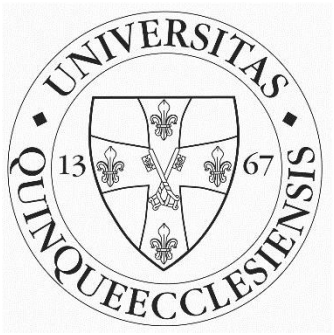
INVESTIGATION OF SENSORY-IMMUNE INTERACTIONS IN VASCULAR, RESPIRATORY AND METABOLIC MECHANISMS

PhD thesis



ZSÓFIA RÉKA HAJNA MD

Pharmacology and Pharmaceutical Sciences Doctoral School
Neuropharmacology Program

Head of the Doctoral School and Program Leader: Erika Pintér MD, PhD, DSc
Supervisors: Erika Pintér MD, PhD, DSc; Zsolt Biró MD, PhD, DSc

University of Pécs, Medical School
Department of Pharmacology and Pharmacotherapy

2017

INTRODUCTION

1. Capsaicin-sensitive sensory nerves and neurogenic inflammation

The subpopulation of capsaicin-sensitive sensory nerves are composed of primary sensory afferents of the dorsal root and trigeminal ganglia expressing the Transient Receptor Potential Vanilloid 1 (TRPV1) receptor. These polymodal nociceptors possess three different functions (Szolcsányi, 1996). Their *classical afferent* function is the mediation of sensory inputs towards the central nervous system, resulting in pain sensation. The *local efferent* function is the formation of **neurogenic inflammation** through the release of proinflammatory neuropeptides (calcitonin gene-related peptide (CGRP) and tachykinins) from the peripheral nerve endings, leading to vasodilatation, plasma protein extravasation and activation of inflammatory cells (Szolcsányi, 1988). Due to the *systemic efferent* function, antiinflammatory neuropeptides (e.g. somatostatin, PACAP) are also released, reaching the systemic circulation and exerting antiinflammatory and analgesic effects in distinct parts of the body (Szolcsányi et al., 1998). TRPV1 is a polymodal cation channel that can be gated by noxious heat (>43°C), high proton concentration (pH<6), plant-derived vanilloids (e.g. capsaicin, resiniferatoxin) and endogenous agonists (e.g. anandamide, arachidonic acid metabolites) (Tominaga et al., 1998; Yoo és mtsai., 2014). Other compounds (e.g. bradykinin, prostaglandins) are able to sensitize the TRPV1 by acting on their own receptor (Szallasi és mtsai., 2007). The activation of the receptor causes Na^+- and Ca^{2+}-influx, resulting in membrane depolarization and consequent formation of action potential and the release of sensory neuropeptides from nerve endings, respectively.

2. Transient Receptor Potential Ankyrin 1 (TRPA1) receptor

Another member of the TRP receptor family, the Transient Receptor Potential Ankyrin 1 (TRPA1) is also expressed on capsaicin-sensitive sensory fibres (Jaquemar et al., 1999). TRPA1 is often colocalized with the TRPV1 receptor, as well as with CGRP and substance P (SP) (Julius, 2013; Chen et al., 2012). Recently, the non-neural expression of TRPA1 has also been descibed, e.g. on fibroblasts, keratinocytes, macrophages and the endothelial cells of cerebral arteries (Fernandes et al., 2012). The exogenous agonists of TRPA1 are several plant-derived compounds, such as allylisothiocyanate (AITC, the pungent agent of mustard oil) and cinnamaldehyde (Jordt et al., 2004; Macpherson et al., 2006), but also certain toxic gases, such as crtotonaldehyde and acrolein found in cigarette smoke (Bautista és mtsai., 2006; André et al., 2008). It is important to note, that numerous endogenously formed compounds, such as reactive oxygen species (ROS) and several lipid peroxydation products (e.g. 4-hydroxynonenal (HNE), 4-oxo-2-nonenal (4-ONE)) are able to stimulate the TRPA1 (Trevisani et al., 2007;Graepel et al., 2011). Among the physical activators of the receptor, the role of mechanical stimuli and noxious cold (<17°C) has been proposed (Story et al., 2003; Jordt et al., 2004; Brierley et al., 2011). The TRPA1 receptor is a ligand-gated non-selective cation channel, the activation of which occurs through the binding of the agonists to the N-terminal

cysteine residues. This results in conformation changes and the opening of the ion channel, followed by the depolarization of the cell membrane and the elevation of the intracellular Ca^{2+}-level (Macpherson et al., 2007; Chen and Hackos, 2015). The subsequent release of SP and CGRP produces neurogenic inflammation (Trevisani et al., 2007). Several activators of the TRPA1 has been suggested to elicit vasodilatation, e.g. cinnamaldehyde and acrolein, as well as the endogenous 4-ONE (Pozsgai et al., 2010; Kunkler et al., 2011; Graepel et al., 2011). Moreover, TRPA1 plays a key role in the development of cold-induced vascular response (Aubdool et al., 2014). However, the precise mechanisms of the vasodilatory effect of TRPA1 is currently unknown (Earley, 2012). Another important role of the TRPA1 is the formation of physiological reactions to airway irritants (Bessac and Jordt, 2008). The acute inhalation of toxic compounds induces protective reflexes (e.g. cough), but the permanent stimulation of the receptor by endogenous activators produced in inflammatory processes results in further release of neurotransmitters and cytokines (Chen et al., 2012). Moreover, the mediator role of TRPA1 has also been suggested in certain effects induced by bacterial endotoxins (Meseguer et al., 2014). Therefore, investigating the function of the TRPA1 receptor is highly reasonable not only in local microcirculation, but also in acute and chronic inflammatory processes of the respiratory system.

3. Neuropeptides released from capsaicin-sensitive nerve endings: CGRP and tachykinins

CGRP is a 37-amino acid peptide, which exists in two isoforms: α-CGRP, found predominantly in the central and peripheral nervous system, and β-CGRP, being present mainly in the enteric nervous system. The expression of CGRP has also been described in non-neuronal cells, e.g. immune- and endothelial cells (Russell et al., 2014). The CGRP receptor is a heterodimeric complex composed of two peptide molecules (CLR-RAMP1) (Barwell et al., 2012). CGRP is released from the peripheral endings of dorsal root and trigeminal ganglia neurons upon stimulation, and its major role is the regulation of microcirculation. CGRP is an extremely potent vasodilator molecule, exerting its effects predominantly in the skin and the trigeminal system, that are the main anatomical targets of the peptide (Brain and Cox, 2006).

The family of **tachykinins** includes the Tac1 gene-encoded SP and neurokinin A (NKA), the Tac3-gene-derived neurokinin B (NKB), as well as the Tac4 gene-encoded hemokinins and endokinins (Steinhoff et al., 2014). The effects of the tachykinins are mediated by three identified G-protein-coupled receptors: the neurokinin 1 (NK_1), the neurokinin 2 (NK_2) and the neurokinin 3 (NK_3) receptors. SP shows the greatest affinity to the NK_1 receptor, whereas NKA binds predominantly to the NK_2, and NKB to the NK_3 receptor (Maggi, 1995). The best known central effect of SP - expressed mainly in the capsaicin-sensitive primary afferents but also found in several immune cells – is the modulation of pain sensation (Zubrzycka and Janecka, 2000). The peripheral actions of SP include plasma protein extravasation, leukocyte infiltration and mast cell degranulation during neurogenic inflammation (Grant, 2002). These effects occur through the activation the NK_1 receptors found on

endothelial and inflammatory cells (Cao et al., 1999). NKA causes plasma protein extravasation, smooth muscle contraction and activation of inflammatory cells (de Swert and Joos, 2006). The Tac4 gene was cloned in 2000, and it encodes several related peptides in different species: hemokinin-1 (HK-1) in mice and rats, as well as human HK-1 (hHK-1) and the group of endokinins (EKA, EKB, EKC, EKD) in humans (Zhang et al., 2000; Page, 2004). HK-1 binds predominantly to the NK1 receptor (Bellucci et al., 2002), moreover, it also shows structural similarities to SP resulting in strong immunological crossreactivity with anti-SP antibodies (Page, 2004). However, numerous effects induced by HK-1 differ from those of SP. This feature might be explained by the hypothesis that certain actions of HK-1 are not mediated by the NK_1 receptor (Endo et al., 2006). Unlike classical tachykinins, HK-1 is mainly expressed in non-neural tissues (Duffy et al., 2003), such as B- and T-cells, macrophages and dendritic cells, suggesting its possible role in inflammatory processes. According to previous results of our workgroup, SP and NKA play an important role in the development of acute pneumonitis (Helyes et al., 2010), however, it is also known, that the expression level the Tac4 gene is remarkably higher in the lung than that of the Tac1 (Duffy et al., 2003). The fact, that the structural and immunological similarities of SP and HK-1 impede to make a correct distinction between the specific functions of the two peptides, has raised the possibility that the group of hemokinins and endokinins may be the missing link in the pathophysiological mechanism of tachykinin-mediated pro-inflammatory actions.

4. The relationship between hydrogen-sulfde and the capsaicin-sensitive sensory neurons

Hydrogen-sulfide (H_2S) is the third known gaseous mediator of the mammalian organism beside nitric oxide (NO) and carbon monoxide (CO) (Wang R, 2012). In the recent years, knowledge has increased concerning the biological functions of H_2S with established activities in the cardiovascular system and in inflammatory processes (Kimura, 2014). Although the molecular targets of H_2S are not precisely clarified, it has been proposed that ion channels (e.g. K^+_{ATP}-channels) might participate in the vasodilatory actions of the gasotransmitter (Liang et al., 2011). The relation between H_2S and the capsaicin-sensitive primary afferents was first shown in the lung (Prior et al., 1990). Later, it has also been suggested that the peptidergic primary afferents may also play a role in the H_2S-induced effects in smooth muscle tissues described in the urinary tract (Patacchini et al., 2004) and in isolated rat mesenteric arteries (White et al., 2013). According to *in vitro* experiments, Ca^{2+}-signal could be detected upon H_2S-stimulus in TRPA1-expressing CHO cells (Streng et al., 2008), moreover, the elevation of $[Ca^{2+}]_i$ was absent after the application of the TRPA1 antagonist in dorsal root ganglion cells (Miyamoto et al., 2011). These data have raised the possibility, that capsaicin-sensitive sensory nerves and the TRPA1 receptors are putative mediators of the effects induced by H_2S.

