SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation the pharmacological properties of the Transient Receptor Potential Melastatin 3 Ion Channel and its role in somatosensory functions

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INTRODUCTION

Both pain and itch can be described as sensory phenomena associated with negative emotions. Our laboratory, together with our collaboration partners, has been studying various aspects of the physiological processes of human skin and various sensory functions, such as pain and itch, for many years. Our previous experimental results have contributed to a better understanding of the function of several members of the Transient Receptor Potential (TRP) ion channel familiy that play prominent roles in sensory functions.

Pain and itch are detected by receptors expressed at the nerve endings of somatosensory neurons. A significant proportion of the molecules involved in pain perception and transduction belong to the group of heat-sensitive TRP ion channels. Several heat-sensitive TRP channels, such as TRPV1 and TRPA1, have been shown to play a role not only in pain and thermosensation but also contribute to the perception and transduction of itch. In our studies, we aimed at further investigating the functional and pharmacological features of the heat-sensitive Transient Receptor Potential Melastatin 3 (TRPM3) ion channel. It is already known to play a role in the development of pain but, its functional characteristics are less known. In our research projects, we investigated the potential effect of various volatile anesthetics on the TRPM3 ion channel and the role of TRPM3 in the development of pruritus which was not studied yet. Our results further expand our knowledge about the functional roles and pharmacological properties of the channel and hopefully can contribute to the future development of novel, TRPM3 targeting drugs.

AIMS

To investigate the potential effect of anesthetics, our experiments were performed on HEK293T cells overexpressing recombinant TRPM3 and sensory neurons from mouse dorsal root ganglia expressing native TRPM3. The effect of halothane, chloroform, isoflurane and sevoflurane were investigated during the chemical activation of TRPM3. Under investigating the effect of volatile anesthetics on TRPM3, we sought answers to the following questions:

1. Do volatile anesthetics activate, or inhibit the TRPM3 ion channels as they do targeting other TRP channels?
2. Do volatile anesthetics affect the chemical or thermal activation of TRPM3?
3. Do volatile anesthetics affect transmembrane currents flowing through the alternative pore of the TRPM3 ion channel activated by CIM0216?
4. In addition to the recombinant TRPM3, how do volatile anesthetics influence the function of native TRPM3 ion channels?

To investigate the role of TRPM3 ion channel in pruritus, *in vivo* behavioral tests, such as the “cheek” and “nape” models were used. In addition, we investigated the function of the TRPM3 ion channel in the cellular effects of various pruritogenic substances by pharmacological methods using sensory neurons from the trigeminal ganglia of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice. In our experiments, we were interested in the following questions:

1. Like other heat and pain sensing TRP channels, does the TRPM3 ion channel play a role in the development of pruritus?
2. Do algogenic TRPM3 agonists induce itch, as well?
3. Does TRPM3 involved in the effect of endogenous pruritogenic mediators in trigeminal somatosensory neurons?

MATERIALS AND METHODS

Preparation of stable solutions of VAs and its monitoring by GC/MS

To investigate the effects of volatile anesthetics on the TRPM3 ion channel, 10 mM stock solutions were prepared by dissolving the pure anesthetics in the working solution used in the measurements (Ca^{2+} buffer or patch-clamp extracellular solution). To completely dissolve the compounds, the stock solutions were stirred in an airtight glass flask overnight. The final working solutions were freshly prepared by further diluting these stock solutions and used for measurements within 45 min. If needed, new working solutions were freshly diluted. The stability of the 10 mM stock solutions in an open, freely ventilated vial was checked by GC/MS method in the Laboratory of Forensic Toxicology of the Department of Forensic Medicine at UD Clinical Center. For stability control, stock solutions were prepared as described above. During the gas chromatographic measurement, the solutions were stored in an open vial, simulating the experimental conditions in an open perfusion system. During the measurement protocol, samples were taken at 0, 10, 20, 30, 45 minutes. Anesthetics were identified using Agilent 7980B-5977A GC-MS instruments (Agilent Technologies, Santa Clara, CA, USA).

Animals

For both *in vitro* and *in vivo* experiments, we used 10–14-week-old mice stored under conventional animal housing conditions at 21°C with 12-h light-dark cycles with unrestricted access to water and food. The primary sensory trigeminal and dorsal root ganglion (TG and DRG) neurons used in our projects were isolated from *Trpm3*$^{+/+}$ (wild-type C57Bl6) and C57Bl6-derived *Trpm3*$^{-/-}$ mice. Male and female mice were both used for isolation. *Trpm3*$^{+/+}$ mice used for *in vitro* experiments were provided by the Experimental Animal House provided by the Institute of Biochemistry and Molecular Biology of the University of Debrecen and by the Ion Channel Research Laboratory headed by Prof. Thomas Voets (KU

Leuven, Belgium). For the *cheek* model used to study the role of TRPM3 in itch, only male mice of the *Trpm3*$^{-/-}$ and *Trpm3*$^{+/+}$ strains were used, which were provided by the Ion Channel Research Laboratory (KU Leuven Belgium) led by Prof. Thomas Voets. During the *nape* model, we used both male and female individuals of the *Trpm3*$^{-/-}$ and *Trpm3*$^{+/+}$ mice, which animals were used in the laboratory of our collaborator, prof. Tibor Rohács. The *in vivo* behavioral animal experiments were supervised by the Laboratory Animal Ethics Committee of the Catholic University of Leuven (License No. P021 / 2018) or the Laboratory Animal Ethics Committee of Rutgers New Jersey Medical School.

Cell culturing and isolation of primary sensory neurons

Our *in vitro* experiments were performed on native HEK293T cells and HEK293T cells stably overexpressing the mouse TRPM3α2 variant (HEK-M3 cells), and on primary DRG sensory neurons isolated from *Trpm3*$^{+/+}$ mice and on primary TG sensory neurons isolated from both *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice.

