deficient mice

In TRPA1^{+/+} animals considerable mechanical hyperalgesia and hindpaw edema developed after the adjuvant injection reaching their maximum on day 3 (65%) and 14 (83%), respectively. Significantly reduced mechanical hyperalgesia and edema was observed in the TRPA1^{-/-} group starting on day 2 and 1, respectively. The thermonociceptive thresholds of the inflamed paws of both groups were similar to the baseline values of naive mice, while cold tolerance similarly decreased in all groups during the experimental period independently of the inflammation.

1.2. Neutrophil MPO activity is reduced in the early phase and vascular leakage is decreased in the late stage of the arthritis in TRPA1^{-/-} animals

Luminol-derived bioluminescence revealed an increase in neutrophil MPO activity in the arthritic ankle joints of TRPA1^{+/+} and TRPA1^{-/-} groups, being significantly smaller in the knockout strain in the early phase (day 2). The fluorescence was similarly high in the ankle joints of both groups in the early phase, demonstrating a remarkable enhancement of plasma extravasation. In the late phase (day 7), plasma extravasation diminished in both groups compared to the early phase, but significant difference was detected in TRPA1^{-/-} mice.

1.3. CFA-induced histopathological severity was reduced in the tibiotarsal joint of TRPA1^{-/-} mice

The tibiotarsal joints of TRPA1^{+/+} mice showed remarkably enhanced inflammatory cell infiltration into the areolar tissue, marked synovial cell lining hyperplasia and minimal cartilage destruction. In contrast, TRPA1^{-/-} mice showed reduced infiltration of inflammatory cells into the areolar tissue and moderate hyperplasia of the synovial cell lining, but cartilage damage was not detected. Semiquantitative scoring of composite arthritic changes in CFA-injected tibiotarsal joints found the severity of arthritis was significantly decreased in knockout animals.

1.4. K/BxN serum-induced late mechanical hyperalgesia is attenuated, but edema is increased in arthritic TRPV1^{-/-} mice

Mechanical hyperalgesia and paw edema in wildtype arthritic mouse groups reached the maximum of 25-31% on day 9 and 50% on days 7-9, respectively. Significantly smaller hyperalgesia and greater edema were measured in TRPV1^{-/-} mice from day 17 and from day 5 to 9, respectively. Meanwhile, there was no difference in the TRPA1^{-/-} group compared to their wildtypes.

1.5. K/BxN serum-induced MPO activity in the arthritic tibiotarsal joints is not influenced by TRPA1 and TRPV1 deletion

Luminol-derived bioluminescence alterations showing neutrophil MPO activity in the arthritic ankle joints and reaching its maximum on day 2, was similar in wildtype, TRPV1^{-/-} and TRPA1^{-/-} mice.

1.6. K/BxN serum-induced plasma extravasation in the arthritic joints is reduced in TRPV1^{-/-} and increased in TRPA1^{-/-} mice

In the late phase, when vascular leakage reached its maximum, significantly smaller plasma extravasation was detected in TRPV1^{-/-} mice (on day 9), but significantly greater in TRPA1^{-/-} ones (on day 6).

1.7. Similar mechanical hyperalgesia, paw edema, thermal hyperalgesia and cold tolerance were detected in TRPA1^{+/+} and TRPA1^{-/-} mice after carrageenan administration

Remarkable mechanical hyperalgesia, paw edema, thermal hyperalgesia and cold sensitivity developed both in the TRPA1^{+/+} and TRPA1^{-/-} groups after the i.pl. injection of carrageenan, but we did not observed significant difference between the groups.

1.8. Mechanical hypersalgesia and edema similarly developed in CFA-induced acute knee joint inflammation of TRPA1^{+/+} and TRPA1^{-/-} mice

Considerable mechanical hyperalgesia and knee joint edema developed in TRPA1^{+/+} and TRPA1^{-/-} mice after i.a. injection of CFA, but we did not detect significant difference between the groups.

2. Role of SSAO in the mouse models of arthritis and pain

2.1. SSAO inhibitors reduce K/BxN serum-induced mechanical hyperalgesia and inflammatory signs

In vehicle-treated arthritic mice 20% decrease in the mechanonociceptive thresholds and 35% increase in the paw volume developed 5 days after the K/BxN serum injection. Repeated daily treatment with 20 mg/kg i.p. SzV-1287 and LJP-1207 similarly and significantly reduced mechanical hyperalgesia, paw edema and clinical arthritis score in the arthritic groups. The time spent on the grid as an indicator of grasping ability was reduced, but this parameter was not affected by the treatments.

2.2. LJP-1207 significantly reduces K/BxN serum-induced early neutrophil MPO activity

Luminol-derived bioluminescence revealed an increase in neutrophil MPO activity in the arthritic ankle joints of all groups, being significantly smaller in the LJP-1207-treated arthritic groups in the early phase as compared to vehicle-treated mice (day 2).

2.3. Both SSAO inhibitors attenuate vascular leakage in the K/BxN serum-induced arthritis model

Plasma extravasation, as shown by the fluorescence signal, was significantly smaller in the ankle joints of SSAO inhibitor-treated arthritic groups in the early phase. In the late phase (day 6), plasma extravasation enhanced in all groups compared to the early phase, but significant reduction was observed only in LJP-1207-treated mice.

2.4. SSAO inhibitors decrease CFA-induced mechanical hyperalgesia and edema

All groups showed a remarkable mechanical hyperalgesia and paw edema after CFA-injection, which were significantly attenuated in both LJP-1207- and SzV-1287-treated mice.

