Biochemical Pharmacology

The TRPM3 ion channel mediates nociception but not itch evoked by endogenous pruritogenic mediators

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Abstract:	During the molecular transduction of itch, the stimulation of pruriceptors on sensory fibers leads to the activation or sensitization of ion channels, which results in a consequent depolarization of the neurons. These ion channels mostly belong to the transient receptor potential (TRP) channels, which are involved in nociception and thermosensation. In particular, TRPV1 and TRPA1 were described in the transduction of both thermal nociception as well as histaminergic and non-histaminergic itch. The thermosensitive TRPM3 plays an indispensable role in heat nociception together with TRPV1 and TRPA1. However, the role of TRPM3 in the development of pruritus has not been studied yet. Therefore, in this study we aimed at investigating the potential role of TRPM3 in the transduction of pruritus and pain by investigating itch- and nociception-related behavior of Trpm3+/+ and Trpm3-/- mice, and by studying the activation of somatosensory neurons isolated from trigeminal ganglia upon application of algogenic and pruritogenic substances. Activators of TRPM3 evoked only nocifensive responses, but not itch in Trpm3+/+ animals, and these nocifensive responses were abolished in the Trpm3-/- strain. Histamine and endogenous non-histaminergic pruritogens induced itch in both Trpm3+/+ and Trpm3-/- mice to a similar extent. Genetic deletion or pharmacological blockade diminished TRPM3 mediated Ca2+ responses of sensory neurons, but did not affect responses evoked by pruritogenic substances. Our results demonstrate that, in contrast to other thermosensitive TRP channels, TRPM3 selectively mediates nociception, but not itch sensation, and suggest that TRPM3 is a promising candidate to selectively target pain sensation.

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21 October 2020

To: S.J. Enna
Editor-in-Chief, Biochemical Pharmacology

Dear Professor Enna,

Hereby, we are submitting the revised version of our manuscript **BP-D-20-01533** entitled "**The TRPM3 ion channel mediates nociception but not itch evoked by endogenous pruritogenic mediators**" for publication in *Biochemical Pharmacology*.

This paper is an original work and is not under consideration for publication elsewhere. All authors have read and approved the manuscript, its content, and its submission to the *Biochemical Pharmacology*. By this submission, we declare our willingness to pay to pay all applicable charges and fees in case the manuscript is accepted for publication

We are very grateful for the positive evaluation of our manuscript, and all the constructive critiques and the very useful comments of the Editor and the Reviewers on the original version. We followed their instructions and revised the original manuscript. To improve the quality of the study and address all the requests we initiated the following changes:

- We carefully checked and added the missing suppliers and their locations for all chemicals used.
- We provided gray scale version of the figures for the printed issue as detailed in our response to the editorial query.
- We added a new data to Figure 1E and a new panel Figure 1F, as requested by the Reviewer 1
- We inserted vehicle group data into Figure 2A-D.
- We recalculated the statistics and added extra statistical analysis ti Figure 1 and Figure 2, as requested by the reviewers
- We provided corresponding information into figure legends.
- We added the requested information to the Materials and methods.
- We added new paragraphs to the text and related references cited in which we discuss the issues raised by the reviewers.
- We removed redundancies and corrected the discovered typos in the MS.
- We changed the term "pain" to "nociception" when discussing animal behavior.

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To help in following our corrections, we clearly highlighted all changes with red coloration in the text.

Beyond the revision of the manuscript, we answered the questions and comments of the Editor and the Reviewers point-by-point, explaining all in details. Please find it below attached to this letter.

We hope, that the Editor and the Reviewers will appreciate our efforts and will agree, that the revised manuscript is substantially improved. We hope that you can accept it for publication in its current form in your distinguished journal.

We are greatly looking forward to your feedback.

Best regards,

Balázs István Tóth

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Answers to the comments of the Editor and the Reviewers

Editor comments:

1. Please confirm that the suppliers and their location are provided in the manuscript text for every chemical used in this study.

We carefully checked and corrected our manuscript and confirm that the suppliers and their location are provided for every chemical used in the study.

2. As you may be aware, Elsevier publishes all color figures for free in the web-based version of the journal (see "Publisher's Note" Volume 65, Issue 5, 1 March 2003, Page v). However, authors are required to pay the hard copy production costs for color illustrations in the printed version. The cost for each color figure is EU€295 (~US$425) effective January, 2009 plus applicable VAT (tax).

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Indicate in your response to the reviewers' letter if you agree to the charges associated with publishing the color figure(s) OR you prefer for the figure(s) to appear in the printed version of the journal in grayscale, OR you are providing two versions of the figure(s) (one set in color and one set in grayscale) to accommodate both.

Thank you for calling attention for the possibilities. We would prefer color figures in the online version and grayscale figures in the printed versions. We have provided both version of the figures with the current submission.

Reviewers' comments:

Reviewer #1: The authors examined whether the TRPM3 ion channel plays a role in pain and itch modulation (through histaminergic and non-histaminergic mechanisms) as previously reported for TRPV1 and TRPA1 channels.

Major concerns:

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1. The authors should explain why they chose to use pregnenolone sulfate and CIM0216 as TRPM3 receptor activators. It would seem that CIM0216 should be the agonist for all the experiments as the steroid-like nature of pregnenolone makes it likely to interact with more sites than just TRPM3, complicating interpretation of the findings.

Thank you for your comment. Pregnenolone sulfate (PregS) was identified as a TRPM3 agonist in 2008 (Wagner et al., Nat Cell Biol, 2008, doi: 10.1038/ncb1801) and since then it is the most widely used and therefore best characterized agonist of the channel. Although it can act on other, TRPM3-independent targets as well (for review see: Harteneck, Molecules, 2013, doi: 10.3390/molecules181012012; Smith et al, Psychopharmacology, 2014, doi: 10.1007/s00213-014-3643-x), PregS is highly relevant to investigate the physiological function of TRPM3 for multiple reasons: (1) PregS is an endogenous activator of TRPM3. Although in most of the in vitro studies it is applied at pharmacological concentration to activate TRPM3 at ambient temperature, in our previous study (Vriens et al, Neuron, 2011, doi: 10.1016/j.neuron.2011.02.051) we have shown that PregS at low concentrations (100 nM) evokes robust TRPM3 activation at 37°C, which is in the range of the observed physiological plasma concentrations. (2) The mechanism of action how PregS activates TRPM3 is very similar to the action of temperature in physiological circumstances. Both PregS and heat evokes a strongly outwardly rectifying current mediated by the opening of the canonical pore of TRPM3, and they did not induce opening of the alternative permeation pathway (Vriens et al, Nat Chem Biol, 2014, doi: 10.1038/nchembio.1428). (3) PregS exerts its effects in DRG neurons predominantly through TRPM3, as Ca^{2+} signals are eliminated in the vast majority of DRG neurons of *Trpm3*$^{-/-}$ mice, even though a small percentage of neurons display some reduced amplitude Ca^{2+} signals (Vriens et al, Neuron, 2011, doi: 10.1016/j.neuron.2011.02.051; Held et al, PNAS, 2015, 10.1073/pnas.1419845112; Kelemen et al, Biochem Pharmacol, 2020, doi: 10.1016/j.bcp.2020.113826). Moreover, the PregS induced nociception is practically abolished in the *Trpm3*$^{-/-}$ strain as shown by our current (Fig. 1.) and previous results (Vriens et al, Neuron, 2011, doi: 10.1016/j.neuron.2011.02.051). In contrast, CIM0216 is a potent exogenous agonist which also opens the alternative permeation pathway of TRPM3 (Held et al, PNAS, 2015, 10.1073/pnas.1419845112). Although the opening of this alternative pathway results in exacerbation of nocifensive responses (Vriens et al, Nat Chem Biol, 2014, doi: 10.1038/nchembio.1428; Held et al, PNAS, 2015, 10.1073/pnas.1419845112) and can contribute to the development of pathological conditions in certain mutations (Van Hoeymissen et al, eLife, 2020, doi: 10.7554/eLife.57190; Zhao et al, eLife, 2020, doi: 10.7554/eLife.55634), its activation has not been described yet by endogenous activators or temperature in the wild type channel. Moreover, although CIM0216 is very potent and relatively selective activator of TRPM3 over other TRP channels, it moderately activates TRPA1, as well (Held et al, PNAS, 2015, 10.1073/pnas.1419845112). It induces Ca^{2+} signals even in some sensory neurons isolated from *Trpm3*$^{-/-}$ animals, likely via a TRPA1-mediated pathway. Therefore, we do not think that CIM0216 is superior to PregS in *in vitro* applications and our results obtained using PregS are better comparable with previous findings.

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2. The authors also need to justify the concentrations of histamine, serotonine and endothelin-1 used in the cheek (5 ug/ml, 1 ug/ml and 15 ng/ml) and nape (4 ug/ml, 0.2 ug/ml y 5 ng/ml) assays to help clarify why when using lower concentrations than in the cheek assay a greater enhancement of scratch is obtained.

The applied doses correspond to previous literature data in case of both the cheek and the nape assays as indicated in the tables below. Although the concentrations applied in the nape assay were indeed lower, the doses (i.e. the total amount of the compound) were equal, or higher in the nape assay than applied in the cheek. Moreover, we did not aim at quantitatively, statistically comparing results obtained in the two assays. We consider them as two independent paradigms because of several differences e.g. anatomical differences in site of administration, innervation (DRG vs. TG), applied/tolerable volume, potential differences in behavioral responses, etc. Therefore, when the experiments were designed, we aimed at following the available literature in both assays for better comparability with previous results. However, we carried out some pilot experiments in a few wild type animals (also covered by the cited ethical approvals), which also influenced the final experimental design. For example, in the wild type strain, we also tested 10 µg / 10 µl histamine in the cheek assay which was found less effective at evoking scratching responses. Therefore, we decided to apply a higher dose. 50 µg / 50 µl serotonin caused mild gastrointestinal symptoms, which affected the scratching responses in the nape assay. Therefore we decided to apply a lower dose in the final experiments. Importantly, during the final experiments, we did not observe any side effects that might influence the behavioral responses.

Table 1: Application of the tested compounds in the "Cheek assay"

Our application		**Literature data**		
Dose	***Concentration***	***Dose***	***Concentration***	***Reference***
HISTAMINE				
50 µg	**5 µg/µl**	50 µg	5 µg/µl	Shimada & LaMotte, 2008, doi.org/10.1016/j.pain.2008.08.002
		60 µg	3 µg/µl	Morita et al, 2015, 10.1016/j.neuron.2015.05.044
		100 µg	10 µg/µl	Kido-Nakhara, 2014, 10.1172/JCI67323
		5-50 µg	0,5-5 µg/µl	Gomez et al, 2012, 10.1016/j.lfs.2012.03.020
SEROTONIN				
10 µg	**1 µg/µl**	0.352-3.52 µg	0.0176-0.176 µg/µl	Morita et al, 2015, 10.1016/j.neuron.2015.05.044

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		10 µg	1 µg/µl	Akiyama et al, 2019, 10.1038/JID.2015.388
ENDOTHELIN 1				
150 ng	**15 ng/µl**	249 ng	24.9 ng/µl	Kido-Nakhara, 2014, 10.1172/JCI67323
		150 ng	15 ng/µl	Gomez et al, 2012, 10.1016/j.lfs.2012.03.020

Table 2: Application of the tested compounds in the "Nape assay"

Our application		**Literature data**		
Dose	***Concentration***	***Dose***	***Concentration***	***Reference***
HISTAMINE				
200 µg	**4 µg/µl**	100 µg	10 µg/µl	Mishra & Hoon, 2013, 10.1126/science.1233765
		100 µg	5 µg/µl	Kardon et al, 2014, 10.1016/j.neuron.2014.02.046
		500 µg	5 µg/µl	Liu et al, 2012, 10.1172/JCI45414
		150 µg	3 µg/µl	Morita et al, 2015, 10.1016/j.neuron.2015.05.044
		500 µg	10 µg/µl	Kühn et al, 2020, 10.1038/s41598-020-59092-2
		500 ug	50 µg/µl	Pandey et al, 2017, 10.1097/j.pain.0000000000000860
		1.11 mg	11 ug/µl	Liang et al, 2011, 10.1016/j.neulet.2011.02.009
		500 µg	10 µg/µl	Huang et al, 2018, 10.1016/j.jid.2018.02.019
		30-50 µg	3-5 µg/µl	Akiyama et al, 2019, 10.1038/JID.2015.388
SEROTONIN				
10 µg	**0.2 µg/µl**	30 µg	3 µg/µl	Mishra & Hoon, 2013, 10.1126/science.1233765
		30 µg	1.5 µg/µl	Kardon et al, 2014, 10.1016/j.neuron.2014.02.046
		20 µg	0.4 µg/µl	Liu et al, 2012, 10.1172/JCI45414
		0.88-8.8 µg	0.0176-0.176 µg/µl	Morita et al, 2015, 10.1016/j.neuron.2015.05.044
		10 µg	0.2 µg/µl	Kühn et al, 2020, 10.1038/s41598-020-59092-2

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		10 µg	1 µg/µl	Pandey et al, 2017, 10.1097/j.pain.0000000000000860
		8.2-10 µg	0.82-1 µg/µl	Akiyama et al, 2019, 10.1038/JID.2015.388
ENDOTHELIN 1				
250 ng	**5 ng/µl**	25 ng	2.5 ng/µl	Mishra & Hoon, 2013, 10.1126/science.1233765
		125ng	2.5 ng/µl	Kühn et al, 2020, 10.1038/s41598-020-59092-2
		25 ng	0.5 ng/µl	Liu et al, 2012, 10.1172/JCI45414
		25 ng	2.5 ng/µl	Pandey et al, 2017, 10.1097/j.pain.0000000000000860
		250 ng	2.5 ng/µl	Liang et al, 2011, 10.1016/j.neulet.2011.02.009

3.To more clearly define the intensity level of the itching-inducing response the authors should include the vehicle group in Figure 2, A-D sections, as it is only included in E.

For the figure 2 A-D, the vehicle group was the same as used and presented in Figure 1 A-D. In the originally submitted version, we did not insert these data again into the Figure 2 to decrease redundancy. In Figure 2, we provided the results of the vehicle group only in Figure 2 E, because it shows the result from another technique (nape model). But we totally agree with the reviewer's comment and have added the control data to figure 2 A-D. in the current revised version. For better clarity, we mention in the figure legend that the vehicle groups are the same in Figure 1 A-D and Figure 2 A-D.

Minor comments:

1.The authors need to justify the use of only male mice for the itch and pain experiments.

In our pilot experiments when we set the injection protocol, we also tested a few female mice and did not observe marked differences in their responses to histamine and capsaicin compared to male mice. In the final experiments, we followed the original experimental design of Shimada & LaMotte (Pain, 2008, doi.org/10.1016/j.pain.2008.08.002) and used only male mice in the cheek assays to eliminate any potential influence of the estrus cycle on the scratching behavior.

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2. In the whole manuscript, please use The authors should use only 'nocicpetion' throughout the report, deleting their references to 'pain' as the latter is a term that should be reserved for human studies only.

Thank you for your comment. We have corrected the MS accordingly. We replaced “pain” with “nociception” especially when reporting our own results from animal studies. However, in the introduction and in the discussion sometimes we kept the term “pain” or pain sensation when it refers in general or specially for human sensory phenomena.

