2020-06-26

Mechanisms Regulating Transient Receptor Potential Cation Channel A1 (TRPA1) and Their Roles in Nociception and Nociceptive Sensitization

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MECHANISMS REGULATING TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL A1 (TRPA1) AND THEIR ROLES IN NOCICEPTION AND NOCICEPTIVE SENSITIZATION

A Master's Thesis Presented

By

Ye Shang

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

June 26th, 2020

NEUROSCIENCE

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Program
NEUROSCIENCE

Month, Day and Year
June 26th, 2020

Acknowledgements

I would first like to thank Dr. Yang Xiang for his mentorship in all these years. He taught me how to do science, showed me the skills and determined spirit to tackle hard but important questions, discussed with me the appropriate controls for high-quality data, and supported and encouraged me when I met difficulties. I am fortunate enough to have joined his lab and worked on some exciting projects in these years.

I would also like to thank my lab members for their help and support during these years. Thanks to Pengyu for giving me critical reagents for my projects and discussing the problems I met. Thanks to Jiaxin for always providing new insights and useful suggestions for my projects. Thanks to Kendra for sharing her experience as a senior fellow student and offering help when I needed it. Thanks to Fei for being a super supportive lab member, sitting next to me and chatting together on science and life during these years in the lab.

I am especially grateful to my TRAC committee, present, and past, for their time and guidance. Dr. Patrick Emery has been a part of this journey from my rotation, qualifying exam to the final thesis writing, and for always lending words of support and encouragement. Dr. Mary Munson provided valuable suggestions on my projects, especially on the biochemistry part of them, which our lab knows

little about. Dr. Cole Haynes and Dr. William Kobertz offered guidance on my projects and experiment designs. Thanks to my thesis committee member Dr. Mary Ellen Lane for her time in thesis evaluation and for her effort to make GSBS such a friendly and supportive environment.

Finally, thanks to my family for their unconditional support throughout my years of study. They are my firmest backing during my adventure. Thanks to my colleagues in Neurobiology Department and my friends for being awesome.

Abstract

Nociception is the sensory nervous system that detects harmful stimuli including excessive heat, cold, toxic chemicals, and noxious mechanical stimulations. Transient receptor potential (TRP) channels are a group of evolutionarily conserved ion channels consisting of 4 subunits, each with 6 transmembrane spans, and detect a variety of external and internal nociceptive stimuli. Due to their critical roles in nociception, it is essential to understand the mechanisms that regulate TRP channels and subsequent nociception. Here, I investigated two distinct types of regulation of *Drosophila* transient receptor potential cation channel A1 (TrpA1): regulation via the expression of different TrpA1 isoforms, and via its binding with associated proteins. I found that one of the TrpA1 isoforms, TrpA1(E), inhibits the thermal responses of other TrpA1 isoforms *in vitro*. I also identified potential TrpA1 binding partners through Co-immunoprecipitation (Co-IP) and mass spectrometry analysis. These binding partners need further validation and characterization through biochemical, cellular, and behavioral assays to illustrate their roles in nociception, and may serve as potential drug targets for chronic pain.

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List of Symbols, Abbreviations or Nomenclature

TRP: transient receptor potential

TrpA1: transient receptor potential cation channel A1

DRG: dorsal root ganglia

GWAS: whole-genome association study

NGF: nerve growth factor

PIP_2: phosphatidylinositol-4,5-biphosphate

PLC: phospholipase C

Co-IP: Co-immunoprecipitation

IP: immunoprecipitation

CGRP: calcitonin gene-related peptide,

TAC: TRP ankyrin cap

Preface

Figure 2.3 in Chapter II was generated by Dr. Pengyu Gu and has been included in this thesis with permission.

TrpA1 isoform-specific knock-out flies and FLAG-tagged flies were generated by Dr.Pengyu Gu.

The work presented in Chapter III represents unpublished work

Chapter I

GENERAL INTRODUCTION

1.1 Nociception and its physiological basis

Animals sense and respond to harmful cues to protect themselves and to survive potential dangerous environments. During evolution, animals developed robust peripheral and central components in their nervous systems to fulfill this function. In mammals, a group of specialized peripheral neurons called nociceptors first detect harmful stimuli. These neurons innervate a large portion of the skin and internal organs to detect dangerous signals and send alarms efficiently. The cell bodies of these neurons are mostly clustered in the dorsal root ganglia (DRG), and they send axons to the spinal cord to relay alarm signals to the central nervous system to cause subjective pain (Berta et al., 2017). Several brain areas are activated in pain conditions, including the secondary somatosensory cortex, insula, midcingulate cortex, and thalamus (Xu et al., 2020). However, their roles in cognitive, emotional, and sensory components of pain are not clear yet (Xu et al., 2020).

In the peripheral nervous system, nociceptors need to first convert noxious stimuli, including heat, cold, chemical, or mechanic stimuli, into electrical signals. In the last two decades, researchers identified TRP channels as pivotal transducers of harmful stimuli (Julius, 2013). Mammalian TRPV1 was the first TRP channel to be related to nociception (Caterina et al., 1997). It is expressed in the heat-sensitive nociceptors, activated by the noxious heat temperature of

~43°C and by pungent chemical capsaicin, and transduces noxious stimuli into electrical signals (Caterina et al., 1997). Meanwhile, mice lacking TRPV1 showed behavioral defects in response to the heat plate and capsaicin (Caterina et al., 2000). Later TRPM8 was identified as a cold and menthol sensor (McKemy et al., 2002; Peier et al., 2002) and TRPA1 is a sensor for several noxious chemicals and reactive oxygen species (Bandell et al., 2004; Jordt et al., 2004; Sawada et al., 2008). The sensor for mechanical nociception is still elusive. DEG/ENaC channels, TRP channels, Piezo channels, and KCNK channels were proposed to be the candidates for mechanical nociception (Basbaum et al., 2009; Zhang et al., 2019). After the initial transition of the external stimuli into electrical signals, the signals need to be further amplified and transmitted in the nociceptor, which requires other channels, including voltage-gated sodium channels and voltage-gated calcium channels. Individuals with mutations in Nav1.7, a voltage-gated sodium channel, have a severe loss of pain perception (Goldberg et al., 2007).

1.2 Chronic pain, its mechanisms and the current treatments for it

In certain conditions, people can suffer from maladaptive nociception. For example, chronic pain, which usually means pain persisting for more than three months, is a common problem (Crofford, 2015). About 1 in 5 Americans had or are experiencing chronic pain (Crofford, 2015). Depending on the cause of chronic pain, it can be classified into four different types--nociceptive pain, inflammatory pain, neuropathic pain and dysfunctional pain (Woolf, 2020).

Normal nociceptive pain can last for a long time if noxious stimuli are continually present, for instance, in the case of a damaged joint where joint movements produce excessive pressure every time and activate nociceptors. Inflammatory pain occurs when there are tissue injury and active inflammation. Immune cells, nociceptors, and damaged cells release various factors, collectively called the inflammatory soup, and those factors act on the nociceptors to make them more sensitized towards stimuli. Neuropathic pain is caused by damage or disease in the sensory nervous system, such as nerve trauma, stroke, or diabetic- or chemotherapy-induced neuropathy. Patients with this condition often experience spontaneous pain without an identifiable external stimulus. Dysfunctional pain is pain in the absence of detectable pathology. It may result from psychosomatic factors or plastic changes in the central nervous system (Woolf, 2020).

Nervous system changes induced by injury or damage mentioned above can happen in both peripheral and central regions, representing two main mechanisms for chronic pain--peripheral sensitization and central sensitization. In peripheral sensitization, ion channels in nociceptors are often the molecular targets of modulation. For example, nerve growth factor (NGF) is released in the inflammatory site, and it increases the expression level of TRPV1, causing the nociceptors to become more sensitive towards pain (Ji et al., 2002). In neuropathic pain, the downregulation of potassium channels and the upregulation of sodium channels change the membrane potential and electrical properties of nociceptors, which contributes to the spontaneous firing of

nociceptors and hence, spontaneous pain (Costigan et al., 2010; Costigan et al., 2009; Tulleuda et al., 2011). Central sensitization can happen after a direct injury in the central nervous system, or after a prolonged exposure to pain which leads to central circuit changes and is regarded as secondary effects in chronic pain. Several mechanisms were proposed to explain central sensitization, such as activation of glutamate/NMDA receptor and synapse potentiation, spinal inhibitory interneuron degeneration, and recruitments of central immune cells (Basbaum et al., 2009; Vardeh et al., 2016).

Although the understanding of nociception and nociceptive sensitization advanced tremendously in the last two decades, the application of this knowledge in clinical practice was not very successful. Most analgesic drugs were found in a serendipitous way, usually long before the drug target was identified (Woolf, 2020). Controversial opioids are known for pain-relieving effects and right now are still frequently used to treat moderate and severe pain, though their usage brings a lot of other problems, including drug addiction, tolerance and overdose. Anti-inflammatory drugs such as aspirin or ibuprofen are also often prescribed as painkillers, which most likely function through inhibiting factors in the inflammatory soup. Recently new drug targets and specific treatments have emerged for different pain syndromes. One successful translational example is the remarkable pain-preventing effect of recently approved migraine drugs targeting calcitonin gene-related peptide (CGRP), a neurotransmitter released during migraine attacks (Goadsby and Edvinsson, 1993). Several clinical trials

are also evaluating other targets such as NGF and its receptors, sodium channels, and TRP channels (Vardeh et al., 2016; Woolf, 2020). We argue that we need to investigate these existing targets in detail and gain deep understanding of them, which might improve our successful rate of translational attempts. We also need to identify and characterize more novel therapeutic targets in nociception and nociceptive sensitization process for future drug developments.

1.3 TRP channel modulation in pathological pain

As mentioned above, TRP channels function as pivotal transducers in nociception. During pathological conditions, the expression, trafficking, subcellular distribution, ubiquitination, as well as functional regulation of TRP channels could be modified, which contributes to pathological pain states (Patapoutian et al., 2009). In inflammation, released NGF boosts TRPV1 expression on nociceptors (Ji et al., 2002). Kinases such as PKA and PKC, which are often activated during inflammation, were shown to phosphorylate TRP channels, leading to channel functional sensitization or subcellular translocation to the plasma membrane (Bhave et al., 2003; Schmidt et al., 2009; Wang et al., 2008). The lipid environment around TRP channels also affects channel sensitivity. Activation of phospholipase C (PLC) changes the concentration of phosphatidylinositol-4,5-biphosphate (PIP_2) and subsequently modulates TRP channel activity (Nilius et al., 2008). In neuropathic pain, TRPV1 expression is

increased and TRPV1 antagonists reverse pain sensitization caused by nerve injury (Kanai et al., 2005; Urano et al., 2012; Vilceanu et al., 2010).

Although many studies tried to find regulatory mechanisms of TRP channels during pain, data are sometimes contradictory due to the difference of pain models, mice strains, and stimulus types (Hatcher and Chessell, 2006; Lukacs et al., 2007). Besides, evidence for the *in vivo* roles of these regulations is limited (Nilius et al., 2008; Schmidt et al., 2009).