2.5. SSAO inhibitors do not influence the neutrophil MPO activity and vascular leakage in the tibiotarsal joints on the CFA-injected side

All groups showed intensive luminol-derived bioluminescence signal in the tibiotarsal joints on the CFA-injected side, but it was not significantly different between any of the groups either on day 2 or day 6. Vascular leakage was similarly high in the arthritic ankle joints of all groups in the early phase, which increased further by day 6. However, significant difference was not observed between the groups.

2.6. SSAO inhibitors reduce the production of KC, but not other inflammatory cytokines in the arthritic tibiotarsal joints

In the K/BxN serum-transfer arthritis model only the concentrations of KC and MIP-2, in the CFA-model IL-6, KC, MIP-2 and TNF- α increased significantly in the ankle joint homogenates of vehicle-treated arthritic mice on day 4 compared to non-arthritic animals. SSAO inhibitors (in K/BxN serum-transfer model SzV-1287, in CFA-model LJP-1207) significantly reduced exclusively the inflammation-evoked tissue KC elevation. IL-1 β in the joint homogenates and any of the 5 investigated cytokines in the plasma did not change in either models in any of the groups at this timepoint.

2.7. SzV-1287 reduces histopathological severity of arthritis

Histopathological evaluation of the left tibiotarsal joints (CFA-injected side) of the vehicle-treated animals revealed synovial lining hyperplasia, infiltration of mononuclear cells into the synovium and cartilage destruction, which are all characteristic histopathological alterations in arthritis. Mice treated with SzV-1287 showed markedly reduced inflammatory cell infiltration into the synovium and minimal damage of the cartilage, animals treated with LJP-1207 showed slightly reduced synovial infiltration of inflammatory cells, but showed substantial cartilage destruction and synovial cell lining hyperplasia. Semiquantitative scoring and composite

arthritis scores revealed that arthritis severity was only significantly decreased in SzV-1287-treated animals, compared to the vehicle-treated group.

2.8. LJP-1207, but not SzV-1287 administration decreases the metachromatic matrix production in micromass cell cultures

In line with our *in vivo* results, SzV-1287 exerted only a moderate effect on cartilage matrix production *in vitro*. No significant differences were detected on day 10 between the cultures treated with SzV-1287 as compared to vehicle. In contrast, the application of LJP-1207 significantly decreased the amount of metachromatic cartilage matrix produced in chondrifying micromass cultures by day 10, following initiation of treatment at days 6 and 8.

3. Relationship of TRPA1 and TRPV1 receptors and SSAO in the mouse models of arthritis and pain

3.1. SzV-1287 significantly inhibits TRPV1 activation-evoked acute thermal and mechanical hyperalgesia of the mouse paw

I.pl. administration of the TRPV1 agonist RTX induced a robust, approximately 8°C thermal and 45% mechanical hyperalgesia in the vehicle-treated group. The acute thermal hyperalgesia was significantly lower in mice pretreated with i.p. SzV-1287, while the late mechanical hyperalgesia characterized by central sensitization was significantly and similarly reduced in SzV-1287- and LJP-1207-pretreated mice.

3.2. SzV-1287 significantly decreases TRPA1 activation-induced acute nocifensive behaviors and hyperalgesia

The total duration of paw lickings and liftings in response to i.pl. injection of the TRPA1 agonist formalin was significantly reduced in mice pretreated with SzV-1287 in the early phase (0–5 min), but the late one (20–45 min) was not affected by either compounds. An approximately 35% mechanical hyperalgesia developed 2 h after formalin injection in vehicle-treated mice, which was significantly and similarly reduced both in SzV-1287- and LJP-1207-pretreated groups.

3.3. SzV-1287 significantly reduces chronic arthritic hyperalgesia and edema

In the vehicle-treated arthritic WT mice an approximately 25–30% mechanical hyperalgesia and 50% hindpaw edema developed 5 days after the serum injection. In WT and TRPA1^{-/-} group hyperalgesia and edema were significantly attenuated by repeated daily treatments of 20 mg/kg i.p. SzV-1287, but these anti-hyperalgesic and anti-edema effects were not observed in TRPV1^{-/-} arthritic mice.

3.4. SzV-1287 significantly inhibits early MPO-activity in the arthritic tibiotarsal joints of WT and TRPV1^{-/-} mice

Intensive bioluminescence signal was detected in the arthritic tibiotarsal joints of WT mice on day 2, then remarkably decreased by day 9. This early MPO activity was significantly smaller in the SzV-1287-treated WT and TRPV1^{-/-}, but not in TRPA1^{-/-} mice.

3.5. SzV-1287 did not influence K/BxN serum-induced vascular leakage

In contrast, the fluorescence signal indicating plasmaprotein extravasation from the leaky venules gradually increased reaching a maximum on day 9, but it was not influenced by SzV-1287 treatment in either the WT or the knockout groups.

3.8. SzV-1287 significantly attenuates neuropathic mechanical hyperalgesia

In traumatic mononeuropathy model, on the 7th postoperative day both SSAO inhibitors resulted in significant reduction of neuropathic hyperalgesia compared to the vehicle-treated controls, while the active metabolite of SzV-1287 (oxaprozin) and the reference NSAID (diclofenac) were ineffective. Different doses of SzV-1287 (2, 5, 10, 20 mg/kg i.p.) did not show any clear dose-dependent effect. Except the smallest dose, all the others resulted in significant, approximately 50-60% anti-hyperalgesic action. In contrast to wildtypes, SzV-1287 had no effect on neuropathic mechanical hyperalgesia in either TRPV1^{-/-} or TRPA1^{-/-} mice.