HEK293T cells

The native HEK293T used as controls and HEK-M3 cell lines stably overexpressing mTRPM3α2 were provided by Prof. Thomas Voets (Ion Channel Research Laboratory (KU Leuven Belgium)). The HEK-M3 cell line was constructed by using the Flp-In transfection system. Cell lines were cultured at 37° C with 5% CO_2 in endotoxin-free DMEM medium. The medium was prepared by adding 10% fetal bovin serum (FBS), 50 IU / ml Penicillin, 50 μg/ ml Streptotmycin (P/S), 25 mg/ ml L-glutamine and an appropriate amount of 10x non-essential amino acid solution. The medium of HEK-M3 cells was supplemented with 200 μg/ ml Hygromycin as a selection antibiotic. Cell cultures at 70-80% confluence were subcultured using a 0.25% trypsin solution and seeded for experiments. In the experiments on the HEK293T and HEK-M3 cell lines, the cells were plated in poly-1-lysine HBr treated special dishes compatible with the experiment.

Isolation of TG and DRG sensory neurons

To investigate the effect of the VAs, sensory neurons were enzymatically isolated from the dorsal root ganglia of *Trpm3* $^{+/+}$ mice, while to investigate the effect of pruritogens were isolated from the trigeminal ganglia of *Trpm3* $^{+/+}$ and *Trpm3* $^{-/-}$ mice. Both male and female mice were used to isolate the neurons. During the isolation of DRG neurons, mice were terminated by using CO_2 gas, and cervical dislocation was used to isolate TG neurons. The DRG and TG neurons were collected in NeuroBasal medium +10% FBS +P/S solution which were kept on ice throughout the preparation. Enzymatic digestion of neurons was started no later than 30 min after termination of the animals in a solution containing 2 mg/ ml collagenase and 2.5 mg/ ml dispase in a sealed eppendorf tube kept in a water bath at 37°C for 40 min. Following enzymatic digestion, the enzymatic solution was removed and the cells were washed three times with neuron culture medium (NeuroBasal + 10% FBS + P / S). Subsequently, the ganglia were mechanically dispersed into individual cells using medical syringe needles of decreasing diameter (18G-> 20G-> 22G-> 26G). Following isolation, DRG neurons were plated on poly-L-lysine HBr-coated glass-bottomed petri dishes and cultured in NeuroBasal medium supplemented with 10% FBS, 2% B-27, 2 mM L-glutamine, 100ug / ml P / S, and 100 ng / ml β –NGF at 37° C in the presence of 5% CO_2. TG neurons were plated on poly-L-ornithine and laminin coated glass-bottomed petri dishes and cultured in NeuroBasal medium supplemented with 10% FBS, 2% B-27, 2 mM L-glutamine, 100 ug/ ml P/S, 2 ng / ml GDNF,10 ng/ ml NT4 at 37 ° C in the presence of 5% CO_2. Isolated sensory neuron cultures were used for experiments within 24-36 hours after isolation.

Intracellular Ca^{2+} measurements

Although the experimental design of the different intracellular Ca^{2+} measurements used for our projects differed in terms of the measurement setups and the used

experimental protocols, the principle of the measurements were similar in all cases. Fluorescent Ca^{2+} sensitive Fluo-4-AM or Fura-2-AM dyes were used to measure the change in intracellular Ca^{2+} levels. The fluorescence intensity of Fluo-4 is significantly increased in the presence of free Ca^{2+}, (excitation: 490 nm; emission: 520 nm). In the case of Fura-2 dye, the excitation maximum of the dye is shifted if it binds Ca^{2+} from 380 nm to 340 nm, while the emission maximum is 510 nm in both cases. Changes in intracellular Ca^{2+} levels of HEK293T and HEK-M3 cells were measured in a population of cells plated in a 96-well plate in equal number/wells and using a Flex Station III automated plate reader. To investigate the effect of heat stimulation , the cells were collected in PCR tubes and a Q-PCR machine was used for simultaneous heat stimulation and fluorescence measuremt. Intracellular Ca^{2+} measurements on primary DRG and TG neurons were performed in a one-cell configuration using open-system gravitational perfusion with a Zeiss LSM 5 Live fluorescent confocal microscope.

Patch Clamp

The effect of VAs on the electrophysiological properties of the TRPM3 were investigated in whole-cell patch-clamp experiments using an Axopatch 1.D amplifier and Clampex 10.2 software (Molecular Devices). HEK-M3 cells were cultured on 12 mm diameter glass coverslips coated with poly-l-lysine HBr, and measurements were performed on adherent cells 2-3 hours after passage. During the experiment, the external solution contained 150 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES buffer, and its pH was adjusted to 7.4 with NaOH. The internal solution in the pipettes contained 100 mM aspartate, 45 mM CsCl, 1,144 mM $MgCl_2$, 10 mM HEPES, and 10 mM EGTA. Its pH was adjusted to 7.2 with CsOH, resulting in a final concentration of approximately 100 mM Cs-aspartate in the pipette solution. Micropipettes were made from a borosilicate glass capillary with a programmable electrode puller with a measured resistance (pipette resistance) ranging from 2 to 5 MΩ. After the whole-cell configuration was established, the

cell capacity typically showed a value around 10 pF, while the serial resistance was below 10 MΩ, which was compensated by 50–70%. In our measurement protocol, to record TRPM3 mediated currents, the holding potential was 0 mV and cells were ramped every 2 s from −150 to +150 mV over the course of 200 ms.

Behavior assays

The effects of different pruritogens and algogens were studied in wild-type (*Trpm3* $^{+/+}$) and Trpm3 knockout (*Trpm3* $^{-/-}$) C57Bl6 mice *in vivo*, using the *"nape"* and the *"cheek"* model. The "*cheek"* model allows for the differentiation between pain- and itch-related behavior and quantitative characterization of the responses. Unlike to the original protocol of LaMotte et al., subcutaneous injections were used in both the "*cheek"* and the *"nape"* model, instead of intradermal injections. The protocols used for the two different behavioral tests are the same except for the injected area. Male *Trpm3* $^{+/+}$ and *Trpm3* $^{-/-}$ individuals aged 8-14 weeks were used for the tests. Animals were adapted to the experimental conditions once a day, for 7 days before to the experiment. One day before the experiment, for the nape model, we shaved the hair from the nape of the animals, while during the cheek model, we shaved the hair from the cheek of the animals. On the day of the experiment, mice were placed into the observation chamber 10 min before the injection and spontaneous behavior was recorded using a video camera. In the cheek model, pruritogenic compounds were injected subcutaneously into the pre-shaved cheek. In the nape model, pruritogenic compounds were injected subcutaneously into the pre-shaved nape. Following injections, mice were placed back into the observation chamber immediately. Specific behavioral responses were recorded for 30 min using a video camera. During the cheek model, the amount of time each mouse spent scratching, the number of scratch bouts and the number of wipes on the injected site were quantified over the course of a 30-min period following the injection while during

