THE EFFECT OF DMTS IN SOMATIC AND NEUROPATHIC PAIN MODELS

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INTRODUCTION

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage". Pain can be divided into sensory and emotional components, resulting from perceived or actual tissue damage. Sensory pain can be nociceptive, usually caused by some kind of tissue injury [1]; and neuropathic, which is caused by injury or disease of the somatosensory nervous system [2]. Nociceptive pain can be somatic and can be visceral, accompanying a disease of internal organs. Whatever the type of pain, its long-term persistence leads to a deterioration in quality of life.

LITERATURE REVIEW

1. Transient Receptor Potential (TRP) channels

The transient receptor potential gene family encodes integral membrane proteins that act as ion channels. The proteins show structural similarity to a photosensitive receptor involved in phototransduction described in *Drosophila*, and this topographical similarity is the reason for their inclusion in the TRP family, but most of them do not show other similarities to transient receptor potential. TRP channels are found in almost all tissues in most eukaryotic species, from yeasts to vertebrates. Since most of them are expressed on both excitable and non-excitable cells, they are involved in a wide range of physiological processes. They play a role in sensing various stimuli (temperature, pain, chemical, mechanical, electrical), muscle contraction, inflammation and the regulation of ionic balance, among others. Their signalling is often G-protein coupled and bound to growth factor receptors and phospholipase C (PLC) [3].

2. Transient Receptor Potential Ankirin 1

TRPA is the only TRP subfamily to which only one cation channel has been classified. The gene encoding the channel was originally cloned from lung fibroblast cells in 1999 [4]. The team identified a gene encoding a protein of 1119 amino acids on human chromosome 8, containing two distinct domains. They found that the N-terminal domain of the protein consists of 18 segments, each of which has an ankyrin repeat domain. The gene encoding the protein was named ANKTM1. Later, after further studies, it was discovered that the protein shares a high degree of similarity with TRP channels, and was classified as one of them after more precise structural identification.

3. Occurrence of Transient Receptor Potential Ankirin 1

TRPA1 was first identified as a nociceptive channel sensitive to noxious cold and pungent compounds, expressed in dorsal root ganglia (DRG) and trigeminal ganglia (TG) [5]. Studies since then have elucidated the structure and function of the receptor and the ion channel in more detail. These studies have revealed that TRPA1 is expressed over a very wide range of cell types and is found in the membranes of a large number of cell types as well as in the membranes of various cellular components.

4. Function of neuronal TRPA1 ion channels

TRPA1 ion channels are multimodal, non-selective cation channels expressed mainly on primary sensory nociceptors, CNS neurons and glia. Activation of TRPA1 and/or TRPV1 at peripheral nerve terminals leads to Na^+ and Ca^{2+} influx. Na^+ influx results in an action potential when the activation threshold is reached. Activation of ion channels at nerve endings leads to the sensation of pain, nociception. At the same time, neuropeptides (e.g. calcitonin gene-related peptide, CGRP; substance P, neurokinin A and neurokinin B) are released locally [6]. The resulting proinflammatory mediators cause a strong vasodilatation of the arterioles, an outflow of plasma proteins from the venules and the activation of inflammatory cells. All this leads to local inflammation. Proinflammatory and algogenic mediators (e.g. serotonin, bradykinin) and some lipid peroxidation products (e.g. 4-hydroxyhexanal, 4-hydroxynonenal, 4-oxononenal) released from injured or inflamed tissue can regulate the function of the TRPA1 channel through PLC, PKA and PKC pathways by phosphorylating it [7].

These ion channels, like other channels expressed in peptidergic sensory neurons, also have a systemic efferent function [6]. In addition to the release of inflammatory neuropeptides responsible for local efferent function, ion channel activation leads to the release of anti-inflammatory peptides (e.g. somatostatin). When released into the circulation anti-inflammatory peptides induce systemic anti-inflammatory and analgesic effects [8].

5. Function of non-neuronal TRPA1 ion channels

In the brainstem, TRPA1 is expressed in the visceral afferent pathway and regulates glutamate release [9]. In astrocytes, which contribute to the formation and function of synapses, TRPA1 expression may be associated with the regulation of inhibitory synapses. TRPA1 is also important in the inner ear, where it is expressed in the stria vascularis, the organ of Corti and the outer and inner hair cells of the cochlea (OHC and IHC) and its unique ankyrin repeat domain structure suggests that it is likely to function as a mechanosensitive channel [10-12].