3. The authors need to briefly explain in Material and Methods the animal shaving conditions (substance/machine/day) as this could influence pain and itch related behaviors.

Thank you for your comment. The shaving of the mouse cheek always happened a day before the experiment. The mouse was held tightly by the experimenter and the fur was shaved at the site of the administration with a small electric Aesculap Isis hair clipper, with a single movement, carefully avoiding the whiskers and not hurting the skin. The skin of mice involved in the experiment was never injured during shaving. The nape was also shaved a day in advance to the experiment, very similar to the cheek. The experimenter restrained the mouse with one hand and carefully shaved the nape area behind the ears using small electronic clippers (PepPet, Guangdong, China). Care was taken to avoid skin and ear injury. We added these details to the revised MS.

4. The authors need to justify in Materials and Methods the use of pregnenolone sulfate, capsaicin, CIM0216, histamine, serotonin, endothelin-1 and cinnamaldehyde. That this, they need to specify whether each is TRPM3 agonists or itching inducer (histaminergic and non-histaminergic).

Thank you for your comment. We added the requested information to the Materials and Methods. Pregnenolone sulfate and CIM0216 were used as TRPM3 agonists, capsaicin was used as a TRPV1 agonist, cinemmaldehyde as a TRPA1 agonist and histamine, serotonin, and endothelin served as endogenous pruritogenic mediators.

5. There are sentences in Results repeated that are identical to those appearing in Material and Methods.

Thank you for the comment we have corrected the redundancies.

6. The authors need to be consistent when assessing statistical differences among data shown on figures. For example, the comparisons against the vehicle group and between the same treatment group, but different TRPM3 expression (+/+ vs -/-), are shown on Figure 1A-C, but not Figure 1D.

Thank you for your comment. We added the requested statistical comparisons to Figure 1D. To be consistent when displaying statistical differences among the figures, we also added the

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statistical comparison against the vehicle group within the same genotype. To be even more consistent, we also corrected the post hoc analysis used after Kruskal-Wallis ANOVA: we replaced Dunn-Bonferroni test with Mann-Whitney test (as we used when comparing *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ groups within the same treatment) with a Bonferroni correction for multiple groups (https://www.ibm.com/support/pages/post-hoc-comparisons-kruskal-wallis-test).

7. The authors need to be consistent in graphs in their use of '(+/+ vs -/+)' or 'WT vs KO'.

Thank you, we have corrected and used +/+ or -/- overall in the MS.

8. The statistical tests performed for part D and E is not indicated in the legend to Figure 1.

Thank you for the comment. We have added the requested information.

9. The authors need to include the CIM time course in Figure 1E and include the significant differences in the 0-5 min period.

Thank you for your suggestion. We added the CIM0216 dataset to the figure. For better visibility, we provided the significant differences within both genotypes in a new panel (panel F) in the Figure. Other relevant statistics are included in the corresponding text in the Results section.

10. The authors need to use the same scale on Figure 2B as Figure 2E to facilitate the comparisons specified in the text.

Thank you for your comment. We changed the scale in Fig 2B and Fig 2E as requested. Moreover, we also arranged the scale in Fig 1B accordingly. However, we consider the cheek and nape assays as two independent paradigm, as detailed above.

Reviewer #2: BP-D-20-01533

Kelemen et al describe differential responses to allogenic and pruritogenic challenges in TRPM3+/+ vs TRPM3-/- mice leading them to conclude that, in contrast to other thermoactivated TRP channels, TRPM3 appears to not to be involved in itch-related behavioural responses.

Minor concerns:

1. The image quality of the figures is poor. These need to be improved to enhance clarity and understanding.

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We apologize for the low quality of the figures. Originally we have submitted high resolution .tiff files. Probably the conversion into .pdf format resulted in a decreased quality. In the revised version we submitted the figures in vector graphic .eps format. We hope it improves the visibility and clarity of the figures even in the .pdf version

2. Itch can be triggered/aggravated by non-noxious heat whereas activation of pain pathways occurs at noxious heat levels. The authors need to discuss the physical properties of TRPM3 in this context and explain how the experimental design takes this into consideration.

The chemical agonists used in our study activate TRPM3 at room temperature as well as at body temperature. Although TRPM3-mediated cellular responses are potentiated already at 33 °C, its activation is more prominent by noxious heat. Compared to TRPV1, the current-temperature relationship curve of TRPM3 is shifted slightly towards higher temperatures (Vriens et al, Nat Rev Neurosci, 2014, doi: 10.1038/nrn3784). In line with this characteristic, behavioral experiments verified that TRPM3 plays an essential role in painful heat sensation, but its genetic ablation had only moderate effect on warm sensation in neutral temperature zone (Vriens et al, Neuron, 2011, doi: 10.1016/j.neuron.2011.02.051; Vandewauw et al, Nature, 2018, doi: 10.1038/nature26137; Paricio-Montesinos, Neuron, 2020, doi: 10.1016/j.neuron.2020.02.035). In general, painful stimuli (including heat) are known to inhibit itch (for a current review see Chen and Sun, Nat Commun, 2020, doi: 10.1016/j.neuron.2010.02.025). In contrast, moderate warming can indeed amplify pruritus, as reported especially in atopic dermatitis (Fruhstorfer, Pain, 1986, doi: 10.1016/0304-3959(86)90048-5; Murota et al J Allergy Clin Immunol. 2012, doi: 10.1016/j.jaci.2012.05.027), although the effect of temperature changes on itch is controversial. The role of thermosensitive ion channels in this sensitization is not revealed in details, but a recent study described that warmth induced pruritus and pruritogen release from atopic keratinocytes is mediated by TRPV3 (Seo et al, J Invest Dermatol, 2020, doi: 10.1016/j.jid.2020.02.028). In our study, we did not aim at investigating warm induced pruritus. Therefore, our experiments were carried out at constant room temperature. However, it can be considered that compounds act at body temperature when applied *in vivo*.

3. The authors need to indicate whether tunnelling has been reported previously for similar animal models. If so, they need to provide details and conclusion from this work..

Several behavioral signs are associate with pain/nociception in experimental animals. Therefore, the assessment and objective, quantitative measurement of pain is challenging (Recognition and Alleviation of Pain in Laboratory Animals, National Academies Press (US), 2009, ISBN-13: 978-0-309-12834-6) The advantage of the cheek model is a clear distinction between pain related (wipes) and non-pain related behavior (scratch, grooming) which are relatively easy to quantify (Shimada and LaMotte, Pain, 2008, doi: 10.1016/j.pain.2008.08.002). However, it is based on the quantification of one specific behavior and may ignore other behavioral signs of pain/nociception. Taking into account this limitation,

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we thought it is important to report other observed behavioral signs of pain, even if those are not involved in the quantitative analysis. Based on our best knowledge, previous publications assessing pain behavior in the cheek model are restricted to the quantification of wipes (as a pre-defined measure) and did not report additional signs of nociception. However, we think that the observed "tunneling" (and the following lethargic behavior) may be related to pain sensation/nociception as a form of counter irritation, analogue to pressing or rubbing a painful area aiming at alleviating pain.

4. The authors need to source of the animals used in this study.

The *Trpm3*$^{+/+}$ BL/6J strain was originally purchased from the Janvier labs (Le Genest Saint Isle, France) and the *Trpm3*$^{-/-}$ strain was established in our laboratory at KU Leuven (Leuven, Belgium) as described in our previous publication (Vriens et al, Neuron, 2011, doi: 10.1016/j.neuron.2011.02.051). We added the requested content to the methods section.

5. The authors need to justify their use of only male mice studying itch and pain behaviour in the cheek assay.

In our pilot experiments, when we set the injection protocol, we also tested a few female mice and did not observe marked differences in their responses to histamine and capsaicin compared to male mice. In the final experiments, we followed the original experimental design of Shimada & LaMotte (Pain, 2008, doi.org/10.1016/j.pain.2008.08.002) and used only male mice in the cheek assays to eliminate any potential influence of the estrus cycle on the scratching behavior.

6. An explanation needs to be provided as to why compounds were dissolved in divalent free buffer.

Divalent free saline is a generally used vehicle during in vivo injections. Choosing the vehicle, we followed the original protocol of Shimada & LaMotte (Pain, 2008, doi.org/10.1016/j.pain.2008.08.002original) who described the cheek model.

7. The current manuscript contains a number duplications, typos and spelling errors, undermining the quality of the report.

Thank you for your comment. We have carefully checked the MS text and corrected typos and spelling errors, and removed duplications decreasing the redundancy in the text.

8. As 50uL seems like a very large volume to inject in a mouse nape the authors need to provide references in support of this protocol.

We carefully checked the literature prior to designing the experiments, and we found that compounds injected into the nape of mice are generally applied in 10-100 µl in itch studies (for references please see the above table summarizing previous data about applications in nape assay). We have tested 50 µl in our pilot experiments and did not observe any adverse effect

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related to the volume applied. Importantly, vehicle injected in the same volume did not induce any scratching or marked disadvantageous behavioral signs that might potentially influence the findings.

9. The authors need to explain the rationale and repercussions of using different Ca2+ dyes (Fura-2-AM and Fluo-4-AM) in different experiments.

We used two fluorescent Ca^{2+} indicator dyes for practical reasons. Comparing the responsiveness of sensory neurons isolated from *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ animals (Figure 3) we used Fura-2-AM with a compatible imaging setup in the laboratory of Prof. Voets at KU Leuven, Belgium. The potential role of TRPM3 in the cellular responses evoked by the pruritogen compounds (Figure 4) was investigated using Fluo-4-AM with a setup available in the Dept. of Physiology, University of Debrecen, Hungary that is optimized for that indicator. Importantly, the two series of experiments were carried out and analyzed independently and signal amplitudes obtained using the different dyes are never compared to each other. However, both dyes possess a Kd value for Ca^{2+} in submicromolar range, and are therefore equally suitable to detect relevant Ca^{2+} signals.

10. The authors need to make certain all abbreviations and acronyms are defined on first use.

We checked the MS and provided the definition of all abbreviations on first use.

11. P22/L7: What does the phrase "…inflammatory pain evoked at the body surface innervated form DRGs…" mean? This needs to be explained or re-written for clarity.

Thank you for your comment. We apologize for the ambiguous phrase. In this sentence, we wanted to explain, that the different forms of the pain are generated in that part of the skin, which part is innervated from the DRGs. We rephrased the sentence as: “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.”

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BIOCHEMICAL PHARMACOLOGY

The TRPM3 ion channel mediates ~~pain~~ nociception but not itch evoked by endogenous pruritogenic mediators

by

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ABSTRACT

During the molecular transduction of itch, the stimulation of pruriceptors on sensory fibers leads to the activation or sensitization of ion channels, which results in a consequent depolarization of the neurons. These ion channels mostly belong to the transient receptor potential (TRP) channels, which are involved in nociception and thermosensation. In particular, TRPV1 and TRPA1 were described in the transduction of both thermal ~~pain~~ nociception as well as histaminergic and non-histaminergic itch. The thermosensitive TRPM3 plays an indispensable role in heat nociception together with TRPV1 and TRPA1. However, the role of TRPM3 in the development of pruritus has not been studied yet. Therefore, in this study we aimed at investigating the potential role of TRPM3 in the transduction of pruritus and pain by investigating itch- and ~~pain~~nociception-related behavior of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice, and by studying the activation of somatosensory neurons isolated from trigeminal ganglia upon application of algogenic and pruritogenic substances. Activators of TRPM3 evoked only nocifensive responses, but not itch in *Trpm3*$^{+/+}$ animals, and these nocifensive responses were abolished in the *Trpm3*$^{-/-}$ strain. Histamine and endogenous non-histaminergic pruritogens induced itch in both *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice to a similar extent. Genetic deletion or pharmacological blockade diminished TRPM3 mediated Ca^{2+} responses of sensory neurons, but did not affect responses evoked by pruritogenic substances. Our results demonstrate that, in contrast to other thermosensitive TRP channels, TRPM3 selectively mediates ~~pain~~ nociception, but not itch sensation, and suggest that TRPM3 is a promising candidate to selectively target pain sensation.

KEYWORDS

nociception; itch; TRP channels; TRPM3; cheek model; endogenous pruritogens

HIGHLIGHTS

- Thermosensitive TRPV1 and TRPA1 play role in the transduction of pain and itch
- Thermo-nociceptor TRPM3 ion channel has not been investigated in itch sensation yet
- TRPM3 agonists ~~cause pain~~ result in nociception but not itch in Trpm3$^{+/+}$ mouse using the cheek model
- Endogenous mediators evoke itch independently of TRPM3
- Endogenous pruritogenic mediators activate sensory neurons independently of TRPM3

1. INTRODUCTION

Although pain and itch are associated with clearly different subjective sensations, their general definitions emphasize common features: both can be described as an unpleasant sensation associated with protective somatosensory responses[1]. Our knowledge about how the somatosensory system manages pain and itch sensation have advanced a lot in the last two decades, but there are still several controversies and open question regarding the relationship between nociception and pruriception, as well as the molecular mechanisms underlying their sensory transduction. Nowadays the "selectivity theory of itch" is increasingly accepted, which states that a subpopulation of nociceptive fibers innervating the skin can be activated by various pruritogenic substances, and that the selective activation of these fibers results in itch sensation, whereas the more general activation of nociceptors evokes pain[2]. Although serious efforts were made ~~there have been several attempts~~ to ~~identify~~ reveal molecular markers of itch-specific neurons[3–7], the molecular mechanisms of the sensory transduction of painful and pruritic stimuli seems to overlap[8–13].

The activation of nociceptors is initiated by the opening of nociceptive ion channels, including thermosensitive transient receptor potential (TRP) channels, acid sensing ion channels (ASICs)[14] or P2X ionotropic purinoreceptors[15–17], which results in depolarization and consequent discharge of the nociceptive neurons. These ion channels can be directly activated by painful stimuli (extreme temperatures, acidosis or pain-evoking chemical ligands) or indirectly, via intracellular signaling pathways initiated by algogenic or inflammatory signals[18]. Thermosensitive TRP channels play an especially important role as multimodal integrators of various painful stimuli[19]. For example, TRP vanilloid 1 (TRPV1), probably the most studied thermo-TRP channel, is directly activated by noxious heat, acidosis or its potent chemical ligand

capsaicin (each evoking pain) and can also be sensitized by inflammatory mediators[20–25]. Beyond TRPV1~~,~~ and TRPA1, TRPM8 and the recently characterized TRPM3 also play important roles in the transduction of thermal, chemical and inflammatory pain in somatosensory neurons[19,26–32]. Although the activation of pruriceptive (itch sensitive) sensory neurons is also related to the activation of ion channels partially overlapping with the nociceptor channels, they are typically activated via an indirect way: pruritic ligands bind to their metabotropic receptors thereby activating the pruritic channels via downstream signaling pathways[8,9]. A particular role of the heat-pain mediating TRPV1 and TRPA1 was also described in the transduction of histaminergic and non-histaminergic itch[11,12,33–36].

