GENERATION OF TRANSGENIC MICE AND CELL LINES TO STUDY TRPA1 AND SST_4 RECEPTORS FOR MEDICAL DRUG RESEARCH

Doctoral (PhD) thesis

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1. General introduction

1.1. Generating transgenic animals and cell lines

For a better understanding of the physiological and pathological functioning of the human organism at the molecular level (fundamental research) and for preclinical research on new drugs, it is essential to select appropriate model organisms that mimic the function of human proteins to a sufficient extent. In many cases, the most suitable organism does not yet exist, but rapidly evolving molecular biological tools are making it increasingly feasible for research laboratories to design and generate their own model organisms. The simplest and most widely used method is the transfection of mammalian cells, such as Chinese hamster ovary epithelial cells (CHO) and human embryonic kidney cells (HEK293), to express human protein and thus allow to study the function of human proteins *in vitro*. However, *in vitro* experiments do not give a complete picture of the complex function of proteins in living organisms, and experimental animals are essential to understand the mechanisms accurately. Mice and rats show a high degree of homology with humans for many proteins, so the function of wild type proteins in mice and rats is often studied first and then compared with human results. A common tool is the use of knockout (KO) animals, which usually show changes (predominantly impaired function) compared to wild type (WT) animals, which are used to infer the function of the protein. It is a relatively new and as yet uncommon method to replace the missing gene in these animals with its human homologues gene in order to test human protein function in animals. Of course, this is not a perfect reflection of the function of the human protein in humans, as the human protein still functions in the environment of the animal organism. However, we can still gain valuable insights into the racial differences between human and animal protein function and, in drug discovery, gain predictive results on what to expect in the human body during clinical trials of a new drug.

In the Institute of Pharmacology and Pharmacotherapy, Medical School, University of Pécs, our research group is interested in the mechanisms of the Transient Receptor Potential Ankyrin 1 (TRPA1) and Somatostatin receptor 4 (SST_4) receptors involved in the modulation of pain and inflammation. During my PhD research, I generated model organisms for current research projects related to these receptors, and tested transgene expression and protein function.

1. The SST_4 receptor is a promising drug target for pain and inflammation reduction. For preclinical testing of novel SST_4 agonist drug candidate molecules, we needed

humanized SST_4 mice. To this end, we introduced the *human* homologous gene (*hSSTR4) into* somatostatin receptor 4 gene-deficient (*Sstr4* KO) mice. The first transgenic mice for this project had already been generated by the time I started my PhD work, so I participated in the thorough study of these mice.

2. Organic polysulfides are analgesic and anti-inflammatory TRPA1 agonists and are promising drugs. To better understand their mechanism of action, we wanted to identify their binding site on the human TRPA1 receptor. For this purpose, we generated several TRPA1 variants by PCR-based site-directed mutagenesis, in which certain cysteines were replaced by alanine. The TRPA1 variants were expressed in CHO cells and their function was investigated by *in vitro* experiments. The binding site for organic polysulfides was identified in the mutant in which organic polysulfides failed to induce any effect, caution should be taken that this is not caused by a global loss of function, which was checked by the agonist effect of a positive control.
3. We investigated the role of astrocytes in TPRA1-mediated cuprizone-induced demyelination in a multiple sclerosis (MS) disease model. For this purpose, we made astrocyte-specific *Trpa1* gene knockout using the Cre-loxP system. The *Trpa1* gene coding sequence between loxP sites was excised and rendered inoperable in cells in which Cre recombinase was expressed. In turn, expression of Cre recombinase was linked to an astrocyte-specific gene (*Gfap*), ensuring that gene knockout only occurs in astrocyte cells. In the mice thus generated, we examined the characteristics of cuprizone-induced demyelination.

1.2. Pain and inflammation

Pain is defined by the *International Association for the Study of Pain* (IASP) as discomfort associated with potential or actual tissue damage. It can be distinguished from nociception, as pain is not solely the result of sensory neuron function, but is a complex subjective experience influenced by biological, psychological and social factors. Although pain has a primarily adaptive role, chronic pain can have detrimental effects on bodily function and on social and psychological well-being (1).

Inflammation is an important defense mechanism whereby the body recognizes and removes harmful and foreign stimuli (primarily pathogens) and then begins the healing process. Persistent inflammation, however, has detrimental effects on the body and is a key factor in most chronic degenerative diseases (e.g. cancer, diabetes, rheumatoid arthritis,

allergic asthma, Alzheimer's disease, multiple sclerosis) (2). Particular attention should be paid to cases where the inflammation itself causes more severe tissue damage than the original stimulus, such as tuberculosis, silicosis, atherosclerosis, allergies and autoimmune diseases. Although there are many mechanisms for eliminating inflammation, by its nature it tends to create a self-perpetuating process, as inflammation can lead to tissue damage and cell necrosis stimulates inflammation. Thus, inflammation may be able to persist after the original stimulus has ceased and develop into *non-resolving* inflammation (3).

The treatment of persistent inflammation is therefore paramount in eliminating the associated pain, restoring impaired function, preventing or reducing further complications and allowing the healing process to continue unhindered.

However, conventional medicines used to treat chronic pain and persistent inflammation (e.g. opioids, steroids, non-steroidal anti-inflammatory drugs - NSAIDs) are often not effective enough or can cause serious side effects during long-term treatment (4–8). It is therefore necessary to understand these pathomechanisms in more detail to identify better drug targets. Thus, we focused our research on TRPA1 and SST_4 receptors, which are promising drug targets for the treatment of chronic pain and persistent inflammation.

1.3. TRPA1

The **transient receptor potential ankyrin 1 (TRPA1)** receptor is a non-selective, sodium- and calcium-permeable cation channel that is predominantly expressed on capsaicin-sensitive peptidergic nociceptive primary sensory neurons, co-localizing with the transient receptor potential vanilloid 1 (TRPV1) receptor in more than 90% (4,9,10). The TRPA1 receptor is capable of sensing a wide range of irritants, such as mechanical stimuli, extreme cold and heat, acidity, reactive oxygen species, and thousands of already identified agonists (11). Activation of the TRPA1 receptor induces acute nociception and the secretion of pro-inflammatory neuropeptides (e.g.: substance P, calcitonin gene-related peptide) that cause vasodilatation and tissue swelling, promoting the development of neurogenic inflammation. However, following TRPA1 activation, the nociceptive neuron also counter-regulates by releasing analgesic and anti-inflammatory neuropeptides such as somatostatin (12–15). The systemic analgesic and anti-inflammatory effects of **somatostatin** are mediated by the SST_4 receptor (16–25).

1.4. Somatostatin

Somatostatin is a cyclic neuropeptide with two isoforms: SST-14 and SST-28. It inhibits the secretion of several excitatory and inhibitory mediators such as somatotropin, glucagon, insulin, acetylcholine, glutamate and gamma-aminobutyric acid (GABA) (26). It regulates a wide range of physiological functions, such as sleep, motor activity, emotion, learning and memory; it also has a role in the regulation of various pathological conditions, such as pain and inflammation (16–25), neurodegeneration (27–30), anxiety and depression (31–34). In the central nervous system, there are long protruding and short proximal GABAergic interneurons that secrete somatostatin (35–37). In the periphery, somatostatin secretion has been observed on capsaicin-sensitive peptidergic sensory neurons, as well as its systemic anti-inflammatory and analgesic effects, which have been termed as "sensocrine" mechanism (38,39).

1.5. Somatostatin receptor 4 (SST_4) receptor

The broad effects of somatostatin are mediated by five inhibitory G_i -protein-coupled receptors, designated SST_{1-5}. These receptors can be divided into two groups, $SRIF_1$ includes SST_2, SST_3 and SST_5, these are specifically activated by octreotide , while SST_1 and SST_4 receptors belonging to $SRIF_2$ are specifically activated by the agonist CGP 23996 (40–42). Previous results of our research group demonstrate that the activation of the SST_4 receptor induces analgesic, anti-inflammatory, anti-anxiety and antidepressant effects without affecting the secretion of other hormones (16–25,27–34,38,39,43,44). Thus, the SST_4 receptor has emerged as a promising new drug target, and recently several pharmaceutical companies have initiated the development of SST_4 agonists (45–51). There are no known antagonists of the SST_4 receptor, therefore, *Sstr4 knockout* animals have been used as negative controls for *in vivo* functional studies (15,20–22,45).

2. Generation of humanized somatostatin receptor 4 (SST_4) mice and characterization of the transgene expression

2.1. Introduction

There is a lot of research going on, including in our institute, to investigate the functions of SST_4. Since there is no known SST_4 specific antagonist, therefore, we have used *Sstr4 knockout* and wild type mice in our previous research (15,20–22,45). Using the *Sstr4* KO mouse model and the synthetic SST_4 receptor agonist J-2156, several research groups, including our institute, have demonstrated that the SST_4 receptor is a unique and novel drug target for the treatment of chronic pain and depression (16,24,52–56). Current drugs used to treat these conditions are often ineffective and cause severe side effects during long-term treatment (4–8). Thus, the SST_4 receptor has become a focus of interest in drug development, and pharmaceutical companies have already begun the research of non-peptide SST_4 agonists (51,54). The design of agonists has been greatly aided by *in silico* 3D modelling of the human protein structure (57,58).

Our aim was to test these new agonists (J-2156 and pyrrolopyrimidine derivatives) in SST_4 humanized mice. A mouse model expressing the human receptor is particularly useful in translational drug discovery, as it can provide more predictive results for human diseases and is a more relevant model animal for testing drug candidates. (59,60). Humanized mice are predominantly generated by transplantation of human cells, tissues or tumors, mainly for immunological and oncological research (61–66) but genetic modification is also more and more frequently the method of choice (67–70). Genetic modification has already been used to successfully replace the mouse bradykinin B_1 receptor gene with its human counterpart to test *in vivo* the human B_1 receptor specific antagonist NVP-SAA164 following *in vitro* results. NVP-SAA164 induced anti-hyperglycemic effects in humanized mice, but not in WT and KO mice (71). In another experiment, differences between mouse and human melanocortin receptors were found using MC1R humanized mice, such as strongly ligand-dependent eumelanogenesis in humanized mice. In contrast, the mouse Mc1r receptor in WT mice *in vivo*, and in transfected cell lines, both mouse and human receptors showed ligand-independent signaling (72). These demonstrate that the different functions of the protein between *in vitro* and *in vivo* conditions, therefore, the humanized model animals can provide valuable information in fundamental and preclinical research.