In vascular endothelial cells, TRPA1 function is associated with endothelium-derived hyperpolarizing factor (EDHF) responses [13,14]. Experiments on its role in the cardiovascular system have found that TRPA1 agonists ingested with food can cause arterial dilatation via two different pathways. Expression of TRPA1 in the pancreas has been demonstrated in rat pancreatic islets of Langerhans [15]. The TRPA1 agonist allyl isothiocyanate and 15-deoxy-Δ-prostaglandin J2 (15dPGJ2) [16,17] significantly enhanced Ca^{2+} influx in β cells. The expression in β cells was also confirmed by specific TRPA1 antagonists, which were shown to block the effects induced by electrophilic activation [18]. TRPA1 also plays an important role in the gastrointestinal system. In the mucosa of the small intestine and colon, TRPA1 probably acts as a chemosensor in cooperation with the intestinal odorant receptors (ORs). TRPA1 is also highly expressed in most non-neuronal cell types that make up the respiratory system. It plays a fundamental role not only in normal airway function but also in modulating disease. In non-neuronal skin cells, such as human keratinocytes and fibroblasts, TRPA1 activation mediates the secretion pattern of eicosanoids such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) and induces prolonged local erythema [19]. In melanocytes, TRPA1 affects the expression of proinflammatory cytokines, including interleukin-1α and β (IL-1α, IL-1β) [20]. In human teeth, TRPA1 is highly expressed in dental pulp fibroblasts, where it may be involved in mechanotransduction-related cold responses and pain [21].

6. Agonist

In general, agonists of the TRPA1 channel can be classified into a large group of thiol-reactive electrophiles that covalently modify the channel; and a group of non-electrophilic compounds that can cause channel activation through non-covalent modifications.

6.1. Electrophil agonists

This group of agonists includes a number of compounds that are also found in everyday spices and herbs. These include allicin in garlic, which is converted to alliin by the enzyme alliinase. Alliin is an odorless and highly unstable compound containing a thiosulfinate group, from which various di-allyl sulfides (DAS), diallyl disulfides (DADS) and diallyl trisulfides (DATS) are formed. Industrial chemicals and volatile irritants, e.g. acrolein and croton aldehyde [22,23]; hydrogen peroxide [24]; various anesthetics, e.g. isoflurane [25], lidocaine [26], propofol [27]; laboratory chemicals, e.g. formalin [28]; and endogenous activators, oxidative stress components, e.g. nitric oxide (NO) [29] also act as agonists. These agonist agents covalently modify the cysteine and lysine side chains on the amino-terminal domain of TRPA1.

6.2. Non-electrophilic agonists

This group of agonists does not exert their activating effect by covalently modifying cysteine side chains. As with the electrophilic agonist group, a number of plant constituents and derivatives can be included. Perhaps the best known of these are menthol [30] and the structurally very similar thymol [31]. The group of non-electrophilic agonists also includes carvacrol [32], found in oregano, thyme and cloves, and a number of other compounds.

7. Gasotransmitters (H_2S and NO)

All three gasotransmitters (CO, H2S, NO) are synthesized in vivo and act as neuromodulators in the body. The potential biological effect of H_2S on capsaicin-sensitive sensory neurons was first described in 1990 in a rat lung injury model [33]. Later, the sulfide donor sodium hydrogen sulfide was identified as an activator of capsaicin-sensitive neurons in isolated rat bladder [34]. Combining the results, Patacchini and colleagues concluded that H_2S acts either on TRPV1 or on a previously unidentified ion channel coexpressed with it on sensory neurons [34]. A few years later, the expression of TRPA1 in the rat bladder was examined by fluorescence immunohistochemistry and real-time PCR and TRPA1 was found to be immunoreactive in the urothelium, suburothelial space and muscle layer, and around blood vessels on unmyelinated nerve fibres of the bladder. Using NaHS, the experiment concluded that the sulfide donor acts on TRPA1 channels in the bladder [35]. A series of experiments investigating the relationship between H_2S and TRPA1 then began. An example is the experiment by Gratzke, who used TRPA1 agonists on human urethral samples and identified the location of the channels in the nerve fibres in different layers. His studies have revealed that TRPA1 colocalizes in neurons expressing both TRPV1 and CGRP, as well as in urothelial and interstitial cells. The results of agonist assays suggest that TRPA1 has a role in human afferent and efferent sensory signaling [36]. The next key finding was made by Miyamoto and colleagues in 2011 [37], who investigated the effect of H_2S on native DRG neurons expressing TRPA1. Their experiments used the TRPA1 specific antagonist HC030031 and Ca^{2+} free extracellular solution. They found that the NaHS-induced Ca^{2+} increase was inhibited by the Ca^{2+}-free solution as well as by the presence of the antagonist, suggesting that NaHS stimulates sensory neurons by activating these channels.

8. Relationship between H_2S and NO

The synergism between the two compounds was first reported in relation to their effects on vascular smooth muscle [38]. The interaction results in the formation of H_2Sn compounds, nitroxyl (HNO) and nitroso persulfide (SSNO), which may be able to activate TRPA1 channels in astrocytes and (DRG) neurons [39].