TRPM3 was recently identified as a heat sensitive TRP channel expressed by somatosensory neurons of the trigeminal and dorsal root ganglia (TGs and DRGs). Its chemical activators evoke ~~pain~~ nocieption, and the channel also plays a role in inflammatory thermal hyperalgesia[30,32,37]. Together with the above mentioned TRPV1 and TRPA1, it has an essential ~~crucial~~ contribution to the ~~sensation of~~ heat-induced ~~pain~~ nociception[38]. However, in contrast to TRPV1 and TRPA1, the potential role of TRPM3 in itch was not ~~yet~~ investigated yet. Therefore, in this study we aimed at describing its role in ~~pain~~ nociception and itch sensation by comparing itch-related and ~~pain-related~~ nocifensive behavior in wild type (*Trpm3*$^{+/+}$) and TRPM3 deficient (*Trpm3*$^{-/-}$) mice *in vivo*, and by exploring the responses of their sensory neurons to algogenic TRP ligands and well known endogenous pruritogens *in vitro*.

2. MATHERIAL AND METHODS

2.1. Animals

8-14 week-old wild-type (*Trpm3*$^{+/+}$, from Janvier labs, Le Genest Saint Isle, France) and TRPM3 deficient (*Trpm3*$^{-/-}$, established in our laboratory at KU Leuven (Leuven, Belgium) as described in our previous publication)[32] C57BL/6J mice weighted 20-30 g were used in all experiments. Only male mice were used to study itch and ~~pain~~ nocifensive behavior in the cheek assay to eliminate any potential influence of the estrus cycle on the scratching behavior, and male and female mice were used in other experiments. Mice were housed in a conventional animal facility at constant 21 °C in a 12-h light–dark cycle with unrestricted access to food and water. All animals were drug and test naïve prior being recruited to experiments. All experiments using animals were carried out in accordance with the European Union Community Council guidelines and approved by the KU Leuven Ethical Committee for Animal Experimentation under project number P021/2018 or by the Institutional Animal Care and Use Committee at Rutgers New Jersey Medical School.

2.2. Behavior assays

2.2.1. Cheek-assay

The itch-induced scratching and ~~pain~~ nociception-related behavior was selectively assessed using the cheek model paradigm[39]. Mice were habituated to a plexiglass recording chamber and the observation room for 30 min, once daily during the week before testing. The fur of the affected cheek was shaved a day in advance of the experiment. The mouse was held tightly by the experimenter and the fur was shaved with a single movement using a small electric hair clipper (Aesculap Isis from Aesculap Suhl GmbH, Shul, Germany), carefully avoiding the whiskers and not hurting the skin. On the day of the experiment, mice were randomly allocated into experimental

groups by a person who was not involved in the further investigation. Mice were placed into the plexiglass recording chamber and video recording was started. 10 minutes later, investigated compounds were applied s.c. via microinjections into the cheek using a 30G needle attached to a 1 ml insulin syringe. The applied doses were selected based on previous literature data and tested in pilot experiments. The following compounds were applied: 10 µg pregnenolone sulfate (PregS), 10 µg capsaicin (Caps), 5 µg CIM0216 (all from Tocris, Bristol, UK), 50 µg histamine (Hist), 10 µg serotonin (5-HT) and 150 ng endothelin-1 (ET-1) (all pruritogens from Sigma-Aldrich, St.Louis, MO, USA) each dissolved in 10 µl of Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) with 7% of Tween-80 (Sigma-Aldrich~~, St.Louis, MO, USA~~) and injected as a 10 µl volume[39]. Then, mice were placed back into the recording chamber, the experimenter left the room and spontaneous behavior was captured for an additional 30 minutes after the injection. 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. One bout of scratching was defined as an episode in which a mouse lifted its hind paw and scratched continuously for any length of time, until the paw was returned to the floor or to its mouth. During treatment and behavioral scoring, investigators were blinded for genotype and treatment.

2.2.2. Calculation of scratch ratio ($R_{scratch}$)

Based on the results of the cheek assay, the pruritogenic and algogenic nature of the compounds applied was characterized by calculating a novel parameter, the scratch ratio ($R_{scratch}$), defined as: $R_{scratch} = N_{scratch} / (N_{scratch} + N_{wipe})$, where $N_{scratch}$ and N_{wipe} are the number of scratches and wipes, respectively, detected during the observation. Value of $R_{scratch}$ can vary between 0 and 1 where 0 indicates pure ~~pain~~ nociception related responses (wipes) without any scratching, and 1 represents exclusively itch-related behavioral responses. Values in the middle range of the scale

are characteristic for neutral compounds inducing neither significant ~~pain~~ nociception nor itch or for compounds inducing similarly frequent ~~pain~~ nocifensive and itch responses.

2.2.3. Nape-assay

In order to further assess the potential role of TRPM3 in mediating itch in different regions of the skin, pruritogenic compounds were also injected into the nape of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice. The animals were acclimated to the test chamber the day before the experiment for 1 hour, and the nape of the neck was shaved. The experimenter restrained the mouse with one hand and carefully shaved the nape area behind the ears using small electronic clippers (PepPet, Guangdong, China). Care was taken to avoid skin and ear injury. Mice were allowed to acclimatize for 10 minutes before the injection took place on the day of the experiment. Pruritogenic compounds were injected s.c. into the nape, and the mouse was immediately placed back into the chamber, the experimenter left the room, and scratching behavior was video recorded for 30 min. As pruritogenic compounds, 200 µg Hist, 10 µg 5-HT, and 250 ng ET-1 dissolved in 50 µl PBS were injected. The applied doses and injection volume were selected corresponding to previous literature data and tested in pilot experiments. The records were analyzed by experimenters blinded to genotype and compound injected.

2.3. Culturing and isolation of sensory neurons

Sensory neurons of trigeminal ganglia (TGs) were obtained from 8-12 week-old *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice, as described before[32,37]. Briefly, mice were euthanized by CO_2, TGs were isolated and digested with collagenase (2 mg/ml) and dispase (2,5 mg/ml) (both from Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA). Suspension of sensory neurons was seeded on laminin (100µg/ml) and poly-L-ornithine HBr (500µg/ml) (both from Sigma Aldrich,

St.Louis, MO, USA) coated glass bottom culture dishes (MatTek, Ashland, MA, USA) and cultured in Neurobasal medium supplemented with 2% B-27 supplement, 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin, 2 ng/ml glial cell line-derived neurotrophic factor (GNDF) (all from Invitrogen/Thermo Fisher Scientific) and 10 ng/ml NT-4 (PeproTech, London, UK) at 37°C in 5% CO_2 containing humidified atmosphere. Neurons were used for experiments within 24 to 36 hrs following isolation.

2.4. Fluorescent measurements of intracellular Ca^{2+} concentration

To measure the cytoplasmic Ca^{2+} concentration in individual sensory neurons, we used microscope-based calcium imaging systems. On the day after the isolation, TG neurons were loaded with acetoxymethyl ester-conjugated fluorescent Ca^{2+} indicators dissolved in culturing medium. 2 µM Fura-2-AM (Invitrogen/Thermo Fisher Scientific) was used in experiments comparing the responses of sensory neurons from *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ animals, and 2 µM Fluo-4-AM (Invitrogen/Thermo Fisher Scientific) was applied when investigating the effect of pharmacological inhibition of TRPM3. Both dyes possess a Kd value for Ca^{2+} in submicromolar range, and are therefore equally suitable to detect relevant changes in cytoplasmic Ca^{2+} concentration. Fura-2-loaded cells were placed on the stage of a Nikon fluorescent microscope and captured with constant setting every 1 second ($\lambda 1_{EX}$:340 nm, $\lambda 2_{EX}$ nm: 380 nm and λ_{EM}: 505 nm) and data were obtained as the ratio of the fluorescence measured at 340 and 380 nm excitation wavelengths (F_{340}/F_{380}). Fluo-4 loaded cells were placed on the stage of a Zeiss LSM 5 Live confocal microscope and captured at $\lambda 1_{EX}$:488 and λ_{EM}: 516 once every second and data were presented as F_1/F_0, where F_0 is the average fluorescence of the baseline (before the first compound application) and F_1 is the actual fluorescence. During the measurements, cells were continuously perfused with Ca^{2+}-buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2x6H_2O$, 2 mM $CaCl_2x2H_2O$,

10 mM glucose xH_2O, 10 mM HEPES, pH 7.4 (all from Sigma-Aldrich)) and different compounds were applied via the perfusion. All experiments were performed at room temperature (21–22°C).

2.5. Materials

The endogenous TRPM3 agonist PregS, the TRPA1 agonist cinnamaldehyde (CA), the TRPV1 agonist Caps, and the exogenous TRPM3 agonist CIM0216 were obtained from Tocris Bioscience (Bristol, UK). The TRPM3 antagonist Isosakuranetin (Isok) was obtained from Carl Roth (Karlsruhe, Germany). The well-characterized pruritogen Hist was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA); and the non-histaminergic endogenous pruritic mediators 5-HT and ET-1 were purchased from Abcam (Abcam, Cambridge, MA, USA) or Sigma Aldrich.

2.6. Data and statistical analysis

Origin 9.0 (OriginLab Corporation, Northampton, MA, USA) was used for analysis and data display for both *in vivo* and *in vitro* data. Statistical analysis was performed using IBM SPSS Statistics 22 software (IBM, Armonk, NY, USA). Kruskal-Wallis test was used to compare multiple groups. Whenever it reported significant differences, Mann-Whitney tests with Bonferroni adjustment were used as ~~Dunn-Bonferroni~~ post hoc analysis for pairwise comparison (e.g. comparing the effect of several treatments within one genotype). ~~and~~ The Mann-Whitney test was also applied to compare only two groups (e.g. *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ within a particular treatment). The distribution of various neurons between the different groups was compared using Chi-squared (X^2) test. In every case, $P < 0.05$ was regarded as showing significant differences between groups. If not mentioned otherwise, individual data were presented in scatterplots marking mean ± SD.

3. RESULTS

3.1. TRPM3 agonists induced ~~pain~~ nociception but not itch in the mouse cheek model

To assess the role of TRPM3 in itch ~~and pain~~ sensation and nociception, we investigated wild type (*Trpm3*$^{+/+}$) and TRPM3-deficient (*Trpm3*$^{-/-}$) C57/Bl6 mice[32] in the "cheek model" paradigm, which allows differentiation between behavioral responses related to nociception and pruriception[39].

First, we injected the TRPM3 agonists PregS and CIM0216 to the cheek of the animals as described in the Methods section. The injection technique and behavioral analysis were optimized in preliminary experiments. As a negative control, we injected the same volume of the vehicle buffer and used the TRPV1 agonist Caps as a well-established algogenic substance to assess TRPM3-independent nocifensive responses. We found that TRPM3 agonists induced marked nocifensive responses in *Trpm3*$^{+/+}$ animals compared to the vehicle control quantified by the number of wiping events on the injected cheek as described in the Materials and methods (Fig. 1A, Suppl. video file 1). These nocifensive responses were abolished in *Trpm3*$^{-/-}$ animals, clearly indicating that PregS- and CIM0216-~~evoked~~induced nociception ~~pain~~ is mediated by TRPM3 in the cheek. Caps also induced marked ~~pain~~ nocifensive behavior but it was not influenced by the deletion of *Trpm3*, demonstrating, as found earlier in other assays[32], that the effect of Caps is independent of TRPM3 in the cheek model. Importantly, similar to Caps, none of the TRPM3 agonists induced significant itch-related behavior in *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ animals, as assessed by the number of scratches and the total time spent with scratching (Fig. 1B-C).

To better characterize the quality of the sensory phenomena evoked by a particular compound, we introduced a new measure, "scratch ratio" ($R_{scratch}$) as defined in the Methods. ~~A high $R_{scratch}$ value (near to 1) is characteristic for pruritogenic but not algogenic compounds and a~~

~~low $R_{scratch}$ value (approaching 0) is a sign of mainly algogenic and not pruritogenic effects. $R_{scratch}$ values in the middle of the scale describe neutral compounds.~~ We found that TRPM3 agonists ~~PregS and CIM0216~~, as well as the TRPV1 agonist Caps behaved as algogenic substances in *Trpm3*$^{+/+}$ animals i.e. they ~~evoked~~ induced mainly ~~pain~~ nociception and hardly any itch. However, in *Trpm3*$^{-/-}$ animals, PregS and CIM0216, but not Caps, were found to behave as neutral compounds, as $R_{scratch}$ was near to 0.5 (Fig. 1D). Interestingly, PregS induced slightly more intense wiping in *Trpm3*$^{+/+}$ animals than Caps (Fig. 1A), however detailed analysis of the observation suggested, that in the applied doses, Caps may induce more intense pain than PregS or CIM0216. For each agonist, the peak response was reached during the first 5 minutes, after which the responses decayed, and this decay was more pronounced in the capsaicin- than in the PregS-treated group (No. of $wipes_{0\text{-}5\ min}$ Caps vs. PregS: 52.38±22.70 vs. 53.38±36.24, U=55.0, p=0.860 and No. of $wipes_{10\text{-}15\ min}$ Caps vs. PregS: 6.00±5.58 vs. 18.13±10.52, U=91.0, p=0.003) (Fig. 1E). However, in the Caps treated group we observed additional ~~pain~~nociception-related behavior including "tunneling" (where the animal digs a tunnel in the bedding while pressing the injected cheek to the bottom of the cage, Suppl. video file 2) and lethargy. Although, as previous studies assessing nociception in the cheek model, we restricted the quantitative analysis to the number of wipes (as a pre-defined measure), these earlier unreported patterns of behavior in the cheek assay may also indicate nociception as a form of counter irritation, analogue to pressing or rubbing a painful area aiming at alleviating pain. These signs of ~~pain~~ nociception were more typical in the Caps injected group in the later phase of the observation, and were less characteristic for the PregS-injected group.

3.2. TRPM3 is not involved in the sensation of itch induced by Hist or non-histaminergic pruritogens 5-HT and ET-1

Although direct activation of TRPM3, similar to TRPV1, ~~evoked~~ resulted exclusively ~~pain~~ 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. Indeed, direct, general activation of TRPV1 is known to induce ~~pain~~ 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[9,11,31,40–42]. 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 both in human and rodent models[43,44]), 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~~ nociception in *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ animals (Fig. 2A-C): 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 (Fig. 2D). 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 ~~pain~~ 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 ~~pain~~ nociception (both induce similar scratching responses), known “pure” pruritogen compound-

induced responses can be interpreted as signs of itch[39,44]. 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 dorsal root ganglia (DRGs) to compare to the results of the cheek injections which affected the innervation area of the trigeminal ganglion (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 (Fig. 2E). The ET-1 induced 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.

3.3. Pruritogens activated trigeminal sensory neurons independently of TRPM3.

Next, we tested whether cellular responses induced by pruritogens are related to TRPM3. For this purpose, we isolated somatosensory neurons from TGs of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice and investigated *in vitro* cellular Ca^{2+} responses evoked by Hist, 5-HT, ET-1 and PregS, CA and Caps, agonists of TRPM3, TRPA1 and TRPV1, respectively. 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 shown in Figure 3A. As expected, the ratio of PregS responsive (PregS+) neurons was strongly reduced in *Trpm3*$^{-/-}$ TG neurons, although, consistent with previous results[32], 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 (Fig. 3B). 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 (Fig. 3C).

3.4. Pharmacological blockade of TRPM3 inhibited PregS evoked activation of trigeminal sensory neurons but did not affect pruritogen induced cellular 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, ~~and~~ 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 (Fig. 4). As shown earlier on DRG derived neurons[45], PregS-induced responses were strongly inhibited by Isok in a reversible way (Fig. 4A-B). In contrast, pharmacological ~~blockade~~ 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 (Fig. 4C-E).

