

HONORS

<i>Title of Award</i>	<i>Year</i>
Doctorate Degree Conferral date, (12/2020)	2020
NIDCR F31 Pre-Doctoral Fellowship, F31DE027270-01	2017-2019
CACPR meeting poster presentation – first place	2016
Graduated from UMB, Masters, <i>Magna Cum Laude</i>	2011
Graduated from GSU, <i>Magna Cum Laude</i>	1993
Finalist for Belle Honors Scholarship, GSU	1990

PUBLICATIONS

Articles

Joseph J, Qu L, Wang S, Kim M, Bennett D, Ro J, Caterina MJ, Chung MK. Phosphorylation of TRPV1 S801 Contributes to Modality-Specific Hyperalgesia in Mice. *J Neurosci*. 2019 Dec 11;39(50):9954-9966. doi: 10.1523/JNEUROSCI.1064-19.2019. Epub 2019 Nov 1. PMID: 31676602; PMCID: PMC6978941

Hall BE, Prochazkova M, Sapio MR, Minetos P, Kurochkina N, Binukumar BK, Amin ND, Terse A, **Joseph J**, Raithel SJ, Mannes AJ, Pant HC, Chung MK, Iadarola MJ, Kulkarni AB. Phosphorylation of the Transient Receptor Potential Ankyrin 1 by Cyclin-dependent Kinase 5 affects Chemo-nociception. *Sci Rep*. 2018 Jan 19;8(1):1177. PMID: 29352128

Wang S, Lim J, **Joseph J**, Wang S, Wei F, Ro JY, Chung MK. Spontaneous and Bite-Evoked Muscle Pain Are Mediated by a Common Nociceptive Pathway with Differential Contribution by TRPV1. *J Pain*. 2017 Jun 29. pii: S1526-5900(17)30628-4. doi: 10.1016/j.j. PMID: 28669862

Wang S, Wang S, Asgar J, **Joseph J**, Ro JY, Wei F, Campbell JN, Chung MK. Ca²⁺ and calpain mediate capsaicin-induced ablation of axonal terminals expressing transient receptor potential vanilloid 1. *J Biol Chem*. 2017 May 19;292(20):8291-8303. doi: 10.1074/jbc.M117.778290. Epub 2017 Mar 30. PMID: 28360106

Wang S*, **Joseph J***, Diatchenko L, Ro JY, Chung MK. Agonist-dependence of functional properties for common nonsynonymous variants of human transient receptor potential vanilloid 1. *Pain*. 2016; Mar 9. PMID: 26967694 (*, co-first author)

Chung MK, Lee J, **Joseph J**, Saloman J, Ro JY. 'Peripheral group I metabotropic glutamate receptor activation leads to muscle mechanical hyperalgesia through TRPV1 phosphorylation in the rat.'

Journal of Pain. 16(1):67-76. Jan 2015. PMID:25451626

Sen Wang*, **John Joseph***, Jin Y. Ro, Man-Kyo Chung. 'Modality-specific mechanisms of PKC-induced hypersensitivity of TRPV1: S800 is a polymodal sensitization site.'

Pain. 2015 May;156(5):931-41.

PMID: 25734989 (*, co-first author)

J. Joseph*, S. Wang*, J. Lee, J. Ro, and M.K. Chung. 'Carboxy-Terminal Domain of Transient Receptor Potential Vanilloid 1 Contains Distinct Segments Differentially Involved in Capsaicin- and Heat-induced Desensitization' The Journal of Biological Chemistry. vol. 288, 50, pp. 35690-35702, December 2013.

PMID:2417452 (*, co-first author)

abstracts

1. Kulkarni A.B., Hall B., Minetos P., Terse A., Amin N., **Joseph J.**, Pant H., Chung M.K. and Prochazkova M.: Cdk5 activity modulates TRPA1 nociceptive responses. 37th Annual Meeting of Society for Neuroscience, Nov. 2017.
2. Wang S., Wang S., **Joseph J.**, Ro J., Wei F., Campbell J., Chung M.K.: Calcium-dependent ablation of TRPV1-lineage axonal terminals by capsaicin. 36th Annual Meeting of Society for Neuroscience, Nov. 2016.
3. Prochazkova M., Hall B., Minetos P., **Joseph J.**, Roque E., Chung M.K., Kulkarni A.B.: Cdk5 modulates functional responses of TRPA1. 16th World Congress on Pain; Sep. 2016.
4. **Joseph J.**, Wang S., Asgar J., Diatchenko L., Ro J. Y., Chung M.K.: Agonist-dependent activation, permeation, and desensitization properties of common nonsynonymous variants of human transient receptor potential vanilloid 1. 34th Annual Meeting of Society for Neuroscience, Nov. 2014.
5. Ro J. Y., Chung M.K., Lee J., **Joseph J.**, Saloman J.L. : Activation of Group I metabotropic glutamate receptors leads to masseter mechanical hypersensitivity through PKC-mediated phosphorylation of TRPV1. 34th Annual Meeting of Society for Neuroscience, Nov. 2014.
6. Ro J.Y., Chung M.K., Lee J.S., Saloman J.L. and **Joseph J.**: Functional interactions between glutamate receptors and TRPV1 in trigeminal sensory neurons. 7th Scientific Meeting of the TMJ Association, Sep 2014.
7. **Joseph J.**, Wang S., Ro J.Y., Chung M.K. : Modality-specific mechanisms of protein kinase C-induced hypersensitivity of transient receptor potential vanilloid 1. 33rd Annual Meeting of Society for Neuroscience, Nov. 2013.

8. Wang S., **Joseph J.**, Ro J., Chung, M.K.: Carboxy terminal domain of TRPV1 is involved in heat-induced desensitization. 32nd Annual Meeting of Society for Neuroscience, Oct. 2012.

Presentations

38th Annual Meeting of Society for Neuroscience, poster. *Phosphorylation of TRPV1 S801 contributes to modality-specific hyperalgesia in mice.* Nov. 2018

Program in Neuroscience, UMB, annual retreat, poster. *Calpain mediates capsaicin-induced ablation of TRPV1-lineage axonal terminals.* 2017.

Annual meeting of Greater Baltimore Chapter of the Society for Neuroscience, poster. *Agonist-dependent activation, permeation, and desensitization properties of common nonsynonymous variants of human transient receptor potential vanilloid 1.* Nov 2014.

34th Annual Meeting of Society for Neuroscience, poster. *Agonist-dependent activation, permeation, and desensitization properties of common nonsynonymous variants of human transient receptor potential vanilloid 1.* Nov. 2014

Johns Hopkins University/University of Maryland, Baltimore Pain workshop. *Agonist-Dependent Properties of Common Variants of Human TRPV1.* Oct 2014.

33rd Annual Meeting of Society for Neuroscience, poster: *Modality-specific mechanisms of protein kinase C-induced hypersensitivity of transient receptor potential vanilloid 1.* Nov 2013.

32nd Annual Meeting of Society for Neuroscience, poster: *Carboxy terminal domain of TRPV1 is involved in heat-induced desensitization.* Oct 2012.

Pain interest group meeting, University of Maryland, Baltimore, Department of Neural and Pain Sciences. *The carboxy-terminus of rTRPV1 has distinct segments affecting agonist-induced desensitization.* Mar 2012.

TEACHING

2018-2019 facilitator, Teaching Research Conference, for UMB Dental students
2018 mediator, Clinical Research Conferences, for UMB Dental students
2017 mediator, Clinical Research Conferences, for UMB Dental students

RESEARCH ACTIVITIES

- 2017-2020 Modality-specific hyperalgesia through post-translational modification of TRPV1
- 2016-2017 The *in vitro* functional effect of a point mutation at a PKC phosphorylation site in TRPV1, TRPV1 modulation during craniofacial neuropathy
- 2015-2016 The CDK5 modification of TRPV1 sensitivity and its contribution to orofacial pain, capsaicin-induced hypoalgesia in murine hindpaw , mechanisms of PKC-dependent TRPV1 sensitization
- 2014-2015 TRPV1 dependent loss of sensory fibers
- 2013-2014 The functional characterization of common polymorphisms for hTRPV1, A role for TRPV1 in muscle mechanical hyperalgesia
- 2012-2013 The modality-specific sensitization of TRPV1 by PKC
- 2011-2012 The characterization of the desensitization observed in the TRP-Vanilloid 1 receptor following agonist application
- 2010-2011 The influence of BK on bladder smooth muscle contraction in select knockout mice

RESEARCH COMMUNITY

- 2012-present *Society for Neuroscience* member, conference poster presentations
- 2012-present UMB Neural Pain Sciences journal club
- 2011-present UMB Pain Investigational Group seminars

COMMUNITY

- 2011 UMB Project Jumpstart Baltimore homeless advocate program, organized by UMB students

Abstract.

Title of Dissertation: The Contribution of TRPV1 S801 Phosphorylation to Nociception and Inflammatory Pain *in Vivo*

John Joseph, Doctor of Philosophy, 2020

Dissertation directed by: Man-Kyo Chung, PhD, Professor,
Department of Neural and Pain Sciences

Transient receptor potential vanilloid subtype 1 (TRPV1) is a nonselective cationic channel activated by painful stimuli such as capsaicin and noxious heat, and enriched in many primary afferent neurons of the pain pathway. During inflammation, chemical mediators activate protein kinases (such as PKC) that phosphorylate TRPV1 and thereby enhance its function, which results in nociceptor sensitization. And this can result in a lower threshold for pain. However, the causal relationships between TRPV1 phosphorylation and pathological pain remain unexplored. To directly investigate the roles of one specific TRPV1 phosphorylation event *in vivo*, we genetically altered a major PKC phosphorylation site, mouse TRPV1 S801, to alanine. The TRPV1 expression pattern in sensory neurons of S801A knock-in (KI) mice was comparable to that in wildtype (WT) controls. In sensory neurons from KI mice, following the activation of PKC, the usual increase of capsaicin-induced currents was substantially impaired. Thermal hyperalgesia induced by PMA or burn injury in KI was identical to WT. Thermal hyperalgesia was only marginally attenuated in KI mice during inflammation. In

contrast, PMA-evoked nocifensive responses and hyperalgesia to capsaicin were significantly attenuated in the hindpaws of KI mice. Ongoing pain from inflamed masseter muscle was also reduced in KI mice, and the pain was further inhibited by the TRPV1 antagonist AMG9810. These results suggest that PKC-mediated phosphorylation of TRPV1 S801 contributes to inflammation-mediated sensitization of TRPV1 to ligand, but not heat, *in vivo*. Further, this suggests that interference with TRPV1 S801 phosphorylation might represent a potential way to reduce inflammatory pain in the clinic, yet spare basal sensitivity and produce fewer side effects than with a more general TRPV1 inhibition.

The Contribution of TRPV1 S801 Phosphorylation to Nociception and Inflammatory
Pain *in Vivo*

by
John C. Joseph

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2020

©Copyright by John Joseph 2020
All rights reserved

Dedication

To my family, wife, and daughter, whose love and encouragement sustained me during my graduate endeavor.

To my father, in memorium, I further give special dedication. His belief in my benefit from a higher education, and commitment to it, enabled my return to graduate school. It was his enduring belief in me, that enabled this achievement.

Acknowledgements

The author would like to gratefully acknowledge the contributions of his advisor, Dr. Man-Kyo Chung. His keen design of experiments, attention to detail, and productivity were essential for this project, and valuable examples for a successful career in research.

For providing a collaborative lab, guidance, enthusiasm and friendship along the way, thank you.

저자는 자신의 지도교수인 정만교 박사 (Dr. Man-Kyo Chung)에게 심심한 감사를 표하고 싶습니다. 그의 예리한 실험 계획, 세부적인 사항에 대한 꼼꼼한 주의, 생산성 등은 본 프로젝트에 필수적인 부분을 차지하였으며, 연구자로서 성공적인 경력을 위한 귀중한 본보기였습니다.

본 프로젝트를 수행하는 동안 그가 제공한 협력적인 실험실과 지도, 열정과 우정에 대해 깊이 감사드립니다.

To Dr. Michael Caterina and Dr. Lintao Qu, for assisting with initial development of the project, and helpful discussions throughout, I am grateful.

My thanks to Dr. Jin Ro and Dr. Feng Wei, for thorough review and commentary on the writing of the dissertation.

Thanks to members of the Chung lab, for contributions to experiments for this and other studies in the lab.

Finally, thanks to my brother Dr. Robert Joseph, Jr., for sharing his professional experience and giving a different perspective on a graduate education.

Table of Contents

Acknowledgements	iv
List of Figures	viii
List of Abbreviations	ix
Chapter 1. Introduction	1
1.1. Pain and nociception	1
1.2. TRPV1.....	3
1.3. TRPV1 structure.....	4
1.4. Capsaicin	5
1.5. Inflammation and peripheral sensitization	6
1.6. The TRPV1 membrane ménage	7
1.7. Kinases and PKC.....	9
1.8. Protein Kinase C substrates include TRPV1	14
1.8.1. PKC Phosphorylation sites on TRPV1.....	14
1.8.2. Analysis of PKC sites on TRPV1.....	16
1.9. Hypothesis.....	21
1.10. Specific Aims.....	21
Chapter 2. Generation of TRPV1 S801A Knock-in Mice	25
2.1. Introduction.	25
2.2. CRISPR/Cas9 Genome Editing Overview	26
2.3. Generation of TRPV1 S801A Mutant Mice.....	33
2.3.1. Primer sequence design.....	34
2.3.2. Confirmation of mutation.....	35
2.3.3. Immunohistochemistry	37
2.3.4. Western blot assay.....	37
2.3.5. Statistical analysis	38
2.4. Results	38
2.4.1. TRPV1 S801A knock-in mice were generated using CRISPR/Cas9.....	38
2.4.2. The effects of the S801A mutation on TRPV1 expression	42

2.5. Discussion	48
Chapter 3. The Effect of the S801A Mutation on Functional Properties of TRPV1	51
3.1. Introduction	51
3.2. Methods	52
3.2.1. Dissociation of mouse sensory neurons	52
3.2.2. Whole-cell patch clamp.....	52
3.2.3. Statistical analysis	53
3.3. Results	53
3.3.1. TRPV1 S801A nociceptors showed impaired sensitization of capsaicin-evoked currents	53
3.3.2. TRPV1 S801A nociceptors show attenuated PMA mediated recovery from desensitization to capsaicin	56
3.4. Discussion	59
Chapter 4. Effect of TRPV1 S801A Mutation on Nociceptive and Pathological Pain	62
4.1. Introduction	62
4.2. Methods	63
4.2.1. Laboratory Animals.....	63
4.2.2. Acute hindpaw nocifensive behaviors.....	63
4.2.3. Capsaicin-induced eye wiping.....	63
4.2.4. Complete Freund’s adjuvant (CFA)-induced inflammation	64
4.2.5. Carrageenan-induced hindpaw inflammation	64
4.2.6. Hargreaves’ radiant paw heating assay	64
4.2.7. Measuring noxious heat threshold.....	65
4.2.8. Von Frey measurement on hind paw.....	65
4.2.9. Paw pinching assay	66
4.2.10. Tail immersion assay.....	66
4.2.11. Hot plate assay.....	67
4.2.12. PMA-induced thermal hyperalgesia of hindpaw.....	67
4.2.13. Mild hindpaw thermal injury.....	67
4.2.14. Mouse grimace scale measurements (MGS).....	67

4.2.15. Bite force assay	68
4.2.16. Conditioned Place Aversion	69
4.2.17. Statistical analysis	70
4.3. Results	71
4.3.1. TRPV1 S801A mutation only marginally attenuates inflammatory thermal hyperalgesia	71
4.3.2. TRPV1 S801A mutation attenuates PMA-induced nociception and sensitization of capsaicin responses	74
4.3.3. TRPV1 S801A attenuates spontaneous pain from inflamed masseter muscle	77
4.3.4. Conditioned Place Aversion	80
4.4. Discussion	86
Chapter 5. Conclusion	92
References	96

List of Figures

Figure 1. Modality-specific contribution of PKC-phosphorylation residues of TRPV1	18
Figure 2. CRISPR/Cas9 transcription and processing	29
Figure 3. Location of the PAM sequence used by Cas9.....	30
Figure 4. Sequence alignment of carboxy terminal domains of TRPV1	33
Figure 5. Generation of TRPV1 S801A KI mice using CRISPR/Cas9 method.....	40
Figure 6. TRPV1 S801A KI mice show no altered expression of TRPV1.....	42
Figure 7. Size distribution of TRPV1+ neurons is unchanged in KI mice	45
Figure 8. Triple labeling of TRPV1, IB4 and CGRP in the spinal cord.....	46
Figure 9. Western blot analysis of TRPV1 using whole DRG from WT or KI mice.....	47
Figure 10. Sensory neurons from KI mice show attenuated sensitization of capsaicin-evoked current response by PMA	55
Figure 11. Sensory neurons from KI mice show attenuated PMA mediated recovery from desensitization to capsaicin.....	58
Figure 12. KI mice show normal heat sensitivity and modestly attenuated CFA-induced thermal hyperalgesia.....	73
Figure 13. KI mice show attenuation of acute PMA-induced nocifensive behavior and PMA-induced sensitization of capsaicin-mediated nocifensive behavior	76
Figure 14. KI mice show attenuated spontaneous ongoing pain	79
Figure 15. Preliminary conditioned place avoidance with PMA in C57BL/6 mice	81
Figure 16. Flooring cue effect on CPA	82
Figure 17. Olfaction cue effect on CPA	83
Figure 18. CPA after injection of capsaicin in the hindpaw.....	84
Figure 19. CPA after hindpaw injection of CFA and capsaicin	85

List of Abbreviation

AKAP	A-kinase anchoring protein
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
BK	bradykinin
CaM	calmodulin
CaMKII	calcium/calmodulin dependent kinase II
Cas	CRISPR-associated gene
CCI	chronic constriction injury
CDK5	cyclin dependent kinase 5
CFA	complete Freund's adjuvant
CGRP	calcitonin-gene-related peptide
CNS	central nervous system
CPA	conditioned place aversion
CRISPR	clustered regularly interspersed short palindromic repeats
crRNA	CRISPR ribonucleic acid
DAG	diacylglycerol
DRG	dorsal root ganglia
DSB	double strand breaks
ET-1	endothelin 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GPR30	GPCR 30, estrogen membrane receptor
GRK	G-protein coupled receptor kinase
HDR	homology directed repair
HEK	human embryonic kidney cell
HODE	hydroxyoctadecadienoic acid
IASP	International Association for the Study of Pain
IB4	isolectin beta subunit 4

KI	TRPV1 S801A Knock-in mice
mGluR5	metabotropic glutamate receptor 5
MGS	mouse grimace scale
MIA	monoiodoacetate
MOR	mu opioid receptor
NF200	neurofilament 200
NHEJ	nonhomologous end-joining
NMDA	glutamate-N-methyl-D-aspartate receptors
PAM	protospacer adjacent motif
PBS	phosphate-buffered saline
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PIPKI	phosphatidylinositol 4-phosphate kinase
Pirt	phosphoinositide interacting regulator of TRP
PKA	protein kinase A
PKC	protein kinase C
PKCεV1-V2	PKCε inhibitor peptide
PMA	phorbol 12-myristate 13-acetate
PTM	post-translational modification
RACK	receptor of activated C-kinase
S801A	mutation of TRPV1 serine 801 to alanine
TG	trigeminal ganglia
TM	transmembrane
tracrRNA	transactivating crRNA
TRPA1	transient receptor potential ankyrin subfamily member 1
TRPV1	transient receptor potential vanilloid subfamily member 1
WT	wildtype C57BL/6J mice

Chapter 1. Introduction

1.1. Pain and nociception

Pain is sometimes a thief, robbing from the enjoyment of life, but a necessary bane. Animals detect harmful stimuli to prevent tissue damage. This is accomplished through a dazzling array of receptors in select sensory nerve fibers. The receptors are enriched at the distal terminals, for initial detection and interaction with the environment; and each which might respond to a different noxious stimulus. If the receptor response is strong enough, it will depolarize the sensory neuron and initiate an action potential.

Nociception is an attempt by these sensory neurons to signal the central nervous system (CNS), provide specifics about the offending stimulus, and possibly 'request' appropriate action to limit further damage. This can initiate behavioral responses from the perception of pain.

There is a formal distinction between pain and nociception in neuroscience. Though often considered one in the same, the two are not necessarily inclusive of one another. The detection and neural encoding of a noxious stimulus defines nociception, according to the International Association for the Study of Pain (IASP). Pain represents an interpretation of the encoded information that often results from nociception. It is an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (IASP). Acute pain can be considered essential to maintaining proper health of an organism, serving as an alarm for potential or existing harm. This can often evoke a withdrawal response, without which tissue damage

might occur. The relative perception of pain results from processing of sensory information within the context of cognitive awareness; which then also depends on the detection and signaling intensity of a noxious stimulus. The neurons which detect and encode a noxious stimulus, or one with the potential to be harmful, are termed nociceptors (IASP).

Nociceptors are a specific class of sensory neurons, all of which are part of the peripheral nervous system. The afferent neurons that innervate segregated regions of the body (dermatomes) have somata which are usually clustered in structures termed ganglia. Sensory ganglia are enlargements along peripheral nerves. The somata each extend one axon that bifurcates, sending a long branch to the periphery which contributes to peripheral nerves, and a much shorter branch that is part of the ganglia rootlets connecting to the central nervous system. The paired trigeminal ganglia are largely responsible for sensory innervation of the face. The dorsal root ganglia (DRG) are present on the dorsal root, as the name implies, of each nerve that emerges from a spinal segment. The DRG are then paravertebral to the spinal column, and largely responsible for sensory innervation of the body. Within the ganglia there are many types of neurons. These can be classified by size, neurochemistry, conduction velocity, and response to different stimuli. Nociceptors associated with the slow conducting C-fiber class are usually neurons with small, peptidergic soma and unmyelinated fibers.

Studies with electrophysiological recordings show that most C-fiber nociceptors are polymodal, responding to more than one noxious stimulus, such as mechanical and thermal; still these stimuli are perceived as distinct. This class of afferent nerves must

then contain distinct subclasses (1-4), or a variety of mechanisms for detection and signaling outputs.

And the basal sensitivity of the protein receptors in the terminals can be of key importance to setting nociceptive (and thereby pain) thresholds.

1.2. TRPV1

The Transient Receptor Potential, Vanilloid subfamily, member 1 (TRPV1) is one such molecular receptor. It is expressed in several classes of sensory neurons, the majority of which are slow-conducting unmyelinated C-type fibers, associated with pain. TRPV1 is an integral membrane channel which is permeable to cations, with a strong selective preference for calcium conduction. Due to the ionic gradients, TRPV1 is therefore excitatory for nociceptive neurons when activated. An effect elicited by the transient rise in calcium of these TRPV1-expressing neurons includes release of modulatory neuropeptides (substance P, calcitonin gene-related peptide, etc.) from local terminals and, subsequently, other cell types near the neuronal peripheral terminals. TRPV1 conduction is activated by heat in the 40-45°C range, acidification of the interstitial fluid (pH<6.5 at 25°C), and several endogenous agonists including anandamide (5-9).

TRPV1 distribution is overwhelmingly in sensory ganglia, yet reports of its distribution throughout other tissue has varied (5, 10-12). It is predominantly expressed in the C-type polymodal nociceptors, which are small unmyelinated fibers. The peptidergic neurons are a subset of these often expressing TRPV1. These neurons contain

peptides which are often pro-nociceptive, such as calcitonin-gene-related-peptide (CGRP). However, in the mouse, C-type sensory neurons which bind the isolectin-B4 are largely exclusive of TRPV1 (13, 14), as are most myelinated fibers.

1.3. TRPV1 structure

Microscopy studies have confirmed TRPV1 is composed of four identical polypeptide subunits joining to form a functional channel (15, 16). Each of the subunits contains six transmembrane domains (TM) that are helices, similar to many other membrane channels, and intracellular N and C terminals. The pore is formed by TM domains 5-6 and a re-entrant loop between them (15). However, consistent with the arrangement of voltage-gated channels, the TM 1-4 associate with the TM 5-6 of an adjacent subunit in the membrane. A C-terminus domain could contribute to tetramer assembly since it, along with one from a linker region in the N-terminus of the same subunit, contacts ankyrin-repeat domains in an adjacent subunit (15). The ankyrin-repeat domains are located in the N-terminus.

The 'TRP-domain' in the C-terminus, conserved in many TRP channels, is a kinked helix which interacts with the TM 4-5 linker and thus can be involved in allosteric modulation of TRPV1 conduction (15). This association of a C-terminus domain with the TM 4-5 linker is another structural interaction shared with voltage-gated channels.

Despite the fact that the fourth TM domain lacks the multiple positively charged residues typical of voltage-gated channels, TRPV1 is activated upon strong depolarization, and changes in temperature can shift its voltage-dependent activation curve (17). Therefore one activator of the channel potentially can act as a modulator for others as well (18).