1.4 Animal models for chronic pain study

In order to investigate mechanisms of chronic pain, researchers developed various animal models with different advantages and limitations. Since pain is a subjective feeling, the report from patients on a questionnaire is the most straightforward way to evaluate pain. Combined with GWAS or family lineage study, it provided valuable insights into pain-relevant genes (Goldberg et al., 2007; Kremeyer et al., 2010; Mahajan et al., 2018). It used to be hard to perform further mechanistic studies in humans on those pain-relevant genes. Recently, however, researchers developed iPSC-derived nociceptors from chronic pain patients, which overcame some of these hindrances and revealed mechanisms for pain-causing $Na_V1.7$ mutations (McDermott et al., 2019; Mis et al., 2019).

Pain studies in mice and rodents provided a foundation for our understanding of pain. Conventional methods to induce pathological pain in these animals include nerve injuries, irritative chemical injections, transgenic mice with gene

mutation causing pain or disease, and feeding of pharmacological agents (Bravo et al., 2020). The most frequently used method to evaluate pain response in these models is measuring reflex withdrawal thresholds induced by thermal or mechanical stimuli (Bravo et al., 2020). While studies in these pain models produced fruitful insights, recent failures in translating preclinical findings in rodent models into positive clinical outcomes prompted researchers to examine these models more carefully (Hill, 2000; Huggins et al., 2012; Yekkirala et al., 2017). The reflex withdrawal response might have little correspondence to the clinical spontaneous pain and constant discomfort. Therefore, assays evaluating affective and cognitive components of pain were proposed, including open field test or burrowing behaviors (Bravo et al., 2020; Calvo et al., 2019). Models to mimic and phenocopy chronic widespread pain such as low back pain are also urgently needed. Alternatively, researchers have begun to test non-human primates as pain models, arguing that it will produce results more translational to humans (Hama et al., 2018).

Although it is difficult to assign the subjective feeling of pain to invertebrate animals, researchers still use them to study nociception or nociceptive sensitization. For example, conducting a genetic screen is much easier in invertebrates than in mice or rodents, and it provides important insights into mammalian pain studies. *Drosophila* is an excellent example to highlight the importance of invertebrate pain models. Fly larvae were firstly used to screen the genes that are important to nociception (Tracey et al., 2003). Later, a large-scale

screen on adult fruit flies identified a set of genes that affect thermal nociception. Among them, the calcium channel subunit α2δ3 was found to have conserved nociceptive function from flies to mice and humans (Neely et al., 2010). To study nociceptive sensitization, an UVC-injured fly larvae model was developed, which shows hypersensitivity towards thermal stimuli and shares some key signaling pathways with mammal pain sensitization, including tumor necrosis factor α and substance P (Babcock et al., 2009; Im et al., 2015). A leg removal model in adult flies was developed to mimic neuropathic pain. In this model the peripheral injury induced GABAergic cell death in the central nervous system, while blocking the GABAergic cell death alleviated the nociceptive sensitization (Khuong et al., 2019).

1.5 Thesis overview

Although evidence indicates that TRP channels and their modulation are essential to pain sensitization, the *in vivo* mechanisms through which they are involved are still controversial (Julius, 2013; Patapoutian et al., 2009). We used *Drosophila* as a model organism to study nociception and nociceptive sensitization. With its powerful genetic tools, we can dissect the detailed mechanisms on the molecular, cellular and behavioral levels. Contrary to its homologs in mammals, *Drosophila* TRPV subfamily is not involved in nociception (Fowler and Montell, 2013). TrpA1 is thought to be the critical nociceptive TRP channel in *Drosophila* (Im et al., 2015; Viswanath et al., 2003), which I chose as

the main subject in this thesis. The first part of this thesis tested the effect of the interaction between TrpA1 isoforms, a topic rarely touched in previous studies. The *in vivo* significance of TrpA1 isoform expression and interaction is also explored. The second part of this thesis aimed to identify the *in vivo* TrpA1 interactome through Co-IP and mass spectrometry analysis, using an unbiased method to reveal novel molecules in nociception and nociceptive sensitization. Those molecules could provide hints for mechanisms through which TrpA1 is involved in these processes, and may serve as potential therapeutic targets for pain treatment.

Chapter II

The roles of TrpA1 isoforms in nociception and nociceptive sensitization

2.1 Abstract

Proteins are regulated in different ways during their synthesis in order to fulfill their functions. While many researchers focused on expression regulation and post-translational modifications of TRP channels, the effect of their isoform regulation on nociception or nociceptive sensitization is rarely known. *Drosophila* TrpA1 has five different isoforms. Here I expressed the five isoforms in HEK 293 cells and tested their thermal responses through calcium imaging. Contrary to a previous study, one isoform, which was thought to be heat-insensitive, showed thermal response in our hands. In addition, since several isoforms are co-expressed in larval nociceptors called C4da neurons that innervate larval skins, I also co-transfected isoforms in HEK 293 cells and tested the effect of isoform interaction. I found that one isoform has a dominant-negative impact on the thermal responses of other isoforms. Although the following behavioral studies suggested that this isoform is not involved in the nociception or UVC-induced nociceptive sensitization models *in vivo*, it provided insights into how a channel could be functionally regulated by its isoform expression and interaction.

2.2 Introduction

Protein isoforms generated by alternative splicing of pre-mRNA are considered as an important source for their functional diversity. TRP channels also utilize this mechanism to detect various noxious stimuli. A study on vampire bats showed that this animal expresses a low-temperature threshold TRPV1 isoform in heat-sensitive neurons that innervate a specialized pit organ around the nose and detect infrared signals (Gracheva et al., 2011). Similarly, a short and truncated TrpM8 isoform is expressed specifically in mammalian keratinocytes and regulates proliferation and differentiation of keratinocytes when it is activated by mild cold (Bidaux et al., 2015). Mouse TRPA1 also generates a short isoform which modulates the activity of full length TRPA1 isoform by upregulating its membrane expression (Zhou et al., 2013). Many other splice variants of mammalian TRP channels were identified, but not functionally tested (Ramsey et al., 2006; Vázquez and Valverde, 2006). *Drosophila* TrpA1 has five different isoforms that were named as TrpA1(A)-(E). These isoforms are generated through alternative promoter usage and alternative splicing (**Figure 2.1A**). Previous studies showed that these isoforms have different expression patterns and respond differently to noxious stimuli (Guntur et al., 2015; Kang et al., 2011; Zhong et al., 2012). One puzzle left from the previous studies is that while the TrpA1(C) isoform was shown to be heat-insensitive, the overexpression of this isoform in larval nociceptors rescued thermal behavioral defects in the TrpA1 mutant (Zhong et al., 2012). This leads to the question of whether TrpA1 is the direct heat sensor in nociceptors (Fowler and Montell, 2013). Besides, the

role of TrpA1 isoforms in nociceptive sensitization is not well understood. Our lab generated a series of TrpA1 isoform-specific knock-in lines (Gu et al., 2019), which puts us in a unique position to answer these questions.

2.3 Results

Thermal response profiling of TrpA1 isoforms in a heterologous system

To test the function of TrpA1 isoforms, we first cloned TrpA1 cDNAs from w^{1118} wild-type flies. All five isoforms were retrieved from our RT-PCR experiments and were sequenced to confirm that they do not contain any mutations compared to the sequences in Flybase (data not shown). I transiently transfected the different TrpA1 isoforms and GCaMP6s, a genetically encoded calcium indicator, into HEK 293 cells. After 24 hours of transfection, the glasses with the cells were placed onto a silicone chamber and heated external saline solution was perfused through the chamber (**Figure 2.1B**). When the channel is activated by heat, calcium ion will pass through TrpA1 from the external saline solution to the cytosol and bind to GCaMP6s which releases fluorescent signals and is recorded by a confocal microscopy (**Figure 2.1B**). I found that four TrpA1 isoforms, TrpA1(A)-(D), are activated by a temperature increase (**Figure 2.1C-E**). Interestingly, I found that the TrpA1(C) isoform is also heat sensitive, compared to control vectors or non-responsive TrpA1(E). This may explain why, when overexpressed in nociceptors, it rescued the thermal defect in TrpA1 mutant flies (Zhong et al., 2012). The TrpA1(B) isoform, which had never been tested before,

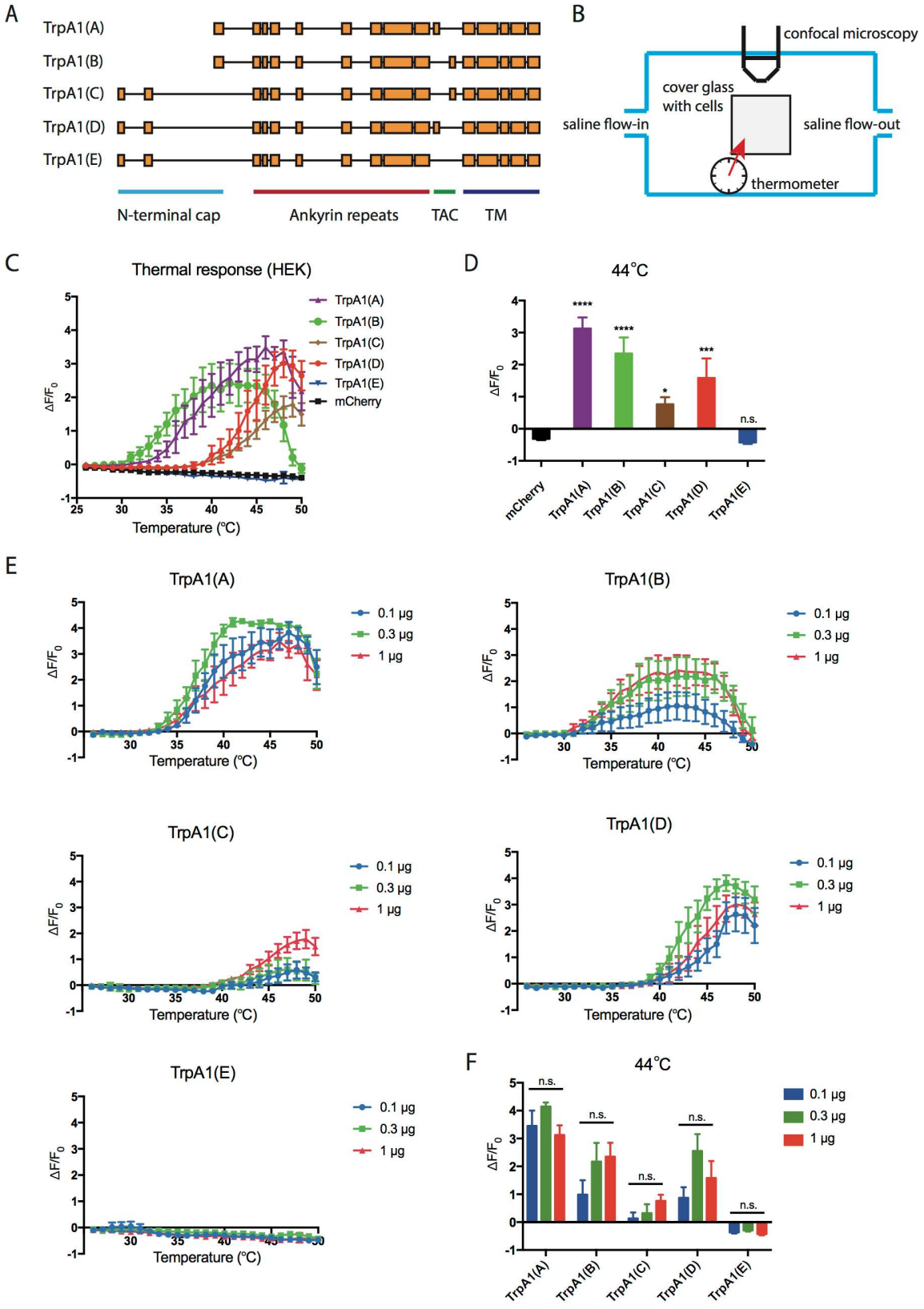
A
TrpA1(A)
TrpA1(B)
TrpA1(C)
TrpA1(D)
TrpA1(E)
N-terminal cap
Ankyrin repeats
TAC
TM
B
confocal microscopy
cover glass
with cells
saline flow-in
saline flow-out
thermometer
C
Thermal response (HEK)
TrpA1(A)
TrpA1(B)
TrpA1(C)
TrpA1(D)
TrpA1(E)
mCherry
ΔF/F0
Temperature (°C)
D
44°C
****
****
*
***
n.s.
mCherry
TrpA1(A)
TrpA1(B)
TrpA1(C)
TrpA1(D)
TrpA1(E)
E
TrpA1(A)
TrpA1(B)
TrpA1(C)
TrpA1(D)
TrpA1(E)
0.1 µg
0.3 µg
1 µg
F
44°C
n.s.