4. DISCUSSION

Emerging evidence suggests that pruriceptive neurons form a subpopulation within nociceptive neurons, rather than forming a purely pruritogen-specific peripheral sensory neuron population,[46–49] 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[2], 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[2,6,41,48,50,51]. TRP channels are long-chased targets for analgesic therapies but they seem to be promising targets in the management of pruritus, as well[10,52–54]. 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[55–59]. The role of TRPV4 was described in both allergic and non-allergic pruritus by mediating 5-HT release from mast cells and keratinocytes, respectively[60], and its activation in keratinocytes results in ET-1 release, as well, which is thought to play a role in sunburn-associated pain[61]. Beyond 5-HT release, TRPV4 is also involved in the sensory transduction of 5-HT-mediated itch in the pruriceptive fibers[62], and as an osmo-mechanoreceptor it plays a role in the development of mechanical hyperalgesia[63–66]. 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[58,67–70], similar to non-neuronal TRPV4.

TRPM3 is also a thermosensitive nociceptor ion channel activated by noxious heat and the endogenous neurosteroid PregS. Its selective activation results in neuropeptide release from the sensory terminals and evokes ~~pain sensation~~ nociception in rodents[30,32]. 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[30,37]. 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~~-evoked pain~~ 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[32,38]. 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 a recent review see[9]). For example, TRPV1 was found to be involved in histamine receptor and protease activated receptor 2 (PAR2) signaling[31] and TRPA1 was shown to transmit the pruritic effect of 5-HT[71], bile acid[34], activators of Mas-Related G Protein–Coupled Receptors (Mrgprs)[35], and thymic stromal lymphopoietin (TSLP)[72]

In our study, we challenged the pruriceptive role of TRPM3 for the first time and we investigated its selective role in itch and ~~pain sensation~~ nociception using the generally accepted cheek model[44,49]. Our results clearly indicate that TRPM3 is involved in nociception but not in pruriception.

The role of TRPM3 in thermal nociception and inflammatory heat hyperalgesia is well established by previous results[32,37,38,73], and TRPM3 antagonists evoked promising analgesic effects in animal studies[45,74,75]. Our *in vivo* results obtained in the cheek model further support the previous conclusion, and demonstrate that the selective pharmacological activation of TRPM3 ~~evoked pain~~ 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 ~~pain~~ 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 ~~a~~ certain subpopulation of the somatosensory neurons, TRPM3 can contribute to pruritic signaling and take part in the transduction of itch. For example, it is also known from TRPV1 that its general activation by Caps evokes a burning pain but not itch sensation[44]. However, as discussed above, TRPV1 is expressed in pruriceptive ~~neurons~~ nociceptors, and takes part in the transduction of histaminergic, and non-histaminergic itch[31], as well. ~~As discussed above, in these neurons, TRPV1 takes part in the transduction of histaminergic, and PAR2-mediated non-histaminergic itch.~~ Furthermore, if TRPV1 is expressed exclusively in pruriceptive, MrgprA3 expressing neurons, its activation with Caps results in itch but not pain sensation[41]. Therefore, we tested 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[76,77]. In the last decade, the underlying spinal circuits were also revealed involving vesicular glutamate transporter type 2 (Vglut2) expressing nociceptors[4,5], Bhlhb5 transcription factor expressing inhibitory interneurons and kappa opioid receptor signaling[78,79] (for a current review see[80]). 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[81,82], although in our experiments it initiated only moderate nocifensive behavior (wiping) and was characterized by high scratch ratio as a mainly pruritogenic substance.

Our results led to the conclusion that TRPM3 is exclusively related to ~~pain~~ nociception but not itch transmission, 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.

The effect of temperature changes on itch is controversial. Noxious heat (as other noxious stimuli) are known to inhibit itch[80], but moderate warming can amplify pruritus, as reported especially in atopic dermatitis[83,84]. However, the role of TRPM3 is less likely in warm induced atopic pruritus. Although chemical activation of TRPM3 is already potentiated at 33 °C, its heat-induced activation is more prominent at noxious temperatures[32]. Compared to TRPV1, the current-temperature relationship curve of TRPM3 is shifted slightly towards higher temperatures[85]. In line with this characteristic, behavioral experiments indicated that TRPM3 plays an essential role in noxious heat sensation, but also that its genetic ablation had only a moderate effect on warm sensation in neutral temperature zone [32,38,86]. A recent study described that warm induced pruritus and pruritogen release from atopic keratinocytes is mediated by TRPV3[68].

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, thermal and inflammatory pain evoked at the body surface innervated form DRGs~~ chemical and thermal nociception, as well as inflammatory pain in the innervation area of DRGs[32,45,73,87]. Our results also demonstrated that PregS- or CIM0216-~~evoked pain~~induced nociception is diminished in *Trpm3*$^{-/-}$ animals in the cheek model, in the trigeminal innervation area, as well. Moreover, we also demonstrated that it does not take part in itch sensation and its inhibition or deletion hardly influence pruritic responses. With the previous results, our findings suggest that TRPM3 may be a superior target in pain therapies than other TRP channels, including the long pursued TRPV1. Indeed, activation or inhibition of TRPV1 can

drastically influence core body temperature, which was not found in case of TRPM3[32,88–90], and our results suggest that the role of TRPM3 is more selective for nociception over pruriception than TRPV1 or TRPA1. Moreover, this latest result can have an impact on the better understanding of the molecular organization of nociceptive and pruriceptive systems.

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 ~~pain~~ nociception as subjective reporting of human subjects[44] and the applied Hist, 5-HT, and ET-1 are known to induce itch both in mice and humans[9,43,91,92]. 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 ~~is~~ are functionally identical: they share agonists, antagonists, and regulation by phospholipids, as well as by βγ subunits of G proteins[87,93–96]. These data suggest that selective targeting of nociception via TRPM3 may be a promising approach even in human analgesia.

5. CONFLICT OF INTEREST

TB and AO provide consultancy services to Phytecs Inc. (TB) and Botanix Pharmaceuticals Ltd. (AO). TV is co- inventor on patents entitled “Treatment of pain” derived from WO2012149614, and his lab has received research funding for pain-related research from industrial parties. Botanix Pharmaceuticals Ltd, Phytecs Inc., and the founding sponsors had no role in conceiving the study, designing the experiments, writing of the manuscript, or in the decision to publish it. Other authors declare no conflict of interest.

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7. REFERENCES

[1] A. Ikoma, M. Steinhoff, S. Ständer, G. Yosipovitch, M. Schmelz, The neurobiology of itch, Nat. Rev.. 7 (2006) 535–547. https://doi.org/10.1038/nrn1950.

[2] R.H. LaMotte, X. Dong, M. Ringkamp, Sensory neurons and circuits mediating itch, Nat. Rev. Neurosci. 15 (2014) 19–31. https://doi.org/10.1038/nrn3641.

[3] D.M. Barry, X.-T. Liu, B. Liu, X.-Y. Liu, F. Gao, X. Zeng, J. Liu, Q. Yang, S. Wilhelm, J. Yin, A. Tao, Z.-F. Chen, Exploration of sensory and spinal neurons expressing gastrin-releasing peptide in itch and pain related behaviors, Nat Commun. 11 (2020) 1397. https://doi.org/10.1038/s41467-020-15230-y.

[4] M.C. Lagerström, K. Rogoz, B. Abrahamsen, E. Persson, B. Reinius, K. Nordenankar, C. Olund, C. Smith, J.A. Mendez, Z.-F. Chen, J.N. Wood, A. Wallén-Mackenzie, K. Kullander, VGLUT2-dependent sensory neurons in the TRPV1 population regulate pain and itch, Neuron. 68 (2010) 529–542. https://doi.org/10.1016/j.neuron.2010.09.016.

[5] Y. Liu, O. Abdel Samad, L. Zhang, B. Duan, Q. Tong, C. Lopes, R.-R. Ji, B.B. Lowell, Q. Ma, VGLUT2-dependent glutamate release from nociceptors is required to sense pain and suppress itch, Neuron. 68 (2010) 543–556. https://doi.org/10.1016/j.neuron.2010.09.008.

[6] S.K. Mishra, M.A. Hoon, The cells and circuitry for itch responses in mice, Science. 340 (2013) 968–971. https://doi.org/10.1126/science.1233765.

[7] M. Pagani, G.W. Albisetti, N. Sivakumar, H. Wildner, M. Santello, H.C. Johannssen, H.U. Zeilhofer, How Gastrin-Releasing Peptide Opens the Spinal Gate for Itch, Neuron. 103 (2019) 102-117.e5. https://doi.org/10.1016/j.neuron.2019.04.022.

[8] T. Akiyama, E. Carstens, Neural processing of itch, Neuroscience. 250 (2013) 697–714. https://doi.org/10.1016/j.neuroscience.2013.07.035.

[9] X. Dong, X. Dong, Peripheral and Central Mechanisms of Itch, Neuron. 98 (2018) 482–494. https://doi.org/10.1016/j.neuron.2018.03.023.

[10] C. Moore, R. Gupta, S.-E. Jordt, Y. Chen, W.B. Liedtke, Regulation of Pain and Itch by TRP Channels, Neurosci Bull. 34 (2018) 120–142. https://doi.org/10.1007/s12264-017-0200-8.

[11] W.-S. Shim, M.-H. Tak, M.-H. Lee, M. Kim, M. Kim, J.-Y. Koo, C.-H. Lee, M. Kim, U. Oh, TRPV1 Mediates Histamine-Induced Itching via the Activation of Phospholipase A2 and 12-Lipoxygenase, J. Neurosci. 27 (2007) 2331–2337. https://doi.org/10.1523/JNEUROSCI.4643-06.2007.

[12] S.R. Wilson, D.M. Bautista, Role of Transient Receptor Potential Channels in Acute and Chronic Itch, in: E. Carstens, T. Akiyama (Eds.), Itch: Mechanisms and Treatment, CRC

Press/Taylor & Francis, Boca Raton (FL), 2014.
http://www.ncbi.nlm.nih.gov/books/NBK200927/ (accessed February 12, 2020).

[13] B.I. Tóth, A.G. Szöllősi, T. Bíró, TRP Channels in Itch and Pain, in: Itch and Pain. Similarities, Interactions, and Differences, IASP - Wolters Kluwer, 2020. https://shop.lww.com/Itch-and-Pain/p/9781975153038.

[14] C.-H. Lee, C.-C. Chen, Roles of ASICs in Nociception and Proprioception, Adv. Exp. Med. Biol. 1099 (2018) 37–47. https://doi.org/10.1007/978-981-13-1756-9_4.

[15] T. Bele, E. Fabbretti, P2X receptors, sensory neurons and pain, Curr. Med. Chem. 22 (2015) 845–850. https://doi.org/10.2174/0929867321666141011195351.

[16] F. De Logu, P. Geppetti, Ion Channel Pharmacology for Pain Modulation, Handb Exp Pharmacol. 260 (2019) 161–186. https://doi.org/10.1007/164_2019_336.

[17] G. Stephan, L. Huang, Y. Tang, S. Vilotti, E. Fabbretti, Y. Yu, W. Nörenberg, H. Franke, F. Gölöncsér, B. Sperlágh, A. Dopychai, R. Hausmann, G. Schmalzing, P. Rubini, P. Illes, The ASIC3/P2X3 cognate receptor is a pain-relevant and ligand-gated cationic channel, Nat Commun. 9 (2018) 1354. https://doi.org/10.1038/s41467-018-03728-5.

[18] A.I. Basbaum, D.M. Bautista, G. Scherrer, D. Julius, Cellular and molecular mechanisms of pain, Cell. 139 (2009) 267–284. https://doi.org/10.1016/j.cell.2009.09.028.

[19] D. Julius, TRP channels and pain, Annu. Rev. Cell Dev. Biol. 29 (2013) 355–384. https://doi.org/10.1146/annurev-cellbio-101011-155833.

[20] M.J. Caterina, A. Leffler, A.B. Malmberg, W.J. Martin, J. Trafton, K.R. Petersen-Zeitz, M. Koltzenburg, A.I. Basbaum, D. Julius, Impaired nociception and pain sensation in mice lacking the capsaicin receptor, Science. 288 (2000) 306–313.

[21] M.J. Caterina, M.A. Schumacher, M. Tominaga, T.A. Rosen, J.D. Levine, D. Julius, The capsaicin receptor: a heat-activated ion channel in the pain pathway, Nature. 389 (1997) 816–824. https://doi.org/10.1038/39807.

[22] J.B. Davis, J. Gray, M.J. Gunthorpe, J.P. Hatcher, P.T. Davey, P. Overend, M.H. Harries, J. Latcham, C. Clapham, K. Atkinson, S.A. Hughes, K. Rance, E. Grau, A.J. Harper, P.L. Pugh, D.C. Rogers, S. Bingham, A. Randall, S.A. Sheardown, Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia, Nature. 405 (2000) 183–187. https://doi.org/10.1038/35012076.

[23] M.J.M. Fischer, P.W. Reeh, Sensitization to heat through G-protein-coupled receptor pathways in the isolated sciatic mouse nerve, Eur. J. Neurosci. 25 (2007) 3570–3575. https://doi.org/10.1111/j.1460-9568.2007.05582.x.

[24] R. Rukwied, B.A. Chizh, U. Lorenz, O. Obreja, S. Margarit, M. Schley, M. Schmelz, Potentiation of nociceptive responses to low pH injections in humans by prostaglandin E2, J Pain. 8 (2007) 443–451. https://doi.org/10.1016/j.jpain.2006.12.004.

[25] M. Tominaga, M.J. Caterina, A.B. Malmberg, T.A. Rosen, H. Gilbert, K. Skinner, B.E. Raumann, A.I. Basbaum, D. Julius, The cloned capsaicin receptor integrates multiple pain-producing stimuli, Neuron. 21 (1998) 531–543. https://doi.org/10.1016/s0896-6273(00)80564-4.

[26] M. Bandell, G.M. Story, S.W. Hwang, V. Viswanath, S.R. Eid, M.J. Petrus, T.J. Earley, A. Patapoutian, Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin, Neuron. 41 (2004) 849–857. https://doi.org/10.1016/s0896-6273(04)00150-3.

[27] D.M. Bautista, S.-E. Jordt, T. Nikai, P.R. Tsuruda, A.J. Read, J. Poblete, E.N. Yamoah, A.I. Basbaum, D. Julius, TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents, Cell. 124 (2006) 1269–1282. https://doi.org/10.1016/j.cell.2006.02.023.

[28] R.W. Colburn, M.L. Lubin, D.J. Stone, Y. Wang, D. Lawrence, M.R. D'Andrea, M.R. Brandt, Y. Liu, C.M. Flores, N. Qin, Attenuated cold sensitivity in TRPM8 null mice, Neuron. 54 (2007) 379–386. https://doi.org/10.1016/j.neuron.2007.04.017.

[29] A. Dhaka, A.N. Murray, J. Mathur, T.J. Earley, M.J. Petrus, A. Patapoutian, TRPM8 is required for cold sensation in mice, Neuron. 54 (2007) 371–378. https://doi.org/10.1016/j.neuron.2007.02.024.

[30] K. Held, T. Kichko, K. De Clercq, H. Klaassen, R. Van Bree, J.-C. Vanherck, A. Marchand, P.W. Reeh, P. Chaltin, T. Voets, J. Vriens, Activation of TRPM3 by a potent synthetic ligand reveals a role in peptide release, Proc. Natl. Acad. Sci. U.S.A. 112 (2015) E1363-1372. https://doi.org/10.1073/pnas.1419845112.