AIMS

The identification of H_2S, previously thought to be a toxic gas, as an endogenous gasotransmitter has opened a new chapter in the history of physiological research. Since then, its multimodal effects on many biological systems have been mapped. It undergoes a number of chemical transformations in the body and the products generated during these processes also play an important regulatory role. Although H_2S, its endogenous transformation and the role of the resulting molecules in complex processes have been widely studied, many questions remain in this area. Research on the effects of polysulfides on various processes is being carried out in many parts of the world, but the relative instability of most polysulfides makes their use in disease models problematic. Based on these considerations, a polysulfide with rather stable properties under physiological conditions, dimethyl trisulfide, was chosen.

The TRPA1 receptor and its biological role have been a long-standing area of research. Its precise structure, its activation by several ligands and its location in many parts of the body are known. Literature suggests that H_2S can activate TRPA1, so we proposed to study the relationship between the two sites.

Our aim was to investigate the effects of DMTS in an inflammatory and a neuropathic pain model and whether the effects are related to the presence or absence of the TRPA1 receptor.

EXPERIMENTAL MODELS AND TESTING METHODS

1. Laboratory animals

All our experiments were performed on 8-15 weeks old, 20-30 g male, functional TRPA1 ($TRPA1^{-/-}$) and somatostatin 4 receptor ($SST4^{-/-}$) gene-deficient (KO) mice and their wild-type (WT) mates ($TRPA1^{+/+}$ and $SST4^{+/+}$). Both strains of genetically modified mice are C57BL/6-based.

2. Ethical aspects

All experimental procedures and studies complied with the requirements of Act XXVIII of 1998 on the Protection and Welfare of Animals and Government Decree No.40/2013 (14.II.) on the performance of animal experiments, as well as the recommendations of Directive 2010/63/EU of the European Parliament and of the Council and the International Society for the Prevention of Cruelty to Animals. The experiments have been approved by the Ethics Committee for Animal Experiments of the University of Pécs, licence number BA02/2000-30/2016.

3. Preparation of DMTS solution

First, a 1 M DMTS solution was prepared in dimethyl sulfoxide (DMSO) and then diluted to 100 mM in saline containing 2% v/v polysorbate 80. After slow dissolution, this was further diluted to 25 mM in physiological saline. The 25 mM solution was administered intraperitoneally at a volume of 10 ml/kg, resulting in a dose of 250 μmol/kg. The vehicle contained DMSO instead of 1 M DMTS solution. The final DMTS solution contained 2.24% v/v DMSO and 0.45% v/v polysorbate 80. The DMSO content of the vehicle was 2.5% v/v DMSO.

4. Experimental models

4.1. Seltzer mononeuropathy

To study spontaneous pain due to partial nerve injury, we used the method described in Seltzer's 1990 article. The rat model was converted to mice. Although in humans we can usually speak of so-called polyneuropathy involving several nerves, in our experiments we used a generally accepted, simplified, single-nerve version of mononeuropathy with partial tying of the sciatic nerve.

4.2. K/BxN serum transfer arthritis

In our experiments, joint inflammation was induced by a single 300 µL injection of K/BxN serum from the respective arthritic mice (Attila Mócsai, Semmelweis University, Budapest). Animals in the control group received the same amount of non-arthritic BxN serum.

5. Test methods

5.1. Detection of mechanical allodynia

The mechanical pain threshold of the hind paws was determined preoperatively and postoperatively. The threshold was measured using a dynamic plantar aesthesiometer (DPA, Ugo Basile 37400, Comerio, Italy).

5.2. Hanging test

The mice were placed on a rigid metal grid, and then the grid was lifted and inverted to test their hanging behaviour. In a healthy animal, clinging to the grid and thus hanging upside down can take up to minutes, so when the hanging time reached 60 seconds, the animals were returned to their original body position.

5.3. Luminescence imaging of macrophages

Imaging studies were performed using lucigenin (pH 7.4). Lucigenin visualizes extracellular superoxide produced by macrophage NADPH oxidase [40]. Mice were first anaesthetized with a mixture of ketamine (120 mg/kg) and xylazine (12 mg/kg) given intraperitoneally, and then lucigenin dissolved in phosphate buffered saline (PBS) was administered intraperitoneally (25 mg/kg). Luminescence imaging was performed 10 min after lucigenin application using an IVIS Lumina II (PerkinElmer, Waltham, USA) with the following settings: acquisition time = 120 s, F/stop = 1, binning = 8 [41]. The luminescence signal measured in our study area was expressed in photons per second..

5.4. Measurement of microglial activation using ionized calcium-binding adapter molecule 1 (IBA1)

Calcium ions exert their signaling activity through binding to various calcium-binding proteins, many of which belong to the EF hand family of proteins. Iba1 is a 17 kDa protein [42], also a member of this family [43]. It is expressed in macrophages/microglia, which, upon activation, increase protein production. Activated cells can be detected using an Iba1-specific antibody in immunohistochemistry.