5. The relationship between the chronic complications of diabetes mellitus and the peptidergic primary afferents, as well as the problems of appropriate modeling of these complications

In 2015, approximately 415 million people suffered from diabetes mellitus all over the world. One third of the patients develop diabetic neuropathy (DN) or diabetic nephropathy (DNP); the latter one is the leading cause of chronic kidney disease (Reutens and Atkins, 2011). Diabetic retinopathy (DR) occurs in 50-75% of the patients, and its development is correlating with both the duration of diabetes and the poor glycemic control (Yau és mtsai., 2012). The TRPV1 and TRPA1 receptors expressed on peptidergic afferent fibres, as well as SP and NKA released from the nerve endings participate in the development of neuropathic pain syndromes associated with diabetes (Coudoré-Civiale et al., 2000; Bölcskei et al., 2005; Koivisto et al., 2012). Besides, the importance of certain TRP ion channels and the antiinflammatory neuropeptide PACAP has been suggested in the pathomechanism of DNP (Colsoul et al., 2013; Bánki et al., 2013). During the formation of DR, several neuropeptides such as SP and CGRP as well as somatostatin and PACAP play an important role (Yang et al., 2013a,b; Szabadfi et al., 2014). In the preclinical development of drugs against chronic complications of diabetes it is strongly needed to use reliable animal models correlating with the alterations of the human disease. The main limitation of the usually applied experimental models is the poor health condition of the experimental individuals, rapidly developing in the absence of any antidiabetic therapy and leading to very short survival (Chen et al., 2005). In order to achieve the formation of detectable functional and morphological diabetes-related complications, the duration of diabetic state has to be extended long enough within the frame of such an experimental design that enables the tolerable general status of the animals parallelly. Another risk factor of the chronic complications of diabetes is the degree of hyperglycemia (Calcutt, 2004). The application of subcutaneously implantable sustained release insulin pumps provides good opportunity for the investigation of chronic glycemic state-dependent alterations by the establishment of several different glycemic states.

6. The significance of sensory-immune interactions in the diseases of several organ systems

Disorders of the interactions existing between the nervous and immune system have been investigated predominantly in neurological and autoimmune diseases; for instance, it is well-known that neurogenic inflammation participates in the pathogenesis of migraine, rheumatoid arthritis, allergic contact dermatitis and inflammatory bowel disases (Pintér és mtsai., 2014). However, increasing data are available suggesting that the defective functions of sensory-immune cooperation contribute to the development of the local vascular diseases of the skin (Aubdool and Brain, 2011), the formation of acute and chronic diseases of the respiratory system (Otmishi et al., 2008), as well as the development of diabetic neuropathy and retinopathy (Bennett, 1999; Yu et al., 2015).

AIMS

Capsaicin-sensitive sensory nerves, as well as TRP ion channels and neuropeptides expressed by both neuronal and non-neuronal structures may play an important role in several diseases of the vascular, respiratory and metabolic systems, providing potential pharmacological targets. Therefore, the appropriate animal modeling and the *in vivo* investigation of these mechanisms are essential.

Our primary aims were the following:

I. To investigate the role of capsaicin-sensitive primary afferents in the H_2S-induced vasodilatory response of the skin.

II. To analyse the involvement of the TRPA1 receptor in the mechanisms of acute and chronic inflammation of the airways.

III. To examine the role of the Tac4 gene-encoded hemokinin-1 in the endotoxin-induced acute pneumonitis.

IV. The setting and optimisation of a chronic rat model suitable for the investigation of the microvascular and sensory complications of diabetes.

EXPERIMENTAL MODELS AND INVESTIGATIONAL TECHNIQUES

1. Animals

Microcirculatory investigations were performed on Balb/c, C57Bl/6 (Charles-River, Hungary) wild type, TRPA1 receptor gene-deficient ($TRPA1^{-/-}$), TRPV1 receptor gene-deleted ($TRPV1^{-/-}$, Jackson Laboratories, USA) and NK_1 receptor gene-deficient ($Tacr1^{-/-}$), as well as α–CGRP wild type (α–$CGRP^{+/+}$) and α–CGRP gene-deleted (α–$CGRP^{-/-}$) mice. In the acute and chronic airway inflammation models C57Bl/6, $TRPA1^{+/+}$ wild type, as well as $TRPA1^{-/-}$ and Tac4 gene-deficient ($Tac4^{-/-}$) mice were used. The original heterozygotic $TRPA1^{+/-}$ breeding pairs were donated by Prof. Pierangelo Geppetti (Firenze), whereas $Tacr1^{-/-}$ mice by Prof. John Quinn (Liverpool), and $Tac4^{-/-}$ mice by Alexandra Berger (Toronto). The induction of diabetes was carried out on Sprague-Dawley CFY rats. Animals were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy and the Laboratory Animal House of the Medical School, University of Pécs, as well as the Laboratory Animal Centre of the King's College, London at 24-25°C, under a 12 h light-dark cycle, provided with standard chow and water *ad libitum*.

2. Protocols and methods applied during the investigation of microcirculation

2.1. RTX-desensitization

One group of C57Bl/6 mice was pre-treated with the ultrapotent TRPV1 agonist resiniferatoxin (RTX; 10, 20, 30, 70 and 100 μg/kg s.c. on 5 consecutive days). This leads to long-lasting defunctionalization of the capsaicin-sensitive nerve endings throughout the body (desensitization). The effect of systemic RTX-desensitization was checked 14 days later and considered satisfactory if capsaicin eye drops (50 μl 0.1%) did not cause eye-wiping movement ("wiping test").

2.2. Investigation of the molecular mediators of H_2S-effects with the help of genetic and pharmacological tools

The results of $TRPA1^{-/-}$, $TRPV1^{-/-}$, α-$CGRP^{-/-}$ and $Tacr1^{-/-}$ mice was compared to those of wild type controls. The selective TRPA1 receptor antagonist HC-030031 (30-100 mg/kg, i.p.), the CGRP receptor antagonist BIBN4096 (0.1-10 mg/kg, i.p.), the NK_1 receptor antagonist CP99994 (10-50 mg/kg, i.p.), the combination of BIBN4096 and CP99994, as well as the K^+_{ATP}-channel blocker glibenclamide (50 mg/kg, i.p.) were administered to Balb/c mice 30 min before the local application of NaHS. The results gained with these different treatments were compared to those of the vehicle-treated group.

2.3. Assessment of the cutaneous blood flow in the mouse ear

The *in vivo* investigation of the microcirculation was performed with laser Doppler imaging technique (Periscan PIM-II; FLPI). Mice were anaesthetised with the combination of ketamine and xylazine (100 mg/kg, 5 mg/kg, s.c.) and colour-coded perfusion photos were taken of them. After taking three control images, the right ear was treated with NaHS, AITC, NaOH, $NaSO_3$ or NaCl, whereas the left ear received respective vehicle. Changes of microcirculation were then recorded for 50 min in the case of NaHS-, NaOH-, $NaSO_3$- and NaCl-treatments, and for 30 min in the case of AITC. For the quantitative evaluation of cutaneous blood flow, two regions of interest (ROIs) were determined representing the total area of the two ears. The changes of microcirculation are expressed in percentage of the initial control value. Areas under the blood flow curves (AUCs) demonstrate the total response of cutaneous blood vessels during the whole period of measurement.

2.4. Induction of cutaneous vasodilatation in the mouse ear

The increase of skin blood flow was elicited by treating the dorsal surface of the right ear with NaHS (15 μl, 5%, pH=12.61, dissolved in saline containing 1.25% methylcellulose) or AITC (15 μl, 2%, dissolved in liquid paraffin). In order to exclude the non-specific physico-chemical effects of pH and osmotic pressure, NaOH (15 μl, 0.16%, pH=12.61), $NaSO_3$ (15 μl, 5%) and NaCl (15 μl, 5%) were also applied (all of them were prepared by using saline containing 1.25% methylcellulose as solvent). Contralateral ears were treated with respective vehicle and served as controls.

3. Methods applied in the acute and chronic airway inflammation models

3.1. Endotoxin-induced acute airway inflammation

Acute interstitial pneumonitis was elicited by intranasal (i.n.) inhalation of *Escherichia coli* (serotype: O83) endotoxin (lipopolysaccharide, LPS; 60 µl, 167 µg/ml), performed under light ether anaesthesia. Non-inflamed intact mice received the same volume of sterile phosphate buffered saline (PBS).

3.2. Smoking-induced chronic airway inflammation

Chronic airway inflammation was elicited by whole body cigarette smoke exposure. Mice were placed into the smoke exposure chamber (Teague Enterprises) twice daily, 10 times/week for 3 months. For this purpose, "3R4F Kentucky Research Cigarette" was used, and the mice were exposed to cigarette smoke for 30 min followed by a ventilation period of another 30 min on every occasion.

3.3. Functional investigation of airway responsiveness

Airway responsiveness was determined by unrestrained whole body plethysmography (Buxco) in conscious, spontaneously breathing animals. In the endotoxin model, bronchoconstriction was elicited by increasing concentrations of aerosolized carbachol 24 h after PBS/LPS treatment, and we determined breathing frequency, as well as the enhanced pause (Penh) parameter correlating with bronchoconstriction and consequent airway resistance. In the cigarette smoke exposure model, changes of the airway function were measured monthly and breathing frequency, tidal volume (TV), peak expiratory flow (PEF) and Penh were determined.

3.4. Bronchoalveolar lavage

In the cigarette smoke exposure model, the lungs of the ketamine-xylazine (100 mg/kg, 5 mg/kg, s.c.) anaesthetized mice were flushed with 5x1 ml PBS with the help of a trachea cannula. Cell profile of the collected bronchoalveolar lavage fluid (BALF) samples were analyzed with CyFlow Space flow cytometer (Partec).

3.5. Histological examination of the lungs

Lung samples were fixed, embedded in paraffin, sectioned and stained with hematoxylin-eosin (HE) or periodic acid-Schiff (PAS) reaction. Semiquantitative evaluation of the inflammatory changes was performed by an expert pathologist blinded from study. Score values were given on the basis of certain histopathological parameters defined separately for the acute and chronic models. These parameters were then added to create composite inflammatory score.

3.6. Determination of myeloperoxidase (MPO) enzyme activity from lung homogenates

MPO activity of the lung homogenates correlating with the count of neutrophil granulocytes and macrophages was measured by spectrophotometry.

3.7. *In vivo* bioluminescence imaging of myeloperoxidase activity

Bioluminescence of the luminol analogue L-012 is proportional to the ROS/RNS production during acute pneumonitis, which was determined with IVIS Lumina II (PerkinElmer) device 24 h after PBS/LPS-treatments. During the quantitative evaluation, the luminescent sign intensity of each ROI was expressed as total radiance (foton flux/s).