[31] N. Imamachi, G.H. Park, H. Lee, D.J. Anderson, M.I. Simon, A.I. Basbaum, S.-K. Han, TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 11330–11335. https://doi.org/10.1073/pnas.0905605106.

[32] J. Vriens, G. Owsianik, T. Hofmann, S.E. Philipp, J. Stab, X. Chen, M. Benoit, F. Xue, A. Janssens, S. Kerselaers, J. Oberwinkler, R. Vennekens, T. Gudermann, B. Nilius, T. Voets, TRPM3 is a nociceptor channel involved in the detection of noxious heat, Neuron. 70 (2011) 482–494. https://doi.org/10.1016/j.neuron.2011.02.051.

[33] B.M. Kim, S.H. Lee, W.S. Shim, U. Oh, Histamine-induced Ca(2+) influx via the PLA(2)/lipoxygenase/TRPV1 pathway in rat sensory neurons, Neurosci. Lett. 361 (2004) 159–162. https://doi.org/10.1016/j.neulet.2004.01.019.

[34] T. Lieu, G. Jayaweera, P. Zhao, D.P. Poole, D. Jensen, M. Grace, P. McIntyre, R. Bron, Y.M. Wilson, M. Krappitz, S. Haerteis, C. Korbmacher, M.S. Steinhoff, R. Nassini, S. Materazzi, P. Geppetti, C.U. Corvera, N.W. Bunnett, The bile acid receptor TGR5 activates the TRPA1 channel to induce itch in mice, Gastroenterology. 147 (2014) 1417–1428. https://doi.org/10.1053/j.gastro.2014.08.042.

[35] S.R. Wilson, K.A. Gerhold, A. Bifolck-Fisher, Q. Liu, K.N. Patel, X. Dong, D.M. Bautista, TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch, Nat. Neurosci. 14 (2011) 595–602. https://doi.org/10.1038/nn.2789.

[36] S.R. Wilson, A.M. Nelson, L. Batia, T. Morita, D. Estandian, D.M. Owens, E.A. Lumpkin, D.M. Bautista, The ion channel TRPA1 is required for chronic itch, J. Neurosci. 33 (2013) 9283–9294. https://doi.org/10.1523/JNEUROSCI.5318-12.2013.

[37] J. Vriens, K. Held, A. Janssens, B.I. Tóth, S. Kerselaers, B. Nilius, R. Vennekens, T. Voets, Opening of an alternative ion permeation pathway in a nociceptor TRP channel, Nat. Chem. Biol. 10 (2014) 188–195. https://doi.org/10.1038/nchembio.1428.

[38] I. Vandewauw, K. De Clercq, M. Mulier, K. Held, S. Pinto, N. Van Ranst, A. Segal, T. Voet, R. Vennekens, K. Zimmermann, J. Vriens, T. Voets, A TRP channel trio mediates acute noxious heat sensing, Nature. 555 (2018) 662–666. https://doi.org/10.1038/nature26137.

[39] S.G. Shimada, R.H. LaMotte, Behavioral differentiation between itch and pain in mouse, Pain. 139 (2008) 681–687. https://doi.org/10.1016/j.pain.2008.08.002.

[40] R. Costa, D.M. Marotta, M.N. Manjavachi, E.S. Fernandes, J.F. Lima-Garcia, A.F. Paszcuk, N.L.M. Quintão, L. Juliano, S.D. Brain, J.B. Calixto, Evidence for the role of neurogenic inflammation components in trypsin-elicited scratching behaviour in mice, Br. J. Pharmacol. 154 (2008) 1094–1103. https://doi.org/10.1038/bjp.2008.172.

[41] L. Han, C. Ma, Q. Liu, H.-J. Weng, Y. Cui, Z. Tang, Y. Kim, H. Nie, L. Qu, K.N. Patel, Z. Li, B. McNeil, S. He, Y. Guan, B. Xiao, R.H. Lamotte, X. Dong, A subpopulation of nociceptors specifically linked to itch, Nat. Neurosci. 16 (2013) 174–182. https://doi.org/10.1038/nn.3289.

[42] B.I. Tóth, A. Szallasi, T. Bíró, Transient receptor potential channels and itch: how deep should we scratch?, Handb Exp Pharmacol. 226 (2015) 89–133. https://doi.org/10.1007/978-3-662-44605-8_6.

[43] M. Kido-Nakahara, J. Buddenkotte, C. Kempkes, A. Ikoma, F. Cevikbas, T. Akiyama, F. Nunes, S. Seeliger, B. Hasdemir, C. Mess, T. Buhl, M. Sulk, F.-U. Müller, D. Metze, N.W. Bunnett, A. Bhargava, E. Carstens, M. Furue, M. Steinhoff, Neural peptidase endothelin-converting enzyme 1 regulates endothelin 1-induced pruritus, J. Clin. Invest. 124 (2014) 2683–2695. https://doi.org/10.1172/JCI67323.

[44] R.H. LaMotte, S.G. Shimada, P. Sikand, Mouse models of acute, chemical itch and pain in humans, Exp. Dermatol. 20 (2011) 778–782. https://doi.org/10.1111/j.1600-0625.2011.01367.x.

[45] I. Straub, U. Krügel, F. Mohr, J. Teichert, O. Rizun, M. Konrad, J. Oberwinkler, M. Schaefer, Flavanones that selectively inhibit TRPM3 attenuate thermal nociception in vivo, Mol. Pharmacol. 84 (2013) 736–750. https://doi.org/10.1124/mol.113.086843.

[46] H.O. Handwerker, C. Forster, C. Kirchhoff, Discharge patterns of human C-fibers induced by itching and burning stimuli, Journal of Neurophysiology. 66 (1991) 307–315. https://doi.org/10.1152/jn.1991.66.1.307.

[47] M. Schmelz, R. Schmidt, C. Weidner, M. Hilliges, H.E. Torebjork, H.O. Handwerker, Chemical response pattern of different classes of C-nociceptors to pruritogens and algogens, J. Neurophysiol. 89 (2003) 2441–2448. https://doi.org/10.1152/jn.01139.2002.

[48] B. Namer, R. Carr, L.M. Johanek, M. Schmelz, H.O. Handwerker, M. Ringkamp, Separate Peripheral Pathways for Pruritus in Man, J Neurophysiol. 100 (2008) 2062–2069. https://doi.org/10.1152/jn.90482.2008.

[49] C. Ma, H. Nie, Q. Gu, P. Sikand, R.H. LaMotte, In vivo responses of cutaneous C-mechanosensitive neurons in mouse to punctate chemical stimuli that elicit itch and nociceptive sensations in humans, J Neurophysiol. 107 (2012) 357–363. https://doi.org/10.1152/jn.00801.2011.

[50] P. Geppetti, N.A. Veldhuis, T. Lieu, N.W. Bunnett, G Protein-Coupled Receptors: Dynamic Machines for Signaling Pain and Itch, Neuron. 88 (2015) 635–649. https://doi.org/10.1016/j.neuron.2015.11.001.

[51] S.-K. Han, M.I. Simon, Intracellular signaling and the origins of the sensations of itch and pain, Sci Signal. 4 (2011) pe38. https://doi.org/10.1126/scisignal.2002353.

[52] T. Bíró, B.I. Tóth, R. Marincsák, N. Dobrosi, T. Géczy, R. Paus, TRP channels as novel players in the pathogenesis and therapy of itch, Biochim. Biophys. Acta. 1772 (2007) 1004–1021. https://doi.org/10.1016/j.bbadis.2007.03.002.

[53] M.M. Moran, M.A. McAlexander, T. Bíró, A. Szallasi, Transient receptor potential channels as therapeutic targets, Nat Rev Drug Discov. 10 (2011) 601–620. https://doi.org/10.1038/nrd3456.

[54] Z. Xie, H. Hu, TRP Channels as Drug Targets to Relieve Itch, Pharmaceuticals (Basel). 11 (2018). https://doi.org/10.3390/ph11040100.

[55] B. Nilius, T. Bíró, TRPV3: a “more than skinny” channel, Exp. Dermatol. 22 (2013) 447–452. https://doi.org/10.1111/exd.12163.

[56] B. Nilius, T. Bíró, G. Owsianik, TRPV3: time to decipher a poorly understood family member!, J. Physiol. (Lond.). 592 (2014) 295–304. https://doi.org/10.1113/jphysiol.2013.255968.

[57] M. Szántó, A. Oláh, A.G. Szöllősi, K.F. Tóth, E. Páyer, N. Czakó, Á. Pór, I. Kovács, C.C. Zouboulis, L. Kemény, T. Bíró, B.I. Tóth, Activation of TRPV3 Inhibits Lipogenesis and Stimulates Production of Inflammatory Mediators in Human Sebocytes-A Putative Contributor to Dry Skin Dermatoses, J. Invest. Dermatol. 139 (2019) 250–253. https://doi.org/10.1016/j.jid.2018.07.015.

[58] A.G. Szöllősi, N. Vasas, Á. Angyal, K. Kistamás, P.P. Nánási, J. Mihály, G. Béke, E. Herczeg-Lisztes, A. Szegedi, N. Kawada, T. Yanagida, T. Mori, L. Kemény, T. Bíró, Activation of TRPV3 Regulates Inflammatory Actions of Human Epidermal Keratinocytes, J. Invest. Dermatol. 138 (2018) 365–374. https://doi.org/10.1016/j.jid.2017.07.852.

[59] B.I. Tóth, A. Oláh, A.G. Szöllősi, T. Bíró, TRP channels in the skin, Br. J. Pharmacol. 171 (2014) 2568–2581. https://doi.org/10.1111/bph.12569.

[60] J. Luo, J. Feng, G. Yu, P. Yang, M.R. Mack, J. Du, W. Yu, A. Qian, Y. Zhang, S. Liu, S. Yin, A. Xu, J. Cheng, Q. Liu, R.G. O'Neil, Y. Xia, L. Ma, S.M. Carlton, B.S. Kim, K. Renner, Q. Liu, H. Hu, Transient receptor potential vanilloid 4-expressing macrophages and keratinocytes contribute differentially to allergic and nonallergic chronic itch, J. Allergy Clin. Immunol. 141 (2018) 608-619.e7. https://doi.org/10.1016/j.jaci.2017.05.051.

[61] C. Moore, F. Cevikbas, H.A. Pasolli, Y. Chen, W. Kong, C. Kempkes, P. Parekh, S.H. Lee, N.-A. Kontchou, I. Yeh, I. Ye, N.M. Jokerst, E. Fuchs, M. Steinhoff, W.B. Liedtke, UVB radiation generates sunburn pain and affects skin by activating epidermal TRPV4 ion channels and triggering endothelin-1 signaling, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) E3225-3234. https://doi.org/10.1073/pnas.1312933110.

[62] T. Akiyama, M. Ivanov, M. Nagamine, A. Davoodi, M.I. Carstens, A. Ikoma, F. Cevikbas, C. Kempkes, J. Buddenkotte, M. Steinhoff, E. Carstens, Involvement of TRPV4 in Serotonin-Evoked Scratching, J. Invest. Dermatol. 136 (2016) 154–160. https://doi.org/10.1038/JID.2015.388.

[63] N. Alessandri-Haber, O.A. Dina, J.J. Yeh, C.A. Parada, D.B. Reichling, J.D. Levine, Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat, J. Neurosci. 24 (2004) 4444–4452. https://doi.org/10.1523/JNEUROSCI.0242-04.2004.

[64] N. Alessandri-Haber, E. Joseph, O.A. Dina, W. Liedtke, J.D. Levine, TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator, Pain. 118 (2005) 70–79. https://doi.org/10.1016/j.pain.2005.07.016.

[65] N. Alessandri-Haber, J.J. Yeh, A.E. Boyd, C.A. Parada, X. Chen, D.B. Reichling, J.D. Levine, Hypotonicity induces TRPV4-mediated nociception in rat, Neuron. 39 (2003) 497–511.

[66] P. Zhao, T. Lieu, N. Barlow, M. Metcalf, N.A. Veldhuis, D.D. Jensen, M. Kocan, S. Sostegni, S. Haerteis, V. Baraznenok, I. Henderson, E. Lindström, R. Guerrero-Alba, E.E. Valdez-Morales, W. Liedtke, P. McIntyre, S.J. Vanner, C. Korbmacher, N.W. Bunnett, Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4, J. Biol. Chem. 289 (2014) 27215–27234. https://doi.org/10.1074/jbc.M114.599712.

[67] C.W. Park, H.J. Kim, Y.W. Choi, B.Y. Chung, S.-Y. Woo, D.-K. Song, H.O. Kim, TRPV3 Channel in Keratinocytes in Scars with Post-Burn Pruritus, Int J Mol Sci. 18 (2017). https://doi.org/10.3390/ijms18112425.

[68] S.H. Seo, S. Kim, S.-E. Kim, S. Chung, S.E. Lee, Enhanced Thermal Sensitivity of TRPV3 in Keratinocytes Underlies Heat-Induced Pruritogen Release and Pruritus in Atopic Dermatitis, J. Invest. Dermatol. (2020). https://doi.org/10.1016/j.jid.2020.02.028.

[69] H. Zhang, X. Sun, H. Qi, Q. Ma, Q. Zhou, W. Wang, K. Wang, Pharmacological Inhibition of the Temperature-Sensitive and Ca2+-Permeable Transient Receptor Potential Vanilloid TRPV3 Channel by Natural Forsythoside B Attenuates Pruritus and Cytotoxicity of Keratinocytes, J. Pharmacol. Exp. Ther. 368 (2019) 21–31. https://doi.org/10.1124/jpet.118.254045.

[70] J. Zhao, A. Munanairi, X.-Y. Liu, J. Zhang, L. Hu, M. Hu, D. Bu, L. Liu, Z. Xie, B.S. Kim, Y. Yang, Z.-F. Chen, PAR2 Mediates Itch via TRPV3 Signaling in Keratinocytes, J. Invest. Dermatol. (2020). https://doi.org/10.1016/j.jid.2020.01.012.

[71] T. Morita, S.P. McClain, L.M. Batia, M. Pellegrino, S.R. Wilson, M.A. Kienzler, K. Lyman, A.S.B. Olsen, J.F. Wong, C.L. Stucky, R.B. Brem, D.M. Bautista, HTR7 Mediates Serotonergic Acute and Chronic Itch, Neuron. 87 (2015) 124–138. https://doi.org/10.1016/j.neuron.2015.05.044.

[72] S.R. Wilson, L. Thé, L.M. Batia, K. Beattie, G.E. Katibah, S.P. McClain, M. Pellegrino, D.M. Estandian, D.M. Bautista, The Epithelial Cell-Derived Atopic Dermatitis Cytokine TSLP Activates Neurons to Induce Itch, Cell. 155 (2013) 285–295. https://doi.org/10.1016/j.cell.2013.08.057.

[73] O. Alkhatib, R. da Costa, C. Gentry, T. Quallo, S. Bevan, D.A. Andersson, Promiscuous G-Protein-Coupled Receptor Inhibition of Transient Receptor Potential Melastatin 3 Ion Channels by Gβγ Subunits, J. Neurosci. 39 (2019) 7840–7852. https://doi.org/10.1523/JNEUROSCI.0882-19.2019.

[74] S. Jia, Y. Zhang, J. Yu, Antinociceptive Effects of Isosakuranetin in a Rat Model of Peripheral Neuropathy, Pharmacology. 100 (2017) 201–207. https://doi.org/10.1159/000478986.