DISCUSSION AND CONCLUSIONS

In the first part of our work we provided new data on the regulatory role of TRPA1 and TRPV1 receptors in adjuvant- and serum transfer-induced chronic arthritis, respectively. Involvement of TRPV1 in inflammation and pain is well-known [1], but only few data are available about TRPA1 in these processes, particularly in chronic arthritis and arthritis-related pain. Although TRPA1-deficient mice are also valuable tools to investigate the role of this receptor *in vivo*, only few arthritis studies have been performed with these [42,49]. Therefore, in the present study, we aimed to analyze its involvement in chronic arthritis of different mechanisms and related nociception in comparison with acute models using TRPA1-deficient mice.

In adjuvant-induced arthritis model in agreement with previous data we detected significantly smaller mechanical hyperalgesia in TRPA1^{-/-} mice as compared with their wildtypes [42,49]. Unlike these data we found similar differences between the groups regarding joint swelling and histopathological changes and we reported first about the regulatory role of TRPA1 in adjuvant-induced neutrophil activation and vascular leakage. The mentioned differences can be explained by the distinct features of models and different investigational techniques [42]. Our results are confirmed by a study performed with selective TRPA1-antagonist (HC-030031) [50]. We previously also showed decreased mechanical hyperalgesia, paw swelling and histopathological alterations in TRPV1^{-/-} mice in the same CFA-model [41]. These results suggest that both TRPA1 and TRPV1 have relevant regulatory roles in long-term inflammation and hyperalgesia [51]. Like human condition thermal hyperalgesia did not develop in our CFA-model, the noxious heat threshold did not significantly change in response to the inflammatory reaction. Data showing no difference in thermosensitivity of TRPA1^{+/+} and TRPA1^{-/-} mice are in agreement with previous reports suggesting that TRPA1 is not a heat sensor [6,52]. The functional relevance of TRPA1 in cold sensation is well-characterized [2,50,52], but in our experimental paradigm cold sensitivity increased in all groups independently of the inflammation suggesting the induction of cold hypersensitivity by the repeated measurements. Therefore, this investigational technique is not suitable for testing nociception in this model.

In contrast to CFA-model involved active immunization mechanisms, in K/BxN serum-transfer arthritis model based on passive immunization mechanical hyperalgesia, paw swelling and neutrophil MPO activity also were similar in TRPA1^{+/+} and TRPA1^{-/-} groups. TRPA1 should have a partial role only in moderation of late vascular leakage, because we found significantly increased plasmaprotein extravasation in TRPA1^{-/-} mice on day 6. In contrast, we showed significantly smaller K/BxN serum-induced mechanical hyperalgesia and plasmaprotein

extravasation, and more serious inflammatory signs in TRPV1^{-/-} mice. However the lack of this receptor did not influence the ROS production. These results are agreement with our previous results obtained in mice where the capsaicin-sensitive sensory nerves were defunctionalized with RTX confirming the complex regulatory and duration-dependent role of TRPV1 in this model. The protective effect of these nerves might be explained by the release of somatostatin, which is a potent anti-inflammatory and antinociceptive peptide [38].

In the present work we showed that TRPA1 does not have a role in acute carrageenan-induced paw edema, mechanical and thermal hyperalgesia. In contrast, previous reports demonstrated that the selective TRPA1-antagonist HC-030031 and genetic deletion of TRPA1 inhibited the development and maintenance of carrageenan-induced inflammatory hypersensitivity in rats, and paw edema in mice, respectively [53,54]. Distinct species, measuring timepoints, volumes, concentrations, and investigational techniques might be possible explanations for the differences. Since TRPA1 and TRPV1 form functional unit on capsaicin-sensitive sensory nerves [51], we have to emphasize that similarly to what we found in TRPA1-deficient mice, we previously described no difference in the carrageenan model in TRPV1-deficient mice [41]. Similarly to the carrageenan model and previous reports, acute i.a. CFA-evoked mechanical hyperalgesia and swelling were not altered by the deletion of the TRPA1 receptor over a 24-hour period [42,55].

The distinct functional outcomes between the roles of the TRPA1 receptor in our four models can be explained by the wide distribution of TRPA1 on sensory nerves and non-neuronal cells [3]. Several data suggest that there is a broad range of endogenous TRPA1 agonists produced locally during inflammatory processes that might differently modulate the receptor on the sensory nerves and non-neuronal structures. This would consequently trigger or inhibit the inflammatory cascades [56,57]. Our findings demonstrate an important regulatory role of TRPA1 and TRPV1 receptor in chronic arthritis and related pain behaviors in the mouse. Therefore, it might be a promising target for novel analgesic and anti-inflammatory drugs.

In the second part of our work we provide the first data on potent analgesic effect of SSAO inhibition in chronic mouse arthritis models. The pivotal role of SSAO in inflammation and the anti-inflammatory effect of SSAO inhibitors, e.g. in mouse models of arthritis, are well-known [13,16,17], but there were no data about its function in pain and the potential analgesic effect of its inhibitors has also never been addressed. Therefore, we aimed to investigate the analgesic effect of SSAO-inhibitor using a newly developed, innovative, metabolism-activated multi-targeting compound patented by our group. Our novel SSAO inhibitor, SzV-1287, similarly to reference compound, LJP-1207 showed significant antinociceptive effect in chronic mouse

arthritis models of different mechanisms. Beside their anti-hyperalgesic effects both compounds reduced significantly the K/BxN serum- and CFA-induced paw edema and the K/BxN serum-induced early plasmaprotein extravasation. In K/BxN serum-transfer arthritis model the late plasmaprotein extravasation was decreased only by reference compound, while the neutrophil MPO activity was influenced by either compounds, similarly to CFA-induced neutrophil activation and increase of vascular leakage. Among the 5 characteristic inflammatory cytokines (IL-1 β , IL-6, KC, MIP-2, TNF- α) in the arthritic tibiotarsal joints only the inflammation-induced increased production of neutrophil chemoattractant KC produced by macrophages, endothelial cells and synoviocytes was significantly reduced by the SSAO inhibitors. However, the inhibitory effect of SzV-1287 reached the level of statistical significance in the K/BxN serum-transfer model and that of LJP-1207 in the CFA-model, they exerted a similar action on this inflammatory parameter. These data suggest that the anti-inflammatory and anti-hyperalgesic actions of SSAO inhibitors are not predominantly related to cytokine production inhibition.

