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Molecular Mediators of Acute and Chronic Itch in Mouse and Human Sensory Neurons

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ABSTRACT OF THE DISSERTATION

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by

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Professor Robert W. Gereau, IV, Chair

Itch is a distinct sensation that arises from the activation of small-diameter pruriceptive nerve fibers innervating the skin. Recent strides in the field have identified several histamine-dependent and -independent pruriceptive pathways and receptors that contribute to acute and chronic itch. The work presented in this thesis further investigates the molecular mechanisms involved in the signaling, development, and sensitization of itch in mouse and human. Most pruritogen receptors are G_q-Protein Coupled Receptors (G_qPCR), which canonically activate protein kinase C (PKC); however, little is known about whether specific PKC isoforms regulate itch. The first study in this thesis demonstrates that the isoform PKCδ contributes to histamine-induced scratching, but not histamine-independent itch. Our studies show that PKCδ is expressed in dorsal root ganglia (DRG), where it mediates sensory neuron responses to histamine.

To investigate the mechanisms behind a common form of chronic pruritus, the second study in this thesis applied a mouse model of dry skin itch to test changes in sensory neuron structure and function. We found that dry skin was marked by a significant increase in epidermal nerve fiber innervation independent of scratching. Furthermore, dry skin was associated with a selective increase in non-peptidergic, Ret-positive fibers and a functional expansion of the proportion of chloroquine-sensitive neurons. Epidermal hyperinnervation and increased growth factor levels in the skin of patients with pruritic skin diseases suggest a potential role for

neurotrophic factors (NTFs) in itch. In our third study, we tested the hypothesis that NTF signaling modulates pruritogen-evoked itch. Pretreatment with nerve growth factor (NGF) selectively potentiated histamine-induced scratching and increased the proportion of histamine-responsive sensory neurons. Artemin pretreatment, on the other hand, potentiated scratching induced by chloroquine, and increased the proportion of chloroquine-responsive neurons. Interestingly, aberrations in endogenous TrkA-NGF signaling significantly impacted normal pain sensation, but did not play a role in histamine- and chloroquine-induced itch.

In the final study of this thesis, we developed a protocol to surgically extract human DRG from organ donors and culture dissociated human primary sensory neurons. Using this approach, we performed functional studies to characterize the pruritogen- and algogen- responsive sensory neuron subpopulations in humans. We found that NGF and artemin pretreatment did not change histamine and chloroquine responses *in vitro*, indicating a potential functional difference between mouse and human sensory neurons.

Chapter 1

Introduction

The clinical burden of itch

Itch, or pruritus, is a sensation that arises from small diameter nerve fibers innervating the skin and mucosa [1]. Normally, itch is an unpleasant sensory and emotional experience that evokes the desire to scratch, thus providing the necessary drive to avoid actual or potential skin damage. But if it becomes pathological, itch can lead to severe skin injury and emotional distress. Chronic pruritus, defined as itch that lasts longer than 6 weeks, affects more than 15% of the world's population and can lead to sleep disturbances, severe anxiety, self-mutilation, and impaired overall quality of life that may be comparable to chronic pain conditions [2-4]. Major causes of chronic pruritus include dermatological diseases such as atopic dermatitis and psoriasis, where itch is reported by more than 70% of patients [4-7]. Neuropathic causes of chronic pruritus include conditions such as post-herpetic neuralgia and notalgia paresthetica [8]. Systemic diseases such as HIV, uremia due to kidney failure, cholestasis due to liver failure, and lymphomas are also associated with a high prevalence of itch [4, 9, 10]. Drug-induced pruritus presents as a side effect of the anti-malarial drug chloroquine, and more commonly the epidural application of opioids [11-13]. Psychogenic pruritus is another cause of chronic itch, but remains poorly characterized in the general and psychiatric populations [8].

Recent advances in basic and clinical research have begun to uncover some of the molecular mechanisms underlying pathological itch, though much remains unclear, and the current treatment options for patients with chronic pruritus are limited in number and efficacy [3, 14]. First-line treatment often consists of topical remedies such as emollients, topical anesthetics, or coolants, and over-the-counter antihistamines, although clinical data on their efficacy is limited. Itch secondary to skin disorders is often associated with substantial skin inflammation and infiltration by immune cells, and can respond to immunomodulatory treatments including corticosteroids and biologics such as TNF-alpha or interleukin blockers [3]. Neuropathic and systemic itch may respond to neuro-modulatory medications such as gabapentin and pregabalin, selective serotonin-reuptake inhibitors (SSRIs), or mu-opioid receptor antagonists. However,

uremic and cholestatic pruritus respond best to resolution of the systemic insult by kidney or liver transplant. Ultimately, there is no magic bullet for the treatment of chronic pruritus. Many of the available therapies provide only limited relief and are associated with significant side effects, further underlining the importance of research to expand the current understanding of pruriceptor physiology and identify novel potential treatment targets.

The neurobiology of itch

Primary sensory nerve fibers can be broadly categorized into three groups: fast-conducting myelinated Aβ fibers, thinly-myelinated Aδ fibers, and slow-conducting unmyelinated C fibers. Pruriceptors, much like nociceptors, consist of a subpopulation of small diameter C-fibers and some Aδ fibers that innervate the epidermis and upper part of the dermis [1, 15, 16]. Microneurography studies in humans have been particularly useful for identifying subtypes of itch-sensitive fibers as they allow researchers to directly correlate sensory fiber activation with the reported perceptions of itch and pain by human subjects in real time. Early studies by Handwerker and Schmelz discovered that superficial skin application of the well-known pruritogen histamine activated a population of C-mechano-insensitive fibers, as well as a population of C-mechano-heat sensitive fibers [17-19]. These histamine-sensitive fibers responded to other inflammatory compounds known to sensitize nociceptors, including prostaglandin-E2 and serotonin, and most were also responsive to capsaicin [19]. While histamine plays a key role in allergic and wheal-and-flare reactions, many forms of clinically-relevant itch appear to work via histamine-independent mechanisms. Microneurography studies using the spicules of cowhage (*mucuna pruriens*) have demonstrated another distinct subset of C-mechano-sensitive nociceptive fibers that are activated to produce histamine-independent pruritus [20, 21].

In the skin, pruriceptive signals are transduced at the peripheral terminals of sensory neurons whose cell bodies are located within dorsal root ganglia (DRG) or trigeminal ganglia (TG). Information is then relayed via central projections which synapse onto cells in lamina I and II of

the spinal cord dorsal horn or spinal trigeminal nucleus in the brain stem, where peptide signaling through the gastrin-related peptide receptors (GRPR) and B natriuretic peptide (BNP) receptor NPRA play a key role [1, 22-24]. Pruriceptive information is then relayed contralaterally via distinct subsets of spinothalamic tract neurons that project to nuclei within the thalamus, and subsequently to higher cortical structures [25-28]. Although the itch neuraxis shares the neuroanatomical structure of pain processing, the mechanisms behind the transmission of these two distinct sensations are still under investigation.

Itch receptors at the primary afferents

Histamine is perhaps the most well-known and widely-studied pruritogen, but also plays prominent physiological roles in sleep-wake cycles, vasodilation, gastric acid secretion, and immune cell function. Histamine receptors are G protein-coupled receptors that can be classified into four subtypes (H1-4) and can be found throughout the nervous system and many non-neuronal tissues. H1R is the main histamine receptor expressed on primary afferents, but can also be found in neurons in the central nervous system (CNS), smooth muscle cells, and endothelial cells [29-31]. H2R is expressed in many cell types, including gastric parietal cells where it is a key mediator of gastric acid release. H3R couples mainly to $G\alpha_{i/o}$ and acts as an autoreceptor throughout the nervous system [30]. H4R is also coupled to $G\alpha_{i/o}$ and has a major role in regulating immune cell function, but whether it has a role in the nervous system remains under investigation [32, 33]. Studies using highly selective pharmacological antagonists have demonstrated that H1R is the main receptor responsible for itch sensation and axon reflex neurogenic vasodilation that results in the wheal and flare induced by histamine skin prick [34, 35]. Although H4R has not been investigated as extensively in humans, preclinical studies demonstrate that H4R antagonists were also able to reduce histamine-induced scratching in mice [3, 36-42]. Functional studies of sensory neurons utilizing ratiometric fluorescent calcium indicators demonstrate that histamine activates 10-15% of rodent DRG neurons [43-45].

Recent studies have identified several histamine-independent itch pathways that signal through receptors in the mas-related G protein receptor (MRGPR) family. This is a diverse family of receptors, consisting of the conserved subfamilies D to G, and several subfamilies found only in rodents (A, B, C, and H) or primates (X). Initial studies found that most MRGPR subtypes are expressed almost exclusively in a small subset of sensory neurons that bind the plant lectin isolectin B4 (IB4) [46]. A key study using Mrgpr cluster knock-out mice, which lacked most MrgprA and MrgprC genes, found that despite normal pain sensation, itch responses to chloroquine were greatly reduced. Chloroquine is an anti-malarial drug that induces robust itch in mice and can induce itch in humans of African descent [13]. Experiments in heterologous cells found that chloroquine asserts its effects by activating the MrgprA3 receptor [47]. Chloroquine-responsive DRG neurons were found to comprise a very small subset of sensory neurons (approximately 5%), and also responded to histamine and the transient receptor potential (TRP) channel V1 agonist capsaicin. The same group also found that MrgprC11 is a receptor expressed in a subset of the MrgprA3-positive cells and is activated by the peptides SLIGRL and bovine adrenal medulla 8-22 pepetide (BAM8-22) to induce itch in rodents [48].

As mentioned previously, MRGPRX subfamily receptors are found exclusively in primates and in humans they are mostly restricted to DRG and TG tissues. Studies using heterologous systems demonstrate that MRGPRX1 can be activated by the peptides BAM8-22 and BAM1-22 [49, 50]. MRGPRX1 can also be activated by chloroquine, though at a much higher EC_{50} [47]. BAM8-22 injection induced itch along with a stinging sensation in human volunteers, supporting a role for MRGPRX1 in the signaling of itch in humans [51]. While MRGPRX1 is not a true human ortholog of MrgprC11 or MrgprA3, it may play a key role in the signaling of non-histaminergic itch in humans. MRGPRX2 RNA has been identified in both DRG and mast cells, though its potential role in itch remains unknown [52-55]. No ligands have been described yet for MRGPRX3 and 4 subtypes, but a study overexpressing MRGPRX3 under the β-actin promoter in rat found several skin and eye abnormalities [56, 57].

MrgprD is another pruritogen receptor subtype that is conserved in rodents and primates, and is found specifically in non-peptidergic C-fiber nociceptors that synapse onto lamina II neurons of the spinal cord dorsal horn [58]. A subset of MrgprD-positive neurons can be activated by β-alanine, an amino acid commonly found in muscle building supplements, to induce itch in both mice and humans [59]. Some MrgprD-positive neurons are also heat- and mechano-sensitive, but the complexities of this subpopulation are not yet well characterized [60-62]. In addition to the histamine and Mrgpr family receptors, various other itch receptors have been discovered, including proteinase-activated receptors (e.g. PAR2), toll-like receptors (TLR7), interleukin receptors (IL-31), and thymic stromal lymphopoetin receptor (TSLPR) [63].