[75] U. Krügel, I. Straub, H. Beckmann, M. Schaefer, Primidone inhibits TRPM3 and attenuates thermal nociception in vivo, Pain. 158 (2017) 856–867. https://doi.org/10.1097/j.pain.0000000000000846.

[76] H.H. Andersen, J. Elberling, N. Sharma, L.E. Hauberg, P. Gazerani, L. Arendt-Nielsen, Histaminergic and non-histaminergic elicited itch is attenuated in capsaicin-evoked areas of allodynia and hyperalgesia: A healthy volunteer study, Eur J Pain. 21 (2017) 1098–1109. https://doi.org/10.1002/ejp.1013.

[77] G. Yosipovitch, K. Fast, J.D. Bernhard, Noxious heat and scratching decrease histamine-induced itch and skin blood flow, J. Invest. Dermatol. 125 (2005) 1268–1272. https://doi.org/10.1111/j.0022-202X.2005.23942.x.

[78] A.P. Kardon, E. Polgár, J. Hachisuka, L.M. Snyder, D. Cameron, S. Savage, X. Cai, S. Karnup, C.R. Fan, G.M. Hemenway, C.S. Bernard, E.S. Schwartz, H. Nagase, C.

Schwarzer, M. Watanabe, T. Furuta, T. Kaneko, H.R. Koerber, A.J. Todd, S.E. Ross, Dynorphin acts as a neuromodulator to inhibit itch in the dorsal horn of the spinal cord, Neuron. 82 (2014) 573–586. https://doi.org/10.1016/j.neuron.2014.02.046.

[79] S.E. Ross, A.R. Mardinly, A.E. McCord, J. Zurawski, S. Cohen, C. Jung, L. Hu, S.I. Mok, A. Shah, E.M. Savner, C. Tolias, R. Corfas, S. Chen, P. Inquimbert, Y. Xu, R.R. McInnes, F.L. Rice, G. Corfas, Q. Ma, C.J. Woolf, M.E. Greenberg, Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice, Neuron. 65 (2010) 886–898. https://doi.org/10.1016/j.neuron.2010.02.025.

[80] X.-J. Chen, Y.-G. Sun, Central circuit mechanisms of itch, Nat Commun. 11 (2020) 3052. https://doi.org/10.1038/s41467-020-16859-5.

[81] G. Hans, B.L. Schmidt, G. Strichartz, Nociceptive sensitization by endothelin-1, Brain Res Rev. 60 (2009) 36–42. https://doi.org/10.1016/j.brainresrev.2008.12.008.

[82] T.P. Smith, T. Haymond, S.N. Smith, S.M. Sweitzer, Evidence for the endothelin system as an emerging therapeutic target for the treatment of chronic pain, J Pain Res. 7 (2014) 531–545. https://doi.org/10.2147/JPR.S65923.

[83] H. Fruhstorfer, M. Hermanns, L. Latzke, The effects of thermal stimulation on clinical and experimental itch, Pain. 24 (1986) 259–269. https://doi.org/10.1016/0304-3959(86)90048-5.

[84] H. Murota, M. Izumi, M.I.A. Abd El-Latif, M. Nishioka, M. Terao, M. Tani, S. Matsui, S. Sano, I. Katayama, Artemin causes hypersensitivity to warm sensation, mimicking warmth-provoked pruritus in atopic dermatitis, J Allergy Clin Immunol. 130 (2012) 671-682.e4. https://doi.org/10.1016/j.jaci.2012.05.027.

[85] J. Vriens, B. Nilius, T. Voets, Peripheral thermosensation in mammals, Nat. Rev. Neurosci. 15 (2014) 573–589. https://doi.org/10.1038/nrn3784.

[86] R. Paricio-Montesinos, F. Schwaller, A. Udhayachandran, F. Rau, J. Walcher, R. Evangelista, J. Vriens, T. Voets, J.F.A. Poulet, G.R. Lewin, The Sensory Coding of Warm Perception, Neuron. 106 (2020) 830-841.e3. https://doi.org/10.1016/j.neuron.2020.02.035.

[87] S. Dembla, M. Behrendt, F. Mohr, C. Goecke, J. Sondermann, F.M. Schneider, M. Schmidt, J. Stab, R. Enzeroth, M.G. Leitner, P. Nuñez-Badinez, J. Schwenk, B. Nürnberg, A. Cohen, S.E. Philipp, W. Greffrath, M. Bünemann, D. Oliver, E. Zakharian, M. Schmidt, J. Oberwinkler, Anti-nociceptive action of peripheral mu-opioid receptors by G-beta-gamma protein-mediated inhibition of TRPM3 channels, Elife. 6 (2017). https://doi.org/10.7554/eLife.26280.

[88] A. Garami, Y.P. Shimansky, E. Pakai, D.L. Oliveira, N.R. Gavva, A.A. Romanovsky, Contributions of different modes of TRPV1 activation to TRPV1 antagonist-induced hyperthermia, J. Neurosci. 30 (2010) 1435–1440. https://doi.org/10.1523/JNEUROSCI.5150-09.2010.

[89] N.R. Gavva, A.W. Bannon, S. Surapaneni, D.N. Hovland, S.G. Lehto, A. Gore, T. Juan, H. Deng, B. Han, L. Klionsky, R. Kuang, A. Le, R. Tamir, J. Wang, B. Youngblood, D. Zhu, M.H. Norman, E. Magal, J.J.S. Treanor, J.-C. Louis, The vanilloid receptor TRPV1 is tonically activated in vivo and involved in body temperature regulation, J. Neurosci. 27 (2007) 3366–3374. https://doi.org/10.1523/JNEUROSCI.4833-06.2007.

[90] N.R. Gavva, J.J.S. Treanor, A. Garami, L. Fang, S. Surapaneni, A. Akrami, F. Alvarez, A. Bak, M. Darling, A. Gore, G.R. Jang, J.P. Kesslak, L. Ni, M.H. Norman, G. Palluconi, M.J. Rose, M. Salfi, E. Tan, A.A. Romanovsky, C. Banfield, G. Davar, Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans, Pain. 136 (2008) 202–210. https://doi.org/10.1016/j.pain.2008.01.024.

[91] L.O. Gomes, D.B. Hara, G.A. Rae, Endothelin-1 induces itch and pain in the mouse cheek model, Life Sciences. 91 (2012) 628–633. https://doi.org/10.1016/j.lfs.2012.03.020.

[92] R. Katugampola, M.K. Church, G.F. Clough, The neurogenic vasodilator response to endothelin-1: a study in human skin in vivo, Exp. Physiol. 85 (2000) 839–846.

[93] D. Badheka, I. Borbiro, T. Rohacs, Transient receptor potential melastatin 3 is a phosphoinositide-dependent ion channel, J. Gen. Physiol. 146 (2015) 65–77. https://doi.org/10.1085/jgp.201411336.

[94] D. Badheka, Y. Yudin, I. Borbiro, C.M. Hartle, A. Yazici, T. Mirshahi, T. Rohacs, Inhibition of Transient Receptor Potential Melastatin 3 ion channels by G-protein βγ subunits, Elife. 6 (2017). https://doi.org/10.7554/eLife.26147.

[95] B.I. Tóth, M. Konrad, D. Ghosh, F. Mohr, C.R. Halaszovich, M.G. Leitner, J. Vriens, J. Oberwinkler, T. Voets, Regulation of the transient receptor potential channel TRPM3 by phosphoinositides, J. Gen. Physiol. 146 (2015) 51–63. https://doi.org/10.1085/jgp.201411339.

[96] L. Vangeel, M. Benoit, Y. Miron, P.E. Miller, K. De Clercq, P. Chaltin, C. Verfaillie, J. Vriens, T. Voets, Functional expression and pharmacological modulation of TRPM3 in human sensory neurons, Br. J. Pharmacol. 177 (2020) 2683–2695. https://doi.org/10.1111/bph.14994.

FIGURE LEGENDS

Figure 1. Pain and itch behaviour induced by TRPM3 agonist and capsaicin in the mouse cheek model

Trpm3$^{+/+}$ and *Trpm3*$^{-/-}$ mice were injected in the cheek with the agonists indicated in the figure. (A) Number of wipes, (B) bouts of scratches, and (C) total time of scratching as determined within 30 minutes following the injection. (D) Scratch ratio ($R_{scratch}$) calculated as described in the methods. (E) Mean time courses of the number of wipes after injecting the agonists in 5 minute-long intervals within 35 minutes after injection. (F) Statistical comparison of the number of wipes in the 0-5 min interval, as indicated in in panel (E). Dots mark data from individual animals, boxes indicate 25-75 percentile, thick lines and thin lines in the boxes point the mean and median, respectively, whiskers show SD. n.s.: $p>0.05$ (non-significant), *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared either to the vehicle treated group within the same genotype using Kruskal-Wallis test with ~~Dunn-~~Bonferroni adjusted Mann-Whitney U test as post hoc analysis (grey marking) or compared between *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ within the same treatment, as indicated, using Mann-Whitney U test (black marking). ~~(E) Mean time courses of the number of wipes after injecting the agonists in 5 minute-long intervals within 35 minutes after injection. WT: *Trpm3*$^{+/+}$, KO: *Trpm3*$^{-/-}$~~

Figure 2. Quantification of the itch and pain related behavior induced by Hist, 5-HT, and ET-1 in *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice

Trpm3$^{+/+}$ and *Trpm3*$^{-/-}$ mice were injected in the cheek with the pruritogens indicated in the figure. (A) Number of wipes, (B) bouts of scratches, and (C) total time of scratching as determined within 30 minutes following the injection. (D) Scratch ratio ($R_{scratch}$) calculated as described in the methods. (E) Bouts of scratches after injecting indicated compounds in the nape of *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice. Vehicle treated control groups presented in panel (A)-(D) are identical with those presented in Figure 1A-D. Dots mark data from individual animals, boxes indicate 25-75 percentile, thick lines and thin lines in the boxes point the mean and median, respectively, whereas whiskers show SD. n.s.: p>0.05 (non-significant), *p<0.05, **p<0.01, ***p<0.001, between *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ within the same treatment, as indicated, using Mann-Whitney U test (black marking) or compared to the vehicle treated group within the same genotype using Kruskal-Wallis test with Bonferroni adjusted Mann-Whitney U test as post hoc analysis (grey marking). ~~WT: *Trpm3*$^{+/+}$, KO: *Trpm3*$^{-/-}$~~

Figure 3. Effect of pruritogens in TG neurons isolated from *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ animals.

(A) Representative traces showing typical changes in intracellular Ca^{2+} concentration of TG neurons isolated from *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice in response to 100 µM Hist, 20 µM PregS, 100 µM CA, 1 µM Caps as indicated in the panel. 25 mM KCl was used as positive control to depolarize the neuronal cell membrane. (B) Percentage of TG neurons form *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice responding to PregS (PregS+), CA (CA+) and Caps (Caps+). The measurements were carried out as in panel (A). (C) Percentage of TG neurons form *Trpm3*$^{+/+}$ and *Trpm3*$^{-/-}$ mice responding to 100 mM Hist, 100 mM 5-HT and 100 nM ET-1 in the experiments. Sample size (n) is indicated over the columns~~, WT: *Trpm3*$^{+/+}$, KO:~~

~~*Trpm3*$^{-/-}$~~. The measurements were carried out as in panel (A). In each group, neurons were isolated from ≥3 mice, each measured in independent experiment.

Figure 4. Effect of Isok on the Ca^{2+} signals evoked by PregS and pruritogens in *Trpm3*$^{+/+}$ mice

(A) Averaged traces of several PregS+ TG neurons from *Trpm3*$^{+/+}$ mice showing mean changes in intracellular Ca^{2+} concentration in response to 20 µM PregS in the presence or absence of 3 µM IsoK. 25 mM KCl was used as positive control to depolarize the neuronal cell membrane. (B) Statistical analysis of Isok effect on PregS induced Ca^{2+} transients in PregS+ *Trpm3*$^{+/+}$ TG sensory neurons as shown in panel (A). Values are given as percentage of the first PregS-induced Ca^{2+} transient. Dots represent individual neurons, and horizontal lines indicate mean values. Effect of Isok was compared to the vehicle treated control group by Mann-Whitney test, n.s: $p>0.05$ (non-significant), ***$p<0.001$. (C) Representative traces showing typical changes of intracellular Ca^{2+} concentration in TG neurons from *Trpm3*$^{+/+}$mice in response to 100 µM serotonin and 20 µM PregS in the presence and absence of 3 µM Isok. 25 mM KCl was used as positive control to depolarize the neuronal cell membrane. (D) Statistical analysis of Isok effect on the pruritogens induced Ca^{2+} transients in pruritogen responsive TG sensory neurons from *Trpm3*$^{+/+}$ animals. Values are given as Δ(F1/F0), dots represent individual neurons, and horizontal lines indicate mean values. Effect of Isok was compared to the vehicle treated control group by Mann-Whitney test, n.s: $p>0.05$ (non-significant). (E) Percentage of TG neurons form WT mice responding to Hist, 5-HT and ET-1 in the presence and absence of 3 µM Isok. The measurements were carried out as in panel (C). The distribution of the pruritogen responders among TG sensory neurons was compared using Chi squared test, n.s.: $p>0.05$

(non-significant). Responders are marked with the indicated colors and non-responders are marked with grey. In each group, neurons were isolated from ≥3 mice and measured in independent experiments.