Figure 2.1 TrpA1 isoform thermal responses in HEK 293 cells.

A, The schematic of five TrpA1 isoforms. Orange boxes are exons and black lines are introns in the genomic locus. Domains corresponding to the TrpA1 exons were labelled by color lines. TAC, TRP ankyrin cap; TM, transmembrane domain. **B,** Custom-made chambers for calcium imaging of HEK 293 cells. HEK 293 cells were grown on the cover glasses and transiently transfected with TrpA1 plasmids. Heated saline flowed through the chamber, and the temperature and fluorescent signals were simultaneously recorded by the thermometer and confocal microscopy respectively. **C,** Calcium imaging of TrpA1 isoform activity induced by perfusion of hot saline solution. The transfection amount was 1μg for each isoform's plasmid. GCaMP6s background signals were affected by the increasing temperature, showing a dropping baseline in the control group. **D,** The responses of the five TRPA1 isoforms at 44ºC, which was the temperature used in our nociception behavioral assay. Different isoforms were compared to the mCherry control group (n>6). One-way ANOVA with Bonferroni's multiple comparisons test. n.s, *,***, **** represent $p > 0.05$, $p < 0.05$, $p < 0.001$, $p < 0.0001$. **E,** The responses of the TrpA1 isoforms with different transfection amounts. **F,** The responses of the TrpA1 isoforms with different transfection amounts at 44ºC (n>6). One-way ANOVA with Bonferroni's multiple comparisons test. No significant difference was detected among different transfection amounts.

is also highly sensitive to a mild temperature increase (**Figure 2.1C**). To test whether the expression level affects the thermal responses, I varied the amount of plasmid transfected. The overall channel activity patterns remained mostly unchanged (**Figure 2.1E**). Calcium signal amplitudes were not correlated with plasmid quantities, mainly due to a narrow dynamic range of the calcium imaging (maximal ΔF/F around 4) (**Figure 2.1F**). I also tested the responses of the five TRPA1 isoforms in *Drosophila* S2 cells, observing similar thermal responses (data not shown). Lastly, we also used whole-cell patch clamp to more precisely examine ion channel activation parameters for each isoform, including activation temperature threshold and temperature coefficient Q10 that evaluates channels' activity dependency on temperature (Gu et al., 2019).

TrpA1(E) dominant negatively regulates the thermal responses of other isoforms in a heterologous expression system

We generated TrpA1 isoform-specific reporter lines which showed the expression pattern of each isoform (Gu et al., 2019). We found that TrpA1(C), TrpA1(D), TrpA1(E) are co-expressed in fly larval C4da nociceptors. One question that arose from this observation is whether these isoforms interact with each other in the nociceptor to modulate overall channel activity. We were particularly interested in the TrpA1(E) isoform since it was not investigated in previous studies and did not seem to respond to classic TrpA1 specific stimuli. We wondered whether it would play a regulatory role in the nociceptors. To test this possibility, I transfected TrpA1(E) together with TrpA1(C) or TrpA1(D).

Interestingly, I found that TrpA1(E) inhibits the thermal responses of the other two isoforms (**Figure 2.2A-D**). This inhibition effect is enhanced when the ratio of TrpA1(E) to TrpA1(D) is increased (**Figure 2.2C-D**). These data suggested that TrpA1(E) have an inhibitory regulatory effect.

TrpA1(E) is not involved in nociceptive rolling behavior or UVC-induced nociceptive sensitization.

To test whether the inhibitory effect of TrpA1(E) plays a role in regulating animal nociception and nociceptive sensitization, we used two established behavioral assays. Fly larvae show a typical rolling behavior when stimulated with a heat probe (Tracey et al., 2003), which can be used to evaluate thermal nociception. Also, when they are injured by UVC light, their rolling behavior become more sensitive, and they respond to the heat probe at a lower temperature (Babcock et al., 2009), which can be used to evaluate nociceptive sensitization. We tested the behavioral assays of TrpA1(E) isoform-specific knock-out larvae, with only TrpA1(E) in a wrong reading frame while the four other isoforms are in the correct reading frame (Gu et al., 2019). We found that TprA1(E) specific knock-out did not affect larval nociception or nociceptive sensitization (**Figure 2.3A-B**). As a positive control, TrpA1 whole-gene knock-out larvae showed severe defects in these two behavioral assays (**Figure 2.3A-B**). This suggested that the inhibitory effect of the TrpA1(E) isoform does not participate in the regulatory process of nociceptive rolling behavior or UVC-injured nociceptive sensitization.

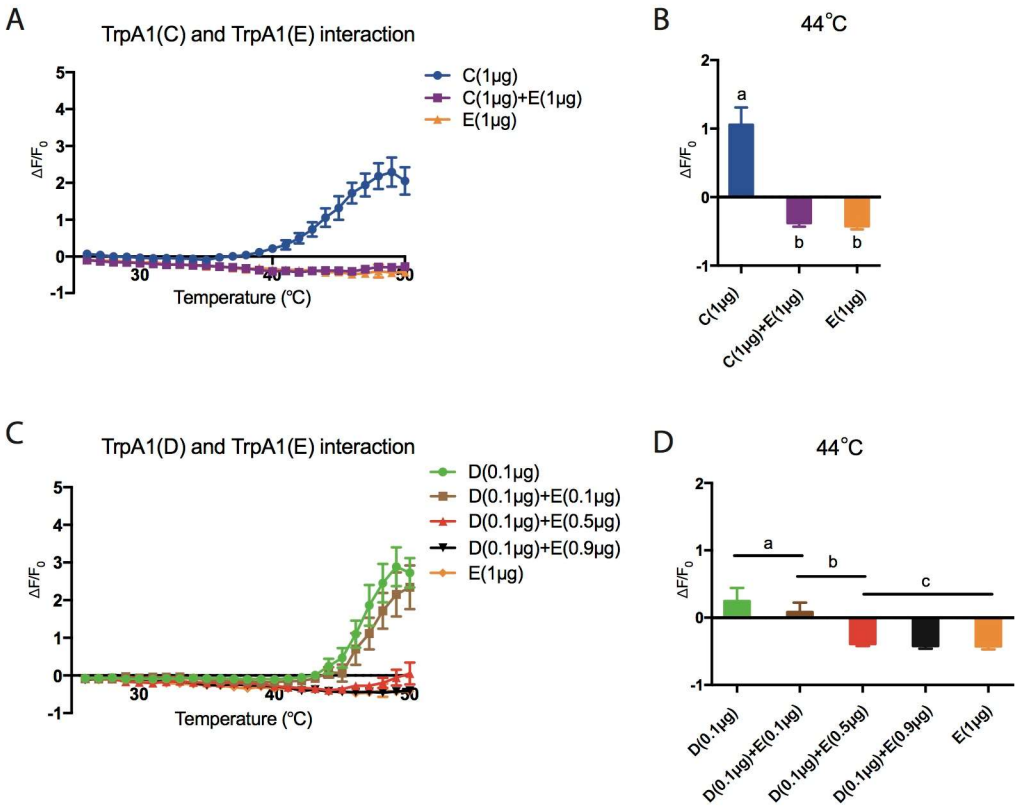

Figure 2.2 TrpA1(E) has a dominant-negative impact on the thermal responses of TrpA1(C) and TrpA1(D)

A-B, The thermal response resulting from TrpA1(C) and TrpA1(E) co-expression in HEK 293. Calcium imaging was recorded (**A**) and statistical analysis was performed at 44ºC (**B**). **C-D,** The thermal response resulting from TrpA1(D) and TrpA1(E) co-expression. One-way ANOVA with Bonferroni's multiple comparisons test (B,D). Bars with different superscript (a, b, and c) are significantly different from each other ($p < 0.05$)