The expression pattern of pruritogen receptors identifies at least three functionally distinct subtypes of pruriceptive fibers (Fig. 1) [64-66]. Calcium imaging studies have identified a histamine-responsive subgroup (Fig.1A) and a β-alanine-responsive group (Fig. 1B), representing two distinct subpopulations that do not respond to other pruritogens [43, 47, 59, 67-69]. The third pruriceptor subtype is a chloroquine-responsive group, representing only 5-10% of sensory neurons. This group includes all SLIGRL/BAM8-22 responsive neurons. In addition, more than half of all chloroquine-sensitive neurons also respond to histamine (Fig. 1C). Interestingly,

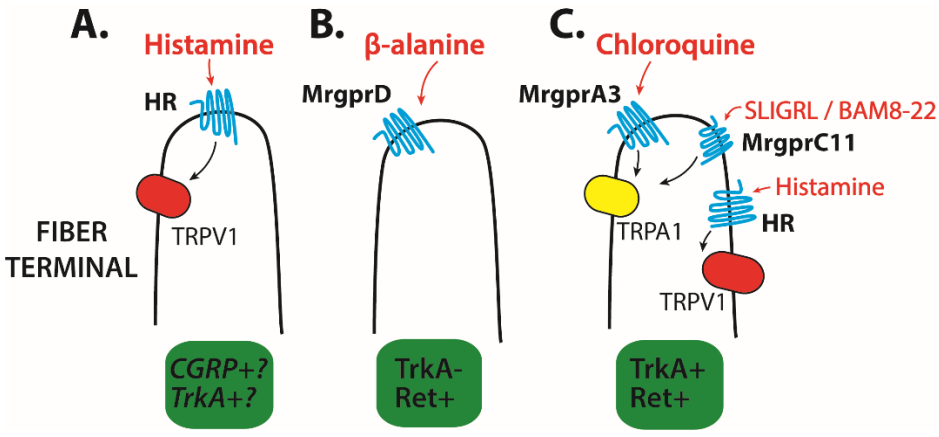

Figure 1. Subtypes of pruriceptive fibers. A. Histamine-responsive subset of pruriceptors is functionally linked to TRPV1. **B.** β-alanine-responsive cells are a distinct subset of MrgprD+ non-peptidergic neurons. **C.** MrgprA3-expressing neurons are both TrkA/Ret+ and represent a subset of pruriceptors that can respond to a number of pruritogens.

ablation of MrgprA3-positive neurons had no effect on pain behavior, but caused profound deficits in scratching induced by chloroquine, SLIGRL, BAM8-22, and in models of dry skin and allergic contact dermatitis [70]. Histamine-induced scratching was reduced to a lesser extent, likely due to the remaining histamine-specific subpopulation (Fig 1A). These studies suggest that the MrgprA3-positive population of chloroquine-responsive neurons may be particularly tuned for sensing itch.

Intracellular mediators of itch

The majority of recently identified pruritogen receptors are Gq protein-coupled receptors (GqPCRs), which exert their physiological effects via protein kinase C (PKC) activation and release of intracellular calcium stores [63, 64, 71]. The specific signaling cascades immediately downstream of pruritogen receptor activation remain less clear. Sensory neuron activation via H1R appears to be dependent on PLCβ3, as demonstrated by the findings that PLCβ3 knock-out mice scratch less when injected with histamine and almost all DRG calcium responses to histamine are ablated [43]. However, this study also found that over 85% of IB4-positive neurons and more than 30% of peptidergic neurons expressed PLCβ3, posing the question of what mechanisms confer specificity to histaminergic signaling.

The canonical PLC pathway generates inositol triphosphate (IP_3) and diacylglycerol (DAG), which act to release intracellular calcium stores and activate downstream targets, respectively. Gq protein-coupled receptors induce PKC activation via the generation of DAG and release of intracellular calcium stores [72]. Several PKC isoforms are expressed in sensory neurons [73-75] and are known to directly modulate ion channels, pointing to one possible mode of regulation of itch signaling [75-77]. The novel PKC subtype PKCδ depends on DAG for activation and is found in sensory neurons [72, 73, 78, 79]. PKCδ does not play an important role in acute pain behavior but was demonstrated to be involved in CFA-induced thermal hyperalgesia - a TRPV1-dependent process [80]. Furthermore, studies of histamine-induced signaling in

human aortic endothelial cells (HAECs) and HeLa cells (all of which express H1R endogenously) demonstrate that PKCδ is phosphorylated in response to histamine [81, 82]. PKCδ also appears to be important for histamine-induced H1R mRNA up-regulation and activation of ERK1/2 and p38 [81, 82]. In chapter 2, we investigate whether PKCδ plays a role in the signaling of histaminergic and histamine-independent itch.

Several studies indicate that pruritogen receptors are functionally linked to specific transient receptor potential (TRP) channels, a family of non-selective cation channels that can be activated by diverse environmental and physiological stimuli [83-87]. Early microneurography studies in humans demonstrate that most histamine-sensitive fibers respond to heat, suggesting the expression of the heat-sensitive channel TRPV1 [17, 19]. *In vitro* animal studies confirmed this, demonstrating that 30-60% of histamine-responsive sensory neurons can also be activated by the TRPV1 agonist capsaicin [47, 68, 86, 88]. TRPV1 deletion and pharmacological inhibition significantly reduced histamine-evoked scratching and sensory neuron responses to histamine, indicating a functional link between histamine receptor and TRPV1 [88, 89]. TRPV1 is also expressed in other subsets of pruriceptors, including MrgprA3- and MrgprC11-positive neurons, but it does not appear to play a functional role in histamine-independent itch [47, 48, 68, 86, 89, 90].

Several findings indicate that histamine-independent itch is instead mediated by TRPA1, a promiscuous TRP channel activated by a number of environmental irritants. The TRPA1 agonist mustard oil (MO) activates a large proportion of CQ- and BAM8-22-responsive neurons [68, 86, 90]. Both genetic deletion and pharmacological inhibition of TRPA1 significantly reduced scratching responses and neuronal activation by chloroquine and BAM8-22 [90, 91]. Interestingly, it appears that the Mrgpr receptors engage different mechanisms to activate TRPA1, where MrgprA3 signals via Gβγ, while MrgprC11 signals through PLC [90]. TRPA1 also mediates chronic itch and oxidative stress-induced itch, further demonstrating its role in histamine-independent mechanisms of pruritus [90, 92, 93].

Differentiating between itch and pain

Itch and pain are two distinct sensory experiences. The prototypical behavioral response that inhibits itch is a noxious stimulus in the form of a scratch. Conversely, inhibition of pain by epidural anesthetics is frequently associated with itch. The polymodal nature of pruriceptive neurons has given rise to two major competing theories regarding the physiological basis of itch. The first, known as “the intensity theory”, postulates that itch arises from the same population of neurons that signal pain. It hypothesizes that stimulus intensity determines whether a stimulus is perceived as “itchy” or painful, with the expectation that a low-intensity noxious stimulus produces itch. The alternative, “labeled-line theory” proposes that itch- and pain-sensitive pathways are anatomically separate and specialized for transducing only one sensation.

Recent studies of the molecular mechanisms that mediate itch and pain have uncovered evidence to support the labeled-line theory. Even though pruriceptors resemble nociceptors in their ability to respond to painful stimuli like heat and chemical irritants, a recent finding demonstrates that selective activation of pruriceptive neurons results in itch, not pain. In an elegantly designed study, Han et al. used TRPV1 global knock-out mice engineered to express TRPV1 solely in the MrgprA3-positive subset of neurons. In these animals, cheek application of the TRPV1 agonist capsaicin induced itch-specific scratching instead of the nociceptive wiping response induced by capsaicin in wild type controls [70]. These results demonstrate that the selective activation of MrgprA3-positive neurons specifically transduces itch and further support the idea that pruriceptors are a distinct subset of sensory neurons specialized for sensing itch. Even so, psychophysical studies in humans have demonstrated that the sensation experienced after application of the pruritogens histamine, BAM8-22, or β-alanine may not be a true pure itch, as participants report it is frequently accompanied by prickling or burning undertones, suggesting more complex underlying mechanisms.

Development and sensitization of pruriceptors

Primary afferents are pseudounipolar neurons derived from neural crest cells that migrate out of the neural tube and differentiate into a heterogeneous pool of proprioceptors, mechanoreceptors, thermoreceptors, nociceptors, and pruriceptors [94, 95]. The survival and phenotype specification of these functionally distinct groups is regulated by transcription factors and neurotrophic factors. Nerve growth factor (NGF) signals via the receptor tyrosine kinase A (TrkA) to support the survival of small diameter sensory neurons during embryogenesis [96-103]. Those neurons which retain TrkA expression into adulthood become the peptidergic subset known for their expression of calcitonin gene-related peptide (CGRP) and substance P [100, 104, 105]. However, about half of small diameter neurons downregulate TrkA in the early post-natal period and begin to express the receptor tyrosine kinase Ret [104, 106]. This subset is dependent on the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) GDNF, neurturin (NRTN), and artemin (ARTN). Ret activation by the GFLs requires a cell surface co-receptor known as GDNF Family Receptor α (GFRα). Analysis of GFL and co-receptor null mice demonstrates highly restricted physiological pairing of ligand and high affinity co-receptors in vivo: GFRα1/GDNF, GFRα2/NRTN, and GFRα3/ARTN [107, 108]. The small diameter Ret-positive neurons constitute the non-peptidergic subset of nociceptors, which are largely identified by their ability to bind the plant lectin isolectin B4 (IB4). Additionally, neurotrophic factor signaling via TrkA and Ret is necessary for the establishment of peripheral target innervation during development and the maintenance of innervation throughout adulthood [101, 109, 110]. The expression of TrkA and Ret determine neuronal dependence on different neurotrophic factors, contributing to the development and maintenance of anatomically and functionally distinct populations of sensory neurons.

Sensory nerve fibers in the skin are in close proximity to keratinocytes, dermal fibroblasts, and immune cells, which can release pruritogens, various pro-inflammatory mediators, and neurotrophic factors (NTFs) [1, 111-119]. Studies have identified multiple mechanisms by which

inflammation and growth factors directly activate and sensitize nociceptors in the context of pain. NGF and the GFLs have been shown to directly sensitize and upregulate the expression of TRPV1 and TRPA1 [120-125]. Overexpression of select growth factors in keratinocytes results in epidermal hyperinnervation, sensory neuron cell body hypertrophy, and enhanced expression of TRP channels, which contributes to a state of hyperalgesia [126-130]. In addition, neurotrophic factors regulate the expression and function of sodium channels, which directly contribute to the regulation of nociceptor excitability [131-135]. In conclusion, these findings indicate a clear role for neurotrophic factors in the sensitization of pain, but whether and how they directly affect pruriceptors remains unknown.

Several key observations suggest that neurotrophic factors could contribute to chronic itch. First, epidermal hyperplasia and inflammatory cell infiltration are hallmarks of many pruritic skin diseases, serving as a source of increased neurotrophic factor levels. Studies of atopic dermatitis and psoriasis have shown that the severity of itching correlates with increased serum levels of NGF [136-139]. Lesional skin from atopic dermatitis patients is marked by increased levels of NGF in the skin and increased TrkA in epidermal nerve fibers [137-140]. Another recent study found that artemin is also increased in the skin of patients with atopic dermatitis [141]. Epidermal hyperinnervation of lesional skin is another common feature of pruritic skin diseases that suggests neurotrophic factor involvement [140, 142, 143]. Co-culturing human keratinocytes from patients with atopic dermatitis with porcine sensory neurons resulted in increased local NGF release and greater axon growth when compared to a co-culture using healthy human keratinocytes [144]. NGF and TrkA inhibition in a mouse model of atopic dermatitis reduced both scratching and epidermal fiber density [145, 146]. Human TrkA deficiency results in congenital insensitivity to both pain and itch, confirming that NGF-TrkA signaling plays a key role in the development and maintenance of pruriceptors [147].