Although there was no significant difference between the action of SzV-1287 and LJP-1207 in our chronic *in vivo* experiments, regarding CFA-induced histopathological alterations we found prominent distinction. The most remarkable was the significant reduction in the inflammatory histopathological alterations (mononuclear cell infiltration and cartilage damage) in SzV-1287-treated mice, while chronic LJP-1207 treatment actually worsened the cartilage destruction at the 20 mg/kg i.p. dose, resulting in similarly worsened therapeutic outcomes. This *in vivo* observations were supported by the decreased metachromatic cartilage matrix production detected after LJP-1207, but not SzV-1287 treatment, in chondrocyte cultures. The cartilage damaging action of LJP-1207 in the chronically inflamed joint is not likely to be due to its SSAO inhibitory effect, since a very recently published paper reports that SSAO inhibition delayed the chondrocyte differentiation without any alteration of cell viability *in vitro* [58]. This might be related to some other mechanisms due to its different structure, since as an allylhydrazine derivate it belongs to the toxic compounds [21]. The unique features of SzV-1287 compared to other SSAO inhibitors are potent dual antagonistic property at the TRPA1/TRPV1 receptor, which may explain its beneficial effect against cartilage destruction in our models supported by recent data showing a potent protective action of TRPA1 activation on human and mouse chondrocytes, against monosodium iodoacetate (MIA)-evoked inflammatory damage [49]. In conclusion, these are the first data describing that SSAO inhibition exerts analgesic effects. We have shown that our novel SSAO inhibitor, SzV-1287, with a unique complex mechanism of action inhibits chronic arthritis with special emphasis on

related pain and cartilage destruction. Therefore, it opens promising perspectives for the development of a novel analgesic and anti-inflammatory drug.

In the third part of our work we examined the involvement of TRPA1 and TRPV1 receptors in the analgesic and anti-inflammatory actions of SzV-1287. Previously our group addressed first the link between SSAO and nociception based on the ability of highly reactive SSAO products to stimulate the TRPA1 receptor, than we described that our newly developed and patented SSAO inhibitor, SzV-1287 has a potent dual antagonistic effect on TRPA1 and TRPV1 receptors on primary sensory neurons [25]. *In vivo* SzV-1287 significantly decreased both the faster developing, TRPV1 and TRPA1 activation-induced acute thermal hyperalgesia and nocifensive behavior and the slower developing mechanical hyperalgesia. In contrast, the reference compound LJP-1207 decreased only mechanical hyperalgesia involving central sensitization in both models. Therefore, SSAO inhibition is likely to be involved in the similar inhibitory actions of the two compounds on mechanical hyperalgesia, while the direct TRPV1/TRPA1 antagonistic effect of SzV-1287 seems to play a major role in the inhibition of rapid peripheral activation and sensitization of sensory nerve terminals.

In autoimmune arthritis model evoked by repeated injection of K/BxN serum SzV-1287 had no effect on chronic mechanical hyperalgesia associated with neuropathic components in TRPV1^{-/-}, on edema in both knockout and on neutrophil MPO activity in TRPA1^{-/-} group. Conclusively, the anti-hyperalgesic effect of SzV-1287 is likely to be TRPV1-, its anti-edema effect TRPV1- and TRPA1- and its neutrophil MPO inhibitory action TRPA1-dependent. In the traumatic mononeuropathy model, where peripheral inflammatory mechanisms are not involved, a single administration of 20 mg/kg i.p. SzV-1287 induced a maximal of 65% anti-hyperalgesic effect as compared with vehicle-treated group. Repeated injections during the 7-day period did not increase the effect compared to the single administration, which suggests that it is an acute analgesic-like action and not related to the development of the neuropathy. SzV-1287 did not decrease neuropathic mechanical hyperalgesia in either TRPV1^{-/-} or TRPA1^{-/-} mice pointing out that its analgesic action is mediated by both receptors forming a functional dimer on the peptidergic nerve terminals. We did not find any difference between the anti-hyperalgesic actions of the reference SSAO inhibitor LJP-1207 and SzV-1287 either in this model, which clearly prove that SSAO inhibition play a major role in the analgesic actions of SzV-1287. Among the reference compound neither diclofenac nor the active metabolite oxaprozin decreased the neuropathic mechanical hyperalgesia confirming that COX inhibition does not play a role in the analgesic action of SzV-1287.

In conclusion, SzV-1287 exerts potent analgesic effects in chronic pain models with neuropathic mechanisms independently of its anti-inflammatory actions. This analgesic action is TRPV1-mediated in the arthritis and TRPV1/TRPA1-mediated in the traumatic nerve injury model. Our results provided an excellent basis to start the preclinical development of a new drug candidate, which we would like to achieve within the framework of a project entitled “Development of a new, multi-targeting, innovative analgesic drug: pharmacodynamic, preclinical and human phase I. studies” (GINOP-2.2.1-15-2016-00020). The completion of the preclinical dossier is expected for the end of 2019, and hopefully the human phase I. studies can be finished in 2020. We hope that SzV-1287 can open promising perspectives for the development of a completely new analgesic drug to target neuropathic pain, which is a currently unmet medical need.