1.4. Capsaicin

One notable specific agonist of this receptor is capsaicin. Known to evoke a painful response in humans and other mammals, capsaicin is an active compound present in many types of *Capsicum* plants commonly known as red peppers. TRPV1 was first cloned through a screening approach using capsaicin. The description of capsaicin-induced irritation is a burning pain, a well-known example of which is the use of peppers in chili that can irritate the mouth. It was found the burning sensation that associates with capsaicin is carried by C-type fibers (19). Application to the skin typically causes a flare, with erythema of the surrounding skin, the neurogenic inflammation brought about through the release of neuropeptides like CGRP. Though considered a noxious compound, capsaicin has been used in several cultures for medicinal remedies and as a flavoring, where some find the spiciness of red pepper pleasant in certain foods. As there is variability in the amount of capsaicin within types of peppers, there is also variability among people in preference, or tolerance, for the taste. There does not seem to be an exclusivity of pain as the interpretation from this nociceptive signaling. Thus there is a 'second nature' to the signaling received from capsaicin-sensitive (TRPV1) fibers. It is noted that not all TRPV1+ sensory neurons are nociceptive (20). Whether a pleasant or painful sensation is caused could depend on the emotional state of an animal, learned experience, or another physiological basis — which may include the graded encoding or duration of the nociceptive signal by nociceptors.

1.5. Inflammation and peripheral sensitization

Again, sensitivity of the nociceptors is of key importance to setting nociceptive (and thereby pain) thresholds. During a pathological condition, such as inflammation, a noxious stimulus can produce an exaggerated pain response, which is termed hyperalgesia. Furthermore, allodynia defines a perception of pain to a usually non-painful stimulus. These are clinically important features of many patients seeking medical treatment. Inflammation causes rubor (redness), calor (heat), turgor (swelling), and dolor (pain). This can be quite evident on a cool day when there is inflammation in part of the skin on the hand, for example. While the rest of the hand is pale and cool to the touch, the affected part is warmer and red due to increased skin blood flow. The release of cytokines, histamine, prostaglandins, and neuromodulators from irritated tissue evoke an immune response—termed neurogenic inflammation. The culmination of these many signaling processes initiated in the tissue gives these hallmarks of inflammation, and ‘the network of signal transduction’ can result in hyperalgesia. The change in pain perception can often be attributed to significantly increased nociceptor sensitivity, i.e., more response to the same amount of noxious stimulus; and would result in stronger signaling to the second order neurons in the spinal cord. Notably, TRPV1 has a well-established contribution to peripheral sensitization during inflammation, as TRPV1 deficient mice do not exhibit inflammatory thermal hyperalgesia (21, 22).

The possibility for selective interference of nociception by inhibition of TRPV1 has been alluring. Indeed, inflammatory pain was reversed in a rat model using a vanilloid receptor antagonist (23). Clinical trials of an inhibitor were used to assess pain

relief following a dental procedure including a molar tooth extraction (24). Several of the patients receiving treatment incurred fever though. This unexpected effect has precluded further use, and stymied pre-clinical development of a useful TRPV1 antagonist.

Furthermore, basal sensitivity of a nociceptor is important for maintaining a protective physiological response to harmful stimuli. The general inhibition of TRPV1, with its contribution to thermal hyperalgesia, could also allow for increased risk of thermal injury; or compromise another TRPV1-involved process such as corneal wound healing (25) .

TRPV1 is a receptor activated by multiple ligands and expressed in polymodal nociceptors (C-fibers), and as such likely contributes to distinct signaling pathways.

1.6. The TRPV1 membrane ménage

There are many binding sites for interaction with modulators spread across the channel. The N and C-termini are both intracellular, making a basket-like structure (16). Calmodulin (CaM) has been shown to bind at least two sites on TRPV1 which can influence sensitivity of the channel. Near the first of six ankyrin-repeat domains in the N-terminus, calcium-dependent binding of CaM decreases the open probability of the channel (26), and contributes to tachyphylaxis (desensitization) of the channel. This is a multi-ligand site (27) as adenosine-triphosphate competes for binding and can attenuate capsaicin-induced tachyphylaxis. Two residues (K155 and K160) key for the TRPV1 N-terminus interaction with calmodulin and ATP were identified in mutagenesis experiments, and a change of either (K155A, K160A) abrogated tachyphylaxis (27).

While in the C-terminus, CaM binding affects desensitization kinetics (28), with this latter site very near the S801 residue. Though the TRPV1-CT has no classical calmodulin recognition motif, it has a higher CaM affinity of the two sites, and there are two residues (W787, L796) critical for interaction with the lobes of CaM (29). There is biochemical evidence that suggests calmodulin could be weakly attached to TRPV1 at both these sites, foregoing the calcium requirement, poised to inhibit when the channel is strongly activated (30). A recent report suggests capsaicin competitively inhibits the TRPV1-calmodulin interaction (31). In addition the kinase anchor AKAP150 associates with rodent TRPV1 to promote phosphorylation by PKA and PKC, and its disruption by CaM reduces channel activity (32). The human orthologue (AKAP79) interaction site has been localized to the 736-749 region of TRPV1 as well (33).

TRPV1 is regulated by phospholipids as well, for example phosphatidyl-inositol-(4,5)- biphosphate (PIP₂). It was initially considered an inhibitor of activation (34), though later, substantial evidence from electrophysiology experiments of excised patches suggests its binding positively affects the channel opening (35). Also, functional recovery of TRPV1 from desensitization requires PIP₂ (36). There are at least two interaction sites on TRPV1 for this modulator. If still an inhibitor under some conditions, PIP₂ might then have a dual role as a TRPV1 modulator (37, 38). This could also involve competitive inhibition of capsaicin as a phosphoinositide can occupy the vanilloid binding site (39), which is in a hydrophobic pocket of the intracellular loop near TM3 of each subunit (40, 41).

The extent of PIP₂ potentiation of TRPV1 activity could be influenced by another member of the TRPV1 membrane complex, *phosphoinositide interacting regulator*

of TRP (Pirt) (42). This regulator is expressed specifically in the peripheral nervous system and binds both PIP₂ and TRPV1. A recent study also suggests Pirt binds calmodulin and interacts with cholesterol-derivatives (43), suggesting Pirt could be a liaison of the CaM and PIP₂ balance of TRPV1 modulation. A region of TRPV1 (amino acids 777–820) has been identified as a motif involved in regulation by PIP₂, though there are several. Interestingly, this region includes Ser 801, and overlaps with a 35 amino-acid segment (767-801) necessary for CaM binding.

Furthermore, the S100A1 calcium-binding protein, which is a calcium effector analogous to calmodulin, has been reported to compete with PIP₂ and CaM for the same C-terminal region of rat TRPV1 (44). Though primarily expressed in neuroglia, it is expressed in human and rat peripheral neurons, and can interact with the cytoskeletal system (45).

The C-terminus is packed with modulatory sites and structural motifs, and presents a ‘landscape’ for interaction of different signaling pathways/modulators. Small changes could determine which holds sway over this key receptor.

1.7. Kinases and PKC

The post-translational modification of a membrane channel can allow for a rapid alteration of its sensitivity, conductance, or signaling pathway. Several mechanisms have been identified by which sensitivity of transduction and voltage-gated channels in nociceptive neurons could be altered. Changes may occur at the transcriptional, translational, or post-translational levels. Where transcription and translation of mRNA

and regulating protein expression amounts provide for alternative, stable, and ‘cost-effective’ regulation, this could take hours or longer to be impactful, and likely would contribute at a later stage of inflammation to maintain peripheral sensitization. This would seem to be the case particularly for some sensory neurons, whose peripheral terminal processes can be many centimeters away from the cell body. There would be a delay for the transit of new membrane channels. Alternatively the plasticity of nociceptive neurons can be affected by local translational control of mRNA, where evidence supports local synthesis and retrograde transport of a DNA-binding protein that contributes to acute mechanical hyperalgesia (46). The activity-dependent local protein synthesis can thus modify signaling pathways in local compartments (47), although this has not been demonstrated for function of nociceptive channels. Moreover, a reserve pool of membrane receptors held at the terminals might aid in keeping surface expression consistent, but could still be depleted during persistent stimulus if receptors are removed from the membrane and degraded to prevent excessive signaling. Thus it would seem (of the three) post-translational modification would have a more profound effect on dynamic responses to membrane stimuli, and by inference the rapid sensitization of a physiological response.

Phosphorylation is an important post-translational modification (PTM) for many membrane receptors. It is a post-translational modification whereby a phosphoryl group is attached to an amino acid side chain. Phosphoryl transfer reactions are involved in many key biological processes such as energy transduction, signal transduction and protein/nucleic acids synthesis (48). This is effected by protein kinases that are site-directed to serine, threonine, and tyrosine residues. This modification of the side chain

can cause a change in conformation, assembly between protein subunits, or expose binding sites for interaction with different signaling pathways.

A multitude of kinases within the nerve terminals are activated under different conditions, including inflammation, and directed to phosphorylation sites on various proteins that can be distinct or overlapping. There can be redundancy between kinases for phosphorylation sites. These sites can also have the phosphoryl group removed by phosphatases. Importantly, phosphorylation offers a reversible short-term control of membrane proteins.

Kinases are mediators in signaling transduction networks, including those for pain. They can quickly modify protein functions to influence cellular events. The kinases can build upon themselves to produce kinase cascades, providing great amplification in signaling. These then also can be regulatory points in a signal transduction process and are considered candidates for targeted therapeutics. Important to their efficacy is localization of the kinase near its intended substrate. A PKC is often held away from the neuronal membrane until an activation signal has been initiated. Subsequently a translocation peptide can bind and direct the kinase to the neurolemma (49). The translocation of a kinase to the neuronal membrane can be critical to its signaling effect. Once at the neurolemma, this is improved through interaction with scaffolding proteins that can place the kinase near its substrates, and which serves as another point for control of signal transduction (32). A scaffold is nonspecific in that it can be used by many different kinases, and phosphorylation of the scaffold itself can cause loss of binding partners. The assembly and translocation of activated complexes to and from the plasma

membrane further exemplifies the dynamic range of intracellular signaling influenced by phosphorylation.

Protein kinase C (PKC) is one family of serine/threonine kinases and implicated in mediating a wide range of physiological processes. There are 12 isoforms identified at present, which can be separated into four classes, including the type of coactivator required. Although a degree of redundancy has been suggested, evidence now supports individual, perhaps subtle roles for many of the PKC members. Second messengers or allosteric modulators can bind the regulatory domain on a PKC molecule and activate that specific isoform. The first type of PKC identified were those requiring calcium and diacylglycerol (DAG) binding while at the membrane. These were termed conventional PKC's after novel PKC members were found not to require calcium, and the atypical which do not require either calcium or DAG phospholipid. The remaining class can be activated allosterically by G-proteins , for review (50). There is evidence supporting a critical role for PKC in diabetes, some autoimmune diseases, psoriasis, and other human diseases, for review (49).

A plethora of studies have demonstrated PKC has a significant role in pain hypersensitivity (51-53). Protein kinase C is typically activated in the local sensory neuron fibers during peripheral inflammation. Several PKC family members have been localized in neurons at both peripheral and spinal cord terminals, with activation contributing to different effects. The kinase can phosphorylate components in several neuronal pathways, including nociception. For sympathetic neurons *in vitro*, PKC activation in the periphery inhibits neurite outgrowth (54). Following a chronic constriction nerve injury (CCI) in rats, PKC inhibition in the spinal cord attenuates tactile

allodynia (55). A mouse model of inflammation using peripheral administration of the phorbol ester Phorbol Myristate Acetate (PMA) produces overt nociception when injected to the hindpaw of mice. This is largely relieved by pretreatment with a PKC-inhibitor (53). Given this location and the involvement with regulation of intracellular network signaling and amplification, PKCs and associated adapter proteins have been targets for development of new therapeutics for nearly two decades (32, 50, 56). Further evidence supports contributions of single isoforms of PKC in nociception and hyperalgesia (57-59). This includes contribution to chronic pain, defined as pain lasting more than three months (IASP), which is often resistant to standard relief medications such as opioids (60). This is another motive in possibly targeting the PKC family for novel therapeutic intervention.

However, with the widespread participation (of PKC's) in signal cascades, both in neuronal and non-neuronal cells, it should be anticipated that unintended effects are likely to manifest. For example, PKC's are involved in hormonal receptor regulation (61), and inhibition may impact normal hormone responses. Even single isoforms can be promiscuous, as Protein Kinase C epsilon (PKC ϵ) is involved in signaling pathways for other cell-types (62) or within the sensory neuron itself (63). Additionally the PKC-associated scaffold proteins serve other kinases. Thereby I have great concerns over the clinical applicability of small molecule inhibitors of both PKC and its translocation peptides. It seems specificity of the inhibition for nociceptive signaling should be paramount to clinical intervention.

1.8. Protein Kinase C substrates include TRPV1

One of the many targets of PKC is the aforementioned TRPV1, with an established role in inflammatory pain, at least in rodents. TRPV1 phosphorylation by PKC occurs downstream from G_q-coupled receptor activation by several inflammatory mediators, including prostaglandins and bradykinin (64, 65). In one report, carrageenan and complete Freund's adjuvant-induced inflammation caused upregulation of the specific isotype PKCε in DRG neurons, where double-labeling showed that activation of PKCε was predominantly in TRPV1 small diameter DRG neurons (66).

1.8.1. PKC Phosphorylation sites on TRPV1

The role of phosphorylation on TRPV1 at various sites is well-studied using recombinant heterologous expression systems and individual neurons from rodents. The ability of capsaicin to induce desensitization of sensory neurons has been of clinical use and interest for many years. Some of the first electrophysiological studies revealed that capsaicin application causes a complex desensitization, which could be modified by calcineurin inhibition (67) and activation of kinases. There are many potential target sites on TRPV1 for phosphorylation by protein kinases, which can affect TRPV1 sensitivity in different ways. NGF can increase kinase activity and rapidly increases membrane expression of TRPV1 heat-gated ion channels (68). The tyrosine 200 site, near an N-terminal ankyrin repeat, is important in modulating the neuronal membrane trafficking of the channel. The phosphorylation of the threonine 407 residue (T407) is

under control of cyclin-dependent kinase 5 (Cdk5), requiring the coactivator p35 (69). Its inhibition reduces TRPV1-dependent calcium influx in sensory neurons, and the conditional knockout of Cdk5 causes reduced basal thermal sensitivity in the mouse hindpaw. Kinase activity at this site affects the gating mechanism of the channel; with effects on desensitization, ligand sensitivity, and voltage-dependence (70). Of the many potential sites for PKA to phosphorylate, S117 and S503 seem to have the largest effect on TRPV1 function, with S117 reversing desensitization and S503 potentiating the current response to capsaicin (71-73). It has been reported that PKC β II acts as an accessory subunit to TRPV1 in a subset of sensory neurons, and controls the heat sensitivity via T705 phosphorylation, as mutation of this site almost eliminates heat sensitivity (74). It should be pointed out that TRPV1 itself (without PKC β II) acts as a heat sensor since patch-clamp studies with HEK293 showed heat-evoked openings at 44°C (30). The PKC ϵ isoform facilitates the sensitization of DRG neurons to heat caused by the inflammatory mediator bradykinin (57). For TRPV1, PKC activation potentiates the membrane currents gated by anandamide, low pH, and capsaicin (75). From a mechanistic aspect, PKC phosphorylation increases the open probability of TRPV1 (76). Furthermore the phosphorylation of the S801 site can restore the whole-cell responses of sensory neurons to capsaicin after tachyphylaxis, which is a diminished current from repeated capsaicin applications (73).

Analysis of TRPV1 following activation of PKC with PMA demonstrated three primary sites of phosphorylation (77, 78), the residues serine 502, serine 800, and threonine 704 in rat TRPV1 (S503, S801, and T705 respectively in mice) (S502, S801, T705 respectively in human). There are several other sites for phosphorylation on

TRPV1, but those are not the focus of Protein kinase C. The S503 residue is available to protein kinase A (PKA) (79) , calcium calmodulin-dependent kinase II (CaMKII) and PKC; T705 is available to both CaMKII (80) and PKC ; however the TRPV1 S801 phosphorylation site (S800 in rat, S801 in human) is unique to PKC. Thus, there is some redundancy and overlap in control of TRPV1 phosphorylation, and it is a balance between these kinases and a dephosphorylated state that influences TRPV1 sensitivity.

Despite a plethora of phosphorylation and modulatory sites, no common mutations found in human *TRPV1* occur at any of these (81). All prior investigations into mutations of TRPV1 phosphorylation sites and the effect on function have been performed *in vitro*. The contribution of TRPV1 phosphorylation, at a single-site, to *in vivo* function and inflammatory hyperalgesia, however, has yet to be examined.

1.8.2. Analysis of PKC sites on TRPV1

The primary intent of our next study was then to identify which of these residues, if any, would be superior for selective attenuation of TRPV1-dependent hyperalgesia. For a polymodal nociceptor, an activator of the channel has potential to uncover specific contributions to nociception or pain; in other words, the mode of TRPV1 activation could determine between downstream signaling effects. It had been suggested the C-terminus of TRPV1 is involved with thermal sensitivity (82, 83). Our study further showed the C-terminus has distinct structural segments that affect the heat and capsaicin modes for activation of TRPV1 (84). However the N-terminus contains an ATP binding site that sensitizes the response to low dose capsaicin (27) . There are segments throughout the

channel which are contributors to the varied modalities of TRPV1 activation. We chose to investigate the protein kinase C sites because of a well-validated role for this kinase in acute inflammatory pain. The three PKC sites are dispersed along the channel with S503 at the first intracellular loop between transmembrane domains, T705 in the C-terminus, and S801 in the distal C-terminus. Moreover TRPV1 sensitization has been attributed to all three PKC target residues (77, 78). Given this, and that TRPV1 has separate structural domains for distinct modalities of channel activation, we determined the contribution of each PKC phosphorylation site to various modes of channel sensitization.

The molecular mechanisms of PKC-mediated TRPV1 sensitization have been derived largely from in vitro studies using transformed heterologous expression vectors, such as human embryonic kidney cells (HEK293). Our approach was to first evaluate the role of each residue for PKC-mediated sensitization of receptor response to varied modalities of activation, using a similar heterologous expression approach with site-directed mutagenesis of the rat TRPV1 orthologue (85). Using electrophysiology studies, we found the S502 residue is largely involved in sensitization of TRPV1 current to capsaicin. The T704 residue contributes more to that for heat, though it reduces initial sensitivity to acid activation as well. It is the S800 residue that is polymodal, contributing to PKC-mediated sensitization of all three modes tested for activation of TRPV1 (Figure 1).

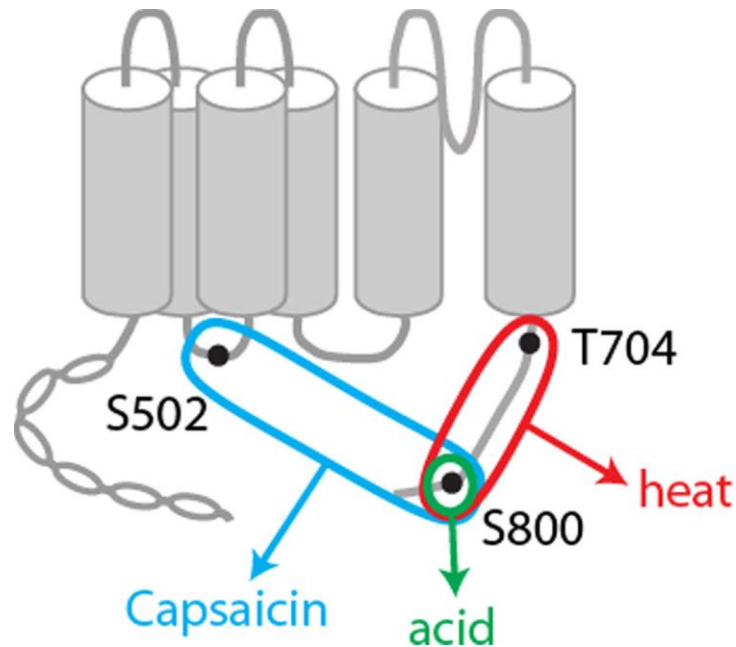


Figure 1. Modality-specific contribution of PKC-phosphorylation residues of TRPV1

While this provided a breadth of details about these PKC phosphorylation sites, the integrated TRPV1 signaling output may differ or be more complex in a native expression vector. We followed with similar experiments using expression of the three TRPV1 phosphorylation site mutants in the more native environment of sensory neurons. The rat TRPV1 mutants were expressed in DRG neurons cultured from TRPV1 knockout mice. Our findings largely replicated those from our heterologous expression experiments. The TRPV1 S800A mutant also showed impairment of sensitization to capsaicin caused by bradykinin, an endogenous inflammatory mediator in humans and

rodents. Importantly the initial response of the receptor to capsaicin was not reduced with the S800A mutation, making this phosphorylation site a potential means for selective treatment of inflammatory hyperalgesia.

Furthermore, for a rodent model of orofacial pain, the activation of glutamate receptors increases TRPV1 S800 (rat) phosphorylation in TG neurons (86). The metabotropic glutamate receptor 5 (mGluR5) is expressed in primary afferents innervating the masseter muscle of the rat, and glutamate is a transmitter released in the muscle during inflammation. The application of an mGluR5 agonist along with stimulation of the masseter, simulating a chronic pain condition like temporomandibular disorder, increases mechanical hyperalgesia. Inhibition of either PKC, a scaffold protein for docking, or TRPV1 attenuates the nocifensive response (52). After treatment with an mGluR5 agonist, a western blot of trigeminal ganglion neurons showed increased S800 phosphorylation, when hyperalgesia was prominent. There is further support for this interaction at pre-synaptic terminals of nociceptors in the spinal cord, where central terminals contain both TRPV1 and mGluR5 (87). Mice lacking TRPV1 had reduced mechanical hyperalgesia following activation of spinal mGluR5. Additional evidence from two different models shows that S801 phosphorylation is increased in peripheral neurites during inflammation. An intraplantar injection of endothelin-1 (ET-1) in mice causes thermal hypersensitivity, which is PKC dependent. The ET-1-induced thermal hyperalgesia was also attenuated in TRPV1-deficient mice, compared with that in wild-type mice. The electrophysiology experiment in HEK293 showed increased capsaicin response with co-application of an ET-1 activator, when both TRPV1 and the endothelin type A receptor were expressed. This was attenuated by a S502A/S800A mutant TRPV1

(88). From a rat mono-iodoacetate (MIA)-induced joint pain model (osteoarthritis), spontaneous pain-related behavior induced by intra-articular injection of capsaicin (TRPV1 agonist) was significantly increased in MIA rats compared to sham rats. The TRPV1 protein amount remained unchanged in the dorsal root ganglion neurons corresponding to the affected joint, but phosphorylated TRPV1 at Ser800 increased (89). The intra-joint pre-injection of a PKC inhibitor lowered the pain response to capsaicin. Considering these studies, there is ample evidence to suggest a role for S801 phosphorylation in nociception, especially under inflammatory conditions.

1.9. Hypothesis

Studies with knockout mice and inhibitors provide compelling support of a role for TRPV1 in hyperalgesia after tissue inflammation. A broad inhibition of channel function has adverse effects. We therefore contend a more discrete targeting of TRPV1 signaling would be necessary to prevent the exaggerated pain that occurs with inflammation, yet leaves normal nociception intact. A selective inhibition of TRPV1 signaling might hold the key to successful development of a clinical analgesic adjunct. Several studies indicate TRPV1 phosphorylation is increased during experimental inflammatory conditions which cause hyperalgesia. The effect of preventing phosphorylation of a single site *in vivo* has never been examined previously.

We hypothesize that preventing phosphorylation of S801 will result in reduced neuronal TRPV1 sensitivity and hyperalgesia during inflammatory conditions, while leaving normal, protective nociceptive thresholds intact.

1.10 Specific Aims

The aim of this proposal is to determine the *in vivo* effect of blocking phosphorylation at a single site on TRPV1. In keeping with this we have genetically engineered a mouse strain with a point mutation, the TRPV1 S801A mouse, referred to here as the knock-in mouse (KI). As outlined in the following, we will detail the effect on TRPV1 localization, expression, channel function, and also behavioral hyperalgesia.

Aim 1. Determine the effects of the S801A mutation on TRPV1 expression and phosphorylation.

Working hypothesis: The mutation prevents S801 phosphorylation, and the KI shows no difference compared to its wild-type (WT) littermates for expression and localization of TRPV1.

1.1. To demonstrate the lack of S801 phosphorylation, we will use a TRPV1 S801 phospho-specific antibody in western blot assays of dissociated dorsal root ganglion neurons. Following PKC activation, there should be increased phosphorylation in WT as compared to vehicle controls. For the KI there should be no phosphorylation in either vehicle or PKC activator treatment.

1.2. Using immunohistochemistry to identify markers of neuron class, we will demonstrate that the basal expression pattern of TRPV1 within sensory neurons has not been altered in KI mice. Samples of trigeminal ganglion and nerve will be labeled for CGRP, NF-200, IB4, along with TRPV1. TRPV1 is coexpressed significantly more with CGRP in WT. The immunolabeling will be quantified and compared between KI and WT littermates, predicting little difference of TRPV1 expression.

Aim 2. Determine the effect of the S801A mutation on TRPV1 function in sensory neurons.