Acutely, intradermal injection of NGF in human subjects enhanced itch produced by the non-histaminergic pruritogen cowhage [148]. In a Phase II clinical study investigating artemin as

a potential treatment for unilateral sciatica, the most commonly reported adverse reactions to intravenous or subcutaneous artemin administration were changes in temperature perception and pruritus [149, 150]. In animal studies, artemin treatment induced warmth-provoked scratching behavior that was dependent on the expression of the artemin co-receptor GFRα3 [141]. Finally, neurotrophic factors can directly induce mast cell degranulation, stimulating local histamine release to induce itch [151-154]. These findings suggest that NTFs regulate itch, but the mechanisms of this modulation are poorly understood.

Immunohistochemical characterization of pruriceptive neurons suggests a potential role for both NGF-TrkA and GFL-Ret signaling in the regulation of pruriceptor physiolgy. Histamine-responsive neurons co-express TRPV1 and their activation results in the axon-reflex vasogenic reaction, suggesting they express peptides, likely making them TrkA-positive. On the other hand, Ret is expressed in both the β-alanine- and chloroquine-responsive subsets of pruriceptive neurons (Fig 1B-C) [58, 70]. Interestingly, the MrgprA3-positive group was also found to express CGRP, indicating that MrgprA3 pruriceptors represent a small subpopulation of neurons that have retained both peptidergic and non-peptidergic markers [70]. In chapter 4 of this manuscript, we investigate the role of both NGF and the GFLs GDNF, neurturin, and artemin in histamine- and chloroquine-evoked itch.

Translational approaches to preclinical studies of itch and pain

For decades, animal models have been the backbone of preclinical research. Studies using mice provide key advantages, most notably precise genetic manipulation and quick breeding, that allow thorough hypothesis testing in a complex biological system. As an *in vitro* model, primary cultures of dissociated rodent sensory neurons have been used to study pain, itch, nerve injury, regeneration, and axonal transport. Many candidate molecular targets and genes have been identified using this approach, yet few of these findings have directly translated into effective and safe clinical treatments [155-159]. Several notable failures in translation suggest

prominent differences in fundamental biological mechanisms between humans and rodents [160-166]. MrgprA3 and MrgprC11 pruriceptor physiology is one important example of fundamental receptor differences between species. Due to divergent evolutionary mechanisms, these receptor families are greatly expanded in the mouse, but are not found in primates. While the human MRGPRX1 receptor can be activated by the same agonists, key differences in receptor structure and pharmacology demonstrate that these receptors are not true orthologs [47, 48, 70, 167]. In addition, the functional link between mouse MrgprA3 and C11 receptors and TRPA1 may not be present in primates. Studies using heterologous expression models suggest that MRGPRX1 may actually engage TRPV1 as a downstream mechanism for cell activation [167, 168]. Thus, key differences in the molecular mechanisms underlying human and rodent sensory neuron processing demonstrate the importance of preclinical target validation in human cells [169-172].

A major obstacle impeding the validation of basic research findings is access to human tissues. Human neurons are notoriously difficult to obtain and maintain in culture for use in experiments. That is most likely the reason why very little is currently known about the physiology of human pruriceptive subpopulations. In the final study in this dissertation, we describe a protocol we developed for the surgical extraction of human dorsal root ganglia from deceased organ donors. This approach has provided us with access to human tissue with a minimal post-mortem interval, allowing the successful culturing of human sensory neurons. In chapter 5, we characterize the human pruriceptive subpopulations that respond to histamine and chloroquine, and directly test the effects of neurotrophic factors on pruriceptor calcium responses.

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Chapter 2

Protein kinase Cδ mediates histamine-evoked itch and responses in pruriceptors

This chapter contains the manuscript:
Valtcheva MV, Davidson S, Zhao C, Leitges M, Gereau RW. Protein kinase Cδ mediates histamine-evoked itch and responses in pruriceptors. Molecular pain. 2015;11(1):1. doi: 10.1186/1744-8069-11-1. PubMed PMID: 25558916; PMCID: PMC4298070

Abstract

Itch-producing compounds stimulate receptors expressed on small diameter fibers that innervate the skin. Many of the currently known pruritogen receptors are G_q-Protein Coupled Receptors (G_qPCR), which activate Protein Kinase C (PKC). Specific isoforms of PKC have been previously shown to perform selective functions; however, the roles of PKC isoforms in regulating itch remain unclear. In this study, we investigated the novel PKC isoform PKCδ as an intracellular modulator of itch signaling in response to histamine and the non-histaminergic pruritogens chloroquine and β-alanine. Behavioral experiments indicate that PKCδ knock-out (KO) mice have a 40% reduction in histamine-induced scratching when compared to their wild type littermates. On the other hand, there were no differences between the two groups in scratching induced by the MRGPR agonists chloroquine or β-alanine. PKCδ was present in small diameter dorsal root ganglion (DRG) neurons. Of PKCδ-expressing neurons, 55% also stained for the non-peptidergic marker IB4, while a smaller percentage (15%) expressed the peptidergic marker CGRP. Twenty-nine percent of PKCδ-expressing neurons also expressed TRPV1. Calcium imaging studies of acutely dissociated DRG neurons from PKCδ-KO mice show a 40% reduction in the total number of neurons responsive to histamine. In contrast, there was no difference in the number of capsaicin-responsive neurons between KO and WT animals. Acute pharmacological inhibition of PKCδ with an isoform-specific peptide inhibitor (δV1-1) also significantly reduced the number of histamine-responsive sensory neurons. Our findings indicate that PKCδ plays a role in mediating histamine-induced itch, but may be dispensable for chloroquine- and β-alanine-induced itch.

Introduction

Itch, clinically known as pruritus, is an unpleasant sensory and emotional experience that leads to the desire to scratch [1, 2]. Chronic itch can result in severe anxiety, self-mutilation, and impaired overall quality of life that is comparable to chronic pain [3-5]. Several histamine-dependent and histamine-independent itch receptors have been recently identified; however, few of the intracellular mediators downstream of these receptors have been characterized. Elucidating the intracellular mediators that activate pruriceptors may provide a new set of targets to aid in the generation of more specific and efficacious treatments.

Intradermal histamine induces itch via direct activation of the H1 histamine receptor, which is expressed in sensory neurons [6-10]. Additionally, several subtypes of the recently characterized class of Mas-related gene protein receptors (Mrgpr) have been shown to respond selectively to a variety of non-histaminergic, itch-producing compounds. For example, MrgprA3 is activated by the anti-malarial drug chloroquine (CQ) [11, 12], and β-alanine induces itch by activating a subset of nonpeptidergic MrgprD-expressing sensory neurons [13, 14].

A common property of many of the identified pruritogen receptors, including the H1 histamine receptor (H1R), MrgprA3, and MrgprD, is that they are G_q-protein coupled receptors (G_qPCRs) [10-12, 14]. Canonically, G_qPCRs activate phospholipase C (PLC), which cleaves phosphatidylinositol (PIP_2) into inositoltriphosphate (IP_3) and diacylglycerol (DAG), resulting in release of intracellular calcium stores and activation of downstream targets. However, the itch-mediating factors downstream of PLC are largely unknown [10, 15]. Protein kinase C (PKC) is coupled to the canonical G_qPCR/PLC pathway via activation by DAG and/or calcium and therefore may play a role in the signaling of itch.

A number of PKC isoforms are expressed in sensory neurons [16-19]. One of these isoforms is PKCδ, a member of the “novel” PKC isozymes, which depends on DAG but not calcium for its activation. Previously, we demonstrated that PKCδ is dispensable for withdrawal responses to acute noxious mechanical and thermal stimuli [20]. However, studies of H1R

signaling in human aortic endothelial cells and HeLa cells showed that PKCδ is phosphorylated in response to histamine [21, 22]. PKCδ also mediated histamine-induced H1R mRNA upregulation and downstream activation of ERK1/2 and p38 [21, 22]. These lines of evidence suggest that PKCδ could play a specific role as an intracellular modulator of itch in sensory neurons.

In this study, we tested the hypothesis that PKCδ contributes to pruritogen-induced itch. We determined the role of PKCδ in histaminergic and non-histaminergic itch by examining scratching responses to histamine and the non-histaminergic pruritogens chloroquine and β-alanine, which activate separate pruriceptor subpopulations. We characterized the distribution of PKCδ in sensory neurons and show that both genetic deletion and pharmacological inhibition of PKCδ significantly decrease the proportion of histamine-responsive neurons.

Materials and Methods

Subjects and ethical approval

All experiments were conducted in accordance with the National Institute of Health guidelines and received the approval of the Animal Care and Use Committee of Washington University School of Medicine. Eight to twelve-week old male littermate mice were housed on a 12 hour light-dark cycle and allowed ad libitum access to food and water.

PKCδ-KO mice were obtained from Dr. Michael Leitges [23]. These mice were generated using a standard gene targeting approach to insert a LacZ/neo cassette in the first transcribed exon of the PKCδ gene to abolish transcription, resulting in a global knock-out [23]. PKCδ-KO mice were backcrossed on a C57BL/6 background for at least 6 generations prior to use. PKCδ-KO mice were then crossed with wild type C57BL/6 mice to generate heterozygous mice, which were used to generate wild type and KO littermates.

Pruritogen-induced scratching behavior

The nape of the neck and upper back were shaved with electric clippers one day prior to behavioral experiments. On the day of experiment, mice were placed in individual plexiglass observation boxes and allowed to acclimate in the presence of white noise for 2 hours. Using gentle restraint, 50µl consisting of pruritogen dissolved in 0.9% normal saline was injected intradermally at the nape of the neck using a 29½ gauge insulin syringe. The following pruritogen amounts were used: 1mg histamine (Sigma Aldrich, St. Louis, MO), 200µg chloroquine (Sigma Aldrich, St. Louis, MO), and 223µg β-alanine (Sigma Aldrich, St. Louis, MO). A single scratch bout was defined as one or more rapid back-and-forth motions of the hindpaw directed at the injection site, ending with either a pause, licking, or biting of the toes or placing of the hindpaw on the floor. Scratch bouts by the hind-paw directed at the injection site were counted over a period of 30 minutes. Experimenters were blinded to mouse genotype.

PKCδ immunohistochemistry and Western Blotting

For Western blotting, mice were euthanized by swift decapitation and lumbar spinal cord and lumbar DRG were removed. Tissue samples were homogenized in homogenization buffer (20mM Tris-HCl, ph7.4, 1mM EDTA, 1mM sodium pyrophosphate, 25µg/ml aprotinin, 25µg/ml leupeptin and 100µM phenylmethylsufonyl fluoride) on ice. 7µg of spinal cord and DRG protein were separated using 4-12% SDS-PAGE, then transferred to nitrocellulose membrane. Membrane was blocked in Odyssey blocking buffer for 1 hour, then incubated in rabbit anti-PKCδ (1:1000, Santa Cruz) and mouse anti-β-Tubulin (1:1000, Sigma-Aldrich) primary antibodies in Odyssey buffer with 0.1% Tween-20 at 4°C overnight. Blots were then washed in TBS-0.1% Tween-20, and incubated for 1 hour at room temperature in secondary antibodies (goat anti-rabbit Alexa Fluor 680 (1:20,000, Sigma Aldrich); goat anti-mouse IR800 (1:20,000, Sigma Aldrich)). Blots were washed and scanned using an Odyssey infrared scanner.