the nape model, only the number of scratch bouts. were quantified. During treatment and behavioral scoring, investigators were blinded for genotype and treatment. A scratching unit is considered to be a series of full-length events in which an animal raises its hind leg on the same side as the injected area, scratches the injected area with its paw and then finally places it back on the ground or lifts it to its mouth. A painful unit is a unique movement in which the animal touches and rubs the injected area lifting only the first paw on the same side. Compounds used in the methods were dissolved in 7% TWEEN-80 Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) and used at the following concentrations: 200 µg/ 50 µl histamine, 30 µg/ 50 µl serotonin and 250 ng/ 50 µl endothelin-1 for the nape model, while 10 µg/ 10 µl PregS, 10 µg/ 10 µl capsaicin, 5 µg / 10 µl CIM0216, 50 µg / 10 µl histamine, 10 µg / 10 µl Serotonin, 150 ng / 10 µl endothelin- 1 when using the cheek model. Subcutaneous microinjections were performed with a 30G medical syringe needle attached to a 1 ml Insulin syringe.

Statistics

Experimental data were processed using OriginPro 9.0 software (OriginLab Corporation, Borthampton, MA, USA). Our results were presented as mean ± SD. To investigate the dose-response relationships, logistic dose-response curves were fitted according to the following equation: y = A2 + (A1- A2) / (1 + (x / x0) ^ p), where A1: value of the minimum response (y_{min}), A2: value of the maximum response (y_{max}), x0: the semi-effect concentration (EC_{50}/IC_{50}), p: the calculated slope determining the slope. The y-axis shows the amplitude of the Ca^{2+} signals, while the x-axis shows the logarithm of the concentration of the applied substance based on 10.

Statistical analyzes were performed using IBM SPSS Statistic 23.0 (IBM, Armonk, NY, USA) and OriginPro 9.0 (OriginLab Corporation) software. To test the antagonist effect of isosakuranetine, agonist-induced Ca^{2+} signals in the presence and absence of isosakuranetine were compared with Student's two-tailed

t-test. To control unwanted variances of measured currents between HEK-M3 cells, TRPM3 currents measured in the presence of VAs were normalized, and compared to the agonist induced current (considered as 100%) in the same cell and two-tailed Student's t-test for one sample was used for statistical evaluation. In case of Ca^{2+} signals recorded on sensory neurons, if it is not mentioned otherwise, signal amplitudes were normalized to the 1st agonist evoked signal, considered as 100% to control variances experienced between individual neurons. Then, data were subjected for statistical analysis using one-way ANOVA with Dunnett post-hoc test to compare the effect of VAs to vehicle control. In our *in vivo* behavior experiments, the Mann-Whitney test was used for statistical comparison, where appropriate. In every case, $p < 0.05$ was considered as significant difference between the group means. Data for *in vivo* and *in vitro* experiments are presented as mean ± SD

RESULTS

Effect of volatile anesthetics on TRPM3 ion channel function

Investigation of the stability of volatile anesthtetics

In our experiments, we investigated the effects of various volatile anesthetics (VAs) with different chemical structures on the TRPM3 ion channel. Due to their volatile and lipophilic nature, we assumed that it might be challenging to prepare a stabile aqueous solution of VAs suitable for measurements, although they reportedly can be solved >10 mM in water at 25 °C. For checking this, we performed GC/MS measurements on 10 mM stock solutions of the VAs, which were prepared by rigorous overnight stirring in an airtight vial, simulating our experimental setups with an open air perfusion system. GC/MS measurements were performed by dr. János Posta in Toxicology Laboratory of the Institute of Forensic Medicine of the University of Debrecen, faculty of General Medicine. For the experiment, 10 mM stock solutions were prepared one day before the measurements. Our experimental results showed that the concentration of 10 mM stock solutions of anesthetics in an open vial did not change significantly within a 45-min period.

Effect of VAs on chemical activation of recombinant TRPM3

First, we tested the effect of VAs on the recombinant TRPM3 by investigating the chemical activation of recombinant TRPM3 in HEK-M3 cells in the presence of VAs during intracellular Ca^{2+} measurements. In our experiments, after the treatment with different concentrations of VAs, we activated TRPM3 using the endogenous agonist PregS or the exogenous agonist CIM0216 in the presence of VAs. Our results clearly demonstrated that the investigated VAs do not activate the recombinant TRPM3 ion channel, but they inhibited the TRPM3-mediated Ca^{2+} transients induced by endogenous PregS and the more potent exogenous activator CIM0216 in a dose-dependent manner. Moreover, the EC50 value of PregS (applied in the concentration range of 0.1 to 300 μM) was shifted towards

higher concentration ranges. Although halothane was found to be the most potent inhibitor of TRPM3 among the tested VAs, it also evoked robust Ca^{2+} transients when applied alone at high concentrations (≥5 mM). This stimulatory effect of halothane was found to be independent of TRPM3, because (i) it was equally observed in native HEK293T cells and HEKM3 cells and (ii) the halothane-induced Ca^{2+} signals were not inhibited by the TRPM3 antagonist isosakuranetin.

Effect of VAs on TRPM3-mediated transmembrane currents

The effect of VAs' on the TRPM3-mediated transmembrane currents was further investigated using whole-cell patch-clamp method. In our experiments, the outwardly rectifying currents activated by PregS were partially inhibited by anesthetics at a concentration of 1 mM and completely inhibited at a concentration of 5 mM, which effects were proved to be rapid and reversible. In our patch clamp experiments, similar to our previous IC Ca^{2+} measurement experiments, sevoflurane was found to be the least potent inhibitor, inhibiting PregS-activated TRPM3 currents only very slightly at 1 mM, but increased however not complete inhibition was observed at 5 mM.

