TRPC3 ANTAGONIZES CONTACT DERMATITIS-INDUCED PRURITUS

Katherine C. Beattie

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Supervisor of Dissertation

_____

Wenqin Luo, M.D., Ph.D.
Associate Professor of Neuroscience

Graduate Group Chairperson

_____

Joshua I Gold, Ph.D.
Professor of Neuroscience

Dissertation Committee:

Michael Granato, Ph.D., Professor of Cell and Developmental Biology
Minghong Ma, Ph.D., Professor of Neuroscience
Brian Capell, M.D., Ph.D., Assistant Professor of Dermatology and Genetics
Zhaolan Zhou, Ph.D., Professor of Genetics
Sarah Ross, Ph.D., Associate Professor of Neurobiology and Anesthesiology, University of Pittsburgh

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DEDICATION

This thesis is dedicated to the memory of my mother, Judith Beattie, and to my father, Mark Beattie. I would not be in this position without your constant love and support.

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ABSTRACT

TRPC3 ANTAGONIZES CONTACT DERMATITIS-INDUCED PRURITUS

Katherine C. Beattie

Wenqin Luo

Somatosensation allows us to perceive sensory stimuli at the surface of and inside the body. One sensation, itch, plays a protective role under acute conditions to alert us to potentially harmful stimuli, such as mosquitos, parasites, or irritating chemicals. Although helpful in these situations, the itch pathway can become chronically activated, leading to debilitating itch in patients suffering from chronic pruritus. Despite the many years of research on the molecular mediators and transmission of itch sensation, there is still much to learn about the complicated underpinnings of pruritus, particularly in the setting of dermatological illnesses. In **Chapter 1**, I present an overview of the molecular and cellular mediators of itch sensation, highlighting the role of immune cells in itch. After describing the initial activation of itch transmission, I discuss models of itch circuits. I end the chapter with a review of methods used to study itch sensation, focusing on mouse models of dermatological diseases used to study pruritus. In **Chapter 2**, I investigate the role of *TrpC3* in antagonizing itch induced by a mouse model of contact dermatitis. Transient Receptor Potential Cation Channel Subfamily C Member 3

(TRPC3) is a cation-permeable ion channel that is expressed in cellular subtypes in a wide variety of systems (e.g., nervous system, immune system, cardiac system). *TrpC3*'s high level of expression in primary sensory neurons made it a prime candidate to function in modulating somatosensation, however previous studies identified little deficits in acute somatosensation in *TrpC3* null mice. I show *TrpC3* null mice display significantly increased pruritus with contact dermatitis (CD), pinpoint the importance of *TrpC3* expression in DRG to antagonize itch sensation, and identify a decrease in nonpeptidergic subtype 1 (NP1^{+}) MRGPRD^{+} neurons in *TrpC3* null mice with CD. In sum, our findings identified a novel role of *TrpC3* and NP1 afferents in CD pathology, which resulted in the proposal of a new model of modulation of itch sensation.

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CHAPTER 1: An introduction to itch

1.1 An overview of somatosensation

Elementary school students are classically taught that humans have 5 senses: sight, hearing, smell, taste, and touch. Although true, the colloquial title of touch only covers one aspect of the sensations transmitted by the skin and mucous membranes, which comprise the field of somatosensation. In addition to touch (detection of light mechanical stimuli), somatosensation encompasses the sensations of nociception (pain), pruritoception (itch), thermosensation (detection of hot and cold), proprioception (detection of mechanical displacement of muscle and joints), and interoception (the sensation of our internal organ systems) (Lumpkin and Caterina, 2007). Positive sensations (e.g., the touch of a mother), and negative sensations (e.g., the painful prick of a vaccination needle), are all initiated by primary somatosensory neurons that innervate our skin (and internal organs). Cell bodies of primary sensory neurons are located in trigeminal ganglia at the base of the skull (mediating sensations of the head and face) and dorsal root ganglia (DRG) along the spinal column (mediating sensation below the head).

DRG neurons are pseudounipolar neurons that form one axon that bifurcates to send one branch into the spinal cord (to the CNS) and another branch to the spinal nerve to innervate the periphery (skin and other tissues). DRG neurons have been categorized by the size of their cell body, degree of myelination, conduction velocity (inversely related to myelination level), innervation of sensory organs, and central projection in the spinal cord (Abraira and Ginty, 2013; Basbaum et al., 2009). There are four widely accepted categories of somatosensory neurons: small, unmyelinated C fibers that detect

noxious stimuli including itch, pain, and temperature (Basbaum et al., 2009); medium-diameter, lightly-myelinated Aδ fibers that primarily mediate acute pain; and large-diameter, highly myelinated Aα and Aβ fibers which mediate proprioception and light touch, respectively (Lumpkin and Bautista, 2005). The activation of somatosensory neurons is dependent on the activation of specialized receptors and channels at peripheral terminals, with certain nociceptors being responsive to multiple sensory modalities. In this introductory chapter I will discuss itch, common dermatological diseases associated with itch and key findings that have illuminated the molecular and cellular mechanisms underlying itch sensation. I will then examine the theories of itch transduction, highlighting the work done examining the gating of itch by pain, before closing with a section on behavioral methods utilized to study itch sensation.

1.2 Itch and contact dermatitis

Itch

Pruritus, or itch, was defined as "an unpleasant sensation that elicits the desire to scratch" in 1660 by the German physician Samuel Hafenreffer (Ikoma et al., 2006). Itch is a symptom of systemic diseases (e.g., liver cholestasis and kidney disease), neurological (e.g., postherpetic neuralgia), psychological disorders (e.g., obsessive compulsive disorder) and many dermatological diseases (e.g., atopic dermatitis, psoriasis, and contact dermatitis) (Patel and Yosipovitch, 2010; Ständer et al., 2007; Sun and Dong, 2016). Acute itch, lasting <6 weeks, serves an adaptive purpose of alerting the organism to harmful external threats (e.g., toxins, parasites, and insects) (Mishra and Hoon, 2015).

Chronic itch, lasting ≥ 6 weeks (Ständer et al., 2007), results in a negative cycle of itch sensation leading to scratch, scratch causing skin excoriations, and inflammatory mediators in the damaged skin activating nociceptive neurons responsible for itch sensation. This cycle negatively affects the patients' quality of life, and leads to sleep deprivation, anxiety, and depression (Grundmann and Stander, 2011; Matterne et al., 2009; Pereira and Stander, 2017; Stander et al., 2007; Weisshaar and Dalgard, 2009).

Societal impact of itch

In addition to the detrimental effect of itch on patients' quality of life and their financial health, pruritus has a significant burden on the medical system. One study, examining 9 years of data from the Medical Expenditure Panel Survey, found that using conservative national estimates of chronic pruritus (6.1%), pruritus is associated with >$90 billion per year in population-level expenditures in the United States (Tripathi et al., 2019). Analysis of data from the National Ambulatory Medical Care Survey (NAMCS) found that 7 million patients present to outpatient care with itch as a symptom every year in the U.S. (Shive et al., 2013). In addition, approximately 0.8% of all physician visits per year were due to itch, with the most common primary diagnosis being contact dermatitis (12.8%; Shive et al., 2013). Despite the existence of several drug classes to treat chronic pain, there is not a single medication approved by the FDA for the treatment of chronic itch (Wang and Kim, 2020). These findings support the need for more effective therapeutic interventions for chronic itch. In this section I will provide a brief overview of the two

most common types of contact dermatitis (which are associated with irritating itch): allergic contact dermatitis (ACD) and irritant CD (ICD).

Contact dermatitis

Irritant Contact Dermatitis (ICD) is the most common type of contact dermatitis and comprises ~80% of occupational contact dermatitis (Clark and Zirwas, 2009). Specifically, ICD is a localized inflammatory skin response to a variety of environmental agents (Ale and Maibach, 2014). The clinical severity of ICD is influenced by the physical and chemical properties and strength of the irritating substance, frequency of exposure, host-related susceptibility factors (e.g., skin susceptibility), and environmental factors (e.g., temperature) (Ale and Maibach, 2014; Jakasa et al., 2006; Ohlenschlaeger et al., 1996). Unlike ACD, ICD does not involve adaptive immunity, but instead results from the direct cytotoxic effect of irritants (activating the innate immune system). Specifically, ICD development is attributed to skin barrier disruption, keratinocyte damage, and the release of pro-inflammatory mediators that recruit and activate T lymphocytes (Ale and Maibach, 2014).

ACD is a common dermatological disease that results from epidermal re-exposure of an allergen, such as poison ivy or nickel. The pathophysiology of ACD is characterized by two distinct phases, the sensitization phase and the elicitation phase. The sensitization phase occurs after the first contact of the hapten with the skin. Haptens enter the epidermis and react with endogenous proteins to form hapten-protein complexes that are taken up by Langerhans cells (LCs), the antigen presenting cells (APCs) of the skin.

While LCs migrate to lymph nodes they mature into dendritic cells (DCs). DCs interact with T cells to generate memory T cells (T_{mem}) that circulate throughout the body and are subsequently recruited into the skin during the elicitation phase upon re-exposure of the skin to the offending hapten (Vocanson et al., 2009). The inflammatory reaction, mediated by activation of hapten-specific T_{mem} cells, results in tissue damage of the skin and the sensation of itch (Vocanson et al., 2009). The pathophysiology of ACD is regulated by T_{reg} cells which can lower or suppress the process of sensitization and can suppress memory T cells in the elicitation phase (Honda et al., 2011; Ring et al., 2006). Cytokines released by immune cells activate primary itch-sensing afferents to drive itch sensation and behaviors, as indicated by greatly increased spontaneous scratching (Wenning et al., 2011).

1.3 Peripheral itch mediators and cellular receptors

During an episode of acute itch, the epidermis often suffers a physical breach or chemical insult. In response, keratinocytes and resident immune cells of the skin detect damage or foreign substances and respond by releasing chemical mediators, triggering an inflammatory cascade (Pasparakis et al., 2014). Mast cells undergo degranulation and release the content of their cytosolic granules, which include histamine, serotonin, leukotriene, proteases, cytokines and chemokines (Rao and Brown, 2008). These inflammatory mediators can be detected by pruritoceptive nerve endings and cause itch sensation. In this section I will lay out the organization of itch subtypes as histaminergic and nonhistaminergic itch and discuss well-known endogenous and exogenous

pruritogenic substances. I will end by highlighting the role of pruitogens secreted from epidermal and immune cells.

Histaminergic Itch

Itch-sensitive neurons are frequently classified according to their sensitivity to histamine, an amine that is released from keratinocytes and immune cells (mast cells and basophils), which was first studied 120 years ago (Dale and Laidlaw, 1910). Histamine acts on a subset of sensory neurons that express histamine receptors ($H_{1-4}R$, GPCRs) and the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) (Bautista et al., 2014; Rossbach et al., 2011). Three out of the four histamine receptors have been shown to mediate histamine induced itch in unique ways (namely H_1R, H_3R, & H_4R). While H_1R and H_4R are key components to the development of histamine-dependent itch, and blocking these receptors greatly reduces itch, H_3R serves to inhibit itch (Rossbach et al., 2011). TRPV1 plays a critical role in mediating histaminergic itch by working downstream of histamine receptors. This is evidenced in both *in vitro* and *in vivo* experiments: 1) Treatment of DRG cultures with a TRPV1 blocker, capsazepine, inhibited histamine-evoked signals (Shim et al., 2007); 2) TRPV1 deficient mice display significantly less scratching to histamine than wild type littermates, suggesting the involvement of additional channels in mediating histaminergic itch (Imamachi et al., 2009; Shim et al., 2007). In addition to TRPV1, another TRP family member, TRPV4 has been implicated in mediating itch sensation through its expression in pruritoceptors (Kim et al., 2016) and keratinocytes (Chen et al., 2016), and macrophages (Luo et al., 2018).

Deletion of TRPV4 specifically in macrophages and keratinocytes was found to reduce allergic and non-allergic chronic itch in mice (Luo et al., 2018).

Non-histaminergic Itch

Due to the ineffective nature of antihistamines in providing itch relief to patients suffering from chronic itch (unless that itch resulted from urticaria), much work in the field has shifted to studying non-histaminergic itch (to identify the mechanisms underlying chronic itch). This has led to the discovery of numerous exogenous and endogenous pruritogens, including: mucunain (from the cowhage plant), chloroquine (anti-malarial drug), β-alanine (body building supplement), the peptide Ser-Leu-Ile-Gly-Arg-LeuSLIGRL-NH2 (product of proteolytic cleavage of PAR2), endothelin-1 (an endogenous vasoconstrictor), bovine adrenal medulla peptide (BAM) 8–22 (product of proteolytic cleavage of proenkephalin), bile acid (synthesized in the liver, accumulates in cholestasis), and various cytokines including thymic stromal lymphopoietin protein (TSLP) & Interleukin-31 (IL-31)(Bautista et al., 2014; Han and Dong, 2014).