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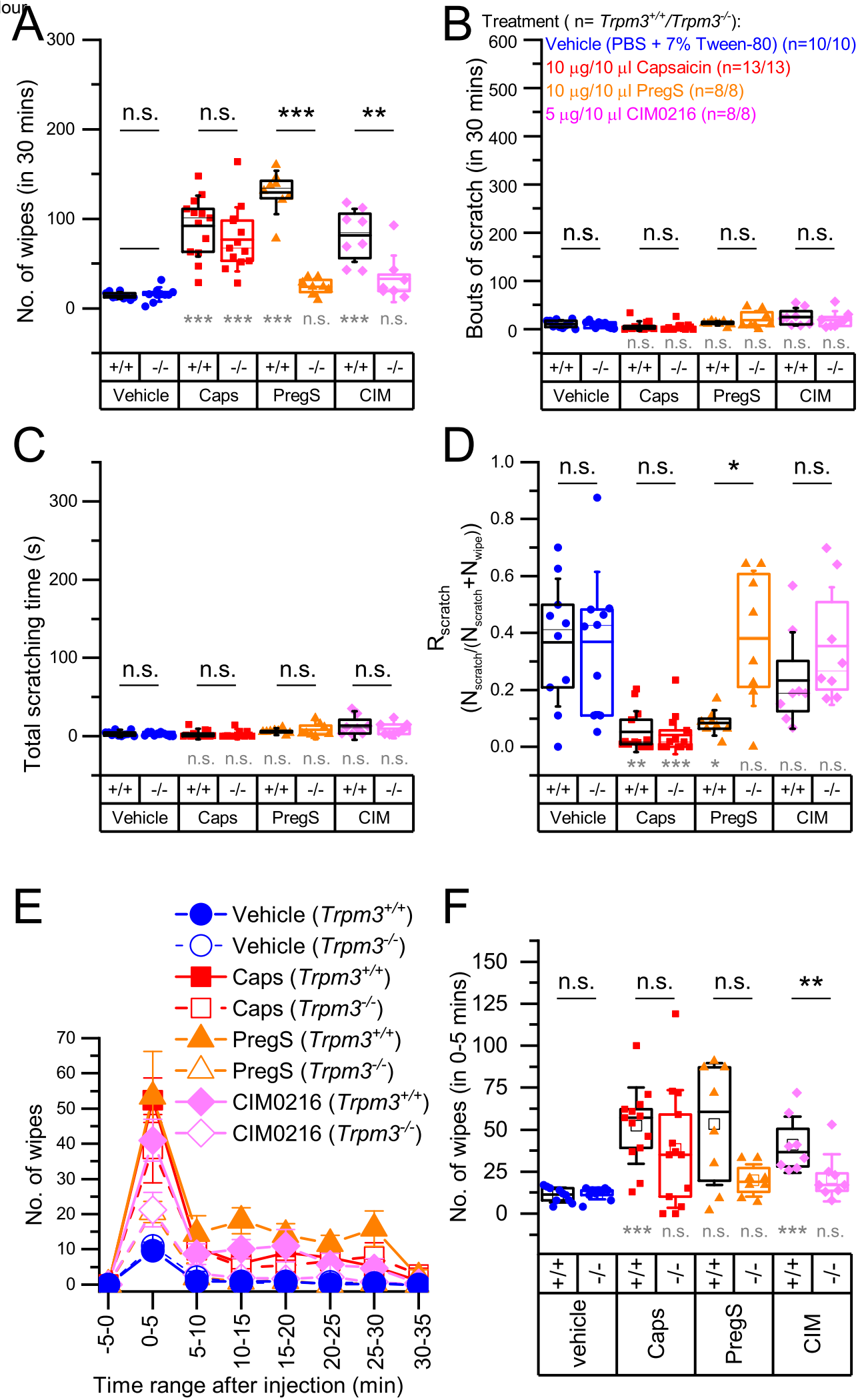

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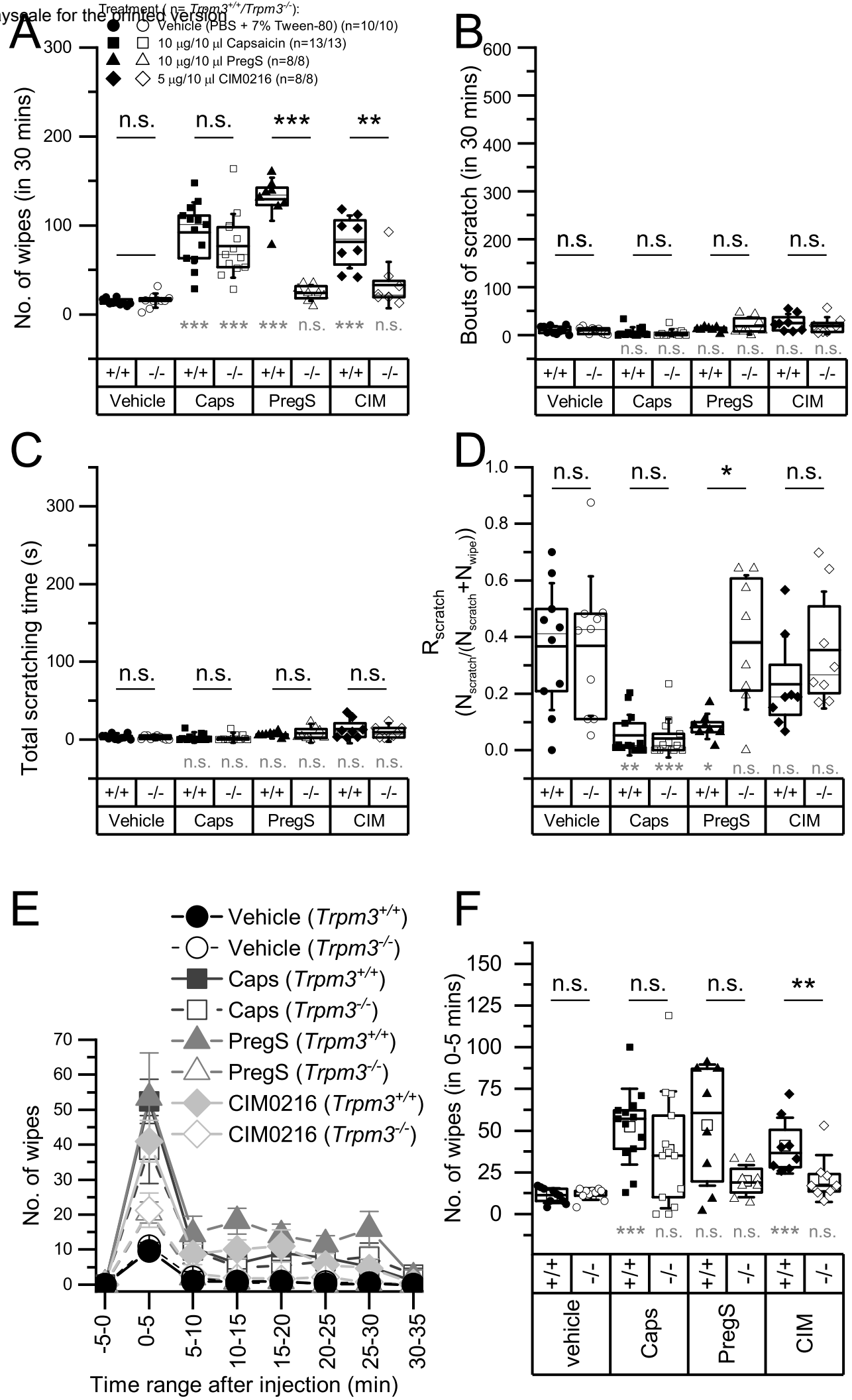

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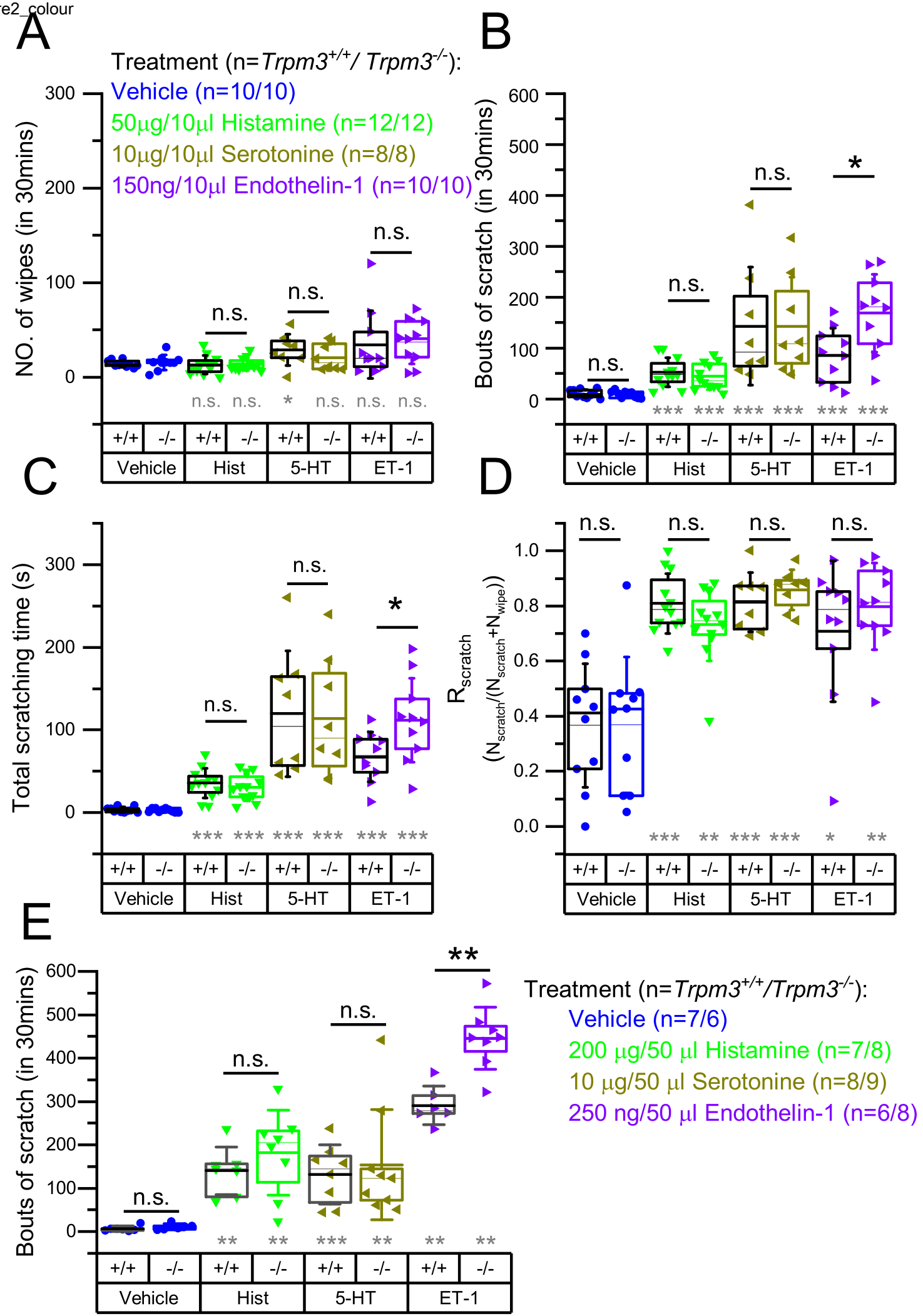

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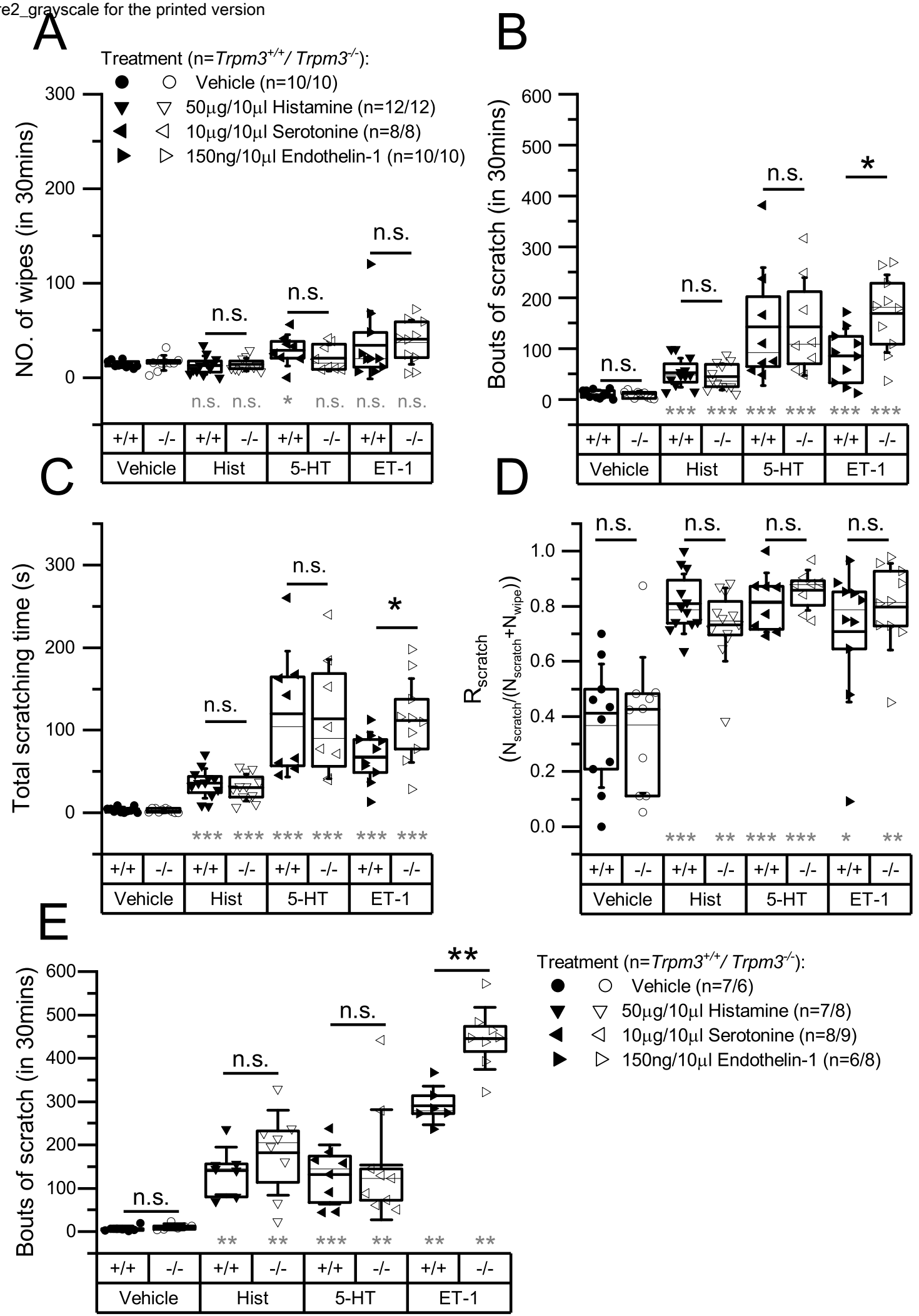

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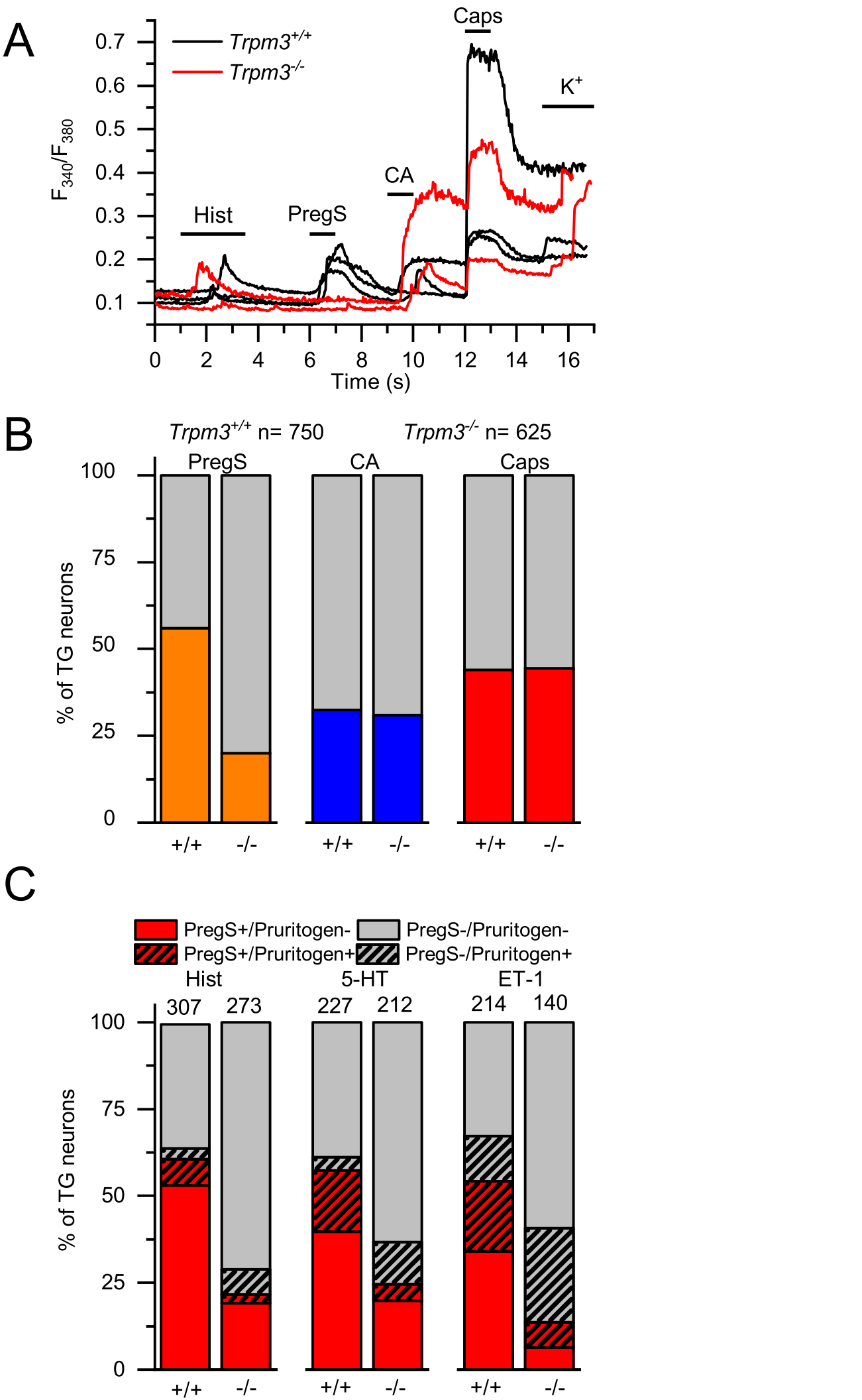