PregS-induced transmembrane currents flow through the main pore of TRPM3, while the more potent exogenous activator CIM0216 opens an alternative permeability pathway in the TRPM3 voltage sensor domain with an inward current flowing at a negative membrane potential, in addition to the main pore. Our experimental results demonstrated that the currents flowing through the main pore and the alternative permeability pathway activated by CIM0216 were partially inhibited by VAs when applied at 1 mM, and completely inhibited while applied at 5 mM, similarly to the effects observed during activation by PregS.

Effect of VAs on native TRPM3 ion channels expressed by sensory neurons isolated from mouse dorsal root ganglion

We investigated the effects of VAs on the native TRPM3 ion channel on sensory neurons isolated from mouse dorsal root ganglia. During experimental protocol

used for IC Ca^{2+} measurements on neurons, 2 minute-long PregS stimulations were repeated three times, and VAs (1 mM) were applied for 2 minutes before and during the 2nd PregS application. When analyzing the results, cells which were activated by depolarizing 25 mM KCl solution applied at the end of the experimental protocol were considered as neurons. Neurons responding to PregS (PregS+) were considered as TRPM3-expressing (TRPM3+) neurons. Similar to previous research, our results demonstrated that VAs directly activated some sensory neurons according to the following distribution: 15.3%, 21%, 5.1% and 1.8% of neurons were activated by 1mM halothane, chloroform, isoflurane and sevoflurane treatment respectively. The distribution of neurons activated by VAs was practically the same in TRPM3+ and TRPM3- neurons, and the vast majority of TRPM3+ neurons were not activated by VAs (VA- neurons). This result clearly confirmed our hypothesis that activation of neurons by VAs is independent of the TRPM3 ion channel. To exclude the effect of VAs-induced TRPM3-independent Ca^{2+} transients on PregS-induced responses, we analyzed only TRPM3+ and VA- neurons. Our results demonstrated that the investigated VAs used at 1 mM concentration, effectively inhibited PregS-induced TRPM3-mediated Ca^{2+} transients. The inhibitory effect of the anesthetics is considered reversible in 4 minutes after application.

Investigation of the role of TRPM3 ion channel in pruritus

To investigate the role of TRPM3 in pruritus and pain, we performed *in vivo* behavioral studies and *in vitro* experiments using sensory neurons isolated from mice. In both cases wild-type (*Trpm3* $^{+/+}$) and TRPM3-deficient (*Trpm3* $^{-/-}$) mice were used. One part of the *in vitro* intracellular Ca^{2+} measurements of the project was performed at the University of Debrecen, while other part of the in vitro experiments and mouse behavior tests using the cheek model were performed in the Laboratory of Ion Channel Research led by Thomas Voets at the Catholic

University of Leuven in Belgium. The experiments related to the nape model were performed by Nawoo Kim PhD student at Tibor Rohács' laboratory, in Rutgers New Jersey Medical School, Newark, NJ, USA.

PregS induces pain through TRPM3 but not itching

We used a previously presented method by LaMotte et al. (2008). After we established the cheek model protocol as it was detailed in the methods section, we studied different algogenic and pruritogenic substances in *Trpm3* $^{+/+}$ and *Trpm3* $^{-/-}$ mice. Previous researches have shown that both the endogenous TRPM3 activator PregS and the exogenous agonist CIM0216 induce pain related behavioral responses in different mouse models. Thus, we examined the effect of PregS and CIM0216 in the cheek model compared to the well-known algogenic capsaicin and the negative control vehicle (PBS + 7% TWEEN-80). Capsaicin elicited pain-related behavioral responses in both strains, while it did not elicit pruritus-related responses in either strain. The vehicle used as control did not induce pain or itch related behavior in either type of animals. Our experimental results demonstrated that neither the endogenous nor the exogenous TRPM3 agonists induced itch-related behavioral responses in either type of animal, whereas pain-related responses were observed in both PregS and CIM0216 treated groups in *Trpm3* $^{+/+}$ mice, which behavioral responses were abolished in *Trpm3* $^{-/-}$ animals. To better characterize the quality of the sensory phenomena evoked by a particular compound, we introduced a new measure, ratio of scratch ($R_{scratch}$), which describes the specificity of the test substances on the pain-itch axis. To calculate $R_{scratch}$, the total number of itchy responses was divided by the total number of behavioral responses (sum of painful and itchy responses). A low (0 or close) $R_{scratch}$ value refers to behavioral responses to purely pain, while a high (1 or close) $R_{scratch}$ value refers to purely itching but not pain-induced behavioral responses. Our results demonstrate that the TRPM3 agonist PregS and CIM0216, together with the TRPV1 agonist capsaicin, in *Trpm3* $^{+/+}$ animals behaved as an

algogenic substance, with low $R_{scratch}$ values. However, in *Trpm3*$^{-/-}$ animals, in contrast to capsaicin, PregS and CIM0216 showed a general distribution similar to the vehicle control, with an $R_{scratch}$ value of about 0.5.

Effects of pruritogen compounds in Trpm3$^{-/-}$*animals*

Although direct activation of TRPM3, similar to TRPV1, resulted exclusively in nociception and not itch, these results cannot exclude that TRPM3 signaling can also contribute to the sensory transduction of pruritus, as has been described for TRPV1 or TRPA1. Indeed, direct, general activation of TRPV1 is known to induce nociception and not itch, but TRPV1 expressed locally in pruriceptive sensory neurons takes part in the transduction of both histaminergic and some forms of non-histaminergic pruritus. To investigate the role of TRPM3 in the sensory transduction of pruritus evoked by the endogenous mediators Hist, 5-HT and ET-1 (each known to evoke severe itch in both human and rodent models), we tested these compounds in the cheek model paradigm in *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice. We found that Hist, 5-HT and ET-1 induced pronounced itch but hardly any pain related responses in *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ animals: the number of wipes detected was comparable to vehicle whereas the number of scratches and the time spent scratching were strongly elevated by each pruritogenic compound, irrespective of genotype. Most importantly, the number of scratches induced by the pruritogens was not decreased in the *Trpm3*$^{-/-}$ strain compared to wild type animals. High $R_{scratch}$ values also indicated that Hist, 5-HT, and ET-1 evoked a predominant pruritogenic and not algogenic effect in both strains. Interestingly, ET-1 induced itch was found to be significantly more intense in the *Trpm3*$^{-/-}$ group than in the *Trpm3*$^{+/+}$ group. Since high $R_{scratch}$ values indicated that Hist, 5-HT and ET-1 evoked mainly itch and hardly induced nociception, we also tested their effect injected in the nape of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ animals. Although behavioral reactions after nape injection cannot clearly discriminate between itch and nociception (both results in similar scratching responses), known “pure”