In the late 1990s, a transcription factor called *Neurogenin 1* (*Ngn1*) was found to be critical for the development of a subset of neurons in the DRG that express TrkA, the receptor for nerve growth factor (Ma et al., 1999). In the absence of *Ngn1* most nociceptive DRG neurons failed to develop (Ma et al., 1999). This opened up an opportunity to identify nociceptor-specific genes, by performing subtractive hybridization of the cDNAs in newborn wild-type and *Neurogenin1* knockout mice (Dong et al., 2001). This screen identified many genes known to be involved in nociception (e.g., TRPV1 and

CGRP) as well as 5 previously unknown genes. One of these unknown genes encoded a protein with 7 transmembrane segments, characteristic of GPCR, which was found to be expressed in small diameter neurons. Due to its significant homology to MAS1 and the mammalian GPCR Mas-related gene 1 (*Mrg1*), this receptor was named Mas-related gene A1 (*MrgA1;* Dong et al., 2001). After this initial discovery, further experiments and bioinformatic analysis found that of 50 *Mas-related G protein–coupled receptors (Mrgprs)* sequences detected, 27 have open reading frames, (Meixiong and Dong, 2017). Although originally hypothesized to modulate nociception due to MRGPRA1's function as a receptor for RFamide neuropeptides, which were previously linked to pain, further studies highlighted the role of MRGPRs in primarily modulating itch sensation (Dong et al., 2001). In this large family of murine GPCRs, three were found to mediate various types of non-histaminergic itch: MRGPRA3, MRGPRD, and MRGPRC11. MRGPRA3 is a receptor for the antimalarial drug chloroquine (CQ) which is necessary to mediate neuronal activation by CQ (Liu et al., 2009). BAM-22 has been shown to activate both murine MRGPRC11 (Liu et al., 2009) and human MRGPRX1 (Lembo et al., 2002). Chloroquine induced itch in humans is also transmitted by activation of MRGPRX1 (Lembo et al., 2002). *MrgprA3* and *MrgprC11* are both expressed in the same subset of pruritogenic neurons and rely on TRPA1 for downstream calcium transduction, with *MRGPRC11* signaling through phospholipase C (PLC) and MRGPRA3 signaling with GβY (Wilson et al., 2011). MRGPRA3 and MRGPRC11 are required for both chloroquine- and BAM-evoked depolarization of somatosensory neurons (and induced scratching behavior in mice) (Wilson et al., 2011). MRGPRD is a receptor for β-alanine in humans and rodents (Liu et al., 2012a; Shinohara et al., 2004). The intradermal

injection of β-alanine in mice and humans resulted in itch, with humans also reporting pain related symptoms including stinging and burning sensations (Liu et al., 2012a). In addition to itch sensation, MRGPRD^{+} neurons have been shown to play a role mediating additional sensations, including painful mechanosensation (Cavanaugh et al., 2009) and cold allodynia (in a neuropathic pain model) (Wang et al., 2019).

Searches of human genomic databases yielded 8 *Mrgprs*, 4 of which (*Mrgprs-D, E, F, & G*) were clear, single gene mouse orthologs (Dong et al., 2001; Lembo et al., 2002). The other 4 genes, *MrgprX1-4* or SNSRs (sensory neuron specific receptors), share homology with the murine subfamilies *MrgprA-C*. For example, the human *MrgprX1* shares 54% amino acid identity with the mouse *MrgprC11* and less than 50% with mouse *MrgprA3* (McNeil and Dong, 2014). After the identification of the human orthologs, there was an emphasis on identifying the involvement of human MRGPRs in mediating itch sensation.

For many years bile acids, which accumulate in the liver of patients with cholestasis, have been hypothesized to elicit cholestatic itch. In mice, bile acids were found to selectively bind to the GPCR TGR5, expressed in small diameter nociceptors, which activates the TRPA1 channel to induce itch (Alemi et al., 2013; Kirby et al., 1974; Lieu et al., 2014). In human DRG, which do not express TGR5, bile acids act as ligands for MRGPRX4 to elicit a Ca^{2+} response in cultured hDRG (Yu et al., 2019).

Epithelial and Inflammatory mediators of itch

As previously stated, itch sensation is due to a combination of molecular and cellular events contributed by neurons, resident epidermal cells, immune cells, proteases, cytokines, and neuropeptides. No one system (e.g., nervous system, immune system, or integumentary system) induces itch sensation in a vacuum, however there are subsets of itch that rely on immune cells, and their products (e.g., histamine) to be activated. This section will focus on itch that is induced by inflammatory mediators such as cytokines and proteases, which can be secreted by immune cells and keratinocytes. The end of the section highlights the role of immunomodulatory agents secreted by mast cell degranulation in itch sensation.

Members of the protease activated receptor (PAR) family have been shown to be expressed in DRG and keratinocytes and mediate nonhistaminergic itch through their activation by tethered ligands, such as SLIGRL and AYPGKF, or by protease-induced cleavage of part of their extracellular domain that acts as tethered ligand (Ramachandran and Hollenberg, 2008; Shimada et al., 2006; Steinhoff et al., 1999; Steinhoff et al., 2003). Several endogenous (released from keratinocytes and immune cells) and exogenous proteases (plant, fungi, and bacteria) have been identified as activating PAR and inducing itch sensation (Akiyama et al., 2015). Key proteases frequently studied in the literature include: 1) mucunain, the active component of cowhage, which acts at PAR-2 and PAR-4 (Reddy et al., 2008); 2) kallikreins, serine proteases expressed in the epidermis, produce itch in humans upon injection through multiple PARs (Hägermark, 1974); 3) the serine protease tryptase, stored in mast cell granules, and activates PAR-2 (Ui et al., 2006). 4)

cathepsin S, a cysteine protease expressed in keratinocytes and a variety of antigen presenting cells, cleaves PAR-2 and PAR-4, and activates MrgprC11 (Akiyama et al., 2015; Reddy et al., 2010; Reddy et al., 2015). PAR2, cathepsin S, and tryptase levels were significantly increased in the skin of patients with atopic dermatitis, suggesting activation of PAR2 plays a role in chronic itch (Reddy et al., 2010; Steinhoff et al., 2003). Activation of PAR2 by SLIGRL, tryptase or cathepsin S promotes keratinocyte release of the cytokine thymic stromal lymphopoietin (TSLP), a robust pruritogen associated with atopic dermatitis that activates TRPA1^{+} neurons through binding the TSLP receptor (Wilson et al., 2013).

Toll-like receptors (TLRs) are important mediators of the innate immune response through their recognition of pathogen-associated molecular patterns (Kawai and Akira, 2010). Two TLRs expressed in DRG have been found to be involved in itch sensation in mice, TLR7 and TLR3. Activation of TLR7 by imiquimod elicited scratching behavior in mice (Liu et al., 2010a). Deletion of TLR3 and knock down of TLR3 in DRG of wild type mice both led to attenuated pruritus (Liu et al., 2012b). Notably, excitatory synaptic transmission in the intact spinal cord was found to be impaired in *Tlr3*$^{-/-}$ mice, but not in *Tlr7*$^{-/-}$ mice, suggesting a critical role for TLR3 in regulating synaptic cord transmission of itch sensation (Liu et al., 2012b).

Mast cells and itch

Many dermatologic illnesses associated with chronic itch have a mast-cell dependent mechanism, making these cells of considerable interest to the field. Mast cells, which reside near sensory fibers across multiple barrier surfaces (Egan et al., 1998), are granulocytes filled with a variety of immunomodulatory factors (e.g., histamine, prostaglandins, and cytokines) (Pasparakis et al., 2014). In addition to the canonical activation of mast cells, which occurs through antigen binding to immunoglobulin E (IgE) antibody and crosslinking of the IgE receptor, Fc epsilon RI (FcεRI), numerous additional peptides can activate the degranulation of mast cells, including various toxins, neuropeptides, and hormones (Meixiong and Dong, 2017; Metcalfe et al., 1997).

In addition to histamine, another pruritogenic molecule secreted from mast cells is the neurotransmitter serotonin (5-HT) (Morita et al., 2015). Interestingly, the transient receptor potential vanilloid type-4 (TRPV4), a temperature sensitive cation channel (Güler et al., 2002), was found to mediate 5-HT induced scratching in mice (Akiyama et al., 2016). Substance P (SP) is secreted by nerves and inflammatory cells such as macrophages, eosinophils, lymphocytes, and dendritic cells (O'Connor et al., 2004). SP produces itch in humans and scratching in mice, but the itch is transduced through different mechanisms in these species (Andoh et al., 1998; Hägermark et al., 1978). In human skin, SP promotes the release of histamine through mast cell degranulation (Hägermark et al., 1978). In mice, SP elicits scratching by directly activating pruriceptive neurons and promoting the release of pruritogenic agents, such as leukotriene B4 (LTB4), from keratinocytes (Andoh et al., 2001; Andoh et al., 1998).

The tick salivary peptide IP defensin 1 (IPDef1) induced itch in mice by activating MrgprC11/MRGPRX1 to sensitize TRPV1 in pruriceptive neurons (Li et al., 2020). In addition to sensitization of TRPV1, IPDef1 also activated MRGPBR2, and the human ortholog MRGPRX2, which are expressed on mast cells, inducing the mast cells to secrete inflammatory cytokines that promoted acute inflammation in mice, but did not directly contribute to IPDef1-induced itch (Li et al., 2020). Compound 48/80 is a mast cell degranulation agent that elicits itch in humans and mice (Inagaki et al., 2002; Rukwied et al., 2013). In humans, compound 48/80 utilizes MRGPRX1 and MRGPRX2 for mast cell degranulation (Kashem et al., 2011). Further evidence linking mast cell degranulation-induced itch to Mrgprs comes from work conducted in the Dong lab. They found that mast cell activation via MRGPBR2 evoked non-histaminergic itch and that depletion of MRGPBR2, the mouse homolog of human MRGPRX2, resulted in decreased itch in multiple ACD models (Meixiong et al., 2019).

Basophils, Neutrophils, and Itch

In addition to mast cells, recent research has illuminated the important role of basophils and neutrophils in promoting itch in mouse models of atopic dermatitis (AD). The Bautista lab found that depletion of neutrophils in a mouse model of AD led to attenuated scratching (Walsh et al., 2019). Additionally, they identified that neutrophils are required for the activation of sensory neurons through their induction of CXCL10, a ligand of the CXCR3 receptor (Walsh et al., 2019). Work in the Kim demonstrated that basophils were required in promoting acute itch flares in mice with AD-like disease (Wang et al., 2021).

In the setting of AD, basophils released leukotriene C4 which was found to bind to the receptor CysLTR2 that is expressed in pruritoceptive neurons (Sasaki and Yokomizo, 2019; Wang et al., 2021). Pharmacological blockade of CysLTR2 signaling significantly reduced acute itch flares in mice with AD-like disease (Wang et al., 2021).

Cytokines and Itch

Cytokines are immunomodulatory molecules utilized by immune cells for communication. T helper type 2 (Th2) type cytokines (including IL-4, IL-13, IL-31). have been strongly associated with multiple forms of dermatitis, including atopic dermatitis (Brandt and Sivaprasad, 2011). In addition to maintaining inflammation, these cytokines can directly or indirectly activate itch sensing neurons, which likely serves as an adaptive mechanism to warn the body of potentially harmful stimuli.

Thymic stromal lymphopoietin (TSLP) is primarily expressed by epithelial cells, including epidermal keratinocytes, and is released in response to a range of stimuli, including protease activation by PAR2. High levels of TSLP were detected in the skin lesions of patients with atopic dermatitis and were found to be associated with activation and migration of antigen presenting cells within the dermis (Soumelis et al., 2002). TSLP can directly activate nociceptive neurons via the TSLP receptor (composed IL7Ra and TSLPR) (Wilson et al., 2013). Notably, TRPA1 is required for downstream signaling of TSLP, again demonstrating recurrent role of TRP channels in mediating somatosensation.

Another cytokine that has been associated with atopic dermatitis is Interleukin-31 (IL-31), which is predominantly produced by Th2 type T cells (Cevikbas et al., 2014; Nattkemper et al., 2018a). Small diameter itch-sensing neurons express both subunits of the IL-31 receptor, IL-31Rα and the oncostatin M receptor (OSMR), as well as receptors for 5-HT (Usoskin et al., 2015). In mice, subcutaneous injection of IL-31 triggered scratching behavior through downstream activation of TRPV1 and TRPA1 (Cevikbas et al., 2014).

In addition to its role in driving immune cells to produce Th2 cytokines (Brandt and Sivaprasad, 2011; Han et al., 2017), IL-33 is a mediator of itch caused by poison ivy (urushiol) induced allergic contact dermatitis (Liu et al., 2016). The IL-33 receptor, composed of the IL-33-specific ST2 chain and an accessory IL-1 receptor-like 1 (IL-1RAcP) chain, is expressed in pruritoceptors that are sensitive to chloroquine and histamine (Liu et al., 2016). Direct IL-33 application triggers a mild increase in neuronal Ca^{2+} and promotes itch in mice that already have urushiol-induced itch but does not promote pruitis in naïve mice. Notably, both TRPV1 and TRPA1 are required for IL-33 signaling in pruritoceptors (Liu et al., 2016)).