3.8. Measurement of the cytokine concentrations of the lung homogenates

Lung samples were homogenized, centrifuged and the concentrations of inflammatory cytokines were determined with the help of Luminex xMAP technique. The results are given in pg/g wet tissue.

4. Protocols and methods applied in long-term modeling of diabetes

4.1. Treatment protocols

Diabetes was induced by single injection of 60 mg/kg streptozotocin (STZ, i.p.). Blood glucose levels were checked 48 h after STZ-injection and rats with blood glucose over 14 mmol/l were involved in the study and divided into 4 groups. Diabetic 1, 2 and 3 groups received s.c. implantable insulin implants (Linplant 2 NE/24 h, LinShin): the Diabetic 1 group got 1 + 1, the Diabetic 2 group got 1 + 0.5, whereas the Diabetic 3 group got 0.5 + 0.5 implants on Weeks 1 and 9. The animals of the Diabetic 4 group received 2 IU Humulin N (i.p.) intermediate-acting insulin injection every second day. Control animals got vehicle (citrate buffer, i.p.) at the beginning of the experimental period, and blank implants (Palmitic Acid Micro Crystal) on Weeks 1 and 9. Investigation of the animals could be performed for 12 weeks after the induction of diabetes in case of the Diabetic 4 group, and for 16 weeks in the other groups.

4.2. Monitoring of blood glucose level, body weight, as well as food and water consumption

Blood samples, collected from the tail vein, were tested by Accu-Check Active blood glucose tester twice a week in the diabetic groups and once a month in the Controls. Body weight changes were recorded twice and once a week in the Diabetic and the Control groups, respectively. Relative food and water consumption were measured once a week and daily, respectively. These *in vivo* data were compared to the starting values recorded on one occasion during the pretrial period.

4.3. Eye examination and ophthalmoscopy

In vivo investigation of the eyes was carried out in ketamine-diazepam (30 mg/kg ill. 3 mg/kg) anaesthesia, and the pupils were dilated with 5 mg/ml tropicamide. Colour photos of the anterior segment – including cornea and iris - were taken. The proportion of the area affected by neovascularization was calculated. For histological examination of the cornea, eyes were fixed, embedded into paraffin, sectioned and stained with HE. Posterior pole of the eye was investigated with an indirect binocular ophthalmoscope (IBO) connected to a computer (Optibrand Clear View optical imaging system, USA); The evaluation of the retina was performed by semiquantitative scoring on the basis of certain pre-defined parameters. These parameters were then added to assess the composite Total Retinal Score (TRS) value.

4.4. Histology and immuno-histochemistry of the retina

For histological examination, eyes were fixed in 4% paraformaldehyde and embedded into Durcupan ACM resin. Semi-thin (2 μm) sections were stained with toluidine blue and morphometric analysis of the samples was performed. Immuno-histochemistry was carried out on another group of samples using the following antibodies: anti-tyrosine-hydroxylase (TH), anti-glial fibrillar acidic protein

(GFAP) and anti-Brn3a antibodies, as well as FITC-conjugated peanut agglutinin (PNA). Colour photos taken during the microscopic examination were evaluated qualitatively and quantitatively. A third group of samples served for the determination of apoptotic cell amount with the help of TUNEL assay.

4.5. Urine analysis

In order to collect urine samples, animals were individually housed in metabolic cages for 16 h and deprived of food during sampling. Samples were centrifuged at room temperature (6000 rpm, 5 min) and the supernatants were stored at -20°C. Concentrations of creatinine and total protein were measured.

4.6. Histology of the kidneys

Kidney samples were fixed in 4% formaldehyde and embedded into paraffin; the 3 μm sections were stained with HE and PAS. Following microscopic investigation, the proportion of PAS-positive glomerular area related to the total glomerular area was calculated. Another group of the samples was fixed in absolute alcohol, and the sections were stained with PAS- and diastase digested PAS-reaction in order to prove the glycogen content of tubular granules (Armanni-Ebstein phenomenon).

4.7. Electron microscopic investigations

Samples of the eyes and kidneys were fixed in 4% paraformaldehyde supplemented with glutaraldehyde, treated with OsO_4, dehydrated and embedded in Durcupan ACM resin or Araldit. Ultra-thin (70 nm) sections were cut and counter-stained with Reynold's lead citrate. Samples were photographed during electron microscopic investigation.

4.8. Investigation of mechanosensitivity and cold allodynia

Touch senstivity of the plantar surface of the hindpaws was measured by dynamic plantar aesthesiometry (Ugo Basile). Since this mechanical stimulus is not painful in rats, the drop of the mechanonociceptive threshold is regarded as allodynia. Pressure senstivity of the hindpaws was determined with Randall-Selitto test using the Ugo Basile analgesimeter. Since this stimulus is painful in rats, the drop of this mechanonociceptive threshold is considered as hyperalgesia. Cold allodynia was investigated by immersing the hindpaw or the tail into 0°C water, and assessed by the latency time of withdrawal reaction (cut-off time was 180 sec).

Statistical analysis

The results of microcirculation measurements were analyzed with unpaired t-test or one-way ANOVA followed by Bonferroni's post hoc test. The evaluation of airway function parameters, MPO activity, total flux values and cytokine concentrations was performed with one- or two-way ANOVA + Bonferroni's post test. Composite inflammation scores were analyzed with Kruskal-Wallis test followed by Dunn's post test. The results of the diabetic animals were evaluated with one-way ANOVA + Bonferroni's post hoc test. In all cases, $p<0.05$ value was considered as statistically significant.

Animal welfare

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 (243/1988), as well as the European Parliament and Council Directive of 2010/63/EU. The studies were approved by the Ethics Committee on Animal Research of the University of Pécs and by the King's College London Animal Care and Ethics Committee (license No. BA 02/2000-2/2012, PPL No. 70/7959).

RESULTS AND DISCUSSION

I. Investigation of the role of capsaicin-sensitive primary afferents, as well as the sensory neuropeptides in the H_2S-induced vasodilatation

Results

Capsaicin-sensitive primary afferents play an important role in the H_2S- and AITC-induced vasodilatation

In C57Bl/6 mice, NaHS continously increased the cutaneous microcirculation of the ear during the total examination period (42% increase in perfusion at 48 min). The vasodilatory effect of NaHS was significantly attenuated by RTX-desensitization: the decline of the total vascular response was 41.5%. The RTX-pretreatment significantly decreased the AITC-induced vasorelaxation as well.

TRPA1 and TRPV1 are differently involved in NaHS- and AITC-evoked vasodilatation

In $TRPA1^{-/-}$ mice, the increase of microcirculation elicited by NaHS was significantly lower than in the C57Bl/6 group: the total vascular response was decreased by 47%. On the contrary, there was no difference between the blood flow changes of the $TRPV1^{-/-}$ and C57Bl/6 mice. AITC-induced vasodilatation was totally absent in the $TRPA1^{-/-}$ group, but in the $TRPV1^{-/-}$ mice it was similar to that of the C57Bl/6 group.

The TRPA1 receptor antagonist inhibits the NaHS- and AITC-induced vasorelaxation

Vasodilatation elicited by NaHS was greater in Balb/c mice than in the C57Bl/6 group (67% increase in perfusion at 48 min). HC-030031 attenuated the increase of skin blood flow significantly and in a concentration-dependent manner. The two different doses of the antagonist decreased the total vascular response by 24.6% and 51.4%. HC-030031 also attenuated AITC-induced vasorelaxation significantly.

CGRP plays an important role in the H_2S-induced increase of cutaneous microcirculation

The NaHS-evoked increase of microcirculation was significantly lower in the $\alpha\text{-CGRP}^{-/-}$ mice than in the $\alpha\text{-CGRP}^{+/+}$ animals: the total vascular response was 55.5% lower in the absence of α-CGRP. BIBN4096 decreased NaHS-induced vasodilatation significantly and in a concentration-dependent

manner: the dose of 0.1 mg/kg caused only tendentious, but the higher doses (≥ 1 mg/kg) caused significant difference.

The activation of NK_1 receptor contributes to the development of H_2S-evoked vasodilatation

NaHS-induced vasodilatation was significantly lower in the $Tacr1^{-/-}$ mice than in the wild types. The total vascular response was 48.2% lower in the absence of NK_1 receptor. Attenuation of the NaHS-evoked increase of blood perfusion was significant and concentration-dependent: the lower dose (10 mg/kg) did not, but the higher doses (≥ 25 mg/kg) did significantly decrease the vasorelaxation.

The effect of simultaneous blockade of CGRP és NK_1 receptors on H_2S-induced vasodilatation

Combined treatment with BIBN4096 and CP99994 resulted in a significant decline of NaHS-evoked vasodilatation. The initial phase of the vasodilatory response resembled the single inhibition of the NK_1 receptor, whereas the second phase of the reaction was rather similar to the inhibition of the CGRP receptor.

K^+_{ATP}-channels are also involved in the H_2S-induced vasodilatation

The K^+_{ATP}-channel blocker glibenclamide significantly attenuated the increase of skin blood flow elicited by NaHS. The kinetics and extent of the inhibitory effect was similar to the decrease of vasorelaxation after the treatment with HC-030031.

The pH and osmotic pressure do not influence cutaneous microcirculation

The effects of NaOH, $NaSO_3$ and NaCl on cutaneous microcirculation were significantly lower than the vasodilatory action of NaHS, and practically did not influence the local perfusion of the mouse ear.