2.2. Objective

SST_4 expression and function are relatively well characterized in the mouse brain but little is known about the human receptor (73). Therefore, our aim was to create a humanized mouse with a transposon vector containing the human *hSSTR4* gene together with its regulatory elements, map its random insertion sites, characterize its expression (pattern and level), identify the neuronal cell types expressing it in the brain, and select the most suitable transgenic mouse line for further functional experiments.

2.3. Results

A key achievement of this project is the successful generation of *SSTR4* humanized mouse lines by random insertion of the PB transposon vector and the characterization of human receptor-expressing neurons in brain regions associated with pain and mood regulation. These mice may be useful model animals for preclinical research on the SST_4 receptor, a novel target for the development of analgesic, anti-inflammatory and antidepressant drugs (16,20,21).

Instead of the popular *knock-in* technique, random insertion was chosen to avoid the influence of regulatory elements of the mouse *Sstr4* gene on the transgene. In addition to the coding region of the human gene, the human regulatory elements were inserted into the transposon vector and the positional effect was inhibited by using insulators at the ends of the transgene to hopefully make the transgene show an expression pattern as similar as possible to the expression in human organism. The drawback of this method, however, is that mapping the integration site in the transgenic mice generated can be problematic, and the insertion can interrupt mouse genes (74,75).

Random insertion of the PB transposon in our case resulted in more integration sites in the F0 mouse generation. The location of three of these copies was successfully determined by LM-PCR technique (76) (Chr3, Chr10 and ChrX), but the location of two copies is still unknown (U1 and U2). The failure to locate the integration sites of U1 and U2 leads us to conclude that the transgenes may have integrated into repetitive regions of the genome, which makes gene mapping significantly more difficult. Knowing the exact location of the Chr3 copy, we were able to design a site-specific PCR assay that not only allows us to determine the presence of the transgene, but also to distinguish between heterozygous and homozygous mice.

In the F0 generation, all three transgenic female mice experienced complications during pregnancy and delivery, which eventually resulted in their death. This leads us to conclude that in all three cases the transgene may have been inserted into the genome multiple times and the resulting overexpression of SST_4 may have caused the problem, as this did not occur in the offspring carrying a single copy. This observation supports a role of SST_4 in pregnancy, as this somatostatin receptor has been found predominantly in the human placenta (77,78).

In vivo bioluminescence imaging showed *hSSTR4*-linked expression of the luciferase enzyme in different organs, with the strongest luminescence signal in the brain. Chr3 mice showed the strongest expression in the cerebrum area, while U1 and U2 mice showed weaker expression here, but stronger expression in the *bulbus olfactorius* and posterior brain area. RT-qPCR confirmed these results, as we measured the highest *hSSTR4* expression levels in the cerebral cortex and BO. Mouse *Sstr4* gene expression in WT mice was slightly lower overall than *hSSTR4* in Chr3 mice, except in the lung, in which the expression was much higher in WT mice, but much lower in the cerebellum and brainstem. These results are in agreement with previous comprehensive expression study databases comparing mouse and human receptors (73,78–80). Expression of U1 and U2 copies showed similarity to each other in both luciferase IVIS and RT-qPCR results, leading us to conclude that perhaps these two copies are in fact identical. Further supporting this hypothesis, we genotyped 100 offspring in the F2 generation in a cross between the U1 and U2 mouse lines and found no *hSSTR4* KO individuals. Regardless of the transgene integration site, *hSSTR4* expression levels and patterns also varied between individuals in the abdomen and pelvis. The databases also documented variable SST_4 expression levels (from undetectable to moderate) in both human and mouse gastrointestinal and reproductive organ systems (81–85). These data are supported by the relatively high *hSSTR4* expression levels measured by RT-qPCR in Chr3 mice. In contrast to the database, however, low *hSSTR4* expression was measured in the stomach and intestine. In the brain area, the strongest luciferase luminescence signal was found in Chr3, almost three times stronger than in U1 and U2 mice. Furthermore, in contrast to U1 and U2 mice, we were able to distinguish between heterozygous and homozygous individuals in Chr3 mice by genotyping, which we compared *in vivo* imaging and found that the luminescent signal strength in homozygotes was twice as strong as in heterozygotes.

The tdTomato showed no detectable signal in any mouse line, neither *in vivo* imaging nor fluorescence microscopy, probably due to the generally low expression of the transgene. Fluorescent reporter proteins are usually driven by a very strong viral promoter (e.g. cytomegalovirus) or a mammalian housekeeping gene promoter (e.g. elongation factor 1

alpha) to be expressed in sufficient quantities to give a strong signal (86). Furthermore, although tdTomato is more tolerant to N-terminal protein modifications than its mRFP1 precursor (87), we found that the fluorescence of tdTomato is significantly attenuated in the fusion protein form compared to the native tdTomato protein, probably because luciferase binding interferes with tdTomato protein folding or tetramerization (88,89).

Due to the strong homology between species and within the SST receptor family, there is no reliable *hSSTR4*-specific antibody, so we chose the RNA *in situ* hybridization technique RNAscope instead of immunohistochemistry. Based on the previous results, Chr3 copies were found to be the most suitable for further experiments and *hSSTR4* expression was characterized in these copies using the RNAscope technique. The *hSSTR4* showed the strongest signal in the hippocampus (CA1 and CA2 regions) and cortex (Pir, S1, PrL), which is consistent with the mouse and human expression databases (81–85). In Chr3 mice, *hSSTR4* is expressed mainly in *Vglut1*-positive glutamatergic excitatory neurons, similarly to *Sstr4* in WT mice, but at visibly lower expression level than that. *hSSTR4* was also expressed in GABAergic interneurons in the same brain regions, whereas mouse *Sstr4* expression was observed in GABAergic cells located in the nucleus of the central amygdala. In the primary somatosensory cortex, *hSSTR4* was most strongly expressed in layers II-III, which is significantly different from mouse *Sstr4* expression in WT mice, which is most strongly expressed in layer V (73). In a previous study, *Sstr4* was expressed in the BO glomerular layer of WT mice, but not in the granular layer (90), whereas in transgenic mice, *hSSTR4* was expressed mainly in the granular layer of BO.

These differences in expression between human *SSTR4* and mouse *Sstr4* may be due to species differences, but may also be due to limitations of the humanized mouse model, such as the position effect (75). Therefore, these differences needs to be further investigated.

We conclude that the Chr3 *hSSTR4* mouse transgene is expressed primarily in excitatory glutamatergic neurons of brain regions responsible for pain and mood regulation, showing several similarities and some differences compared to *Sstr4* expression in WT mice. Following further in-depth investigation of the function of the human receptor, the Chr3 mouse line may be a suitable translational research tool for exploring the potential of the SST_4 receptor as an analgesic, antidepressant and anti-inflammatory drug target, as well as for preclinical testing of novel SST_4 agonist drug candidates.

3. Identification of organic polysulfide binding sites on the human TRPA1 receptor

3.1. Introduction

Chronic pain and persistent inflammation are a serious problem in modern society, affecting 20-45% of people worldwide (3,91–95). The direct impact of chronic pain is a reduction in quality of life and even loss of body function (1,96–98). If persistent inflammation is not accompanied by pain, it can easily remain hidden, but even so, it can contribute to the development of many other chronic diseases, such as type 2 diabetes, allergies, cardiovascular disease and several types of cancer (2,99,100). Traditional painkillers and anti-inflammatory drugs such as steroids, NSAIDs and opioids are not suitable for long-term treatment because they are gradually outweighed by their side effects (4–8). Therefore, there is a great need to develop new drugs with new mechanisms of action for the treatment of chronic pain and persistent inflammation. Polysulfides are promising agents for this purpose and their effects are increasingly being studied in drug research. In the past, analgesic and anti-inflammatory effects were attributed to hydrogen sulfide (H_2S), a gaseous endogenous signal transducer. It is now well established that H_2 S released locally during inflammation is spontaneously oxidized to sodium hydrogen sulfide (NaSH) and sodium sulfide (Na_2S) and spontaneously polymerized to inorganic polysulfides (e.g.: Na_2S_3). These agents are reactive enough to covalently bind to the cysteines of the transient receptor potential ankyrin 1 (TRPA1) receptor and activate it (12,101–103). Activation of TRPA1 results in the release of somatostatin, which exerts systemic analgetic and anti-inflammatory effects mediated by the SST_4 receptor (16–25). These effects of polysulfides are abolished by knocking out the *Trpa1* or *Sstr4* gene (12). These data suggest that the analgesic and anti-inflammatory effects of polysulfides are at least partly mediated by activation of the TRPA1 receptor. Despite their beneficial effects, inorganic polysulfides are not suitable as drugs because they are highly reactive and unstable molecules. Their delivery is very difficult, either by direct administration or by endogenous synthesis via H_2S donor delivery (e.g.: GYY4137). Thus, our attention has turned to organic polysulfides, which have similar biological activity but are much more stable, such as dimethyl trisulfide (DMTS), diallyl trisulfide (DATS) and diallyl disulfide (DADS), which are naturally found in garlic (12,15,104–108).

The molecular mechanism of action of inorganic polysulfides is already widely studied, but very little is known about inorganic ones. Therefore, as a first step in our research, we set out to identify the binding site of inorganic polysulfides on the TRPA1 receptor using site-specific mutagenesis. Out the 28 cysteines of human TRPA1, we investigated those composing the conventional binding site of electrophilic agonists at the N-terminal domain (C621, C641 and C665) (109–112), and protein surface cysteines located in the transmembrane region, which are theoretical binding sites of highly hydrophobic agonists (C727 and C834) (113,114). We have successfully generated TRPA1 mutant variants with reduced or completely abolished sensitivity to organic polysulfides, but other functions remain intact (e.g., action of non-electrophilic agonists and antagonists). The binding properties of the mutant receptors were preliminarily investigated by *in silico* molecular docking techniques. Functional changes were investigated *in vitro* by calcium-sensitive fluorescence flow cytometry, radioactive Ca-45 liquid scintillation counting and *whole-cell patch-clamp* technique.

3.2. Objective

The binding site for endogenous, predominantly inorganic polysulfides (e.g. Na sulfide) is already known at the TRPA1 receptor but the binding site for exogenous organic polysulfides remains to be mapped (115). To this end, we aim to create a mutant TRPA1 receptor variant that is not activated by organic polysulfides but is activated by other binding site agonists (e.g.: carvacrol, thymol, menthol). The binding properties of the TRPA1 mutants will first be investigated by computational modelling, the designed mutations will be generated by site-directed mutagenesis, and the function of the TRPA1 variants will be investigated by *in vitro* methods.