IL-4 and IL-13 are closely related in their sequences and function in initiating Th2 immunity by driving the differentiation of naïve $CD4^+$ helper T cells into Th2 cells (Brandt and Sivaprasad, 2011). These Th2 cells can produce large amounts of IL-4 and IL-13 to maintain the Th2 phenotype. Activation of IL-4 and IL-13 receptors of itch sensing neurons result in elevation of intracellular Ca^{2+} through the Janus kinase (JAK) pathway, as well as TRPV1 and TRPA1 (Oetjen et al., 2017; Usoskin et al., 2015). Although injection of IL-4 and IL-13 into the skin did not trigger acute scratching,

researchers found these Th2 cytokines act to sensitize sensory neurons to many different pruritogens (Oetjen et al., 2017). These observations are consistent with human studies that found amplified itch responses to pruritogenic challenges of lesioned skin of patients with AD (compared to control subjects) (Andersen et al., 2017; Ikoma et al., 2003).

1.4 Organization of the itch pathway and theories of itch transduction

Overview of the itch pathway

Itch occurs after primary sensory neurons are exposed to exogenous and endogenous stimuli including allergens, proteases, neuropeptides, and cytokines (Akiyama and Carstens, 2013). These pruritogens activate receptors on the cutaneous nerve endings of pruritoceptors, causing calcium influx and activation of intracellular signaling pathways that result in the transmission of an electrical impulse from the skin to the DRG or Trigeminal ganglia (TG) to the primary somatosensory cortex. Specifically, itch information is transmitted by DRG or TG to the thalamus and the primary somatosensory cortex through the ascending spinothalamic tract (STT) of the spinal cord (from DRGs) or the trigeminothalamic tract of brainstem (from TGs) (Han and Dong, 2014). Although most literature focuses on the role of unmyelinated C-fibers in itch, electrophysiological studies have shown thinly myelinated Aδ fibers have been found to contribute to a small subset of itch sensation as well (LaMotte et al., 2014; Ringkamp et al., 2011). Importantly, Aδ and C nociceptors have 'polymodal' response properties, meaning they respond to multiple stimuli (mechanical, thermal, and/or chemical) (Liu et al., 2012a). In this section I will discuss the classification of nociceptive neurons based on their

molecular identities, describe the innervation pattern of peptidergic and nonpeptidergic nociceptors in the skin and the spinal cord, and briefly summarize the transmission of itch sensation from the spinal cord to the brain.

Classification of mammalian nociceptor populations

Immunostaining has identified various molecular markers expressed by mature DRG neurons in rodents and used these markers to classify nociceptors into two categories (peptidergic and nonpeptidergic) (Han et al., 2013). Peptidergic neurons typically contain the neuropeptides calcitonin gene-related peptide (CGRP) and substance P and are marked by persistent expression of the nerve growth factor (NGF) receptor tryptomyosin receptor kinase 1 (TRK1) (Ennett et al., 1996; Rosenfeld et al., 1983). Nonpeptidergic neurons express the glial-derived neurotrophic factor receptor RET, and most of this population binds isolectin B4 (IB4) (Molliver et al., 1997). Transgenic mouse lines were utilized to conduct targeted studies of these neuronal classes. Researchers have targeted the *Calca* locus, which encodes for CGRP, to study the function of peptidergic neurons (McCoy et al., 2013; McCoy et al., 2012). Nonpeptidergic neurons have been commonly studied by utilizing transgenic mice targeting the *Mrgprd* locus (Zylka et al., 2005), which encodes for the Mas-related gene product receptor, a GPCR (Dong et al., 2001) that is expressed in ~75% of IB4+ non-peptidergic nociceptors (Zylka et al., 2005).

In the last decade numerous laboratories have conducted transcriptome profiling of mouse DRG neurons which has aided in the molecular categorization of nociceptors (Chiu et al., 2014; Li et al., 2018; Sharma et al., 2020; Thakur et al., 2014; Usoskin et al., 2015). Although RNAseq analysis of single-cell and population levels of DRG from

different groups yielded small discrepancies on the expression of certain genes, there is a general consensus of assignment to neuronal subtypes. In the following sentences, I will use the nomenclature proposed by the Ernfors group in their principal component analysis of single cell transcriptomes of DRG cells to describe the subcategorization of nonpeptidergic and peptidergic somatosensory neurons (Emery and Ernfors, 2018; Usoskin et al., 2015). There are two subtypes of peptidergic nociceptors, both of which express Tac1. PEP1 are primarily thermosensitive C nociceptors and PEP2 are lightly myelinated Aδ nociceptors. Three subtypes of nonpeptidergic (NP) neurons, NP1, NP2, and NP3 are likely itch sensory neurons based on their molecular identities. NP1 neurons express Mrgprd. NP2 and NP3 neurons are overlapping populations that both express histamine receptors and the IL-33 receptor. The NP2 and NP3 populations are distinguished by the expression of MrgprA3 and MrgprC11 in NP2 neurons, and NP3 neurons were characterized by the expression of neuropeptides involved in spinal cord transmission of itch (natriuretic polypeptide B, Nppb; somatostatin, Sst) as well as receptors for serotonin (5-hydroxytryptamine receptor 1F (Htr1f), leukotrienes (cysteinyl leukotriene receptor 2 (Cysltr2)), and IL-31 (Emery and Ernfors, 2018).

Innervation patterns of peptidergic and nonpeptidergic neurons

Nonpeptidergic and peptidergic neurons exhibit anatomically distinct innervation patterns in both the skin and the spinal cord. The majority of nonpeptidergic nociceptors preferentially innervate the epidermis, while peptidergic nociceptors innervate the skin and deeper tissues including the dermis, muscle, and visceral organs (Yang et al., 2013;

Zylka et al., 2005). Within the skin, NP1 MrgprD-expressing neurons innervate the stratum granulosum, the most superficial layer of the epidermis, while peptidergic neurons innervate the underlying stratum spinosum (Zylka et al., 2005).

Itch transmission

Nociceptive fibers enter the spinal cord through the dorsal root, travel rostrocaudally in Lisseur's tract of the spinal cord, and then dive ventrally to innervate the distinct laminae of the dorsal horn of the spinal cord. Peptidergic fibers innervate laminae I, V, and the outer region of laminae II, while non-peptidergic fibers innervate layer II ventral to these peptidergic fibers (Braz et al., 2005). Specifically, MrgrdEGFP fibers (NP1) were seen to innervate a boundary between the outer and inner layers of laminae II, which the Zylka group identified as the middle region of laminae II (Zylka et al., 2005). From the dorsal horn, projection fibers transmitting itch sensation ascend the spinal cord via the spinothalamic tract, where they project contralaterally to the ventrobasal and posterior thalamus (Davidson et al., 2012). Over the past decade several groups of spinal neurons have been identified as mediating itch sensations, including spinothalamic tract (STT) neurons (Andrew and Craig, 2001), gastrin-releasing peptide receptor $(GRPR)^{+}$ neurons (Sun and Chen, 2007; Sun et al., 2009), and neurons that express natriuretic peptide receptor A and gastrin releasing peptide ($Npra^{+}$/ GRP^{+} (Mishra and Hoon, 2013). Several neurotransmitters have been shown to activate spinal itch sensing neurons, including gastrin releasing peptide GRP (Sun and Chen, 2007), neuromedin B (NMB) (Mishra et al., 2012), and NPPB (Mishra and Hoon, 2013). In addition to the excitatory spinal

neuronal types mentioned above, researchers have identified inhibitory interneurons in the spinal cord (e.g., $BHLHB5^{+}$ (Ross et al., 2010) and $GLYT2^{+}$ (Foster et al., 2015).

Anterograde tracing of itch-selective GRP^{+} neurons in rodents have identified several regions of the brain thought to mediate itch sensation, including the thalamus, parabrachial nucleus (PBN), amygdala, S1, periaqueductal gray (PAG), and rostral ventromedial medulla (RVM) (Albisetti et al., 2019). Spinal projection neurons project to the thalamus as well as the parabrachial nucleus (PBN), which serves as a key itch processing nucleus (Dong and Dong, 2018). Another area of the brain which has been frequently studied in the field of itch is the PAG, a midbrain area shown to function in pain, analgesia, fear, anxiety, localization, and cardiac control (Behbehani, 1995). A recent study found that activation of PAG GABAergic neurons or inhibition of glutamatergic neurons resulted in attenuation of scratching in both acute and chronic pruritus (Samineni et al., 2019). In addition, they discovered that PAG GABAergic neurons, but not glutamatergic neurons, may encode the aversive component of itch. Taken together with previous studies linking activation of PAG to scratching, this suggests that PAG could represent a critical neuromodulatory hub for itch sensation.

Cortical activation of itch in humans

Functional magnetic resonance imaging in human subjects experiencing itchy stimuli revealed activation of the primary somatosensory cortical regions (S1) topographically corresponding to the stimuli (Dong and Dong, 2018). In addition to the activation seen in S1, itch stimuli were found to elicit activity in the secondary somatosensory cortex (S2)

and a variety of cortical regions in numerous imaging studies (Darsow et al., 2000; Hsieh et al., 1994; Leknes et al., 2007; Mochizuki et al., 2003). These cortical regions were found to be involved in emotional processing and reward (cingulate and prefrontal cortex, amygdala and the limbic systems), evaluation and self-awareness (insular cortex); as well as motor areas which are likely involved in initiating scratch behavior (Chen and Sun, 2020; Dong and Dong, 2018). Notably, the activation pattern seen in the brain of patients suffering from chronic itch differs from healthy controls, with cortical areas involved in affect and emotion (anterior and posterior cingulate cortex, insular cortex) showing greater activity in patients suffering from atopic dermatitis than healthy controls after iontophoresis of histamine (Ishiuji et al., 2009).

Theories of itch transduction

The existence of separate itch and pain pathways, and how these pathways interact, have been highly debated topics in the field of somatosensation. Primary sensory neurons known to convey itch stimuli also express TRPV1, a channel that is typically associated with capsaicin induced pain (Caterina et al., 1997). Thus, although the TRPV1 ligand capsaicin elicits pain sensation by activating neurons that only sense pain, it also simultaneously activates itch sensing neurons without producing pruritus. The temporary alleviation of itch by scratching has long been thought to occur through the activation of the pain pathway (Braz et al., 2014). In this section I will discuss three theories of itch transmission and include evidence that supports or contradicts those theories.

The intensity theory proposed that itch sensing neurons are polymodal and transmit itch when weakly activated and pain when strongly activated (von Frey, 1922). Evidence against this theory was provided by a study that showed pruritus induced by electrical stimulation of human skin did not convert to pain sensation with increased frequency of stimulation (Tuckett, 1982). Alternatively, decreasing the frequency of painful stimuli was not found to provoke itch sensation (Ochoa and Torebjörk, 1989).

The labeled line model proposed that itch and pain pathways are functionally and anatomically segregated in the central and peripheral nervous system. The concept of labeled-line coding for itch is supported by studies that demonstrate $GRPR^+$ dorsal horn neurons selectively mediate itch and not pain (Sun et al., 2009). Recently, the discovery of itch selective $MRGPRA3^+$ population of C fibers has also strongly supported the existence of labeled lines as deletion of these neurons leads to attenuated itch but not pain behavior (Han et al., 2013).

However, it is recognized that the majority of pruritogen-sensitive primary afferents and second-order spinal neurons also respond to noxious stimuli, suggesting the labeled-line theory does not fully explain itch transmission (Akiyama and Carstens, 2013). This led to the selectivity model (also known as population coding), which proposes that itch and pain neurons are polymodal, with itch sensation occurring when selective subsets of itch neurons are activated alone and pain sensations dominating when itch and pain neurons are activated together (Ma, 2012). This theory incorporated new findings of spinal inhibition into the labeled line theory. Two studies of the vesicular glutamate transporter 2 (VGLUT2) in nociceptors provided supporting evidence for the

selectivity model by identifying a population of peripheral neurons that include the itch-selective subset (Lagerström et al., 2010; Liu et al., 2010b).

1.5 Studying itch

The classic definition of itch is an irritating sensation that elicits a desire to scratch. As such, rodent models for pruritus use scratching behavior as an indication of itch sensation. Initial experiments of acute itch were conducted by injecting pruritogens in the nape of the neck of the mouse, and quantifying scratching behavior (Kuraishi et al., 1995). One limitation of injecting in the nape of the neck was the finding that neck injection of the pain-inducing compound capsaicin also resulted in scratching behavior (Shimada and LaMotte, 2008). The desire for researchers to be able to distinguish between pain and itch in mice led to the development of the "cheek injection model" (Shimada and LaMotte, 2008). This model found that mice exhibit distinct behaviors in response to cheek injection of pruritogens versus algogens (cause pain), as cheek injection with a pruritogen evoked hindpaw scratching and cheek injection with an algogen elicited facial wiping.