5.5 Histological assessment

The number of IBA1-positive cells and the activation status of microglia in 1-2 lamellae of the dorsal horn of the spinal cord were assessed using software.

5.6. Measurement of paw swelling by plethysmometry

The volume of the hind paws and its changes due to inflammation were measured using a plethysmometer (Ugo Basile, Gemonio, Italy). The volume of fluid displaced by the leg was expressed in cm^3.

5.7. Arthritis score

The independent expert assessment was based on the degree of oedema, redness and mobility of the lower ankle joint.

5.8. RNAScope In situ hybridization of mouse dorsal root ganglion

The experimental animals were anesthetized with intraperitoneally administered pentobarbital injection (2.4 g/kg, i.p.) and transcardially perfused with Millonig phosphate buffer containing 4% paraformaldehyde. After reaching a properly fixed state, the L4 DRG was removed and after postfixation, 20 μm sections were made from it. RNAscope analysis was performed according to the manufacturer's protocol. DAPI binds to the adenine and thymine-rich region of DNA to form a fluorescent complex and can be visualized under a microscope.

5.9. Patch clamp

The whole cell currents of voltage-coupled cells were recorded by manual patch clamp electrophysiology according to standard protocols using Axopatch 200B amplifiers connected to a computer and digitized using a Digidata 1550A (Molecular Devices, San Jose, California, USA). GFP-positive TRPA1 transfected CHO (Chinese hamster ovary) cells were identified using a Nikon Eclipse TE2000-U fluorescence microscope (Auro-science LLC, Budapest, Hungary).

5.10. Measurement of plasma extravasation by fluorescence

IR676 fluorescent dye was used for the measurements. The solution contained Kolliphor HS 15 (5% v/v) to achieve a particle size suitable for plasma leakage detection. The mice were first anesthetized with a combination of intraperitoneally administered ketamine (120 mg/kg) and xylazine (12 mg/kg), followed by an injection of IR676 into the retrobulbar venous plexus. Leakage was then detected using an IVIS Lumina II (PerkinElmer, Waltham, Massachusetts, USA). The magnitude of the recorded signal was expressed as total irradiance = (photons/s)/μW/cm2) [44].

5.11. Histological examination of the ankle joints

The tibiotarsal joints of the treated animals were removed 7 days after serum administration and fixed in 4% buffered paraformaldehyde. The specimens were first decalcified to allow incision of the bones and then embedded in paraffin to produce 3-5 μm thick sections. The sections were stained with hematoxylin-eosin and evaluated by an independent expert who scored the following histopathological lesions: cartilage damage, mononuclear cell count, synovial tissue proliferation, fibroblast count, collagen deposition rate.

5.12. Data analysis, statistics

For the K/BxN arthritis model, Clampfit 10.7 (Molecular Devices, California, USA) and GraphPad Prism 5 (GraphPad, California, USA) were used for data visualization and analysis, and GraphPad Prism 8 (GraphPad, California, USA) was used for the analysis of the neuropathic pain model.

RESULTS

1. Concentration-dependent effects of DMTS on TRPA1 ion channels

The activation of the TRPA1 ion channel by DMTS was investigated in vivo in Chinese hamster ovary (CHO) cells transfected with human trpa1 using a patch clamp method. A microelectrode was attached to the membrane of the cells and the ionic current generated upon opening of the ion channel was recorded. DMTS induced a current similar to that elicited by the known TRPA1 activator allyl isothiocyanate (AITC). The current increased much more slowly for DMTS than for AITC. At the end of the experiment, we checked the functionality of the ion channels by using selective TRPA1 antagonist HC030031. A concentration-response curve was constructed using currents normalized to the AITC-induced responses. The EC_{50} value was 6.92 μmol/L.

2. Analysis of Trpa1 mRNA in mouse dorsal root ganglion neurons

We used RNAscope in situ hybridization on the dorsal root ganglion of mice. Fluorescent signals were photographed with a confocal microscope. We also recorded Trpa1 mRNA and Calca (encoding CGRP) mRNA fluorescent signal in mouse L4 dorsal root ganglion. Detailed analysis of the sections revealed that Trpa1 is expressed on CGRP positive sensory neurons in DRG, i.e. Trpa1 and Calca genes show partial colocalization in this region. The study showed that although colocalization was significant, not all neurons expressing Trpa1 were peptidergic, just as not all peptidergic sensory neurons were Trpa1-positive.