pruritogen compound-induced responses can be interpreted as signs of itch. Studying the behavioral responses evoked by the aforementioned pruritogens in the nape, we aimed at investigating the role of TRPM3 in the innervation area of the DRGs to compare to the results of the cheek injections which affected the innervation area of the TG. We found that Hist and 5-HT evoked similarly intense pruritus in *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice, as we observed in case of cheek injection, as well. The ET-1 induce responses were also in line with the scratches evoked in the cheek model: *Trpm3*$^{-/-}$ animals showed significantly stronger ET-1-induced itch responses than *Trpm3*$^{+/+}$ mice.

Activation of trigeminal sensory neurons by pruritogens is independent of TRPM3

To investigate the role of the TRPM3 ion channel in the pruritogen-induced cellular responses and to validate our *in vivo* behavioral results, we isolated somatosensory neurons from TGs of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice and investigated the distribution of sensory neurons responding to Hist, 5-HT, ET-1 and PregS, CA and Caps, agonists of TRPM3, TRPA1 and TRPV1, respectively. We compared pruritogen treatments in TG neuron cultures isolated from *Trpm3* $^{+/+}$ and *Trpm3* $^{-/-}$ mice. Different pruritogens were tested in individual experiments to avoid potential interactions. Only those cells were considered sensory neurons and included in the subsequent analysis which responded to depolarizing KCl solution or Caps applied at the end of the measurements. As expected, the ratio of PregS responsive (PregS+) neurons was strongly reduced in *Trpm3*$^{-/-}$ TG neurons, although, consistent with previous results, some neurons still responded to PregS suggesting other, as yet unidentified targets available in *Trpm3*$^{-/-}$ animals. The ratios of CA+ and Caps+ neurons were practically identical in the presence and absence of TRPM3. Pruritogens activated a subpopulation of both PregS+ and PregS- neurons in *Trpm3*$^{+/+}$ animals, indicating that the pruritogen-induced responses do not correlate with TRPM3 expression. Most importantly, the ratios

of the neurons responding to Hist (10.7 vs. 9.9%; $X^2 = 0.115$, $p = 0.735$), 5-HT (21.6 vs. 17.0%; $X^2 = 1.489$, $p = 0.222$) and ET-1 (33.2 vs. 34.3%; $X^2 = 0.006$, $p = 0.939$) were not different between the *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ groups

Pharmacological inhibition of TRPM3 has no effect on pruritogen-induced neural responses

Finally, we investigated how the pharmacological blockade of TRPM3 influences cellular activation of sensory neurons isolated from TGs of *Trpm3*$^{+/+}$ animals. TRPM3 agonist PregS, as well as Hist, 5-HT, and ET-1 were applied during intracellular Ca^{2+} measurements in the presence and absence of the TRPM3 antagonist Isok. PregS-induced responses were strongly inhibited by Isok in a reversible way. In contrast, pharmacological inhibition of TRPM3 did not affect the neural activation induced by the endogenous pruritogens: neither the amplitude of the pruritogen-induced Ca^{2+} signals nor the ratio of the Hist+, 5-HT+ and ET-1+ neurons were significantly changed in the presence of 3 μM Isok.

DISCUSSION

The recently characterized TRPM3 ion channel, similarly to TRPV1, is highly expressed in the dorsal root ganglia and plays a similar role in the perception of painful warm temperatures and the development of thermal hyperalgesia in inflammation, as well. Numerous studies have shown that heat-sensitive TRP channels play a prominent role in the thermosensation and through their versatile activation play a central role in sensing pain and pruritus. Due to these characteristics, they can be promising targets in the development of new types of analgesic and antipruritic drugs. Over the past decade, a several pharmaceutical companies have initiated research to achieve this goal, targeting the TRPV1 ion channel primarily as an analgesic target. Both TRPV1 inhibitors and TRPV1 activators may have analgesic effects, such as causing desensitization of TRPV1 after channel activation. However, the use of TRPV1 agonists and antagonists results in a number of intolerable side effects. These side effects e.g. a burning, painful sensation at the application site and an increase in body temperature or relative insensitivity to hot temperature, which increases the tissue damage caused by painfully warm stimuli and the risk of scalding accidents. As this strategy is not perfect, the need for alternative therapeutic approaches, e.g. targeting other molecules involved in nociception, arise. One possible target is the TRPM3, which role in the development of pain and temperature sensation is known. Previous research has shown that, in contrast to TRPV1, activation or inhibition of TRPM3 does not affect body temperature, and antagonists such as flavones, like isosakuranetin, hespertin, and liquirigenin, inhibited TRPM3, thereby effectively reduceds pain related to TRPM3 activation.

Further research demonstrated that many pain-sensing receptors have been shown to play a role in the development and transduction of pruritus. Such receptors are the pain and heat-sensitive TRPV1, -3, -4, and TRPA1 ion channels. However, the role of TRPM3 ion channel in pruritus has not been previously investigated.