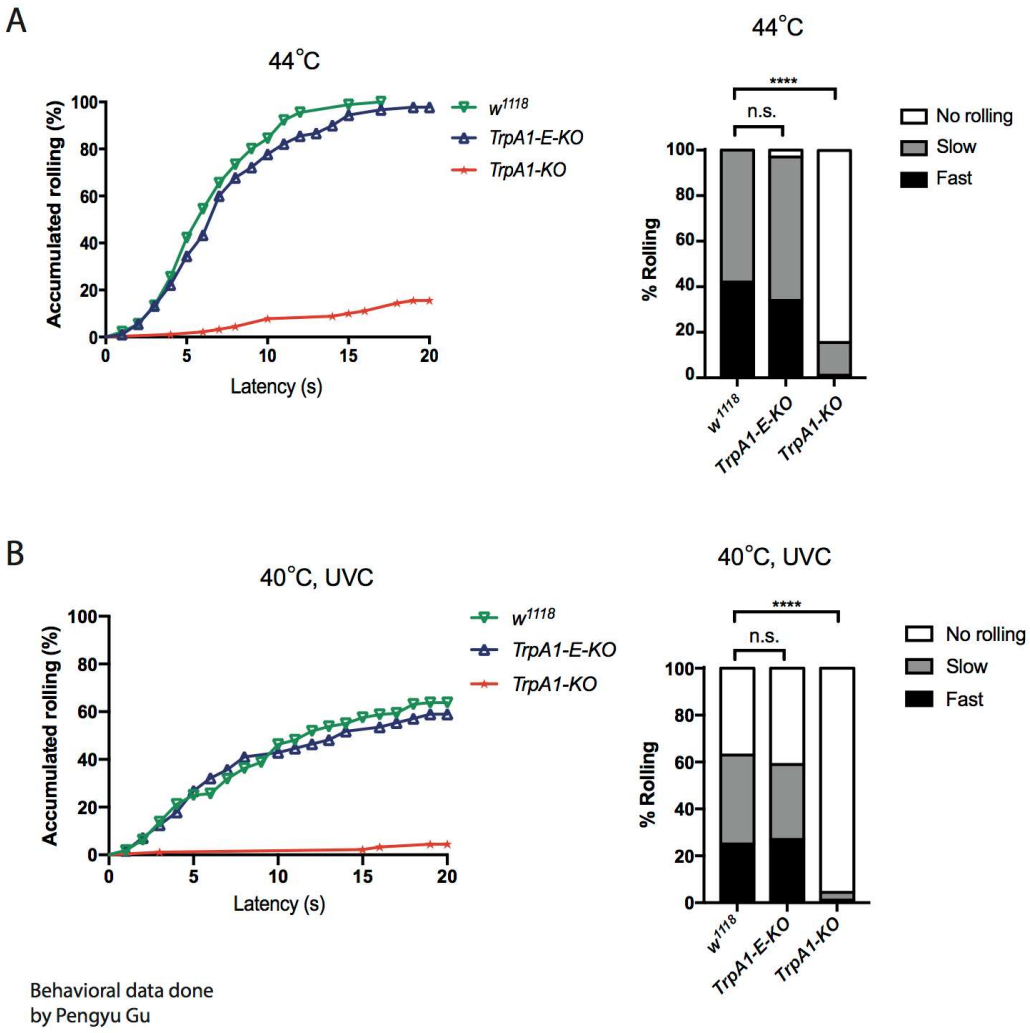

Figure 2.3 TrpA1(E) does not regulate nociceptive rolling behavior and UVC-induced nociceptive sensitization

A-B, Rolling behavior responses of larvae to heat stimulation at 44ºC (**A**), or at 40ºC after UVC injury (**B**). Results were displayed in both ‘‘non-categorial’’ accumulated curve and ‘‘categorial’’ bar graphs. Accumulated curve showed percentage of larvae exhibiting rolling behavior. ‘‘Categorial’’ bar graphs divided rolling behavior into three categories, fast (≤5 s), slow (>5 but ≤20 s), and no

rolling (>20 s), and the numbers of larvae in each category were counted. At least 60 larvae were tested for each group. Log-rank test was performed to compare the datasets displayed in accumulated response curves. For clarity, results of statistical analysis were actually marked in the accompanying bar graphs. n.s., no significant difference. **** $p < 0.0001$.

2.4 Discussion

Many proteins including TRP channels undergo alternative splicing. The effect of expressing multiple TRP isoforms on channel functionality is not clear, and the *in vivo* effect of isoform expression on nociception is also mostly unknown. In this chapter, we tested TrpA1 isoform function in an *in vitro* cell system and tested the TrpA1(E) isoform's regulatory roles in both cells and *in vivo* animal models. Interestingly, we found that TrpA1(C), which was thought to be heat-insensitive, actually responds to the temperature increase. One possible reason for the discrepancy between our results and the previous data could be that the plasmids we used are different. We sequenced the plasmids used in the previous publication (Zhong et al., 2012), and found that compared to the fly genome in the database, the plasmid of TrpA1(C) that the authors used contains two mutations (E699Q, and A709T). These two mutations are located near the TAC domain that connects the cytoplasmic ankyrin repeats and the transmembrane helixes (**Figure 2.1A**). The TrpA1(E) isoform, which lacks the TAC domain compared to TrpA1(C) or TrpA1(D), also lacks heat sensitivity, suggesting an indispensable role of this domain in heat sensation. Therefore, these two mutations near the TAC domain might comprise the TrpA1(C) thermal response and cause the data discrepancy. Retesting the thermal response of TrpA1 containing these two mutations in our settings will determine whether these mutations indeed affect channel functionality. Differences in the rate of temperature increase, which has been shown to affect TrpA1 thermal responses

(Luo et al., 2017), could also explain the different outcomes in our and previous studies.

Meanwhile, the mechanisms for the thermal activation of TRP channels remain largely unknown. Previous research proposed that a PIP_2 interaction domain in the C-terminal domain of TRPV1 regulates its thermal response (Prescott and Julius, 2003). Here we determined the distinct thermal profiles of five TrpA1 isoforms. A detailed structural analysis of these isoforms before and after thermal activation would provide insights into the biophysical basis of the channel's thermal sensitivity.

Another interesting finding from our *in vitro* cell assays is that when we co-expressed the TrpA1-C/D isoforms together with TrpA1(E), the latter dominant negatively inhibited the other isoforms' thermal responses. This observation suggested that isoform regulation could be a source of channel functionality modulation. Most mammalian TRP channels have splice variants, and some isoforms were also proposed to have dominant negative effects, suggesting that the phenomenon we observed could be conserved in mammals (Vázquez and Valverde, 2006). Future mechanistic studies could determine whether different isoforms form homo- or heteromeric TRP channels, and whether the expression of TrpA1(E) affects the biophysical properties or the membrane expression of the channels. Moreover, *in vivo* evidence for a dominant-negative effect of specific TRP channel isoforms is lacking. By utilizing the powerful genetic tools in *Drosophila*, we generated TrpA1(E) isoform-specific knock-out flies and

attempted to answer this question. Surprisingly, TrpA1(E) inhibitory regulation did not appear to affect sensitivity or sensitization in the behavioral assays we established in the lab. This could be due to the low expression of the TrpA1(E) isoform in larval nociceptors (Gu et al., 2019). TrpA1(E) is also expressed in the central nervous system (Gu et al., 2019). Behavioral assays that involved TrpA1 positive cells in the central nervous system could be used to test the function of TrpA1(E) (Rosenzweig et al., 2005). The differences between the *in vitro* assays and *in vivo* knock-out experiments also indicated the importance of testing TRP channel regulatory mechanisms in animal models. Although TrpA1(E) specific knock-out did not have any effect in our behavioral assays, TrpA1(E) overexpression in C4da nociceptors indeed inhibited nociceptive rolling behavior *in vivo* (Gu et al., 2019), consistent with the *in vitro* data. One way to further investigate TrpA1(E) function is to find situations in which TrpA1(E) is modulated, and to investigate the role of TrpA1(E) in these situations. For example, TrpA1(E) expression in pathological pain models such as the leg removal model could be examined to check whether its expression gets reduced, and the impact of loss of TrpA1(E) determined. (Khuong et al., 2019). Another way is to screen for genes that specifically upregulate TrpA1(E) expression, and test the roles of these genes in nociception. This could lead to the identification of novel nociceptive genes.

TRP channels sense various noxious stimuli in the environment. Alternative isoforms and the interaction among isoforms provide multiple layers of

complexities for TRP channels to achieve and fine-tune their versatile detection abilities, and will deserve further attention (Vázquez and Valverde, 2006). The present study is an exciting exploration of TRP channel regulation through the generation of multiple isoforms, and of the isoform's *in vivo* function that had rarely been examined before.

2.5 Materials and methods

Generation of TrpA1 isoform expression plasmids

Full-length cDNAs of five *Drosophila* TrpA1 isoforms were cloned from the total RNA extracted from w^{1118} larvae by RT-PCR, using SuperScript III (Invitrogen) and PrimeStar GXL DNA polymerase (Takara). The translated amino acids corresponding to each of the five alternative isoforms were identical to the Flybase annotation. Each of the five TrpA1 cDNAs was then cloned into the vector pcDNA3.1(+). A Kozak consensus sequence (GCCACC) was introduced immediately before ATG translational start site. For the cloning of full-length cDNAs of TrpA1-A/B and TrpA1-C/D/E, primer sets TrpA1-AF/TrpA1-R and TrpA1-DF/TrpA1-R were used, respectively.

TrpA1-AF: gGAATTCgccaccATGACTTCGGGCGACAAGGAGACT.

TrpA1-DF: gGAATTCgccaccATGCCCAAGCTCTACAACGGAG

TrpA1-R: ataagaatGCGGCCGCctaCATGCTCTTATTGAAGCTCAGGG

HEK293 cell culture and transfection

HEK cells were grown on gelatin coated cover glasses in 3.5-cm culture dishes with DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and transfected with TrpA1 isoform plasmids as indicated in each assays, 1 μg of GCaMP6s plasmids and 5 μg of polyethylenimine. The total amount of transfected plasmids was equilibrated to be 2μg with empty vector plasmids.

Perfusion of heated solution and calcium imaging

The saline solution (pH 7.2) contained the following ingredients: 130 mM NaCl, 3 mM KCl, 0.6 mM $MgCl_2$, 1.2 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, and 1 mM $CaCl_2$. HEK cells in cover glasses were placed in a custom-made chamber. Additional saline solution went through a condenser with pre-heated water around the pipe and then mixed with the saline solution in the chamber. The temperature of the saline solution in the chamber rose from 25ºC to 50ºC in ~80 s. A temperature probe (IT- 24P) was positioned adjacent to recorded cells, and the temperature was measured by a Physitemp BAT-10 thermometer (Physitemp Instruments). Temperature values were recorded by Clampex simultaneously with calcium imaging. Calcium imaging data were collected by Zeiss LSM700 confocal system. The basal fluorescent intensities of transfected cells without heat stimuli were around 800 A.U.(arbitrary unit), while the maximal attainable value in the microscopy system was 4096 A.U. The maximal ΔF/F is thus around 4. The average peak response was calculated with Zeiss Zen

software. At least 7 cells in 1 cover glass were measured and each data point contains at least 6 biological repeats of cover glasses.

Generation of TrpA1 isoform-specific lines

Drosophila TrpA1 gene knockout procedure was performed as previously described (Huang et al., 2009; Huang et al., 2008; Zhou et al., 2012). Briefly, about four kilobases homolog arms of the *TrpA1* gene were cloned and inserted into P element-based transforming vector pGX-attP-WN. This targeting vector was inserted into the fly genome by a standard transgenic procedure to generate transgenic donor fly stocks.

Targeting crosses. A line with transgenic donor DNA on the 2nd chromosome was chosen for targeting cross to delete the entire *TrpA1* gene of ~10kb. In the targeting cross, 40 vials of crosses were set up. Each vial contained 20 virgin females of transgenic donor flies mated with 20 *6934-hid* males (BL#25680). Crosses were maintained at room temperature and flies were transferred to new vials every 24 h. Eggs in vials were maintained on 0.2 mg/mL G418-containing food at 25ºC, and were heat-shocked at both 48 h and 72 h after egg-laying. Heat shock was carried out at 38ºC for 90 min in a water bath.

Screening crosses. Ten virgin females from the targeting crosses were mated with ten *GAL477[w-] (w; GAL477[w-]; TM2/TM6b)* males in each vial. Flies were transferred to fresh vials every two days at 25ºC with a total of five transfers.