Working hypothesis: S801A reduces PKC-mediated TRPV1 sensitization. We will use whole-cell patch clamp of dissociated trigeminal neurons to evaluate the functional effect of the mutation in TRPV1.

2.1. We will compare channel sensitization properties. Using the PKC activator phorbol myristate (PMA) to sensitize TRPV1, we predict a subsequent capsaicin response is reduced in KI as compared to WT. We will also evaluate the response to the endogenous inflammatory mediator bradykinin, a known sensitizer of TRPV1. We predict KI will show reduced response.

2.2. We will compare channel desensitization properties; predicting no difference between KI and WT with repeated applications of a large dose of capsaicin.

Aim 3. Determine the contribution of TRPV1 Ser801 phosphorylation to *in vivo* capsaicin sensitivity and pathological pain.

Working hypothesis: The mutation prevents phosphorylation at S801, reducing TRPV1 sensitization and pathological pain.

3.1. Determine capsaicin sensitivity using the PKC-mediated TRPV1 sensitization paradigm. Capsaicin will be applied to the cornea alone, or following an initial application of PMA, and nocifensive response quantified by eye wiping in KI and WT. In a separate cohort, capsaicin will be injected into the hindpaw, or following an initial injection of PMA, and nocifensive response quantified. We predict a significant reduction of nocifensive response in KI mice to capsaicin only after the PMA in both models.

3.2. Evaluate the effect of the S801A mutation for inflammatory pain.

We will establish basal responses for WT and KI in several pain models, to compare nociception for different pain modalities. This will be repeated under inflammatory conditions. For example, following injection of complete Freund's adjuvant into

hindpaw, we will use a Hargreaves' assay to determine paw withdrawal latency to radiant heating over several time points. We predict KI mice show significantly less reduction in latency than WT, and thus are more resistant to inflammatory thermal hyperalgesia.

Chapter 2. Generation of TRPV1 S801A Knock-in Mice

2.1. Introduction.

In experimental therapeutics, it's difficult to deliver drugs to specific body systems without causing havoc in other systems. Developing a drug with the ability to act only on specific biological pathways is an even more thorny challenge. In evaluating the contribution of S801 phosphorylation to nociception, the huge hurdle for us then is specificity of inhibition --- to prevent its phosphorylation without disturbing the entirety of the PKC signaling network or TRPV1 signaling within the nociceptive neurons.

Though inhibitors for PKC, scaffold proteins, and PKC translocation peptides have been developed, there are none which solely prevent the phosphorylation of S801. Even isoform-specific PKC inhibitors should conceivably prevent phosphorylation at the other TRPV1 PKC sites, as well as other proteins. There are no common polymorphisms in human or rodent that affect this site, or any phosphorylation site (81). Thus the approach used was genetic modification of the *Trpv1* gene, producing a new strain of mice carrying a mutation to prevent phosphorylation at S801.

A concern can be raised if disrupting a single phosphorylation site (of many), on a single protein, in a complex signaling pathway could be enough to have behavioral consequences. Two previous studies provide evidence that indeed this could happen. The major excitatory neurotransmitter receptors in the vertebrate central nervous system are glutamate receptors. The AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) subtype of glutamate receptors mediate the majority of the fast-excitatory synaptic

transmission in the central nervous system. In mice with a mutation of two serine residues on the AMPA receptor-GluR1 subunit, which are targeted by kinases, caused a deficit in synaptic long-term-depression (LTD) and learning tasks (90). It was later reported that the mutation also attenuates response in a post-surgical pain model (91)

The phosphorylation at serine 897 of the NR1 subunit of glutamate-N-methyl-D-aspartate receptors (NMDAR) is markedly reduced in schizophrenia patients. A knock-in mutation in mice at this site caused impaired NMDAR-mediated synaptic transmission and long-term potentiation. Furthermore the mutant mice showed behavioral deficits in social interaction and sensorimotor gating (92). These studies indicate that an impairment in single-site phosphorylation can contribute to behavioral outcomes.

2.2. CRISPR/Cas9 Genome Editing Overview

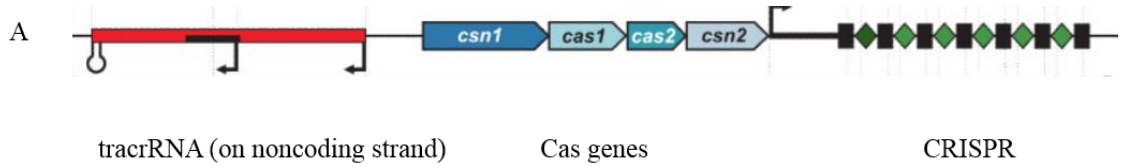
CRISPRCas9 is a system that can make site-specific modifications in the genomes of organisms. This gene editing technique is derived from a bacterial immune defense against viral and plasmid invasions (93, 94). CRISPR refers to a genetic sequence comprised of clustered regularly interspaced short palindromic repeats (CRISPR) of similar size, which are separated by unique spacers. The spacers are acquired individual samples from invading plasmids and viruses, and which provide a recognition sequence for a foreign genetic material previously encountered. There are genes near the CRISPR that encode CRISPR-associated proteins (Cas) which have or contribute to an endonuclease function. The CRISPR arrays are transcribed into RNAs, which recognize and promote the cleavage of invading viruses when part of a

ribonucleoprotein complex with the Cas, termed the CRISPR-Cas system. Three different types (types I–III) of systems have been defined (95), and are determined by conserved clusters of Cas genes that are signature for each. The type II system is more simplistic in that it has a reduced number of Cas genes near the CRISPR array, and is only present in bacteria. It was later determined that Cas9 is the only protein in the type II system required for the endonuclease function, which favored this system as more readily adapted for use in mammalian genome editing.

A protospacer is a nucleic acid fragment from an invading source that can be used to create a spacer in the CRISPR array. This essentially provides a sample sequence of the foreign nucleic acid for the bacterial host immune system to retain. When transcription of the CRISPR array occurs (Figure 2), these spacers are processed into RNA guide sequences (crRNA) which are used by Cas9 to identify invading DNA. The processing requires a transactivating CRISPR RNA (tracrRNA) segment. This is derived from the noncoding DNA strand upstream of the CRISPR array (96). The tracrRNA forms a duplex with the direct repeats on the CRISPR transcript, and that allows RNase III to process the spacers into the mature guide-crRNA used by Cas9.

A necessary requirement of the Cas9 system is a protospacer-adjacent motif (PAM). Immediately adjacent to the 3' region of the protospacer sample sequence (taken for the array), there is a PAM. This PAM is a trinucleotide sequence that is present on the noncoding strand of the invading DNA, and importantly is not part of the spacer (Figure 3). It therefore can be used by Cas9 as a starting point for binding to and separating DNA duplexes, either invading or host genomic DNA since the trinucleotide sequence occurs in both, and then interrogating the 5' sequence on the coding strand for a

match to the crRNA (containing the spacer sequence). The PAM recognition also facilitates the transition of Cas9 to its nuclease function for scission of the target. If there is a match to both the PAM and crRNA, the Cas9 dual-endonuclease function is initiated and makes a double strand break, on both the noncoding (PAM) and coding strands (spacer match) of the DNA (97). The crRNA guide is left intact for possible further target recognition. It is important to note there are no PAM sequences contained in the direct repeats that separate the spacers, which prevents self-targeting of the CRISPR array by an active CRISPRCas9 complex.



(black boxes are the repeats)

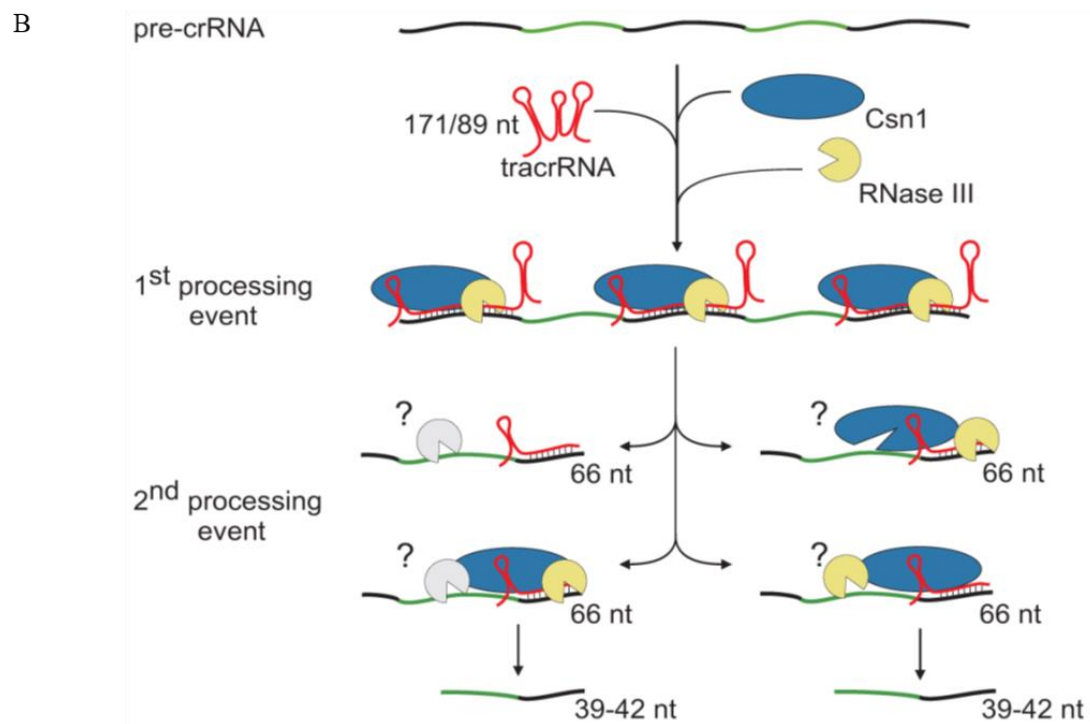


Figure 2. CRISPR/Cas9 transcription and processing

A. Genomic organization of tracrRNA and CRISPR01/Cas.

B. Model for tracrRNA-mediated crRNA maturation.

(both adapted from Deltcheva et al, 2011)

If a double strand break occurs in a host genomic segment, this initiates repair of the broken DNA through two systems, homology directed repair (HDR) or non-homologous end joining (NHEJ). DNA damage often results from normal cellular processes, and organisms have developed checkpoint mechanisms to ensure the integrity of their DNA. The DSBs can cause deletions, translocations, and fusions in the DNA if not repaired correctly. This is referred to as genomic rearrangement, and often associated with deleterious consequences.

Of the two systems, HDR provides a higher level of fidelity to ensure an exact replacement of the original sequence. The NHEJ pathway is an effective repair pathway where DSBs are fixed by re-ligating their two ends together, without requiring a homologous sequence. However small deletions or insertions at the break site are a general result, and larger rearrangements can occur. The repair fidelity is better if the DSB's are short breaks with complementary overhangs; but those with blunt end breaks or extensive overhangs may require gap filling prior to ligation, which may produce small insertions and deletions at the DSB location. For mammalian cells, with two copies of a chromosome, homologous repair is an alternative. DSB repair by HDR corresponds to a transfer of identical sequences from an intact DNA copy to the one carrying the DSB. Homology-directed repair is a faithful mechanism if the template used for repair is an exact match to the original DNA sequence present at the DSB. The initial step of 5' nuclease activity by repair complexes on the DSB creates 3' sequence overhangs of appropriate length that can search the homologous chromosome for nucleotide base complementarity. If a match is found, a process known as strand invasion can occur, allowing the DSB overhang to interpose in the copy DNA duplex. The repair proceeds

with the intact copy as a template for the removed sequence on the interposed DSB strand. The opposing strand on the DSB chromosome is then also restored. If the donor sequence is not an identical match to the original, HDR repair of DSBs can also lead to local mutations of the sequence (as with the NHEJ repair). This has been exploited with CRISPR/Cas9 to make a gene editing system.

By introducing homologous repair templates in mammalian cells along with a modified type II system, coding sequences of target genes can be altered to create selected mutations. As mentioned, CRISPR relies on crRNA's to direct degradation of complementary sequences. An *in vitro* study demonstrated that a crRNA fused with a tracrRNA (also necessary for Cas9 activation) was sufficient for Cas9 activity, so a single guideRNA (crRNA with tracrRNA) could be transfected along with Cas9 into mammalian cells for making a DSB.

Additionally, the target cell can be given large quantities of a donor template for repair. This sequence has the desired insertion or modification, flanked by segments of DNA that are homologous to the blunt ends of the cleaved DNA. These serve as substitute templates for homology-directed repair. The large quantities increase the likelihood that a donor template is encountered and used in the repair, rather than the homologous chromosome. Thus following the RNA-guided cleavage of a specific site on the gene of interest, the natural repair mechanisms of the cell can be used to insert the desired genetic substitution, editing the genome of a target cell with high-precision (98).

2.3. Generation of TRPV1 S801A Mutant Mice

Using this strategy to engineer a mutation in *TRPV1*, we generated a knock-in (KI) mouse line in which the serine residue 801 was mutated into alanine (S801A) by genome editing to prevent its PKC-mediated phosphorylation.

The rat TRPV1 S800 site equivalent is conserved in many TRPV1 orthologs across different species (Figure 4), including all common variants of human TRPV1. We chose the mouse for our study as it considered an excellent animal model, with very high homology between mouse, human, and rat TRPV1; and most *in vitro* studies have been done with rat TRPV1.

Red fox (<i>Vulpes vulpes</i>)	FSLVPLLRDA	STRERHPAQP	EEVHLRHFAG	821
Brown bat (<i>Eptesicus fuscus</i>)	FALVPLLRDA	STRERQPAQH	EEVHLRHFAG	821
Mouse (<i>Mus musculus</i>)	FALVPLLRDA	STRDRHSTQP	EEVQLKHYTG	820
Rabbit (<i>Oryctolagus cuniculus</i>)	FALVPLLRDA	STRDRHPXPP	EDVHLRPFVG	823
Human (<i>Homo sapiens</i>)	FALVPLLRDA	SARDRQSAQP	EEVYLRQFSG	820
Gharial (<i>Gavialis gangeticus</i>)	- TLVPLLRDG	SRREEAPKLT	EEVKFKSVLE	832
Chicken (<i>Gallus gallus</i>)	- TLVPLLRDG	SRREETPKLP	EEIKLKP ILE	826

Figure 4. Sequence alignment of carboxy terminal domains of TRPV1 from different species. The orthologous mouse S801 residue is shown in red. A critical consensus residue necessary for PKC-phosphorylation is highlighted in yellow.

Methods

2.3.1. Primer sequence design

The TRPV1 locus was edited using the CRISPR/Cas9 technique (99). A sgRNA recognition sequence (GGGACGCAAGCACTCGAGAT; highlighted in yellow at top sequence of (Figure 5A) adjacent to a protospacer adjacent motif (PAM) sequence (AGG) was selected to target the vicinity of the TRPV1 S801 codon. Complementary oligonucleotides encoding the recognition sequence (5_-CAC CGG GAC GCA AGC ACT CGA GAT-3_ and 5_-AAA CAT CTC GAG TGC TTG CGT CCC-3_) were annealed together and subcloned into pX330 (99) using BbsI sites. A DNA sequence encoding the full-length sgRNA was subsequently PCR amplified from this construct using the following two primers: 5_-TTA ATA CGA CTC ACT ATA GGG GGG ACG CAA GCA CTC GAG AT-3_ and 5_- AAAGCACCGACTCGGTGCC-3_, in which the underlined portion of the first primer encodes an attached T7 promoter. The full-length sgRNA was then generated by in vitro reverse transcription using the T7 Quick High Yield RNA synthesis kit (New England Biolabs) and the MEGAclean clean-up kit, followed by precipitation with ammonium acetate and resuspension in nuclease free water. A 150 nucleotide custom synthesized single stranded DNA repair template (Dharmacon) (5_-TGC CTT TCA GTT TCA GGG AGA AAC TGG AAG AAC TTT GCC CTG GTT CCC CTT CTG AGG GAC GCA GCC ACG CGT GAT AGA CAT AGC ACC CAG CCG GAA GAA GTT CAG CTG AAG CAC TAT ACG GGA TCC CTT AAG CCA GAG GAT GCT GAG GTC-3_;Figure 5A) was designed to span a region including part of TRPV1 exon 15 and a portion of the preceding intron. This

template was identical to the corresponding TRPV1 locus, with the exception of a mutation of the codon encoding TRPV1 serine 801 to alanine, silent mutations changing an XhoI site to an MluI site, and disruption of the PAM sequence from AGG to AGA.

The template was resuspended in nuclease free water to 1 ug/ml before use.

Polyadenylated Cas9 mRNA was produced by in vitro transcription from NotI-linearized plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid # 42230) (99) using the mMACHINE T7 ULTRA IVT kit (ThermoFisher, Cat# AM1345), precipitated using lithium chloride, and resuspended in nuclease-free water.

The sgRNA, Cas9 mRNA and the repair template were diluted in microinjection buffer (100). Two hundred and seventy single-cell embryos from C57BL/6 mice were microinjected into one pronucleus. Injected zygotes were implanted into 10 pseudopregnant ICR female mice to produce founder offspring.

2.3.2. Confirmation of mutation

Initially, genomic DNA from the founders was amplified with two primers (5_-AGAACTTTG CCCTGGTTC-3_ and 5_-TCAACCCGGCTCTATTGCTC-3_) to yield a 247 bp fragment that spanned the intended mutagenesis site. This product was subsequently digested with either XhoI (to detect the WT allele) or MluI (to detect the mutant allele). Subsequent analysis of the mutated TRPV1 genomic sequence was performed in founders and in the offspring resulting from founder mating with WT C57BL6 mice by sequencing

a 560 bp genomic PCR product that extended beyond either end of the homology repair template (using primers 5'-TCCGTGACC CATGGATCTCT-3' and 5'-GCAGAGTACAGCCAGCCAACA-3'). To further confirm proper targeted recombination, we performed reverse transcription of mRNA harvested from dorsal root ganglia (DRG) of homozygous KI/KI mice from each of two founder-derived lines, followed by PCR amplification of the mutation-containing region using two primers (5_-ACACCAACGTGGGCATCATC-3_ and 5_-TGGTT AGATTCACAGCTCGCTTC-3_) annealing to exons 14 and 15, respectively (to exclude amplification of genomic DNA). The PCR products were sequenced to confirm the sole presence of the KI allele. Routine allele-specific genotyping was later performed on the colonies using a common forward primer (Comm-for, 5_-TCCGTGACCCATGGA TCTCT-3_) that anneals upstream of the repair template with either a WT-specific reverse primer (WT-rev, 5_-ATGCCTATCTCGAGTGCT- 3_) or a mutant-specific reverse primer (KI-rev, 5_-ATGCCTA TCACGCGTGGC 3_) (Figure 5B). We obtained two founder lines. To minimize the impact of potential unintended mutations from the CRISPR/Cas9 gene editing, both founders were backcrossed with C57BL/6 mice. For the hindpaw CFA inflammatory pain model, we used mice backcrossed a minimum of two generations. For all other experiments, we used mice backcrossed a minimum of four generations. Both S801A mouse lines were used in all experiments, and the data were pooled as we did not find a difference between them.

2.3.3. Immunohistochemistry

Immunostaining was performed as described previously (101). After sedation with ketamine and xylazine, mice were transcardially perfused with 3.7% paraformaldehyde. TG and DRG were dissected, cryoprotected, and sectioned at 12 μm intervals. The lumbar spinal cord was sectioned at 30 μm intervals. Conventional immunohistochemical procedures were performed with rabbit anti-TRPV1 (1:1000) (6), guinea anti-CGRP (1:1000; Peninsula Labs, Inc), and mouse anti-NF200 (1:1000, Sigma) followed by appropriate secondary antibodies (Invitrogen). To label non-peptidergic nociceptive afferents, sections were exposed to IB4-biotin (10 $\mu\text{g}/\text{ml}$, Invitrogen) followed by streptavidin conjugated fluorophore (Invitrogen). Images were acquired by optical sectioning fluorescence microscopy (Zeiss Axiovert, Carl Zeiss MicroImaging).

2.3.4. Western blot assay

Mice were euthanized and whole DRG were excised into 1.5 mL cold Ca^{2+} and Mg^{2+} -free Hank's balanced salt solution. The buffer was removed and replaced with 0.2 mL of radioimmunoprecipitation assay buffer (Thermo Scientific, 89900) containing protease inhibitor cocktail (Cell Signaling, 5872). The DRG were mixed in the buffer and sonicated (25 amplitude setting, 10 pulses, Qsonica, Q55). Samples were centrifuged at 12000g for 10' in a table-top centrifuge pre-cooled to 4°C to remove debris. The supernatant was collected into 1.5 mL tubes. Sample lysates were loaded onto 4-12% bis-tris NuPAGE gels (Invitrogen) at 30 $\mu\text{g}/\text{well}$ and blotted onto polyvinylidene difluoride (PVDF) membranes. The blot was blocked and then probed with antibodies against

TRPV1 (Proteintech, 22686-1-AP, rabbit, 1:700; custom, (6) rabbit, 1:800) and GAPDH (EMD, CB1001, mouse, 1:10000), and incubated at 4°C overnight. The blot was washed and fluorescently labeled with goat-anti-mouse (1:20000) (Li-Cor, 926-68020) and goat-anti-rabbit (1:1000) (Li-Cor, 926-32211). Following a wash, the wet blot was scanned using an Odyssey imager and ‘Image studio software’, v 5.2. The images were quantified using the ‘Image J’ program.

2.3.5. Statistical analysis

The method of statistical analysis used in each data set is indicated in the figure legend. Data from two groups were compared using Student’s t-test. Data from three or more groups were compared using one-way ANOVA followed by Bonferroni post hoc test. Data are presented as mean±s.e.m. The criterion for statistical significance was $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6.0.

2.4. Results

2.4.1. TRPV1 S801A knock-in mice were generated using CRISPR/Cas9

To assess the role of TRPV1 phosphorylation at a single residue in hyperalgesia, we focused our attention on S801 in mouse TRPV1. We selected this residue because our previous study suggested it as a polymodal sensitization site (85), and alanine mutation at this site does not produce basal functional changes in TRPV1 (85, 102). To generate a mouse line lacking phosphorylation at TRPV1 S801, we edited the TRPV1 locus using

the CRISPR/Cas9 technique following the strategy shown in Figure 5A. A guide RNA sequence was selected to direct Cas9-mediated cleavage approximately 6 nucleotides downstream of the TRPV1 S801 codon to be targeted. A homology repair template used to direct repair of the resulting double strand DNA break was designed to introduce several changes in the TRPV1 gene: mutation of the S801 codon to one encoding alanine; the silent replacement of an XhoI site with an MluI site; and the disruption of the PAM sequence. Candidate founder mice and their offspring were genotyped using three complementary methods. As an initial step to detect successful knock-in of the repair template, we performed enzymatic digestion of PCR products amplified from the targeted locus with XhoI or MluI, to identify mice in which the MluI site had been successfully inserted. Whereas the PCR product from wild-type mice could be digested only with XhoI, we identified several founder mice that also exhibited partial MluI sensitivity within the corresponding PCR product, suggestive of the presence of one copy of the desired mutant allele (Figure 5B).

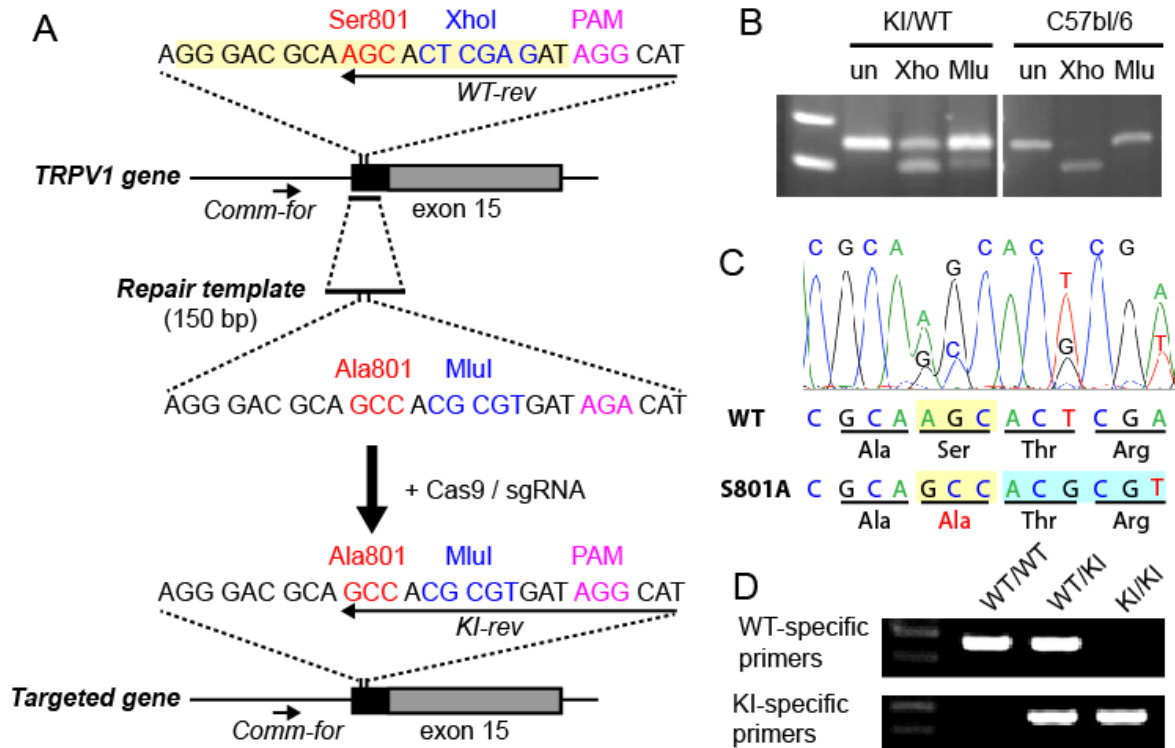


Figure 5. Generation of TRPV1 S801A KI mice using CRISPR/Cas9 method

A, Sequences of region of interest in *trpv1* gene (top), the repair template (middle), and the resulting targeted *trpv1* gene (bottom). Note that the PAM site was not mutated as intended. **B**, Enzymatic digestion of PCR products amplified from genomic DNA of a heterozygous founder (KI/WT) or C57BL/6 using XhoI (Xho) or MluI (Mlu) or undigested (un). Upper size marker, 300 bp; lower size marker, 200 bp. **C**, Sanger sequencing of PCR products amplified from genomic DNA of a heterozygous founder. **D**, Example of genotyping assay in WT, heterozygote KI, and homozygote KI using primers indicated in A, WT-specific primers, Comm-for and WT-rev; KI-specific primers, Comm-for and KI-rev. Upper size marker, 300 bp; lower size marker, 200 bp.