Discussion

In the present study we have provided clear *in vivo* evidence that capsaicin-sensitive sensory nerves play a major role in the mediation of H_2S-induced vasodilatation. The role of primary sensory afferents in the vascular effects of H_2S was investigated *in vitro* by White et al. (2013), and the data of their isolated organ experiments are in accordance with our present results obtained in the mouse ear microcirculation model. Previous data suggested that the TRPA1 receptor expressed on sensory nerve endings is involved in the mediation of neuronal (Pozsgai et al., 2010; Kunkler et al., 2011; Aubdool et al., 2014) and endothelium-dependent vasodilatation (Earley et al., 2009). The hypothesis, that H_2S may activate TRPA1, was first proposed based on *in vitro* experiments (Streng et al., 2008; Miyamoto et al., 2011). Our present *in vivo* results clearly demonstrate that the activation of TRPA1 plays a key role in the H_2S-evoked increase of cutaneous microcirculation, highlighting that the TRPA1 receptor should be considered as an important mediator of the vasoactive effects of H_2S. The potential molecular mechanism of the interaction between H_2S and TRPA1 might be the S-sulfhydration of the cysteine residues found on the ion channel (Macpherson et al., 2007; Mustafa et al., 2009); the intermediate species of this process are presumably polysulfide compounds generated in aqueous solutions of H_2S (Greiner et al., 2013; Hatakeyama et al., 2015). On the other hand,

specific disulfide bonding between critical cysteine residues and major conformational changes on the N-terminus might also contribute to the activation of TRPA1 (Eberhardt et al., 2014). Concerning the role of TRPV1 receptor in H_2S-induced activation of sensory nerves, contradictory data are available in the literature (Trevisani et al., 2005; Miyamoto et al., 2011). According to our results, the extent of vasodilatation was identical in $TRPV1^{-/-}$ mice and in wild types, suggesting that the TRPV1 receptor does not participate in the mediation of H_2S-induced vasodilatory response. These *in vivo* results have been confirmed by other members of our workgroup with the help of radioactive $^{45}Ca^{2+}$-uptake experiments performed on TRPA1 as well as TRPV1 receptor expressing CHO cells. It is also known that not only TRPA1, but also CGRP takes part in the mediation of 4-ONE-induced vasorelaxation (Graepel et al., 2011). On the basis of *in vitro* data, it also has been suggested that CGRP may be involved in the H_2S-evoked smooth muscle relaxation and vasodilatation (Fernandes et al., 2013; Eberhardt et al., 2014). Moreover, H_2S has also been proposed to elicit acute pneumonitis in a SP- and NK_1 receptor-dependent manner (Bhatia et al., 2006), raising the possibility that the NK_1 receptor mediated signaling pathway may also contribute to the H_2S-evoked increase of microcirculation. Our present study provides clear *in vivo* evidence that both CGRP and SP are important mediators of H_2S-induced cutaneous vasorelaxation. Combined treatment with the CGRP and NK_1 receptor antagonists did not result in an additive effect, however, the inhibition of H_2S-evoked microcirculatory changes was similar to the NK_1 receptor inhibition in the first 10-15 min, and resembled the CGRP receptor inhibition in the later phases of the experiment. Our observations can be explained by faster cell signaling in the case of NK_1 receptor activation, but potentially different pharmacokinetic characteristics of the two antagonists cannot be excluded. Regarding the role of K^+_{ATP}-channels in the relaxation of blood vessels and other smooth muscle tissues, conflicting results have been published (Fernandes et al., 2013; White et al., 2013; Eberhardt et al., 2014). Our present findings show that K^+_{ATP}-channels of vascular smooth muscle cells also contribute to the H_2S-induced relaxation of skin arteries. Since glibenclamide-sensitive K^+_{ATP}-channels have been shown to be involved in the mediation of certain CGRP effects (Santicioli and Maggi, 1994), the interaction between capsaicin-sensitive sensory nerves and K^+_{ATP}-channels is also presumable in the molecular mechanisms of H_2S-induced cutaneous vasodilatation. Although alkaline pH has been proposed to activate TRPA1 receptors (Fujita et al., 2008), we did not experience any changes in response to high pH or osmotic effects, suggesting that these non-specific physico-chemical stimuli do not influence the microcirculatory effects of H_2S. It can be concluded that the activation of capsaicin-sensitive primary afferents through the TRPA1 receptor, as well as the vasoactive neuropeptides released from sensory nerve endings play a key role in the development of H_2S-evoked increase of local microcirculation.

II. Involvement of the TRPA1 receptor in acute and chronic airway inflammations

Results

TRPA1 exerts protective function in the endotoxin-induced airway hyperreactivity

Penh was increased upon LPS stimulation in both $TRPA1^{+/+}$ and $TRPA1^{-/-}$ mice in a concentration-dependent manner, indicating the development of inflammatory airway hyperreactivity. Bronchial hyperresponsiveness was significantly greater in $TRPA1^{-/-}$ mice than in the wild types. LPS-treatment caused a significant decrease of the baseline value of breathing frequency in both $TRPA1^{+/+}$ and $TRPA1^{-/-}$ mice. Carbachol inhalation evoked a concentration-dependent decline of breathing frequency in the $TRPA1^{-/-}$, but not in the $TRPA1^{+/+}$ group.

TRPA1 plays an important role in the increase of inflammatory MPO activity of the lung

Basal activity of the MPO enzyme was practically the same in the two control groups. The treatment with endotoxin resulted in a remarkable increase of the MPO activity, which was significantly greater in mice lacking TRPA1 than in the wild types.

TRPA1 is not involved in the endotoxin-induced histopathological alterations of the lung

LPS-treatment caused significant perivascular/peribronchial edema, inflammatory cell accumulation and goblet cell hyperplasia in wild type mice compared to the PBS-treated groups. These histopathological alterations were similar in the $TRPA1^{+/+}$ and $TRPA1^{-/-}$ animals.

TRPA1 does not influence cigarette smoke-induced changes of the airway function parameters

Breathing frequency was significantly decreased in both $TRPA1^{+/+}$ and $TRPA1^{-/-}$ mice from the first month of cigarette smoke exposure. Between the frequency values of the two animal groups no significant difference could be observed at any time point. TV, PEF and Penh did not alter significantly during the 3-month examination period.

TRPA1 plays a complex role in the cigarette-smoke induced inflammatory changes of the lung

In the first month of the smoke exposure model, remarkable perivascular edema developed in both examined mouse groups which gradually and significantly decreased in the second and third months. This parameter showed no difference between the $TRPA1^{+/+}$ and $TRPA1^{-/-}$ mice. However, the amount of accumulating neutrophil granulocytes was greater in the first month, whereas the extent of macrophage infiltration and alveolar-interstitial edema was lower in the second month in the $TRPA1^{-/-}$ strain compared to the wild types.

Accumulation of lymphocytes is increased in the absence of the TRPA1 receptor

The amount of lymphocytes in the bronchoalveolar lavage fluid was tendentiously higher at the end of the second month, however, it was significantly greater at the end of the third month in $TRPA1^{-/-}$ mice compared to the wild types. There was no difference in the amount of neutrophils and macrophages of the two examined mouse groups.

Discussion

The TRPA1 receptor has been suggested to take part in the development of several airway diseases (e.g. bronchial asthma, COPD) (Grace et al., 2014), but data concerning its activation mechanisms and its precise pathophysiological role are few and contradictory. In our present study, we have provided evidence that the TRPA1 receptor exerts clear protective effects in the bronchial hyperreactivity as well as the granulocyte- and macrophage-derived MPO production associated with endotoxin-induced acute pneumonitis. Although some recent publications suggested that TRPA1 could mediate bronchoconstriction in different asthma models (Jha és mtsai, 2015; Devos és mtsai., 2016), our observation demonstrating the protective role of TRPA1 during acute inflammation is confirmed by previous investigations of our workgroup, where the activation of peptidergic primary afferents through the TRPV1 receptor exerted protective effects in the development of endotoxin-induced acute airway inflammation and consequent bronchial hyperresponsiveness (Elekes et al., 2007; Helyes et al., 2007). The similar functions of the two ion channels are not surprising, since they are co-expressed on the sensory nerve endings, and their physical and functional interaction is also presumable (Lee et al., 2015). The TRPA1-mediated protective effects can be explained with the release of antiinflammatory neuropeptides (e.g. somatostatin) from the primary sensory afferents (Helyes et al., 2009). Previous *in vitro* data suggested that the cigarette smoke extract (CSE) causes CGRP- and SP-release from peptidergic sensory nerves, as well as that TRPA1 takes part in the formation of CSE- or nicotine-induced plasma extravasation and bronchoconstriction (André et al., 2008; Talavera et al., 2009). Besides, ROS and several lipid peroxydation products generated in oxidative stress associated with COPD are also able to activate this ion channel (Bessac és Jordt, 2008). In the present *in vivo* model of chronic cigarette smoke exposure we found that the histopathological picture of the first month (acute phase, dominated by peribronchial and perivascular edema) changed in the second month (intermediate phase) and it was then characterized by infiltration of inflammatory cells, in accordance with the newest results of our workgroup (Kemény et al., under review). Furthermore, our present results provided evidence that in the $TRPA1^{-/-}$ mice the accumulation of granulocytes was significantly higher in the first month, whereas macrophage-dominated cell infiltration and alveolar-interstitial edema was significantly lower at the end of the second month. These results suggest that the activation of TRPA1 exerts protective effects in the acute phase of the cigarette smoke-evoked airway inflammation, but later it presumably contributes to the progression of inflammation as a member of the proinflammatory cascade. The possible explanation of this observation is that the transient activation of TRPA1 by toxic irritants of the cigarette smoke typically induces protective reflexes, but the permanent stimulation of the receptor by its endogenous activators produced under chronic inflammatory conditions continously maintains the neurogenic inflammation state (Chen et al., 2012). On the other hand, beside the capsaicin-sensitive sensory nerves TRPA1 is also expressed on non-neuronal cells, e.g. on macrophages (Kun et al., 2017), and the stimulation of TRPA1 on different structures may activate different intracellular

signaling mechanisms leading to diverse outcomes in certain phases of chronic inflammation. Since the absence of TRPA1 receptor resulted in an increased accumulation of lymphocytes in the third month of cigarette smoke-exposure (lymphocyte-dominated chronic phase), and since the ion channel is also expressed on lymphocyte cells (Stokes et al., 2006), it is presumable that the TRPA1 receptor plays a regulatory role in the functions of lymphocytes as well. The complex investigation of airway function performed in our study showed that these parameters are not influenced by activation of TRPA1, therefore we cannot confirm the hypothesis based on *in vitro* data that suggested a mediator role for the receptor in the cigarette smoke-induced changes of airway function. Nevertheless, it is important to note that H_2S exerts antiinflammatory and bronchodilatory effects in chronic smoke exposure (Chen et al., 2011). The fact that not only the toxic irritants of cigarette smoke are able to activate TRPA1 but also the gaseous mediator H_2S (see Results and discussion/Chapter I), raises the possibility of an interaction between the different signaling pathways at this point. Therefore, the role of TRPA1 in the development of chronic bronchitis seems to be much more complicated than it was supposed before. Moreover, since our experiments proved the protective function of the ion channel in endotoxin-induced acute pneumonitis, it is strongly presumable, that the activation of capsaicin-sensitive sensory neurons and inflammatory cells through the TRPA1 receptor results in complex modulatory effects during the acute and chronic inflammatory processes of the lung.