3.3. Results

The TPRA1 receptor activating effect of garlic-derived organic polysulfides is well known (12,15,104,105,116–119), however, our experiments were the first to validate site-specific TRPA1 mutant variants for the identification of organic polysulfide binding sites. Our results were obtained by computer modeling of mutant TRPA1 variants and three functional assays: calcium-sensitive fluorescence flow cytometry, radioactive Ca-45 liquid scintillation counting, and *whole-cell patch-clamp.* Our results overlap significantly and support each other. In this study, we have demonstrated that the organic polysulfides DMTS, DADS and DATS covalently bind to amino acids C621, C641 and C665, thereby activating the TRPA1

receptor. Of these, C621 plays the most important role, but the other two cysteines also contribute to the binding of electrophilic agonists. Only the combined mutation of the three cysteines led to complete insensitivity of TRPA1 to organic polysulfides.

Of the three organic polysulfides, the largest molecule, DATS, showed the most favorable calculated free energy of binding, but, as the experimental results highlighted, it is the formation of a covalent bond with C621 that is important, not the strength of the bond. It should be noted that the apo conformation of TRPA1 is not favorable for binding electrophilic agonists, as seen in the prerequisite docking calculations. In the prerequisite docking, the distance between atoms involved in covalent bonding on the holo structure was smaller. We can explain this observation by the fact that the A-loop covers the binding site from the ligands in the apo structure but not in the holo structure. As has been shown in previous articles (120,121), the upward motion of the A-loop after the prerequisite binding of the agonist to P666 and F669 is necessary for the accessibility of the actual binding site.

C621, C641 and C665 compose the known binding sites for most electrophilic agonists, except for AITC, which also binds to lysine K710 for activation, and JT010, which binds to C621 alone for TRPA1 activation (109–112,122).

In contrast to radioactive Ca-45 liquid scintillation and *whole-cell patch-clamp* measurements, Fluo-4 calcium-sensitive fluorescence flow cytometry showed no difference between single TRPA1 mutants. This was probably due to the ability of the Fluo-4 dye to saturate at high calcium concentrations, which could be due to the relatively high concentration of organic polysulfides (100 μM). Therefore, Ca-45 liquid scintillation counting and *whole-cell patch-clamp* techniques were considered to be more accurate and reliable methods and could be used to explore the differences in function of the three cysteines.

Among the three cysteines, the most important role of C621 in the binding of organic polysulfides was predicted by computer modelling and confirmed by radioactive Ca-45 liquid scintillation counting and *whole-cell patch-clamp* results. C621 is known to play a key role in the binding of several electrophilic TRPA1 agonists such as JT010, iodoacetamide, BODIPY-iodoacetamide, AITC and BITC (110,122–125).

Whole-cell patch-clamp showed that C665 is the second most important cysteine for binding organic polysulfides, while C641 has the least important role. The role of C665 has already been demonstrated for other electrophilic agonists such as iodoacetamide, BODIPY-iodoacetamide, N-ethylmaleimide and BITC (122,124–126).

Although the single cysteine mutations only reduced the effect of organic polysulfides in TRPA1, the effect of JT010 was completely lost in the case of the C621A single mutation.

Several lines of evidence suggest that JT010 does not covalently bind to other cysteines, only to C621 (122,127,128). Nevertheless, our calcium-sensitive fluorescence flow cytometry results showed that single mutations in C641A and C665A reduced the effect of JT010, leading us to conclude that they have a role other than covalent binding of the agonist. Presumably, C641 and C665 help to maintain the functional structure of the binding pocket or help to create an attractive environment for electrophilic agonists, or possibly are involved in both.

Since a single mutation of C621 was not enough to completely abolish the effect of organic polysulfides, it can be concluded that C641 and C665 are also involved in the covalent binding of these active compounds. Only the combined triple mutation of the three cysteines was able to completely abolish the effect of the organic polysulfides. Other electrophilic agonists are also known to lose their effect in triple mutants of these three cysteines, except for AITC, which is able to retain a minimal effect with intact K710 lysine (109–112). In our calcium-sensitive fluorescence flow cytometric results, AITC showed a stronger effect in WT and single mutant TRPA1 variants than effect of carvacrol. In contrast, in the triple mutant, there was only a minimal effect of AITC, while the effect of carvacrol was preserved.

Neither single nor double mutations of amino acids C727 and C834 together caused a change in the effect of DMTS compared to WT TRPA1. This result refutes the hypothesis that C727 and C834 would form a binding site of the highly hydrophobic electrophilic agonists on the transmembrane domain (113,114).

In HEK293 cells expressing the mouse Trpa1 receptor, amino acids C415 and C422 have been shown to be involved in the binding of inorganic polysulfides (disodium trisulfide) and other electrophilic agonists (102,123,129). Whether the human homologues of these (C414 and C421) or possibly the remaining 21 of the 28 cysteines play a role in the binding of organic polysulfides remains to be further investigated.

Of the organic polysulfides we used, the factory-made DMTS was almost completely pure (≥98%), but the DADS and DATS we synthesized were inseparable companions, with DADS at 90% and DATS at 63.5%. They are also constant contaminants of each other in their natural source, garlic oil. In the *whole-cell patch-clamp* results, the effect of DADS was found to be stronger than the other two organic polysulfides in WT TRPA1, however, it was most affected by single cysteine mutations. As long as the effect of DMTS and DATS was reduced in C621A and C665A single mutants, the effect of DADS was completely abolished. Single mutation C641A reduced the effect of DADS, but not the effect of DMTS and DATS. These findings suggest that of the three organic polysulfides, DADS is the most potent TRPA1

agonist, but this was not supported by the results of our other assays, where the three organic polysulfides showed identical effects, and thus needs further investigation.

The *whole-cell patch-clamp* also showed that the C621A/C641A/C665A triple mutant was insensitive to the HC-030031 antagonist, for reason which remains to be clarified, whether it was caused by the triple cysteine mutation or possibly a hidden random mutation. The insensitivity of the triple mutant to the organic polysulfides and the HC-030031 antagonist could have been due to a global loss of function caused by the mutations, but the effect of carvacrol remained intact, demonstrating that the receptor remained properly functional and was suitable for our experiments.

Using three different functional assays, we demonstrated that organic polysulfides covalently bind to cysteines C621, C641 and C665 to activate TRPA1, and that only a triple mutation of these cysteines together was able to completely abolish the effect of organic polysulfides. We further identified a key role for C621 in this and that C655 is the second most important cysteine. This overlaps significantly with the known binding sites of other electrophilic agonists, and their results may therefore further clarify the binding mechanism of other electrophilic agonists.

In this study, we have not only shown that TRPA1 is an important target of organic polysulfides, but also identified their exact binding sites using mutant receptor variants.

4. Generation and expression assay of astrocyte-specific *TRPA1* conditional *knockout* mice

4.1. Introduction

Multiple sclerosis (MS) is a severe neurodegenerative autoimmune disease of the central nervous system in which persistent inflammation and massive cell death cause the myelin sheath that covers the axons of neurons to break down, resulting in impaired neural function (130,131). A widely used method for modelling MS is the long-term treatment of mice with cuprizone, an oligodendrocyte-specific cell-toxic agent, which induces demyelination, mainly in the *corpus callosum*, associated with macrophage invasion and astrocyte reaction (132,133). Previous studies have shown that TRPA1 KO mice are more resistant to cuprizone-induced demyelination (133,134). It was concluded that TRPA1 regulates the mitogen-activated protein kinase pathway leading to cell apoptosis, and therefore, in the absence of TRPA1, there is much less adult oligodendroglia death in response to cuprizone. TRPA1 inhibition represents a potential new drug mechanism for the treatment of MS.

TRPA1 receptor is expressed mainly in capsaicin-sensitive nociceptive sensory neurons (4,9,10), but is also expressed at lower levels in non-neuronal cells such as keratinocytes, endothelial cells and gastrointestinal mucosal cells (135–138), and in the brain in oligodendroglia (139) and astrocytes (140–142). In the mouse cerebral cortex, low levels of TRPA1 expression have been detected in neurons, astrocytes, oligodendroglia and microglia (143). In our experiments, we investigated the role of astrocytes in the pathomechanism of MS, as reactive astrocytes are already known to contribute to neuroinflammatory processes in neurodegenerative diseases (144,145).

4.2. Objective

In the present study, we aim to demonstrate the key role of TRPA1-mediated processes leading to cuprizone-induced oligodendrocyte apoptosis in astrocyte cells. To this end, we aim to generate a mouse model using the Cre-Lox recombination system with astrocyte-specific knockout of the *Trpa1* gene (conditional *knockout* - cKO). We will investigate the demyelination effect of cuprizone in the mice thus generated and compare the results with those obtained in *Trpa1* WT and global KO mice.

4.3. Results

The TRPA1 receptor contributes to neuroinflammatory processes in neurodegenerative diseases. Our previous research has shown in a disease model of MS that *Trpa1* global KO mice are more resistant to cuprizone-induced demyelination (133,134). We concluded that TRPA1 regulates the mitogen-activated protein kinase pathway leading to cell apoptosis, and therefore in the absence of TRPA1 there is much less adult oligodendroglia death in response to cuprizone. TRPA1 inhibition represents a potential new drug mechanism for the treatment of MS.

Since reactive astrocytes are known to contribute to neuroinflammatory processes in neurodegenerative diseases (144,145), we therefore wanted to investigate the role of astrocytes in TRPA1-mediated processes involved in the pathomechanism of MS in a disease model of cuprizone-induced demyelination in MS. To this end, we designed astrocyte-specific TRPA1 cKO mice, which we planned to generate using a Cre-loxP system. The most widely used astrocyte marker is the *Gfap* gene, so we chose mice in which the Cre recombinase gene is driven by the *Gfap* promoter. Gfap-Cre$^{+/-}$ mice were pretested by crossbreeding them with tdTomato reporter mice. In the resulting offspring, the tdTomato red fluorescent protein was expressed in cells where Cre recombinase was expressed. We assessed tdTomato fluorescence in the whole body by *in vivo* imaging and in the brain area by fluorescence microscopy. Cre recombinase was shown to be expressed predominantly in the brain area, in astrocytic cells, but also showed a small amount of activity in neurons. We considered this to be appropriately specific expression, and if Trpa1 is accordingly deleted in the experimental Gfap-Cre$^{+/-}$ *Trpa1*$^{Fl/Fl}$ mice, then the expected loss of function may indeed be related to astrocytes. Other research groups have also observed in *Gfap-Cre* mice that recombination occurs in the vast majority of astrocytes, but small amounts were also observed in neurons and oligodendroglia (146–148). Presumably this is due to intercellular mass transfer and low levels of unspecific gene expression. We have attempted to identify the unknown insertion site of the *Gfap-Cre* transgene in the mouse genome using ligation-mediated and inverse PCR techniques, but unfortunately without success. We concluded that it was probably integrated into a long repeat sequence.