Effect of anesthetics on the TRPM3 ion channel

Activation of several members of the superfamily of the voltage gated ion channels is influenced by VAs, and a number of these channels, in particular the hyperpolarization activated and cyclic nucleotide gated channel 1 (HCN1), shaker-related delayed rectifier K^+ channels (K_v1) and two-pore-domain K^+ channels (K_2P) have been implicated in the induction of general anesthesia. Voltage gated Na^+ and Ca^{2+} channels can also be inhibited by VAs. Moreover, recent studies reported the effect of VAs on thermosensitive TRP channels, as well. The cold- and menthol-activated TRPM8, after an initial activation, was inhibited by VAs. Likewise TRPC5, another cold-sensitive member of the TRP family, was inhibited by halothane and chloroform. The warmth sensor TRPM2 was not influenced by halothane or chloroform, whereas the noxious heat sensor TRPV1 was sensitized, or, if applied at higher concentration, even activated by VAs. Moreover, irritant VAs, isoflurane and desflurane directly activated TRPA1, a general target of several irritant chemicals, whereas the non-irritating halothane and sevoflurane did not induce TRPA1 activation. These results can explain some adverse effects often associated with general anesthesia induced by certain VAs. Indeed, irritant VAs evoke mechanical hyperalgesia and bronchoconstriction, impaired respiratory pattern, augmented laryngeal C-fiber activity and stimulate tracheal CGRP release mainly mediated by TRPA1.

In our current study, we investigated the effect of VAs on TRPM3, a less characterized thermo-nociceptive TRP channel, which together with TRPV1 and TRPA1 plays a crucial role in acute heat pain sensation. In contrast to the other two heat-pain sensors TRPV1 and TRPA1, TRPM3 was found to be neither sensitized nor activated by any of the investigated VAs. In contrast, activation of TRPM3 by both chemical ligands and heat was markedly inhibited by the investigated VAs. Among those, halothane was found to be the most potent, inhibiting PregS-evoked TRPM3 activity with an IC_{50} of approximately 0.5 mM,

equivalent to ca. 2-times the minimal alveolar concentration (2 MAC) that induces anaesthesia in different species. Although the IC_{50} of chloroform was slightly higher (ca. 1.67 mM), this value also corresponded to approximately 1.5 MAC. Other VAs were less effective in clinically relevant concentrations: the IC_{50} value for isoflurane (≈1.1 mM) is equivalent to about 3 MAC, whereas the IC_{50} of sevoflurane approached 10 MAC against PregS-evoked activation. Applied at 1 mM, each VA shifted the PregS activation curve of TRPM3 toward higher concentrations. Again, halothane was found to be the most effective, as it increased the EC_{50} of PregS approximately 50-fold. Importantly, at 1 mM, all investigated VAs inhibited the activation of native TRPM3 in sensory neurons of mouse DRGs, as well. Moreover, VAs inhibited not only the activity of TRPM3 induced by PregS, but they also inhibited the effect of the synthetic agonist CIM0216 and the heat-induced TRPM3 responses with very similar potencies. The sensitivity of TRPM3 toward some VAs seems to be slightly lower than sensitivity of ion channels generally believed to mediate anaesthesia. Clinically relevant concentrations (≈1 MAC) of volatile anaesthetics activate several members of the K_2P channel family known to conduct background K^+ currents, which crucially contribute to the negative membrane potential. For example, the EC_{50} of halothane and sevoflurane that induce TASK-1 mediated K^+ currents were 0.23 mM and 0.29 mM (near to 1 MAC), respectively. Moreover, NMDA receptor mediated currents were also effectively inhibited by isoflurane and sevoflurane with reported IC_{50} values between 0.25 and 1.3 MAC and ca. 1.25 MAC, respectively. The EC_{50} of isoflurane and sevoflurane potentiating GABA induced activity of $GABA_A$ receptor was found also around 1 MAC (0.29 mM and 0.33 mM, respectively). However, the potency of halothane inhibiting NMDA receptor mediated postsynaptic excitatory currents (IC_{50} = 0.57 mM, equivalent with ca. 2 MAC) and potentiating GABA induced $GABA_A$ currents (EC_{50} = 0.67 mM, ca. 2 MAC) were very close to the value we found for TRPM3.

Although there is a growing body of evidence suggest that VAs can directly affect the function of different TRP channels, we still have limited knowledge of the specific mechanism and the possible molecular binding sites. Previous researches targeting TRPV1 and TRPA1 has highlighted that the pore-forming domain may play an important role in VAs binding site, whereas other research raise the possibility of multiple binding sites for the TRPV1 ion channel. Although in the current project we did not investigate the potential binding site, our electrophysiological measurements indicated that VAs not only inhibited the transmembrane currents through the classical pore activated by the endogenous PregS, but they also reduced TRPM3 currents flowing through the alternative pathway activated by the CIM0216. These results clearly suggest that the investigated VAs are not selective for any of the pores, but they likely inhibit channel gating by promoting some activation-inhibiting conformation.

Investigating the effect of VAs on native TRPM3, we found that, applied in 1 mM, all the studied VAs inhibited the native TRPM3 ion channel expressed on sensory neurons of dorsal root ganglia. Our data also demonstrated that the TRPM3 inhibitory effect of VAs is reversible and can be washed away quickly.

Our results not only introduced TRPM3 as a new ion channel influenced by VAs, but also revealed new mechanisms of possible analgesic effects of VAs, and increased our knowledge about the pharmacological interactions of the TRPM3 ion channel. Thus, they can also provide valuable information for the development of new types of analgesics acting on TRPM3. Namely, inhibition of TRPM3, as confirmed by previous results, may be an effective strategy in alleviating pain and treating pain-related syndromes. Among TRPM3 inhibitors, the herbal flavovone derivative isosakuranetin and the antiepileptic drug primidone were indeed verified to inhibit pain sensation in animal models *in vivo*, as well.

Because the affinity of VAs to TRPM3 has been shown to be lower than to their other targets, such as $GABA_A$ and K_2P channels, their clinical use as an actual

TRPM3 inhibitor is limited. However, our results may provide valuable information for future research on TRPM3 as a potential target of novel analgesic drugs.