Preliminary targeting candidates from targeting crosses were screened based on eye color.

Mapping crosses. A single male candidate was crossed with double balancer virgin females *(w; sp/CyO,Wee-P; TM2/TM6b)*. Targeting candidates with white selection marker on the 3rd chromosome were selected for further genotyping. *TrpA1* full gene knockout flies were established and confirmed by genotyping and RT-PCR. To remove the white selection marker and vector backbone, these white+ lines were crossed with Cre recombinase line (BL#851). A single male progeny with white eyes was selected and further balanced to remove Cre. Next, we backcrossed these lines to change their X and 2nd chromosomes into w^{1118} background, to establish the final *TrpA1-KO* founder line.

The following primers were used in genotyping of *TrpA1-KO* founder line.

Mcm7-F: CAGGATCGTACAGCCATTC Mcm7-R: CTTAGACATCTCCAGCAGAC

mfr-F: ATCAAGCAGAGCAACTACAT mfr-R: CATTAGAAGACGACCAACATAG

TrpA1(5')-F: CGACTTCAAGCGACTCTTC TrpA1(5')-R:

CGATGACATTGCGGTACTG

TrpA1(3')-F: GATAATACTTGGCAACTTGGT TrpA1(3')-R:

TTAGCTTCGATGGAATGAG

To generate *TrpA1* isoform-specific knock-in or knockout flies, a full length *Drosophila TrpA1* genome fragment (-10 kb) was cloned from bacterial artificial chromosome (Pacman BAC Ch322-152I23, BACPAC Resources Center, Children's Hospital Oakland Research Institute) and was inserted into pGE-attB-

GMR vector (Huang et al., 2009). Wild-type *TrpA1* allele on pGE-attB-GMR vector was modified *in vitro*. DNA fragments, amplified by PCR with primers containing designated mutations, were merged together by In-Fusion HD cloning kit (Cat#638909, Takara). Vectors with modified *TrpA1* genome were injected into *TrpA1-KO* founder line embryos to insert at the native *TrpA1* locus, by phiC31 mediated attB-attP recombination. Integration events were scored by the white selection marker. These lines were then balanced to confirm that modified *TrpA1* was inserted on the 3rd chromosome. To remove unnecessary vector backbone sequences and selection markers, single male of these lines was crossed with flies bearing Cre recombinase strain (BL#851). A single male offspring with white eyes was selected and further balanced to remove Cre. Next, we backcrossed these lines to change their X and 2nd chromosomes into w^{1118} background, to establish final *TrpA1-isoform-KI/KO* alleles.

The following RT-PCR primers were used in detection of *TrpA1* alternative transcripts.

TrpA1-Ex1,2-F: GCCGGAACAGCAAGTATT

TrpA1-Ex3,4-F:GTGGACTATCTGGAGGCG

TrpA1-Ex4,5-R: TATCCTTCGCATTAA AGTCGC

Rpl-F: CGGATCGATATGCTAAGCTGT

Rpl-R: GCGCTTGTTCGATCCGTA

TrpA1-Ex10,11-F: GGTGGAC AAGGATGGGAACA

TrpA1-Ex14,15-R: TCAGCTGCTCCCATCCCA

Behavioral assays

Thermal nociception assay was performed as previously described (Babcock et al., 2009). A custom-made heat probe with a proportional–integral–derivative (PID) control unit was used to deliver heat stimulation. An early 3rd instar larva was rinsed with distilled water and stimulated along with the segment A4-6. One larva was only stimulated once and the positive response behavior was defined as at least one 360º roll. The response latency to each stimulus was recorded up to a 20 s cutoff. According to the responding time, the behavioral responses were divided into 3 categories, fast rolling (<=5 s), slow rolling (> 5 but <=20 s) and no rolling (> 20 s). The assay was conducted at room temperature under a dissection scope (Nikon SMZ800) and a light source (Fostec ACE).

UVC-induced nociceptive sensitization behavioral assay was performed as previously described (Babcock et al., 2009). Early 3rd instar larvae were rinsed with distilled water and lightly anesthetized with ether. Larvae were placed in a Petri dish, put inside a UVC crosslinker (254 nm, Spectorlinker XL-1000) and exposed to UVC radiation of 20 mJ/cm^2. UVC intensity (5 mW/cm2) was measured by a hand-held UV spectrophotometer (AccuMAX XS-254, Spectroline). After treatment, larvae were returned to fly food at 25ºC for 24h and then rolling assay was performed as thermal nociception assay.

Quantification and statistical analysis

Statistical analysis was performed using Graphpad Prism software. GCaMP6s imaging of HEK 293 cells were analyzed using one-way ANOVA with Bonferroni's multiple comparisons test. Latency of rolling behavior under stimulation were analyzed using the Log-rank (Mantel-Cox) test. Error bars are standard errors of the mean (SEM).

Chapter III

Proteomic analysis of TrpA1 to interrogate nociception

3.1 Abstract

Ion channels frequently bind to other proteins to form complexes and fulfill their functions. Understanding the molecular and cell biology of TRP channels' interactomes should prove important to develop next-generation mechanism-based analgesics. However, very little is known about the identity of *in vivo* TRP channel binding partners and how they regulate TRP channel physiology and subsequently nociception, partially due to the extremely low endogenous expression of TRP channels. To tackle this problem, we employed a CRISPR-based genome editing technique to knock-in FLAG-tagged TrpA1, followed by mass spectrometry-based interactome analysis in *Drosophila*. So far, we have successfully identified the interactome of TrpA1. Follow-up experiments at the molecular, cellular, and behavioral levels are needed to reveal the roles of these hits on nociception or sensitization.

3.2 Introduction

The function of TRP channels is not only determined by core channel proteins but is also regulated by accessory proteins including scaffolds, adaptors, and regulators. *Drosophila* transient receptor potential (trp), the founding member of TRP channels, interacts with INAD, a PDZ domain scaffold protein, and forms a macromolecular complex to achieve light sensation function (Shieh and Zhu,

1996; Tsunoda et al., 1997). Later, research in mammalian TRP channels revealed more protein-protein interactions that regulate TRP channel function. For example, an interactome analysis identified GABAB1 receptors as a TRPV1 binding partner, and its activation inhibited TRPV1-related pathological pain (Hanack et al., 2015). TRPV1 also interacts with other TRP channels including TRPA1 and forms assembly (Staruschenko et al., 2010; Weng et al., 2015). About 700 potential mammalian TRP channel interactions were summarized in a network database (http://www.trpchannel.org/), though many of the interactions lacked *in vivo* data or functional outcomes (Shin et al., 2012). Meanwhile, while TRPA1 is an important ion channel in nociception, little is known about its binding partners (Shin et al., 2012). Here I conducted an *in vivo* TrpA1 interactome study using Co-IP and mass spectrometry analysis.

3.3 Results

Detect endogenous TrpA1 from TrpA1-FLAG knock-in flies through western blots

TrpA1 is expressed at an extremely low level in flies, and the available anti-TrpA1 antibody is not good enough to detect or purify endogenous TrpA1 (Rosenzweig et al., 2005). In order to perform biochemical analysis of TrpA1, we generated FLAG-tagged TrpA1 knock-in lines (Gu et al., 2019). TrpA1-FLAG fly larvae showed normal rolling behavior compared to wild-type larvae, suggesting that the FLAG tag does not affect TrpA1 function (Gu et al., 2019). Even with the

well-developed FLAG tag and anti-FLAG antibodies, TrpA1 was still undetectable in western blot using larval skins containing C4da nociceptors (data not shown). After comparing the TrpA1 expression level in different fly tissues, I chose adult fly heads for the following biochemical analysis because fly heads have a relatively high expression of TrpA1 and it is easier to collect a large number of adult heads. To enrich TrpA1 in western blot samples, I used immunoprecipitation (IP) with anti-FLAG beads followed by western blot. With SDS denaturing gel, TrpA1 bands were observed at the size of ~120kD, which is around the estimated size of a TrpA1 monomer (**Figure 3.1A**). With native gel which preserves protein-protein interactions, a relatively homogeneous TrpA1 complex band at around 1100kD was observed (**Figure 3.1B**). According to the band intensities and homogeneity, the western blot analysis provided tools to evaluate and optimize the following Co-IP procedures in aspects of the enrichment efficiency and the complex stability.

Identify TrpA1 interactome through Co-IP and mass spectrometry analysis

The Co-IP and mass spectrometry procedure was briefly described (**Figure 3.2A**). TrpA1-FLAG, along with its binding proteins, were purified from solubilized membrane fractions prepared from adult fly heads. Then, the sample was run on the gel and sent to the mass spectrometry facility for protein identification. Two repeats were performed and the results were summarized in a volcano plot (**Figure 3.2B**) and **Table 3.1**. TrpA1 was only detected in the TrpA1-FLAG samples (32 and 14 peptides in two repeats, respectively), but not in wild-type

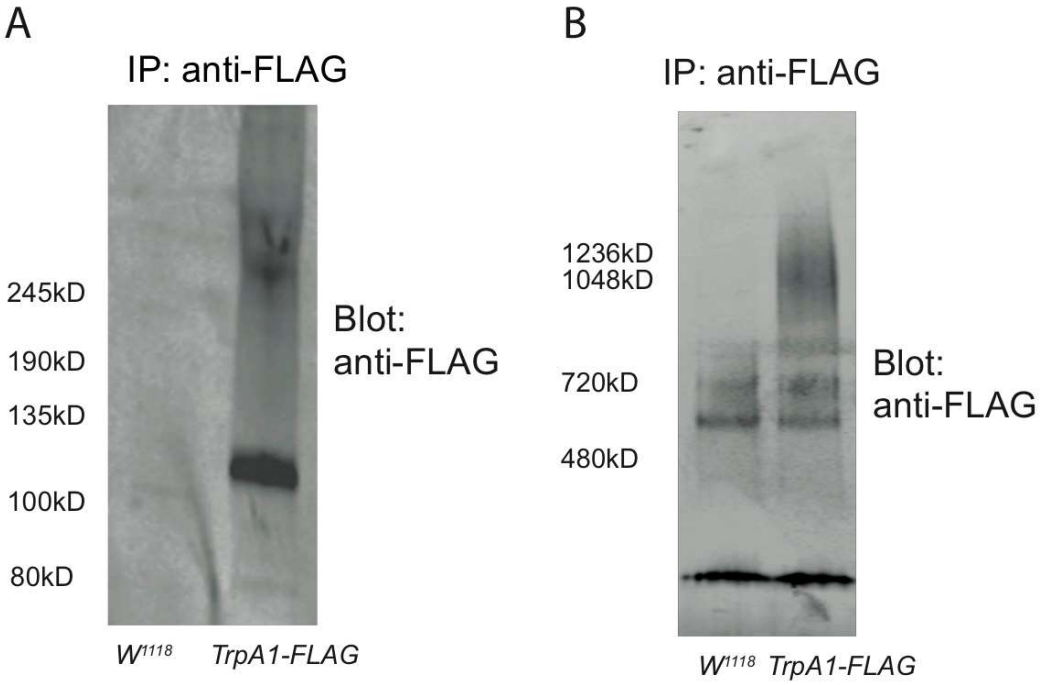

Figure 3.1 Western blots of TrpA1 monomer and TrpA1 complexes

A, Western blot of TrpA1 monomers after electrophoresis in SDS PAGE. *W1118* wild-type flies were used as a negative control. Co-IP was performed before western blot to enrich TrpA1 concentration in the samples. The identity of smearing bands higher than 245kD is unknown. The western blot was repeated three times. **B,** Western blot of TrpA1 complexes after electrophoresis in native PAGE. Similar conditions were used as in (A) except no boiling or denaturing reagents were used during sample preparation or electrophoresis. The western blot was repeated three times.