The mice for the experiment were generated by crossbreeding Gfap-Cre$^{+/-}$ and *Trpa1*$^{Fl/Fl}$ mice. In *Trpa1*$^{Fl/Fl}$ mice, loxP sites were identified by PCR and sequencing. Subsequently, we were able to design genotyping primers to distinguish between mice carrying *Trpa1* genes knocked out from only a subset of cells (conditional) and from all cells (global). Thus, we

were now able to screen animals crossed for the *Trpa1* global KO genotype resulting from the accidental unspecific action of Cre recombinase. In Gfap-Cre$^{+/-}$ *Trpa1*$^{Fl/Fl}$ mice generated at the end of crossbreeding, we measured reduced expression levels of intact *Trpa1* by RT-qPCR. Summarizing the assays, the generated Gfap-Cre$^{+/-}$ *Trpa1*$^{Fl/Fl}$ mice were found to be suitable for the cuprizone-induced demyelination experiment. As control groups, Gfap-Cre$^{-/-}$ *Trpa1*$^{Fl/Fl}$ (loxP control, effectively showing a *Trpa1* WT phenotype) and Gfap-Cre$^{+/-}$ *Trpa1*$^{Fl/-}$ mice (hetero control) were provided.

In the functional experiments led by *Dr. Gábor Kriszta,* astrocyte-specific *Trpa1* cKO mice were significantly more resistant to cuprizone-induced demyelination compared to *Trpa1* WT mice during the period of the most intense pathophysiological lesions, i.e., weeks 3-5 of treatment, and by week 6, both groups showed a reduction in symptoms and differences between groups. Compared to the global *Trpa1* KO mice previously studied, astrocyte-specific *Trpa1* cKO mice were less resistant to cuprizone-induced demyelination, suggesting that astrocytes are not solely involved in TRPA1-mediated demyelination in multiple sclerosis (149). The expression and function of TRPA1 receptors in other brain cells (oligodendrocytes, microglia, neurons) requires further investigation (139,150).

5. Summary

In the Institute of Pharmacology and Pharmacotherapy, Medical School, University of Pécs, our research group is interested mainly in the mechanisms of TRPA1 and SST_4 receptors involved in the modulation of pain and inflammation. In my PhD research I have generated model organisms for current research projects related to these receptors, and tested transgene expression and protein function.

We have successfully generated humanized *SSTR4* mice, which will be used to further explore the species differences between human and mouse homologous SST_4 receptors, to further investigate the physiological and pathological function of the human SST_4 receptor, and to investigate new drug candidates for the SST_4 agonist in preclinical studies. These mice were generated by constructing a transposon vector carrying the human *hSSTR4* gene with all its expression regulatory elements and then inserting the transgene at a random site into *Sstr4* gene-deficient mice. The location of these copies in the mouse genome was identified by ligation-mediated PCR. Based on the luminescence of the luciferase reporter protein, *in vivo* imaging showed that the *hSSTR4* transgene is expressed mainly in the brain. Using RT-qPCR technique, we confirmed that the *hSSTR4* transgene is expressed in the brain and some other peripheral organs, which was consistent with the results obtained from *in vivo* imaging. By RNAscope *in situ* hybridization, we showed that the *hSSTR4* transgene is expressed in glutamatergic excitatory neurons in hippocampal CA1 and CA2, in GABAergic interneurons in the granular layer of the *bulbus olfactorius*, and in both types of neurons in primary somatosensory cortex, piriform cortex, prelimbic cortex and amygdala.

We have successfully generated human TRPA1 single and multiple mutant variants, identifying that organic polysulfides bind covalently to C621, C641 and C665 cysteines to activate the receptor. Only their combined triple mutation results in TRPA1 insensitivity to organic polysulfides, which overlaps with the general binding site of electrophilic agonists. In the transmembrane region, C727 and C834 cysteines, theoretical binding sites for highly hydrophobic electrophilic agonists, are not involved in TRPA1 activation induced by organic polysulfides. The TRPA1 mutant variants used for these experiments were generated by PCR-based site-directed mutagenesis of a plasmid vector expressing human TRPA1 cDNA. The binding properties of the mutant receptors were preliminarily investigated by *in silico* molecular docking technique. Functional changes were investigated *in vitro* by calcium-sensitive fluorescence flow cytometry, radioactive Ca-45 liquid scintillation counting and *whole-cell patch-clamp* technique.

We have successfully generated astrocyte-specific *Trpa1* cKO mice to investigate the role of astrocytes in TRPA1-mediated cuprizone-induced demyelination in a model of MS disease. These animals were generated by crossing Gfap-Cre$^{+/-}$ and *Trpa1*$^{Fl/Fl}$ mice. In a preliminary study using tdTomato reporter mice, we have shown that the Cre recombinase gene driven by the *Gfap* promoter is expressed primarily in the brain in astrocytes, but also shows activity in neurons to a small extent. We were unable to locate the *Gfap-Cre* transgene in the mouse genome. We have identified the location of loxP sequences in *Trpa1*$^{Fl/Fl}$ mice, for which we have developed a routine genotyping method. This allowed us to test Gfap-Cre$^{+/-}$ *Trpa1*$^{Fl/Fl}$ mice generated for the cuprizone-induced demyelination experiment to ensure that no *Trpa1* global KO mice were generated as a consequence of the non-astrocyte-specific activity of Cre recombinase. We measured changes in the expression levels of the intact *Trpa1* gene in the brains of Gfap-Cre$^{+/-}$ *Trpa1*$^{Fl/Fl}$ mice.

In functional animal experiments, it was shown that astrocyte-specific *Trpa1* conditional KO mice were less resistant to cuprizone-induced demyelination compared to *Trpa1* global KO mice. This suggests that astrocytes do not have a sole role in TRPA1-mediated demyelination in multiple sclerosis, but presumably other TRPA1-expressing cells in the brain (oligodendrocytes, microglia, neurons) (139,150). This requires further investigation.

Thanks to the molecular biology and genetics tools currently available, we have been able to generate all the specific model organisms required for our research. Fortunately, molecular technology continues to evolve rapidly and steadily, providing more and more opportunities for therapeutic research.

6. New results

- We have successfully created *SSTR4* humanized mice.
- We identified the location of 3 randomly inserted copies of the transgene in the generated *SSTR4* humanized mice. Routine genotyping methods were developed for these.
- We have determined the expression levels of *hSSTR4* transgenes in the brain and viscera of humanized mice.
- We have characterized the expression of the copy Chr3 of the *hSSTR4* transgene in the humanized mouse brain. Differences were found compared to *Sstr4* expression in WT mice, suggesting racial differences between homologous genes.

- Organic polysulfides bind covalently to the C621, C641 and C665 cysteines to activate the TRPA1 receptor. Only their combined triple mutation causes complete insensitivity of the TRPA1 receptor to organic polysulfides.
- The binding site of organic polysulfides overlaps with the general binding site of electrophilic agonists.
- The C727 and C834 cysteines in the transmembrane region are not part of the organic polysulfide binding site.

- By crossbreeding *Gfap-Cre* and STOP-loxP mice, we have successfully visualized the astrocyte-specific expression of Cre recombinase and, to a small extent, its transmission and activity in neurons.
- By crossbreeding *Gfap-Cre* and *Trpa1-loxP* mice, we generated transgenic mice with astrocyte-specific knockout of the mouse *Trpa1* gene.
- We have demonstrated a reduction in the expression level of the intact *Trpa1* gene in the brains of *Trpa1* cKO mice.
- Compared to *Trpa1* global KO mice, astrocyte-specific *Trpa1* cKO mice were less resistant to cuprizone-induced demyelination, suggesting that astrocyte is not the only cell type involved in this pathomechanism.