For immunohistochemistry (IHC), mice were deeply anesthetized with a ketamine, xylazine, and acepromazine cocktail, then perfused intracardially with cold PBS followed by 4% paraformaldehyde in PBS. Lumbar DRG were removed and cryoprotected in 30% sucrose. Transverse sections were cut at 18µm thickness on a cryostat and collected on slides. To determine the percentage of total neurons that express PKCδ, dual labeling was performed with rabbit anti-PKCδ (1:50, Santa Cruz) and mouse anti-β-tubulin (1:1000, Sigma Aldrich) primary antibodies. Briefly, sections were blocked in 2% BSA, 0.1% Milk powder, 0.05% Tween-20 TBS for 1 hr, then incubated in primary antibodies overnight at 4°C. On day 2, slides were washed and incubated in secondary antibodies for 2-4 hours at 4°C (Alexa Fluor 488 Donkey anti-rabbit 1:200, Alexa Fluor 555 donkey anti-mouse 1:200, Invitrogen). Images were obtained using an upright epifluorescent microscope (Nikon 80i, CoolSnapES camera). Labeled neurons were counted in at least 3 randomly selected sections separated by >50µm per animal. The size distribution of PKCδ+ neurons was determined using ImageJ software to measure cell diameter. The percentage of PKCδ+ neurons that also expressed CGRP or IB4 was determined using dual

labeling for PKCδ and CGRP (goat anti-CGRP 1:400, Serotec) or Alexa-568 conjugated to IB4 (1:400, Invitrogen) using the above-described procedures. PKCδ-TRPV1 coexpression was determined using a goat anti-PKCδ antibody (1:50, Santa Cruz) and a rabbit antibody directed against the TRPV1 C-terminus peptide (1:500) [24].

Calcium imaging

Scratching behavior was evoked with pruritic stimuli applied to the back skin where site directed scratching occurs. We expanded our functional analyses of neuronal physiology to include both thoracic and lumbar DRG. Mice were euthanized rapidly by decapitation and DRG removed and acutely dissociated using previously described methods [25]. Briefly, DRG were incubated in 45U papain/L-cysteine in Hank's buffered saline solution (HBSS) without Ca^{2+} or Mg^{2+} and with 10mM HEPES for 20 minutes at 37°C and 5% CO_2. Ganglia were then washed, followed by 20 minute incubation in 1.5 mg/ml collagenase in HBSS+HEPES. Ganglia were then triturated with fire-polished Pasteur pipettes, the dissociated cells were filtered through a 40 μm cell strainer, and were plated on poly-D-lysine and collagen-coated glass coverslips. Cells were incubated overnight at 37°C in 5% CO_2 humidified air in culture medium (Neurobasal A with B27, pen/strep, 2mM glutamax, 5% fetal bovine serum (Gibco)). All experiments were performed within 24 hours of plating.

Cells were incubated in 3μg/ml Fura-2 AM (Molecular Probes) for 30 minutes and then incubated for 30 minutes in external solution (in mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 Glucose, 10 HEPES. For each recording, a coverslip was placed in a perfusion chamber and perfused with external solution at room temperature. Cells were viewed under an inverted microscope (Olympus Optical, Tokyo, Japan) and images were captured with a Hamamatsu Orca camera. SimplePCI Software was used to draw regions of interest (ROI) around Fura-loaded cells prior to recording. The ratio of fluorescence emission at an excitation wavelength of 357 and 380nm was measured for each ROI. The experimental protocol consisted of a 2 minute baseline

followed by 30 second bath application of histamine (100μM in external solution), >8 minutes of external solution wash, 10 second application of capsaicin (200nM), >8 minutes of external solution wash, and 10 seconds of KCl (50mM) followed by wash (<2 minutes) to determine live neurons. A 10% or greater change from baseline 357nm/380nm ratio was considered a response to histamine. Capsaicin experiments were performed similarly except with 10 second capsaicin application (200nM). For experiments using PKCδ peptide inhibitor and scrambled peptide, cells were incubated in 100μM peptide solution (dissolved in external solution) for 30 minutes prior to recording (δV1-1 peptide inhibitor (Myr-SFNSYELGSL-NH2), peptide inhibitor scramble (Myr-GLSFSEYLSN-NH2), Biomatik).

List of abbreviations

PKC: protein kinase C; G_qPCR: G_q protein-coupled receptor; MRGPR: Mas-related gene protein coupled receptor; CQ: chloroquine; KO: knock-out; WT: wild type; DRG: dorsal root ganglion; SC: spinal cord; PLC: phospholipase C; PIP_2: phosphatidylinositol; IP_3: inositoltriphosphate; DAG: diacylglycerol; H1R: histamine receptor 1; ERK1/2: extracellular signal regulated kinase 1/2; CGRP: calcitonin gene-related peptide; IB4: isolectin B4; Scr: scramble; Inh: inhibitor; PBS: phosphate-buffered saline; HBSS: Hank's buffered saline solution; IHC: immunohistochemistry; TRPV1: transient receptor potential vanilloid receptor 1; TRPA1: transient receptor potential cation channel, subfamily A, member 1; PGE_2: prostaglandin E_2; NGF: nerve growth factor; IL-6: interleukin-6

Results

PKCδ mediates histamine-induced itch

To determine if PKCδ plays a role in behavioral responses to itch, we assessed scratching responses to histamine and non-histaminergic pruritogens in PKCδ knock-out mice (PKCδ-KO) and their wild type littermates. Mice were injected intradermally at the nape of the neck with one of three pruritogens: histamine (1mg), chloroquine (CQ) (200μg), or β-alanine (223μg). PKCδ-KO mice scratched significantly less than their wild type littermates when injected with histamine (Figure 1A, $p<0.05$). On the other hand, chloroquine-induced scratching was not significantly different between WT and PKCδ KO mice (Figure 1B, $p=0.129$). There was also no difference in the number of scratch bouts induced by β-alanine (Figure 1C, $p=0.61$). These results indicate that PKCδ mediates histaminergic itch, but is not necessary for non-histaminergic itch induced by CQ and β-alanine.

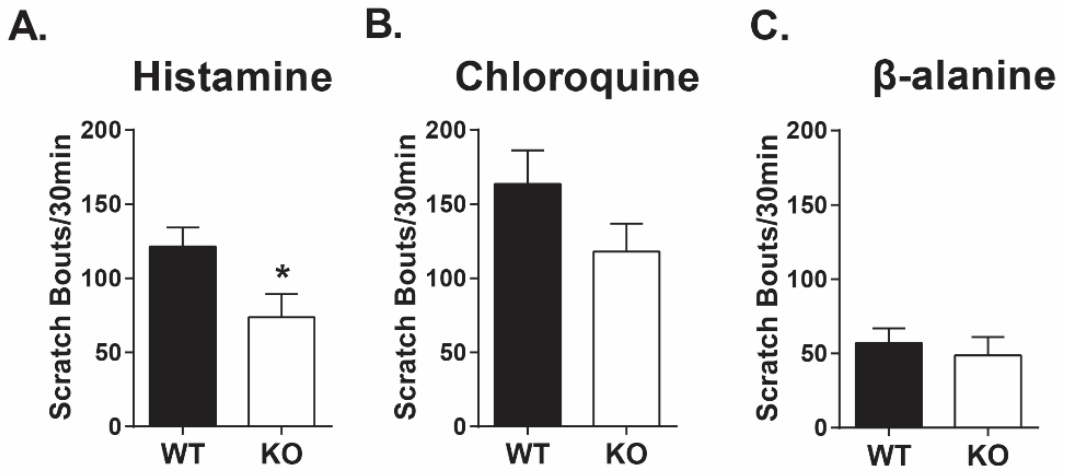

Figure 1. PKCδ-KO and WT scratching responses to pruritogens. A. PKCδ-KO mice scratched less than wild type littermates in response to intradermal histamine injection (WT=121.4±12.8 scratch bouts/30min, n=25; KO=73.8±15.5 scratch bouts/30min, n=19; unpaired t test $p<0.05$). **B.** Chloroquine (CQ)-induced scratching was not different between PKCδ-KO and WT mice (WT=163.7±22.6 scratch bouts/30min, n=23; KO=118.0±18.9 scratch bouts/30min, n=21; unpaired t test $p=0.129$). **C.** Scratch bouts induced by β-alanine were also not different between PKCδ-KO and WT mice (WT=57.0±10 scratch bouts/30min, n=6; KO=48.8±12.3 scratch bouts/30min, n=8; unpaired t test $p=0.61$).

PKCδ is preferentially expressed in small diameter DRG neurons

PKCδ is expressed in a variety of tissues, including the brain and peripheral nervous system [19, 21, 26-33]. To assess whether PKCδ is localized to potential pruriceptive sensory neurons, immunohistochemistry (IHC) was used to characterize the distribution of PKCδ in dorsal root ganglion (DRG) neurons. Antibody specificity was confirmed via western blot using PKCδ-KO DRG and spinal cord tissue. No antibody staining was found corresponding to the 78kD PKCδ band in PKCδ-KO DRG tissue (Figure 2A). This was further confirmed by IHC of knock-out and wild type DRG (Figure 2B). In wild type lumbar DRG, PKCδ was expressed in 43.2% of total neurons labeled with βIII tubulin (567/1314 cells, n=3 animals) and PKCδ expression was

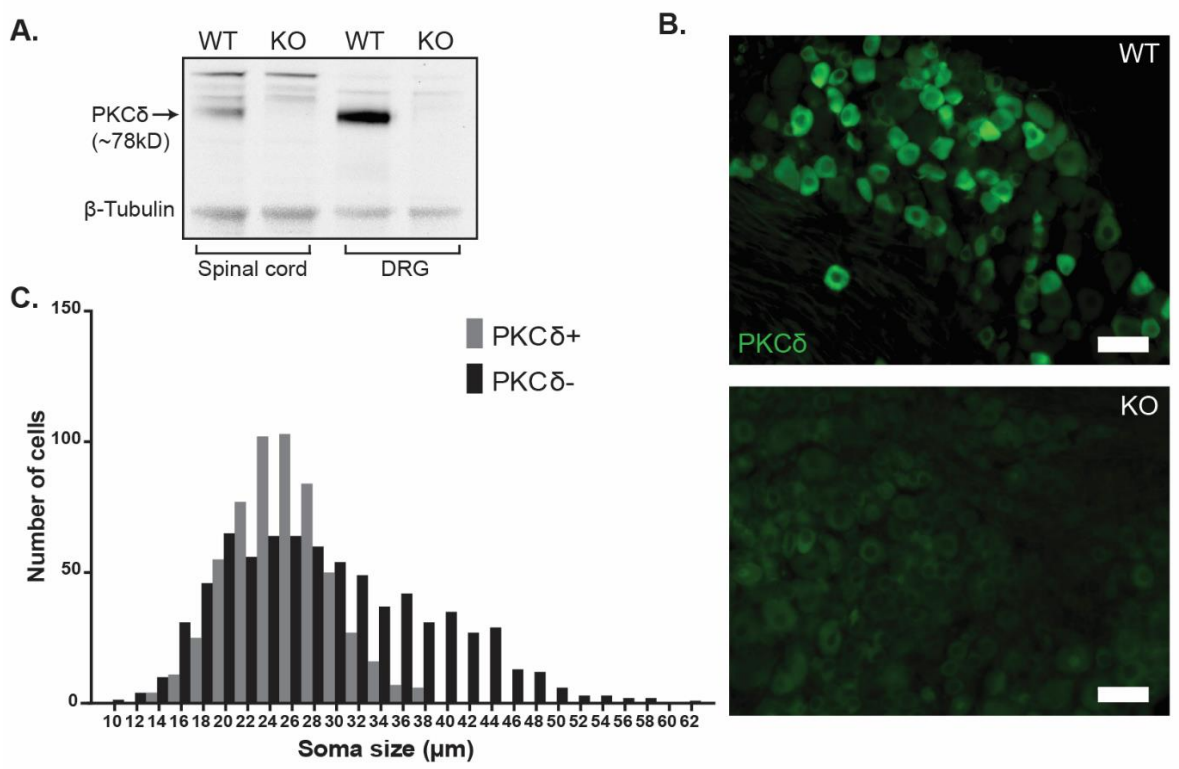

Figure 2. Immunological analyses of PKCδ in the spinal cord and DRG. A. Western blot demonstrating expression of PKCδ in WT DRG and spinal cord (SC) but not in PKCδ-KO tissue confirming the validity of the PKCδ antibody. **B.** Representative images of 18μm sections from WT and PKCδ-KO lumbar DRG. (Scale bar = 50μm) **C.** Histogram of cell diameter measurements of PKCδ+ and PKCδ- neurons illustrates the localization of PKCδ to small and medium diameter soma.

predominantly restricted to small diameter neurons (average diameter 23.3±0.19μm, min 11.6μm, max 36.2μm) (Figure 2C).