SUMMARY OF THE NEW FINDINGS PRESENTED IN THE THESIS

1. Our findings demonstrate an important regulatory role of TRPA1 and TRPV1 receptors in chronic arthritis and related pain behavior in mouse models.
2. We provided the first data that SSAO inhibition exerts analgesic effects showing that our novel SSAO inhibitor, SzV-1287, with a unique complex mechanism of action inhibits chronic arthritis with special emphasis on related pain and cartilage destruction.
3. SzV-1287 exerts potent analgesic effects in chronic pain models with neuropathic mechanisms independently of its anti-inflammatory actions. This analgesic action is TRPV1-mediated in arthritis and TRPV1/A1-mediated in traumatic nerve injury.

REFERENCES

1. **Caterina MJ**, et al. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997;389:816-24.
2. **Story GM**, et al. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 2003;112:819-29.
3. **Fernandes ES**, et al. The functions of TRPA1 and TRPV1: moving away from sensory nerves. *Br J Pharmacol* 2012;166:510-21.
4. **Chen J**, Hackos DH. TRPA1 as a drug target-promise and challenges. *Naunyn-Schmiedberg's Arch Pharmacol* 2015;388:451-63.
5. **Szállási Á**, Blumberg PM. Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol Rev* 1999;51:159-212.
6. **Bautista DM**, et al. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* 2006;124:1269-82.
7. **Holzer P**. Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev* 1991;43: 143-201.
8. **Szolcsányi J**. Capsaicin-sensitive chemoceptive neural system with dual sensory-efferent function. In: Chahl LA, Szolcsányi J, Lembeck F, editors. *Antidromic Vasodilatation and Neurogenic Inflammation*. Budapest, Hungary: Akadémiai Kiadó; 1984. pp. 27-53.
9. **Helyes Zs**, et al. Anti-nociceptive effect induced by somatostatin released from sensory nerve terminals and by synthetic somatostatin analogues in the rat. *Neurosci Lett* 2000;278:185-8.
10. **Helyes Zs**, et al. Regulatory role of sensory neuropeptides in inflammation. In: Kovács M, Merchenthaler I, editors. *Neuropeptides and peptide analogs*. Kerala, India: Research Signpost; 2009. pp. 111-141.
11. **Yu PH**, et al. Physiological and pathological implications of semicarbazide-sensitive amine oxidase. *Biochim Biophys Acta* 2003;1647:193-9.
12. **Andrés N**, és mtsai. Tissue activity and cellular localization of human semicarbazide-sensitive amine oxidase. *J Histochem Cytochem* 2001;49:209-17.
13. **Salmi M**, Jalkanen S. VAP-1: An adhesin and an enzyme. *Trends Immunol* 2001;22:211-16.
14. **Noda K**, et al. Vascular adhesion protein-1 blockade suppresses choroidal neovascularization. *FASEB J* 2008;22:2928-35.
15. **Pannecoeck R**, et al. Vascular adhesion protein-1: Role in human pathology and application as a biomarker. *Crit Rev Clin Lab Sci* 2015;52:284-300.
16. **Salter-Cid LM**, et al. Anti-inflammatory effects of inhibiting the amine-oxidase activity of semicarbazide-sensitive amine oxidase. *J Pharmacol Exp Ther* 2005;315:553-62.
17. **Marttila-Ichihara F**, et al. Vascular amine oxidases are needed for leukocyte extravasation into inflamed joints in vivo. *Arthritis Rheum* 2006;54:2852-62.
18. **Noda K**, et al. Vascular adhesion protein-1 regulates leukocyte transmigration rate in the retina during diabetes. *Exp Eye Res* 2009;89:774:81.
19. **Marttila-Ichihara F**, et al. Small-molecule inhibitors of vascular adhesion protein-1 reduce the accumulation of myeloid cells into tumors and attenuate tumor growth in mice. *J Immunol* 2010;184:3164-73.
20. **Jarnicki AG**, et al. A novel SSAO inhibitor PXS-4728A suppresses inflammation and fibrosis and improves lung function in experimental chronic obstructive pulmonary disease. *Br J Pharmacol* 2016;doi: 10.1111/bph.13573.

21. **Foot JS**, et al. PXS-4681A, a potent and selective mechanism-based inhibitor of SSAO/VAP-1 with anti-inflammatory effects in vivo. *J Pharmacol Exp Ther* 2013;347:365-74.
22. **Mátyus P**, et al. Compounds for inhibiting semicarbazide-sensitive amine oxidase (SSAO)/vascular adhesion protein-1 (VAP-1) and uses thereof for treatment and prevention of diseases. 2010;PCT Int Appl WO/2010/029379.
23. **Helyes Zs**, et al. Semicarbazide-sensitive amine-oxidase inhibitors, as analgesics in traumatic neuropathy and neurogenic inflammation. 2014; Hungarian and USA PCT P1400205.
24. **Mátyus P**, Chai CL. Metabolism-activated multitargeting (MAMUT): An innovative multitargeting approach to drug design and development. *Chem Med Chem* 2015;doi: 10.1002/cmdc.201500406.
25. **Payrits M**, et al. A novel 3-(4,5-diphenyl-1,3-oxazol-2-yl)propanal oxime compound is a potent Transient Receptor Potential Ankyrin 1 and Vanilloid 1 (TRPA1 and V1) receptor antagonist. *Neuroscience* 2016;324:151-62.
26. **Poór Gy**. Áttörés az ízületi gyulladások kezelésében. *Magyar Tudomány* 2014;8:958-64.
27. **Gibovsky A**. Overview of epidemiology, aetiology, and diagnosis of rheumatoid arthritis. *Am J Manag Care* 2012;18:S295-302.
28. **Szekanecz Z**. A rheumatoid arthritis gyakorlati kérdései. *Háziorvos Továbbképző Szemle* 2004;9:17-22.
29. **Smolen JS**, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. *Ann Rheum Dis* 2014;73:492-509.
30. **Aronson JK**. Meyler's side effects of analgesics and anti-inflammatory drugs. Amsterdam: Elsevier; 2009.
31. **Coutinho AE**, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol* 2011;335:2-13.
32. **Ramiro S**, et al. Safety of synthetic and biological DMARDs: a systematic literature review informing the 2013 update of EULAR recommendations for management of rheumatoid arthritis. *Ann Rheum Dis* 2016;75:16-22.
33. **Jensen TS**, et al. A new definition of neuropathic pain. *Pain* 2011;152:2204-5.
34. **Bouhassira D**, et al. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain* 2008;136:380-7.
35. **Attal N**, et al. EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision. *Eur J Neurol* 2010;17:1113-23.
36. **Szabó Á**, et al. Role of TRPV1 receptors in adjuvant-induced chronic arthritis: in vivo study using gene-deficient mice. *J Pharmacol Exp Ther* 2005;314:111 –9.
37. **Jakus Z**, et al. Critical role of phospholipase C γ 2 in integrin and Fc receptor-mediated neutrophil functions and the effector phase of autoimmune arthritis. *J Exp Med* 2009;206:577-93.
38. **Borbély É**, et al. Capsaicin-sensitive sensory nerves exert complex regulatory functions in the serum-transfer mouse model of autoimmune arthritis. *Brain Behav Immun* 2015;45:50-9.
39. **Seltzer Z**, et al. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 1990;43:205-18.
40. **Malmberg AB**, Basbaum AI. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain* 1998;76:215-22.