7. Bibliography

1. Raja SN, Carr DB, Cohen M, Finnerup NB, Flor H, Gibson S, et al. The Revised IASP definition of pain: concepts, challenges, and compromises. Pain. 2020 Sep 1;161(9):1976–82.
2. Pahwa R, Goyal A, Bansal P, Jialal I. Chronic Inflammatio. 2021 [cited 2022 May 16]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK493173
3. Nathan C, Ding A. Nonresolving Inflammation. Cell. 2010 Mar 19;140(6):871–82.
4. Horváth Á, Tékus V, Boros M, Pozsgai G, Botz B, Borbély É, et al. Transient receptor potential ankyrin 1 (TRPA1) receptor is involved in chronic arthritis: in vivo study using TRPA1-deficient mice. Arthritis Res Ther. 2016 Jan 8;18(1):6.
5. Schuelert N, McDougall JJ. Involvement of Nav 1.8 sodium ion channels in the transduction of mechanical pain in a rodent model of osteoarthritis. Arthritis Research & Therapy. 2012 Jan 7;14(1):R5.
6. Schett G, Gravallese E. Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. Nat Rev Rheumatol. 2012;8(11):656–64.
7. Alarcón GS. Methotrexate use in rheumatoid arthritis. A Clinician's perspective. Immunopharmacology. 2000 May;47(2–3):259–71.
8. Cavalli E, Mammana S, Nicoletti F, Bramanti P, Mazzon E. The neuropathic pain: An overview of the current treatment and future therapeutic approaches. Int J Immunopathol Pharmacol [Internet]. 2019 Mar 22 [cited 2021 Jan 4];33. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6431761/
9. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, et al. ANKTM1, a TRP-like Channel Expressed in Nociceptive Neurons, Is Activated by Cold Temperatures. Cell. 2003 Mar 21;112(6):819–29.
10. Bonet IJM, Fischer L, Parada CA, Tambeli CH. The role of transient receptor potential A 1 (TRPA1) in the development and maintenance of carrageenan-induced hyperalgesia. Neuropharmacology. 2013 Feb 1;65:206–12.
11. Liu C, Reese R, Vu S, Rougé L, Shields SD, Kakiuchi-Kiyota S, et al. A Non-covalent Ligand Reveals Biased Agonism of the TRPA1 Ion Channel. Neuron. 2021 Jan 20;109(2):273-284.e4.
12. Pozsgai G, Payrits M, Sághy É, Sebestyén-Bátai R, Steen E, Szőke É, et al. Analgesic effect of dimethyl trisulfide in mice is mediated by TRPA1 and sst4 receptors. Nitric Oxide. 2017 May 1;65:10–21.
13. Zygmunt PM, Högestätt ED. TRPA1. In: Nilius B, Flockerzi V, editors. Mammalian Transient Receptor Potential (TRP) Cation Channels: Volume I [Internet]. Berlin, Heidelberg: Springer; 2014 [cited 2022 Nov 17]. p. 583–630. (Handbook of Experimental Pharmacology). Available from: https://doi.org/10.1007/978-3-642-54215-2_23
14. Kun J, Szitter I, Kemény Á, Perkecz A, Kereskai L, Pohóczky K, et al. Upregulation of the Transient Receptor Potential Ankyrin 1 Ion Channel in the Inflamed Human and Mouse Colon and Its Protective Roles. PLOS ONE. 2014 Sep 29;9(9):e108164.
15. Dombi Á, Sánta C, Bátai IZ, Kormos V, Kecskés A, Tékus V, et al. Dimethyl Trisulfide Diminishes Traumatic Neuropathic Pain Acting on TRPA1 Receptors in Mice. International Journal of Molecular Sciences. 2021 Jan;22(7):3363.
16. Helyes Z, Pinter E, Sandor K, Elekes K, Banvolgyi A, Keszthelyi D, et al. Impaired defense mechanism against inflammation, hyperalgesia, and airway hyperreactivity in somatostatin 4 receptor gene-deleted mice. Proceedings of the National Academy of Sciences. 2009 Aug 4;106(31):13088–93.
17. Helyes Z, Pintér E, Németh J, Sándor K, Elekes K, Szabó, et al. Effects of the somatostatin receptor subtype 4 selective agonist J-2156 on sensory neuropeptide release and inflammatory reactions in rodents. British Journal of Pharmacology. 2006 Oct;149(4):405–15.
18. Markovics A, Szőke É, Sándor K, Börzsei R, Bagoly T, Kemény Á, et al. Comparison of the Anti-inflammatory and Anti-nociceptive Effects of Cortistatin-14 and Somatostatin-14 in Distinct In Vitro and In Vivo Model Systems. J Mol Neurosci. 2012 Jan;46(1):40–50.
19. Qiu C, Zeyda T, Johnson B, Hochgeschwender U, Lecea L de, Tallent MK. Somatostatin Receptor Subtype 4 Couples to the M-Current to Regulate Seizures. J Neurosci. 2008 Apr 2;28(14):3567–76.
20. Scheich B, Gaszner B, Kormos V, László K, Ádori C, Borbély É, et al. Somatostatin receptor subtype 4 activation is involved in anxiety and depression-like behavior in mouse models. Neuropharmacology. 2016 Feb;101:204–15.
21. Scheich B, Csekő K, Borbély É, Ábrahám I, Csernus V, Gaszner B, et al. Higher susceptibility of somatostatin 4 receptor gene-deleted mice to chronic stress-induced behavioral and neuroendocrine alterations. Neuroscience. 2017 Mar;346:320–36.
22. Nemes B, Bölcskei K, Kecskés A, Kormos V, Gaszner B, Aczél T, et al. Human Somatostatin SST4 Receptor Transgenic Mice: Construction and Brain Expression Pattern Characterization. International Journal of Molecular Sciences. 2021 Jan;22(7):3758.
23. Sándor K, Elekes K, Szabó Á, Pintér E, Engström M, Wurster S, et al. Analgesic effects of the somatostatin sst4 receptor selective agonist J-2156 in acute and chronic pain models. European Journal of Pharmacology. 2006 Jun;539(1–2):71–5.
24. Pintér E, Pozsgai G, Hajna Z, Helyes Z, Szolcsányi J. Neuropeptide receptors as potential drug targets in the treatment of inflammatory conditions. British Journal of Clinical Pharmacology. 2014;77(1):5–20.
25. Matsuoka N, Maeda N, Yamaguchi I, Satoh M. Possible involvement of brain somatostatin in the memory formation of rats and the cognitive enhancing action of FR121196 in passive avoidance task. Brain Research. 1994 Apr 11;642(1):11–9.
26. Baraban SC, Tallent MK. Interneuron Diversity series: Interneuronal neuropeptides – endogenous regulators of neuronal excitability. Trends in Neurosciences. 2004 Mar 1;27(3):135–42.
27. Tuboly G, Vecsei L. Somatostatin and Cognitive Function in Neurodegenerative Disorders. Mini Reviews in Medicinal Chemistry. 2013 Jan 1;13(1):34–46.
28. Martel G, Dutar P, Epelbaum J, Viollet CP. Somatostatinergic systems: an update on brain functions in normal and pathological aging. Front Endocrinol [Internet]. 2012 [cited 2020 Dec 15];3. Available from: https://www.frontiersin.org/articles/10.3389/fendo.2012.00154/full
29. Sandoval KE, Farr SA, Banks WA, Crider AM, Morley JE, Witt KA. Somatostatin receptor subtype-4 agonist NNC 26-9100 decreases extracellular and intracellular $A\beta_{1-42}$ trimers. Eur J Pharmacol. 2012 May 15;683(1–3):116–24.
30. Sandoval K, Umbaugh D, House A, Crider A, Witt K. Somatostatin Receptor Subtype-4 Regulates mRNA Expression of Amyloid-Beta Degrading Enzymes and Microglia Mediators of Phagocytosis in Brains of 3xTg-AD Mice. Neurochem Res. 2019 Nov;44(11):2670–80.

31. Lin LC, Sibille E. Somatostatin, neuronal vulnerability and behavioral emotionality. Molecular Psychiatry. 2015 Mar;20(3):377–87.
32. Engin E, Stellbrink J, Treit D, Dickson CT. Anxiolytic and antidepressant effects of intracerebroventricularly administered somatostatin: Behavioral and neurophysiological evidence. Neuroscience. 2008 0 2;157(3):666–76.
33. Lin LCMS, Sibille EPD. Reduced brain somatostatin in mood disorders: a common pathophysiological substrate and drug target? Front Pharmacol [Internet]. 2013 [cited 2020 Dec 7];4. Available from: https://www.frontiersin.org/articles/10.3389/fphar.2013.00110/full
34. Kormos V, Gaszner B. Role of neuropeptides in anxiety, stress, and depression: From animals to humans. Neuropeptides. 2013 0 1;47(6):401–19.
35. Epelbaum J. Somatostatin in the central nervous system: Physiology and pathological modifications. Progress in Neurobiology. 1986 0 1;27(1):63–100.
36. Gulyás AI, Hájos N, Katona I, Freund TF. Interneurons are the local targets of hippocampal inhibitory cells which project to the medial septum. European Journal of Neuroscience. 2003;17(9):1861–72.
37. Tomioka R, Okamoto K, Furuta T, Fujiyama F, Iwasato T, Yanagawa Y, et al. Demonstration of long-range GABAergic connections distributed throughout the mouse neocortex. European Journal of Neuroscience. 2005;21(6):1587–600.
38. Szolcsányi J. Forty years in capsaicin research for sensory pharmacology and physiology. Neuropeptides. 2004 0 1;38(6):377–84.
39. Thán M, Németh J, Szilvássy Z, Pintér E, Helyes Z, Szolcsányi J. Systemic anti-inflammatory effect of somatostatin released from capsaicin-sensitive vagal and sciatic sensory fibres of the rat and guinea-pig. European Journal of Pharmacology. 2000 Jul;399(2–3):251–8.
40. Hoyer D, Bell GI, Berelowitz M, Epelbaum J, Feniuk W, Humphrey PPA, et al. Classification and nomenclature of somatostatin receptors. Trends in Pharmacological Sciences. 1995 Mar 1;16(3):86–8.
41. Patel YC. Somatostatin and Its Receptor Family. Frontiers in Neuroendocrinology. 1999 Jul 1;20(3):157–98.
42. Thoss VS, Piwko C, Probst A, Hoyer D. Autoradiographic analysis of somatostatin SRIF1 and SRIF2 receptors in the human brain and pituitary. Naunyn Schmiedebergs Arch Pharmacol. 1997 Feb;355(2):168–76.
43. Szolcsányi J, Pintér E, Helyes Z, Oroszi G, Németh J. Systemic anti-inflammatory effect induced by counter-irritation through a local release of somatostatin from nociceptors. British Journal of Pharmacology. 1998 Oct;125(4):916–22.
44. Helyes Z, Thán M, Oroszi G, Pintér E, Németh J, Kéri G, et al. Anti-nociceptive effect induced by somatostatin released from sensory nerve terminals and by synthetic somatostatin analogues in the rat. Neuroscience Letters. 2000 Jan;278(3):185–8.
45. Szolcsanyi J, Pinter E, Helyes Z, Petho G. Inhibition of the Function of TRPV1-Expressing Nociceptive Sensory Neurons by Somatostatin 4 Receptor Agonism: echanism and Therapeutical Implications. CTMC. 2011 Sep 1;11(17):2253–63.
46. Hannon JP, Nunn C, Stolz B, Bruns C, Weckbecker G, Lewis I, et al. Drug design at peptide receptors. J Mol Neurosci. 2002 Feb 1;18(1):15–27.
47. Feytens D, Cescato R, Reubi JC, Tourwé D. New sst4/5-Selective Somatostatin Peptidomimetics Based on a Constrained Tryptophan Scaffold. J Med Chem. 2007 Jul 1;50(14):3397–401.
48. Rivier J, Erchegyi J, Hoeger C, Miller C, Low W, Wenger S, et al. Novel sst4-Selective Somatostatin (SRIF) Agonists. 1. Lead Identification Using a Betide Scan. J Med Chem. 2003 Dec 1;46(26):5579–86.
49. Gademann K, Kimmerlin T, Hoyer D, Seebach D. Peptide Folding Induces High and Selective Affinity of a Linear and Small β-Peptide to the Human Somatostatin Receptor 4. J Med Chem. 2001 Jul 1;44(15):2460–8.
50. Prasad V, Birzin ET, McVaugh CT, van Rijn RD, Rohrer SP, Chicchi G, et al. Effects of Heterocyclic Aromatic Substituents on Binding Affinities at Two Distinct Sites of Somatostatin Receptors. Correlation with the Electrostatic Potential of the Substituents. J Med Chem. 2003 May 1;46(10):1858–69.
51. https://www.lilly.com/discovery/pipeline [Internet]. 2020. Lilly.com (2020) Clinical Development Pipeline [online].
52. Markovics A, Szőke É, Sándor K, Börzsei R, Bagoly T, Kemény Á, et al. Comparison of the Anti-inflammatory and Anti-nociceptive Effects of Cortistatin-14 and Somatostatin-14 in Distinct In Vitro and In Vivo Model Systems. J Mol Neurosci. 2012 Jan 1;46(1):40–50.
53. Botz B, Bölcskei K, Helyes Z. Challenges to develop novel anti-inflammatory and analgesic drugs. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2017 May;9(3).
54. Kántás B, Börzsei R, Szőke É, Bánhegyi P, Horváth Á, Hunyady Á, et al. Novel Drug-Like Somatostatin Receptor 4 Agonists are Potential Analgesics for Neuropathic Pain. IJMS. 2019 Dec 11;20(24):6245.
55. Shenoy PA, Kuo A, Khan N, Gorham L, Nicholson JR, Corradini L, et al. The Somatostatin Receptor-4 Agonist J-2156 Alleviates Mechanical Hypersensitivity in a Rat Model of Breast Cancer Induced Bone Pain. Front Pharmacol. 2018 May 15;9:495.
56. Park TSW, Khan N, Kuo A, Nicholson JR, Corradini L, Smith MT. J-2156, a somatostatin receptor type 4 agonist, alleviates mechanical hyperalgesia in a rat model of chronic low back pain. Biomed Pharmacother. 2019 Sep;117:109056.
57. Szőke É, Bálint M, Hetényi C, Markovics A, Elekes K, Pozsgai G, et al. Small molecule somatostatin receptor subtype 4 (sst4) agonists are novel anti-inflammatory and analgesic drug candidates. Neuropharmacology. 2020 0 1;178:108198.
58. Liu Z, Crider AM, Ansbro D, Hayes C, Kontoyianni M. A Structure-Based Approach to Understanding Somatostatin Receptor-4 Agonism (sst4). J Chem Inf Model. 2012 Jan 23;52(1):171–86.
59. Henderson CJ, Kapelyukh Y, Scheer N, Rode A, McLaren A, MacLeod AK, et al. An Extensively Humanized Mouse Model to Predict Pathways of Drug Disposition and Drug/Drug Interactions, and to Facilitate Design of Clinical Trials. Drug Metab Dispos. 2019 Jun 1;47(6):601–15.
60. Ueda O, Tateishi H, Higuchi Y, Fujii E, Kato A, Kawase Y, et al. Novel genetically-humanized mouse model established to evaluate efficacy of therapeutic agents to human interleukin-6 receptor. Scientific Reports. 2013 Feb 1;3(1):1196.
61. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. Nat Rev Immunol. 2007 Feb;7(2):118–30.
62. Manz MG, Di Santo JP. Renaissance for mouse models of human hematopoiesis and immunobiology. Nat Immunol. 2009 Oct;10(10):1039–42.
63. Pearson T, Greiner DL, Shultz LD. Humanized SCID mouse models for biomedical research. Curr Top Microbiol Immunol. 2008;324:25–51.