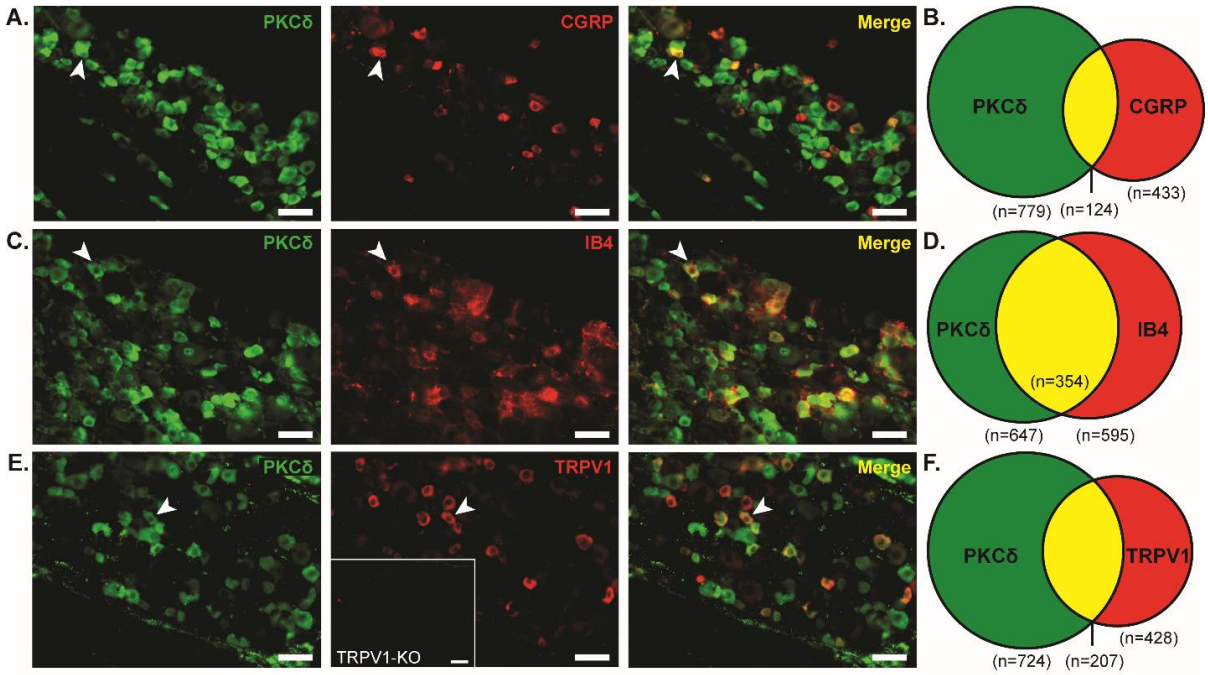

Figure 3. Localization of PKCδ and markers of peptidergic and non-peptidergic dorsal root ganglion neurons. A. Representative images of CGRP+, PKCδ+, and co-expressing (Merge) DRG neurons. **B.** Graphical representation of total neurons counted and degree of overlap (n = number of neurons). **C, D.** Representative images of IB4+, PKCδ+, and IB4+/PKCδ+ neurons and illustration of overlap. **E, F.** Representative images of TRPV1+, PKCδ+, and TRPV+/PKCδ+ neurons and illustration of overlap. Inset demonstrates TRPV1 antibody stain in TRPV1-KO mice. (Scale bar = 50μm; Arrowheads indicate example cells that express both markers).

PKCδ is expressed in peptidergic and non-peptidergic DRG neurons

We further characterized PKCδ expression in small diameter DRG neurons by immunohistochemical analysis of peptidergic and non-peptidergic markers. Of peptidergic neurons identified by anti-Calcitonin Gene Related Peptide (CGRP+) immunoreactivity, 26.8% expressed PKCδ, while 14.7% of PKCδ-expressing neurons were CGRP-positive (Table 1, Figure 3A-B). PKCδ was also expressed in non-peptidergic neurons identified by isolectin B4 (IB4) binding. Of IB4+ DRG neurons, 61.6% expressed PKCδ and 55.0% of PKCδ+ neurons exhibited IB4 binding (Table 1, Figure 3C-D). These findings indicate that PKCδ is expressed in both

peptidergic and non-peptidergic sensory neurons, with greater expression overlap found with non-peptidergic IB4+ neurons.

Behavioral and physiological studies have shown that itch produced by histamine is largely dependent on the non-specific cation channel transient receptor potential vanilloid receptor 1 (TRPV1) [34-36]. Because PKC has previously been demonstrated to modulate TRPV1 function and may present one potential mechanism by which PKCδ regulates itch, we determined the degree of overlap between PKCδ and TRPV1 expression [24, 37]. We first confirmed the specificity of our antibody directed against TRPV1 using TRPV1 knockout mice (Figure 3E, inset). Our data indicate that 29.1% of PKCδ-positive neurons were also TRPV1-positive, and 48.9% of TRPV1-positive neurons also expressed PKCδ, suggesting a potential functional relationship between PKCδ and TRPV1 (Figure 3E-F; Table 1).

Table 1. Percent of DRG neurons in which PKCδ colocalizes with other markers (mean ± SEM). (n = 3-4 mice per marker.)

Marker	% of PKCδ+ neurons expressing marker	% of marker+ neurons expressing PKCδ
CGRP	14.7 ± 2.5	26.8 ± 3.5
IB4	55.0 ± 3.0	61.6 ± 7.9
TRPV1	29.1 ± 2.2	48.9 ± 4.0

PKCδ-KO sensory neurons exhibit diminished responses to histamine

PKCδ is expressed in the brain, spinal cord, and the peripheral nervous system [33, 38-41]. Therefore, global genetic deletion of PKCδ in our knockout mice makes it difficult to pinpoint where PKCδ functions to modulate histamine-induced scratching. The expression of PKCδ in small diameter sensory neurons suggests that it may mediate histamine-evoked itch by signaling in nociceptive neurons responsive to pruritic agents (i.e., pruriceptors). To determine if PKCδ directly modulates neuronal responses to histamine, calcium imaging was performed on acutely dissociated adult mouse DRG neurons (Figure 4A-B). Of the total sensory neurons treated with histamine, 11.1% of wild type neurons responded to bath application of 100μM histamine

(126/1137 total WT neurons, N=5 animals), but only 6.7% of PKCδ-KO neurons responded to histamine (47/706 total KO neurons, N=3 animals), indicating a significant reduction of 39.6% in the proportion of histamine responsive neurons ($p<0.01$, χ^2 test) (Figure 4C). No significant difference in peak calcium responses to histamine was detected between knock-out and wild type cells (WT 33.6±2.8% increase from baseline, n=126 cells; KO 39.9±6.6% increase from baseline, n=47 cells, unpaired t-test, p=0.304) (Figure 4D).

We hypothesized that PKCδ could contribute to neuronal responses to histamine by mediating histamine receptor coupling to TRPV1, or by regulating the normal expression or function of TRPV1. To investigate whether the absence of PKCδ affects the activation of TRPV1 within histamine-responsive neurons, we applied the TRPV1-specific agonist capsaicin after the histamine response. We found that 58.7% of WT histamine-responsive neurons subsequently responded to capsaicin (74/126 total His+ neurons), while only 40.4% of KO histamine-sensitive neurons responded to capsaicin (19/47 total His+ neurons; $p<0.05$, χ^2 test) (Figure 4E). To determine whether the functional expression of TRPV1 is altered in PKCδ-KO neurons, we tested WT and KO sensory neurons for responses to capsaicin. There were no differences in the total number of capsaicin-responsive neurons between KO and WT groups (117/194 (60.3%) WT neurons, 144/259 (55.6%) KO neurons, $p=0.315$, χ^2 test, Figure 4G). Together, these data indicate that the reduction in histamine responses is not due to altered levels of TRPV1 receptors in the KO, and support the idea that PKCδ could modulate TRPV1 downstream of histamine receptor activation.

To further control for the possibility of developmental effects or compensatory mechanisms that may occur with congenital genetic deletion of PKCδ, we performed calcium imaging experiments with the same experimental design using acute pharmacological inhibition in wild type DRG cultures. The δV1-1 peptide inhibitor has been shown to inhibit PKCδ activation *in vitro* and *in vivo* by competitively binding to receptor for activated C-kinase (RACK)