Injection of pruritogens is used to evoke only acute itch-scratching behavior. To study chronic itch, researchers have developed numerous mouse models of dermatological diseases including atopic dermatitis (Li et al., 2006), xerosis (dry skin) (Miyamoto et al., 2002), psoriasis (Sakai et al., 2016), and allergic contact dermatitis (Qu et al., 2014; Scott et al., 2002). Many of these models have been used to probe the function of transgenic mice lacking pruritic candidate genes.

. Studies utilizing these mouse models have highlighted the important role nonpeptidergic neurons, and their expression of TRP channels, have in mediating chronic itch sensation. Loss of MRGPRA3^{+} also led to a significant decrease in scratching in a mouse model of allergic itch, suggesting that these neurons contribute at least partially to chronic itch (Han et al., 2013). Importantly, TRPA1 deficient mice display significantly decreased itch and decreased skin thickening in allergic itch models (Han et al., 2013). An additional study of allergenic itch found that loss of TRPA1 resulted in decreased skin edema, immune cell infiltration, and cytokine levels (Liu et al., 2013). These studies highlight the important role of TRPA1 in itch transduction as well as the inflammatory changes that occur within the skin to promote chronic itch.

1.6 Conclusion

Despite the considerable knowledge accumulated over the last 50 years in the field of somatosensation, much more work is needed to identify the complex molecular and cellular mechanisms underlying itch. Elucidation of these key mediators will allow for the creation of more effective therapeutic treatment for patients suffering from acute and chronic itch. In chapter 2, I will discuss my work on identifying the role of TRPC3 in antagonizing contact dermatitis induced itch sensation and propose a new model of gating itch.

CHAPTER 2

TRPC3 Antagonizes Pruritus in A Mouse Contact Dermatitis Model

This chapter is adapted from:

Katherine Beattie, Haowu Jiang, Mayank Gautam, Mary K. MacVittie, Barbara Miller, Minghong Ma, Qin Liu, and Wenqin Luo**.** *TrpC3* Antagonizes Pruritus in A Mouse Contact Dermatitis Model. *Currently under review at the Journal of Investigative Dermatology.*

2.1 Abstract

Contact dermatitis (CD), including allergic and irritant CD, are common dermatological diseases and characterized by an erythematous rash and severe itch. In this study, we investigated the function of TRPC3, a canonical TRP channel highly expressed in type 1 non-peptidergic (NP1) nociceptive primary afferents and other cell types, in a mouse CD model. Though *TrpC3* null mice had little deficits in acute somatosensation, they showed significantly increased pruritus with CD. In addition, *TrpC3* null mice displayed no differences in mechanical and thermal hypersensitivity in an inflammatory pain model, suggesting that this channel preferentially functions to antagonize CD-induced itch. Using dorsal root ganglia (DRG) and pan-immune-specific *TrpC3* conditional KO (CKO) mice, we determined that *TrpC3* in DRG neurons, but not in immune cells, is required for this function. Furthermore, the number of MRGPRD^{+} NP1 afferents in CD-affected DRGs is significantly reduced in *TrpC3* mutant mice. Taken together, our results suggest that *TrpC3* plays a critical role in NP1 afferents to cope with CD-induced excitotoxicity, and that degeneration of NP1 fibers may lead to an increased itch of CD. Our study identified a novel role of *TrpC3* and NP1 afferents in CD pathology.

2.2 Introduction

Contact dermatitis (CD) is a common group of skin diseases, which include irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) (Nosbaum et al., 2009). ICD is the clinical manifestation of a local inflammatory reaction of the skin following exposure to a physical or chemical agent (Ale and Maibach, 2014). In ACD, activation of memory T cells, by re-exposure to allergens, causes an inflammatory cascade that results in skin injury and enhanced itch (Kostner et al., 2017). Itch sensation is a hallmark and main complaint of patients with CD, which can lead to a negative cycle of scratching, skin excoriations, and even worse itch sensation. These symptoms significantly affect patients' quality of life (Pereira and Ständer, 2017). At present, treatment of CD and CD-induced itch is limited due to our incomplete knowledge of the molecular mechanisms and difficulty in identifying the exact offending agent.

CD-induced itch sensation is generated by interactions of the primary sensory afferents, the immune system, and the integumentary system in the periphery. External and internal pruritogens are detected by free nerve terminals in the skin, whose cell bodies are in the dorsal root ganglion (DRG) and trigeminal ganglion. The signals are then relayed through the spinal cord to the brain to generate the perception of "itch". The immune system plays a critical role in triggering pathological pruritus by releasing inflammatory mediators that can directly activate pruritoceptors (Storan et al., 2015). Keratinocytes, the resident epithelial cells which comprise the epidermis, promote chronic itch through both direct (barrier disruption allowing pruritogen entry) and indirect mechanisms (cytokine release) (Schwendinger-Schreck et al., 2015).

Transient receptor potential (TRP) channels in mammals comprise a superfamily of over 30 membrane-bound proteins that form tetrameric non-selective cation channels and function in a variety of sensory pathways, including itch sensation (Julius, 2013; Montell, 2011; Sun and Dong, 2016). Canonical TRP family members (TRPC) can function downstream of a G-protein coupled receptor (GPCR) or as receptors by themselves (Chen et al., 2020). TRPC channels can integrate several types of intracellular signals into changes in membrane potential and calcium entry (Chen et al., 2020). Indeed, deregulation of TRPC channels can disrupt calcium homeostasis and lead to cell damage and neuronal death (Chen et al., 2020; Jeon et al., 2021).

TRPC3 is a TRPC family member that is highly expressed in DRG neurons (Dong et al., 2017; Luo et al., 2009; Quick et al., 2012). In addition, it is also expressed in Purkinje cells, cholinergic neurons, thalamic glutamatergic neurons, and immune cells (Wenning et al., 2011; Zeisel et al., 2018). Within DRG, *TrpC3* is mainly expressed in non-peptidergic (NP) nociceptors: highest in the NP1 primary afferents, marked by a GPCR MRGPRD, and followed by NP2 and NP3 primary afferents, which are marked by MRGPRA3 and a neuropeptide natriuretic polypeptide B (NPPB), respectively (Usoskin et al., 2015; Zeisel et al., 2018). NP1 is a polymodal afferent for sensing mechanical force, chemicals, and temperature, whereas NP2 and NP3 afferents are itch selective (Cranfill and Luo, 2021). Despite the high expression of *TrpC3* in nonpeptidergic DRG neurons, *TrpC3* null mice exhibited few deficits in a variety of behavioral tests for acute pain and itch sensation, mechanosensation, and thermosensation (Dong et al., 2017; Quick et al., 2012).

Here we examined the role of *TrpC3* in a mouse CD model, which is induced by repeated application of squaric acid dibutylester (SADBE). Though this model was initially established as an ACD model (Qu et al., 2015), a later study using this model also found ICD features, including that itch could be induced in the absence of lymphocytes and that SADBE could directly activate primary sensory neurons (Feng et al., 2017). In contrast to the minor deficits in acute somatosensory assays (Dong et al., 2017; Quick et al., 2012), *TrpC3* null mice displayed a significant increase in spontaneous scratching of this SADBE-CD model, suggesting that TRPC3 functions to antagonize CD-induced itch. We generated DRG neuron and immune cell specific *TrpC3* CKO models and found that *TrpC3* in DRG is required for modulating itch sensation. Moreover, we performed immunohistochemistry with the DRG, skin, and spinal cord of affected and unaffected regions of control and *TrpC3* null mice and revealed a significant reduction in the number of MRGPRD^{+} NP1 nociceptors innervating the affected region of mutant mice. Since TRPC3 is a known mediator of calcium homeostasis and plays a critical role in excitotoxicity (Alkhani et al., 2014; Jeon et al., 2021), we propose a model that *TrpC3* null MRGPRD^{+} NP1 nociceptors are susceptible to excitotoxicity induced by this CD model and that degeneration of NP1 neurons disinhibits the itch pathway, resulting in an increased amount of scratching in *TrpC3* mutant mice.

Results

2.3 *TrpC3* antagonizes itch sensation in a mouse CD model

Given the high expression of *Trpc3* in DRG neurons, it was surprising that almost no behavioral deficits in acute sensation were identified in *TrpC3* null mice (Dong et al., 2017; Hirschler-Laszkiewicz et al., 2012). To further explore its function in somatosensation, we examined *TrpC3* null mice using a CD model (Qu et al., 2014). Briefly, adult male and female *TrpC3* null and wild type (WT) control mice were treated with SADBE on their abdomens and then re-challenged with SADBE at their necks after a week. Their spontaneous behavior was recorded and quantified on Day 16 (Figure 2.1a). As previously described (Dong et al., 2017; Hirschler-Laszkiewicz et al., 2012), the *TrpC3* null allele was created by excision of exons 7 and 8 (Figure 2.1b), which encode the pore-defining region of the channel, resulting in a transcript with premature stop codon and non-functional TRPC3. SADBE treatment induced comparable ulcerative thickness and lymphocytic infiltration in WT and null mice (Figure 2.1d-f), suggesting that the absence of TRPC3 did not impede the typical skin pathology associated with the CD model. Interestingly, ablation of *TrpC3* resulted in a marked increase in spontaneous scratching bouts in two independent cohorts, which were conducted at the laboratories of Washington University (Cohort W) and the University of Pennsylvania (Cohort P) (Figures 2.1g-h), respectively. The average scratching bout numbers in the Cohort P were higher than that in Cohort W because this cohort excluded mice that were inactive for more than 50% of the 1-hour recording time (the same criterion was used for the following experiments). We further analyzed the scratching behavior by examining the

average scratch duration per bout and the number of scratching bouts in 5-minute bins. No difference in bout duration was found between genotypes (Figure 2.1i), but a statistically significant increase in the high scratching bout numbers/bin was seen in *TrpC3* null mice (Figure 2.1j-k). *TrpC3* null mice also displayed a marked increase in wiping behavior in this CD model (Figure 2.1l). In short, our results suggest *TrpC3* ablation leads to an enhanced pathological itch in the SADBE-CD model.

2.4 *TrpC3* null mice display no difference in mechanical allodynia, thermal sensitivity, or gait

To determine whether *TrpC3* is also required in chronic inflammatory pain sensation, Complete Freund's Adjuvant (CFA) was injected in the hindpaw to induce inflammatory pain. Dynamic and static mechanical allodynia, as well as thermal sensitivity, were tested before and after CFA treatment. No significant differences were found for any of these behavioral assays (Figure 2.2a-c), suggesting that *TrpC3* is dispensable for modulating pain sensation in this inflammatory pain model. Of note, a *TrpC3* gain-of-function point mutation mouse line, Moonwalker (*Mwk)* mice, caused Purkinje cell degeneration and cerebellar ataxia (Becker et al., 2009). To test whether *TrpC3* null mice had the same deficit, we performed a footprint assay, measured their gait width, and calculated the alternative coefficient. We found no statistically significant differences in gait width and alternative coefficient (Figure 2.2d-f). These results are consistent with our previous finding that *TrpC3* null mice displayed normal motor coordination on the rotarod assay

(Dong et al., 2017). Taken together, these findings highlight the preferential requirement of *TrpC3* in modulating CD-induced pruritus.

2.5 *TrpC3* in DRG neurons is required to antagonize CD-induced itch

Although *TrpC3* was shown to be expressed in human keratinocytes (Tu et al., 2005), we found its expression in the mouse skin was negligible (data not shown). Thus, we focused on determining TRPC3 function in neuronal and immune cells in modulating CD-induced itch. We generated *TrpC3* conditional knockout (CKO) mice by crossing a genetic allele, in which exons 7 and 8 were floxed by loxP sites (Hirschler-Laszkiewicz et al., 2012), with different Cre lines (Figure 2.3a). Given the high expression level of *TrpC3* in DRG neurons, we first generated a CKO mouse line that specifically ablate the expression of *TrpC3* in DRG nociceptors, including all NP afferents, using the $TrpV1^{Cre}$ mouse line (Figure 2.3b-c) (Cavanaugh et al., 2011). We then induced CD in control ($TrpC3^{f/f}$) and TrpV1-CKO ($TrpV1^{Cre}$; $TrpC3^{f/f}$) mice. Interestingly, TrpV1-CKO mice showed a significant increase in scratching bouts when compared to control mice (Figure 2.3d). Similar to the null mice, the TrpV1-CKO mice scratched with a greater frequency than the control mice (Figure 2.3e-f) but displayed no difference in the average scratch bout duration (Figure 2.3g). The number of wiping bouts was not significantly changed in TrpV1-CKO mice (Figure 2.3h). These results indicate that *TrpC3* in DRG neurons is required for antagonizing CD-induced itch.