41. **Bölskei K**, et al. Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain* 2005;117:368-76.
42. **Fernandes ES**, et al. A distinct role for transient receptor potential ankyrin 1, in addition to transient receptor potential vanilloid 1, in tumor necrosis factor α -induced inflammatory hyperalgesia and Freund' complete adjuvant-induced monarthritis. *Arthritis Rheum* 2011;63:819–29.
43. **Almási R**, et al. Effect of resiniferatoxin on the noxious heat threshold temperature in the rat: a novel heat allodynia model sensitive to analgesics. *Br J Pharmacol* 2003;139:49-58.
44. **Helyes Zs**, et al. Involvement of transient receptor potential vanilloid 1 receptors in protease-activated receptor-2-induced joint inflammation and nociception. *Eur J Pain* 2010;14:351-8.
45. **Tékus V**, et al. A CRPS-IgG-transfer-trauma model reproducing inflammatory and positive sensory signs associated with complex regional pain syndrome. *Pain* 2014;155:299-308.
46. **Botz B**, et al. Differential regulatory role of Pituitary Adenylate-Cyclase Activating Polypeptide in the serum-transfer-induced arthritis model. *Arthritis Rheumatol* 2014;66:2739-50.
47. **Bánki E**, et al. Effect of PACAP treatment on kidney morphology and cytokine expression in rat diabetic nephropathy. *Peptides* 2013;42:125-30.
48. **Matta C**, et al. Cytosolic free Ca²⁺ concentration exhibits a characteristic temporal pattern during in vitro cartilage differentiation: a possible regulatory role of calcineurin in Ca-signalling of chondrogenic cells. *Cell Calcium* 2008;44:310-23.
49. **Moilanen LJ**, et al. Monosodium iodoacetate-induced inflammation and joint pain are reduced in TRPA1 deficient mice--potential role of TRPA1 in osteoarthritis. *Osteoarthritis Cartilage* 2015;23:2017-26.
50. **da Costa DSM**, et al. The involvement of the transient receptor potential A1 (TRPA1) in the maintenance of mechanical and cold hyperalgesia in persistent inflammation. *Pain* 2010;148:431-7.
51. **Spahn V**, et al. Modulation of transient receptor vanilloid 1 activity by transient receptor potential ankyrin 1. *Mol Pharmacol*. 2014;85:335-44.
52. **Kwan KY**, et al. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron*. 2006;50:277–89.
53. **Moilanen LJ**, et al. TRPA1 contributes to the acute inflammatory response and mediates carrageenan-induced paw edema in the mouse. *Sci Rep* 2012;2:380.
54. **Bonet IJM**, et al. The role of transient receptor potential A 1 (TRPA1) in the development and maintenance of carrageenan-induced hyperalgesia. *Neuropharmacology* 2013;65:206-12.
55. **Petrus M**, et al. A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Mol Pain* 2007;3:40.
56. **Atoyan R**, et al. Non-neuronal expression of transient receptor potential type A1 (TRPA1) in human skin. *J Invest Dermatol* 2009;129:2312-15.
57. **Chao LK**, et al. Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling. *Food Chem Toxicol* 2008;46:220-31.
58. **Filip A**, et al. Expression of the semicarbazide-sensitive amine oxidase in articular cartilage: its role in terminal differentiation of chondrocytes in rat and human. *Osteoarthritis Cartilage* 2016;24:1223-34.

PUBLICATIONS

1. Publications related to the thesis:

Horváth Á, Tékus V, Boros M, Pozsgai G, Botz B, Borbély É, Szolcsényi J, Pintér E, Helyes Zs. Transient receptor potential ankyrin 1 (TRPA1) receptor is involved in chronic arthritis: in vivo study using TRPA1-deficient mice. *Arthritis Res Ther* 2016;18:6. (IF: 3,979)

Horváth Á*, Menghis A*, Botz B, Borbély É, Kemény Á, Tékus V, Csepregi J, Mócsai A, Juhász T, Zákány R, Bogdán D, Mátyus P, Keeble J, Pintér E, Helyes Z. Analgesic and anti-inflammatory effects of the novel semicarbazide-sensitive amine-oxidase inhibitor SzV-1287 in chronic arthritis models of the mouse. *Sci Rep* 2017;7:39863. (IF: 5,228) *Co-first author

Horváth Á, Tékus V, Bencze N, Szentes N, Scheich B, Bölcskei K, Szőke É, Mócsai A, Tóth-Sarudy É, Mátyus P, Pintér E, Helyes Z. Analgesic effects of the novel semicarbazide-sensitive amine oxidase inhibitor SzV-1287 in mouse pain models with neuropathic mechanisms: involvement of TRPV1 and TRPA1 receptors. Under review.