Two of these founders were subsequently mated with C57BL/6 mice to generate two parallel lines of candidate knock-in (KI) mice. We amplified PCR products encompassing a genomic DNA segment extending beyond that corresponding to the homology repair template from both the founders and their offspring. Sequencing these

products confirmed the introduction of most of the intended nucleotide substitutions into the TRPV1 locus in both lines (Figure 5C). Namely, the S801 codon was successfully changed to an alanine codon, and the XhoI site was silently replaced with an MluI site, without alteration of the remaining nearby translational code. Interestingly, the intended mutation of the PAM sequence was not achieved in either line, suggesting that in both founders the homologous recombination had resolved itself between the successfully introduced mutations and the PAM site. Heterozygous KI mice within each of the two mutant lines were independently interbred to generate homozygous KI mice from both lines. Inheritance of two copies of the KI allele was confirmed by sequencing of genomic DNA-derived PCR products and separately through the use of allele-specific PCR primers designed to yield a product only in the presence of the wild-type (WT) or KI allele, respectively (Figure 5C, 5D). Finally, to confirm that the KI allele was expressed, and that no residual WT TRPV1 mRNA was produced in homozygous KI animals, we performed RT-PCR of mRNA collected from DRG of KI homozygous mice followed by sequencing of the PCR products. In this assay, we detected only the sequence of the KI allele, without evidence of contaminating WT sequence (not shown). Together, these results confirmed our successful introduction of the S801A mutation into the *Trpv1* gene. Both heterozygous and homozygous KI mice were healthy and fertile, without overt differences in appearance or behavior from their WT littermates. Breeding results suggest a Mendelian inheritance pattern of the mutation.

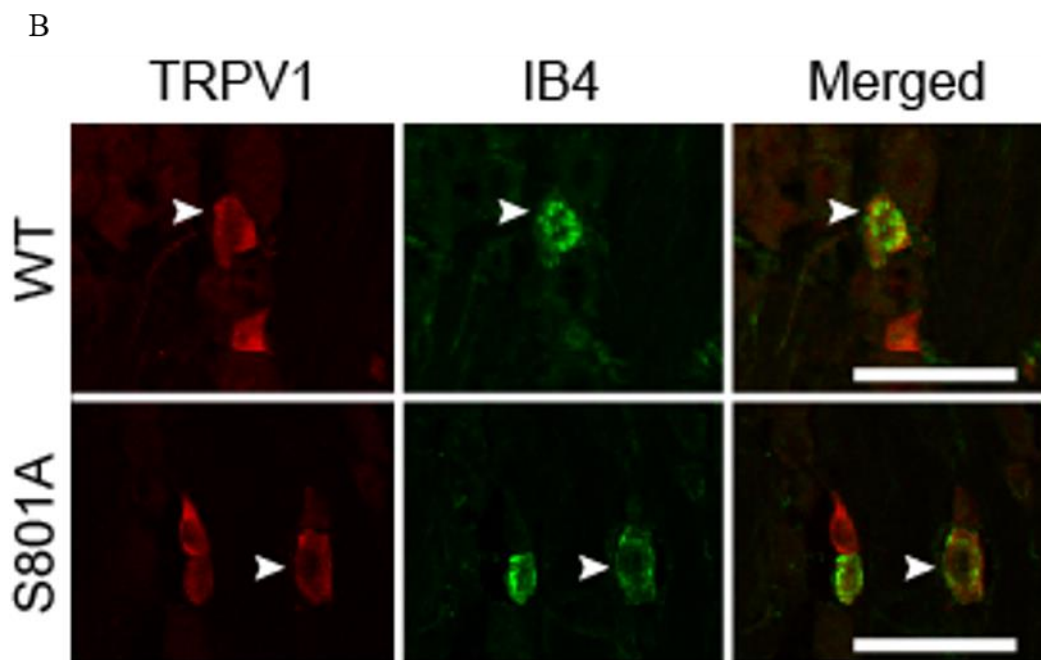
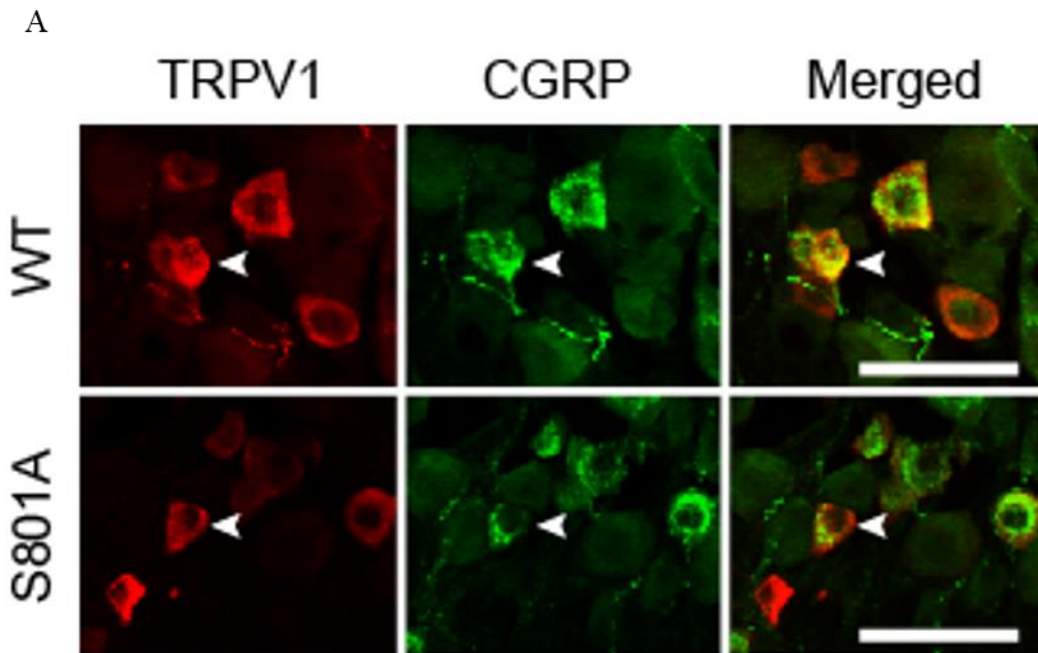
2.4.2. The effects of the S801A mutation on TRPV1 expression

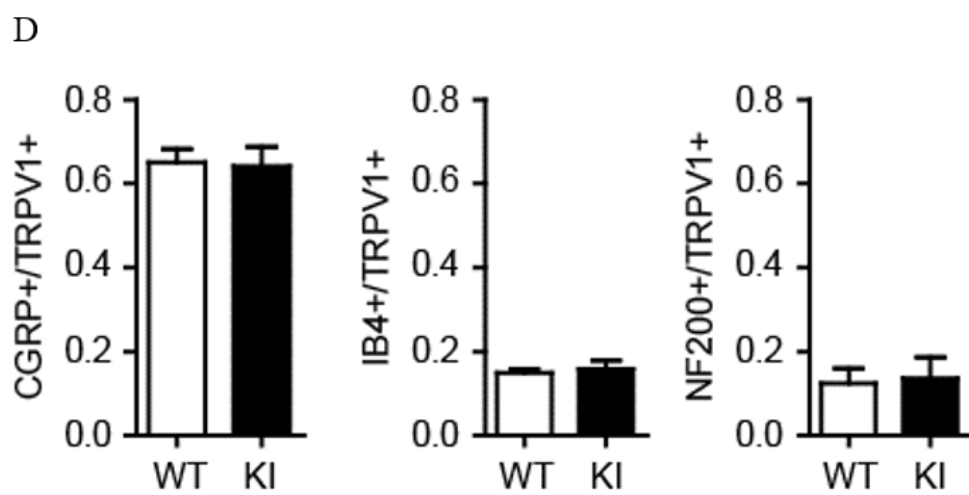
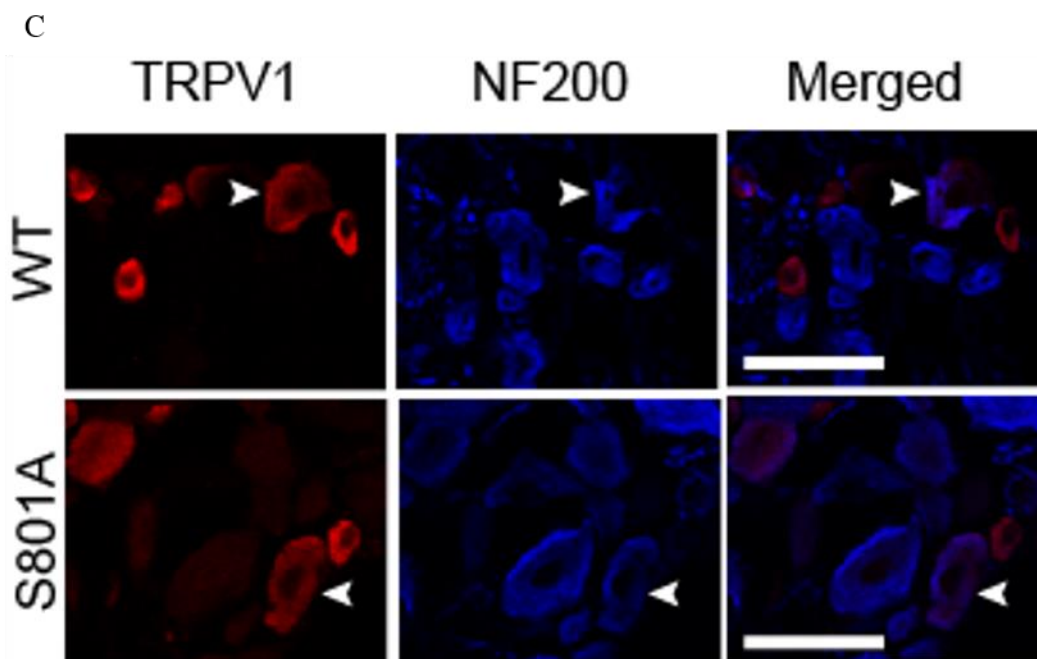
We next investigated whether the expression pattern of the TRPV1 protein is changed in TG of TRPV1 S801A KI mice. Immunohistochemical labeling of TRPV1 in TG showed that neurochemical properties of TRPV1-expressing neurons of KI mice were not different from those of WT (Fig. 6A-D). In both KI and WT, the majority of TRPV1+ neurons (approximately 65%) were colabeled with CGRP but only approximately 15% and 12% of neurons were colabeled with isolectin B4 (IB4) and neurofilament heavy chain (NF200), respectively.

Figure 6. TRPV1 S801A KI mice show no altered expression of TRPV1

A-C. Representative images of double labeling for TRPV1 and CGRP (A), IB4 (B) or NF200 (C) in TG section obtained from WT or TRPV1 S801A KI mice. Arrowheads highlight representative neurons exhibiting colocalization of TRPV1 and the respective markers. Scale bar, 50 μ m.

D. Quantification of the proportion of neurons showing colocalization of TRPV1 and neurochemical markers in TG. n=3-4 mice in each group.





The size of neurons expressing TRPV1 was also comparable between KI and WT (Fig. 7). These findings were also similar in DRG (not shown).

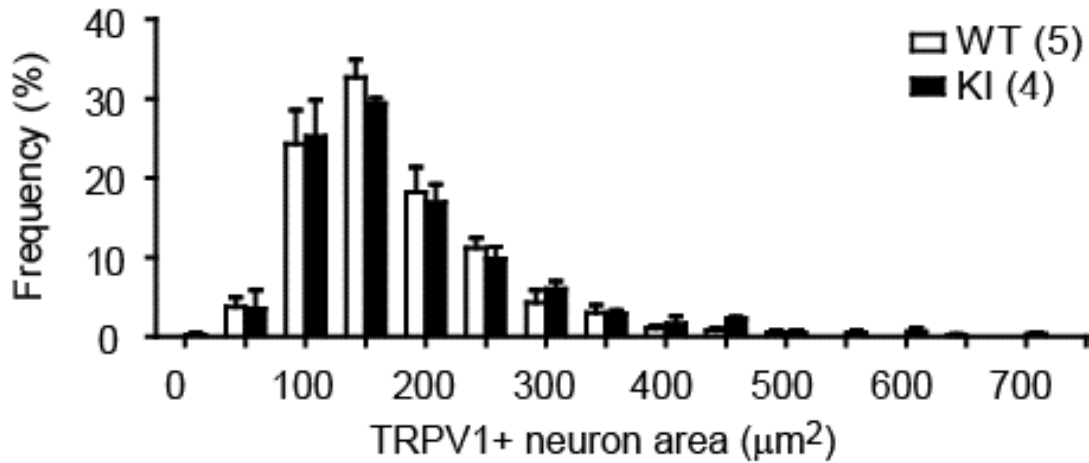


Figure 7. Size distribution of TRPV1+ neurons is unchanged in KI mice

Size distribution of TRPV1+ neurons in TG from WT and TRPV1 S801A KI mice. Number of mice is in parentheses.

TRPV1+ central terminals also appeared similar between WT and KI mice, in both cases projecting into the superficial layer of the spinal cord dorsal horn, and partially overlapping with CGRP and IB4+ terminals (Fig. 8, Scale bar, 100 μ m).

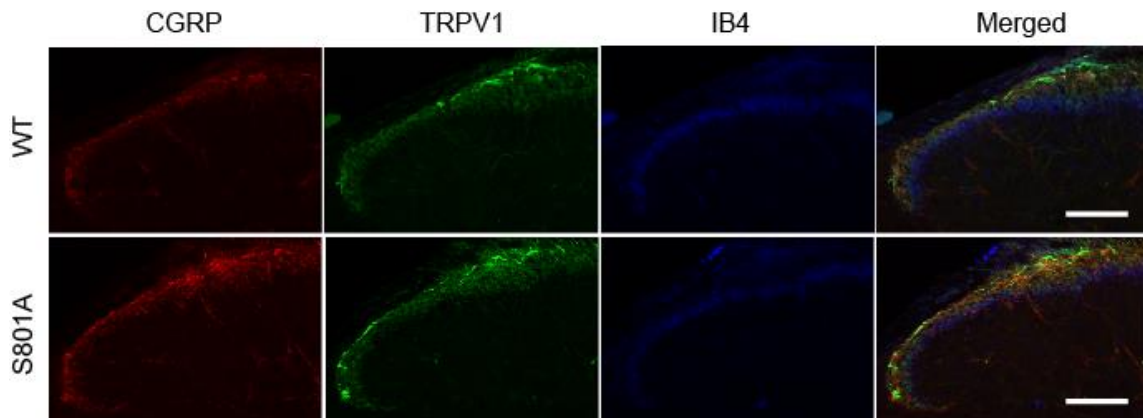


Figure 8. Triple labeling of TRPV1, IB4 and CGRP in the spinal cord of WT and KI mice

The total amount of TRPV1 protein in whole dorsal root ganglion lysates also showed no difference by immunoblot between KI and WT (Fig. 9). These results suggest that TRPV1 expression was not altered in TRPV1 S801A knock-in mice.

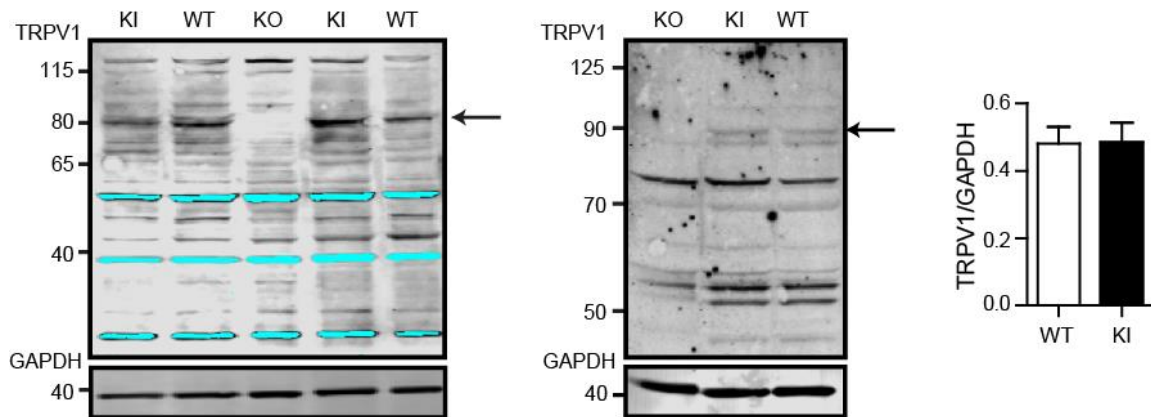


Figure 9. Western blot analysis of TRPV1 using whole DRG from WT or KI mice (left) A representative gel image of western blot using antibodies against TRPV1 (upper) or GAPDH (lower) in WT, KI and TRPV1 KO DRG. (right) Relative quantification of TRPV1, N= 3 per genotype.

2.5. Discussion

Multiple inflammatory mediators are known to induce sensitization of TRPV1 *in vitro* and thermal hyperalgesia *in vivo* (103, 104). Since such effects can be attenuated by the inhibition of protein kinases, aspects of heat hyperalgesia have been attributed to phosphorylation-mediated sensitization of TRPV1. This view is further supported by studies that have sought to prevent TRPV1 phosphorylation by targeting adaptor proteins such as AKAP (105). TRPV1 phosphorylation has been regarded as contributing to inflammatory hyperalgesia. However, there has been no direct examination of the importance of individual TRPV1 phosphorylation events, *per se*, to hyperalgesia *in vivo*. In this study, we examined the *in vivo* importance of phosphorylation of TRPV1 S801 by generating a KI mouse line with that residue mutated to alanine.

CRISPR/Cas9 editing produced the TRPV1 S801A mutation

Three methods were used to confirm the mutation in founder and offspring of two lines of mice. The insertion sequence contained a restriction site that was not in the original WT sequence. Following PCR amplification of genomic samples from the founders, both nucleases could restrict the product, first indicating the insert sequence was present in heterozygous founders. To confirm the coding for alanine at position 801, amino acid sequencing was done on the products from the amplification. Third, a set of genotype specific primers was designed that allowed us to determine the genotype of offspring from breedings. Combined, these three genotyping methods offer substantial support for the successful mutation. By using mRNA from the DRG of mice homozygous for the KI mutation, the sequencing of an RT-PCR product ensured the KI

allele was expressed. Though we could not verify the lack of phosphorylation through the use of an S801-phosphospecific TRPV1 antibody as intended, the alanine mutation has been shown to prevent phosphorylation *in vitro* in several other studies. We thus have confidence the genetic editing prevents phosphorylation of S801 in KI mice.

TRPV1 expression is not different in S801A knock-in mice

Overall TRPV1 expression levels and the TRPV1 expression pattern in sensory ganglia were not affected in the KI mice. Our own previous publication and those of others using heterologous expression systems suggest the S800A mutation should not interfere with TRPV1 expression (73, 85, 106), which undergoes constitutive clathrin-dependent recycling from the surface of the membrane (107). In contrary, agonist-dependent desensitization targets the channel for degradation, and does not involve clathrin (108). We repeatedly attempted to quantify the surface-membrane amounts of TRPV1 phospho-S801 in our dissociated DRG neuron cultures, but were unable to consistently and reliably detect a band in our surface-biotinylation western blot experiments (data not shown). This data clearly would have been useful in confirming similar expression amounts between KI and WT for our electrophysiological recordings. The phospho-antibody used has been previously demonstrated to have S800 TRPV1 specificity in cultures of rat and mouse neurons (73, 86). A difference in labeling and detection methods could have contributed in both cases, in addition to the antibody being polyclonal. Our efforts to quantify TRPV1 in the membrane labeling experiment also failed, as no bands distinct from those in TRPV1KO control samples could be identified. Therefore we cannot exclude the possibility that the membrane expression of TRPV1 is

altered in KI, which needs to be clarified in the future. However the total amount of TRPV1 expression from whole DRG does not differ between KI and WT, as evidenced by our western blot results for TRPV1. The coexpression pattern with a complement of neurochemical markers, localization of central terminals in the spinal cord lamina, and size distribution of neurons expressing the capsaicin receptor bolster our assertion that expression is not different for KI.

The pro-inflammatory peptide bradykinin (BK) enhances TRPV1 activity in a PKC-dependent manner (109), the bradykinin receptor acting first through phospholipase C, which depletes PIP_2 . It is interesting to note that cultured TRPV1-sensory neurons from Pirt knockout mice show reduced bradykinin-potentiation of the capsaicin response in patch clamp experiments, implying an involvement of Pirt in PKC-dependent sensitization of TRPV1. Therefore it would be of interest to quantify S801 phosphorylation in cultured neurons from Pirt-deficient mice, considering 1) the overlap in CaM and PIP_2 binding sites in the C-terminus with S801, 2) the proximity of the AKAP150 binding site (which anchors PKC), 3) the inhibition of AKAP150/TRPV1 interaction by CaM, and 4) the increased association of AKAP150 with TRPV1 following degradation of PIP_2 (32). This might offer insight into a mechanism of integration for these three modulators during TRPV1 desensitization: favored by CaM, opposed by PIP_2 , and recovery by PKC activation.

Chapter 3. The Effect of the S801A Mutation on Functional Properties of TRPV1

3.1. Introduction

The mutation of an amino acid within a protein could have profound effects on function of an ion channel. It can also have little or no effect on structure and function.

Conservative substitutions of residues are usually better tolerated. This is evident in TRPV1 polymorphisms, where many naturally occurring single amino acid differences are essentially reticent. But even if the two residues are similar in properties, there can be a loss of secondary structure, and in the case of phosphorylation, a loss of charge on the protein. For example, both I585M and M315I are conservative substitutions of TRPV1, and alter the function of TRPV1 for specific modes of activation (81). The loss of any phosphorylation site is not among the common TRPV1 polymorphisms. Despite extensive work detailing functional effects of the S800A (rat) mutation in vitro, it is quite possible there are differences with an ex vivo system. Using electrophysiological recordings, we examined several functional consequences of S801A in sensory neurons from mice with this innate genetic mutation. Given that PKC activation can potentiate TRPV1 currents, our specific aim was to compare the response of KI and WT neurons following PKC activation.

3.2. Methods

3.2.1. Dissociation of mouse sensory neurons

Mice (4-9 weeks old) were anesthetized using a cocktail of ketamine (80 mg/kg) and xylazine (10 mg/kg). DRG were dissected out and collected in cold Puck's saline (171 mM NaCl, 6.7 mM KCl, 1.4 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 6.0 mM glucose, pH 7.3). The ganglia were incubated in 5 ml DMEM/F12 medium containing collagenase type IV (1 mg/ml, Millipore Sigma) at 37°C for 30 min. The ganglia were incubated for an additional 15 min following the addition of trypsin (0.25%) and EDTA (0.025%). The tissues were triturated with flame-polished Pasteur pipettes. The neurons were plated onto glass coverslips (8-mm) coated with poly-ornithine and laminin. Dissociated neurons were maintained with DMEM/F12 containing 10% FBS, 1% penicillin/streptomycin at 37°C in a 5% CO₂ incubator. Electrophysiological recordings were performed after 1-3 days.

3.2.2. Whole-cell patch clamp

Whole-cell voltage clamp techniques were performed as described previously (110, 111). The recording pipettes (2-3 MΩ) were pulled from borosilicate glass using a P-97 (Sutter Instrument). In analyses of HEK293 cells, the pipettes were filled with internal solution (150 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, pH 7.4). Unless otherwise indicated, the recording bath contained an external solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4). In analyses of sensory neurons, the pipette was filled with a solution containing 140 mM KCl, 5 mM

NaCl, 1 CaCl₂, 1 MgCl₂, 2.5 Mg-ATP, 10 mM EGTA, and 10 mM HEPES (pH 7.3).