2.6 *TrpC3* in immune cells is not required to modulate CD-induced itch

To determine TRPC3 function in the immune system, we ablated *TrpC3* expression in pan-immune cells utilizing the $Vav1^{iCre}$ line (Figure 2.4a) (Yang et al., 2008) and induced CD in control ($TrpC3^{f/f}$) and Vav1-CKO ($Vav1^{iCre}$; $TrpC3^{f/f}$) mice. No differences in spontaneous scratching behavior (including bout number, duration per bout, and frequency) or wiping were found between the genotypes (Figure 2.4b-d). These findings are consistent with the histological result: similar amount of inflammatory cell infiltration in affected skin of *TrpC3* null and WT mice (Figure 2.1d-f). Together, our data suggest that *TrpC3* in the immune cells is not required to modulate itch behavior and/or skin inflammation in SADBE-induced CD (Figure 2.4b-f).

2.7 Degeneration of $MRGPRD^{+}$ neurons in the CD-affected region of *TrpC3* null mice

To reveal potential mechanisms underlying the increased CD-induced itch sensation of *TrpC3* mutant mice, we performed immunohistochemistry with the DRG, skin, and spinal cord of the affected cervical region of control and *TrpC3* null mice. Since *TrpC3* shows a highly overlapped expression with *Mrgprd* (Dong et al., 2017), we utilized the $Mrgprd^{EGFP(+/-)}$ allele (Zylka et al., 2005) to visualize the NP1 afferents. We found a significant reduction in $IB4^{+}$ (marker of NP1 neurons) and GFP^{+} DRG neurons and a reduction of GFP^{+} fibers innervating the lamina II of the spinal cord (Figure 2.5a-c). In contrast, the number and central terminals of peptidergic ($CGRP^{+}$) neurons, which normally had low expression level of *TrpC3*, did not differ between genotypes (Figure

2.5a-b). Though $CGRP^+$ and GFP^+ fibers normally innervate the epidermis layer of the skin (Zylka et al., 2005), both types of intraepidermal free nerve terminals were completely lost in the SADBE treated neck skin, and only few dermis innervating fibers remained. No significant difference was found in the number of these remaining dermis nerve fibers (Figure 2.5d). Together, these results indicate that *TrpC3* null $MRGPRD^+$ NP1 neurons innervating the CD-affected skin region degenerate and die.

Since our previous data demonstrated a normal number of $MRGPRD^+$ DRG neurons and spinal cord and skin innervation in untreated juvenile *TrpC3* null mice (Dong et al., 2017), this new result suggests either an age-dependent increase in cell death or an increased cell death caused by the SADBE-CD model. To differentiate the two possibilities, we conducted immunohistochemistry on lumbar DRGs that innervate the untreated mouse skin (Figure 2.6a). No differences in $MRGPRD^+$ neurons, nonpeptidergic (IB4), or peptidergic (CGRP) neurons were found (Figure 2.6b). This result suggests that the decrease in cervical $MRGPRD^+$ (NP1) neurons of *TrpC3* null mice is due to an increased cell death caused by the SADBE-CD model.

2.8 Discussion

In this study, we identified a novel function of TRPC3 in somatosensation: antagonizing pathological itch sensation of the SADBE-CD model. We determined that *TrpC3* in DRG neurons, but not in immune cells, is required for this function. Interestingly, in the affected region of *TrpC3* null mice, there is a marked reduction in the number of $MRGPRD^+$ DRG neurons, where *TrpC3* is normally expressed at the highest level. Based

on these results, we propose a new model: in WT mice at the baseline condition, *TrpC3* is highly expressed in NP1 neurons, which antagonize the itch pathway mediated by NP2 and NP3 neurons (Figure 2.6c). Upon CD induction, NP1 neurons degenerate in the absence of *TrpC3*, which leads to the disinhibition of the itch pathway and results in increased itch sensation/behaviors (Figure 2.6c).

TRPC3 is a non-selective cation channel which mediates calcium homeostasis and sensitization of primary nociceptors through its ability to engage in both receptor-operated calcium influx and in store-operated calcium entry (Alkhani et al., 2014). In addition, TRPC3 can be coupled to metabotropic glutamate receptors (Hartmann et al., 2008) and is required for IgG immune complex-induced excitation of DRG neurons (Qu et al., 2012). Disruptions in *TrpC3* expression or TRPC3 activity may lead to dysfunction of calcium homeostasis and cell death. A gain-of-function mutation of TRPC3 in *Mwk* mice led to increased intracellular Ca^{2+} concentration, which resulted in purkinje cell death and ataxia (Becker et al., 2009). An additional mouse model of spinocerebellar ataxia found that *TrpC3* was downregulated before the onset of neural degeneration (Lin et al., 2000). Since NP1 afferents have the highest level of *TrpC3* expression, 2-5-fold higher than its expression in NP2 and NP3 afferents (Zeisel et al., 2018), the ablation of *TrpC3* should disrupt physiological processes, such as the calcium homeostasis, in NP1 afferents to the most extent. We speculate that this deficit renders NP1 neurons susceptible to chronic excitotoxicity induced by SADBE-CD (Figure 2.6c).

MRGPRD is expressed in ~20% of mouse DRG neurons whose afferents form the densest innervation of the epidermis, specifically in the stratum granulosum (Zylka et al., 2005). $MRGPRD^{+}$ (NP1) neurons are polymodal and respond to noxious heat,

mechanical pain, inflammatory pain, and pruritogens such as β-alanine (Abdus-Saboor et al., 2019; Cavanaugh et al., 2009; Liu et al., 2012a; Shinohara et al., 2004). Unlike the NP1 subpopulation, NP2 (which express *Mrgpra3* and *Mrgprc11*) and NP3 (which express somatostatin, the interleukin-31 receptor A (*IL31r*), and *Nppb*) neurons predominantly mediate histaminergic and non-histaminergic itch (Han et al., 2013; Liu et al., 2009; Mishra and Hoon, 2013). Some recent studies further highlighted the critical role of NP2 and NP3 afferents in mediating dermatitis-induced itch (Solinski et al., 2019; Wang et al., 2021; Zhu et al., 2017). Though it is generally believed that the "pain" pathway antagonizes the "itch" pathway (Lagerström et al., 2010; Liu et al., 2010b), whether the NP1 pathway could inhibit the NP2/NP3 itch pathway has not been directly tested and established. Our study revealed that *TrpC3* mutant mice had increased itch sensation but decreased number of NP1 neurons when challenged with the SADBE-CD model. A possible model to explain these data is that the NP1 pathway normally inhibits the NP2/NP3 itch pathway, so NP1 afferent degeneration leads to disinhibition of the itch pathway, resulting in an increased itch phenotype. Future experiments by directly manipulating NP1 neuronal activities while stimulating NP2 and/or NP3 afferents will be needed to test and establish this model.

In summary, our study discovered a new function of TRPC3 in modulating itch sensation of a mouse CD model. The molecular and cellular mechanisms we identified here bring novel insights into pathology and treatment strategy of CD.

2.9 Materials and Methods

Mice

Three to four-month-old male and female mice were used for all experiments. *TrpC3* null mice and floxed TrpC3 mice were imported from Dr. Barbara Miller's lab at Pennsylvania State College of Medicine (Hirschler-Laszkiewicz et al., 2012). C57BL/6J (000664), *Trpv1*^{Cre}(017769), and *Vav1*^{iCre}(008610) mice were purchased from Jackson Laboratories (Cavanaugh et al., 2011; Yang et al., 2008). All experiments were conducted in accordance with the National Institute of Health guidelines and with approval from the Animal Care and Use Committee of University of Pennsylvania and Washington University School of Medicine. Mice were housed in a 12-hour light/dark cycle with food and water *ad libitum*.

Behavior

Contact dermatitis model

A murine model of contact dermatitis was produced with repeated topical application of 25 µl of squaric acid dibutyl ester (SADBE, 1% in acetone, Sigma, 339792) on shaved skin. The protocol timeline was adapted from previously described procedures as summarized in Figure 2.1 (Qu et al., 2014; Scott et al., 2002). On day 16, spontaneous behavior was recorded for 1 hour (23 hours after the treatment), and scored for scratching behavior, wiping behavior, and inactive periods by researchers blinded to mouse genotype. Scratching bouts were defined as an uninterrupted movement of the hindpaw directed at the treated site and ended when paws were placed on the floor, licked, or

paused in the air for more than 1 second. A wipe was defined as a downward movement of the forepaw to the head/neck. Scratch frequency distribution graphs were made by dividing the 1-hour video into 5-minute bins, quantifying the number of bouts that occurred in each interval, and sorting the interval into bins based on bout number. Then the percentage of low (< 60 bouts/bin) and high (≥ 60 bouts/bin) scratch bins were calculated and compared. Experiments on two cohorts of mice were independently conducted at the Washington University of St. Louis (Cohort W) and the University of Pennsylvania (Cohort P).

Inflammatory pain behaviors

Baseline behavior recordings were taken 1-3 days before hindpaw injection of 10 µl CFA (Sigma, St. Louis; suspended in a 1:1 emulsion of saline and heat-killed Mycobacterium tuberculosis in mineral oil, final concentration 5µg/10 µl) or saline (vehicle). For static mechanical sensitivity assay, mice were placed under plexiglass chambers on a raised wire mesh platform (Ugo Basile, Italy) daily for 2 days for habituation and 45 minutes prior to testing day. The up-down method was used to assess paw withdrawal threshold (Chaplan et al., 1994). Eight calibrated and logarithmically spaced von Frey monofilaments (Bending forces: 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6 and 1 g; Stoelting, Wood Dale, IL) were applied perpendicular to the plantar hind paw (until the filament slightly buckled) for 3-4 seconds. The middle filament (0.16 g) was applied to the hind paw. Depending on the mouse response, incrementally lower filaments were applied (after withdrawal response) or incrementally higher filaments were applied (no response). The experimenter waited 3-4 minutes between filament

administrations to avoid sensitization or learning. Rapid withdrawal of paw away from the fiber within 4-5 seconds (flicking or licking the stimulated paw) was considered a positive response. After the initial elicited response, testing continued for until 4 filaments were applied (heavier or lighter depending on the last response). The final 50% withdrawal threshold was obtained using the following equation, where X_f= value of the final von Frey filament used (in log units); k = tabular value for the pattern of positive/ negative value and δ = mean difference (in log units) between stimuli (Chaplan et al., 1994):

$$50\% \text{ withdrawal threshold (g)} = (10[X_f+k\delta])/10{,}000$$

Dynamic mechanical hypersensitivity was tested by lightly stroking the plantar surface of the hindpaw with a paintbrush (moving heel to toe) and observing paw withdrawal behavior (Figure 2.2B) (Cheng et al., 2017). The behavioral responses observed were then assigned numerical scores. A brief paw lift (~1 s) would be given a score of 0. Cases of sustained lifting (>2 s) or slight flinching of the stimulated paw received a 1. Lateral lifting above the body or jumping were scored as 2. The highest score, 3, was reserved for multiple flinching responses or licking of the paw. The test was repeated 3X, with 3–4-minute rest intervals, and the average score per mouse was calculated.

Thermal hyperalgesia was measured using Hargreaves apparatus (Cheah et al., 2017). For the Hargreaves plantar test, animals were placed in Plexiglas chambers over the glass surface of Hargreaves apparatus (UCSD instruments, San Diego, CA, USA) and acclimatized for 45 minutes prior to testing. A thermostat was used to check the constant

temperature of the glass plate (~25°C). Heat was applied to the middle plantar surface by a bulb beneath the glass. A positive stimulus response was characterized as paw flick or licking. Cutoff time was 20 s. The latency period was determined by averaging three measurements (taken at 10-minute intervals).

Gait

Gait analysis of paw prints were conducted as previously published (Becker et al., 2009; Clark et al., 1997). Gait width was measured as the average lateral distance between right and left step pairs. The alternative coefficient was calculated by determining the mean of the absolute value of 0.5 minus the ratio of right-left step distance to right-right step distance for every right-left step pair.

Hematoxylin and eosin staining, Imaging, and Quantification

On day 17, treated and untreated neck skin was collected and fixed overnight in 4% buffered paraformaldehyde at 4°C (Thermo Scientific, AAJ19943K2). Tissue was then trimmed, placed in cassettes (Azer Scientific, ES6010-W), and processed into paraffin wax (Paraplast Extra, Leica, 39603002) using a tissue processor (Sakura, VIP5B) with the following schedule: 70% ethanol, 80% ethanol, 95% ethanol (Azer Scientific, ES753), 3X 100% ethanol (Azer Scientific, ES631), 3X xylene, 4X paraffin wax (Paraplast Plus, Leica, 39602004). All steps were carried out at ambient temperature except for paraffin which was at 60 °C. Following embedding (Leica, EG1160) in paraffin wax (Paraplast Extra, Leica, 39603002), wax blocks were cooled on ice and

sectioned at a thickness of 5 μM using a rotary microtome (Leica, RM2125). Ribbons were floated on water at 41 degrees C and two sections were collected per positively charged glass slide (Fisher Scientific, 12-550-15). After drying overnight, slides were heated at 60 degrees C for 30 minutes and then stained with hematoxylin (Leica, cat#3801540) and eosin (Leica, 3801600) using an automated stainer (Leica, Autostainer XL). Images were collected at 20X magnification utilizing brightfield microscopy. Analysis of cellular infiltration and ulcer thickness occurred in Fiji.