2. Publications not related to the thesis:

Tékus V, **Horváth Á**, Hajna Zs, Borbély É, Bölcskei K, Boros M, Pintér E, Helyes Zs, Pethő G, Szolcsányi J. Noxious heat threshold temperature and pronociceptive effects and pronociceptive effects of allyl isothiocyanate (mustard oil) in TRPV1 or TRPA1 gene-deleted mice. *Life Sci* 2016;154:66-74. (IF: 2,685)

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Horváth Á. Tranziens Receptor Potenciál Vanilloid 1 (TRPV1) és Ankyrin 1 (TRPA1) ioncsatornák szerepének vizsgálata a termonocicepcióban. Kari TDK konferencia, Pécs, Magyarország, 2012.

Horváth Á. Tranziens Receptor Potenciál Vanilloid 1 (TRPV1) és Ankyrin 1 (TRPA1) ioncsatornák szerepének vizsgálata gyulladásoos fájdalom egérmodelljeiben. Kari TDK konferencia, Pécs, Magyarország; XXXI. OTDK, Szeged, Magyarország; XX. Tudományos Diákköri Konferencia, Marosvásárhely, Románia; V. Nemzetközi és XI. Országos Interdiszciplináris Grastyán Konferencia, Pécs, Magyarország, 2013.

Horváth Á. Tranziens Receptor Potenciál Ankyrin 1 (TRPA1) ioncsatorna szerepének vizsgálata akut és krónikus gyulladásoos egérmodelljeiben. Kari TDK konferencia, Pécs, Magyarország; XXI. Tudományos Diákköri Konferencia, Marosvásárhely, Románia; Pécsi Tudományegyetem Idegtudományi Centrum és Szentágothai János Kutatóközpont PhD és TDK konferencia, Pécs, Magyarország, 2014.

Horváth Á. Tranziens Receptor Potenciál Ankyrin 1 (TRPA1) ioncsatorna szerepének vizsgálata gyulladásoos fájdalom egérmodelljeiben. Korányi Frigyes Tudományos Fórum, Budapest, Magyarország; VI. Nemzetközi és XII. Országos Interdiszciplináris Grastyán Konferencia, Pécs, Magyarország, 2014.

Horváth Á., Menghis A., Botz B., Borbély É., Csepregi J., Mócsai A., Mátyus P., Pintér E., Szolcsányi J., Helyes Zs. Szemikarbazid-szenzitív aminoszén-dioxid gátlók gyulladáscsökkentő és fájdalomcsillapító hatásai krónikus artritisz egérmodelljeiben. VII. Nemzetközi XIII. Országos Interdiszciplináris Grastyán Konferencia; IV. Interdiszciplináris Doktorandusz Konferencia; II. Magyar Neuroendokrinológiai Szimpózium, Pécs, Magyarország, 2015.

Horváth Á. Tranziens Receptor Potenciál Ankyrin 1 (TRPA1) ioncsatorna szerepének vizsgálata akut és krónikus gyulladás egérmodelljeiben. XXXII. OTDK, Budapest, Magyarország, 2015.

Horváth Á., Menghis A., Botz B., Borbély É., Csepregi J., Mócsai A., Czompa A., Ludmerczki R., Mátyus P., Pintér E., Szolcsányi J., Helyes Zs. Anti-inflammatory and analgesic effects of semicarbazide-sensitive amine-oxidase (SSAO) inhibitors in chronic arthritis mouse models. International CEEPUS Summer School on Complex Diseases, Portoroz, Szlovénia, 2015.

Horváth Á., Tékus V, Boros M, Pozsgai G, Botz B, Borbély É, Szolcsányi J, Pintér E, Helyes Zs. Mediator role of the Transient receptor potential ankyrin 1 (TRPA1) receptor is involved in chronic arthritis. Neuroptides 2015, Aberdeen, Skócia, 2015.

Tékus V, Helyes Z, Hajna Z, **Horváth Á.**, Kun J, Bölcskei K, Pintér E, Szolcsányi J. Transient Receptor Potential Vanilloid 1 (TRPV1), but not Ankyrin 1 (TRPA1) ion channels mediate mustard oil-induced hyperalgesia in mice. I. International Doctoral Workshop of Natural Sciences, Pécs, Magyarország, 2012.

Helyes Zs, Scheich B, **Horváth Á.**, Botz B, Tékus V, Czompa A, Ludmerczki R, Pozsgai G, Boros M, Pintér E, Szolcsányi J, Mátyus P. A Tranziens Receptor Potenciál Ankyrin 1 (TRPA1) ioncsatorna szerepe és aktivációs mechanizmusa gyulladás és neuropátia egérmodelljeiben. Magyar Kísérletes és Klinikai Farmakológiai Társaság Experimentális Farmakológiai szekciójának IX. szimpóziuma, Velence, Magyarország, 2015.

Helyes Zs, Scheich B, **Horváth Á.**, Botz B, Tékus V, Czompa A, Ludmerczki R, Pintér E, Csepregi J, Szolcsányi J, Mócsai A, Mátyus P. Role and activation mechanism of the Transient Receptor Potential Ankyrin 1 (TRPA1) ion channel in mouse models of chronic pain. Neuroptides 2015, Aberdeen, Skócia, 2015.