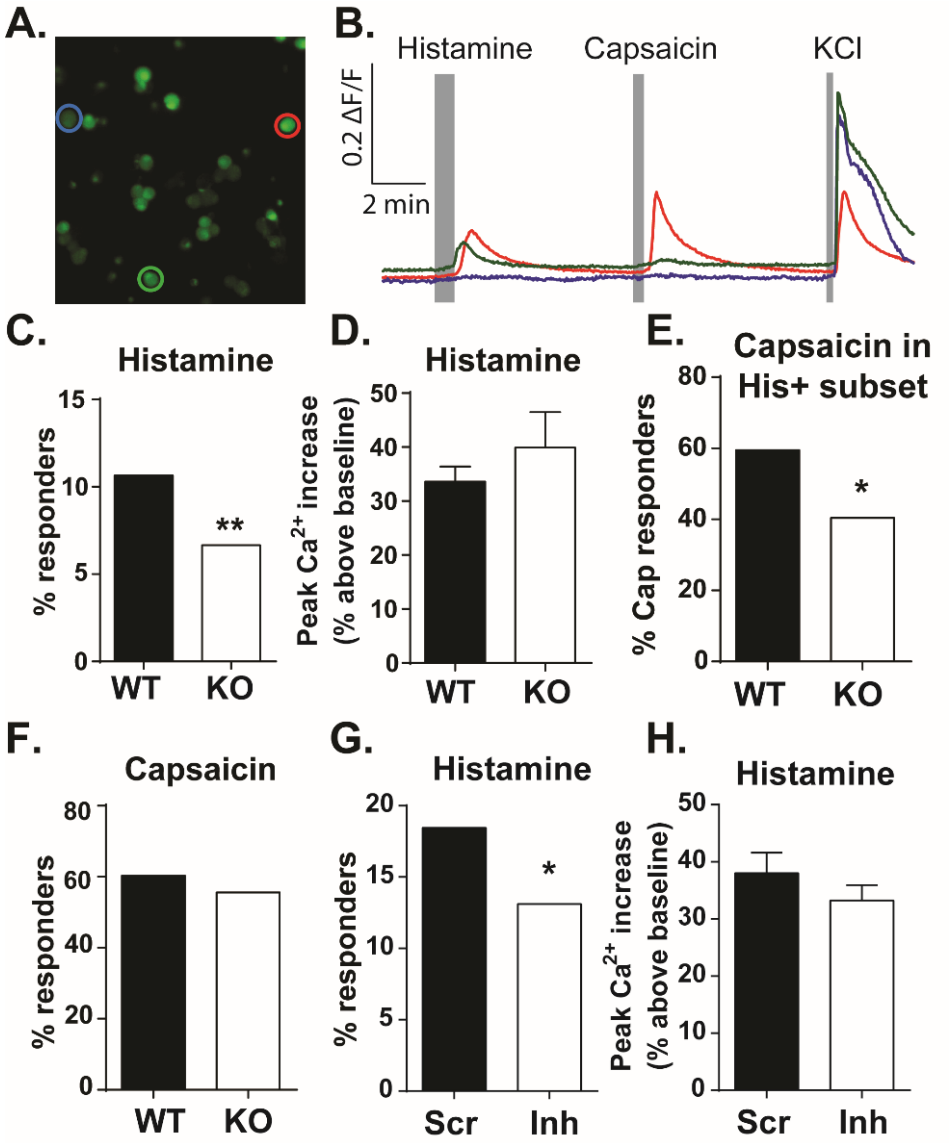

Figure 4. PKCδ mediates sensory neuron responses to histamine. A. Representative image of dissociated DRG neurons loaded with Fura2-AM. **B.** Representative traces of selected cells (corresponding colored circles in **A**) in response to histamine, capsaicin, and KCl. **C.** Proportion of total histamine responders in WT and KO DRG neurons (WT=11.1% (126/1137 total WT neurons); KO=6.7% (47/706 total KO neurons); ** $p<0.01$, χ^2 test). **D.** Peak calcium increase in response to histamine, defined as % signal increase above baseline. WT 33.6±2.8% change from baseline, n=126 cells; KO 39.9±6.6% change from baseline, n=47 cells, unpaired t-test, p=0.304. **E.** Percent of histamine-responsive neurons that responded to capsaicin (74/126 WT His+ neurons; 19/47 KO His+ neurons; $p<0.05$, χ^2 test). **F.** Proportion of capsaicin-responsive neurons (117/194 WT neurons, 144/259 KO neurons, p=0.315, χ^2 test). **G.** Proportion of histamine-responsive neurons in scramble- vs. inhibitor-treated groups (18.5% (75/405) of scramble-treated neurons vs. 13.1% (69/527) of peptide-treated neurons, $p<0.05$, χ^2 test). **H.** Peak calcium increase in response to histamine (inhibitor: 33.23±2.7% change from baseline, n=69 cells; scramble: 41.0±3.7% change from baseline, n=75 cells, p=0.095, unpaired t test).

proteins, which confer PKC isoform substrate specificity [31, 42-45]. DRG neurons were incubated with the peptide inhibitor δV1-1 for 30 minutes prior to recording. Consistent with our findings in PKCδ-KO neurons, there was a significant reduction in the total number of histamine-responsive neurons treated with peptide inhibitor when compared to scramble peptide-treated control neurons (18.5% (75/405) of scramble-treated neurons vs. 13.1% (69/527) of peptide-treated neurons, N=4 animals, $p<0.05$, χ^2 test) (Figure 4F). Peak calcium responses induced by histamine were not different between inhibitor- and scramble-treated groups (inhibitor 33.23±2.7% change from baseline, n=69 cells; scramble 41.0±3.7% change from baseline, n=75 cells, p=0.095, unpaired t test) (Figure 4G).

Discussion

Pruritic stimuli can activate sensory neurons via specific intracellular signaling cascades, which represent potential targets for anti-pruritics, but these cascades remain poorly understood. Characterizing signaling components is a significant challenge, in part because of the great diversity of recently identified pruritic receptors. Some receptors involved in pruritus are coupled to $G_{i/o}$ cascades such as the H4 histamine receptor, while others utilize kinase signaling pathways like the TSLP/IL-7 receptors [6, 7, 46, 47]. However, the majority of identified pruritic receptors, including the H_1 receptor and the "orphan" family of MRGPR receptors, are linked by a common $G_{\alpha q}$ signaling mechanism [10, 48-51]. In this study we focused on a component downstream of the canonical $G_{\alpha q}$ signaling pathway, the serine/threonine kinase PKC.

PKC isozymes are divided into three groups: classic (activated by DAG and Ca^{2+}), novel (activated by DAG but not Ca^{2+}), and atypical (activated by neither DAG nor Ca^{2+}) [52, 53]. Specific PKC isoforms have been shown to selectively regulate nociceptive behavior and nociceptor physiology [16, 17, 19, 54-60]. We previously showed that the novel isozyme PKCδ is dispensable for acute mechanical and thermal nociceptive behaviors [20]. Previous reports have implicated PKCδ in H_1R signaling in non-neuronal cells [21, 22], but the specific role of PKCδ in pruriceptor signaling and itch had not been explored. In this study we found that PKCδ was necessary for the full expression of histamine-induced itch, but it did not have significant effects on histamine-independent itch produced by the MRGPR ligands chloroquine or β-alanine.

To determine whether the scratching deficit we observed in PKCδ null mice could be attributed to loss of function within sensory neurons, we examined anti-PKCδ staining in mouse lumbar DRG. Previous studies indicated that PKCδ is expressed in murine spinal cord and DRG, but the precise subset of PKCδ-positive neurons had not been characterized [33, 38-41]. We found that PKCδ expression was restricted to small diameter dorsal root ganglion neurons. Furthermore, although PKCδ was expressed in both peptidergic and non-peptidergic sensory neurons, it was greatly enriched in the non-peptidergic subset. Both peptidergic and

nonpeptidergic fibers have been shown to play a role in pruritus. Histaminergic itch is largely dependent on CGRPα-positive neurons [61], however, a subset of histamine-responsive neurons also express the nonpeptidergic marker IB4 [11, 62].

Calcium imaging studies of dissociated DRG neurons demonstrated that genetic deletion of PKCδ resulted in a significant reduction of the proportion of adult sensory neurons that were histamine-responsive. We further confirmed these results using acute pharmacological inhibition, supporting the hypothesis that PKCδ functions within normal wild type sensory neurons to mediate acute histaminergic signaling. The expression of PKCδ in small diameter sensory neurons, along with the reduction of histamine-responsive sensory neurons, suggest a peripheral mechanism for the behavioral effects of global PKCδ deletion on histamine-induced scratching.

Following histamine release, sensory neuron signaling to produce itch is thought to depend on functional coupling of H_1R to TRPV1. Supporting this idea, mice lacking TRPV1 exhibit greatly reduced scratching behavior and cellular responses to histamine, and blocking TRPV1 channel function likewise abolishes the response of sensory neurons to histamine [34, 36, 63]. The mechanisms by which H_1R recruits TRPV1 are complex and several different signaling pathways have been implicated [35, 36, 64, 65]. One possible mechanism by which histamine could couple to TRPV1 in sensory neurons is through PLC-induced PKC activation. This is further supported by an expanding body of literature indicating that PKC directly modulates TRPV1 function [24, 37]. Indeed, inhibitors for PLC and PKC prevent histamine-induced TRPV1-potentiation [64]. We previously found that acute mechanical and thermal pain were independent of PKCδ. In contrast, PKCδ was necessary for the full expression of thermal hyperalgesia during Complete Freund's Adjuvant-induced inflammation, which is a TRPV1 dependent process [20].

In this study, 49% of TRPV1-expressing neurons were PKCδ-positive and we showed a significant reduction in the proportion of capsaicin-responsive neurons within the subset of neurons responsive to histamine. Additionally, sensory neurons from PKCδ-KO mice not previously treated with histamine responded similarly to wild type neurons when challenged with

capsaicin. These observations indicate that acute detection of heat stimuli by TRPV1 is not dependent on PKCδ, but suggest that PKCδ could function downstream of the histamine receptor to modulate TRPV1 function. In further support of this idea, several other inflammatory mediators including PGE_2, NGF, and IL-6 have been shown to activate PKCδ [41, 66-68]. It is possible that pruritic dermatoses marked by inflammation may recruit PKCδ, resulting in sensory neuron modulation that could potentiate itch.

In contrast to histaminergic signaling, the MRGPR receptors appear to produce their pruritic effects through an alternative, PKCδ-independent pathway. Chloroquine activates MRGRPA3 which couples to the irritant receptor TRPA1 to produce itch. Neuronal responses to chloroquine were prevented by inhibiting Gβγ subunit activity, suggesting that the $G_{\alpha q}$ pathway is not necessary for chloroquine-induced itch [15]. However, MRGPRA3 was also recently shown to sensitize TRPV1 via a PKC mechanism likely dependent on $G_{\alpha q}$ signaling, suggesting a possible mechanism for thermal sensitization [69]. This suggests that MRGPRA3 may have biased signaling mechanisms that lead to itch and/or TRPV1-related sensitization. Thus, the histamine receptor and MRGPRs may share a pathway leading to sensitization of TRPV1 via PKC signaling, despite an alternative Gβγ mechanism for MRGPRA3 signaling of itch [48, 70].

In summary, we found that PKCδ is a mediator of histaminergic itch signaling in sensory neurons. Although we specifically investigated PKCδ in this study, other PKC isoforms may also be involved in modulating the response to itch. For example, another novel PKC isozyme, PKCε, is expressed in largely IB4+ neurons, and has been shown to also modulate TRPV1 responses [16, 59, 60, 71]. The sensory neuron responses involved in itch are complex, involving multiple molecular cascades which may be differentially mediated by specific PKC isoforms. Future studies that investigate the roles of PKC isozymes in itch may contribute to better therapeutic specificity for the treatment of acute and chronic pruritus.

Author contributions and Acknowledgements

MVV, SD, and CZ performed experiments and analyzed data. MVV, SD, CZ, and RWG conceived the study and contributed to writing the manuscript. ML provided PKCδ-KO mice. All authors read and agreed to the final version of the manuscript. This work was supported in part by NIH funding NS089130 & GM007200 (MVV); NS076324 (SD); NS48602 & NS42595 (RWG). The authors would like to thank Sherri Vogt for exceptional mouse colony care and biochemistry technical support; Dr. Judith Golden for helpful discussions and critical reading of the manuscript; Dr. Vijay Samineni for experiment support; and Gereau lab members for helpful discussions. We thank Dr. George Wilcox for the PKCδ inhibitor.