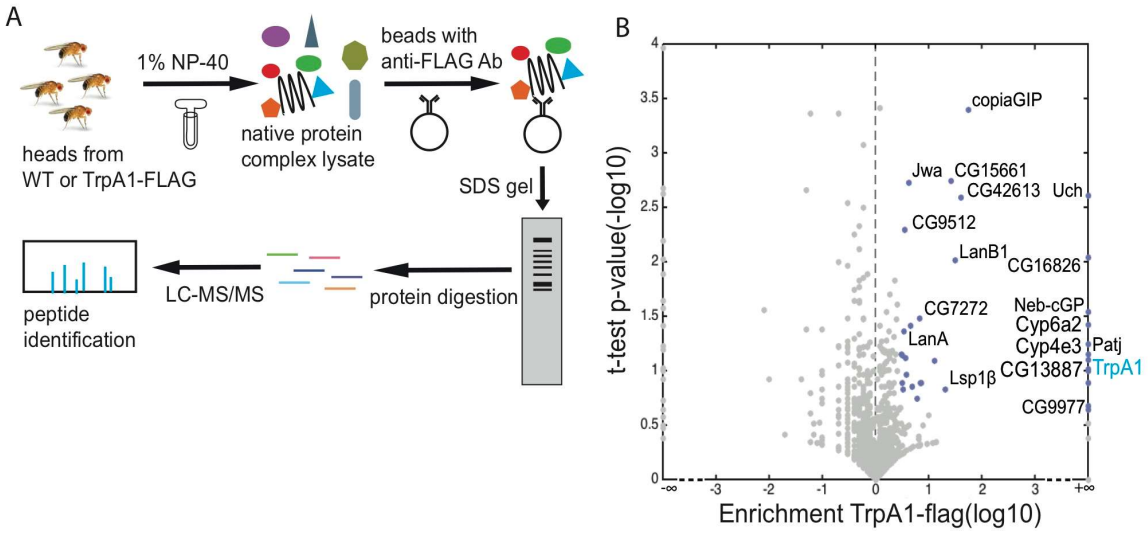

Figure 3.2 Proteomic analysis of TrpA1.

A, Schematic illustration of Co-IP and mass spectrometry process. Adult fly heads were homogenized, and membrane components were dissolved in 1% NP-40 lysis buffer. The lysate was incubated with beads conjugated with anti-FLAG antibody. Binding proteins to the beads were eluted and run on a short gel, which was cut and analyzed by mass spectrometry. **B,** *in vivo* binding proteins of TrpA1. Two independent biological replicates were performed. TrpA1 binding hits were labeled in the figure. Volcano plot showed enrichment of proteins in TrpA1-FLAG samples versus wild-type controls. TrpA1 was highlighted in cyan, which only exists in TrpA1-FLAG samples showing specificity. Binding hits were selected by (i) the enrichment folds for each protein, and (ii) the statistical significance comparing two replicate experiments. *t*-test with Benjamini-Hochberg correction.

Table 3.1 Summary of identified hits in TrpA1 complexes.

Gene name	Molecular weight	p-value	Enrichment fold	Mammalian homolog	Subcellular localization
TrpA1	135 kDa	0.098	INF	Y	membrane
CG16826	38 kDa	0.0092	INF	N	
Neb-cGP	5 kDa	0.029	INF	Y	mitochondrial membrane
Cyp6a2	59 kDa	0.038	INF	Y	ER or mitochondria membrane
Cyp4e3	61 kDa	0.057	INF	Y	ER or mitochondria membrane
Patj	93 kDa	0.08	INF	Y	plasma membrane
CG13887	26 kDa	0.1	INF	Y	membrane
Cyp309a1	57 kDa	0.1	INF	Y	ER or mitochondria membrane
AhcyL1	57 kDa	0.21	INF	Y	cytosol
CAH1	30 kDa	0.23	INF	Y	cytoplasm
copia	48 kDa	0.00039	56	N	cytosol
CG42613	93 kDa	0.0026	41	N	
LanB1	198 kDa	0.0099	32	Y	extracellular matrix
Ugt49C1	61 kDa	0.0018	27	Y	membrane-bounded organelle
Lsp1β	96 kDa	0.15	21	N	extracellular
CG5676	17 kDa	0.082	13	Y	mitochondrial outer membrane
Hcs	117 kDa	0.26	10	Y	polytene chromosome
Lspα	99 kDa	0.13	7.2	N	extracellular
PPO2	79 kDa	0.13	7.1	N	extracellular
CG7272	25 kDa	0.033	6.9	Y	membrane
Egm	71 kDa	0.18	6.2	Y	Extracellular

Cyp6a23	47 kDa	0.15	5	Y	ER or mitochondria membrane
Tomosyn	158 kDa	0.038	4.6	Y	membrane
Jwa	28 kDa	0.0019	4.2	Y	membrane
Aldh7A1	58 kDa	0.11	3.8	Y	
CG45002	110 kDa	0.076	3.7	Y	membrane
CG9512	69 kDa	0.005	3.6	Y	
LanA	411 kDa	0.044	3.5	Y	extracellular matrix
SPoCk	114 kDa	0.15	3.3	Y	membrane
CCT6	58 kDa	< 0.0001	3.3	Y	cytosol
MRP	173 kDa	0.13	3.2	Y	plasma membrane

flies (0 and 0 peptides, respectively), showing the specificity of the sample preparation (**Figure 3.2B**). Due to the limited number of replications and the considerate quantitative variances in the two replicates, *p*-values for TrpA1 (*p*=0.098) and many other identified proteins are high. Considering the high *p*-value of our bait protein TrpA1, we were concerned that some of the authentic TrpA1 binding partners might also have high a *p*-value. To avoid excluding these binding partners from our follow-up analysis, we set an arbitrary *p*-value of 0.3 and an enrichment fold of 3 as cutoffs for our hits. (**Figure 3.2B, Table 1**). The list retrieved different components in protein complexes, such as LanA, LanB1 in laminin complex and Lspα, Lsp1β in larval serum protein complex, suggesting that the conditions we used are mild enough to keep the complexes intact. Most hits are membrane proteins, which are presumably good candidates as TrpA1 binding partners (**Table 1**). Some hits are proteins located in the extracellular space, including LanA, LanB1, Lspα, and Lsp1β, which could interact with the extracellular part of TrpA1 and regulate its function. About 80% of hits have mammalian homologs, indicating that the interaction we found might be conserved in mammals.

3.4 Discussion

TRP channels play critical roles in nociception. However, it remains unsolved whether and how they function in a complex to participate in pain sensation. In this chapter, I provided an *in vivo* TrpA1 interactome analysis through Co-IP and

mass spectrometry. Compared with strategies using overexpressed tagged-proteins, our endogenous knock-in strategy was more likely to detect real and biologically relevant interactions. TrpA1 is expressed at a very low level. We first generated genetically tagged knock-in flies. Then we developed western blots for TrpA1 and used them as readouts to optimize the Co-IP and mass spectrometry procedures. This genetic-proteomic approach could be adopted to identify *in vivo* interactome of other low expression TRP channels. Western blot of TrpA1 could also be used as a tool to investigate other aspects of TrpA1 such as post-translational modification in *in vivo* conditions.

To further validate that the TrpA1 binding partners we found are authentic *in vivo* and are not artifacts caused by tissue homogenization, biochemical assays including co-immuonstaining of TrpA1 and binding proteins in fly tissues are needed. Binding domain analysis between two proteins is also needed to reveal the interaction mechanism of those proteins.

Certain proteins in our hits are known to form complexes, including LanA, LanB1 in laminin complex and Lspα, Lsp1β in larval serum protein complex. Laminin complex and larval serum protein complex are all components in the extracellular matrix. Laminins were found in all basement membranes which covers the basal side of most animal tissues including peripheral nerves (Urbano et al., 2009). The proximity between basement membranes and neurons makes it possible for laminins to interact with the extracellular part of TrpA1 and regulate its function. Laminins are conserved among different species. The interaction

between mammalian laminins and TRP channels deserves further investigation. Larval serum proteins are one of the most abundant serum proteins in fly larval hemolymph, and were regarded as storage proteins during fly development (Roberts et al., 1991). The detailed function of these proteins in nociception requires further examinations.

Since TrpA1 is essential for nociception, we expect that the hits we got could also affect nociception through regulating TrpA1. We will use mutants or RNAi to test the gene function through behavioral assays and nociceptor cellular response assays. If the protein affects nociception or cellular function of nociceptors, further mutation analysis on the binding domain of that protein could be performed to test whether the binding with TrpA1 is required in this process. To investigate the mechanisms of binding partners regulating TrpA1, we can co-express TrpA1 and binding proteins in cell systems *in vitro* to test whether the hits affect TrpA1 channel expression, trafficking or functionality. TRP channels get modulated by multiple signaling pathways, including growth factors such as NGF and inflammatory signals (Dai et al., 2007; Diogenes et al., 2007; Wang et al., 2008). The role of binding proteins in those signaling pathways could also be an interesting topic.

Chronic inflammatory pain is accompanied with orchestrated interaction between ion channels and their binding proteins (Liu et al., 2008; Tappe et al., 2006). Further investigation of the TrpA1 interactome could be performed in different pathological models, and the binding partners could be compared with

those in healthy condition. The differentially regulated hits could be critical molecules that are responsible for the channel functionality modulation and nociceptive sensitization.

One important goal of identifying TRP channel interactomes is to find novel therapeutic targets. Drugs directly targeting TRP channels seem to provide only limited benefits, mainly due to side effects (Kaneko and Szallasi, 2014). For example, due to the central role of TRPV1 in thermal sensation, TRPV1 antagonists cause hyperthermia, which is the main reason for their clinical failures (Brederson et al., 2013). Targeting specific protein interactions could potentially minimize these side effects. Our study provided clues for future TrpA1-interaction studies and drug developments.