3. Effect of DMTS treatment in a K/BxN serum transfer arthritis model

3.1. Examination of the effect of DMTS on the mechanical sensitivity of the hind legs

The mechanical sensitivity of the hind paw of mice receiving inflammatory serum transfer was measured using a DPA (Dynamic Plantar Aestesiometer, Ugo Basile 21036, Gemonio, Italy). One habituation and 3 control measurements were performed, and the test was repeated on days 5 and 7 after serum injection. As expected, the values of animals injected with K/BxN serum were significantly reduced compared to animals injected with BxN in the control group. This decrease was not affected by daily treatment with DMTS (125 μmol/L) in either TRPA1 WT or TRPA1 KO animals.

3.2. Examination of the effect of DMTS treatment on the hanging performance of mice

Mice in the K/BxN or BxN treatment groups were subjected to a hanging test on days 5 and 7 after the corresponding serum injection. The time spent in the hanging position was significantly reduced in the arthritis-serum-treated group in both tested strains. These values were not changed by daily treatment with DMTS or vehicle.

3.3. Effect of DMTS treatment on paw swelling and arthritis severity

Administration of K/BxN serum increased hind paw volume and arthritis score compared to the control group treated with non-arthritic BxN serum. Daily treatment with DMTS reduced hind paw swelling in the KBxN group on days 5 and 7 after the onset of arthritis, with the effect in both TRPA1 WT and KO animals. Similarly, the arthritis score calculated from ankle joint redness, swelling and passive mobility was reduced by DMTS treatment. DMTS had no effect on any of the parameters tested in the control group. The protective effect was also observed in TRPA1 WT and KO animals, suggesting that DMTS exerts its effects on these parameters independently of TRPA1.

3.4. Effect of DMTS treatment on MPO activity

MPO activity was detected by luminescence imaging in the hind paws at 2 and 6 days after the onset of arthritis. A control measurement was performed before serum injection and compared to the values measured on days 2 and 6 after treatment. Both TRPA1 WT and KO mice with serum-transfer arthritis had elevated MPO activity compared to control values, which was independent of treatment with DMTS or vehicle, i.e. DMTS treatment had no effect on myeloperoxidase activity. TRPA1 KO animals treated with vehicle had higher MPO levels compared to the TRPA1 WT group receiving the same treatment..

3.5. Effect of DMTS treatment on plasma extravasation

The actual plasma extravasation rate in the hind paws was measured by IR676 fluorescence imaging on days 2 and 6 after treatment with K/BxN or BxN serum. Using this method, the rate of plasma extravasation can be determined over a 20 min time interval. Control values were recorded before the treatments and compared to values after the treatments.

Serum-transfer arthritis increased the extravasation rate compared to baseline values recorded as controls in both TRPA1 WT and KO mice. Measured on day 6 after serum injection, we detected reduced plasma extravasation in DMTS-treated groups (WT and KO), suggesting that DMTS can reduce plasma extravasation rates independently of TRPA1. TRPA1 KO animals treated with vehicle showed higher extravasation on day 6 compared to the TRPA1 WT group also treated with vehicle. The effect of DMTS treatment did not differ between TRPA1 WT and KO mice.

3.6. Effect of DMTS treatment on collagen deposition in the ankle joint

On day 7 after K/BxN serum administration, the tibiotarsal joints of the sacrificed animals were removed and histological sections were taken for further studies. The prepared histological sections were scored. The 4 parameters studied were: number of mononuclear cells, synovial cell proliferation, number of fibroblasts, degree of collagen deposition. With the exception of the cartilage destruction score, the other three parameters were statistically significantly increased in KBxN serum-injected mice compared to BxN controls, regardless of genotype. No difference in cartilage degradation values was observed between arthritic TRPA1 WT mice compared to DMTS and vehicle-treated groups. That is, while there was an increase in cartilage loss in KO animals, this increase was not observed in WT animals. The number of fibroblast-like synoviocytes (FLS) and collagen deposition were found to be lower in DMTS-treated TRPA1 KO animals than in their wild-type counterparts. The extent of collagen deposition was not different in arthritic DMTS-treated animals in either mouse strain compared to the vehicle-treated group.

4. Effect of DMTS treatment in a neuropathic pain model

4.1. Study of the effect of DMTS treatment on neuropathic pain

Mechanical pain thresholds were determined using a DPA device after one habituation and 3 control measurements. Based on the control measurements, it is clear that the presence or absence of TRPA1 ion channels does not affect the mechanical sensitivity of intact paws. Measurements were repeated 24 hours after suture implantation in the sciatic nerve and animals with no change in mechanical sensitivity of the operated leg were excluded from further studies. Repeating the test 7 days after surgery, we found that the implantation of a suture in the sciatic nerve caused a significant decrease in the mechanical pain threshold after 7 days in a TRPA1 independent manner, compared to the values detected before surgery.

The vehicle slightly increased the pain threshold, the difference is not statistically significant.