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Chapter 3

Enhanced non-peptidergic intraepidermal fiber density and an expanded subset of chloroquine-responsive neurons in a mouse model of dry skin itch

This chapter contains the manuscript:
Valtcheva MV, Samineni VK, Golden JP, Gereau RW, Davidson S. Enhanced nonpeptidergic intraepidermal fiber density and an expanded subset of chloroquine-responsive trigeminal neurons in a mouse model of dry skin itch. The journal of pain: official journal of the American Pain Society. 2015;16(4):346-56. doi: 10.1016/j.jpain.2015.01.005. PubMed PMID: 25640289; PubMed Central PMCID: PMC4385451

Abstract

Chronic pruritic conditions are often associated with dry skin and loss of epidermal barrier integrity. In this study, repeated application of acetone and ether, followed by water (AEW) to the cheek skin of mice produced persistent scratching behavior with no increase in pain-related forelimb wiping, indicating the generation of itch without pain. Cheek skin immunohistochemistry showed a 64.5% increase in total epidermal innervation in AEW-treated mice compared to water-treated controls. This increase was independent of scratching, because mice prevented from scratching by Elizabethan collars showed similar hyperinnervation. To determine the effects of dry skin treatment on specific subsets of peripheral fibers, we examined Ret-positive, CGRP-positive, and GFRα3-positive intraepidermal fiber density. AEW treatment increased Ret-positive fibers, but not CGRP-positive or GFRα3-positive fibers, suggesting that a specific subset of non-peptidergic fibers could contribute to dry skin itch. To test whether trigeminal ganglion neurons innervating the cheek exhibited altered excitability after AEW treatment, primary cultures of retrogradely labeled neurons were examined using whole-cell patch clamp electrophysiology. AEW treatment produced no differences in measures of excitability compared to water-treated controls. In contrast, a significantly higher proportion of trigeminal ganglion neurons were responsive to the non-histaminergic pruritogen chloroquine after AEW treatment. We conclude that non-peptidergic, Ret-positive fibers and chloroquine-sensitive neurons may contribute to dry skin pruritus.

Introduction

Pruritus is a primary complaint associated with xerosis (dry skin) and other dermatoses that compromise skin barrier integrity such as atopic dermatitis and psoriasis [1, 2]. A rodent model of persistent and ongoing dry skin pruritus was previously developed by application of equal parts acetone and ether followed by water (AEW) to the rostral back skin [3]. Adaptation of the AEW model to the hind-limb, where biting and licking behaviors were used to indicate itch, showed an absence of behavioral sensitization to heat and mechanical stimuli, suggesting the dry skin produced itch but not hyperalgesia [4]. Recently, clear differentiation between pain and itch behaviors was achieved by application of algogens or pruritogens to the rodent cheek [5, 6]. When repeated AEW treatments were applied to the cheek, hind-limb scratching behavior was evoked, indicating that dry skin produced ongoing itch [7].

The mechanisms by which dry skin generates itch are unclear. Intriguingly, human pruritic dermatoses are frequently associated with increased intraepidermal fiber density [8-11]. Increased intraepidermal fiber density was also reported after a single, acute application of acetone in rodents [12, 13], and has been hypothesized to occur in AEW-induced itch [14]. However, scratching after AEW treatment develops with a latency of 3 days, suggesting that the sprouting fibers observed after a single treatment may be insufficient to induce itch [3, 4, 15]. The identity of the expanded peripheral fibers is not known, nor is it understood whether fiber hyperinnervation directly contributes to pruritus.

In addition to possible changes in epidermal innervation, enhancement of pruritic receptor function and phenotypic switching of sensory neurons into pruriceptors may contribute to the increased itch generated by dry skin. Like many intractable pruritic conditions, AEW-induced itch is thought to be histamine-independent [3]. Novel non-histaminergic neural pathways and pruritic receptors have recently been identified [16]. Importantly, members of the Mas-related gene protein receptor family (Mrgprs) are activated by the non-histaminergic pruritogen chloroquine, and ablation of MrgprA3 resulted in decreased scratching after AEW treatment [17, 18]. MrgprA3

is functionally coupled to TRPA1, a channel that exhibits sensitivity to a wide range of irritants including mustard oil and formalin [19-22]. A significant reduction in scratching was observed in TRPA1 knock-out mice exposed to AEW treatment. Furthermore, AEW treatment induced upregulation of MrgprA3 mRNA, suggesting that these receptors may contribute to dry skin-induced itch [7].

The aim of this study was to determine the effects of dry skin pruritus on peripheral fiber anatomy and the physiological properties of sensory neurons innervating dry skin. Intraepidermal innervation was characterized and quantified with and without scratch-preventing Elizabethan collars to determine whether scratching itself contributes to changes in nerve fiber density in dry skin. We tested the hypothesis that dry skin produces peripheral sensitization by enhancing the excitability of trigeminal neurons. Finally, we monitored calcium responses evoked by chloroquine and mustard oil from AEW-treated and control animals to test for altered expression or function of itch-related receptors.

Materials and Methods

Animals and Acetone-Ether-Water (AEW) treatment

All experiments were conducted in accordance with the National Institutes of Health guidelines and received the approval of the Animal Care and Use Committee of Washington University, School of Medicine. 8-12 week old littermate mice (C57BL/6 (Jackson lab) or Ret-EGFP [23, 24]) were housed on a 12 hour light-dark cycle and allowed ad libitum access to food and water. Ret-EGFP reporter mice (129/SvJ:C57BL/6) were obtained from Dr. Jain [23]. Cheek skin was shaved with electric clippers one day prior to the start of acetone-ether-water treatments. The acetone-ether group was treated with a 1:1 mixture of acetone and diethyl ether (Sigma, St. Louis, MO) for 30 seconds by soaking and then applying a gauze-wrapped cotton tip to the cheek, followed by similarly applied water for 30 seconds. Control animals were treated with water only. After 6 days of twice per day treatments (morning and evening), scratching behavior was quantified 6-8 hours after the final AEW treatment. Mice were placed in individual observation chambers and allowed to acclimate for 1 hour prior to observation. Bouts of scratching were then counted for 1 hour with experimenters blinded to treatment. A bout of scratching was defined as any number of individual scratch events separated by a pause. During the pause, behaviors such as licking or biting of the hind-limb, holding the limb motionless, or putting the limb down on the surface, could occur. Wiping behavior was taken to indicate pain and was defined as a rostrally-directed movement of the ipsilateral forelimb across the cheek starting from the ear [5, 6]. Scratching behavior with continued AEW treatment has been reported to persist for at least 2 weeks [4, 15].

Immunohistochemistry

After six days of AEW treatment, mice were deeply anesthetized (ketamine-xylazine-acepromazine: 38-1.92-0.38 mg/mL; 2.7 mL/kg). The treated skin was dissected and immersion-fixed in Zamboni's fixative for 4 hours, rinsed in PBS, and cryoprotected in 30% sucrose, then

sectioned at 30μm and collected on slides. Wild type C57BL/6 mice were used to determine β-III tubulin, CGRP, and GFRα3 fiber innervation, while a separate strain of Ret-EGFP reporter mice[23, 24] were used to determine Ret-positive fiber density. Antibodies and dilutions: rabbit anti-βIII tubulin (1:1000, Covance), goat anti-CGRP (1:1000, Serotec), goat anti-GFRα3 (1:100, R&D Systems), rabbit anti-GFP (1:1000, Invitrogen), Alexa Fluor 488/555 donkey anti-rabbit (1:200-400, Invitrogen), Alexa Fluor 488 donkey anti-goat (1:200-350, Invitrogen). These concentrations are based on previous demonstrations of intraepidermal fiber staining [24-26]. Specificity of the GFRα3 antibody was previously shown using a GFRα3 knock-out mouse [27]. All slides were stained with bisbenzamide (1:40,000, Sigma, St. Louis, MO.) and MetaMorph Software (Molecular Devices, Sunnyvale, CA.) was used to measure the length of the dermal-epidermal border. In each examined section labeled fibers crossing the dermal-epidermal border were counted on an upright epifluorescent microscope (Nikon 80i; CoolSnapES camera). Six examined sections separated by >60µm were analyzed for each animal and the mean fiber density was calculated. To determine whether changes in fiber innervation were dependent on scratching, modified Elizabethan collars (Harvard Apparatus, Holliston, MA.) were secured at the start of treatment in both AEW and water-only groups. For hematoxylin and eosin (H&E) staining, tissue was fixed in Zamboni's fixative for 2-4 hours, then embedded in paraffin. Sections 10 µm thick were stained using standard H&E methods [28]. Representative images of fibers stained with the above-described methods were obtained using a Leica SPE Confocal microscope. Images were collected across the z-plane at 1µm width and maximum projection images were generated using ImageJ software.

Culture of trigeminal ganglion neurons

Wild type mice were sacrificed by decapitation after nine days of treatment and the ipsilateral trigeminal ganglia (TG) were removed and cut into several pieces. Ganglia were incubated in 45U papain (Worthington, Lakewood, NJ) in 3 mL Hank's buffered saline solution

without Ca^{2+} or Mg^{2+} and with 10 mM HEPES at 37°C, 5% CO_2 for 20 minutes. TG were washed and then incubated in collagenase (1.5 mg/ml) for 20 minutes. TG were triturated with a fire-polished Pasteur pipette, then passed through a 40μm filter, and the dissociated cells were plated on poly-D-lysine and collagen coated glass coverslips. Cells were cultured overnight in Neurobasal A media supplemented with B27, 100U/mL penicillin/streptomycin, 2 mM Glutamax, and 5% fetal bovine serum (Gibco). No additional growth factors were added to the media. All experiments were performed within 24 hours of plating.

Whole-cell patch clamp electrophysiology

For electrophysiology experiments, FastDiI (Sigma, St. Louis, MO.) was injected intradermally into the cheek of wild type mice on day 2 of AEW or water treatment to label trigeminal neurons innervating the skin at the treatment site. After seven additional days of AEW or water treatment to allow maximum retrograde labeling of trigeminal neurons, including sprouting terminals, trigeminal ganglia were cultured as described above. Retrogradely labeled trigeminal neurons from AEW- or water-treated mice were then identified using an Olympus BX-50 epifluorescence microscope and subsequently examined for differences in membrane excitability. Cells were tested in an external recording solution consisting of (in mM): 145 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 7 Glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH and 305 mOsm with sucrose. Borosilicate, filamented glass electrodes with 2-5 MΩ resistance (Warner Instruments, Hamden, CT) contained internal solution (in mM): 130 K-gluconate, 5 KCl, 5 NaCl, 3 Mg-ATP, 0.3 EGTA, 10 HEPES, adjusted to pH 7.3 with KOH and 294 mOsm with sucrose. After acquiring gigaseal and break-in, neurons were given 2 minutes to stabilize and then a series of protocols to determine membrane excitability were performed. Action potentials were evoked in current clamp mode using a series of increasing 1 second ramp current injections. The first action potential of a train was used to determine threshold, defined as the voltage at which the first derivative of the membrane potential increased by 10 V/s. Rheobase was established from

the step current pulse at which the first action potential was triggered. Data were collected with a HEKA EPC 10 amplifier, digitized at 20 kHz, and recorded on a PC running Patchmaster software (v2x-71). Series resistance was kept below 10 MΩ in all recordings and only DiI labeled cells with a diameter less than 30 µm were studied.