III. Investigation of the role of HK-1 in endotoxin-induced acute pneumonitis

Results

HK-1 does not influence endotoxin-induced airway hyperreactivity

In comparison with the PBS-treated controls, Penh value of the LPS-treated mice showed a significant and concentration-dependent increase. This airway hyperreactivity was similar in the $Tac4^{-/-}$ mice and in the wild types. Breathing frequency decreased in both LPS-treated groups in a concentration-dependent manner, and no difference was detectable between the $Tac4^{-/-}$ and wild type mice.

HK-1 exerts proinflammatory effect in the endotoxin-evoked histological alterations of the lung

One day after the treatment with endotoxin, remarkable peribronchial/perivascular edema, neutrophil accumulation, mononuclear cell infiltration and goblet cell hyperplasia developed in wild type mice. The severity of inflammation was significantly reduced in the $Tac4^{-/-}$ group. In the lung samples of LPS-treated $Tac4^{-/-}$ mice, large, dense, follicle-like lymphoid structures were detectable peribronchially and perivascularly. This feature could not be observed either in the wild types or in other knockout strains examined previously by our workgroup.

HK-1 plays an important role in the neutrophil-derived MPO activity in the lung

In the LPS-treated mouse groups, bioluminescent sign intensity of the lungs was remarkably increased compared to the PBS-treated mice. The lungs of the LPS-treated $Tac4^{-/-}$ mice showed significantly lower bioluminescence compared to the wild types.

HK-1 enhances myeloperoxidase activity in the lung

MPO activity was significantly higher in the wild type mouse lungs 24 h after LPS inhalation. In the Tac4$^{-/-}$ mice, MPO activity was attenuated in comparison with that of the wild type mice.

HK-1 increases the proinflammatory cytokine production in the lung

In wild type mice, the levels of all examined cytokines remarkably elevated 24 h after the treatment with LPS. The concentrations of IL-1β, IL-6, KC, TNFα and MCP-1 were reduced significantly, whereas the level of MIP-1α was tendentiously lower in the lungs of Tac4$^{-/-}$ mice compared to the wild types.

Discussion

We have provided here the first evidence that HK-1 plays an important role in the development of endotoxin-induced interstitial pneumonitis. Our experiments revealed that edema formation and inflammatory cell accumulation as well as the neutrophil granulocyte- and macrophage-derived MPO activity were significantly reduced in the absence of HK-1. However, previous investigations of our workgroup demonstrated that the lack of NK_1 receptor caused no alterations in these parameters (Helyes et al., 2010). On the basis of these results, it can be concluded that HK-1 plays an important role in the acute inflammatory processes of the lung, but this effect is not mediated by the NK_1 receptor. Our present findings are in accordance with the results of our workgroup gained in chronic murine arthritis, showing that the development of joint inflammation is independent from NK_1 receptor activation (Borbély et al., 2013). Therefore, our results strongly support the existence of another, specific receptor for HK-1 mediating inflammatory actions at the periphery. Since the results of MPO activity measurements and bioluminescent imaging of ROS showed perfect correlation in our model, it is strongly presumable that the luminescent sign intensity of the lung developed due to the activation of MPO. It is known that numerous proinflammatory cytokines are released during the LPS-evoked inflammatory reaction (e.g. TNFα, IL-1β, IL-6, KC) and the relation between HK-1 and the cytokine system was also suggested based on *in vitro* investigations (Berger et al., 2007). The results of our present *in vivo* experiments clearly show, that the LPS-induced elevation of proinflammatory cytokines is significantly attenuated in the Tac4$^{-/-}$ mice, further indicating that HK-1 plays a crucial role in cytokine release and consequent immune cell activation during pulmonary inflammation. Since the large, dense, follicle-like structures associated to the vessels and bronchi were observed only in the lungs of LPS-treated Tac4$^{-/-}$ mice and were not detectable in other previously examined knockout mouse strains, it can be presumed that HK-1 also contributes to the regulated activation of lymphocytes under pathophysiological conditions. This first *in vivo* observation is supported by the earlier described B-lymphopoesis promoting effect of HK-1 (Berger et al., 2010). In contrast to the previously published role of SP and NKA (Helyes et al., 2010), and in contrast to the mediator function of HK-1 in peripheral inflammatory reactions, HK-1 does not influence airway hyperreactivity. Although hHK-1 has been suggested to evoke bronchoconstriction

(Grassin-Delyle et al., 2010), we could not confirm a similar effect for HK-1 in this murine model of acute pneumonitis. One explanation for this contradiction is that hemokinins and endokinins make up a more complicated system in humans than in mice: the human TAC4 gene has four different splice variants, and their sequences are different from their murine counterparts, moreover, they also show different expression characteristics (Page, 2004). Another explanation is that the inflammatory bronchoconstriction is predominantly mediated by the NK_2 receptor (Elekes et al., 2007; Helyes et al., 2010), but HK-1 shows remarkably lower affinity to the NK_2 receptor, than it does to the NK_1 (Bellucci et al., 2002). In summary, we have demonstrated the first data for an important mediator role of HK-1 in airway inflammation. Furthermore, HK-1 seems to have a specific regulatory role on lymphocyte functions under inflammatory conditions, the precise mechanism of this process needs further investigation.

IV. The development of a rat diabetes model for the complex investigation of chronic complications

Results

The changes of blood glucose level, body weight, as well as water and food consumption

Blood glucose level of the Control rats was 5-7 mmol/l during the total examination period. Average blood glucose level was 8-10 mmol/l in the Diabetic 1 group, 16-18 mmol/l in the Diabetic 2 group, 22-25 mmol/l in the Diabetic 3 group, and it reached 30-33 mmol/l in the Diabetic 4 group. Weight gain of the Diabetic 1-3 groups was slightly less than that of the Controls, whereas in the Diabetic 4 group a weight loss of 13.4% was observed instead of the physiological weight gain. Relative water and food consumption was markedly increased in the Diabetic 3-4 groups. The results of the Diabetic 3-4 groups showed statistically significant differences in all parameters compared to the Controls.

Neovascularization of the anterior segment and the pathological changes of the posterior pole

Diabetic 3-4 animals developed remarkable anterior segment neovascularization. The severity of neovascularization was significantly greater in the Diabetic 4 group, than it was in the Diabetic 2-3 animals. The TRS was significantly elevated in the Diabetic 2-4 groups compared to the Control and Diabetic 1 animals, moreover, TRS values of the Diabetic 3-4 groups were significantly higher than that of the Diabetic 2 group.

Histological alterations of diabetic retinopathy

In Diabetic retinas, a slight decrease of the OLM-ILM distance, as well as of the OPL and IPL widths was detectable compared to the Controls. Besides, the total number of cells and the ganglion cell number were significantly reduced in 100 μm GCL. GFAP immunoreactivity of the retina was increased in all Diabetic groups. The amount of cone terminals in the OPL was significantly lower in the Diabetic 2-4 groups than in the Control animals. Processes of the dopaminergic amacrine cells were remarkably thinner in the Diabetic 3 and 4 groups, moreover, in the latter one they were even

absent. The number of TUNEL-positive cells was significantly higher in the Diabetic 3-4 groups than in the Control retinas, the extent of apoptosis was most severe in the ONL. Besides, the degeneration of the pigment epithelium, as well as blood vessels extending into the retina from the choroid were detected. Glial cell membranes forming the tight junctions of the ILM were separated in the Diabetic 3 retinas, moreover, the degeneration of the IPL was also especially notably in this group. In the retina samples of the Diabetic 3-4 groups, macrophages were observable in the OPL, INL and IPL as well as microglial cells in the IPL.

Histological alterations of diabetic nephropathy

The relative ratio of the PAS-positive glomerular area was significantly increased in all Diabetic groups compared to the Controls, moreover, the glomerular PAS-positivity in the Diabetic 4 animals was significantly higher than in any other examined group. The presence of the Armanni-Ebstein phenomenon could be detected in the Diabetic 2-4 animals. Besides, segmental thickening of the glomerular basement membrane, multifocal fusion of the podocyte foot processes and expansion of the mesangial matrix were also observed.

Urine investigations for the detection of DNP

At the end of the examination period, the excreted urine amount was significantly higher in the Diabetic 2-3 groups than in the Control and Diabetic 1 animals (polyuria). In Diabetic 4 rats, after an initial polyuric phase, the urine amount declined by the end of the experiment (threatening anuria). The total protein/creatinine ratio slightly increased in the Diabetic 1-2 groups. In Diabetic 3 and 4 rats, this ratio changed differently: it was remarkably elevated at the middle time point of the experiment, but markedly decreased by the end of the examination period.

Mechanical hyperalgesia and cold allodynia

The mechanonociceptive thresholds in touch and pressure sensitivity, as well as the withdrawal latency from 0°C water of the hindpaws were significantly reduced in all Diabetic groups compared to the Controls. These changes were similar in Diabetic 1-3 animals, however, the tail withdrawal latency from ice-cold water was hyperglycemia-dependent.

Discussion

In our present study we tested different insulin treatment protocols in order to keep diabetic animals in relatively good condition but to allow different levels of hyperglycemia to remain for a long time. For this purpose, we performed targeted glycemic control with the help of sustained release insulin implants (Havel et al., 2000). Our findings provide clear evidence that the anterior segment neovascularization, as well as retinal alterations defined with TRS proved to be glycemic state-dependent. During the development of **DR**, the damaged inner blood-retinal barrier results in microaneurysms and intraretinal bleeding. Besides, it is presumable that the increased production of vascular endothelial growth factor (VEGF) is responsible for the neovascularization affecting both the anterior and posterior segments of the eye (Klaassen et al., 2013). In contrast to the physiological