3.5 Materials and methods

Generation of TrpA1-FLAG lines

Same procedures were performed as the generation of TrpA1 isoform-specific lines in Chapter II except that the *TrpA1* genome fragment in pGE-attB-GMR vector was modified to add the 3×FLAG tag to the TrpA1 C-terminal.
Following oligo nucleotide of 3×FLAG was added before stop codon:
cctagggactataaggaccacgacggagactacaaggatcatgatattgattacaaagacgatgacgataag

Co-IP of TrpA1

Pierce™ Co-Immunoprecipitation Kit (Thermo Fisher, 26149) was used for Co-IP and the protocol was modified from the manufacturer's manuals. About 20 Trays of large bottles of W^{1118} or TrpA1-FLAG adult flies were raised, collected in 50ml centrifuge tubes, frozen in -80ºC refrigerator overnight, and then vortexed. All of the following procedures were carried out at 4°C. Parts of bodies were separated with metal sieves (DUAL MFG. CO., US2.5-25S and US2.5-40S) to get heads. 6g heads were homogenized in 100ml PBS with 2 tablets of protease inhibitor (Sigma, 11697498001) using a 50ml glass homogenizer on ice. The lysate was centrifuged twice at 500×g for 5mins. Discard precipitation of nuclear fraction and cell debris after each centrifugation was done. The supernatant was then centrifuged in ultracentrifuges (Beckman Coulter Optima XL-100k, SW-28) at 28000rpm for 1.5h and the crude membrane fraction in the precipitation was collected. The membrane pellet was solubilized in 30ml lysis buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH7.4) with protease inhibitor in a 4ºC cold room for 0.5h, centrifuged at 10000rpm for 10mins and the supernatant was kept. Beads (Thermo Fisher, 26149) conjugated with anti-FLAG M2 antibody (Sigma, F1804) were prepared according to Co-IP kit manuals. 25µl beads conjugated with 75µg antibodies were added to the supernatant and were incubated in a 4ºC cold room for 6h. The beads were collected by centrifugation at 1000×g for 10mins, washed 2 times with 300µl lysis buffer, and incubated with elution buffer (30µl lysis buffer with 150ng/µl FLAG peptides (Sigma, F4799)) for 20mins.

Western blots with SDS PAGE or native PAGE

5µl elution sample from Co-IP was used for western blots. For denaturing SDS PAGE, protein samples were mixed with Laemmli sample buffer (6x) and β-mercaptoethanol, boiled at 95ºC for 5mins, and loaded in 4–20% Mini-Protean TGX precast SDS gel (Bio-Rad) in Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad). Blotting was performed according to LI-COR fluorescence western blot protocol. Immobilon-FL PVDF Membrane (Millipore, IPFL00010) was blocked with Odyssey Blocking Buffer (LI-COR, P/N 927-40000) and subsequently incubated with anti-FLAG M2 antibody (Sigma, F1804, 1:500) at 4°C overnight. Following three washing steps with PBS with 0.1% Triton, the membranes were incubated with IRDye® 800CW Goat anti-Mouse antibody (LI-COR, 925-32210 , 1:10000) for 2hr at RT. After incubation, membranes were washed and imaged on LI-COR ODYSSEY CLx. Native PAGE electrophoresis was performed according to NativePAGE™ Novex® Bis-Tris Gel System protocol, protein samples were mixed with NativePAGE™ Sample Buffer (4X) (Thermo Fisher, BN2003) and 0.1% NativePAGE™ G-250 Sample Additive (Thermo Fisher, BN2004), and loaded in 3-12% NativePAGE™ Novex® Bis-Tris Gel in XCell™ SureLock™ Mini-Cell (Thermo Fisher). NativeMark™ Unstained Protein Standard (Thermo Fisher, LC0725) was used as a marker. After electrophoresis, blotting was performed as in SDS-PAGE.

Mass spectrometry analysis and quantification

25µl elution sample from Co-IP was used for mass spectrometry. Samples were run in a SDS-PAGE gel as mentioned above, but as short gels which only run to 2cm below the bottom of the well. The short gels containing the proteins were then cut and sent to University of Massachusetts Medical School Mass Spectrometry Core for downstream LC/MS/MS analysis. The results were visualized and analyzed by Scaffold 4 (Proteome Software, Portland, OR). Normalized iBAQ was used to calculate enrichment fold. *t*-test with Benjamini-Hochberg correction was used to calculate *p*-value.

Bibliography

Babcock, D.T., Landry, C., and Galko, M.J. (2009). Cytokine signaling mediates UV-induced nociceptive sensitization in Drosophila larvae. Current biology : CB *19*, 799-806.

Bandell, M., Story, G.M., Hwang, S.W., Viswanath, V., Eid, S.R., Petrus, M.J., Earley, T.J., and Patapoutian, A. (2004). Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron *41*, 849-857.

Basbaum, A.I., Bautista, D.M., Scherrer, G., and Julius, D. (2009). Cellular and molecular mechanisms of pain. Cell *139*, 267-284.

Berta, T., Qadri, Y., Tan, P.H., and Ji, R.R. (2017). Targeting dorsal root ganglia and primary sensory neurons for the treatment of chronic pain. Expert opinion on therapeutic targets *21*, 695-703.

Bhave, G., Hu, H.J., Glauner, K.S., Zhu, W., Wang, H., Brasier, D.J., Oxford, G.S., and Gereau, R.W.t. (2003). Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proceedings of the National Academy of Sciences of the United States of America *100*, 12480-12485.

Bidaux, G., Borowiec, A.S., Gordienko, D., Beck, B., Shapovalov, G.G., Lemonnier, L., Flourakis, M., Vandenberghe, M., Slomianny, C., Dewailly, E.*, et al.* (2015). Epidermal TRPM8 channel isoform controls the balance between keratinocyte proliferation and differentiation in a cold-dependent manner. Proceedings of the National Academy of Sciences of the United States of America *112*, E3345-3354.

Bravo, L., Llorca-Torralba, M., Suárez-Pereira, I., and Berrocoso, E. (2020). Pain in neuropsychiatry: insights from animal models. Neurosci Biobehav Rev.

Brederson, J.D., Kym, P.R., and Szallasi, A. (2013). Targeting TRP channels for pain relief. European journal of pharmacology *716*, 61-76.

Calvo, M., Davies, A.J., Hébert, H.L., Weir, G.A., Chesler, E.J., Finnerup, N.B., Levitt, R.C., Smith, B.H., Neely, G.G., Costigan, M.*, et al.* (2019). The Genetics of Neuropathic Pain from Model Organisms to Clinical Application. Neuron *104*, 637-653.

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

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature *389*, 816-824.

Costigan, M., Belfer, I., Griffin, R.S., Dai, F., Barrett, L.B., Coppola, G., Wu, T., Kiselycznyk, C., Poddar, M., Lu, Y.*, et al.* (2010). Multiple chronic pain states are associated with a common amino acid-changing allele in KCNS1. Brain : a journal of neurology *133*, 2519-2527.

Costigan, M., Scholz, J., and Woolf, C.J. (2009). Neuropathic pain: a maladaptive response of the nervous system to damage. Annual review of neuroscience *32*, 1-32.

Crofford, L.J. (2015). Chronic Pain: Where the Body Meets the Brain. Transactions of the American Clinical and Climatological Association *126*, 167-183.

Dai, Y., Wang, S., Tominaga, M., Yamamoto, S., Fukuoka, T., Higashi, T., Kobayashi, K., Obata, K., Yamanaka, H., and Noguchi, K. (2007). Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. The Journal of clinical investigation *117*, 1979-1987.

Diogenes, A., Akopian, A.N., and Hargreaves, K.M. (2007). NGF up-regulates TRPA1: implications for orofacial pain. Journal of dental research *86*, 550-555.

Fowler, M.A., and Montell, C. (2013). Drosophila TRP channels and animal behavior. Life sciences *92*, 394-403.

Goadsby, P.J., and Edvinsson, L. (1993). The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Annals of neurology *33*, 48-56.

Goldberg, Y.P., MacFarlane, J., MacDonald, M.L., Thompson, J., Dube, M.P., Mattice, M., Fraser, R., Young, C., Hossain, S., Pape, T.*, et al.* (2007). Loss-of-function mutations in the Nav1.7 gene underlie congenital indifference to pain in multiple human populations. Clinical genetics *71*, 311-319.

Gracheva, E.O., Cordero-Morales, J.F., González-Carcacía, J.A., Ingolia, N.T., Manno, C., Aranguren, C.I., Weissman, J.S., and Julius, D. (2011). Ganglion-specific splicing of TRPV1 underlies infrared sensation in vampire bats. Nature *476*, 88-91.

Gu, P., Gong, J., Shang, Y., Wang, F., Ruppell, K.T., Ma, Z., Sheehan, A.E., Freeman, M.R., and Xiang, Y. (2019). Polymodal Nociception in Drosophila

Requires Alternative Splicing of TrpA1. Current biology : CB *29*, 3961-3973.e3966.

Guntur, A.R., Gu, P., Takle, K., Chen, J., Xiang, Y., and Yang, C.H. (2015). Drosophila TRPA1 isoforms detect UV light via photochemical production of H2O2. Proceedings of the National Academy of Sciences of the United States of America *112*, E5753-5761.

Hama, A., Natsume, T., Ogawa, S., Higo, N., Hayashi, I., and Takamatsu, H. (2018). Gaps in Understanding Mechanism and Lack of Treatments: Potential Use of a Nonhuman Primate Model of Oxaliplatin-Induced Neuropathic Pain. Pain research & management *2018*, 1630709.

Hanack, C., Moroni, M., Lima, W.C., Wende, H., Kirchner, M., Adelfinger, L., Schrenk-Siemens, K., Tappe-Theodor, A., Wetzel, C., Kuich, P.H.*, et al.* (2015). GABA blocks pathological but not acute TRPV1 pain signals. Cell *160*, 759-770.

Hatcher, J.P., and Chessell, I.P. (2006). Transgenic models of pain: a brief review. Current opinion in investigational drugs (London, England : 2000) *7*, 647-652.

Hill, R. (2000). NK1 (substance P) receptor antagonists--why are they not analgesic in humans? Trends in pharmacological sciences *21*, 244-246.

Huang, J., Zhou, W., Dong, W., Watson, A.M., and Hong, Y. (2009). From the Cover: Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering. Proceedings of the National Academy of Sciences of the United States of America *106*, 8284-8289.

Huang, J., Zhou, W., Watson, A.M., Jan, Y.N., and Hong, Y. (2008). Efficient ends-out gene targeting in Drosophila. Genetics *180*, 703-707.

Huggins, J.P., Smart, T.S., Langman, S., Taylor, L., and Young, T. (2012). An efficient randomised, placebo-controlled clinical trial with the irreversible fatty acid amide hydrolase-1 inhibitor PF-04457845, which modulates endocannabinoids but fails to induce effective analgesia in patients with pain due to osteoarthritis of the knee. Pain *153*, 1837-1846.

Im, S.H., Takle, K., Jo, J., Babcock, D.T., Ma, Z., Xiang, Y., and Galko, M.J. (2015). Tachykinin acts upstream of autocrine Hedgehog signaling during nociceptive sensitization in Drosophila. eLife *4*, e10735.

Ji, R.R., Samad, T.A., Jin, S.X., Schmoll, R., and Woolf, C.J. (2002). p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron *36*, 57-68.