64. Ito M, Kobayashi K, Nakahata T. NOD/Shi-scid IL2rgamma(null) (NOG) mice more appropriate for humanized mouse models. Curr Top Microbiol Immunol. 2008;324:53–76.
65. Legrand N, Weijer K, Spits H. Experimental models to study development and function of the human immune system in vivo. J Immunol. 2006 Feb 15;176(4):2053–8.
66. Zhang B, Duan Z, Zhao Y. Mouse models with human immunity and their application in biomedical research. J Cell Mol Med. 2009 Jun;13(6):1043–58.
67. Devoy A, Bunton-Stasyshyn RKA, Tybulewicz VLJ, Smith AJH, Fisher EMC. Genomically humanized mice: technologies and promises. Nature Reviews Genetics. 2012 Jan;13(1):14–20.
68. Zhu F, Nair RR, Fisher EMC, Cunningham TJ. Humanising the mouse genome piece by piece. Nature Communications. 2019 Apr 23;10(1):1845.
69. Lampreht Tratar U, Horvat S, Cemazar M. Transgenic Mouse Models in Cancer Research. Front Oncol [Internet]. 2018 [cited 2021 Feb 18];8. Available from: https://www.frontiersin.org/articles/10.3389/fonc.2018.00268/full
70. Moriwaki T, Abe S, Oshimura M, Kazuki Y. Transchromosomic technology for genomically humanized animals. Experimental Cell Research. 2020 May 15;390(2):111914.
71. Fox A, Kaur S, Li B, Panesar M, Saha U, Davis C, et al. Antihyperalgesic activity of a novel nonpeptide bradykinin B1 receptor antagonist in transgenic mice expressing the human B1 receptor. Br J Pharmacol. 2005 Apr;144(7):889–99.
72. Jackson IJ, Budd PS, Keighren M, McKie L. Humanized MC1R transgenic mice reveal human specific receptor function. Human Molecular Genetics. 2007 Oct 1;16(19):2341–8.
73. Kecskés A, Pohóczky K, Kecskés M, Varga ZV, Kormos V, Szőke É, et al. Characterization of Neurons Expressing the Novel Analgesic Drug Target Somatostatin Receptor 4 in Mouse and Human Brains. International Journal of Molecular Sciences. 2020 Jan;21(20):7788.
74. Scheer N, Snaith M, Wolf CR, Seibler J. Generation and utility of genetically humanized mouse models. Drug Discovery Today. 2013 0 1;18(23):1200–11.
75. Davis J, Maillet M, Miano JM, Molkentin JD. Lost in Transgenesis: A Users guide for Genetically Manipulating the Mouse in Cardiac Research. Circ Res. 2012 Aug 31;111(6):761–77.
76. Bryda EC, Pearson M, Agca Y, Bauer BA. Method for detection and identification of multiple chromosomal integration sites in transgenic animals created with lentivirus. BioTechniques. 2006 Dec;41(6):715–9.
77. Caron P, Buscail L, Beckers A, Estève JP, Igout A, Hennen G, et al. Expression of Somatostatin Receptor SST4 in Human Placenta and Absence of Octreotide Effect on Human Placental Growth Hormone Concentration during Pregnancy1. The Journal of Clinical Endocrinology & Metabolism. 1997 0 1;82(11):3771–6.
78. Møller LN, Stidsen CE, Hartmann B, Holst JJ. Somatostatin receptors. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2003 Sep 22;1616(1):1–84.
79. Schwabe W, Brennan MB, Hochgeschwender U. Isolation and characterization of the mouse (Mus musculus) somatostatin receptor type-4-encoding gene (mSSTR4). Gene. 1996 0 1;168(2):233–5.
80. Regard JB, Sato IT, Coughlin SR. Anatomical profiling of G protein-coupled receptor expression. Cell. 2008 Oct 31;135(3):561–71.
81. SSTR4 protein expression summary - The Human Protein Atlas [Internet]. [cited 2021 Jan 21]. Available from: https://www.proteinatlas.org/ENSG00000132671-SSTR4
82. Sstr4 MGI Mouse Gene Detail - MGI:105372 - somatostatin receptor 4 [Internet]. [cited 2021 Jan 21]. Available from: http://www.informatics.jax.org/marker/MGI:105372
83. Sstr4 RT-PCR Gene Expression Assay - GXD [Internet]. [cited 2021 Jan 21]. Available from: http://www.informatics.jax.org/assay/MGI:1204215
84. Sstr4 RT-PCR Gene Expression Assay - GXD [Internet]. [cited 2021 Jan 21]. Available from: http://www.informatics.jax.org/assay/MGI:1204217
85. Sstr4 RT-PCR Gene Expression Assay - GXD [Internet]. [cited 2021 Jan 21]. Available from: http://www.informatics.jax.org/assay/MGI:1204442
86. Teschendorf C, Warrington KH, Siemann DW, Muzyczka N. Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus. Anticancer Res. 2002 Dec;22(6A):3325–30.
87. Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol. 2004 Dec;22(12):1567–72.
88. Lauf U, Lopez P, Falk MM. Expression of fluorescently tagged connexins: a novel approach to rescue function of oligomeric DsRed-tagged proteins1. FEBS Letters. 2001;498(1):11–5.
89. Palmer E, Freeman T. Investigation Into the use of C- and N-terminal GFP Fusion Proteins for Subcellular Localization Studies Using Reverse Transfection Microarrays. Comparative and Functional Genomics. 2004;5(4):342–53.
90. Nocera S, Simon A, Fiquet O, Chen Y, Gascuel J, Datiche F, et al. Somatostatin Serves a Modulatory Role in the Mouse Olfactory Bulb: Neuroanatomical and Behavioral Evidence. Front Behav Neurosci [Internet]. 2019 [cited 2021 Jan 18];13. Available from: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00061/full
91. Mha AN, Peleg R, Singer Y, Sherf M, Shvartzman P. Chronic Pain: A Population-Based Study. 2008;10:5.
92. Apkarian AV, Baliki MN, Geha PY. Towards a theory of chronic pain. Progress in Neurobiology. 2009 Feb 1;87(2):81–97.
93. Harden RN, Bruehl S, Perez RSGM, Birklein F, Marinus J, Maihofner C, et al. Validation of proposed diagnostic criteria (the "Budapest Criteria") for Complex Regional Pain Syndrome. Pain. 2010 Aug;150(2):268–74.
94. Gaborit M, Massotte D. Therapeutic potential of opioid receptor heteromers in chronic pain and associated comorbidities. British Journal of Pharmacology. 2023;180(7):994–1013.
95. Réthelyi JM, Berghammer R, Kopp MS. Comorbidity of pain-associated disability and depressive symptoms in connection with sociodemographic variables: results from a cross-sectional epidemiological survey in Hungary. Pain. 2001 Aug 1;93(2):115–21.
96. Keeley P, Creed F, Tomenson B, Todd C, Borglin G, Dickens C. Psychosocial predictors of health-related quality of life and health service utilisation in people with chronic low back pain. PAIN®. 2008 Mar 1;135(1):142–50.
97. Niv D, Kreitler S. Pain and quality of life. Pain Pract. 2001 Jun;1(2):150–61.