GFAP expression selectively localized to the end feet of Müller glial cells, we observed increased GFAP immunoreactivity in the entire retina of Diabetic 3 rats indicating severe metabolic damage of the retina (Chang et al., 2007). Malfunctioning cellular metabolism leads to apoptosis of the retinal neurons (Szabadfi et al., 2014). The present study also showed that not only the cone terminals of the photoreceptors were degenerated in the Diabetic 3 rats, but also dopaminergic amacrine cells which retinal cell type is most sensitive to diabetic changes. Furthermore, our investigations also revealed that the amount of TUNEL-positive apoptotic cells is proportional to the severity of hyperglycemia. Severe alterations of synaptic profiles and open glial tight junctions in the ILM results in easy access to the retinal structures for migrating immunocompetent cells. The apoptosis and degeneration of retinal cells led to remarkable thinning of the retina corresponding to the later phase of human diabetes (Takahashi and Chihara, 2008). In the present study we observed remarkable glomerular PAS positivity, increased tubular glycogen accumulation, mesangial matrix expansion, segmentally thickened glomerular basement membrane, as well as podocyte foot effacement in the kidney samples of the Diabetic 3 animals, which alterations are also characteristic for human **DNP** (Dronavalli et al., 2008). The elevation of the urine total protein/creatinine ratio in the initial phase of the examination period and its subsequent decrease in the later phase correlates with the clinical experience in human diabetic patients suffering from end-stage renal failure. This apparent improvement of proteinuria occurs as a sign of serious glomerular damage and occlusion. From the examined parameters of DNP both glomerular PAS-positivity and urine total protein/creatinine ratio showed glycemic state-dependence. Human **DN** occurs mostly as distal symmetrical polyneuropathy and often causes hyperalgesia and allodynia (Sun et al., 2012). Major contributing factor of the development of DN is hyperglycemia, but the defective direct signaling of insulin and the possible effects of STZ on sensory neurons has also been postulated in rodents (Brussee et al., 2004; Pabbidi et al., 2008). Since cold allodynia of the tail was clearly hyperglycemia-dependent, the investigation of this parameter could be a reliable opportunity for the evaluation of diabetic neuropathy associated with long-term continous or intermittent hyperglycemia. In summary, the application of different “doses” of insulin implants enabled the setting of different degrees of hyperglycemia severity, and consequently the investigation of several hyperglycemia-dependent pathological conditions. In our complex series of experiments, the Diabetic 3 animals have been proved to be the most suitable model of chronic complications, where the alterations of the eye, kidneys and peripheral nervous system could be investigated for 16 weeks after the induction of diabetes.

SUMMARY OF THE NOVEL FINDINGS

I. We have provided first *in vivo* evidence, that the capsaicin-sensitive sensory nerve endings play a crucial mediator role in the H_2S-induced vasodilatation of the skin. H_2S exerts its vasodilatory effects through the activation of the TRPA1 receptor expressed on the sensory fibres, whereas the TRPV1 receptor does not participate in this process. CGRP and SP released from activated peptidergic primary afferents play a key role in the H_2S-evoked increase of local microcirculation. Activation of the K^+_{ATP}-channels also contributes to the formation of vasodilatation elicited by H_2S, but pH and osmotic effects do not influence this vascular response.

II. The present results clearly demonstrate, that **the TRPA1 receptor mediates complex regulatory effects in the acute and chronic inflammatory processes of the lung**. TRPA1 plays a protective role in the development of acute bronchial hyperreactivity, as well as in the activation of neutrophil granulocytes and mononuclear cells, similarly to the previously described functions of the TRPV1 receptor. Furthermore, during the progression of cigarette smoke-induced airway inflammation, TRPA1 has a complex role in the regulation of granulocyte-, macrophage- and lymphocyte-functions, as well as the formation of the alveolar-interstitial edema. Activation of the receptor does not affect airway function.

III. We have provided here the first evidence for the proinflammatory functions of HK-1 in acute interstitial pneumonitis. HK-1 enhances the accumulation and activation of neutrophil granulocytes and macrophages, as well as the production of proinflammatory cytokines in the lung. Moreover, our observations raised the possibility that HK-1 is involved in the regulation of lymphocyte functions, but its precise mechanism needs further investigation. HK-1 has no influence on inflammatory airway hyperreactivity.

IV. Our present study characterized a preclinical model that is suitable for long-term and parallel evaluation of diabetic eye disorders, nephropathy and neuropathy. The neovascularization of the anterior segment, the Total Retinal Score, the amount of apoptotic cells in the retina, the glomerular PAS-positivity, the urine total protein/creatinine ratio and the cold allodynia were proven to be hyperglycemia-dependent, and the quantitative analysis of these parameters could serve as an essential step in the preclinical development of drugs against chronic diabetic complications.

The disorders of sensory-immune interactions are involved in the development of certain vascular, respiratory and metabolic diseases. Detailed identification of the molecular targets participating in these processes strongly promote the development of novel drugs affecting the interactions between the sensory and immune systems.

REFERENCES

Andrè et al. (2008) *J Clin Invest.* 118: 2574-82.
Aubdool and Brain (2011) *J Investig Dermatol Symp Proc.* 15: 33-9.
Aubdool et al. (2014) *Nat Commun.* 5: 5732.
Bánki et al. (2013). *Peptides.* 42: 125-130.
Barwell et al. (2012) *Adv Exp Med Biol.* 744: 13–24.
Bautista et al. (2006) *Cell.* 124, 1269-1282.
Bellucci et al. (2002) *Br J Pharmacol.* 135: 266–74
Bennett (1999) *Proc Natl Acad Sci U S A.* 96: 7737-8.
Berger et al. (2010) *Blood.* 116: 3792–801.
Berger et al. (2007) *J Neuroimmunol.* 187: 83–93.
Bessac and Jordt. (2008) *Physiology (Bethesda).* 23: 360-70.
Bhatia et al. (2006) *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 291: L896-L904.
Borbély et al. (2013) *PLoS One* 8: e61684.
Bölcskei et al. (2005) *Pain.* 117: 368-376.
Brain and Cox (2006) *Br J Pharmacol* 147 (Suppl. 1): S202–11.
Brierley et al. (2011) *J Physiol.* 589: 3575-93.
Brussee et al. (2004) *Diabetes.* 53: 1824-1830.
Calcutt (2004) *J Neurol Sci.* 220: 137-139.
Cao et al. (1999) *Am J Physiol.* 277(2 Pt 2): R476-81.
Chang et al. (2007). *J Anat.* 210: 54-65.
Chen et al. (2005) *Cardiovasc Diabetol.* 4: 3.
Chen and Hackos (2015) *Naunyn Schmiedebergs Arch Pharmacol.* 388: 451–463.
Chen et al. (2012) *In: TRP channels in drug discovery.* Vol. 1. p. 43-59. online ISBN: 978-1-62703-077-9
Chen et al. (2011) *Cytokine.* 53: 334-41.
Colsoul et al. (2013) *Curr Top Med Chem.* 13: 258-69.
Coudoré-Civiale et al. (2000) *Eur J Pharmacol.* 401: 47-53.
De Swert and Joos (2006) *Eur. J. Pharmacol.* 533: 171-181.
Devos et al. (2016) *Eur Respir J.* 48: 380-92.
Dronavalli et al. (2008) *Nat Clin Pract Endocrinol Metab.* 4: 444–452.
Duffy et al. (2003) *Neuropharmacology.* 45: 242–50.
Earley et al. (2009) *Circ. Res.* 104: 987-994.
Earley (2012) *Br J Pharmacol.* 167: 13-22.
Eberhardt et al. (2014) *Nat Commun.* 5: 4381.
Elekes et al. (2007) *Regul Pept.* 141: 44-54.
Endo et al. (2006) *Neurosci Lett.* 392: 114–7.
Fernandes et al. (2012) *Br J Pharmacol.* 166: 510-21.
Fernandes et al. (2013) *J Urol.* 190: 746-56.
Fujita et al. (2008) *J. Clin. Invest.* 118: 4049-4057.
Grace et al. (2014) *Br J Pharmacol.* 171: 2593-607.
Graepel et al. (2011) *J. Pharmacol. Exp. Ther.* 337: 117-124.
Grant (2002) *Inflammopharmacology* 9: 403-420.
Grassin-Delyle et al. (2010) *Respir Res.* 11: 139.
Greiner et al. (2013) *Antioxid Redox Signal.* 19: 1749-65.
Hatakeyama et al. (2015) *Mol Pain.* 11: 24.
Havel et al. (2000). *Diabetes.* 49: 244-252.
Helyes et al. (2007) *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 292: L1173-81.
Helyes et al. (2010) *Neuropeptides.* 44: 399–406.
Helyes et al. (2009) *Proc Natl Acad Sci U S A.* 106: 13088–93.
Jaquemar et al. (1999) *J Biol Chem.* 274: 7325-33.
Jha et al. (2015) *Can J Physiol Pharmacol.* 93: 171-6.
Jordt et al. (2004) *Nature.* 427: 260-265.
Julius (2013) *Annu Rev Cell Dev Biol.* 29: 355-84.

Kemény et al. Chronic cigarette smoke-induced pulmonary and cardiac co-morbidities in a COPD mouse model. *(under review)*
Kimura (2014) *Nitric Oxide.* 41: 4-10.
Klaassen et al. (2013) *Prog Retin Eye Res.* 34: 19-48.
Koivisto et al. (2012) *Pharmacol Res.* 65: 149-58.
Kun et al. (2017) *Oral Dis.* 23: 189-198.
Kunkler et al. (2011) *Pain.* 152: 38-44.
Lee et al. (2015) *Pulm Pharmacol Ther.* 35: 87-93.
Liang et al. (2011) *Am. J. Physiol. Heart Circ. Physiol.* 300: H2088-H2095.
Macpherson et al. (2006) *Mol Cell Neurosci.* 32: 335-43.
Macpherson et al. (2007) *Nature.* 445: 541-5.
Maggi (1995) *Prog Neurobiol.* 45: 1–98.
Meseguer et al. (2014) *Nat Commun.* 5: 3125.
Miyamoto et al. (2011) *Neurosci. Lett.* 499: 137-142.
Mustafa et al. (2009) *Sci. Signal.* 2: ra72.
Otmishi et al. (2008) *Clin Med Circ Respirat Pulm Med.* 2: 35-44.
Pabbidi et al. (2008) *Mol Pharmacol.* 73: 995-1004.
Page (2004) *Cell Mol Life Sci.* 61: 1652-1663.
Patacchini et al. (2004) *Br J Pharmacol.* 142: 31-4.
Pintér et al. (2014) *Br J Clin Pharmacol.* 77: 5-20.
Pozsgai et al. (2010) *Cardiovasc. Res.* 87: 760-8.
Prior et al. (1990) *Toxicol Pathol.* 18: 279-88.
Reutens and Atkins (2011) *Contrib Nephrol.* 170: 1-7.
Russell et al. (2014) *Physiol Rev.* 94: 1099-142.
Santicioli and Maggi (1994) *Br J Pharmacol.* 113: 588-92.
Steinhoff et al. (2014) *Physiol Rev.* 94: 265-301.
Story et al. (2003) *Cell.* 112: 819-829.
Streng et al. (2008) *Eur. Urol.* 53: 391-399.
Stokes et al. (2006) *Cell Signal.* 18: 1584 −1594.
Sun et al. (2012). *PLoS One.* 7: e39647.
Szabadfi et al. (2014) *Int Rev Cell Mol Biol.* 311: 1-121.
Szallasi et al. (2007) *Nat. Rev. Drug Discov.* 6: 357-72.
Szolcsányi (1988) *Agents Actions.* 23: 4-11.
Szolcsányi et al. (1998) *Br J Pharmacol.* 125: 916-922.
Szolcsányi (1996) *Prog Brain Res.* 113: 343-359.
Takahashi and Chihara (2008) *Invest Ophthalmol Vis Sci.* 49: 687-92.
Talavera et al. (2009) *Nat Neurosci.* 12: 1293-9.
Tominaga et al. (1998) *Neuron.* 21: 531 -543.
Trevisani et al. (2005) *Br J Pharmacol.* 145: 1123–1131.
Trevisani et al. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104: 13519-13524.
Wang (2012) *Physiol Rev.* 92: 791-896.
White et al. (2013) *Br J Pharmacol.* 168: 785-93.
Yang et al. (2013a) *Regul Pept.* 187: 12-6.
Yang et al. (2013b) *J Diabetes Complications.* 27: 531-7.
Yau et al. (2012) *Diabetes Care.* 35: 556-564.
Yoo et al. (2014) *Molecules.* 19: 4708-44.
Yu et al. (2015) *J Neuroinflammation.* 12: 141.
Zhang et al. (2000) *Nat Immunol.* 1: 392–7.
Zubrzycka and Janecka (2000) *Endocr Regul.* 34: 195-201.