The use of DMTS had a positive effect on the mechanical sensitivity of the operated hind paw, especially in TRPA1 WT animals. There was a significant increase in the pain threshold of the operated leg compared to the KO animals. DMTS treatment caused a statistical difference in this parameter not only compared to the gene-deficient control, but also compared to the vehicle-treated one. DMTS treatment was able to mitigate the abnormal pain perception due to sciatic nerve insult to such an extent that the difference was not statistically detectable compared to either the preoperative control value of the operated leg or the non-operated (intact) leg.

In the genetic absence of functional TRPA1 ion channels, the restorative effect of DMTS was not observed. DMTS-treated TRPA1 KO animals had similar pain thresholds to those treated with vehicle.

These findings all suggest that Trpa1 is a key mediator of the effects of DMTS in neuropathic pain.

4.2. Investigation of the role of the somatostatin receptor SST4 in the antihyperglycemic effect of DMTS

The experiment described above was performed on both SST4 WT and KO mice. Comparing their control values with TRPA1 WT and KO mice, respectively, no fundamental strain differences were observed.

Treatment with vehicle did not affect the mechanical sensitivity of either intact or operated legs. In contrast to TRPA1 WT and KO mice, it did not increase the lower pain threshold after surgery.

Measured 7 days after surgery, both Sstr4 WT and Sstr4 KO animals showed a strong decrease in pain threshold compared to control values. Mechanical hyperalgesia was induced by the trauma of nerve ligation. Subsequent DMTS treatment resulted in a statistically significant difference in the mechanical sensitivity of the neuropathic hind legs in Sstr4 WT animals. DMTS also increased pain threshold values compared to both pre-treatment values and post-treatment values in the vehicle -treated group. In mice with a genetic deficiency of somatostatin receptor 4, treatment with DMTS did not induce any antihyperalgesic effects.

The ability of DMTS to exert a positive effect on partial sciatic nerve ligation-induced neuropathic pain only in the presence of Sstr4 suggests that another key player in the effect of DMTS besides TRPA1 is SST4.

4.3. Effect of DMTS treatment on macrophage activity around the injured sciatic nerve

Tissue injury results in macrophage activation in the damaged areas and the release of chemokines and inflammatory cytokines. Literature suggests that these chemokines and radicals released from activated macrophages contribute to the pathomechanism of neuropathic pain [45,46].

Examining macrophage activity in the peri-injury area of nerve-injured hind legs, we found that DMTS treatment reduced luminescence in both TRPA1 WT and KO mice compared to the vehicle-treated group, but the difference was not statistically significant. Macrophage activity was significantly higher in vehicle-treated TRPA1 KO mice than in their wild-type counterparts.

The study was performed on both SST4 WT and KO mice. DMTS treatment did not cause any significant difference in these strains compared to vehicle treated animals. In contrast to TRPA1 strains, SST4 KO animals treated with vehicle did not exhibit increased macrophage cell activity.

4.4. Effect of DMTS treatment on the microglia density of the posterior horn of the spinal cord

The presence of the microglia/macrophage-specific calcium-binding protein IBA 1 was investigated in laminae 1 and 2 of the dorsal horn of the spinal cord. Since the protein is upregulated by microglial/macrophage activation, the number of IBA 1 positive cells and derived activation index directly indicate microglial/macrophage activation in the area under investigation.

Density was higher in animals with sciatic nerve entrapment, regardless of treatment group and genotype, compared to their counterparts without nerve injury. Interestingly, significantly increased microglia density was also observed in treated mice on the non-operated side compared to untreated control animals. Significantly increased density values were observed on the lesion side (right) compared to the contralateral side in untreated and vehicle-treated mice. No such difference was observed in DMTS-treated animals for any of the TRPA1 mouse strains. Although DMTS treatment reduced the number of activated microglia, the results clearly show that their activation status was not reduced. The lack of increased microglia density on the lesion side in the DMTS-treated group suggests a protective effect of DMTS. This effect was observed in both TRPA1 WT and KO mice, suggesting that TRPA1 does not play a role in mediating the effect. Trpa1 WT and KO mice with partial sciatic nerve lesions also showed increased microglial activation compared to the contralateral side. Intact Trpa1 KO animals showed greater microglial activation on both the right and left sides compared to WT animals.

DISCUSSION

Both our in vitro and in vivo experiments confirm our previous observations that DMTS is a potent agonist of TRPA1 ion channels. DMTS treatment reduced the rate of edema formation and plasma extravasation in the K/BxN model, which was independent of the presence of the TRPA1 receptor. TRPA1 WT mice did not develop significant cartilage damage compared to control group members receiving BxN non-arthritic serum, in contrast to KO animals, which showed extensive cartilage damage. DMTS treatment had no effect on either mechanical hyperalgesia of the hind legs or on the hanging performance of the animals. In addition, neither neutrophil granulocyte nor mononuclear cell counts were affected. The amount of fibroblast-like synoviocytes was reduced by DMTS treatment, but only in TRPA1 KO animals.