Jordt, S.E., Bautista, D.M., Chuang, H.H., McKemy, D.D., Zygmunt, P.M., Högestätt, E.D., Meng, I.D., and Julius, D. (2004). Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature *427*, 260-265.

Julius, D. (2013). TRP channels and pain. Annual review of cell and developmental biology *29*, 355-384.

Kanai, Y., Nakazato, E., Fujiuchi, A., Hara, T., and Imai, A. (2005). Involvement of an increased spinal TRPV1 sensitization through its up-regulation in mechanical allodynia of CCI rats. Neuropharmacology *49*, 977-984.

Kaneko, Y., and Szallasi, A. (2014). Transient receptor potential (TRP) channels: a clinical perspective. British journal of pharmacology *171*, 2474-2507.

Kang, K., Panzano, V.C., Chang, E.C., Ni, L., Dainis, A.M., Jenkins, A.M., Regna, K., Muskavitch, M.A., and Garrity, P.A. (2011). Modulation of TRPA1 thermal sensitivity enables sensory discrimination in Drosophila. Nature *481*, 76-80.

Khuong, T.M., Wang, Q.P., Manion, J., Oyston, L.J., Lau, M.T., Towler, H., Lin, Y.Q., and Neely, G.G. (2019). Nerve injury drives a heightened state of vigilance and neuropathic sensitization in Drosophila. Science advances *5*, eaaw4099.

Kremeyer, B., Lopera, F., Cox, J.J., Momin, A., Rugiero, F., Marsh, S., Woods, C.G., Jones, N.G., Paterson, K.J., Fricker, F.R.*, et al.* (2010). A gain-of-function mutation in TRPA1 causes familial episodic pain syndrome. Neuron *66*, 671-680.

Liu, X.J., Gingrich, J.R., Vargas-Caballero, M., Dong, Y.N., Sengar, A., Beggs, S., Wang, S.H., Ding, H.K., Frankland, P.W., and Salter, M.W. (2008). Treatment of inflammatory and neuropathic pain by uncoupling Src from the NMDA receptor complex. Nature medicine *14*, 1325-1332.

Lukacs, V., Thyagarajan, B., Varnai, P., Balla, A., Balla, T., and Rohacs, T. (2007). Dual regulation of TRPV1 by phosphoinositides. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 7070-7080.

Luo, J., Shen, W.L., and Montell, C. (2017). TRPA1 mediates sensation of the rate of temperature change in Drosophila larvae. Nature neuroscience *20*, 34-41.

Mahajan, A., Taliun, D., Thurner, M., Robertson, N.R., Torres, J.M., Rayner, N.W., Payne, A.J., Steinthorsdottir, V., Scott, R.A., Grarup, N.*, et al.* (2018). Fine-mapping type 2 diabetes loci to single-variant resolution using high-density imputation and islet-specific epigenome maps. Nature genetics *50*, 1505-1513.

McDermott, L.A., Weir, G.A., Themistocleous, A.C., Segerdahl, A.R., Blesneac, I., Baskozos, G., Clark, A.J., Millar, V., Peck, L.J., Ebner, D.*, et al.* (2019). Defining the Functional Role of Na(V)1.7 in Human Nociception. Neuron *101*, 905-919.e908.

McKemy, D.D., Neuhausser, W.M., and Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature *416*, 52-58.

Mis, M.A., Yang, Y., Tanaka, B.S., Gomis-Perez, C., Liu, S., Dib-Hajj, F., Adi, T., Garcia-Milian, R., Schulman, B.R., Dib-Hajj, S.D.*, et al.* (2019). Resilience to Pain: A Peripheral Component Identified Using Induced Pluripotent Stem Cells and Dynamic Clamp. The Journal of neuroscience : the official journal of the Society for Neuroscience *39*, 382-392.

Neely, G.G., Hess, A., Costigan, M., Keene, A.C., Goulas, S., Langeslag, M., Griffin, R.S., Belfer, I., Dai, F., Smith, S.B.*, et al.* (2010). A genome-wide Drosophila screen for heat nociception identifies α2δ3 as an evolutionarily conserved pain gene. Cell *143*, 628-638.

Nilius, B., Owsianik, G., and Voets, T. (2008). Transient receptor potential channels meet phosphoinositides. The EMBO journal *27*, 2809-2816.

Patapoutian, A., Tate, S., and Woolf, C.J. (2009). Transient receptor potential channels: targeting pain at the source. Nature reviews Drug discovery *8*, 55-68.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S.*, et al.* (2002). A TRP channel that senses cold stimuli and menthol. Cell *108*, 705-715.

Prescott, E.D., and Julius, D. (2003). A modular PIP2 binding site as a determinant of capsaicin receptor sensitivity. Science (New York, NY) *300*, 1284-1288.

Ramsey, I.S., Delling, M., and Clapham, D.E. (2006). An introduction to TRP channels. Annual review of physiology *68*, 619-647.

Roberts, D.B., Jowett, T., Hughes, J., Smith, D.F., and Glover, D.M. (1991). The major serum protein of Drosophila larvae, larval serum protein 1, is dispensable. European journal of biochemistry *195*, 195-201.

Rosenzweig, M., Brennan, K.M., Tayler, T.D., Phelps, P.O., Patapoutian, A., and Garrity, P.A. (2005). The Drosophila ortholog of vertebrate TRPA1 regulates thermotaxis. Genes & development *19*, 419-424.

Sawada, Y., Hosokawa, H., Matsumura, K., and Kobayashi, S. (2008). Activation of transient receptor potential ankyrin 1 by hydrogen peroxide. The European journal of neuroscience *27*, 1131-1142.

Schmidt, M., Dubin, A.E., Petrus, M.J., Earley, T.J., and Patapoutian, A. (2009). Nociceptive signals induce trafficking of TRPA1 to the plasma membrane. Neuron *64*, 498-509.

Shieh, B.H., and Zhu, M.Y. (1996). Regulation of the TRP Ca2+ channel by INAD in Drosophila photoreceptors. Neuron *16*, 991-998.

Shin, Y.C., Shin, S.Y., Chun, J.N., Cho, H.S., Lim, J.M., Kim, H.G., So, I., Kwon, D., and Jeon, J.H. (2012). TRIP database 2.0: a manually curated information hub for accessing TRP channel interaction network. PloS one *7*, e47165.

Staruschenko, A., Jeske, N.A., and Akopian, A.N. (2010). Contribution of TRPV1-TRPA1 interaction to the single channel properties of the TRPA1 channel. The Journal of biological chemistry *285*, 15167-15177.

Tappe, A., Klugmann, M., Luo, C., Hirlinger, D., Agarwal, N., Benrath, J., Ehrengruber, M.U., During, M.J., and Kuner, R. (2006). Synaptic scaffolding protein Homer1a protects against chronic inflammatory pain. Nature medicine *12*, 677-681.

Tracey, W.D., Jr., Wilson, R.I., Laurent, G., and Benzer, S. (2003). painless, a Drosophila gene essential for nociception. Cell *113*, 261-273.

Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M., and Zuker, C.S. (1997). A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature *388*, 243-249.

Tulleuda, A., Cokic, B., Callejo, G., Saiani, B., Serra, J., and Gasull, X. (2011). TRESK channel contribution to nociceptive sensory neurons excitability: modulation by nerve injury. Molecular pain *7*, 30.

Urano, H., Ara, T., Fujinami, Y., and Hiraoka, B.Y. (2012). Aberrant TRPV1 expression in heat hyperalgesia associated with trigeminal neuropathic pain. International journal of medical sciences *9*, 690-697.

Urbano, J.M., Torgler, C.N., Molnar, C., Tepass, U., López-Varea, A., Brown, N.H., de Celis, J.F., and Martín-Bermudo, M.D. (2009). Drosophila laminins act as key regulators of basement membrane assembly and morphogenesis. Development (Cambridge, England) *136*, 4165-4176.

Vardeh, D., Mannion, R.J., and Woolf, C.J. (2016). Toward a Mechanism-Based Approach to Pain Diagnosis. The journal of pain : official journal of the American Pain Society *17*, T50-69.

Vázquez, E., and Valverde, M.A. (2006). A review of TRP channels splicing. Seminars in cell & developmental biology *17*, 607-617.

Vilceanu, D., Honore, P., Hogan, Q.H., and Stucky, C.L. (2010). Spinal nerve ligation in mouse upregulates TRPV1 heat function in injured IB4-positive nociceptors. The journal of pain : official journal of the American Pain Society *11*, 588-599.

Viswanath, V., Story, G.M., Peier, A.M., Petrus, M.J., Lee, V.M., Hwang, S.W., Patapoutian, A., and Jegla, T. (2003). Opposite thermosensor in fruitfly and mouse. Nature *423*, 822-823.

Wang, S., Dai, Y., Fukuoka, T., Yamanaka, H., Kobayashi, K., Obata, K., Cui, X., Tominaga, M., and Noguchi, K. (2008). Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a molecular mechanism of inflammatory pain. Brain : a journal of neurology *131*, 1241-1251.

Weng, H.J., Patel, K.N., Jeske, N.A., Bierbower, S.M., Zou, W., Tiwari, V., Zheng, Q., Tang, Z., Mo, G.C., Wang, Y.*, et al.* (2015). Tmem100 Is a Regulator of TRPA1-TRPV1 Complex and Contributes to Persistent Pain. Neuron *85*, 833-846.

Woolf, C.J. (2020). Capturing Novel Non-opioid Pain Targets. Biological psychiatry *87*, 74-81.

Xu, A., Larsen, B., Baller, E.B., Scott, J.C., Sharma, V., Adebimpe, A., Basbaum, A.I., Dworkin, R.H., Edwards, R.R., Woolf, C.J.*, et al.* (2020). Convergent neural representations of experimentally-induced acute pain in healthy volunteers: A large-scale fMRI meta-analysis. Neurosci Biobehav Rev *112*, 300-323.

Yekkirala, A.S., Roberson, D.P., Bean, B.P., and Woolf, C.J. (2017). Breaking barriers to novel analgesic drug development. Nature reviews Drug discovery *16*, 545-564.

Zhang, M., Wang, Y., Geng, J., Zhou, S., and Xiao, B. (2019). Mechanically Activated Piezo Channels Mediate Touch and Suppress Acute Mechanical Pain Response in Mice. Cell reports *26*, 1419-1431.e1414.

Zhong, L., Bellemer, A., Yan, H., Ken, H., Jessica, R., Hwang, R.Y., Pitt, G.S., and Tracey, W.D. (2012). Thermosensory and nonthermosensory isoforms of

Drosophila melanogaster TRPA1 reveal heat-sensor domains of a thermoTRP Channel. Cell reports *1*, 43-55.

Zhou, W., Huang, J., Watson, A.M., and Hong, Y. (2012). W::Neo: a novel dual-selection marker for high efficiency gene targeting in Drosophila. PloS one *7*, e31997.

Zhou, Y., Suzuki, Y., Uchida, K., and Tominaga, M. (2013). Identification of a splice variant of mouse TRPA1 that regulates TRPA1 activity. Nature communications *4*, 2399.