PUBLICATIONS

Publications related to the thesis

Hajna Z, Sághy É, Payrits M, Aubdool AA, Szőke É, Pozsgai G, Bátai IZ, Nagy L, Filotás D, Helyes Z, Brain SD, Pintér E. Capsaicin-sensitive sensory nerves mediate the cellular and microvascular effects of H_2S via TRPA1 receptor activation and neuropeptide release. *J Mol Neurosci.* 60: 157-70. (2016) **IF: 2.376*** (50% of the article)

Hajna Z, Szabadfi K, Balla Z, Biró Z, Degrell P, Molnár GA, Kőszegi T, Tékus V, Helyes Z, Dobos A, Farkas S, Szűcs G, Gábriel R, Pintér E. Modeling long-term diabetes and related complications in rats. *J Pharmacol Toxicol Methods.* 78: 1-12. (2016) **IF: 2.171***

Hajna Z, Borbély É, Kemény Á, Botz B, Kereskai L, Szolcsányi J, Pintér E, Paige CJ, Berger A, Helyes Z. Hemokinin-1 is an important mediator of endotoxin-induced acute airway inflammation in the mouse. *Peptides.* 64: 1-7. (2015) **IF: 2.535**

Pozsgai G, **Hajna Z**, Bagoly T, Boros M, Kemény Á, Materazzi S, Nassini R, Helyes Z, Szolcsányi J, Pintér E. The role of transient receptor potential ankyrin 1 (TRPA1) receptor activation in hydrogen-sulphide-induced CGRP-release and vasodilation. *Eur J Pharmacol*. 689: 56-64. (2012) **IF: 2.592 (co-first author)**

Helyes Z, **Hajna Z**. Endotoxin-induced airway inflammation and asthma models. (book chapter) In: *Arpad Szallasi, Tamás Bíró: TRP channels in drug discovery. Methods in Pharmacology and Toxicology*. Vol.1, pp. 301-342. (2012) online ISBN: 978-1-62703-077-9

Cumulative impact factor of publications related to the thesis: 9.674
Number of independent citations: 23

Publications not related to the thesis

Borbély É, **Hajna Z**, Nabi L, Scheich B, Tékus V, László K, Ollmann T, Kormos V, Gaszner B, Karádi Z, Lénárd L, Paige CJ, Quinn JP, Szolcsányi J, Pintér E, Keeble J, Berger A, Helyes Z. Hemokinin-1 mediates anxiolytic and anti-depressant-like actions in mice. *Brain Behav Immun.* 59: 219-232. (2017) **IF: 6.020* (co-first author)**

Tékus V, Horváth Á, **Hajna Z**, Borbély É, Bölcskei K, Boros M, Pintér E, Helyes Z, Pethő G, Szolcsányi J. Noxious heat threshold temperature and pronociceptive effects of allyl isothiocyanate (mustard oil) in TRPV1 or TRPA1 gene-deleted mice. *Life Sci.* 154: 66-74. (2016) **IF: 2.627***

Scheich B, Gaszner B, Kormos V, László K, Ádori C, Borbély É, **Hajna Z**, Tékus V, Bölcskei K, Ábrahám I, Pintér E, Szolcsányi J, Helyes Z. Somatostatin receptor subtype 4 activation is involved in anxiety and depression-like behavior in mouse models. *Neuropharmacology.* 101: 204-15. (2016) **IF: 4.709***

Bánki E, **Hajna Z**, Kemény Á, Botz B, Nagy P, Bölcskei K, Tóth G, Reglődi D, Helyes Z. The selective PAC1 receptor agonist maxadilan inhibits neurogenic vasodilation and edema formation in the mouse skin. *Neuropharmacology*. 85: 538-47. (2014) **IF: 5.106**

Tékus V, **Hajna Z**, Borbély É, Markovics A, Bagoly T, Szolcsányi J, Thompson V, Kemény Á, Helyes Z, Goebel A. A CRPS-IgG-transfer-trauma model reproducing inflammatory and positive sensory signs associated with Complex Regional Pain Syndrome. *Pain*. 155: 299-308. (2014) **IF: 5.213 (co-first author)**

Pintér E, Pozsgai G, **Hajna Z**, Helyes Z, Szolcsányi J: Neuropeptide receptors as potential drug targets in the treatment of inflammatory conditions. *Br J Clin Pharmacol*. 77: 5-20. (2014) **IF: 3.878**

Borbély É, **Hajna Z**, Sándor K, Kereskai L, Tóth I, Pintér E, Nagy P, Szolcsányi J, Quinn J, Zimmer A, Stewart J, Paige C, Berger A, Helyes Z. Role of tachykinin 1 and 4 gene-derived neuropeptides and the neurokinin 1 receptor in adjuvant-induced chronic arthritis of the mouse. *PLoS One*. 8: e61684. (2013) **IF: 3.534**

(*5-year impact factor)

Cumulative impact factor of all publications: 40.761
Total number of independent citations: 59

Abstracts published in cited journals

Borbély É, Tékus V, **Hajna Z**, Hunyady Á, Berger A, Paige CJ, Szolcsányi J, Pintér E, Helyes Z. Nociceptive role of hemokinin-1, the newest member of the tachykinin family, in chronic traumatic neuropathy model of the mouse. *Digestive Diseases and Sciences*, 59: 1649. (2014)

Kemény Á, **Hajna Z**, Szitter I, Kun J, Pintér E, Helyes Z. Role of TRPV1 and TRPA1 ion channels in cigarette smoke-induced chronic airway inflammation model of the mouse. *J Mol Neurosci*. 53 (Suppl 1): S174. (2014)

Reglődi D, Bánki E, **Hajna Z**, Kemény Á, Nagy P, Bölcskei K, Tóth G, Helyes Z. Receptorial mechanisms of the inhibitory action of PACAP on the vascular changes in neurogenic inflammation. *J Mol Neurosci*. 53 (Suppl 1): S181. (2014)

Kun J, Feller D, Szitter I, **Hajna Z**, Kemény Á, Perkecz A, Csöngei V, Ernszt D, Kovács T, Pongrácz J, Helyes Z. Cigarette smoke-induced upregulation of the Transient Receptor Potential Ankyrin 1 ion channel in the mouse lung and in a human pulmonary tissue 3-dimensional model. *Acta Physiol*. 211 (Suppl. 697): 107. (2014)

Pintér E, **Hajna Z**, Pozsgai G, Bagoly T, Helyes Z, Szolcsányi J. Characterization of TRPA1-mediated and TRPA1-independent microcirculatory responses to hydrogen sulfide in mice. *Nitric Oxide*, 31 (Suppl 2): S29-S30. (2013)

Szabadfi K, **Hajna ZR**, Gábriel R, Pintér E. Comparative histological investigation of the retina in wild type and somatostatin 4 receptor knockout mice. *J Neurochem*, 125 (Suppl 1): 272. (2013)

Helyes Z, Scheich B, Kormos V, Tékus V, **Hajna Z**, Gaszner B, Pintér E, Szolcsányi J. Activation of somatostatin receptor subtype 4 (sst4) inhibits anxiety and depression-like behaviours in mouse models. *J Neurochem*, 125 (Suppl 1): 230. (2013)

Borbély É, **Hajna Z**, Nabi L, Tékus V, László K, Ollmann T, Karádi Z, Lénárd L, Quinn JP, Berger A, Paige CJ, Keeble J, Szolcsányi J, Pintér E, Helyes Z. Role of hemokinin-1 and NK1 receptors in anxiety, stress and depression-like behaviour in mice. In: Csillag András (szerk.) *XIV. Conference of the Hungarian Neuroscience Society*. p. 282. (ISBN: 978-963-88224-2-0) (2013)

Borbély É, **Hajna Z**, Berger A, Sándor K, Tóth I, Kereskai L, Pintér E, Szolcsányi J, Paige CJ, Quinn JP, Zimmer A, Helyes Z. Hemokinin-1 plays an important role in adjuvant-induced joint and lung inflammation of the mouse. *Eur J Clin Invest*. 42 (Suppl 1): 66. (2012)

Hajna Z, Pozsgai G, Boros M, Kemény Á, Helyes Z, Szolcsányi J, Pintér E. Transient receptor potential ankyrin 1 (TRPA1) receptors play a role in hydrogen sulphide-induced vasodilation of the mouse ear. *Clinical Neuroscience*, 65:1 p. 26. (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 thermonociception in mice. *Clinical Neuroscience*, 65:1 p. 68. (2012)

Scheich B, Kormos V, Tékus V, **Hajna Z**, Gaszner B, Pintér E, Szolcsányi J, Helyes Z. Somatostatin receptor subtype 4 (sst4) plays an inhibitory role in anxiety and depression-like behaviours of mice. *Clinical Neuroscience*, 65:1 p. 57. (2012)

Szabadfi K, **Hajna Z**, Gábriel R, Pintér E. Analysis of retinal histology in wild type and somatostatin 4 receptor deficient mice. *J Mol Neurosci.* 48 (Suppl 1): S191. (2012)