Investigation of the role of TRPM3 ion channel in pruritus

Severe acute and chronic itch is one of the most common dermatological symptoms in the world, which can ruin the lives of many patients. In recent years, high researches effort was made to identify the most important players, e.g. specific mediators, receptors, molecular interactions and signaling pathways in the development of pruritus, aiming at developing new therapeutic strategies in the treatment of pruritus. It has been an open question for a long time whether we can talk about an autonomous itch sensing (pruriceptive) system separated from the pain-sensing nociceptive pathways. Emerging evidence suggests that pruriceptive neurons form a subpopulation within nociceptive neurons, rather than forming a purely pruritogen-specific peripheral sensory neuron population, but the organization of nociceptive and pruriceptive sensory system is still unclear. Non-pruritogenic nociceptive neurons were identified to be unresponsive to pruritic chemical signals, and there are numerous attempts to identify itch-specific molecular markers. Such studies not only aim at identifying itch-specific/selective neurons and pathways but are also motivated by the medical need to identify molecular targets for pharmacotherapies selectively alleviating itch or pain. Several receptors activated by pruritic substances have been identified in sensory neurons innervating the skin of both humans and mice, e.g. the H1 histamine receptor, the chloroquinine sensitive G protein-coupled receptor (MRGPRA3), the BAM8-22 sensitive receptor (MRGPRC11) or the PAR2 receptor. TRP channels are long-chased targets for analgesic therapies but they seem to be promising targets in the management of pruritus, as well. Among TRP channels, the thermosensitive TRPV1 and TRPA1 are of special importance: they seem to be promiscuously expressed in nociceptive and pruriceptive neurons and were

shown to play role in the sensory transduction of both pain and itch. Moreover, other thermosensitive TRP channels can be also involved in the development of both itch and pain. TRPV4 and TRPV3 are expressed in non-neuronal cells of the skin, and can play roles in the release of endogenous pruritogens and algogens, especially related to inflammation. The role of TRPV4 was described in both allergic and non-allergic pruritus by mediating 5-HT release from mast cells and keratinocytes, respectively. Moreover, its activation in keratinocytes results in ET-1 release, as well, which is thought to play a role in sunburn-associated pain. Beyond 5-HT release, TRPV4 is also involved in the sensory transduction of 5-HT-mediated itch in the pruriceptive fibers, and as an osmo-mechanoreceptor it plays a role in the development of mechanical hyperalgesia. TRPV3 is also highly expressed by keratinocytes and its activation can contribute to inflammation and several forms of itch by inducing the release of inflammatory and pruritic mediators, similar to non-neuronal TRPV4.

Although relatively lot of data are already available about the role of different TRP channels in pruritus, the role of the thermosensitive nociceptor TRPM3 in pruritus has not been investigated yet. TRPM3 is activated by noxious heat and the endogenous neurosteroid PregS. Its selective activation results in neuropeptide release from the sensory terminals and evokes nociception in rodents. Certain ligands and ligand combinations open an extra permeability pathway in the channel, which results in a strong depolarizing current at negative membrane potentials and in the exacerbation of pain sensation, as well. In the nociceptive system, TRPM3 functions seem to partially overlap with other thermosensitive TRP channels. Together with TRPV1 and TRPA1, TRPM3 plays a crucial role in the sensory transduction of noxious heat sensation and it was also found to be involved in inflammatory heat hyperalgesia. Moreover, beyond the functional similarities, its expression in the somatosensory neurons of DRGs largely overlaps with TRPA1 and TRPV1. The functional and anatomical overlap, and the molecular relationship between these thermosensitive TRP channels led to the

plausible preposition that TRPM3 expressed by the somatosensory neurons could share even more functions with TRPV1 and TRPA1, for example in pruriception. As mentioned above, beyond nociception and thermosensation, TRPV1 and TRPA1 are also important players in itch transduction at the sensory terminals: they were shown to be involved in the detection of various forms of itch. For example, TRPV1 was found to be involved in histamine receptor and protease activated receptor 2 (PAR2) signaling and TRPA1 was shown to transmit the pruritic effect of 5-HT, bile acid, activators of Mas-Related G Protein–Coupled Receptors (Mrgprs), and thymic stromal lymphopoietin (TSLP).

Previous results uncovered the role of TRPM3 in heat-induced nociception and inflammatory warm hyperalgesia, and the analgesic effects of TRPM3 antagonists in animal models offer promising possibilities.

To investigate the potential role of the TRPM3 ion channel in the development and regulation of pruritus, *in vivo* mouse behavioral tests and *in vitro* methods for measuring changes in intracellular Ca^{2+} concentration were used. In our experiments, we used wild-type *Trpm3* $^{+/+}$ and *Trpm3* $^{-/-}$ C57Bl6 mice. Our *in vivo* results obtained in the cheek model further support the previous conclusion, and demonstrate that the selective pharmacological activation of TRPM3 resulted in nociception even in the cheek, i.e., in the innervation area of TGs, and this was abolished by the genetic ablation of the channel. As opposed to nociception, TRPM3 agonists did not induce scratching behavior in the animals, suggesting that TRPM3 activation did not evoke itch sensation on its own. Although its general activation resulted exclusively in pain, these findings do not exclude that expressed in certain subpopulation of the somatosensory neurons, TRPM3 can contribute to pruritic signaling and take part in the transduction of itch. Therefore, we investigated whether TRPM3 is necessary for the pruriceptive effect of highly relevant endogenous pruritic mediators, Hist, 5-HT and ET-1. We found that each mediator evoked similarly intense scratching in *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice

injected to either the cheek or the nape, skin areas that are innervated by neurons from the TG and the DRGs, respectively. In good accordance with the *in vivo* findings, the ratio of the trigeminal sensory neurons activated by Hist, 5-HT, and ET-1 was not affected by the deletion of *Trpm3*, although the investigated pruritogens activated both TRPM3 expressing (PregS+) and TRPM3 non-expressing (PregS-) neurons of wild type (*Trpm3*$^{+/+}$) animals. Moreover, the pharmacological blockade of TRPM3 by Isok affected neither the number of pruritogen responsive neurons nor the amplitude of their Ca^{2+} transients evoked by the itch mediators. These results strongly argue for that TRPM3 does not play any significant role in cellular signaling events evoked by Hist, 5-HT or ET-1 that result in the pruritic effect of these compounds.