Osmolarity of each solution was measured by a vapor pressure osmometer (Wescor Inc), and was adjusted with mannitol to 290 to 310 mOsm as necessary. Unless otherwise indicated, all recordings were performed at room temperature.

3.2.3. Statistical analysis

The method of statistical analysis used in each data set is indicated in the figure legend.

Data from two groups were compared using Student's t-test. Data from three or more groups were compared using one-way ANOVA followed by Bonferroni post hoc test.

Data are presented as mean±s.e.m. The criterion for statistical significance was P<0.05.

All statistical analyses were performed using GraphPad Prism 6.0.

3.3. Results

3.3.1. TRPV1 S801A nociceptors showed impaired sensitization of capsaicin-evoked currents

To compare the properties of capsaicin-evoked currents in sensory neurons between WT and TRPV1 S801A mice, we performed whole-cell voltage-clamp recordings on dissociated DRG neurons. Two different doses of capsaicin (0.4 μM and 10 μM) produced current densities that were similar between genotypes when comparing KI to WT (Fig. 10A). Times taken for 10%-90% rise of currents evoked by capsaicin (10 μM) were also not different between WT and KI (Fig. 10B). The proportions of dissociated DRG neurons responsive to capsaicin (1 μM) in Ca²⁺ imaging experiments were also

similar (WT, 49.3%; KI, 46.2%). These data are consistent with the identical capsaicin sensitivities of heterologously expressed rat TRPV1 WT and S800A (77, 102). The application of PMA sensitizes TRPV1. This effect is mediated through the activation of PKC, since a PKC inhibitor blocks the effects of PMA and an inactive PMA analog does not produce sensitization (102). To evaluate whether PKC-mediated sensitization of TRPV1 is altered in TRPV1 S801A KI neurons, we determined the extent of PMA-induced increases of peak-current densities evoked by a low-dose of capsaicin (Fig. 10C-E). Brief application of capsaicin (0.4 μ M) evoked small current responses in WT (Fig. 10C), homozygous TRPV1 S801A KI (Fig. 10D) and heterozygous (Het, not shown) neurons. PMA (0.3 μ M) was subsequently applied for 2 minutes, followed by a second application of 0.4 μ M capsaicin. A saturating dose of 10 μ M capsaicin was applied at the end of the recording period, to permit normalization of the currents evoked by the lower capsaicin dose. Under these conditions, the normalized response (peak-current densities) to the 2nd 0.4 μ M capsaicin application was significantly greater than that to the first application in all experimental groups. However, the extent of sensitization by PMA varied between genotypes (Fig. 10E; time effect, $F_{1,20}=106.68$, $p<0.0001$; genotype effect, $F_{2,20}=11.74$, $p=0.0004$; interaction, $F_{2,20}=11.62$, $p=0.0004$; two-way repeated measure ANOVA). Specifically, post-hoc analysis showed the extent of PMA-induced sensitization was significantly smaller in Het and KI neurons than in WT (Fig. 10E), supporting a role for TRPV1 S801 phosphorylation in this process. The small but significant PMA-induced sensitization in KI neurons might reflect overlapping contributions from one or more additional phosphorylated serine residues, likely including S502 (77, 102).

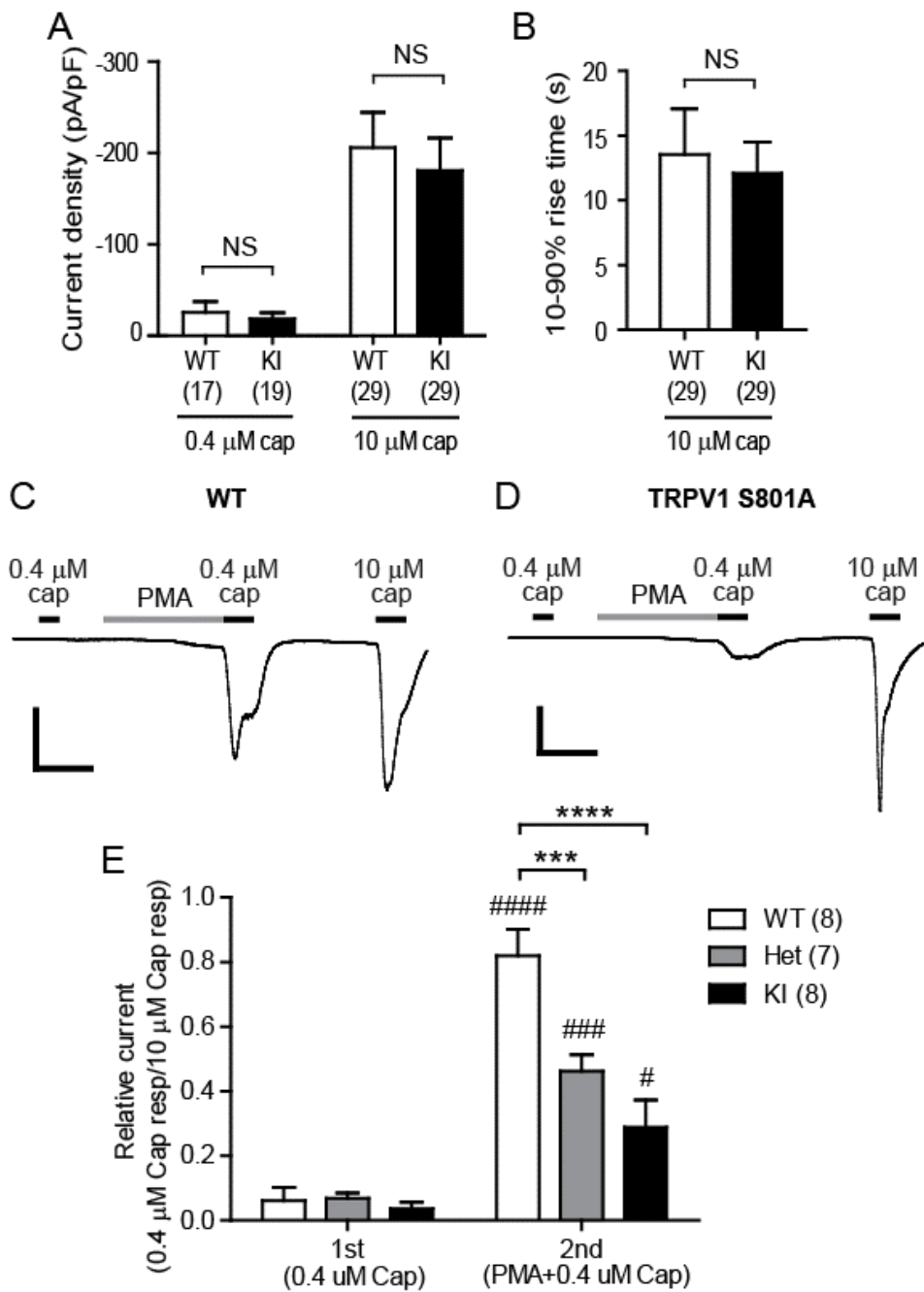


Figure 10. Sensory neurons from KI mice show attenuated sensitization of capsaicin-evoked current response by PMA

A. Densities of currents evoked by 0.4 μ M or 10 μ M of capsaicin in dissociated DRG neurons from WT and KI mice. The numbers within parentheses represent the number of neurons.

B. Rising time (10-90%) of currents evoked by 10 μ M of capsaicin in dissociated DRG neurons from WT and KI mice.

C-D. Representative current responses to capsaicin in dissociated DRG neurons from WT (C) or KI (D) mice. The currents were recorded by whole-cell voltage clamp at -60 mV. 0.4 μ M or 10 μ M capsaicin and 0.3 μ M of PMA was applied as indicated. Scale bars, 200 pA/pF and 1 min.

E. The extent of PMA-mediated sensitization of responses evoked by 1st and 2nd application of 0.4 μ M capsaicin that were normalized to the response by 10 μ M capsaicin in each neuron. ***, $p < 0.001$; ****, $p < 0.0001$ in Bonferroni post-hoc test following two-way repeated measure ANOVA. #, $p < 0.05$; ###, $p < 0.001$; ####, $p < 0.0001$ in Bonferroni post-hoc test between 1st and 2nd currents. Numbers within parentheses represent the numbers of cells analyzed.

3.3.2. TRPV1 S801A nociceptors show attenuated PMA mediated recovery from desensitization to capsaicin

PMA not only sensitizes heterologously expressed TRPV1 but also reverses desensitization produced by a high concentration of capsaicin (73, 112). This reversal phenomenon is abolished in the rat TRPV1 S502A/S800A double mutant (73). However, it is unknown whether this effect of PMA occurs in sensory neurons, or the extent to which it is attributable to phosphorylation of S800. We therefore investigated if PKC-mediated reversal of capsaicin-induced desensitization is affected in DRG neurons dissociated from KI mice. Initial stimulation with 10 μ M capsaicin evoked a robust and reversible activation of TRPV1 in both WT and KI neurons (Fig. 11A). A second application of 10 μ M capsaicin reproducibly evoked a second current response, but the

amplitude of this response was only ~60% that of the initial current, indicative of TRPV1 desensitization. The extent of capsaicin-induced desensitization was not significantly different between genotypes (Fig. 11A, C), suggesting that capsaicin-induced desensitization does not require TRPV1 S801. In WT neurons, PMA application prior to the second application of capsaicin prevented desensitization and resulted in a tendency of the 2nd response to be even larger than the 1st response (Fig. 11B), which was significantly different from the condition without PMA treatment (Fig. 11C). However, in Het neurons, the 2nd 10uM capsaicin response (following PMA) was almost equal to the 1st response. In KI neurons, the 2nd response was smaller than the 1st response, showing desensitization even following PMA treatment. As a result, the ratios of 1st/2nd responses were not significantly different between PMA and Vehicle groups in either the Het or KI neurons (Fig. 11C). These results suggest that PMA-mediated reversal of capsaicin-induced desensitization is impaired in TRPV1 S801A KI mice.

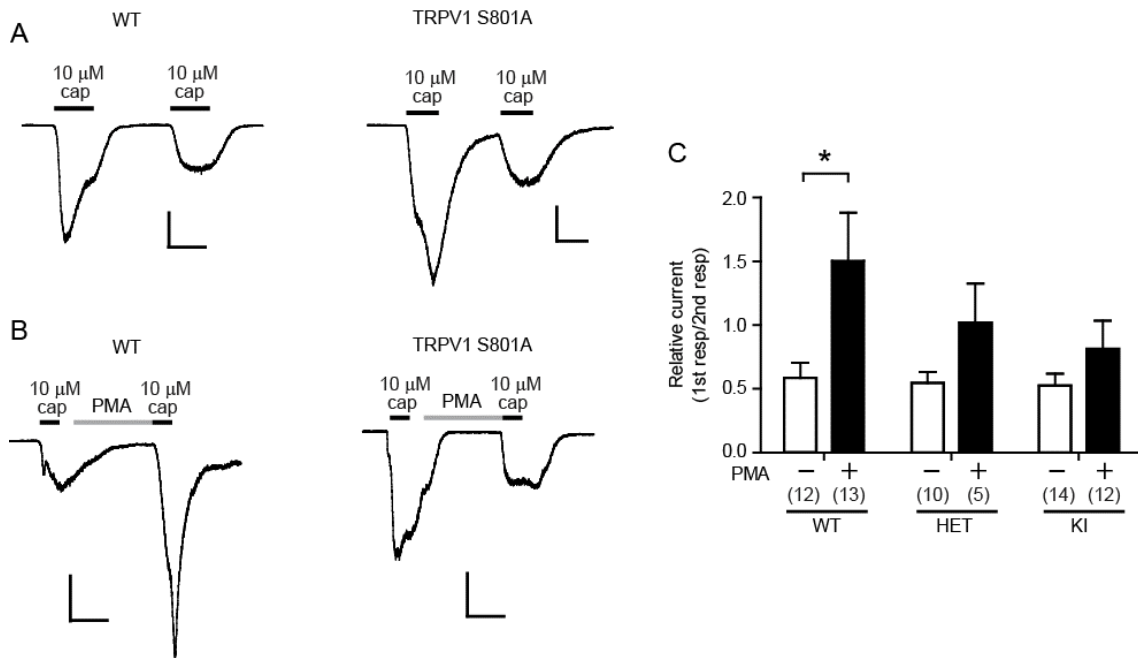


Figure 11. Sensory neurons from KI mice show attenuated PMA mediated recovery from desensitization to capsaicin

A. Representative current responses evoked by consecutive application of 10 μ M capsaicin in dissociated DRG neurons from WT (left) or KI (right) mice. The currents were recorded by whole-cell voltage clamp at -60 mV. Scale bars, 50 pA/pF and 30 sec.

B. Representative current responses by consecutive application of 10 μ M capsaicin with 0.3 μ M PMA prior to the 2nd capsaicin application in dissociated DRG neurons from WT (left) or KI (right) mice. Scale bars, 50 pA/pF and 1 min.

C. The extent of PMA-mediated recovery of desensitization. *, $p < 0.05$ in Bonferroni post-hoc test following two-way ANOVA. Numbers within parentheses represent the numbers of cells analyzed.

3.4. Discussion

A previous investigation from our lab (C-terminal desensitization) indicated that the C-terminal loss of the S800 site would not affect capsaicin sensitivity much, so it should not be surprising there was little effect on basal response. However, sensitization to capsaicin through PKC activation could be affected depending on redundancy of effect with the S502 site.

The electrophysiological recordings in sensory neurons derived from these mice clearly showed attenuation, but not elimination, of PKC-mediated sensitization of capsaicin responses and impaired reversal of capsaicin-induced desensitization by PMA. These findings suggest that sensory neurons of KI mice with the S801A mutation are functionally impaired in PKC-mediated TRPV1 sensitization. The deficit in KI whole-cell currents following PMA is consistent with previous *in vitro* studies (75), which suggested an approximately 2-fold shift (increase) in the capsaicin concentration-response curve for WT TRPV1 following PKC activation. Since bradykinin receptors stimulate PKC, and phorbol esters mimic most effects of bradykinin in sensory neurons and several experimental pain models (113-116), we did not pursue the comparison of bradykinin responses between the KI and WT.

It has been reported that PKC-mediated potentiation of vanilloid-receptor activity is, in part, mediated by SNARE-dependent exocytosis of the receptor (117). As noted earlier, we were unable to quantify TRPV1 membrane expression in our recording condition (discussion, chapter 3). We therefore cannot discount a contribution of reduced surface expression to our results. But the current responses to two doses of capsaicin,

prior to PKC activation, were similar between KI and WT, suggestive of similar basal expression.

The effect of PKC activation on single hTRPV1 channels, as expressed in HEK293 cells, is to increase the open probability when exposed to capsaicin, while the conductance is unaffected (76). This was unaltered in the double mutant S502A/S801A (human). The use of a PKC-inhibitor revealed resting channel conductance receives little contribution from either of these sites. When coupled with our data showing no loss of capsaicin sensitivity for the KI sensory neurons, this bolsters our premise that S801 is a good target for therapeutic intervention as the normal TRPV1 response seems intact. The two key PKC sites for capsaicin sensitization, S503 and S801 (mouse), are located in substantially separated parts of the channel, which suggested to the authors an increase in capsaicin affinity was not a likely explanation for the effect. More recent evidence suggests an interaction between the N and C termini affects desensitization (118). If so, these two phosphorylation sites might be in closer proximity than expected after initial activation of the channel. The S801 site has been demonstrated to be involved in the recovery from capsaicin-induced desensitization and tachyphylaxis (73) as well. Our prior results show S801 is a polymodal PKC sensitization site, yet its mechanism of TRPV1 potentiation following PKC phosphorylation could involve more than one process.

Though it would be of interest to compare the PMA potentiation effect with other agonists, evidence suggests TRPV1 potentiation by phosphorylation appears to be independent of the activator (75). I would be careful to point out however, this does not necessarily support identical response irrespective of which site is phosphorylated,

moreover identical nociceptive signaling subsequent to TRPV1 activation by any activator.

Our macroscopic recording of neurons from HET mice showed an intermediate phenotype between WT and KI, supporting biallelic *trpv1* expression. However, our experiments do not allow us to presume any subunit association events underlying such a phenotype. For example, HET neurons might contain only two homomeric populations of TRPV1, with the S801A mutation or the WT. Alternatively, the assembly of heteromeric tetramers of S801A and WT in varying stoichiometries could exist in HET neurons. The latter possibility could be potentially important if there is a cooperativity between phosphorylation sites, either between S801 sites on different subunits or between S801 and other sites on the same or different subunits. Although we have no evidence for cooperativity of TRPV1 phosphorylation, the possibilities raised by the *in vitro* HET phenotype are intriguing. A recent report indicates capsaicin needs to be bound by at least two subunits for full channel activation (119), and TRPV1 can exist as dimers within the membrane (120), hinting at a functional cooperativity.

Chapter 4. Effect of TRPV1 S801A mutation on nociceptive and Pathological Pain

4.1. Introduction

TRPV1 has been in focus as an alternative pain relief therapeutic for over twenty years. The relevance of this receptor to pathological pain was evident when Caterina (21) and Davis (22) demonstrated a clear lack of inflammatory hyperalgesia during heating of the hindpaw in mice lacking TRPV1. This spurred the development of selective TRPV1 inhibitors which further confirmed a role in hyperalgesia in rodents. Since, the involvement for sensitization of nociceptors has been extended, contributing in some models of mechanical allodynia and chronic neuropathy. Along with its expression in nociceptive C-fibers, it has been shown multiple inflammatory mediators can influence the channel response, further indicating importance in nociception. A common convergence point for many of these mediators is protein kinase C. The inhibition of PKC or its associated scaffolding proteins often has the same effect (on nociception) as TRPV1 inhibition. The evidentiary role of S801 phosphorylation in TRPV1 sensitization, and that the site is unique to PKC, might translate into importance for increased nociceptive signaling. Thus we investigated numerous models to assess the contribution of S801 phosphorylation to pathological pain.

4.2. Methods

4.2.1. Laboratory Animals

Adult (>8 week-old) mice were randomly allocated into different experimental groups. Both male and female mice were used. The experimenter was blinded to the experimental groups.

4.2.2. Acute hindpaw nocifensive behaviors

Twenty μ l of PMA (3 ng/ μ l) in PBS, or PBS alone (vehicle) was injected intraplantarly to a hindpaw. The stock of 3mM PMA in DMSO was diluted prior to injection. An anesthetic was not used for injections. The mice were then immediately put on a lab bench with Whatman paper (Millipore-Sigma, 3030917) and covered with plastic boxes (10X10X14 cm, open at base). The mice were observed for 30 min, with video recordings to evaluate nociceptive behavior and quantify time spent for licking and biting of the injected paw. The mice were then injected with 1 μ g of capsaicin in a buffer containing 135 mM NaCl, 3 mM KCl, 1 mM Na₂HPO₄, 1 mM MgSO₄, 1.2 mM CaCl₂, pH 7.4 (10 μ l) into the same hind paw, and placed back into the box for an additional 15 min of recording. Quantifications were done by an observer blinded to the genotypes of the mice.

4.2.3. Capsaicin-induced eye wiping

To test chemical sensitivity of ophthalmic nociceptors, an eye wiping test was performed. Mice were placed in plastic containers (9 \times 9 \times 13 cm high) with two mirror back walls,

affording the camera a four-sided view. A digital video camera (Sony HDR-CX230/B High Definition Handycam Camcorder) was placed at a fixed distance from the cubicle to record their behavior. Free behaviors of mice were videotaped 5 min before and 5 min after the application of 20 μ l of capsaicin solution (0.03% in H₂O with 3.3% PEG300). The number of eye wipes with the forepaw was counted for five minutes.

4.2.4. Complete Freund's adjuvant (CFA)-induced inflammation

Mice were briefly anesthetized using 3% isoflurane and CFA (20 μ l, 1:1 in PBS) was injected unilaterally into masseter muscle or hindpaw as indicated in each experiment. CFA invariably produced swelling of the masseter muscle and hindpaw.

4.2.5. Carrageenan-induced hindpaw inflammation

Mice were anesthetized with isoflurane (3%) and one hindpaw was cleaned with betadine and alcohol, followed by unilateral carrageenan injection (20 μ L, 1% in PBS, using a 30 gauge needle). The injection site was near the proximal footpad. The carrageenan was prepared by heating PBS to 50°C, then slowly adding the carrageenan and stirring for an hour.

4.2.6. Hargreaves' radiant paw heating assay

Mice were acclimated to the testing environment for at least 30 min each day for two days by placing them on the glass platform (30 °C) of a Hargreaves device (PAW Thermal Stimulator, UC San Diego or Plantar Analgesia Meter, IITC Life Sciences) under an acrylic box. For testing, the mice were placed on the glass platform under an

acrylic box for 10 minutes until they settled. Baseline latency of the radiant heat source was adjusted to a range of 10-12 s with a cutoff time of 20.5 s to prevent tissue damage. Paw withdrawal latency in response to the heat stimulus was measured in both hindpaws 3 times each, with a 10-min interstimulus interval, and the average of the three latencies was used for analysis.

4.2.7. Measuring noxious heat threshold

Hindpaw noxious heat threshold was assessed as described previously (121) using a temperature-ramp hot plate device (IITC Life Science). The temperature of the plate was increased from 30 °C to 50 °C linearly (6 °C/min). The mice were habituated on the metal plate (20cmX15cm) maintained at 30 °C under an acrylic observation chamber (20cmX15cmX20cm) for 30 minutes on each of two days. The test lasted until the animal showed nocifensive behaviors (licking, shaking, or lifting) involving either hindpaw, then the mouse was immediately removed from the plate. The heat threshold measurement was repeated after 30 minutes and the mean of the two thresholds was considered the noxious heat threshold of the mouse.

4.2.8. Von Frey measurement on hind paw

Mice were placed under acrylic boxes on an elevated wire mesh platform and habituated in a behavioral room for at least 30 min per day for 3 days prior to testing. A series of von Frey filaments were applied perpendicularly to the hindpaw plantar surface. The filaments had bending forces ranging from 0.008 g to 4 g. Lifting of the hindpaw or flinching immediately upon removal of the filament was defined as a response. Each

filament was applied 5 times at intervals of a few seconds. The response frequencies [(number of responses/number of stimuli) X100%] to a range of filament forces were determined and stimulus-response frequency curves were plotted. The plots were fitted with a logistic function from which EF50, the mechanical force that produced a 50% response frequency, was obtained.

4.2.9. Paw pinching assay

For the pinch assay (122), each mouse was confined in a plexiglass chamber (7.5cm long × 7.5cm wide × 10cm high) which was placed onto a glass surface, allowing video recording from the bottom. An alligator clip (Generic Micro Steel Toothless alligator test clips 5AMP) was applied to the ventral skin surface between the footpad and the heel. The animal was put back into the chamber and video recorded for 60 s to determine duration of nocifensive behavior towards the hindpaw.

4.2.10. Tail immersion assay

Mice were wrapped in a towel while leaving the tail exposed for most of its length. While the mouse was being held, the last 2 cm of tail was dipped into a water bath maintained at 50°C. The temperature was confirmed throughout the experiment with a thermometer (BAT-12, Physitemp Instruments, Clifton, NJ). The time for the mouse to withdraw or flick its tail was measured, and the average withdrawal latency was determined for each group.

4.2.11. Hot plate assay

Mice were placed on a hot plate apparatus (series 8, PE34) (IITC, Woodland Hills, CA) with the plate set to a temperature of 53°C. The latency was measured for the mouse to show signs of nocifensive behavior, such as licking of hindpaws or jumping from the heated plate. A cutoff of 20 seconds was used to prevent serious burn injury.

4.2.12. PMA-induced thermal hyperalgesia of hindpaw

To test PMA-mediated thermal hyperalgesia, 2 ng of PMA (20 µl of 0.1 µg/ml in PBS) was injected intraplantarly into one hindpaw. A Hargreaves' test was performed before and 3 to 4 hours after the injection.

4.2.13. Mild hindpaw thermal injury

A mild thermal injury model was performed as described previously (121). After measurement of basal heat sensitivity, the mice were anesthetized using isoflurane. The left hind paw was immersed into a water bath at 51 °C for 15 seconds to a level above the ankle. The mice were returned to their home cage and allowed to recover from anesthesia. To evaluate changes in thermal sensitivity induced by mild heat injury, we evaluated thermal threshold again 30 min and 60 min following heat injury.

4.2.14. Mouse grimace scale measurements (MGS)

Previously we showed that masseter injection of CFA increases MGS scores. This was substantially attenuated by pharmacological or genetic inhibition of TRPV1 or transient

receptor potential ankyrin subtype 1 (TRPA1), ablation or chemogenetic inhibition of TRPV1+ primary afferents, or ablation of NK1+ second order neurons (123, 124). These studies suggest that masseter inflammation-induced changes in MGS are mediated by nociceptive inputs from masseter muscle, and supports MGS as a method for assessing spontaneous pain during masseter inflammation. The MGS was utilized as previously described (124, 125). The mice were videotaped for 30 min for each experimental time point. To capture facial images of mice in an unbiased manner, image extraction was performed by blinded experimenters. Images containing a clear view of the entire face were manually captured every 3 min during the video recording (10 images per 30 min session). The scores of the five action units in each photograph were averaged, and a mean MGS score was obtained from the 10 images, which was presumed to reflect the level of spontaneous pain. The mean MGS score of each mouse before CFA treatment was used as the baseline value.