Kemény Á, Boros M, Borbély É, **Hajna Z**, Sétáló G, Pintér E, Szolcsányi J, Helyes Z. Cytokine profiling of inflamed mouse tissues obtained from different in vivo models. *J Mol Neurosci.* 48 (Suppl 1): S199. (2012)

Hajna Z, Móricz A, Elekes K, Kemény Á, Kereskai L, Berger A, Paige C J, Szolcsányi J, Pintér E, Helyes Zs: Role of Hemokinin-1 in a mouse model of acute airway inlammation. *Acta Physiol.* 202 (Suppl. 684): 39. (2011)

Kemény Á, Boros M, Borbély É, **Hajna Z**, Sétáló G, Pintér E, Szolcsányi J, Helyes Z. Cytokine profiling in different inflammatory in vivo mice models. *Acta Physiol*. 202 (Suppl. 684): 53-54. (2011)

Pozsgai G, Bagoly T, **Hajna Z**, Boros M, Helyes Z, Szolcsányi J, Pintér E. Role of transient receptor potential ankyrin 1 (TRPA1) receptors in hydrogen sulphide-evoked calcitonin gene-related peptide (CGRP) release from isolated rat tracheae. *Acta Physiol*. 202 (Suppl. 684): 98-99. (2011)

Hajna Z, Borbély É, Quinn JP, Zimmer A, Pintér E, Szolcsányi J and Helyes Z. Role of preprotachykinin A (TAC1) gene-derived peptides and the neurokinin 1 (NK1) receptor in acute nocifensive behaviours and hyperalgesia. *Front. Neurosci*. Conference Abstract: 13th Conference of the Hungarian Neuroscience Society (MITT). doi: 10.3389/conf.fnins.2011.84.00131 (2011)

Borbély É, **Hajna Z**, Simon G, Berger A, Paige C, Quinn J, Pintér E, Szolcsányi J and Helyes Z Investigation of the role of preprotachykinin A and C (TAC1 and TAC4) gene-derived peptides in anxiety, stress and depression-like behaviour in mice. *Front. Neurosci*. Conference Abstract: 13th Conference of the Hungarian Neuroscience Society (MITT). doi: 10.3389/conf.fnins.2011.84.00099 (2011)

Pozsgai G, Bagoly T, **Hajna Z**, Boros M, Helyes Z, Szolcsányi J and Pintér E. The role of transient receptor potential ankyrin 1 (TRPA1) receptors in the H_2S-evoked calcitonin gene-related peptide (CGRP) release from isolated rat trachea. *Front. Neurosci*. Conference Abstract: 13th Conference of the Hungarian Neuroscience Society (MITT). doi: 10.3389/conf.fnins.2011.84.00022 (2011)

Oral presentations

Hajna Zsófia: Mikrocirkuláció vizsgálatára alkalmas képalkotó módszerek a gyulladáscsökkentő és fájdalomcsillapító gyógyszerek fejlesztésében. *Gyógyszerésztudományok Fóruma, Pécs, 2014*

Helyes Z, **Hajna Z**, Botz B: Kisállat-képalkotó módszerek jelentősége és alkalmazási lehetőségei a gyulladás- és fájdalomkutatásban. *IX. Szentágothai Szeminárium, Pécs, 2014*

Zs Hajna, G Pozsgai, T Bagoly, L Boros, Á Kemény, Zs Helyes, J Szolcsányi, E Pintér: Transient Receptor Potential Ankyrin 1 receptors mediate hydrogen-sulphide-induced calcitonin-gene-related-peptide-release and cutaneous vasodilatation. *37th International Congress of Physiological Sciences (IUPS 2013), Birmingham, UK, 2013*

Helyes Zs, Borbély É, Botz B, Tékus V**, Hajna Zs**, Sándor K, Markovics A, Szolcsányi J, Quinn JP, Berger A and McDougall JJ: Role of capsaicin-sensitive afferents and sensory-immune interactions in arthritis. *Neuroinflammation – A satellite symposium to the FENS Featured Regional Meeting, Prague, Czech Republic, 2013*

E Pintér, **Z Hajna**, G Pozsgai, T Bagoly, Z Helyes, J Szolcsányi: Characterization of TRPA1-mediated and TRPA1-independent microcirculatory responses to hydrogen sulfide in mice. *2nd European Conference on the Biology of Hydrogen Sulfide, Exeter, UK, 2013*

Pintér E., **Hajna Z.**, Pozsgai G., Bagoly T., Helyes Zs., Szolcsányi J. The role of CGRP in the TRPA1 receptor mediated effects. *10th Joint Meeting of the European Neuropeptide Club and the Summer Neuropeptide Conference, Gdynia, Poland, 2013*

Pintér E, **Hajna Z**, Szabadfi K, Gábriel R, Balla Z, Biró Z, Degrell P, Kőszegi T, Tékus V, Helyes Z, Dobos A, Farkas S, Szűcs Gy. Az 1-es típusú diabetes mellitus modellezése diabeteses neuropathia, nephropathia és szemelváltozások vizsgálatára patkányban. *Magyar Élettani, Farmakológiai és Mikrocirkulációs Társaságok 2013. évi közös tudományos Kongresszusa, Budapest, 2013*

Zs Hajna, G Pozsgai, T Bagoly, L Boros, Á Kemény, Zs Helyes, J Szolcsányi, E Pintér: Hydrogen-sulphide (H2S)-induced CGRP-release and cutaneous vasodilatation are mediated by Transient Receptor Potential Ankyrin 1 (TRPA1) receptors. *I. International Doctoral Workshop of Natural Sciences, Pécs, 2012 (III. helyezés)*

E Pinter, **Zs Hajna**, G Pozsgai, T Bagoly, M Boros, A Kemeny, Zs Helyes, J Szolcsanyi: The role of transient receptor potential ankyrin 1 (TRPA1) receptor activation in hydrogen-sulphide-induced CGRP-release and vasodilation. *6th European Congress of Pharmacology, Granada, Spain, 2012*

Z Helyes, I Szitter, **Z Hajna**, K Elekes, Á Kemény, L Kereskai, J Quinn, K Sándor, E Pintér, J Szolcsányi: Role of the TRPV1 ion channel and sensory neuropeptides in cigarette-smoke-induced chronic airway inflammation model of the mouse. *9th Joint Meeting of the European Neuropeptide Club and the Summer Neuropeptide Conference, London, UK, 2012*

Helyes Z, Dezső-Tékus V, **Hajna Z**, Borbély É, Kormos V, Botz B, Gaszner B, Nagy P, Bölcskei K, Szolcsányi J: Neuropátiás állapotok állatkísérletes modellezése szenzoros, motoros és vaszkuláris működések vizsgálati lehetőségei. *A Magyar Élettani, Anatómusok, Biofizikai, Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa, Debrecen, 2012*

Pozsgai G, **Hajna Z**, Bagoly T, Szolcsányi J, Helyes Z, Pintér E. A tranziens receptor potenciál ankyrin 1 receptor szerepe a hidrogén-szulfiddal kiváltott vazodilatációban. *A Magyar Élettani, Anatómusok Biofizikai, Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa, Debrecen, 2012*

É. Borbély, **Z. Hajna**, A. Berger, K. Sándor, I. Tóth, L. Kereskai, E. Pintér, J. Szolcsányi, C.J. Paige, J.P. Quinn, A. Zimmer & Z. Helyes: Hemokinin-1 plays an important role in adjuvant-induced joint and lung inflammation of the mouse. *46th Annual Scientific Meeting of the European Society for Clinical Investigation, Budapest, 2012*

Hajna Zsófia: Endotoxinnal kiváltott légúti gyulladás és hiperreaktivitás preprotachykinin C (TAC4) génhiányos egerekben. *XVI. Korányi Frigyes Tudományos Fórum, Budapest, 2011*

ACKNOWLEDGEMENTS

I would like to acknowledge my gratitude to my supervisor Prof. Erika Pintér for her professional guidance, acquainting me with the significance and beauty of pharmacological sciences as well as the characteristics of precise and systematic research work, opening new perspectives for me in this field. I am also most grateful for the personal support and understanding I have received throughout all these years. I wish to thank my supervisor Prof. Zsolt Biró for supporting my work since my academic years and for showing me a good example of devotion and humility towards medical sciences. I would like to express my sincere thanks to my "honorary supervisor", Prof. Zsuzsanna Helyes for her inspirating and indispensable help as well as her professional instructions I could always rely on. I am also grateful for her particular enthusiasm and passionate commitment to scientific research which always gave me new impulsions. I would like to express my gratitude to Prof. Loránd Barthó and Prof. Gábor Pethő for supporting and helping me on the field of teaching, as well as Prof. János Szolcsányi, whose professional calling has set a good example for all of us. Special thanks go to Éva Borbély and Valéria Tékus for their sincere friendship and for supporting my work with their kind help and enthusiasm. I would like to thank Ágnes Kemény, Gábor Pozsgai, Éva Szőke and Kata Bölcskei for their essential help and professional advices during my research. I also thank Bálint Botz, Éva Sághy, Maja Payrits, Bálint Scheich, Kata Csekő, István Szitter and József Kun for all their help giving me in the experiments, as well as the student research fellows Tímea Gubányi and András Móricz for their eager work. I would like to thank Anikó Perkecz for the preparation of excellent histological slides, as well as Dóra Ömböli, Ildikó Fábián, Nikolett Szentes, Csilla Zádor, Katalin Gógl and Teréz Bagoly for their expert technical assistance. I thank all members of the Department forming such a community that provides a friendly athmosphere for performing successful work. I wish to thank all those colleagues working in other institutes who I had the oppotunity to collaborate with. I would like to acknowledge my most gratitude to Krisztina Szabadfi, who passed away tragically young and whose conscientious attitude to research work remains exemplary for me. I would like to thank to her and Prof. Róbert Gábriel for their professional work provided in histological investigation of the retina. I wish to thank László Kereskai and Prof. Péter Degrell for the evaluation of histological preparations, as well as Eszter Bánki, Zsolt Balla, Tamás Kőszegi, Sándor Farkas and András Dobos for their valuable contributions in research work. Furthermore, I am also grateful to our collaborators abroad: Prof. Pierangelo Geppetti, Prof. John Quinn, Alexandra Berger, Prof. Susan D. Brain, Aisah Aubdool and Fiona Russell for providing us the gene-deficient mice, as well as for their remarkable help in performing experiments and preparing papers. Finally, I would like to express special thanks to my Family I could always rely on during my studies and scientific career. I am most grateful to my Husband for all his love, patience and encouragement he is giving me, as well as to my Mother for her generous and supporting help.