Intriguingly, ET-1 induced more intense scratching in *Trpm3*$^{-/-}$ animals. This finding may be explained by the common observation and experimental findings that painful stimuli inhibit itch. Regarding our results, it is possible that the lack of TRPM3 results in decreased basal activity of the nociceptive neurons, which consequently leads to enhanced itch signaling in certain cases. It cannot be excluded that ET-1 itself causes a minor activation of the nociceptors which partially inhibit itch responses, but this inhibition is diminished in *Trpm3*$^{-/-}$ animals. ET-1 was also reported to mediate nociception, although in our experiments it initiated only moderate nocifensive behavior and was characterized by high scratch ratio as a mainly pruritogenic substance.

Our results led to the conclusion that TRPM3 is exclusively related to nociception but not itch transduction, since it was not involved in the transmission of the pruritic effect of key endogenous itch mediators (i.e., Hist, 5-HT, and ET-1). However, it cannot be excluded that TRPM3 might be necessary for itch evoked by some other mechanisms.

Our results further support the idea that TRPM3 represents a promising candidate target to specifically treat pain. Earlier results already showed that its genetic

ablation or pharmacological inhibition alleviates chemical and thermal nociception, as well as inflammatory pain in the innervation area of DRGs. Our results also demonstrated that PregS- or CIM0216-induced nociception is diminished in *Trpm3*$^{-/-}$ animals in the cheek model, in the trigeminal innervation area, as well.

As for most animal studies, it is important to consider to what extent we can translate these results to humans. In general, the cheek model can similarly discriminate between itch and nociception as subjective reporting of human subjects and the applied Hist, 5-HT, and ET-1 are known to induce itch both in mice and humans. Although, based on our best knowledge, effects of TRPM3 ligands were not published in human *in vivo* studies yet, the available pharmacological and cellular data suggest that the mouse and human wild type TRPM3 are functionally identical: they share agonists, antagonists, and regulation by phospholipids, as well as by βγ subunits of G proteins. These data suggest that selective targeting of nociception via TRPM3 may be a promising approach even in human analgesia.

Based on our *in vivo* and *in vitro* experimental results, it can be concluded that the TRPM3 ion channel is not involved in the development of endogenous pruritogen-induced itch in mice, and that its direct chemical activation contributes to the development of pain rather than pruritus.

SUMMARY

The molecular and functional properties of the TRPM3 ion channel, as well as it's role in thermosensation and nocicepton were described by our collaborators and by other independent research groups. In our current studies, we investigated the effect of volatile anaesthetics on TRPM3 *in vitro*, and studied its role in the development of histamine-dependent and –independent itch elicited by endogenous mediators using both *in vivo* and *in vitro* experiments. Using fluorescence based intracellular Ca^{2+} concentration measurements we found that VAs (chloroform, halothane, isoflurane and sevoflurane) effectively inhibited thermal and chemical activation of the recombinant TRPM3 in a dose-dependent manner. Among them, halothane was identified as the most effective TRPM3 inhibitor. In our studies we showed, that VAs applied in 1 mM concentration partially inhibited, while applied in 5 mM concertation totally abolished TRPM3 mediated transmembrane currents. Our studies revealed that anesthetics not only inhibited the classical pore of TRPM3 opened by PregS, but also inhibited transmembrane currents flowing through an alternative pore that opens in the presence of CIM0216. The TRPM3 ion channel expressed on sensory neurons isolated from the dorsal root ganglia of mice was also effectively and reversibly inhibited by the anesthetics we studied at a concentration of 1 mM.

Investigating the role of TRPM3 ion channels in pruritus, we found that it is not involved in the development of either histamine-dependent or histamine-independent pruritus. Using the *cheek* model, which method allowed us to separate behavioral responses related to pain and itch, we introduced a new parameter, the itch ratio ($R_{scratch}$). The $R_{scratch}$ gives the specificity of the compounds used in the cheek model on the itch-pain axis. In our experiments we demonstrated that TRPM3 ion channel agonists, like the endogenous PregS and exogenous CIM0216, induced exclusively pain in *Trpm3*$^{+/+}$ animals but not in the *Trpm3*$^{-/-}$ strain. Importantly, TRPM3 agonists did not induce pruritus in any

groups of animals. In contrast, endogenous pruritic mediators induced similar intense scratching in both *Trpm3*$^{+/+}$and *Trpm3*$^{-/-}$ strains. Our *in vivo* behavioral results were validated by *in vitro* intracellular Ca^{2+} concentration measurements on sensory neurons isolated from TGs of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice. We have shown that genetic ablation or pharmacological inhibition of TRPM3 did not influence the effect of the studied prutiogenic substances arguing for the conclusion that TRPM3 is not involved in the transduction of pruritic signals.

APPENDIX

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List of publications related to the dissertation

1. **Kelemen, B.**, Pinto, S., Kim, N., Lisztes, E., Hanyicska, M., Vladár, A., Oláh, A., Pénzes, Z., Shu, B., Vriens, J., Bíró, T., Rohács, T., Voets, T., Tóth, I. B.: The TRPM3 ion channel mediates nociception but not itch evoked by endogenous pruritogenic mediators.
   Biochem. Pharmacol. 183, 1-11, 2021.
   DOI: http://dx.doi.org/10.1016/j.bcp.2020.114310
   IF: 4.96 (2019)
2. **Kelemen, B.**, Lisztes, E., Vladár, A., Hanyicska, M., Almássy, J., Oláh, A., Szöllősi, A. G., Pénzes, Z., Posta, J., Voets, T., Bíró, T., Tóth, I. B.: Volatile anaesthetics inhibit the thermosensitive nociceptor ion channel transient receptor potential melastatin 3 (TRPM3).
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   DOI: http://dx.doi.org/10.1016/j.bcp.2020.113826
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List of other publications

3. Kemény, Á., Kodji, X., Horváth, S., Komlódi, R., Szőke, É., Sándor, Z., Perkecz, A., Gyömörei, C., Sétáló, G., **Kelemen, B.**, Bíró, T., Tóth, I. B., Brain, S. D., Pintér, E., Gyulai, R.: TRPA1 Acts in a Protective Manner in Imiquimod-Induced Psoriasiform Dermatitis in Mice.
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