4.2.15. Bite force assay

To assess bite-evoked pain associated with craniofacial muscle inflammation in mice, we performed a bite force assay as previously described (124-126). Mice were acclimated to the testing environment and handling for two days prior to behavioral testing. Mice were placed in a modified 60-ml plastic syringe with a wide opening at one end to accommodate the head of the mouse. In order to prevent the mouse from escaping, the syringe plunger was inserted into the syringe to loosely restrain the mouse inside the syringe. To minimize stress, the mouse was released immediately from the syringe if it vigorously moved or tried to hide inside the syringe. The syringe containing the mouse was held manually and moved slowly at 0.5-1 cm/sec toward bite plates so that the mouse

could bite the plates. Spike 2 software was used to measure the voltage changes from transducer displacement. SigmaPlot 8.0 was used to convert the voltage change into force based on calibration using standard weights. Bite force was recorded for 120 sec per session and the top five force measurements were averaged.

4.2.16. Conditioned Place Aversion

The conditioned place aversion (CPA) apparatus consists of three opaque chambers separated by open doors. A smaller center chamber (60 mm W × 60 mm D × 150 mm H) connects the two end chambers that are identical in size (150 mm W × 150 mm D × 150 mm H) but can be distinguished by the texture of the floor (circular opening vs. diamond pattern mesh), wall color (black walls vs. white walls), and olfactory cues (papaya vs. cherry ChapStick™ applied on a cotton swab beneath the flooring). All flooring was cleaned or replaced between trials. Movement of mice and time spent in each chamber were monitored and recorded using custom-built infrared sensors and software.

Habituation was performed 1-2 days prior to conditioning for 30 min when mice were exposed to the environment with full access to all chambers. A single-trial conditioning protocol was used in the experiments. On conditioning day for the PMA and capsaicin experiments, mice first received vehicle control (intraplantar hindpaw injection of saline, 20 uL) paired with the black chamber in the morning and, 3–4 h later, intraplantar treatment (PMA, 60 ng in 20 uL of PBS) (capsaicin, 0.3 ug in 10 uL saline) in the other hindpaw paired with the other chamber. During the conditioning, mice were allowed to stay only in the paired chamber without access to other chambers for 30 min immediately following saline or treatment injection. On the test day, about 20 h after the treatment pairing, mice were placed in the middle chamber

of the CPA box with all doors open so animals had free access to all chambers. Movement and duration of each mouse spent in each chamber were recorded for 30 min for analysis of chamber aversion. For the CFA/capsaicin experiment there was no vehicle control pairing. In that experiment, conditioning was done 1 day post CFA hindpaw injection (15 uL, 1:1 CFA in PBS), and again 3 days post CFA with a capsaicin injection pairing (ipsilateral hindpaw to CFA, 0.3 ug in 10 uL saline). The mice were injected without anesthesia, except for CFA. The data is presented as group means + SEM, with n=5 in each group for all experiments.

4.2.17. Statistical analysis

The method of statistical analysis used in each data set is indicated in the figure legend. Data from two groups were compared using Student's t-test. Data from three or more groups were compared using one-way ANOVA followed by Bonferroni post hoc test. The effects of pharmacological or genetic manipulations in different time points were analyzed with two-way ANOVA with repeated measures. All multiple group comparisons were performed by Bonferroni post hoc test. Data are presented as mean±s.e.m. The criterion for statistical significance was $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6.0.

4.3 Results

4.3.1 TRPV1 S801A mutation only marginally attenuates inflammatory thermal hyperalgesia

We examined the somatosensory phenotypes of TRPV1 S801A KI mice. Paw withdrawal latency from a radiant heat source (Hargreaves' assay; Fig. 12A-C) or from a hot plate (53 °C; Fig. 12B) was comparable between WT and KI mice. Latency to tail flick following immersion into hot water (50 °C; Fig. 12C) was also not different. Paw licking duration following pinching of the plantar surface of hindpaw using an alligator clip was also comparable between WT and KI mice (Fig. 12D). Mechanical threshold of hindpaw determined by Von Frey assay (Fig. 12A) and nociceptive threshold on a variable-temperature hot plate (Fig. 12D) were also not significantly different between WT and KI mice. Together, these findings argue against a role for TRPV1 S801 phosphorylation in setting basal mechanical or heat pain sensitivity. To examine PKC-related thermal hyperalgesia, we injected PMA (2 ng) into the hindpaw. To our surprise, PMA decreased paw withdrawal latency in the Hargreaves assay in both WT and KI mice, without a significant difference between genotypes (Fig. 12A). To test inflammation-mediated thermal hyperalgesia, we injected CFA into the hindpaw. Paw withdrawal latency was decreased at 1 day following intraplantar CFA in the ipsilateral hindpaws of both WT and KI mice. The extent of decrease was slightly less in KI mice compared to WT (interaction, $F_{2,74}=3.16$, $p=0.0483$, two-way RM ANOVA), but there was no significant difference between groups in post-hoc analysis (Fig. 12B). To further test whether pathological heat hyperalgesia is affected in KI mice, we utilized a mild thermal injury model, in which heat hyperalgesia is partially mediated by TRPV1 (121).

Mild thermal injury of the hindpaw resulted in decreased withdrawal latency (Fig. 12C) and heat pain threshold (Fig. 12D) in both WT and KI mice after 30 minutes, without significant differences between genotypes. These results suggest that ablation of S801 phosphorylation on TRPV1 marginally affects inflammatory heat hyperalgesia but is not sufficient to robustly attenuate it.

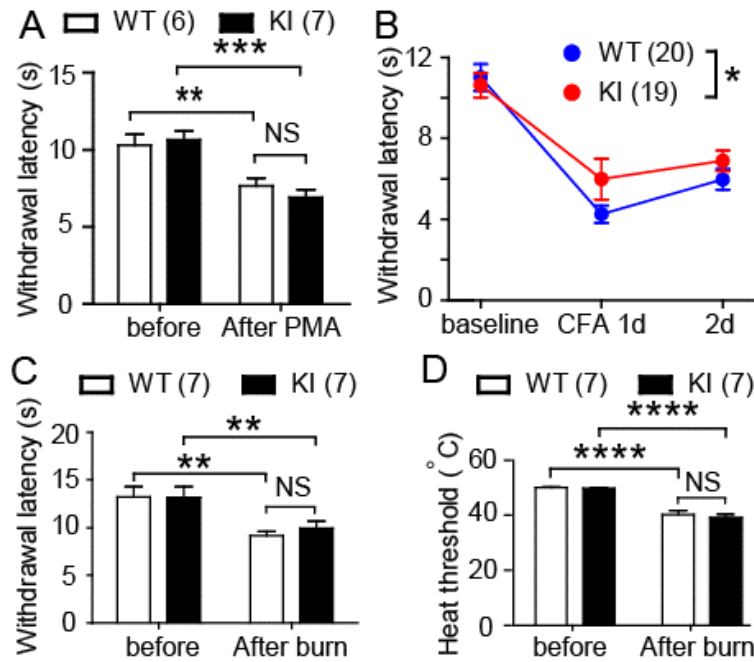


Figure 12. KI mice show normal heat sensitivity and modestly attenuated CFA-induced thermal hyperalgesia

A. Thermal hyperalgesia evaluated using the Hargreaves' test following intraplantar injection of PMA (2 ng). Number of mice is in parentheses.

B. Thermal hyperalgesia evaluated using the Hargreaves' test following intraplantar injection of CFA (20uL, 1:1 in PBS). *, $p < 0.05$ in two-way RM ANOVA. Number of mice is in parentheses.

C-D. Hindpaw withdrawal latency in the Hargreaves' test (C) and nociceptive heat threshold on the variable hot plate (D) in a mild thermal injury model. NS, not significant. **, $p < 0.01$; ****, $p < 0.0001$ in Bonferroni post-hoc analysis following two-way RM ANOVA. Number of mice is in parentheses.

4.3.2. TRPV1 S801A mutation attenuates PMA-induced nociception and sensitization of capsaicin responses

We next assessed the impact of TRPV1 S801 mutation on acute responses to noxious chemical stimulation. The eye wiping behavioral response to capsaicin was not different between WT and KI mice (Fig. 13A). Intraplantar injection of capsaicin (1 μ g) similarly evoked nocifensive behaviors that were not statistically different between genotypes (Fig. 13B). These results suggest the basal sensitivity of KI mice to TRPV1 ligands is comparable to that of WT. PMA injection is known to evoke a nocifensive behavioral response in mice that depends on TRPV1 (53, 121). This response has been proposed to involve protein kinases, since it could be attenuated by a PKC inhibitor (53) or segregation of TRPV1 from the adaptor protein AKAP, which mediates TRPV1-protein kinase interactions (33). When we administered PMA (60 ng) into the hindpaw, WT mice showed robust nocifensive behavior (Fig. 13C). Such behavior was completely absent in TRPV1 KO mice (not shown), a finding consistent with a previous report (121). In KI mice, PMA produced only a modest response that was significantly smaller than that in WT (Fig. 13C). Furthermore, when capsaicin was injected into a hindpaw that had been pre-injected with PMA, nocifensive behaviors were significantly less in KI mice, compared with WT mice (Fig. 13D). Importantly, when the paw was pretreated with vehicle, capsaicin-induced nocifensive behaviors were not significantly different between WT and KI mice (Fig. 13D). Of note, there was a tendency towards greater capsaicin-induced nocifensive behaviors following PMA injection than that following vehicle injection, but this trend did not reach statistical significance. This is in contrast to the

clear PMA-induced sensitization of capsaicin-evoked currents in Fig. 10. Although the source of this discrepancy is not clear, it might be due to the complicated action of PMA *in vivo*. PMA injection evokes broad inflammatory signaling, which may lead to the eventual activation of TRPV1 and, thereby, induce long-lasting nociception (53, 121). However, subsequent TRPV1 desensitization might suppress nocifensive behaviors evoked by later capsaicin injection. It is thus possible that, under our experimental conditions, PMA-induced reversal of the desensitization was not sufficient to overcome the effects of desensitization produced by preceding PMA. Nonetheless, these results suggest that TRPV1 KI and WT mice show identical capsaicin responses under naïve conditions, but that loss of TRPV1 S801 phosphorylation significantly attenuates both acute PMA-mediated nociception and the PKC-mediated sensitization of capsaicin evoked nociceptive responses *in vivo*.

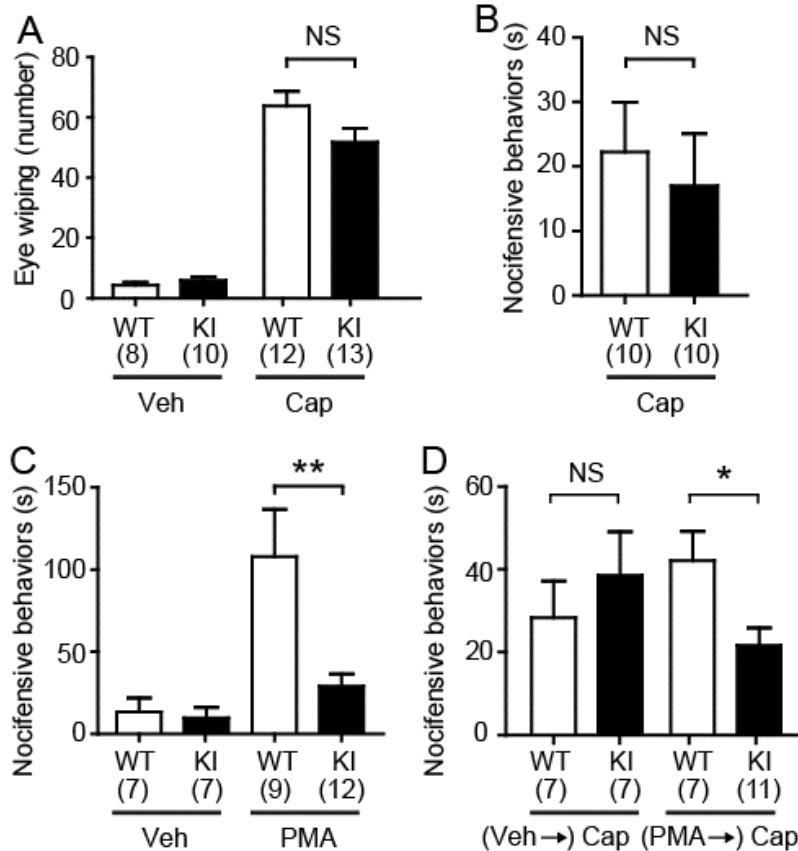


Figure 13. KI mice show attenuation of acute PMA-induced nocifensive behavior and PMA-induced sensitization of capsaicin-mediated nocifensive behavior

A. Number of eye wiping response during 5 min after ocular application of vehicle (20 μ l saline or PBS) or capsaicin (0.03%; 20 μ L). Number of mice is in parentheses. NS, not significant in Student's t-test.

B. Duration of hindpaw nocifensive behaviors (licking and shaking) over 15 min after intraplantar injection of 1 μ g of capsaicin.

C. Duration of hindpaw nocifensive behaviors (licking and shaking) over 30 min after intraplantar injection of vehicle (PBS) or PMA (60 ng). Number of mice is in parentheses. **, $p < 0.01$ in Student's t-test.

D. Duration of hindpaw nocifensive behaviors (licking and shaking) over 5 min following intraplantar injection of capsaicin (1 μ g). The hindpaws were pretreated with either vehicle or PMA (60 ng) 30 min prior to capsaicin injection. Number of mice is in parentheses. *, $p < 0.05$ in Student's t-test.

4.3.3. TRPV1 S801A attenuates spontaneous pain from inflamed masseter muscle

To examine the role of TRPV1 S801 phosphorylation in spontaneous pain, we utilized the mouse grimace scale (MGS) in a masseter inflammation model (Fig. 14A). Unilateral injection of CFA into the masseter muscle of WT mice significantly increased MGS scores on post injection days 1 and 3 compared with vehicle (Fig. 14A), consistent with our previous results (124). The injection of vehicle into the masseter muscle of KI mice slightly increased MGS scores to a similar extent as in WT mice. Further, whereas the injection of CFA into the masseter muscle of KI mice produced significantly greater MGS scores than injection of the vehicle, the extent of increase by CFA was significantly less in KI mice than that in WT mice (interaction, $F_{9,36}=24.4$, $p<0.0001$; genotype effect, $F_{3,12}=46.1$, $p<0.0001$; time effect, $F_{3,36}=235.3$, $p<0.0001$; two-way RM ANOVA), suggesting a partial role for TRPV1 S801 in this model of inflammation-induced spontaneous pain.

To further estimate the proportion of TRPV1-mediated pain that could be attributed to S801 phosphorylation in this model of spontaneous pain, we tested the effects of AMG9810, a small molecule antagonist of TRPV1 function, in both WT and KI mice (Fig. 14B). One day after masseter injection of CFA, MGS scores were evaluated before and 1 hour after the injection of AMG9810 into the masseter muscle. The effects of genotype and AMG9810 on MGS scores were significantly different between KI and WT mice (interaction, $F_{1,18}=5.8$, $p<0.027$; genotype effect, $F_{1,18}=43.88$, $p<0.0001$; time effect, $F_{1,18}=76.66$, $p<0.0001$; two-way RM ANOVA). CFA once again

produced robust increases in MGS scores after 1 day in both genotypes, albeit to a lesser extent in KI mice. Masseter injection of AMG9810 significantly attenuated MGS in both KI and WT mice, but the extent of inhibition was greater in WT than KI, which resulted in a similar level of residual MGS score in WT and KI mice. These results are not likely confounded by differential CFA evoked changes in TRPV1 expression between genotypes, since when we examined TRPV1 mRNA in TG one day after CFA injection, ipsilateral TRPV1 mRNA was not significantly different between KI and WT ganglia (TRPV1/GAPDH; 1.48 ± 0.01 in WT; 1.51 ± 0.01 in KI; $n=5$ in WT and KI; $t_8=2.03$, $p=0.77$, unpaired t-test). Our previous study showed that reduction of bite force during masseter inflammation was modestly attenuated in TRPV1 KO mice (124). We therefore asked whether CFA induced changes in bite-evoked pain might be affected by the lack of phosphorylation at S801 (Fig. 14C). During masseter inflammation, the reduction of bite-force was not significantly different between genotypes (WT, $30.6 \pm 5.3\%$; KI, $34.5 \pm 9.1\%$; $n=10$ in WT and KI; $t_{18}=0.37$, $p=0.71$, unpaired t-test), arguing that this phenomenon does not depend on TRPV1 S801 phosphorylation.

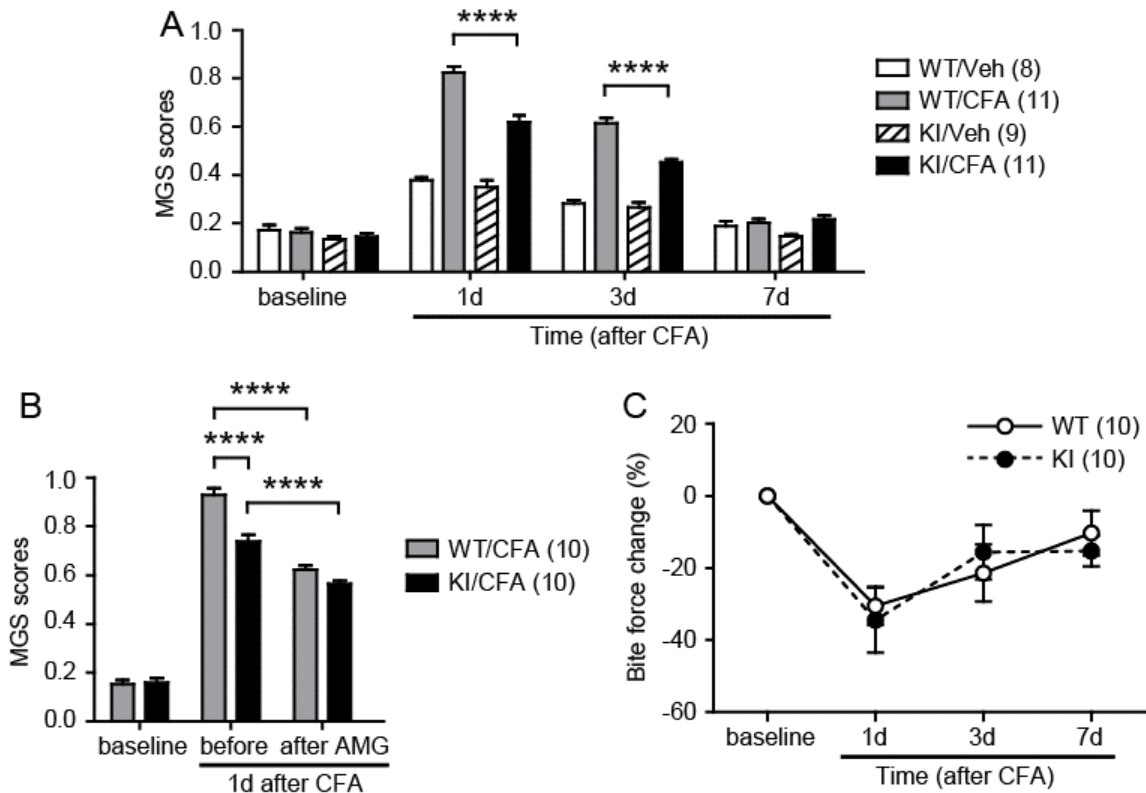


Figure 14. KI mice show attenuated spontaneous ongoing pain following masseter inflammation

- A. MGS score before and after masseter injection of CFA or Veh in WT or TRPV1 S801A KI mice. $P < 0.0001$ in post-hoc analysis following two-way repeated measure ANOVA. Number of mice is in parentheses.
- B. The effects of AMG9810 on MGS scores 1 day after masseter CFA injection in WT and KI mice. The effects of pharmacological manipulation were analyzed with two-way ANOVA with repeated measures. Number of mice is in parentheses. $p < 0.0001$ with Bonferroni post hoc test.
- C. Changes in bite force before and after masseter injection of CFA in WT or TRPV1 S801A KI mice. Number of mice is in parentheses.

4.3.4. Conditioned Place Aversion

As the PMA caused a spontaneous nocifensive response when injected to the hindpaw of most mice, with a noticeable difference between S801A and WT, we tested further for a difference in affective pain. The affective pain referring to a change in behavior, stemming from the acute nociception. A 3-box enclosure (127) was used to assess conditioned place aversion (CPA) in S801A and WT littermates. Preliminary testing for preferences between boxes by C57BL6 mice showed 62.5% and 37.5% occupancy frequency for the black and white boxes, respectively, with no additional cues (Fig. 15). During conditioning, we used both olfaction and flooring cues to assist the mouse in identifying boxes, not just the black or white color, since the boxes are dim when covered for pairing. Following pairing of a hindpaw injection of PMA with confinement in one end box (avoidance conditioning), the mice demonstrated no aversion to the paired box the next day, with a 42% occupancy of the paired box (Fig. 15). This was similar to the initial preference during habituation, thus we did not observe a CPA with these mice in the preliminary PMA experiment (one-way ANOVA, ns). The results shown for all CPA experiments represent group averages plus the SEM, with n=5 for each group of mice in an experiment.

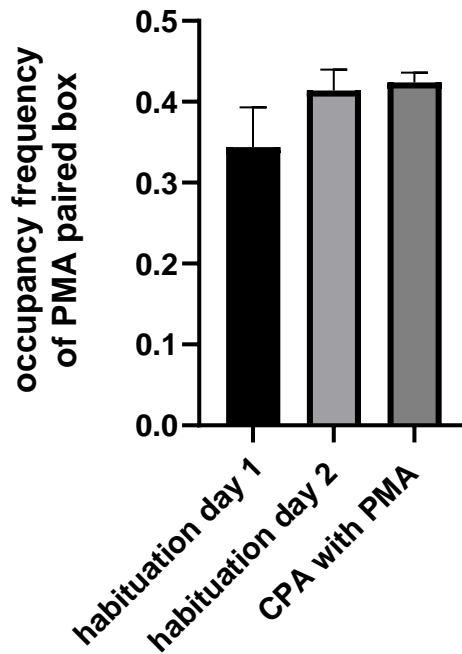


Figure 15. Preliminary conditioned place avoidance with PMA in C57BL/6 mice

A RM 1-way ANOVA indicates no significant avoidance of the PMA paired box in a preliminary experiment for CPA in C57BL/6J mice. The data presented as a mean±SEM, p value .215, n=5.

Additional experiments were done with C57BL/6J to ensure the cues had little effect on preference. There was no pairing for an aversion effect in this experiment. For the flooring cue, a consistent odorant was paired with each box, while flooring was counterbalanced over two experiments. The preferences for the black over white box were consistent between experiments and with habituation results from the preliminary experiment (Fig. 16).

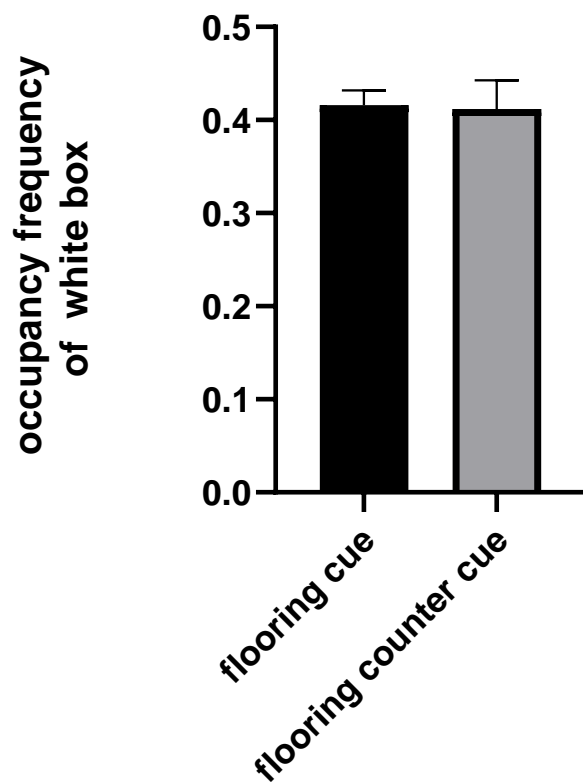


Figure 16. Flooring cue effect on CPA

A paired t-test indicates no significant effect of the olfaction cues on box preference in C57BL6/J mice. The data presented as a mean±SEM, n=5.

For the olfaction cue, a consistent flooring was paired with a colored box, while odorant was countered over two experiments. The preferences for the black over white box were consistent between experiments. The black and white box occupancy were 55% and 45% respectively for both cues (Fig. 17).