RT-PCR

Adult (≥4-month-old) mice were deeply anesthetized with CO_2 and DRGs, cerebellum, and spleen tissue were dissected under RNase free conditions. Tissue was mechanically homogenized, RNA was isolated using the RNeasy Micro Kit (Qiagen 74004), and cDNA was synthesized with oligo-dT primers using the SuperScript First-Strand Synthesis system (Invitrogen 18080051). RT-PCR was performed on cDNA with primers for *TrpC3* (forward primer CCTGGCTTTCATGATTGGCATGTTC for exon 6 and reverse primer CACTCACATCTCAGCACACTGGGG for exon 11).

Immunostaining

Mice were anesthetized with ketamine and xylazine and transcardially perfused with 4% PFA (in PBS) on day 16. Immunostaining was performed according to previously described protocol (Dong et al., 2017; Olson et al., 2017)). Cervical (C2-C5) and lumbar (L2-L5) DRG and spinal cord (SC) were dissected and post-fixed for 2-4 hours in 4%

PFA at 4°C. Neck skin was dissected and post-fixed in 4% PFA O/N. All tissues were cryoprotected in 30% sucrose in PBS O/N at 4°C and embedded in OCT. DRG sections were sliced at 20μm, collected on Superfrost Plus slides, and dried O/N at room temperature. Neck skin and SC were sliced at 20 μm and 30 μm, respectively, and processed as floating sections. Primary antibodies used include chicken anti-GFP (1:2,000 (skin), 1:1,000 (SC, DRG); Aves, GFP-1020), rabbit anti-GFP (1:1,000, Invitrogen, A-11122), rabbit anti-CGRP (1:1,000; Immunostar, 24112), Guinea pig anti-VGLUT1 (1:1,000, Millipore, AB5905), and Alexa 594 conjugated IB4 (1:500; Life Sciences, I21413). Anti-GFP antibodies were used to label the expression of GFP in *Mrgprd*^{EGFP} mice. Secondary antibodies (all 1:500) were Alexa 488 conjugated goat anti-chicken antibody (Invitrogen, A-11039), Alexa 594 conjugated goat anti-rabbit antibody (Invitrogen, A21207), and Alexa 647 conjugated goat anti-guinea pig antibody (Jackson Immunoresearch, 106-605-003). Images were collected using a Leica SP5 confocal microscope.

Data Analysis

All data, except for frequency graphs and Figure 2.2 graphs, are presented as mean ± standard error of mean (SEM) and analyzed using two-tailed *t*-tests. Frequency graphs are presented as the mean percentage of low and high scratch bins and analyzed with Chi-squared tests. Figure 2.2 graphs were analyzed using two-way ANOVA. Statistical analysis was performed using GraphPad Prism. Differences were considered significant if $p < 0.05$.

Acknowledgements

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2.10 Figures

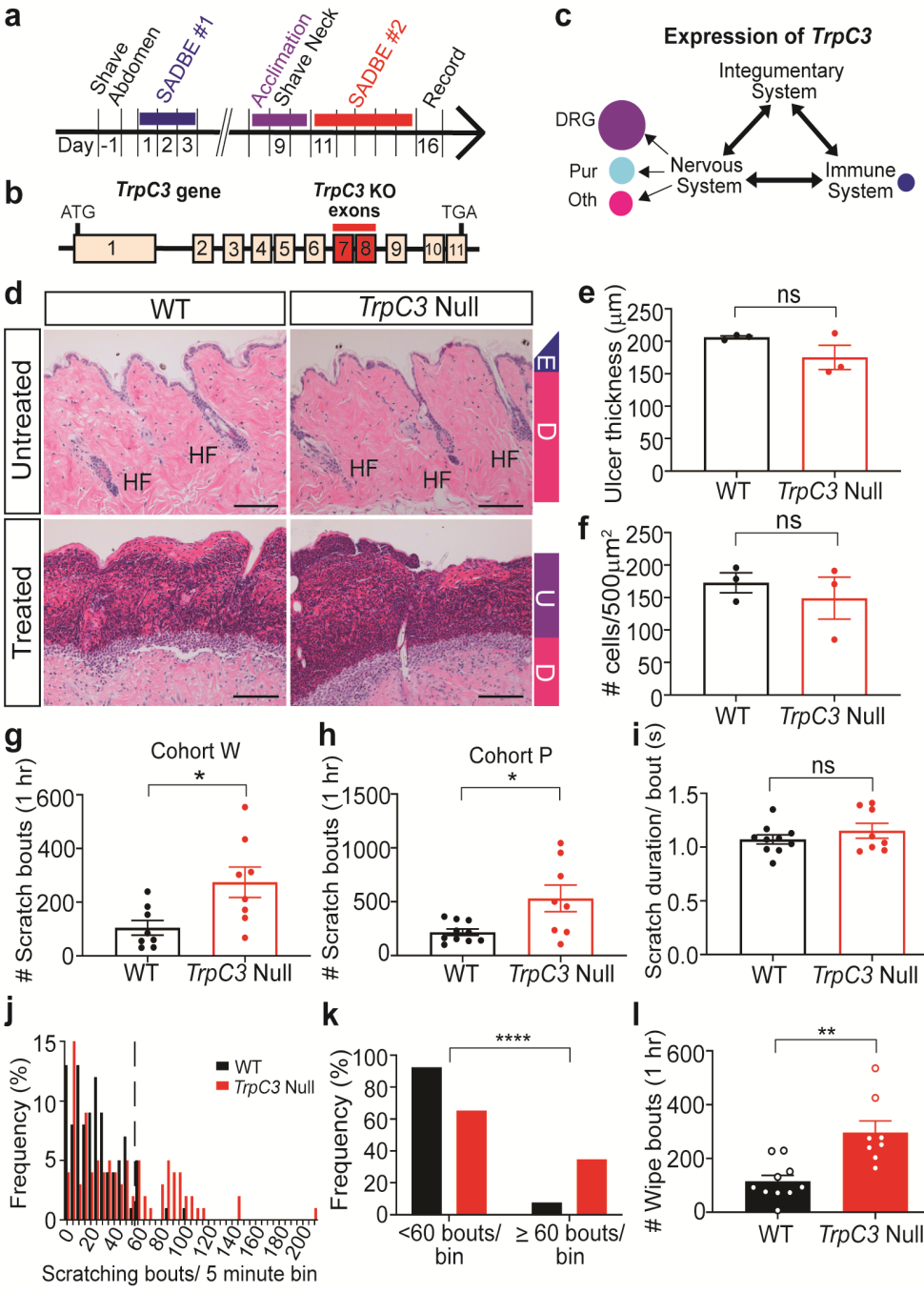

Figure 2.1. *TrpC3* antagonizes itch sensation in the SADBE-CD model. **a.** Protocol and timeline of SADBE-induced CD model. **b.** Diagram of the *TrpC3* gene, depicting

excision of exons 7–8 in null mice. **c.** Schematic representation of relative expression levels of *TrpC3* (based on mouse single neuron RNAseq data) in systems relevant to CD induced itch sensation. **d.** H & E staining of skin from SADBE-treated mice with dermal layers labeled: epidermis (E), dermis (D), ulcer (U). **e-f.** Quantification of ulcer thickness and cellular infiltration of treated neck skin (≥10 measurements/mouse, n=3). **g-h.** Quantification of spontaneous scratch behavior of *TrpC3* null and WT mice on day 16 of the SADBE model (Cohort W, n=8; Cohort P, n=8-10). **i.** Quantification of scratch duration per bout (s). **j.** Frequency distribution of scratch bout numbers in 5-minute bins. **k.** Bar graph comparing the percentage of low (<60 bouts) and high (≥60 bouts) scratching bout bins. **l.** Quantification of wiping bouts**.** Scale bars = 50 μm. Student's two-tailed *t* test (e-i, l). Chi-squared test (k). ns, not significant. *Asterisks* indicate statistical significance. *p<0.05, **p<0.01; error bar, SEM.

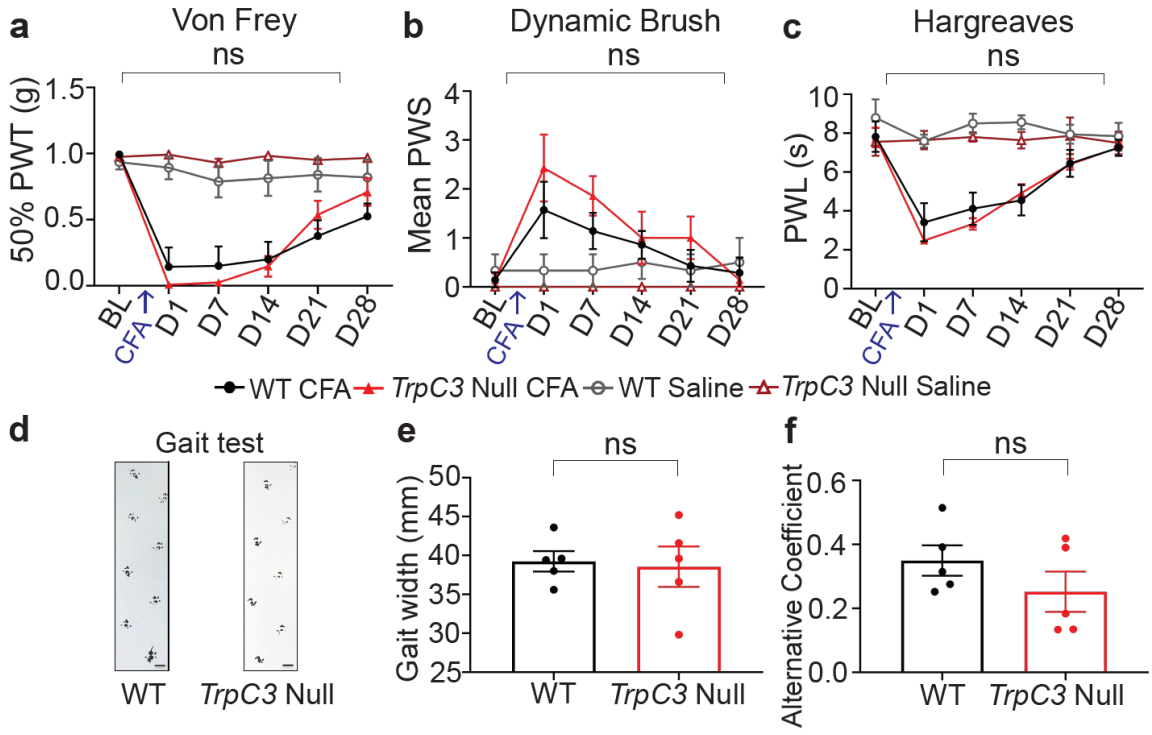

Figure 2.2. TRPC3 ablation does not alter mechanical allodynia, thermal hyperalgesia, or gait. Behavioral assays with WT and *TrpC3* null mice after CFA (*n*=7) or saline (*n*=6) injection. **a.** 50% Paw withdrawal threshold (PWT) in response to von Frey filaments. **b.** Paw withdrawal score (PWS) in response to dynamic brush stimulation. **c.** Paw withdrawal latency (PWL) in Hargreaves test. **d.** Representative footprints of *TrpC*3 null and WT mice in gait assay. **e-f.** Quantification of gait width and the alternative coefficient (indicating step alternation uniformity) (*n*=5). Scale bar = 100 μm. Two-way ANOVA (a-c); Student's one-tail *t* test (e-f); error bar, SEM.