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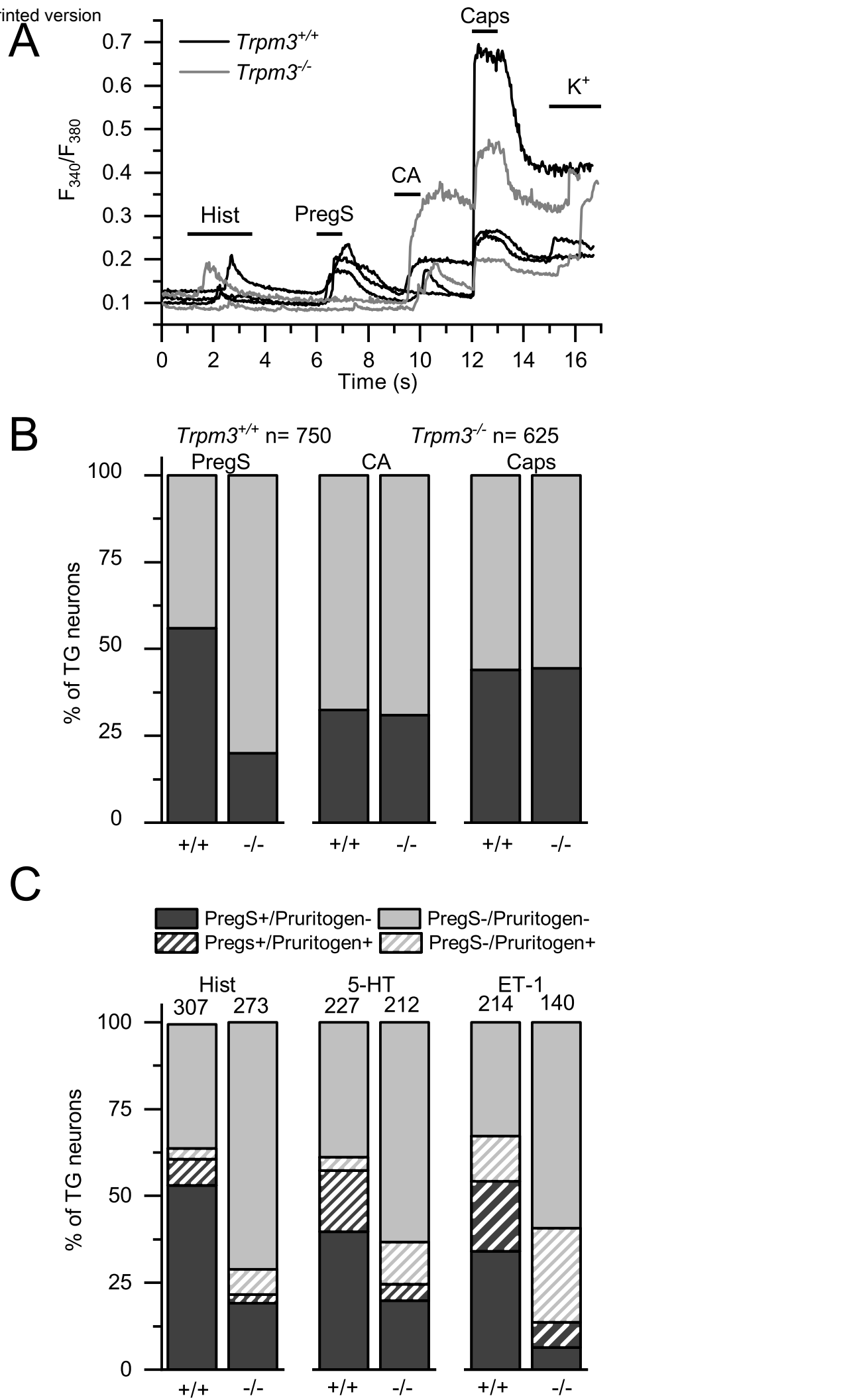

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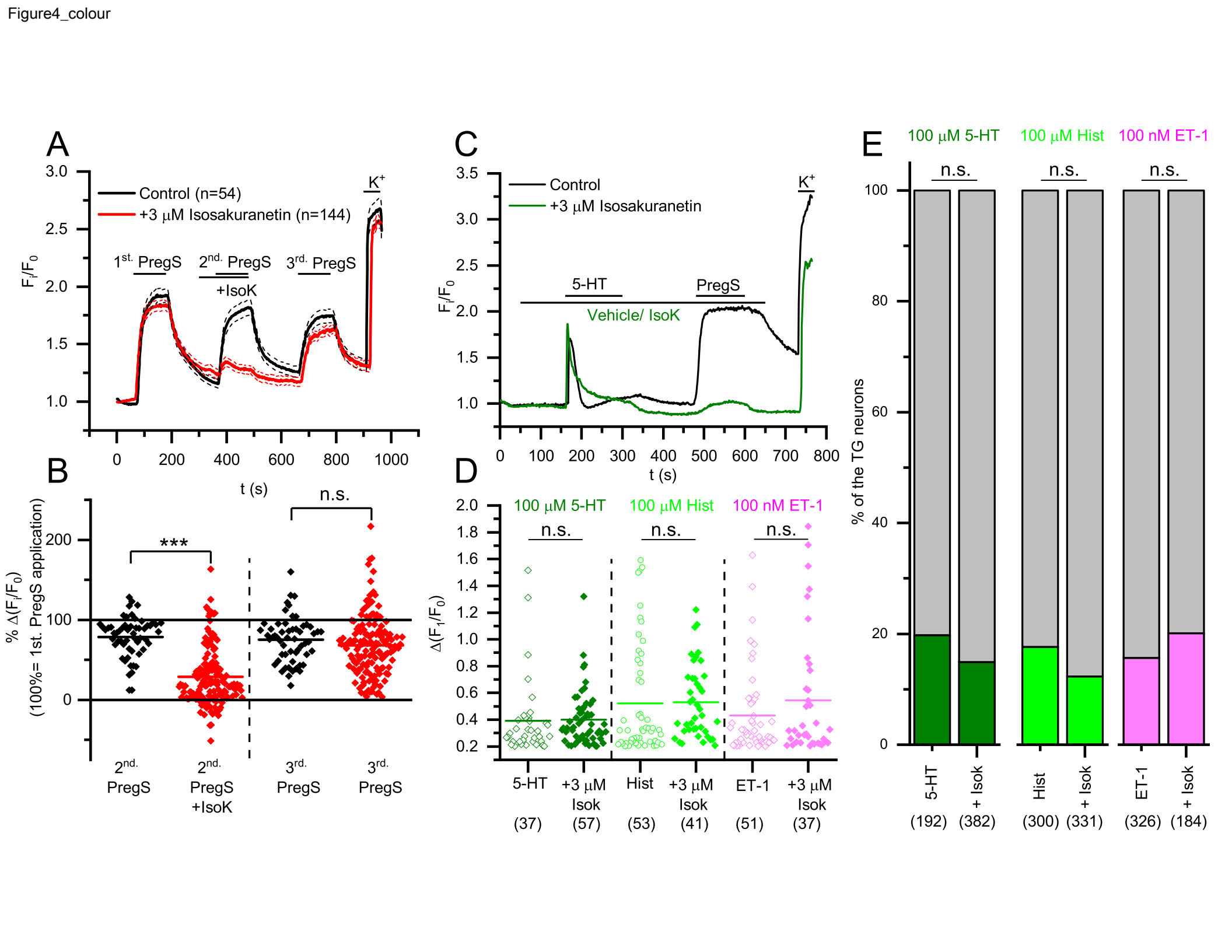

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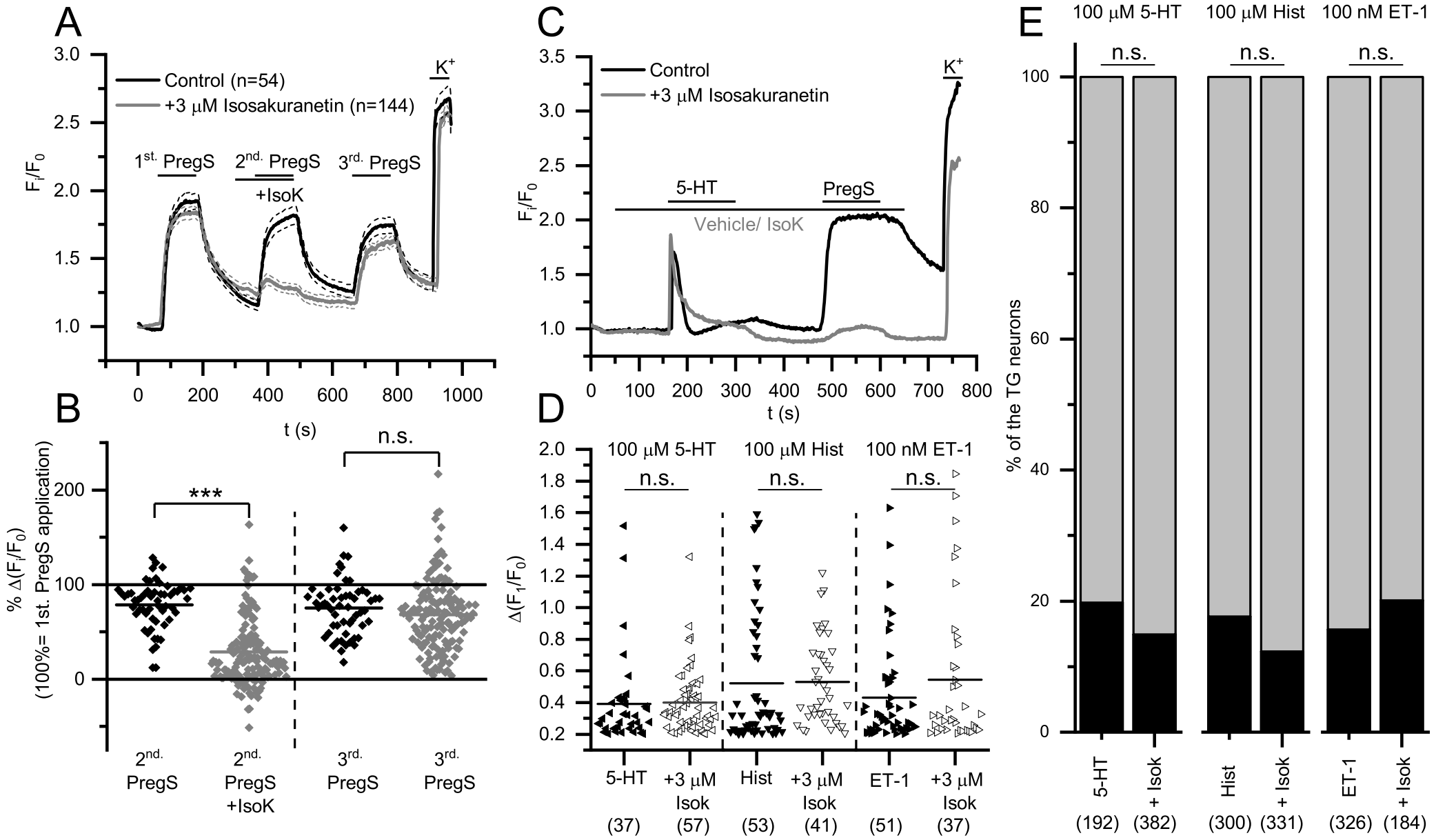

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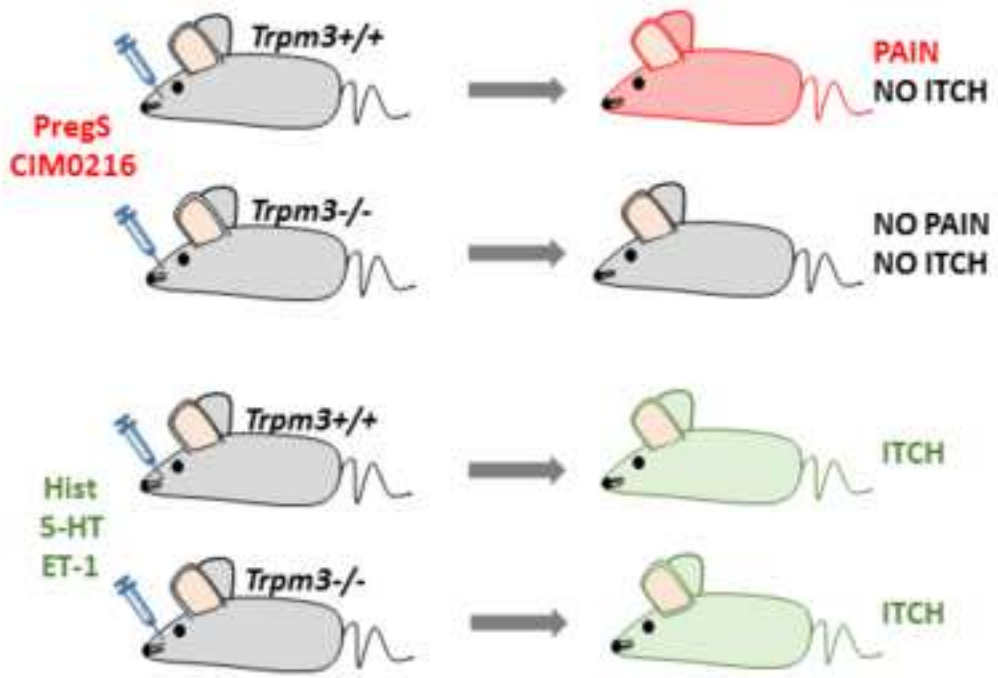

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The TRPM3 ion channel mediates nociception but not itch evoked by endogenous pruritogenic mediators

by

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