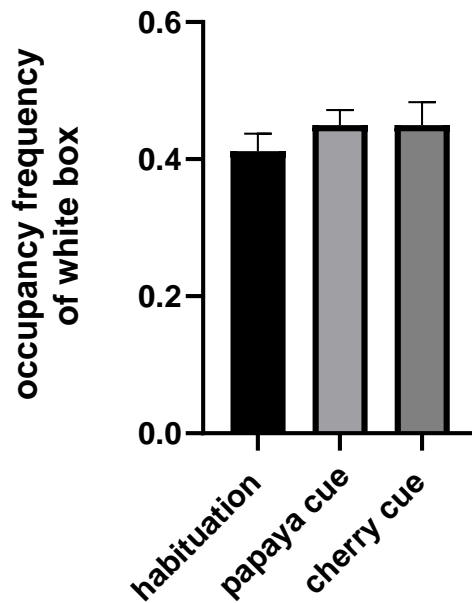


Figure 17. Olfaction cue effect on CPA

A RM 1-way ANOVA indicates no significant effect of the olfaction cues on box preference in C57BL6/J mice. The data presented as a mean±SEM, p value .4, n=5.

Since a conditioned-place-aversion was not shown in the preliminary experiment with PMA and C57BL6 mice, we then tried to establish a positive control for CPA with capsaicin, comparing KI and WT with TRPV1KO. The TRPV1KO mice should not have an aversion to capsaicin pairing, and represented an additional control for the injection and handling. After establishing a pre-pairing preference, the mice had capsaicin injected into a hindpaw and were confined to one of the boxes for conditioning. The following day the mice were observed for CPA. There was little difference between any of the groups, where the occupancy of the paired box prior to capsaicin was 51%, 54%, and 57% for KI, WT, and KO respectively. Following capsaicin the paired box occupancy

was 47%, 55%, 56% for KI, WT, and KO mice (Fig. 18). The single capsaicin hindpaw injection did not establish a place avoidance response in mice.

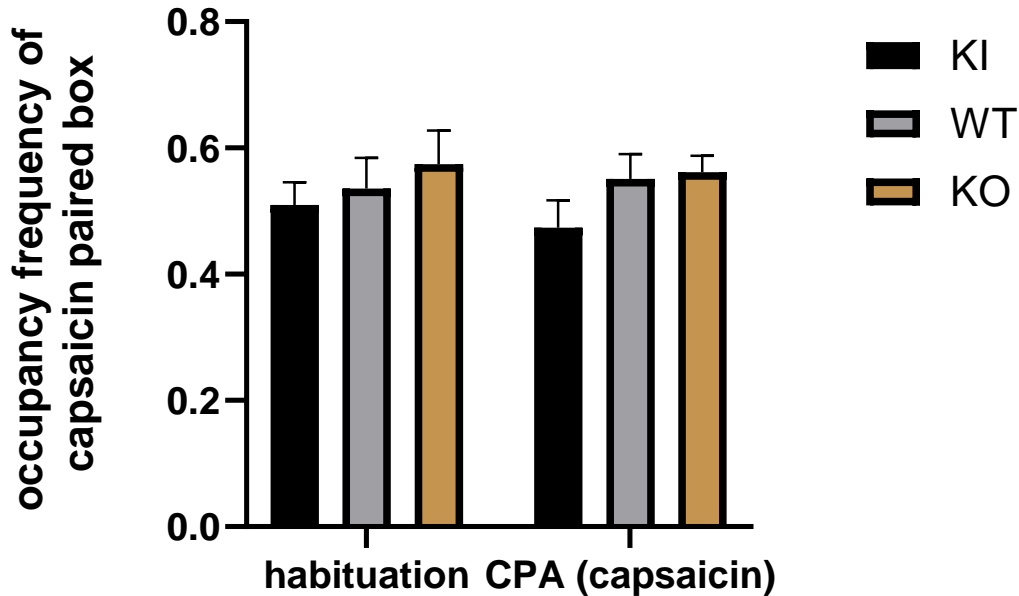


Figure 18. CPA after injection of capsaicin in the hindpaw

A two-way ANOVA indicates no avoidance of a capsaicin paired box in KI, WT, or TRPV1KO mice. There was also no difference in occupancy between genotypes. The data presented as a group mean \pm SEM, n=5.

Another CPA experiment was done using both CFA and capsaicin pairing, with the intent of causing a more aversive condition during pairing. The CFA was administered one day prior to the first pairing, and so the pairing done when thermal hyperalgesia should be near its peak after CFA-induced inflammation. A second pairing was done following an ipsilateral hindpaw injection of capsaicin on the third day after CFA, when inflammation

is still evident. CPA testing was done the next day, and no avoidance response was demonstrated with this protocol either. The occupancy time in the white box was little different after pairing (CFA and capsaicin) for the KI (45% pre-pairing, 43%) and WT mice (45% pre-pairing, 38%) (Fig 19). There was no statistical difference between genotypes.

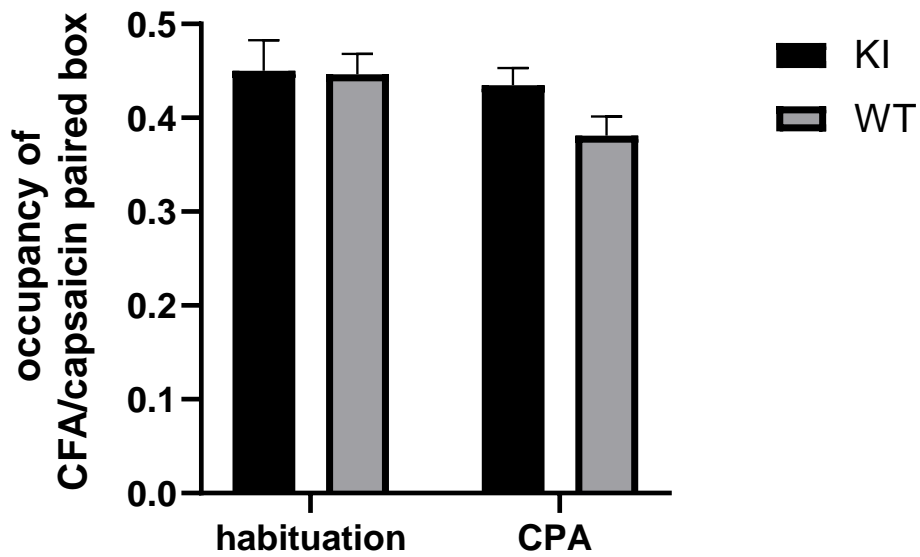


Figure 19. CPA after hindpaw injection of CFA and capsaicin

A RM two-way ANOVA indicates no avoidance of the CFA and capsaicin paired box, and no difference between KI and WT mice. The data presented as a group mean ± SEM, n=5.

4.4. Discussion

Basal withdrawal responses to natural stimuli (heat and mechanical force) were indistinguishable between WT and KI mice, as was the nocifensive response to capsaicin, suggesting that alanine mutation of S801 has little or no effect on normal TRPV1 channel function. This also suggests that basal phosphorylation of TRPV1 S801 does not greatly influence behavioral responsiveness to these stimuli under physiological conditions. KI mice showed only a slight reduction of CFA-mediated thermal hyperalgesia in the hindpaw and no alteration in thermal hyperalgesia in a mild thermal injury model.

It appears the S801 phosphorylation has little to do with heat hyperalgesia. Considering the contribution of TRPV1 and the proposed role of PKC in thermal hyperalgesia and in phosphorylation of TRPV1 S801 (73, 104), such a minor contribution of TRPV1 S801 to thermal hyperalgesia was rather surprising. However, S801 is only one of several PKC phosphorylation sites in TRPV1. Indeed, PMA-induced sensitization of TRPV1 responsiveness to heat is only partially impaired by alanine mutation of rat TRPV1 S800 *in vitro* (85). In contrast, PMA-induced sensitization of TRPV1 to heat was profoundly impaired when another PKC-mediated phosphorylation site T705 (or T704 in rat) was mutated (85). *In vitro* findings have suggested that mutation of rat TRPV1 S800 to alanine alone suppressed the phosphorylation of a c-terminal fragment of TRPV1 to a similar level as quadruple mutations of rat TRPV1 S800/T704/S774/S820 (102), raising the possibility that the rat TRPV1 S800A mutation also affects phosphorylation at T704. Although we could not directly test this possibility, any such influence of S800 on T704 phosphorylation might be weaker in intact channels, limiting the impact of individual S800 or S801 mutations on heat hypersensitivity *in vitro* or heat hyperalgesia *in vivo*.

Although direct *in vivo* evidence is lacking, previous studies support the idea that phosphorylation of mouse TRPV1 T705 plays a dominant role in inflammatory thermal hyperalgesia (74, 85). Ablation of phosphorylation at T705 alone or in combination with that at S801 might therefore be predicted to substantially impair thermal hyperalgesia. This approach, however, would be confounded by the fact that the T705A mutation alters basal TRPV1 heat and acid sensitivity (74, 85). Unlike TRPV1 S801 and T705, mouse TRPV1 S503 can be phosphorylated by both PKC and PKA (72, 102). However, alanine mutation of this residue does not impair PMA-induced sensitization to heat (85). Interestingly, alanine or aspartate mutation of rat TRPV1 S502 impairs forskolin-induced sensitization of TRPV1 to heat (128). Thus, during CFA-induced inflammation, PKA-mediated phosphorylation of TRPV1 S502, along with other PKA-dependent residues, possibly sensitizes TRPV1 to heat and limits loss of function in TRPV1 S801A KI mice. Moreover, PKC and PKA are not the only kinases capable of phosphorylating TRPV1. In fact, inflammation can potentially promote phosphorylation of at least eight residues in TRPV1 through a collection of protein kinases that also include CamKII, cdk5, and src (68, 69, 71, 72, 80). Our experiments do not exclude roles for TRPV1 residues phosphorylated by these protein kinases during CFA-induced thermal hyperalgesia. Additional TRPV1 S801-independent contributors to heat sensation and thermal hyperalgesia likely include inflammation-induced increases in TRPV1 expression (129, 130), receptor tyrosine kinase mediated TRPV1 membrane trafficking (68), and non-TRPV1 heat gated ion channels (131). All these mechanisms of thermal sensation and hyperalgesia could contribute to the lack of a dominant thermal phenotype in the KI mouse. In addition to these results, strong evidence comes from another recent study

(106), where sumoylation of TRPV1 seems to be a key post-translational modification involved in thermal hyperalgesia.

It was considered the inflammatory response to CFA was overwhelming a reduced TRPV1 sensitivity in neurons of KI mice. CFA induces a broad immune response involving release of several mediators. Therefore several kinases could be involved and act on different channels, or residues of TRPV1. The overall effect producing similar sensitization of peripheral neurons in KI and WT for the assay.

We then quantified the eye-wiping response to capsaicin, the oleoresin being a well-known irritant in pepper sprays. The cornea is avascular, and an immune privileged tissue in the naive state, lacking the complement of immune cells present in cutaneous layers. The cornea has a much higher innervation density by C-type peripheral fibers than the skin (132, 133), especially that of mouse hindpaw. Accordingly the TRPV1 expression density of this tissue should be more, and any change in capsaicin sensitivity could have been more evident. This condition presumably gave a ‘cleaner’ result for effect of the mutation on TRPV1-specific peripheral nerve sensitivity, without complication from an immune response. The nocifensive response to capsaicin instillation on the eye was not different for the KI mouse. We had intended to also determine PKC sensitization of this response. The difficulty of keeping the PMA applied to the cornea for any duration, prior to capsaicin, prevented a sufficient interpretation of the experiment. It is interesting to note that in the cornea, nerves and neovessels (new blood vessels in response to poor circulation) inhibit each other in the normal condition (134); in contrary during inflammation, sensory neurons directly promote angiogenesis via substance P signaling (135). TRPV1 sensitization has been demonstrated to affect

release of this neuropeptide in mouse sensory neurons (136). The KI mice might then have a deficit in corneal repair with inflammation.

Although PMA is known to sensitize TRPV1 to other agonists, but not directly activate it (102), the phorbol alone produces nocifensive behavioral responses *in vivo* that are entirely or largely dependent on TRPV1 (53, 121). Such PMA-induced nocifensive behaviors are dependent on PKC, which can affect mast cell degranulation, multiple cytokines, and growth factors at the site of injection (53). PMA strongly activates neutrophil peroxide release, or respiratory burst, which could activate TRPV1 during inflammation. However indications from other studies suggests an inflammatory effect from neutrophils would not be involved in the rapid nocifensive response (<30 minutes) (137) observed from injection of the hindpaw. It should be recognized that the S801A mutation is present in all non-neuronal TRPV1-expressing cells of KI mice, which could affect functions in neutrophils or mast cells. Given these numerous contributors, it is striking that our data support a major role for TRPV1 S801 phosphorylation in acute PMA-induced hindpaw nocifensive behavior. In addition, when capsaicin-induced nocifensive behavior was compared following pretreatment with PMA, the KI mice showed clear attenuation of sensitized behavior. Since PMA itself causes TRPV1-dependent nocifensive behaviors, the responses to subsequent capsaicin application could reflect mixed consequences of sensitization and reversal of desensitization by the preceding PMA application. Interestingly, PMA-mediated thermal hyperalgesia was not affected in KI mice, again suggesting the involvement of S801-independent mechanisms (e.g., T705).

Ablation of TRPV1 S801 phosphorylation clearly attenuated ongoing pain caused by masseter inflammation without altering bite-evoked pain, which is consistent with differential contributions of TRPV1 to spontaneous vs bite-evoked pain (124). Combined pharmacological inhibition of total TRPV1 function with genetic ablation of S801 phosphorylation revealed phosphorylation at this residue accounts for approximately half of all TRPV1-mediated spontaneous pain during masseter inflammation. The magnitude of this effect is surprising, given the multiplicity of TRPV1 sensitization mechanisms described above, and suggests a predominant contribution of phosphorylation at this single site in spontaneous pain in this model. How might the TRPV1 S801 mutation alter ongoing masseter pain following CFA injection? Inflammation upregulates TRPV1 expression in trigeminal ganglia (138). However, the transcriptional level of TRPV1 in the TG of KI mice was not different from that of WT mice following CFA injection, arguing the KI phenotype is not likely due to differential TRPV1 upregulation. Tissue inflammation also produces putative endogenous agonists of TRPV1, such as hydroxyoctadecadienoic acid (HODE) (139), and modulators such as glutamate (86). HODE depletion in inflamed masseter muscle has been reported to attenuate spontaneous pain, suggesting that constitutive activation of TRPV1 by endogenous agonists could lead to spontaneous pain (125). Ongoing pain might, therefore, be mediated by enhanced functionality of cell surface-expressed TRPV1 in this setting, coupled with increased availability of endogenous agonists. However TRPV1 phosphorylation can also enhance its surface localization (68, 140). Our experiment did not dissociate these possible mechanisms of TRPV1 phosphorylation-dependent hyperalgesia.

The contribution of TRPV1 S801 phosphorylation to spontaneous pain that we observed following masseter inflammation may not be generalizable to other inflammatory pain models, since the overall contribution of TRPV1 to spontaneous pain following tissue injury or inflammation varies among models. For example, inhibition of TRPV1 did not affect guarding following skin incision (141) and was variably effective on conditioned place preference in knee joint arthritis (142). TRPV1 inhibition did attenuate mouth rubbing in oral mucositis and guarding in bone cancer (143, 144). The lack of any effect of the mutation in the CPA experiments could reflect this, or the apparently brief duration of the intradermal capsaicin-evoked nociception. The involvement of TRPV1 S801 phosphorylation in different pain models might thus be context-dependent.

Chapter 5. Conclusion

We used the CRISPR/Cas9 editing method to develop a knock-in mouse model with unmatched specificity for inhibition of TRPV1 phosphorylation, removing a single phosphorylation site by mutation S801A. The genetic ablation of S801 attenuates PKC-mediated sensitization of TRPV1, PMA induced acute hindpaw nociception, and inflammation-mediated spontaneous pain from masseter muscle *in vivo*. These results suggest that small molecules selectively interfering with phosphorylation at TRPV1 S801 might provide an effective means of attenuating pain at sites of inflammation or injury, without altering physiological pain sensation. Moreover, TRPV1 S801A mice should serve as a salient model for validating the specificity of such pharmacological modulators.

Our behavioral studies indicate little involvement of S801 phosphorylation for both basal nociceptor responsiveness and during inflammatory thermal hyperalgesia. Several studies provide strong evidence for involvement of the distal C-terminus in affecting the TRPV1 response following heat activation (73, 84, 106, 118). The normal thermal TRPV1 response is strongly influenced by phosphorylation of T407 and T705 (mouse) (69, 74). Our results are supported by recent findings in a conditional knockout mouse model (106), where SUMOylation of TRPV1 at residue K822 in sensory neurons is critical for inflammatory thermal hyperalgesia; and it does not seem to depend on, or interfere with, PKC phosphorylation of S801. There is some hesitation about this result though, as the removal of SEN1P would affect SUMOylation of many proteins within the neurons. This includes PIPKI γ , a key member of the phosphatidylinositol 4-phosphate

kinase (PIPKI) family. A SUMOylation deficiency significantly disrupts PIPKI γ -mediated generation of intracellular PIP₂ (145). The notable influence of PIP₂ on TRPV1 function has been discussed previously. Furthermore, the SUMOylation of AKAP and PKC (146) are also presumably affected. With evidence of crosstalk and phosphorylation between PKC isoforms it is unclear as to how this would affect PKC function and S801 phosphorylation. Though only determined in a heterologous system and not for sensory neurons, there was evidence that the K822 residue affected S800 phosphorylation, and after PMA. The PKC ϵ inhibitor peptide (PKC ϵ V1-V2) prevents association of the isoform with RACK2 (Receptor of Activated C-Kinase), one of the scaffold proteins that can locate PKC ϵ to the neurolemma near its substrates. The sequence of this inhibitor peptide has similarity to the SUMOylation sequence on TRPV1 at K822. Given its effectiveness in reducing inflammatory and thermal hyperalgesia of the rodent hindpaw in various models (57, 147-149), it is interesting to speculate (skeptically) about this inhibitor peptide also interfering with TRPV1 SUMOylation as part of its mechanism in alleviating thermal hyperalgesia. The interplay at the TRPV1 C-terminus of PIP₂, Pirt, AKAP, and SUMOylation (and with its role in PIP₂ production and control of PKC localization), has yet to be explored. But if there is interaction during inflammation, the evidence does not support a role for S801 in thermal hyperalgesia.

The electrophysiology experiments largely corroborated our prior study of PKC-induced capsaicin hypersensitivity of TRPV1 (85). There were no differences noted in determining the effect of S801A on TRPV1 desensitization. Although we did not evaluate the phorbol induced increase in acid-evoked sensitivity in neurons from KI mice, this was a distinct result from the prior study. There are two key residues in the

outer pore loop of TRPV1 shown to affect gating of the acid response (18). A proposed mechanism for heat desensitization has the N-terminus and C-terminus associating in a manner that affects the outer pore selectivity filter, reducing its opening and resembling a closed state (118). This could offer a structural hint at the polymodal sensitization by phosphorylation of S800, where the acid response is affected as well, in that conformation changes in the C-terminus could be relayed to the outer pore. However, that N and C termini interaction is reported to only occur following heat activation. It would seem worthwhile to investigate the PKC-potentiation of the acid response of the neurons from KI mice.

On the other hand, capsaicin-induced tachyphylaxis occurs in part through increased removal of the receptor from the membrane (150). Some *In vitro* results indicate PKC activation and the S801 residue have a role in recovery from this process (73). If reinsertion of the receptor to the neurolemma is PKC and SNARE-dependent, an interaction of phosphoS801 with the microtubules could be involved in restoring expression of the receptor, particularly in peptidergic sensory neurons (151). This could be important for spontaneous pain during an inflammatory or chronic condition with a persistent endogenous TRPV1 activator.

The modality-specific response we observed in KI mice, the significant reduction in phorbol-induced sensitization to capsaicin, is indicative of a polymodal function of TRPV1, in conjunction with its polymodal receptor status. Given the large number of activators and signal cascades influencing this channel, it would be difficult to accept that the output is unitary. With so much being integrated by the channel, it is considered that the output is not limited to one nociceptive pathway, that of mild thermal sensation and

hyperalgesia. The concept of functional selectivity refers to distinct agonists acting at the same receptor, that can modify cellular outcomes differently by engaging different effector subsets. It is almost exclusively applied to GPCRs but might also have relevance for ionotropic channels.

This suggests that for certain pathological conditions, inhibition of different sites on TRPV1 are better for attenuating nociception. The clinical inhibition of a single TRPV1 phosphorylation site is not feasible, yet uncovering a role in nociceptive signaling might illuminate a pathway for pathological pain intervention.

References

1. Cain DM, Khasabov SG, Simone DA. Response Properties of Mechanoreceptors and Nociceptors in Mouse Glabrous Skin: An In Vivo Study. *The Journal of Neurophysiology*. 2001;85(4):1561-74.
2. Cavanaugh DJ, Lee H, Lo L, et al. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *PNAS* 2009;106(22):9075-80.
3. Lawson JJ, McIlwrath SL, et al. TRPV1 Unlike TRPV2 is Restricted to a Subset of Mechanically Insensitive Cutaneous Nociceptors Responding to Heat. *J Pain* 2008 April ; 9(4): 298–30. 2008;9(4):298-308.
4. Mishra SK, Hoon MA. Ablation of TrpV1 neurons reveals their selective role in thermal pain sensation. *Mol Cell Neurosci*. 2010;43(1):157-63.
5. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*. 1997;389(6653):816-24.
6. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron*. 1998;21(3):531-43.
7. Premkumar LS, Ahern GP. Induction of vanilloid receptor channel activity by protein kinase C. *Nature | Letters to Nature*. 2000;408:985-90.
8. Green DP, Ruparel S, Roman L, Henry MA, Hargreaves KM. Role of endogenous TRPV1 agonists in a postburn pain model of partial-thickness injury. *Pain*. 2013;154(11):2512-20.
9. Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, et al. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*. 1999;400(6743):452-7.

10. Sanchez JF, Krause JF, Cortright DN. The Distribution and Regulation of Vanilloid receptor VR1 and VR15 Splice Variant RNA Expression in Rat. *Neuroscience*. 2001;107(3):373-81.
11. Cavanaugh DJ, Chesler AT, Jackson AC, Sigal YM, Yamanaka H, Grant R, et al. Trpv1 reporter mice reveal highly restricted brain distribution and functional expression in arteriolar smooth muscle cells. *J Neurosci*. 2011;31(13):5067-77.
12. Premkumar LS, Sikand P. TRPV1: A Target for Next Generation Analgesic. *Current Neuropharmacology*. 2008;6:151-63.
13. Cavanaugh DJ, Chesler AT, Braz JM, Shah NM, Julius D, Basbaum AI. Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. *J Neurosci*. 2011;31(28):10119-27.
14. Zwick M, Davis BM, et al. Glial Cell Line-Derived Neurotrophic Factor is a Survival Factor for Isolectin B4-Positive, but not Vanilloid Receptor 1-Positive, Neurons in the Mouse. *The Journal of Neuroscience*. 2002;22(10):4057-65.
15. Liao M, Cao E, Julius D, Cheng Y. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature*. 2013;504(7478):107-12.
16. Moiseenkova-Bell VY, Stanciu, Lia A., et al. Structure of TRPV1 channel revealed by electron cryomicroscopy. *PNAS* 2008;105(21):7451-5.
17. Matta JA, Ahern GP. Voltage is a partial activator of rat thermosensitive TRP channels. *J Physiol*. 2007;585(Pt 2):469-82.
18. Jordt S-E, Tominaga M, Julius D. Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proceedings of National Academy of Sciences*. 2000;97(14):8134-9.
19. Heyman I, Rang HP. Depolarizing responses to capsaicin in a subpopulation of rat dorsal root ganglion cells. *Neurosci Lett*. 1985;56(1):69-75.

20. Jo AO, Noel JM, Lakk M, Yarishkin O, Ryskamp DA, Shibasaki K, et al. Mouse retinal ganglion cell signalling is dynamically modulated through parallel anterograde activation of cannabinoid and vanilloid pathways. *The Journal of Physiology*. 2017;595(20):6499-516.
21. Caterina MJ, Leffler A, Malmberg AB, et al. Impaired Nociception and Pain Sensation in Mice Lacking the Capsaicin Receptor. *Science*. 2000;288:306-13.
22. Davis JB, J G, et al. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia
Nature 2000;405:183-7.
23. Gavva NR, Tamir R, Qu Y, Klionsky L, Zhang TJ, Immke D, et al. AMG 9810 [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide], a novel vanilloid receptor 1 (TRPV1) antagonist with antihyperalgesic properties. *J Pharmacol Exp Ther*. 2005;313(1):474-84.
24. Gavva NR, Treanor JJ, Garami A, Fang L, Surapaneni S, Akrami A, et al. Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain*. 2008;136(1-2):202-10.
25. Nidegawa-Saitoh Y, Sumioka T, Okada Y, Reinach PS, Flanders KC, Liu CY, et al. Impaired healing of cornea incision injury in a TRPV1-deficient mouse. *Cell Tissue Res*. 2018;374(2):329-38.
26. Rosenbaum T, Gordon-Shaag A, Munari M, Gordon SE. Ca²⁺/calmodulin modulates TRPV1 activation by capsaicin. *J Gen Physiol*. 2004;123(1):53-62.
27. Lishko PV, Procko E, Jin X, Phelps CB, Gaudet R. The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron*. 2007;54(6):905-18.
28. Numazaki M, Tominaga T, et al. Structural determinant of TRPV1 desensitization interacts with calmodulin. *PNAS*. 2003;100(13):8002-6.
29. Lau SY, Procko E, Gaudet R. Distinct properties of Ca²⁺-calmodulin binding to N- and C-terminal regulatory regions of the TRPV1 channel. *J Gen Physiol*. 2012;140(5):541-55.