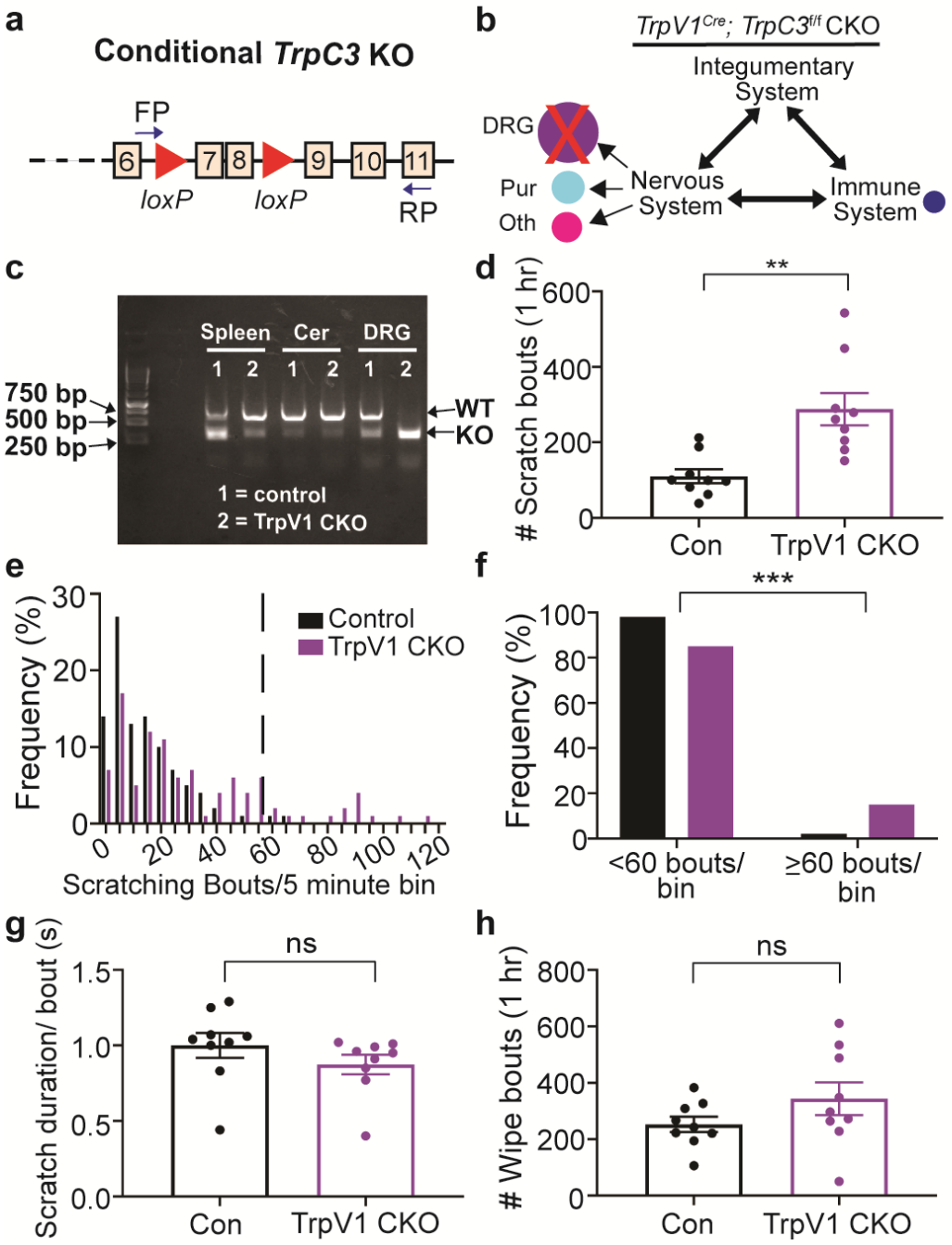

Figure 2.3. *TrpC3* is required in DRG neurons to antagonize CD-induced itch.

a. Diagram of floxed *TrpC3* gene illustrating the loxP sites surrounding exons 7 and 8 of *TrpC3* gene and the location of the forward (FP) and reverse RT-PCR primers (RP). **b.** Schematic representation of knocking out *TrpC3* expression in DRG neurons. **c.** RT-

PCR performed on RNA isolated from floxed control and TrpV1 CKO DRG, cerebellum, and spleen tissue. **d.** Quantification of scratch behavior of *TrpV1* CKO and control mice on Day 16 (n =9). **e.** Frequency distribution of bout numbers in 5-minute bins. **f.** Bar graph comparing the total percentage of low (<60 bouts) and high (≥60 bouts) scratching bout bins. **g.** Quantification of scratch duration per bout (s). **h**. Quantification of wiping bouts. Student's two-tailed *t* test (b, e, f); Chi-squared test (d). *Asterisks* indicate statistical significance. **p<0.01; error bar, SEM.

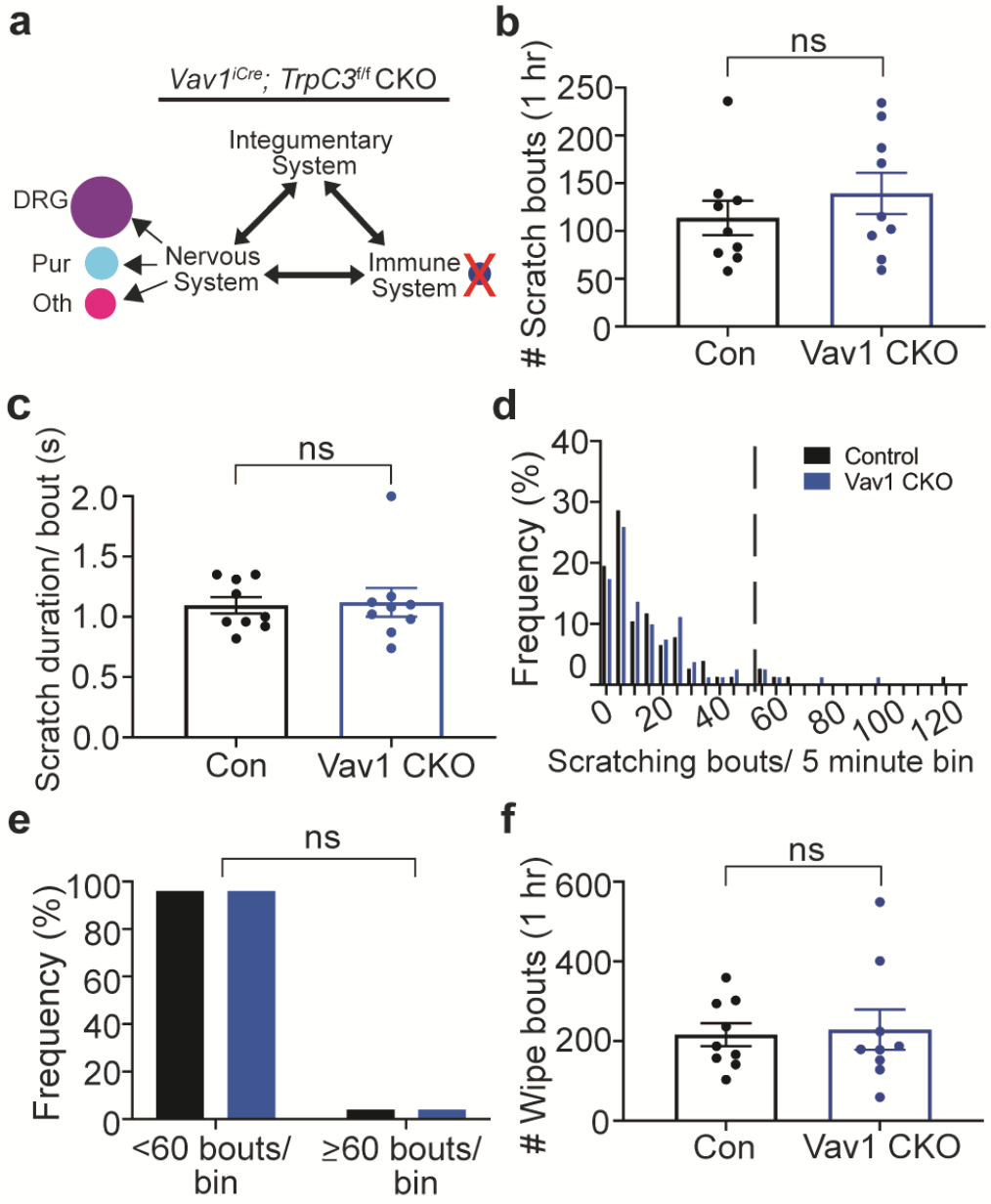

Figure 2.4. *TrpC3* in immune cells is not required to modulate CD-induced itch. a. Schematic illustration showing ablation of *TrpC3* from immune cells**. b.** Quantification of scratch bouts of *Vav1*-CKO and control mice on Day 16 (*n* =9). **c.** Quantification of scratch duration per bout (s). **d.** Frequency distribution of bout numbers in a 5-minute bins. **e**. Bar graph comparing the percentage of low (<60 bouts) and high (≥60 bouts)

scratching bout bins. **f**. Quantification of wiping bouts**.** Student's two-tailed *t* test (c, d, g); Chi-squared test (f); error bar, SEM.

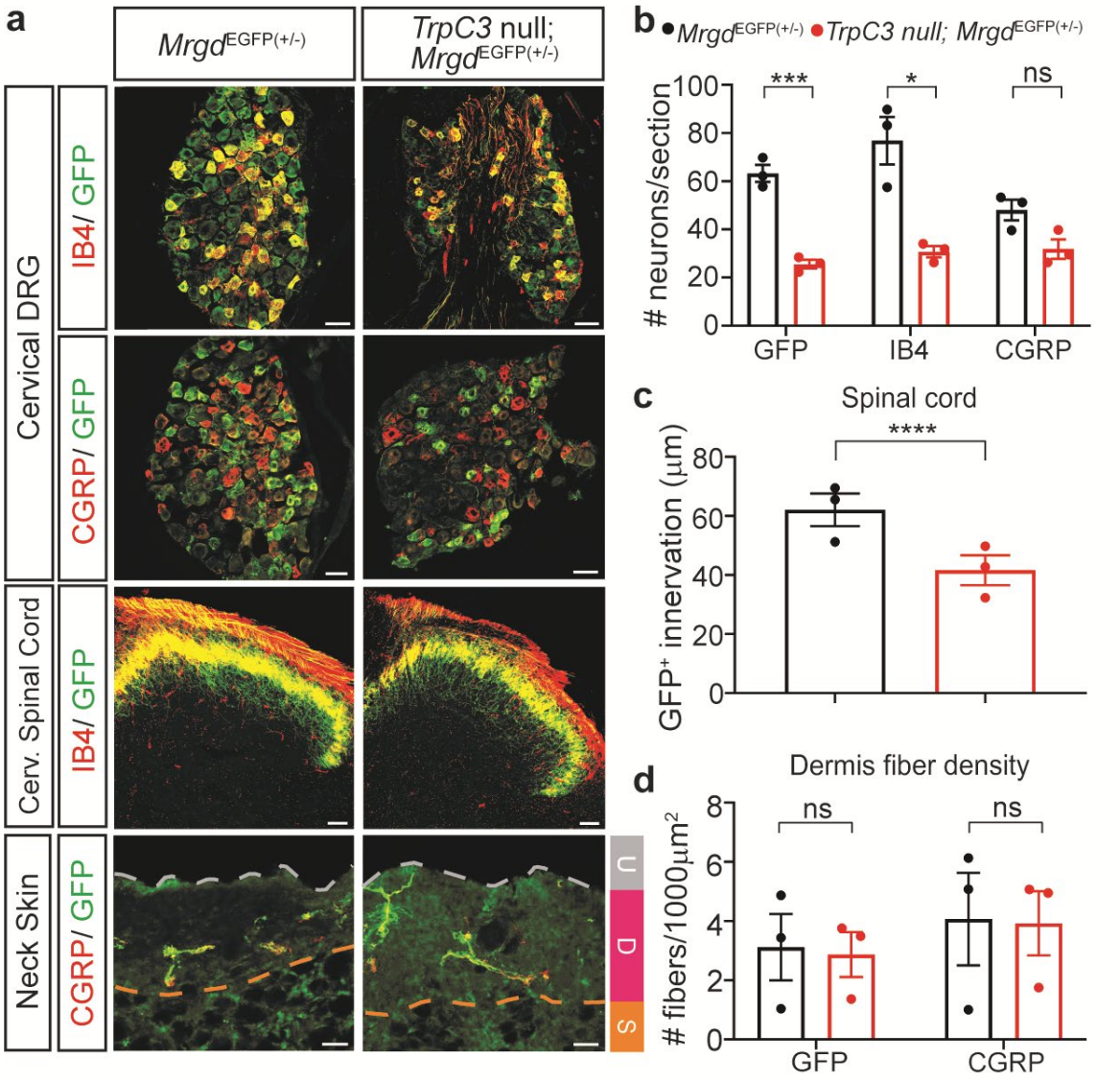

Figure 2.5. Degeneration of MRGPRD$^+$ neurons in the CD affected region of *TrpC3* null mice. a. Immunostaining of adult *Mrgprd*$^{EGFP(+/-)}$ and *TrpC3* null; *Mrgprd*$^{EGFP(+/-)}$ mouse cervical DRG, spinal cord, and treated neck skin sections using IB4 and antibodies against CGRP and GFP. Skin layers: ulcer (U); dermis (D); subcutaneous layer (S). **b.** Quantification of marker positive DRG neuron numbers per section**. c.** Quantification of the innervation thickness of MRGPRD$^+$ central terminals. **d.** Quantification of dermal

innervation of MRGPRD^{+} fibers in 1000μm^2 area ($\geq$4 sections/mouse; *n*=3). Scale bars = 50 μm. Student's two-tailed *t* test. *Asterisks* indicate statistical significance. *p<0.05, ***p<0.001, ****p<0.0001; error bar, SEM.