No antihyperalgesic effect was observed. DMTS treatment in RA seems to have an effect only on edema-dependent mechanisms and humoral inflammation. In neuropathic pain, DMTS had a clearly demonstrable antihyperalgesic effect. Following treatment, the degree of mechanical hyperalgesia due to sciatic nerve injury was significantly reduced. The effect was mediated by the somatostatin receptor SST4 in addition to the TRPA1 ion channel. Somatostatin is a cyclic neuropeptide released upon stimulation mainly from peptidergic pain-sensing neurons. Faris and colleagues [47] found that the release of somatostatin from nerve endings of isolated mouse skin was due to activation of TRPA1 ion channels. The resulting somatostatin efflux is transported by the systemic circulation to somatostatin receptors, where it binds and initiates systemic anti-inflammatory processes. The antinociceptive and anti-inflammatory effects of somatostatin are mediated by receptors 4. Knockout of the sst4 gene abolished the anti-inflammatory effect of somatostatin [48].

In our previous studies, we investigated the effect of GYY4137 sulfide donor in K/BxN serum-induced arthritis. Compared to the effect of inorganic sodium polysulfide (POLY) from GYY4137, that of DMTS is quite different. Treatment with inorganic polysulfide also reduced mechanical hyperalgesia, arthritis score and cartilage damage in the presence of TRPA1. Both mechanical hyperalgesia, plasma extravasation and MPO activity were increased in TRPA1 KO animals. No differences between WT and KO strain were found between the effects of DMTS treatment and POLY. DMTS treatment increased the rate of plasma extravasation in KO animals. This effect was also observed with vehicle treatment. Compared to inorganic sulfide, DMTS did not reduce either mechanical hyperalgesia or neutrophil cell accumulation. DMTS treatment reduced paw swelling and arthritis scores, which is in line with our previous studies investigating the effect of DMTS in carrageenan-induced paw inflammation [49].

The two pain models, although differing in several aspects, have in common that DMTS treatment reduces the rate of plasma extravasation in both autoimmune and non-autoimmune inflammation, which is a good indicator of the status of the blood vessels in the area. Based on these experiments, we conclude that DMTS probably acts through the release of somatostatin from peptidergic sensory nerve endings.

In the present study, we observed a high fibroblast-like synoviocyte (FLS) count and higher collagen deposition in arthritic TRPA1 WT mice treated with DMTS compared to their gene-deficient counterparts. This is in agreement with our previous results, where the effect of the sulfide donor GYY4137 was investigated in K/BxN serum transfer arthritis [50]. TRPA1 ion channel expression has been shown in cardiac fibroblast cells and FLS [51]. These synoviocytes release inflammatory cytokines and matrix metalloproteinases (MMPs) that contribute to the inflammatory processes of arthritis. The cytokines and chemokines attract neutrophils, macrophages and mast cells, which further amplify the autoimmune response [52–54]. TRPA1 activation induces proinflammatory processes in fibroblasts. This claim is supported by several studies. For example, Sieghart's article [55] describing that phenytoin, an antiepileptic drug, is able to activate TRPA1 channels in human fibroblast cells and promote collagen deposition; or even the study by Kloesch and colleagues [56], who described that cytokine degradation from FLS and cartilage destruction is attenuated by administration of a TRPA1 antagonist. These results are all consistent with the increased number of FLS and more severe collagen deposition observed in TRPA1 WT animals. Sulfide-releasing agents have long been known to have anti-inflammatory effects in FLS. S-propyl cysteine inhibits the release of cytokines, oxygen radicals and MMP from human FLS via the Keap1/Nrf2 signaling pathway [57]. Similar results have been published with NaHS in osteoarthritis and RA FLS [55-57]. Diallyl trisulfide from garlic (DATS), an organic sulfide very similar to DMTS, attenuated TNF-α-induced cytokine release in FLS isolated from animals with collagen-induced arthritis. The effect of DATS is probably based on the inhibition of NF-κβ and Wnt signal transduction pathways [58]. These mechanisms may explain the lower FLS density and collagen deposition we observed in TRPA1 KO mice. TRPA1 not only acts on cartilage through the FLS, but is also expressed in chondrocytes. Activation of the ion channel in chondrocytes causes release of inflammatory cytokines and MMPs [59,60].

In previous studies, microglia in the posterior horn of the spinal cord have been identified as being involved in the development of neuropathic pain after partial sciatic nerve ligation [61,62].

On the side of the lesion, we observed an increased ipsilateral microglia density in the posterior horn of the spinal cord, which is in agreement with the results of a previous paper [63], in which increased IBA1-positive microglia numbers were described in lamellae 1 and 2 of the posterior horn of the spinal cord after similar nerve insult. DMTS treatment in TRPA1 KO mice decreased the number of ipsilateral microglia in the dorsal horn, and therefore, based on our immunohistological data, we conclude that microglia are not the main target of the TRPA1-dependent antineuropathic effect of DMTS.