30. Tominaga M, Tominaga T. Structure and function of TRPV1. *Pflügers Arch.* 2005;451(1):143-50.
31. Hetenyi A, Nemeth L, Weber E, Szakonyi G, Winter Z, Josvay K, et al. Competitive inhibition of TRPV1-calmodulin interaction by vanilloids. *FEBS Lett.* 2016;590(16):2768-75.
32. Jeske NA, Por ED, Belugin S, Chaudhury S, Berg KA, Akopian AN, et al. A-kinase anchoring protein 150 mediates transient receptor potential family V type 1 sensitivity to phosphatidylinositol-4,5-bisphosphate. *J Neurosci.* 2011;31(23):8681-8.
33. Zhang X, Li L, McNaughton PA. Proinflammatory mediators modulate the heat-activated ion channel TRPV1 via the scaffolding protein AKAP79/150. *Neuron.* 2008;59(3):450-61.
34. Prescott ED, Julius D. A Modular PIP2 Binding Site as a Determinant of Capsaicin Receptor Sensitivity. *Science* 2003;300(5623):1284-8.
35. Rohacs T. Phosphoinositide regulation of TRPV1 revisited. *Pflügers Arch.* 2015;467(9):1851-69.
36. Liu B, Zhang C, Qin F. Functional recovery from desensitization of vanilloid receptor TRPV1 requires resynthesis of phosphatidylinositol 4,5-bisphosphate. *J Neurosci.* 2005;25(19):4835-43.
37. Lukacs V, Thyagarajan B, Varnai P, Balla A, Balla T, Rohacs T. Dual regulation of TRPV1 by phosphoinositides. *J Neurosci.* 2007;27(26):7070-80.
38. Rohacs T, Thyagarajan B, Lukacs V. Phospholipase C mediated modulation of TRPV1 channels. *Mol Neurobiol.* 2008;37(2-3):153-63.
39. Gao Y, Cao E, Julius D, Cheng Y. TRPV1 structures in nanodiscs reveal mechanisms of ligand and lipid action. *Nature.* 2016;534(7607):347-51.
40. Jordt SE, Julius D. Species-Specific Sensitivity to “Hot” Chili Peppers. *Cell.* 2002;108:421-30.

41. Yang F, Xiao X, Cheng W, Yang W, Yu P, Song Z, et al. Structural mechanism underlying capsaicin binding and activation of the TRPV1 ion channel. *Nat Chem Biol.* 2015;11(7):518-24.
42. Kim AY, Tang Z, Liu Q, Patel KN, Maag D, Geng Y, et al. Pirt, a phosphoinositide-binding protein, functions as a regulatory subunit of TRPV1. *Cell.* 2008;133(3):475-85.
43. Sisco NJ, Luu DD, Kim M, Van Horn WD. PIRT the TRP Channel Regulating Protein Binds Calmodulin and Cholesterol-Like Ligands. *Biomolecules.* 2020;10(3).
44. Grycova L, Holendova B, Lansky Z, Bumba L, Jirku M, Bousova K, et al. Ca(2+) binding protein S100A1 competes with calmodulin and PIP2 for binding site on the C-terminus of the TRPV1 receptor. *ACS Chem Neurosci.* 2015;6(3):386-92.
45. Gonzalez-Martinez T, Perez-Pinera P, Diaz-Esnal B, Vega JA. S-100 proteins in the human peripheral nervous system. *Microsc Res Tech.* 2003;60(6):633-8.
46. Melemedjian OK, Tillu DV, Moy JK, et al. Local translation and retrograde axonal transport of CREB regulates IL-6-induced nociceptive plasticity. *Molecular Pain* 2014;10(45).
47. Jung H, Gkogkas CG, Sonenberg N, Holt CE. Remote control of gene function by local translation. *Cell.* 2014;157(1):26-40.
48. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. *Molecular biology of the cell* (third edition). 1994.
49. Mochly-Rosen D, Das K, Grimes KV. Protein kinase C, an elusive therapeutic target? *Nat Rev Drug Discov.* 2012;11(12):937-57.
50. Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ. PKC and the control of localized signal dynamics. *Nat Rev Mol Cell Biol.* 2010;11(2):103-12.
51. Velázquez KT, Mohammad H, Sweitzer SM. Protein kinase C in pain: Involvement of multiple isoforms. *Pharmacol Res.* 2007;55(6):578-89.

52. Kohno T, Wang H, Amaya F, Brenner GJ, Cheng JK, Ji RR, et al. Bradykinin enhances AMPA and NMDA receptor activity in spinal cord dorsal horn neurons by activating multiple kinases to produce pain hypersensitivity. *J Neurosci*. 2008;28(17):4533-40.
53. Ferreira J, Triches KM, Medeiros R, Calixto JB. Mechanisms involved in the nociception produced by peripheral protein kinase c activation in mice. *Pain*. 2005;117(1-2):171-81.
54. Campenot RB, Draker DD, Senger DL. Evidence that Protein Kinase C Activities Involved in Regulating Neurite Growth Are Localized to Distal Neurites. *Journal of Neurochemistry*. 1994;63:868-78.
55. Hua X-Y, Chen P, Yaksh TL. Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. *Neuroscience Letters* 1999;276:99-102.
56. Aley KO, Messing RO, Mochly-Rosen D, Levine JD. Chronic Hypersensitivity For Inflammatory Nociceptor Sensitization Mediated by the δ Isozyme of Protein Kinase C. *The Journal of Neuroscience*. 2000;12:4680-5.
57. Cesare P, Lodewijk V. Dekker, Sardini A, Parker PJ, McNaughton PA. Specific Involvement of PKC- ϵ in Sensitization of the Neuronal Response to Painful Heat. *Neuron*. 1999;23:617-24.
58. Khasar SG, Lin Y-H, et al. A Novel Nociceptor Signaling Pathway Revealed in Protein Kinase C δ Mutant Mice. *Neuron*. 1999;24:253-60.
59. Honda K, Shinoda M, Kondo M, Shimizu K, Yonemoto H, Otsuki K, et al. Sensitization of TRPV1 and TRPA1 via peripheral mGluR5 signaling contributes to thermal and mechanical hypersensitivity. *Pain*. 2017;158(9):1754-64.
60. Morrone LA, Scuteri D, Rombola L, Mizoguchi H, Bagetta G. Opioids Resistance in Chronic Pain Management. *Curr Neuropharmacol*. 2017;15(3):444-56.
61. González-Arenas A, MÁ P-O, V H-P, et al. PKC α and PKC δ activation regulates transcriptional activity and degradation of progesterone receptor in human astrocytoma cells. *Endocrinology*. 2015;156(3):1010–22.

62. Lessmann E, Leitges M, Huber M. A redundant role for PKC-epsilon in mast cell signaling and effector function. *Int Immunol*. 2006;18(5):767-73.
63. Chen Y, Tian, Q. . . The role of protein kinase C epsilon in neural signal transduction and neurogenic diseases. *Front Med*. 2011;5(1):70-6.
64. Moriyama T, Higashi T, Togashi K, Iida T, Segi E, Sugimoto Y, et al. Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain*. 2005;1:3.
65. Sugiura T, Tominaga M, Katsuya H, Mizumura K. Bradykinin lowers the threshold temperature for heat activation of vanilloid receptor 1. *J Neurophysiol*. 2002;88(1):544-8.
66. Zhou Y, Li GD, Zhao ZQ. State-dependent phosphorylation of epsilon-isozyme of protein kinase C in adult rat dorsal root ganglia after inflammation and nerve injury. *J Neurochem*. 2003;85(3):571-80.
67. Docherty RJ, Yeats JC, Bevan S, Boddeke HW. Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. *Pflugers Arch*. 1996;431(6):828-37.
68. Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J*. 2005;24(24):4211-23.
69. Pareek TK, Keller J, Kesavapany S, Kulkarni AB, et al. Cyclin-dependent kinase 5 modulates nociceptive signaling through direct phosphorylation of transient receptor potential vanilloid 1. *PNAS*. 2007;104(2):660-5.
70. Jendryke T, Prochazkova M, Hall BE, Nordmann GC, Schladt M, Milenkovic VM, et al. TRPV1 function is modulated by Cdk5-mediated phosphorylation: insights into the molecular mechanism of nociception. *Sci Rep*. 2016;6:22007.
71. Mohapatra DP, Nau C. Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem*. 2003;278(50):50080-90.

72. Bhawe G, Zhu W, Brasier DJ, Oxford GS, Gereau RW, IV. cAMP-Dependent Protein Kinase Regulates Desensitization of the Capsaicin Receptor (VR1) by Direct Phosphorylation. *Neuron*. 2002;35:721-31.
73. Mandadi S, Tominaga T, Numazaki M, Murayama N, Saito N, Armati PJ, et al. Increased sensitivity of desensitized TRPV1 by PMA occurs through PKCepsilon-mediated phosphorylation at S800. *Pain*. 2006;123(1-2):106-16.
74. Li L, Hasan R, Zhang X. The basal thermal sensitivity of the TRPV1 ion channel is determined by PKCbetaII. *J Neurosci*. 2014;34(24):8246-58.
75. Vellani V, Mapplebeck S, Moriondo A, Davis JB, McNaughton PA. Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. *Journal of Physiology* 2001;534(3):813-25.
76. Studer M, McNaughton PA. Modulation of single-channel properties of TRPV1 by phosphorylation. *J Physiol*. 2010;588(Pt 19):3743-56.
77. Numazaki M, Tominaga T, Toyooka H, Tominaga M. Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. *J Biol Chem*. 2002;277(16):13375-8.
78. Bhawe G, et al. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *PNAS* 2003;100(21):12480-5.
79. Bhawe G, et al. cAMP-Dependent Protein Kinase Regulates Desensitization of the Capsaicin Receptor (VR1) by Direct Phosphorylation. *Neuron*. 2002;35:721-31.
80. Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H, et al. Phosphorylation of vanilloid receptor 1 by Ca²⁺/calmodulin-dependent kinase II regulates its vanilloid binding. *J Biol Chem*. 2004;279(8):7048-54.
81. Wang S, Joseph J, Diatchenko L, Ro JY, Chung MK. Agonist-dependence of functional properties for common nonsynonymous variants of human transient receptor potential vanilloid 1. *Pain*. 2016;157(7):1515-24.

82. Vlachova V, Teisinger J, Susankova K, Lyfenko A, Ettrich R, Vyklicky L. Functional role of C-terminal cytoplasmic tail of rat vanilloid receptor 1. *J Neurosci.* 2003;23(4):1340-50.
83. Brauchi S, Orta G, Salazar M, Rosenmann E, Latorre R. A hot-sensing cold receptor: C-terminal domain determines thermosensation in transient receptor potential channels. *J Neurosci.* 2006;26(18):4835-40.
84. Joseph J, Wang S, Lee J, Ro JY, Chung MK. Carboxyl-terminal domain of transient receptor potential vanilloid 1 contains distinct segments differentially involved in capsaicin- and heat-induced desensitization. *J Biol Chem.* 2013;288(50):35690-702.
85. Wang S, Joseph J, Ro JY, Chung MK. Modality-specific mechanisms of protein kinase C-induced hypersensitivity of TRPV1: S800 is a polymodal sensitization site. *Pain.* 2015;156(5):931-41.
86. Chung MK, Lee J, Joseph J, Saloman J, Ro JY. Peripheral group I metabotropic glutamate receptor activation leads to muscle mechanical hyperalgesia through TRPV1 phosphorylation in the rat. *J Pain.* 2015;16(1):67-76.
87. Kim YH, Park CK, Back SK, Lee CJ, Hwang SJ, Bae YC, et al. Membrane-delimited coupling of TRPV1 and mGluR5 on presynaptic terminals of nociceptive neurons. *J Neurosci.* 2009;29(32):10000-9.
88. Kawamata T, Ji W, Yamamoto J, Niiyama Y, Furuse S, Namiki A. Contribution of transient receptor potential vanilloid subfamily 1 to endothelin-1-induced thermal hyperalgesia. *Neuroscience.* 2008;154(3):1067-76.
89. Koda K, Hyakkoku K, Ogawa K, Takasu K, Imai S, Sakurai Y, et al. Sensitization of TRPV1 by protein kinase C in rats with mono-iodoacetate-induced joint pain. *Osteoarthritis Cartilage.* 2016;24(7):1254-62.
90. Lee H-K, Takamiya K, Han J-S, et al. Phosphorylation of the AMPA Receptor GluR1 Subunit Is Required for Synaptic Plasticity and Retention of Spatial Memory. *Cell.* 2003;112:631-43.
91. Liu S, Zhao Z, Guo Y, Shu H, Li C, Tang Y, et al. Spinal AMPA Receptor GluA1 Ser831 Phosphorylation Controls Chronic Alcohol Consumption-Produced Prolongation of Postsurgical Pain. *Mol Neurobiol.* 2018;55(5):4090-7.

92. Li B, Devidze N, Barengolts D, Probst N, Sphicas E, Apicella AJ, et al. NMDA receptor phosphorylation at a site affected in schizophrenia controls synaptic and behavioral plasticity. *J Neurosci*. 2009;29(38):11965-72.
93. Bhaya D, Davison M, Barrangou R. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu Rev Genet*. 2011;45:273-97.
94. Fineran PC, Charpentier E. Memory of viral infections by CRISPR-Cas adaptive immune systems: acquisition of new information. *Virology*. 2012;434(2):202-9.
95. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, et al. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol*. 2011;9(6):467-77.
96. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*. 2011;471(7340):602-7.
97. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature*. 2014;507(7490):62-7.
98. Mali P, Yang L, Esvelt KM, et al. RNA-Guided Human Genome Engineering via Cas9. *SCIENCE* 2013;339(15):823-6.
99. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339(6121):819-23.
100. Brinster RL, Chen HY, Trumbauer ME, et al. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc Natl Acad Sci USA, Developmental Biology*. 1985;82:4438-42.
101. Chung MK, Jue SS, Dong X. Projection of non-peptidergic afferents to mouse tooth pulp. *J Dent Res*. 2012;91(8):777-82.
102. Bhawe G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, et al. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci U S A*. 2003;100(21):12480-5.

103. Levine JD, Alessandri-Haber N. TRP channels: targets for the relief of pain. *Biochim Biophys Acta*. 2007;1772(8):989-1003.
104. Huang J, Zhang X, McNaughton PA. Inflammatory pain: the cellular basis of heat hyperalgesia. *Curr Neuropharmacol*. 2006;4(3):197-206.
105. Btesh J, Fischer MJ, Stott K, McNaughton PA. Mapping the binding site of TRPV1 on AKAP79: implications for inflammatory hyperalgesia. *J Neurosci*. 2013;33(21):9184-93.
106. Wang Y, Gao Y, Tian Q, Deng Q, Wang Y, Zhou T, et al. TRPV1 SUMOylation regulates nociceptive signaling in models of inflammatory pain. *Nat Commun*. 2018;9(1):1529.
107. Liu J, Du J, Wang Y. CDK5 inhibits the clathrin-dependent internalization of TRPV1 by phosphorylating the clathrin adaptor protein AP2 2. *Science Signaling* 2019;12(585).
108. Sanz-Salvador L, Andres-Borderia A, Ferrer-Montiel A, Planells-Cases R. Agonist- and Ca²⁺-dependent desensitization of TRPV1 channel targets the receptor to lysosomes for degradation. *J Biol Chem*. 2012;287(23):19462-71.
109. Premkumar LS, Ahern GP. Induction of vanilloid receptor channel activity by protein kinase C. *Nature-letters to nature*. 2000;408.
110. Chung MK, Wang S. Cold Suppresses Agonist-induced Activation of TRPV1. *J Dent Res*. 2011;90(9):1098-102.
111. Wang S, Lee J, Ro JY, Chung MK. Warmth suppresses and desensitizes damage-sensing ion channel TRPA1. *Mol Pain*. 2012;8:22.
112. Mandadi S, Numazaki M, Tominaga M, Bhat MB, Armati PJ, Roufogalis BD. Activation of protein kinase C reverses capsaicin-induced calcium-dependent desensitization of TRPV1 ion channels. *Cell Calcium*. 2004;35(5):471-8.
113. Burgess GM, Mullaney I, McNeill M, al e. Second Messengers Involved in the Mechanism of Action of Bradykinin in Sensory Neurons in Culture. *The Journal of Neuroscience*. 1989;9(9):3314-25.

114. Cesare P, Sardini A, Dekker L, et al. Specific Involvement of PKC-epsilon in Sensitization of the Neuronal Response to Painful Heat. *Neuron*. 1999;23:617-24.
115. Sugiura T, Tominaga M. Bradykinin Lowers the Threshold Temperature for Heat Activation of Vanilloid Receptor 1. *J Neurophysiol* 2002;88:544-8.
116. Petho G, Reeh PW. Sensory and signaling mechanisms of bradykinin, eicosanoids, platelet-activating factor, and nitric oxide in peripheral nociceptors. *Physiol Rev*. 2012;92(4):1699-775.
117. Morenilla-Palao C, Planells-Cases R, Garcia-Sanz N, Ferrer-Montiel A. Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. *J Biol Chem*. 2004;279(24):25665-72.
118. Luo L, Wang Y, Li B, Xu L, Kamau PM, Zheng J, et al. Molecular basis for heat desensitization of TRPV1 ion channels. *Nat Commun*. 2019;10(1):2134.
119. Liu TY, Chu Y, Mei HR, Chang D, Chuang HH. Two Vanilloid Ligand Bindings Per Channel Are Required to Transduce Capsaicin-Activating Stimuli. *Front Mol Neurosci*. 2019;12:302.
120. Storti B, Bizzarri R, Cardarelli F, Beltram F. Intact microtubules preserve transient receptor potential vanilloid 1 (TRPV1) functionality through receptor binding. *J Biol Chem*. 2012;287(10):7803-11.
121. Bolcskei K, Helyes Z, Szabo A, Sandor K, Elekes K, Nemeth J, et al. Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain*. 2005;117(3):368-76.
122. Huang T, Lin SH, Malewicz NM, Zhang Y, Zhang Y, Goulding M, et al. Identifying the pathways required for coping behaviours associated with sustained pain. *Nature*. 2019;565(7737):86-90.
123. Asgar J, Zhang Y, Saloman JL, Wang S, Chung MK, Ro JY. The role of TRPA1 in muscle pain and mechanical hypersensitivity under inflammatory conditions in rats. *Neuroscience*. 2015;310:206-15.

124. Wang S, Lim J, Joseph J, Wei F, Ro JY, Chung MK. Spontaneous and Bite-Evoked Muscle Pain Are Mediated by a Common Nociceptive Pathway With Differential Contribution by TRPV1. *J Pain*. 2017;18(11):1333-45.
125. Wang S, Brigoli B, Lim J, Karley A, Chung MK. Roles of TRPV1 and TRPA1 in Spontaneous Pain from Inflamed Masseter Muscle. *Neuroscience*. 2018;384:290-9.
126. Guo W, Zou S, Mohammad Z, Wang S, Yang J, Li H, et al. Voluntary biting behavior as a functional measure of orofacial pain in mice. *Physiol Behav*. 2019;204:129-39.
127. Ludman T, Melemedjian OK. Bortezomib-induced aerobic glycolysis contributes to chemotherapy-induced painful peripheral neuropathy. *Mol Pain*. 2019;15:1744806919837429.
128. Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, et al. PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia. *J Neurosci*. 2002;22(11):4740-5.
129. Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron*. 2002;36(1):57-68.
130. Chung MK, Asgar J, Jennifer P, Ro JY. Transcriptome analysis of trigeminal ganglia following masseter muscle inflammation in rats. *Mol pain*. 2016.
131. Vandewauw I, De Clercq K, Mulier M, Held K, Pinto S, Van Ranst N, et al. A TRP channel trio mediates acute noxious heat sensing. *Nature*. 2018;555(7698):662-6.
132. Belmonte C, Acosta MC, Gallar J. Neural basis of sensation in intact and injured corneas. *Exp Eye Res*. 2004;78(3):513-25.
133. Rozsa AJ, Beuerman RW. Density and Organization of Free Nerve Endings in the Corneal Epithelium of the Rabbit. *Pain*. 1982;14:105-20.
134. Ferrari G, Hajrasouliha AR, Sadrai Z, Ueno H, Chauhan SK, Dana R. Nerves and neovessels inhibit each other in the cornea. *Invest Ophthalmol Vis Sci*. 2013;54(1):813-20.

135. Liu L, Dana R, Yin J. Sensory neurons directly promote angiogenesis in response to inflammation via substance P signaling. *FASEB J.* 2020;34(5):6229-43.
136. Li F, Yang W, Jiang H, Guo C, Huang AJW, Hu H, et al. TRPV1 activity and substance P release are required for corneal cold nociception. *Nat Commun.* 2019;10(1):5678.
137. Chiu IM, Heesters BA, Ghasemlou N, Von Hehn CA, Zhao F, Tran J, et al. Bacteria activate sensory neurons that modulate pain and inflammation. *Nature.* 2013;501(7465):52-7.
138. Chung MK, Lee J, Duraes G, Ro JY. Lipopolysaccharide-induced Pulpitis Up-regulates TRPV1 in Trigeminal Ganglia. *J Dent Res.* 2011;90(9):1103-7.
139. Patwardhan AM, Akopian AN, Ruparel NB, Diogenes A, Weintraub ST, Uhlson C, et al. Heat generates oxidized linoleic acid metabolites that activate TRPV1 and produce pain in rodents. *J Clin Invest.* 2010;120(5):1617-26.
140. Camprubi-Robles M, Planells-Cases R, Ferrer-Montiel A. Differential contribution of SNARE-dependent exocytosis to inflammatory potentiation of TRPV1 in nociceptors. *FASEB J.* 2009;23(11):3722-33.
141. Wu C, Gavva NR, Brennan TJ. Effect of AMG0347, a transient receptor potential type V1 receptor antagonist, and morphine on pain behavior after plantar incision. *Anesthesiology.* 2008;108(6):1100-8.
142. Okun A, DeFelice M, Eyde N, Ren J, Mercado R, King T, et al. Transient inflammation-induced ongoing pain is driven by TRPV1 sensitive afferents. *Mol Pain.* 2011;7:4.
143. Yamaguchi K, Ono K, Hitomi S, Ito M, Nodai T, Goto T, et al. Distinct TRPV1- and TRPA1-based mechanisms underlying enhancement of oral ulcerative mucositis-induced pain by 5-fluorouracil. *Pain.* 2016;157(5):1004-20.
144. Ghilardi JR, Rohrich H, Lindsay TH, Sevcik MA, Schwei MJ, Kubota K, et al. Selective blockade of the capsaicin receptor TRPV1 attenuates bone cancer pain. *J Neurosci.* 2005;25(12):3126-31.

145. Ni W, Li Y, Cai L, Dong C, Fang H, Chen Y, et al. SUMOylation is required for PIPK1gamma-driven keratinocyte migration and growth. *FEBS J.* 2019;286(23):4709-20.
146. Sun H, Lu L, Zuo Y, Wang Y, Jiao Y, Zeng WZ, et al. Kainate receptor activation induces glycine receptor endocytosis through PKC deSUMOylation. *Nat Commun.* 2014;5:4980.
147. Zhang H, Cang CL, Kawasaki Y, Liang LL, Zhang YQ, Ji RR, et al. Neurokinin-1 receptor enhances TRPV1 activity in primary sensory neurons via PKCepsilon: a novel pathway for heat hyperalgesia. *J Neurosci.* 2007;27(44):12067-77.
148. Gu Y, Li G, Huang LYM. Inflammation induces Epac-protein kinase C alpha and epsilon signaling in TRPV1-mediated hyperalgesia. *Pain.* 2018;159(11):2383-93.
149. Sweitzer SM, Wong SM, Tjolsen A, Allen CP, Mochly-Rosen D, Kendig JJ. Exaggerated nociceptive responses on morphine withdrawal: roles of protein kinase C epsilon and gamma. *Pain.* 2004;110(1-2):281-9.
150. Tian Q, Hu J, Xie C, Mei K, Pham C, Mo X, et al. Recovery from tachyphylaxis of TRPV1 coincides with recycling to the surface membrane. *Proc Natl Acad Sci U S A.* 2019;116(11):5170-5.
151. Devesa I, Ferrandiz-Huertas C, Mathivanan S, Wolf C, Lujan R, Changeux JP, et al. alphaCGRP is essential for algesic exocytotic mobilization of TRPV1 channels in peptidergic nociceptors. *Proc Natl Acad Sci U S A.* 2014;111(51):18345-50.