Pintér E, Pozsgai G, Hajna Z, Sággy E, **Horváth Á.**, Szőke É, Sár C, Bagoly T, Aubdool A, Brain S, Helyes Zs. TRPA1 receptor-mediated effects of H₂S donors and polysulfides. Neuroptides 2015, Aberdeen, Skócia, 2015.

Pethő G, Tékus V, **Horváth Á.**, Bölcskei K, Helyes Zs, Pintér E, Szolcsányi J. A TRP-ioncsatornák szerepe a nociceptív hőküszöb meghatározásában: régi kérdés, új válaszok. Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok közös tudományos konferenciája, Pécs, Magyarország, 2016.

Helyes Z, **Horváth Á.**, Tékus V, Scheich B, Pintér E, Csepregi J, Mócsai A, Bogdán D, Mátyus P. Analgesic effects of the novel semicarbazide-sensitive amine-oxidase inhibitor SzV-1287 in animal models of pain. 7th European Congress of Pharmacology, Isztambul, Törökország, 2016.

4. Poster presentations:

Horváth Á. A Tranziens Receptor Potenciál Ankyrin 1 (TRPA1) ioncsatorna szerepének vizsgálata akut és krónikus gyulladás egérmodelljeiben. Amerikai Magyar Orvosszövetség (HMAA) Hungary Chapter konferenciája, Balatonfüred, Magyarország, 2014.

Horváth Á. Role of the Transient Receptor Potential Ankyrin 1 (TRPA1) ion channel in the acute and chronic inflammatory pain models of the mouse. 15th Biannual Conference of the Hungarian Neuroscience Society, Budapest, Magyarország, 2015.

Horváth Á., Menghis A., Botz B., Borbély É., Csepregi J., Mócsai A., Mátyus P., Pintér E., Szolcsányi J., Helyes Zs. Szemikarbazid-szenzitív aminoszén-dioxid gátlók gyulladáscsökkentő és fájdalomcsillapító hatásai krónikus artritisz egérmodelljeiben. Magyar Kísérletes és Klinikai Farmakológiai Társaság Experimentális Farmakológiai szekciójának IX. szimpóziuma, Velence, Magyarország, 2015.

Horváth Á, Borbély É, Bölcskei K, Botz B, Szentes N, Rauch T, Boldizsár F, Pintér E, Helyes Zs. A kapszaicin-érzékeny érzőideg-végződés komplex szabályozó szerepének vizsgálata krónikus ízületi gyulladás egérmodelljében. Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok közös tudományos konferenciája, Pécs, Magyarország, 2016.

Horváth Á, Scheich B, Botz B, Tékus V, Szentes N, Csepregi J, Mócsai A, Bogdán D, Mátyus P, Pintér E, Helyes Z. Analgesic effects of a novel semicarbazide-sensitive amine-oxidase inhibitor in neuropathic and inflammatory pain models of the mouse. Regulatory Peptides 2016, Rouen, Franciaország, 2016.

Tékus V, Helyes Zs, Hajna Zs, **Horváth Á,** Kun J, Bölcskei K, Pintér E, Szolcsányi J. A Tranziens Receptor Potenciál Vanilloid 1 (TRPV1) és Ankyrin 1 (TRPA1) ioncsatornák szerepének vizsgálata mustárolajjal kiváltott nocifenzív viselkedésben és akut gyulladásos hiperalgégiában. Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok közös tudományos konferenciája, Debrecen, Magyarország, 2012.

Tékus V, Hajna Z, **Horváth Á,** Kun J, Bölcskei K, Szolcsányi J, Helyes Z. Role of the Transient Receptor Potential Vanilloid 1 and Ankyrin 1 (TRPV1 and TRPA1) ion channels in thermociception in mice. IBRO International Workshop, Szeged, Magyarország, 2012.

Tékus V, Helyes Z, Hajna Z, **Horváth Á,** Kun J, Bölcskei K, Pintér E, Szolcsányi J. Transient Receptor Potential Vanilloid 1 (TRPV1), but not Ankyrin 1 (TRPA1) ion channels mediate mustard oil-induced hyperalgesia in mice. British Pharmacological Society (BPS) Focused Meeting on Neuropeptides, London, Egyesült Királyság, 2012.

Tékus V, Scheich B, Kóger T, **Horváth Á,** Hajna Z, Borbély É, Pintér E, Szolcsányi J, Helyes Zs. Role of the Transient Receptor Potential Ankyrin 1 (TRPA1) ion channel in nociceptive processes. Magyar Idegtudományi Társaság XIV. Konferenciája, Budapest, Magyarország, 2013.

Tékus V, **Horváth Á,** Botz B, Szolcsányi J, Pintér E, Helyes Zs. Role of the Transient Receptor Potential Ankyrin 1 (TRPA1) ion channel in the acute and chronic inflammatory pain models using gene-deficient mice. Joint Meeting of FEPS and the Hungarian Physiological Society, Budapest, Magyarország, 2014.

Helyes Zs, Scheich B, **Horváth Á**, Botz B, Tékus V, Czompa A, Ludmerczki R, Pintér E, Csepregi J, Mócsai A, SZolcsányi J, Mátyus P. Analgesic effects of a novel semicarbazide-sensitive amine-oxidase inhibitor in neuropathic and inflammatory pain models of the mouse. IBRO Workshop 2016, Budapest, Magyarország, 2016.

Bölskei K, Tékus V, Scheich B, **Horváth Á**, Czompa A, Ludmerczki R, Mátyus P, Helyes Zs. A szemikarbazid-szenzitív amin oxidáz (SSAO) gátlók antihiperalgetikus hatásának és a TRPA1 ioncsatorna szerepének vizsgálata traumás neuropáthiás fájdalom állatmodelljében. Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok közös tudományos konferenciája, Pécs, Magyarország, 2016.

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