98. Helyes Z, Tékus V, Szentes N, Pohóczky K, Botz B, Kiss T, et al. Transfer of complex regional pain syndrome to mice via human autoantibodies is mediated by interleukin-1–induced mechanisms. Proceedings of the National Academy of Sciences. 2019 Jun 25;116(26):13067–76.
99. Nathan C. Nonresolving inflammation redux. Immunity. 2022 Apr 12;55(4):592–605.
100. Medzhitov R. The spectrum of inflammatory responses. Science. 2021 Nov 26;374(6571):1070–5.
101. Bátai IZ, Sár CP, Horváth Á, Borbély É, Bölcskei K, Kemény Á, et al. TRPA1 Ion Channel Determines Beneficial and Detrimental Effects of GYY4137 in Murine Serum-Transfer Arthritis. Front Pharmacol [Internet]. 2019 [cited 2021 Jun 12];10. Available from: https://www.frontiersin.org/articles/10.3389/fphar.2019.00964/full?utm_source=S-TWT&utm_medium=SNET&utm_campaign=ECO_FPHAR_XXXXXXXX_auto-dlvrit
102. Hatakeyama Y, Takahashi K, Tominaga M, Kimura H, Ohta T. Polysulfide Evokes Acute Pain through the Activation of Nociceptive TRPA1 in Mouse Sensory Neurons. Mol Pain. 2015 Jan 1;11:s12990-015-0023–4.
103. Ogawa H, Takahashi K, Miura S, Imagawa T, Saito S, Tominaga M, et al. H(2)S functions as a nociceptive messenger through transient receptor potential ankyrin 1 (TRPA1) activation. Neuroscience. 2012;218:335–43.
104. Bátai IZ, Horváth Á, Pintér E, Helyes Z, Pozsgai G. Role of Transient Receptor Potential Ankyrin 1 Ion Channel and Somatostatin sst4 Receptor in the Antinociceptive and Anti-inflammatory Effects of Sodium Polysulfide and Dimethyl Trisulfide. Frontiers in Endocrinology [Internet]. 2018 [cited 2022 May 21];9. Available from: https://www.frontiersin.org/article/10.3389/fendo.2018.00055
105. Koizumi K, Iwasaki Y, Narukawa M, Iitsuka Y, Fukao T, Seki T, et al. Diallyl sulfides in garlic activate both TRPA1 and TRPV1. Biochemical and Biophysical Research Communications. 2009 May 8;382(3):545–8.
106. Bautista DM, Movahed P, Hinman A, Axelsson HE, Sterner O, Högestätt ED, et al. Pungent products from garlic activate the sensory ion channel TRPA1. Proceedings of the National Academy of Sciences. 2005 Aug 23;102(34):12248–52.
107. Bai AP, Ouyang Q, Hu RW. Diallyl Trisulfide Inhibits Tumor Necrosis Factor-α Expression in Inflammed Mucosa of Ulcerative Colitis. Dig Dis Sci. 2005 Aug 1;50(8):1426–31.
108. Lee HJ, Lee HG, Choi KS, Surh YJ, Na HK. Diallyl trisulfide suppresses dextran sodium sulfate-induced mouse colitis: NF-κB and STAT3 as potential targets. Biochem Biophys Res Commun. 2013 Jul 26;437(2):267–73.
109. Hinman A, Chuang H hu, Bautista DM, Julius D. TRP channel activation by reversible covalent modification. PNAS. 2006 Dec 19;103(51):19564–8.
110. Eberhardt MJ, Filipovic MR, Leffler A, de la Roche J, Kistner K, Fischer MJ, et al. Methylglyoxal Activates Nociceptors through Transient Receptor Potential Channel A1 (TRPA1). J Biol Chem. 2012 Aug 17;287(34):28291–306.
111. Deering-Rice CE, Romero EG, Shapiro D, Hughen RW, Light AR, Yost GS, et al. Electrophilic Components of Diesel Exhaust Particles (DEP) Activate Transient Receptor Potential Ankyrin-1 (TRPA1): A Probable Mechanism of Acute Pulmonary Toxicity for DEP. Chem Res Toxicol. 2011 Jun 20;24(6):950–9.
112. Shapiro D, Deering-Rice CE, Romero EG, Hughen RW, Light AR, Veranth JM, et al. Activation of Transient Receptor Potential Ankyrin-1 (TRPA1) in Lung Cells by Wood Smoke Particulate Material. Chem Res Toxicol. 2013 May 20;26(5):750–8.
113. Paulsen CE, Armache JP, Gao Y, Cheng Y, Julius D. Structure of the TRPA1 ion channel suggests regulatory mechanisms. Nature. 2015 Apr;520(7548):511–7.
114. Alvarado MG, Thakore P, Earley S. Transient Receptor Potential Channel Ankyrin 1: A Unique Regulator of Vascular Function. Cells. 2021 May;10(5):1167.
115. Kimura H. Signaling Molecules: Hydrogen Sulfide and Polysulfide. Antioxid Redox Signal. 2015 Feb 10;22(5):362–76.
116. Terada Y, Hosono T, Seki T, Ariga T, Ito S, Narukawa M, et al. Sulfur-containing compounds of durian activate the thermogenesis-inducing receptors TRPA1 and TRPV1. Food Chemistry. 2014 Aug 15;157:213–20.
117. Pozsgai G, Bátai IZ, Pintér E. Effects of sulfide and polysulfides transmitted by direct or signal transduction-mediated activation of TRPA1 channels. Br J Pharmacol. 2019 Feb;176(4):628–45.
118. Bátai IZ, Dombi Á, Borbély É, Fehér Á, Papp F, Varga Z, et al. Investigation of the Role of the TRPA1 Ion Channel in Conveying the Effect of Dimethyl Trisulfide on Vascular and Histological Changes in Serum-Transfer Arthritis. Pharmaceuticals. 2022 Jun;15(6):671.
119. Mahajan N, Khare P, Kondepudi KK, Bishnoi M. TRPA1: Pharmacology, natural activators and role in obesity prevention. European Journal of Pharmacology. 2021 Dec 5;912:174553.
120. Zhao J, Lin King JV, Paulsen CE, Cheng Y, Julius D. Irritant-evoked activation and calcium modulation of the TRPA1 receptor. Nature. 2020 Sep;585(7823):141–5.
121. Zsidó BZ, Börzsei R, Pintér E, Hetényi C. Prerequisite Binding Modes Determine the Dynamics of Action of Covalent Agonists of Ion Channel TRPA1. Pharmaceuticals. 2021 Oct;14(10):988.
122. Suo Y, Wang Z, Zubcevic L, Hsu AL, He Q, Borgnia MJ, et al. Structural Insights into Electrophile Irritant Sensing by the Human TRPA1 Channel. Neuron. 2020 Mar 4;105(5):882-894.e5.
123. Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF, et al. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. Nature. 2007 Feb 1;445(7127):541–5.
124. Bahia PK, Parks TA, Stanford KR, Mitchell DA, Varma S, Stevens SM, et al. The exceptionally high reactivity of Cys 621 is critical for electrophilic activation of the sensory nerve ion channel TRPA1. Journal of General Physiology. 2016 Jun 1;147(6):451–65.
125. Habgood M, Seiferth D, Zaki AM, Alibay I, Biggin PC. Atomistic mechanisms of human TRPA1 activation by electrophile irritants through molecular dynamics simulation and mutual information analysis. Sci Rep. 2022 Mar 23;12(1):4929.
126. Wang J, Wolf RM, Caldwell JW, Kollman PA, Case DA. Development and testing of a general amber force field. J Comput Chem. 2004 Jul 15;25(9):1157–74.
127. Takaya J, Mio K, Shiraishi T, Kurokawa T, Otsuka S, Mori Y, et al. A Potent and Site-Selective Agonist of TRPA1. J Am Chem Soc. 2015 Dec 23;137(50):15859–64.
128. Matsubara M, Muraki Y, Hatano N, Suzuki H, Muraki K. Potent Activation of Human but Not Mouse TRPA1 by JT010. International Journal of Molecular Sciences. 2022 Jan;23(22):14297.
129. Fujita F, Uchida K, Moriyama T, Shima A, Shibasaki K, Inada H, et al. Intracellular alkalization causes pain sensation through activation of TRPA1 in mice. J Clin Invest. 2008 Dec 1;118(12):4049–57.

130. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med. 2000 Sep 28;343(13):938–52.
131. Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol. 2001;11(1):107–16.
132. Hiremath MM, Saito Y, Knapp GW, Ting JPY, Suzuki K, Matsushima GK. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. Journal of Neuroimmunology. 1998 Dec 1;92(1):38–49.
133. Sághy É, Sipos É, Ács P, Bölcskei K, Pohóczky K, Kemény Á, et al. TRPA1 deficiency is protective in cuprizone-induced demyelination—A new target against oligodendrocyte apoptosis. Glia. 2016;64(12):2166–80.
134. Bölcskei K, Kriszta G, Sághy É, Payrits M, Sipos É, Vranesics A, et al. Behavioural alterations and morphological changes are attenuated by the lack of TRPA1 receptors in the cuprizone-induced demyelination model in mice. Journal of Neuroimmunology. 2018 Jul 15;320:1–10.
135. Nilius B, Appendino G, Owsianik G. The transient receptor potential channel TRPA1: from gene to pathophysiology. Pflugers Arch. 2012;464(5):425–58.
136. Chen J, Hackos DH. TRPA1 as a drug target--promise and challenges. Naunyn Schmiedebergs Arch Pharmacol. 2015 Apr;388(4):451–63.
137. Talavera K, Startek JB, Alvarez-Collazo J, Boonen B, Alpizar YA, Sanchez A, et al. Mammalian Transient Receptor Potential TRPA1 Channels: From Structure to Disease. Physiol Rev. 2020 Apr 1;100(2):725–803.
138. Fernandes E, Fernandes M, Keeble J. The functions of TRPA1 and TRPV1: moving away from sensory nerves. British Journal of Pharmacology. 2012;166(2):510–21.
139. Hamilton NB, Kolodziejczyk K, Kougioumtzidou E, Attwell D. Proton-gated Ca2+-permeable TRP channels damage myelin in conditions mimicking ischaemia. Nature. 2016 Jan;529(7587):523–7.
140. Shigetomi E, Jackson-Weaver O, Huckstepp RT, O'Dell TJ, Khakh BS. TRPA1 Channels Are Regulators of Astrocyte Basal Calcium Levels and Long-Term Potentiation via Constitutive d-Serine Release. J Neurosci. 2013 Jun 12;33(24):10143–53.
141. Shigetomi E, Tong X, Kwan KY, Corey DP, Khakh BS. TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3. Nat Neurosci. 2012 Jan;15(1):70–80.
142. Verkhratsky A, Reyes RC, Parpura V. TRP Channels Coordinate Ion Signalling in Astroglia. In: Nilius B, Gudermann T, Jahn R, Lill R, Offermanns S, Petersen OH, editors. Reviews of Physiology, Biochemistry and Pharmacology 166 [Internet]. Cham: Springer International Publishing; 2014 [cited 2023 May 5]. p. 1–22. (Reviews of Physiology, Biochemistry and Pharmacology). Available from: https://doi.org/10.1007/112_2013_15
143. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. J Neurosci. 2014 Sep 3;34(36):11929–47.
144. Ben Haim L, Carrillo-de Sauvage MA, Ceyzériat K, Escartin C. Elusive roles for reactive astrocytes in neurodegenerative diseases. Frontiers in Cellular Neuroscience [Internet]. 2015 [cited 2023 May 5];9. Available from: https://www.frontiersin.org/articles/10.3389/fncel.2015.00278
145. Li K, Li J, Zheng J, Qin S. Reactive Astrocytes in Neurodegenerative Diseases. Aging and disease. 2019 Jun 1;10(3):664–75.
146. Hill S, Blaeser A, Coley A, Xie Y, Shepard K, Harwell C, et al. Sonic hedgehog signaling in astrocytes mediates cell-type-specific synaptic organization. 2019.
147. Park YM, Chun H, Shin JI, Lee CJ. Astrocyte Specificity and Coverage of hGFAP-CreERT2 [Tg(GFAP-Cre/ERT2)13Kdmc] Mouse Line in Various Brain Regions. Exp Neurobiol. 2018 Dec;27(6):508–25.
148. Casper KB, McCarthy KD. GFAP-positive progenitor cells produce neurons and oligodendrocytes throughout the CNS. Molecular and Cellular Neuroscience. 2006 Apr 1;31(4):676–84.
149. Kriszta G, Nemes B, Sándor Z, Ács P, Komoly S, Berente Z, et al. Investigation of Cuprizone-Induced Demyelination in mGFAP-Driven Conditional Transient Receptor Potential Ankyrin 1 (TRPA1) Receptor Knockout Mice. Cells. 2020 Jan;9(1):81.
150. Xia M, Chen W, Wang J, Yin Y, Guo C, Li C, et al. TRPA1 Activation-Induced Myelin Degradation Plays a Key Role in Motor Dysfunction After Intracerebral Hemorrhage. Frontiers in Molecular Neuroscience [Internet]. 2019 [cited 2023 May 5];12. Available from: https://www.frontiersin.org/articles/10.3389/fnmol.2019.00098

8. Publications on which the thesis is based

Balázs Nemes, Kata Bölcskei, Angéla Kecskés, Viktória Kormos, Balázs Gaszner, Timea Aczél, Dániel Hegedüs, Erika Pintér, Zsuzsanna Helyes and Zoltán Sándor: "Human Somatostatin SST4 Receptor Transgenic Mice: Construction and Brain Expression Pattern Characterization". International Journal of Molecular Sciences, vol. 22, no. 7 (January 2021): 3758.
https://doi.org/10.3390/ijms22073758

Balázs Nemes, Szabolcs László, Zoltán Zsidó Balázs, Csaba Hetényi, Ádám Fehér, Ferenc Papp, Zoltán Varga, Éva Szőke, Zoltán Sándor, Erika Pintér: "Elucidation of the binding mode of organic polysulfides on the human TRPA1 receptor". Frontiers in Physiology: Insights in Redox Physiology: 2022, vol. 14 (June 2023).
https://doi.org/10.3389/fphys.2023.1180896

Gábor Kriszta, **Balázs Nemes**, Zoltán Sándor, Péter Ács, Sámuel Komoly, Zoltán Berente, Kata Bölcskei and Erika: Pintér "Investigation of Cuprizone-Induced Demyelination in MGFAP-Driven Conditional Transient Receptor Potential Ankyrin 1 (TRPA1) Receptor Knockout Mice". Cells, bound. 9, no. 1 (January 2020): 81.
https://doi.org/10.3390/cells9010081

9. Other publications and conference participations

9.1. Other publications

Zoárd István Bátai, Cecília Pápainé Sár, Ádám Horváth, Éva Borbély, Kata Bölcskei, Ágnes Kemény, Zoltán Sándor, **Balázs Nemes**, Zsuzsanna Helyes, Anikó Perkecz, Attila Mócsai, Gábor Pozsgai and Erika Pintér: "TRPA1 Ion Channel Determines Beneficial and Detrimental Effects of GYY4137 in Murine Serum-Transfer Arthritis". Frontiers in Pharmacology 10 (2019).
https://doi.org/10.3389/fphar.2019.00964.

Erika Pintér, Kata Böcskei, Gábor Kriszta, Zoltán Sándor, **Balázs Nemes**, Péter Ács, Sámuel Komoly, Zoltán Berente: "Examination of the demyelination process in mGFAP-driven conditional transient receptor potential ankyrin 1 (TRPA1) receptor knockout mice". The FASEB Journal, vol. 34, no. S1 (2020).
https://doi.org/10.1096/fasebj.2020.34.s1.04614

9.2. Participation in national and international conferences

III. University of Pécs Centre for Neuroscience PhD and TDK conference, Pécs

2018.11.22-23.

Presentation title: generation of humanized somatostatin receptor 4 (hSSTR4) transgenic mice for neuroscience research

Presenter: Balázs Nemes

Thesis Supervisors: Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

MITT, Hungarian Neuroscience Society Conference 2019, Debrecen

2019.01.17-18.

Poster presentation title: Transgenic mice expressing human somatostatin receptor 4 (hSSTR4): A humanized model for pharmacological research

Balázs Nemes, Kata Bölcskei, Timea Aczél, Adnan Ahmad Alkurdi, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

Remedicon, Pharmaceutical Innovation 2019 Conference, Gárdony

2019.04.01-03.

Poster presentation entitled: Transgenic mice expressing humanized somatostatin receptor 4: A new model for translational medicine

Balázs Nemes, Kata Bölcskei, Timea Aczél, Adnan Ahmad Alkurdi, András Dinnyés, Julianna Kobolák, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

1st Pécs-Osijek Ph.D. Symposium, Pécs

2019.05.10.

Poster presentation title: Human somatostatin receptor 4 expressing transgenic mice generation for pharmacological research

Balázs Nemes, Kata Bölcskei, Timea Aczél, Adnan Ahmad Alkurdi, András Dinnyés, Julianna Kobolák, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

FAMÉ 2019 - MÉT, MFT, MAT, MMVBT joint meeting, Budapest

2019.06.05-08.

Poster presentation entitled: Humanized somatostatin receptor 4 expressing transgenic mice: a new model for translational medicine

Balázs Nemes, Kata Bölcskei, Timea Aczél, Adnan Ahmad Alkurdi, András Dinnyés, Julianna Kobolák, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

7th Summer School on Stress, St Petersburg

2019.06.25-28.

Poster presentation title: Novel humanized model for pharmacological research: generating human somatostatin receptor 4 (hSSTR4) expressing transgenic mice

Balázs Nemes, Kata Bölcskei, Timea Aczél, Adnan Ahmad Alkurdi, Yazan Abuawwad, András Dinnyés, Julianna Kobolák, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

FENS Regional Meeting 2019, Belgrade

2019.07.10-13.

Presentation and poster presentation entitled: Generating human somatostatin receptor 4 (hSSTR4) espressing transgenic mice for pharmacological research

Balázs Nemes, Kata Bölcskei, Timea Aczél, Adnan Ahmad Alkurdi, Yazan Abuawwad, András Dinnyés, Julianna Kobolák, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

MOFT conference 2019, Szeged

2019.11.08-09.

Poster presentation entitled: Humanized somatostatin receptor 4 expressing mice: a novel animal model for the development of somatostatin analogue drugs

Balázs Nemes, Kata Bölcskei, Timea Aczél, Adnan Ahmad Alkurdi, Yazan Abuawwad, András Dinnyés, Julianna Kobolák, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

ISCTICO-HUPHAR-IUPHAR 2021, Pécs

2021.10.27-30.

Poster presentation title: Characterization of transgenic mice expressing the human somatostatin receptor subtype 4

Balázs Nemes, Kata Bölcskei, Angéla Kecskés, Timea Aczél, Adnan Ahmad Alkurdi, Yazan Abuawwad, Erika Pintér, Zsuzsanna Helyes, Zoltán Sándor

MOFT conference 2021, Szeged

2021.11.05-06.

Poster presentation title: Site-specific mutagenesis of TRPA1 receptor binding sites for organic polysulfides

Balázs Nemes, Dr. Ádám Fehér, Dr. Ferenc Papp, Dr. Zoltán Sándor, Dr. Gábor Pozsgai, Prof. Dr. Erika Pintér

MOFT conference 2022, Szeged

2022.11.04-05.

Presentation title: identification of organic polysulfide binding sites on the human TRPA1 receptor for drug development

Balázs Nemes, Dr. Balázs Zsidó, Dr. Csaba Hetényi, Dr. Ádám Fehér, Dr. Ferenc Papp, Dr. Éva Szőke, Dr. Zoltán Sándor, Prof. Dr. Erika Pintér

10. Professional achievements

Number of publications: **5**

Hirsch index (MTMT): **3**

i10 index (Google Scholar): **3**

Impact factor of the publications the thesis is based on: **16.808**

Impact factor of all publications: **21.033**

Number of independent citations (MTMT): **18**

Total number of citations (MTMT): **27**

Total citations (Google Scholar): **39**

11. Professional awards

- 2017-2021. I was awarded the Richter Gedeon Talent Foundation Study Scholarship.
- 10-13.07.2019. I was invited to give a presentation about the subject of my poster at the international FENS conference in Belgrade.
- 25-28.06.2020 I was invited to St. Petersburg for the international "Summer School of Stress" program and to visit the Pavlov Research Institute and Museum in Koltushi.
- 2021-2022. I have been awarded the PhD+1 scholarship of the Medical School, University of Pécs.
- 12.04.2023 I won 2nd place in the Hungarian Pain Society's competition with my poster, and thus won the grant for the upcoming international EFIC conference (2023.09.20-23.).

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