There is a lack of extensive literature on the effects of DMTS, while the effects of DATS and inorganic POLY have been the subject of several studies. The effects of POLY were investigated in the RAW264.7 mouse macrophage cell line. POLY inhibited inflammatory cytokine release, NF-κβ and Toll-like receptor 4 (TLR4) activation [64] and attenuated the activity of calmodulin-dependent protein kinase II (CaMKII). DATS exerted similar effects in lipopolysaccharide (LPS)-stimulated RAW264.7 cells to those of POLY on inflammatory cytokine secretion, NF-κβ and TLR4 (Toll-like receptor 4) signaling. In our experiments, DMTS failed to reduce the activity of cytokine-producing macrophages in the vicinity of the injured sciatic nerve in a statistically significant manner. Huang et al. used mouse BV-2 cell line to test POLY and DATS on microglia. POLY-derived nitroxyl attenuated LPS-induced apoptosis in the cells [65]. Testing DATS in the same model yielded similar results [66]. These findings are partially consistent with our results: DMTS reduced posterior horn microglial density, but did not affect histological markers of microglial activation. Our group has previously investigated the interaction of inorganic POLY and organic DMTS with TRPA1 in CHO cells and in mouse trigeminal ganglion neuronal primary cultures using calcium-sensitive fluorescent markers and patch clamp methods. Their results confirmed the activation of the ion channel by both sulfide compounds [8,50]. Polysulfides are able to react with the thiol group of proteins, thereby altering their function. An example is the work of Braunstein and Greiner [67,68], who have identified actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NF-κβ, ATP-sensitive potassium channel (KATP), protein tyrosine phosphatase 1B (PTP1B), Kelch-like ECH-associated protein-1 (Keap1) and phosphatase and tensin homologous (PTEN) protein for modulation by polysulfides. These data suggest that the differential effects of DMTS on different proteins at different levels of the organism may explain the opposite effects we observed in macrophages.

To summarize our results, we conclude that DMTS treatment reduced paw swelling and plasma extravasation in K/BxN serum-induced rheumatoid arthritis, a valid inflammatory pain model, independently of the TRPA1 ion channel.

DMTS exerted a cartilage protective effect and also affected FLS cells. No analgesic effect was observed, but it had a role in reducing humoral inflammation, also in a TRPA1-independent manner.

The attenuating effect of DMTS on mononeuropathic pain also involves TRPA1 ion channels and SST4 receptors. The relevance of the SST4 receptor has been demonstrated in our model [69,70]. DMTS treatment reduced the number of IBA1-positive microglia in both TRPA1 WT and KO mice, but this anti-inflammatory effect in KO animals was not reflected in the data from behavioral studies.

The apparently contrasting results may be explained by the different kinetics of DMTS administration in the two sets of experiments, with the arthritis model administered once daily for 7 days, achieving a relatively low tissue concentration, which, due to the short half-life of the substance, was likely to be completely depleted by the time of the next dose. In the mononeuropathic model, sulfide was administered hourly for a total of seven times in one day, reaching a relatively high equilibrium tissue concentration 2-2.5 hours after the start of dosing and remaining so throughout the dosing period. Thus, the route of administration may explain the ability of DMTS in the Seltzer model to exert effects at sites and at concentrations that a shorter dose or lower concentration would not allow.

Due to the timing of the experimental series, the effects of DMTS were not tested in neuropathic pain using the administration schedule described in the K/BxN study, nor were the effects on macrophages measured, so no conclusions can be drawn comparing the two papers in this respect.

In an experiment with macrophage-depleted mice, K/BxN serum transfer did not cause arthritis [71], suggesting that macrophages are critical in the development of the disease model. We did not measure macrophage activity here, but their inhibition could explain the milder inflammatory responses observed.

Previously, in the inorganic polysulfide experiment with GYY4137, polysulfide had not only analgesic but also pro-algesic effects, and the analgesic effect was produced independently of TRPA1. We did not see a similar effect in the studies presented in the thesis, probably because DMTS can act not only through TRPA1 but also through other proteins and the two effects can offset each other.

Another important difference between the two models is the neural sensitization and its extent. In the arthritis model, there is mostly peripheral sensitization due to sensitization of nociceptors by inflammatory mediators.

In the case of mononeuropathy, nerve injury results in central sensitization in addition to peripheral one. DMTS is able to cross the blood-one barrier [72] and therefore it is possible that it exerts its analgesic effect by reducing central sensitization.

Our results suggest that DMTS may be a potential adjunctive treatment for patients with both rheumatoid arthritis and neuropathic pain in the future, but much more research is needed.

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