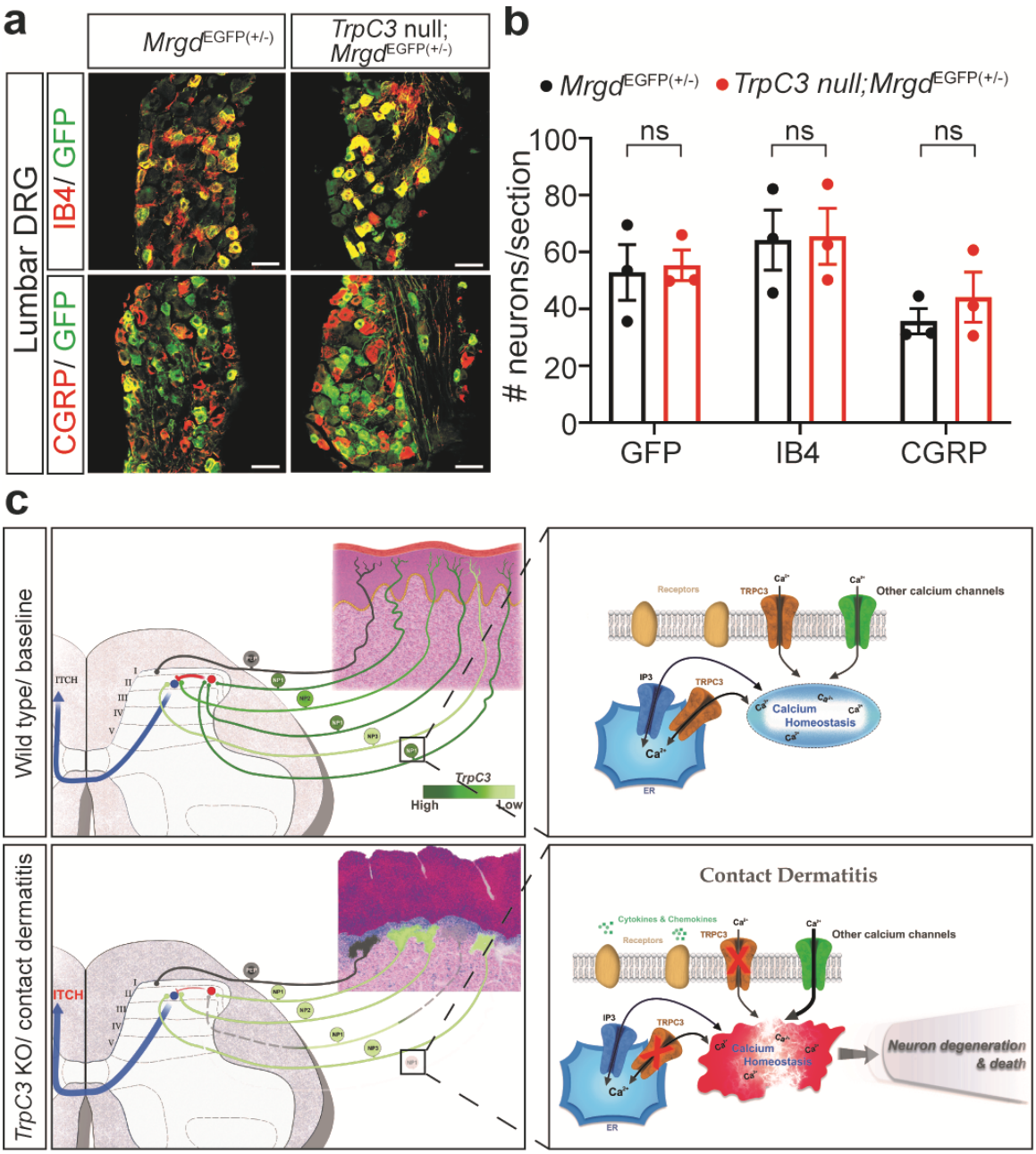

Figure 2.6. MRGPRD^{+} neurons are not affected in the control region of *TrpC3* null mice. a. Immunostaining of adult *Mrgprd*$^{EGFP(+/-)}$ and *TrpC3* null; *Mrgprd*$^{EGFP(+/-)}$ mouse lumbar DRG, sections using IB4 and antibodies against CGRP and GFP. **b.** Quantification of marker positive DRG neuron numbers per section (≥4 sections/mouse,

n=3). **c.** Schematic model of how TRPC3 functions in MRGPRD^{+} neurons to antagonize CD-induced itch. Scale bars = 50 μm. Student's two-tailed *t* test.; error bar, SEM.

CHAPTER 3: Conclusions and future directions

3.1 Conclusions

TRPC3 is a non-selective cation channel that is known to mediate calcium homeostasis and sensitization of primary nociceptors (Alkhani et al., 2014). *TrpC3* is highly co-expressed with *Mrgprd* in polymodal nonpeptidergic neurons (Dong et al., 2017) whose afferents exclusively innervate the epidermis and constitute >90% of all nonpeptidergic cutaneous C-fibers (Zylka et al., 2005). Despite the high expression of *TrpC3* in nonpeptidergic DRG neurons, *TrpC3* null mice exhibited no deficits in a variety of behavioral tests for acute pain, itch sensation, and thermosensation (Dong et al., 2017). Previous research has found MRGPRD^{+} (NP1) neurons display increased excitability following the induction of CD with SADBE, suggesting a potential role for these neurons in mediating chronic itch sensation (Qu et al., 2014). Indeed, I found that *TrpC3* null mice with SADBE induced CD displayed increased scratching behavior, suggesting that TRPC3^{+} neurons modulate chronic itch sensation (Figure 2.1).

To understand the mechanisms underlying the increased scratching behavior exhibited by null mice, we created conditional knockout (CKO) lines to target *TrpC3* expression in cell types of interest. Utilizing these CKO mouse lines, I determined that *TrpC3* in DRG neurons, but not in immune cells, is required for TRPC3 to modulate CD-induced itch (Figures 2.3 and 2.4). Immunohistological staining of cervical spinal cord and DRG, which innervated the treated region, found a marked reduction in the number of MRGPRD^{+} DRG neurons in *TrpC3* null mice (Figure 2.5).

Disruptions in TRPC3 activity have been shown to lead to the dysfunction of calcium homeostasis and cell death (Alkhani et al., 2014; Becker et al., 2009). As NP1

afferents have the highest level of *TrpC3* expression of all the nonpeptidergic neurons (Zeisel et al., 2018), the ablation of *TrpC3* should disrupt calcium homeostasis in NP1 afferents to the greatest extent. Notably, this reduction of NP1$^+$ neurons was not seen in DRG from an untreated region of *TrpC3* null mice, suggesting that SADBE treatment itself promoted the degeneration of these neurons. I hypothesized that the lack of functional TRPC3 in *TrpC3* null mice resulted in increased susceptibility of NP1 neurons to excitotoxicity in the setting of the SADBE induced CD.

NP2 (which express *Mrgpra3* and *Mrgprc11*) and NP3 (which express *Stt*, *IL31r*, and *Nppb*) neurons predominantly mediate itch (Han et al., 2013; Liu et al., 2009; Mishra and Hoon, 2013). In addition to their known role in acute itch, through activation by pruritogens including CQ and BAM-22, several studies have highlighted the critical role of NP2$^+$ and NP3$^+$ afferents in mediating chronic itch utilizing mouse models of dermatitis (Solinski et al., 2019; Wang et al., 2021; Zhu et al., 2017). Specifically, one study showed a significant attenuation of spontaneous scratching behavior in *MrgprA3*$^+$ neuron ablated mice in the setting of induced dry skin or induced allergic itch (Han et al., 2013). A possible model to explain the increased scratching exhibited by *TrpC3* null mice with CD is that the NP1 pathway normally inhibits the NP2/NP3 itch pathway, so NP1 afferent degeneration leads to disinhibition of the itch pathway (Figure 2.6).

Although no deficits in acute pain were seen in *TrpC3* null mice (Dong et al., 2017), studies have suggested the potential involvement of TRPC3 in mediating chronic pain due to its role in calcium homeostasis and activation of pro-inflammatory receptors (Alkhani et al., 2014). Previous work has demonstrated that TRPC3 is required for the cellular response to serum IgG immune complex (via the neuronal Fc-γ receptor), a

compound associated with chronic pain (Qu et al., 2012). We examined the effect of *TrpC3* expression on inflammatory pain and found that *TrpC3* null mice did not display phenotypic differences in mechanical and dynamic allodynia and thermal sensitivity, suggesting that *TrpC3* plays a specific role in modulating itch sensation (Figure 2.2).

3.2 Future Directions

Gating of the itch pathway is traditionally believed to be controlled by the pain pathway (Lagerström et al., 2010; Liu et al., 2010b). In my dissertation, I proposed that nonpeptidergic neurons can modulate the transduction of itch in other subtypes of nonpeptidergic neurons. Specifically, I introduced a model of itch modulation in which the NP1 pathway inhibits the NP2/NP3 itch pathway. This model requires confirmation by directly manipulating NP1 neuronal activities while stimulating NP2 and/or NP3 in the setting of chronic itch.

Several different mouse lines could be used to determine the role of TRPC3 specifically in MRGPRD$^+$ neurons. To confirm the role of MRGPRD$^+$ pruritoceptors in modulating chronic itch induced by CD, the SADBE-CD protocol should be repeated by utilizing mice which have had MRGPRD$^+$ somatosensory neurons selectively ablated. Previously, genetic ablation of unmyelinated sensory neurons expressing *Mrgprd* reduced behavioral sensitivity to noxious mechanical stimuli but not to heat or cold stimuli (Cavanaugh et al., 2009), which demonstrated the effective ablation of this neuronal subtype. In addition, it would be informative to selectively knock out the *TrpC3* expression in MRGPRD$^+$ nociceptors and induce CD with SADBE. The results of this

experiment would identify whether the NP1 population alone was responsible for mediating the increased scratching behavior seen in the DRG *TrpC3* CKO mice with CD.

Future experiments involving the direct manipulation of NP1 neurons are necessary to test our proposed model of antagonism of chronic itch sensation. Specifically, these experiments will elucidate the contributions of different NP subpopulations by selectively stimulating NP1 or NP2 neurons in the setting of chronic itch (in *TrpC3* null and control mice). Due to the demonstrated role of MRGPRA3$^+$ (NP3) pruritoceptive neurons in mouse models of chronic itch (Han et al., 2013; Qu et al., 2014), this neuronal population is an ideal candidate for the neuronal population hypothesized to be inhibited by MRGPRD$^+$ nociceptive neurons. Mouse models of dry skin and SADBE-CD pruritus showed a significant increase in expression of *MrgprA3* and *MrgprC11* in DRG, suggesting these GPCRs may mediate chronic itch sensation (Zhu et al., 2017). *Mrgpr* cluster knockout mice, in which MrgprA3 and MrgprC11 were deleted from the genome, exhibited significantly less scratching behavior under chronic itch conditions (dry skin and SADBE-CD) (Zhu et al., 2017). Although MRGPRC11$^+$ neurons have not been shown to directly mediate chronic itch in hairy skin, the Han lab demonstrated that MRGPRC11$^+$ neurons mediate acute and chronic itch sensation in glabrous skin (Steele et al., 2021).

A recent publication showed that metabotropic chemogenetic stimulation of MRGPRA3$^+$ neurons triggered itch behavior, and that itch transmission was GRP/GRPR dependent (Sharif et al., 2020). This paper also demonstrated that fast ionotropic optogenetic activation of the same neurons resulted in pain responses. However, transmission of pain occurred independently of GRP/GRPR, suggesting that these

sensations are transmitted using different molecular pathways (Sharif et al., 2020). Interestingly, their results contradict the previous characterization of MRGPRA3^{+} afferents as itch-specific (Han et al., 2013). Due to these findings, it would be critical to induce activation of MRGPRA3^{+} afferents utilizing metabotropic chemogenetic stimulation instead of optogenetic activation. It would be particularly interesting to stimulate these afferents on Day 16 of the protocol and observe if any differences in spontaneous scratching occur in mice that lack expression of *TrpC3* in DRG with control mice (upon injection of CNO or vehicle). An increase in spontaneous scratching behavior in mice lacking *TrpC3* expression following activation of MRGPRA3^{+} neurons would suggest that TRPC3 does function to antagonize the itch transmission of MRGPRA3^{+} afferents. If no phenotypic difference is seen in these mice, a similar experiment could be conducted to target the stimulation of MRGPRC11^{+} neurons using *MrgprC11*^{DREADD} mice (Xing et al., 2021). After identifying the subpopulation(s) of nociceptive neurons that are modulated by MRGPRD afferents, electrophysiological experiments should be conducted to further dissect this pathway.

Chronic itch is an example of how sensory responses that are normally employed to remove noxious stimuli can become dysregulated and pathologic. In my dissertation I demonstrated how the absence of TRPC3, which normally functions to maintain calcium homeostasis, resulted in a pronounced increase in spontaneous itch behavior in a mouse model of CD. In addition, I proposed a new model of modulation of chronic itch.

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CONTRIBUTIONS

Chapters 1 to 3 were written by Katherine Beattie and edited by Mary Schreck.

Chapter 2 received substantial input and suggestions from Dr. Wenqin Luo.

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