Calcium imaging

The protocol for calcium imaging was adapted from our previous studies [29-32]. Cells from wild type mice were incubated for 45 minutes in 3 µg/mL of the cell-permeant ratiometric calcium indicator Fura-2 AM (Molecular Probes) and then incubated in external solution (in mM): 130 NaCl, 5 K, 2 $CaCl_2$, 1 $MgCl_2$, 30 Glucose, 10 HEPES for a 30 minute de-esterification prior to recording. Coverslips were positioned in a recording chamber and perfused with external solution at room temperature. Cells were viewed under an inverted microscope (Olympus Optical, Tokyo, Japan) and images were captured with a Hamamatsu Orca camera. Regions of interest encompassing all Fura-loaded cells were identified a priori and the ratio of fluorescence emission at an alternating excitation wavelength of 357 and 380 nm was recorded with SimplePCI Software. The experimental protocol consisted of a 2 minute baseline followed by 30 second bath application of 100 µM mustard oil (MO, Sigma, St. Louis), 8 minutes of external solution, 30 seconds of 1mM chloroquine (CQ, Sigma, St. Louis), 8 minutes of external solution, and 10 seconds of high KCl (50mM). Peak responses were determined by calculating the absolute increase in Fura-2 signal above baseline immediately prior to each stimulus. A change from resting level of ≥20% was set as the threshold for a response to a bath applied chemical. Cells unresponsive to high K+ were excluded from physiological analysis.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.04 (2014). For comparisons between AEW-treated and water-treated scratching behavior, unpaired t-test was

used to compare the total scratch bouts or wipes per 60-minute interval. Electrophysiological data comparisons were performed using unpaired t-test. Differences between the proportion of responders in AEW and water groups were determined using a χ^2 test. Peak calcium increase in response to stimuli was analyzed with unpaired t-test. For all statistical analyses, significance was defined as $p<0.05$. Data are presented as mean ± S.E.M.

Results

AEW-induced dry skin elicits scratching but not wiping behavior

AEW treatment induced grossly visible, dry and scaly skin on the treated cheek of mice, whereas skin from water treated mice appeared unchanged (Figure 1A, B). AEW treatment also induced a hyperproliferation of keratinocytes resulting in thickening of the epidermis that was not observed in the water-only controls. The dry skin group was marked by spongiosis and large pieces of dissociating stratum corneum which still contained nucleated keratinocytes (Figure 1C, D). AEW treatment induced a significant increase in the number of site-directed bouts of scratching (H_2O = 4.0 ± 2.3 scratch bouts, AEW = 60.7 ± 17.0 scratch bouts, unpaired t-test, $p \leq 0.01$; Figure 1E). In contrast, very little wiping behavior was observed in general and no difference in the number of wipes was observed between water-only and AEW treatment groups (H_2O = 1.0 ± 0.8 wipes, AEW = 4.4 ± 1.7 wipes; unpaired t-test, Figure 1F). These results indicate that AEW-induced dry skin elicits ongoing itch without pain.

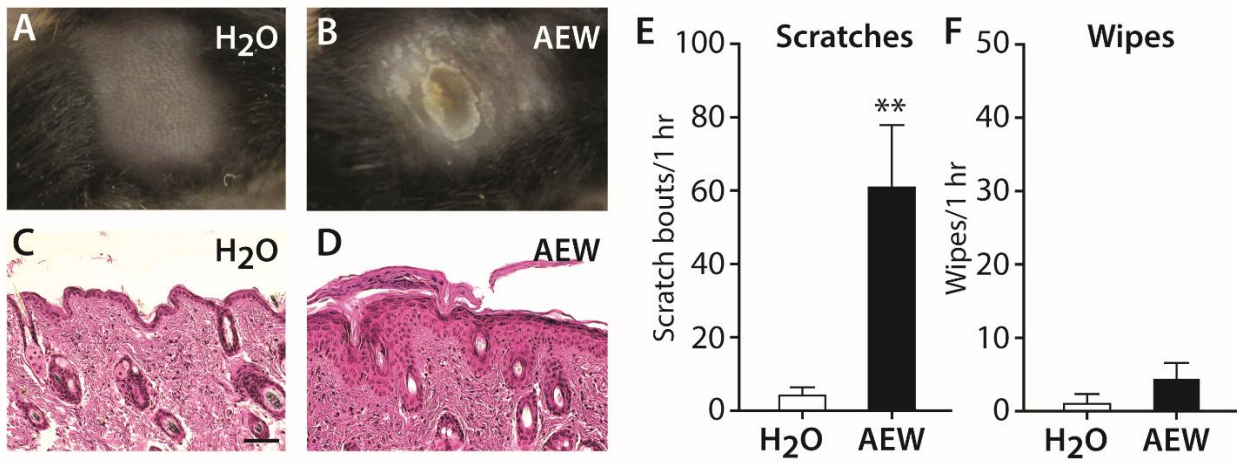

Figure 1. Dry skin treatment induces itch without pain. A, B) Photographs of shaved mouse cheeks after treatment with water or acetone/ether and water. AEW treatment induced scaly, dry skin. **C, D)** H&E staining shows epidermal hyperplasia and hyperkeratosis in the AEW treated group but not in the water treated group. Stratum corneum still containing nucleated keratinocytes can be seen in the process of dissociating from the epidermis. Scale bar = 50µm. **E, F)** Quantification of the mean number of scratch bouts and wipes during 1 hour of observation after 6 days of AEW treatment. AEW treatment greatly enhanced scratching ($p < 0.01$, unpaired t-test, water-treated n=9; AEW-treated n=11) but little wiping occurred and was not significantly different between water and AEW groups (n=5 each group, unpaired t-test).

Dry skin induces intraepidermal hyperinnervation independent of scratching

To determine whether dry skin alters intraepidermal nerve fiber density (IENFD), we quantified fiber innervation in the cheek epidermis from AEW- and water-treated mice. IENFD was measured using an antibody against β-III tubulin, which is specific for neurons and labels axons and their terminals (Figure 2).[33] IENFD was significantly increased in the AEW-treated group compared to water-only controls (H_2O = 26.8 ± 4.2 fibers/mm, AEW=44.1 ± 5.4 fibers/mm, n=5 animals per group, unpaired t-test, $p<0.05$) (Figure 2A - C).

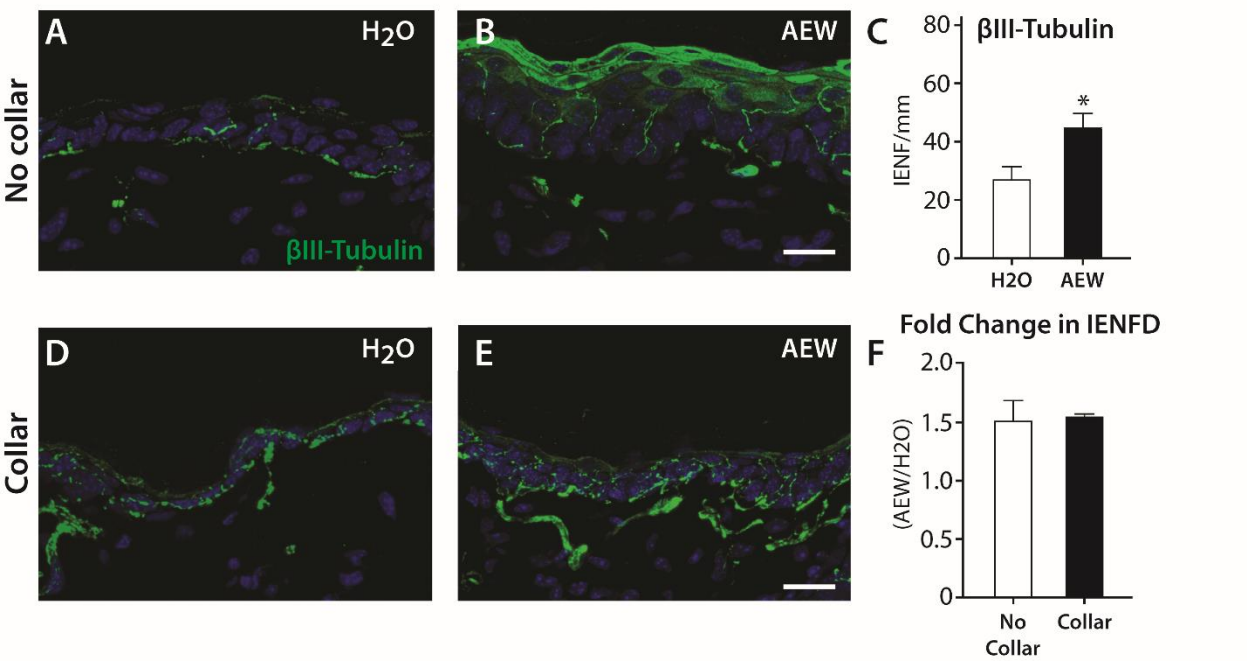

Figure 2. Dry skin induces intraepidermal hyperinnervation independent of scratching. A, B) Mice treated with acetone/ether and then water showed increased fiber innervation and epidermal hyperplasia compared to the water treated animals. Scale bar = 20μm. **C)** Intraepidermal fiber innervation was significantly increased by AEW treatment. **D, E)** Mice with Elizabethan collars placed at the start of treatment also showed increased fiber density and thickening of the epidermis. Scale bar = 20μm. **F)** No change in the magnitude of AEW-induced hyperinnervation relative to water-only controls was observed between collared and non-collared mice after AEW treatment.

Scratching itself is thought to promote itch via a positive-feedback loop known as the "itch-scratch cycle", and could alter fiber innervation. We sought to determine whether AEW-induced dry skin is itself capable of generating epidermal hyperinnervation, or whether scratching behavior was necessary to observe the increased IENFD. To this end, mice were fitted with Elizabethan

collars to prevent scratching of the cheek for the duration of the AEW and water treatments. AEW-treated mice that wore collars also showed an increase in IENFD (H_2O = 45.5 ± 1.8 fibers/mm, AEW = 70.2 ± 1.7 fibers/mm, n=3 animals per group, unpaired t-test, $p<0.001$) (Figure 2D, E). The magnitude of hyperinnervation relative to water controls (Fold Change) was not different between the no collar and collar groups (no collar = 1.5 ± 0.2 fold increase relative to water, n=5 animals; collar = 1.5 ± 0.04 fold increase relative to water, n=3 animals, unpaired t-test, p=0.89) (Figure 2F), indicating that AEW treatment induced epidermal hyperinnervation independent of scratching.

Dry skin selectively induces hyperinnervation by Ret-positive, non-peptidergic fibers

Both peptidergic and non-peptidergic fibers are present in the epidermis and may contribute to pruritus. However, βIII-tubulin is an indiscriminant marker of nerve fibers. Therefore, to determine the subsets of sensory fibers that are increased in AEW-induced dry skin, peptidergic fiber density was assessed with an anti-CGRP antibody. There were no significant changes in CGRP-positive IENFD in the dry skin group when compared to water controls (H_2O = 10.2 ± 1.4 fibers/mm, AEW = 8.9 ± 2.1 fibers/mm, n = 5 animals per group, unpaired t-test, p=0.62) (Figure 3A - C). Ret-EGFP reporter mice were used to identify Ret-positive (non-peptidergic) fibers, which were visualized using an anti-GFP antibody.[24] The density of Ret-positive epidermal fibers was significantly increased in the dry skin group (H_2O = 40.2 ± 1.1 fibers/mm, AEW = 70.1 ± 7.6 fibers/mm, n = 4 animals per group, unpaired t-test, $p<0.01$) (Figure 3D – F). A small subset of fibers that express both peptidergic and non-peptidergic markers can be identified by their expression of the artemin co-receptor GFRα3.[34] Intraepidermal fiber density of these fibers was quantified using an antibody directed against the GFRα3 receptor and no change in innervation after AEW treatment was observed (H_2O = 16.0 ± 1.2 fibers/mm, AEW = 14.9 ± 2.0 fibers/mm, n = 4-5 animals per group, unpaired t-test, p=0.68) (Figure 3G-I). These data suggest that dry skin

induces branching and extension of non-peptidergic, Ret-positive epidermal fibers, which may be important for the development or maintenance of dry skin-induced itch.

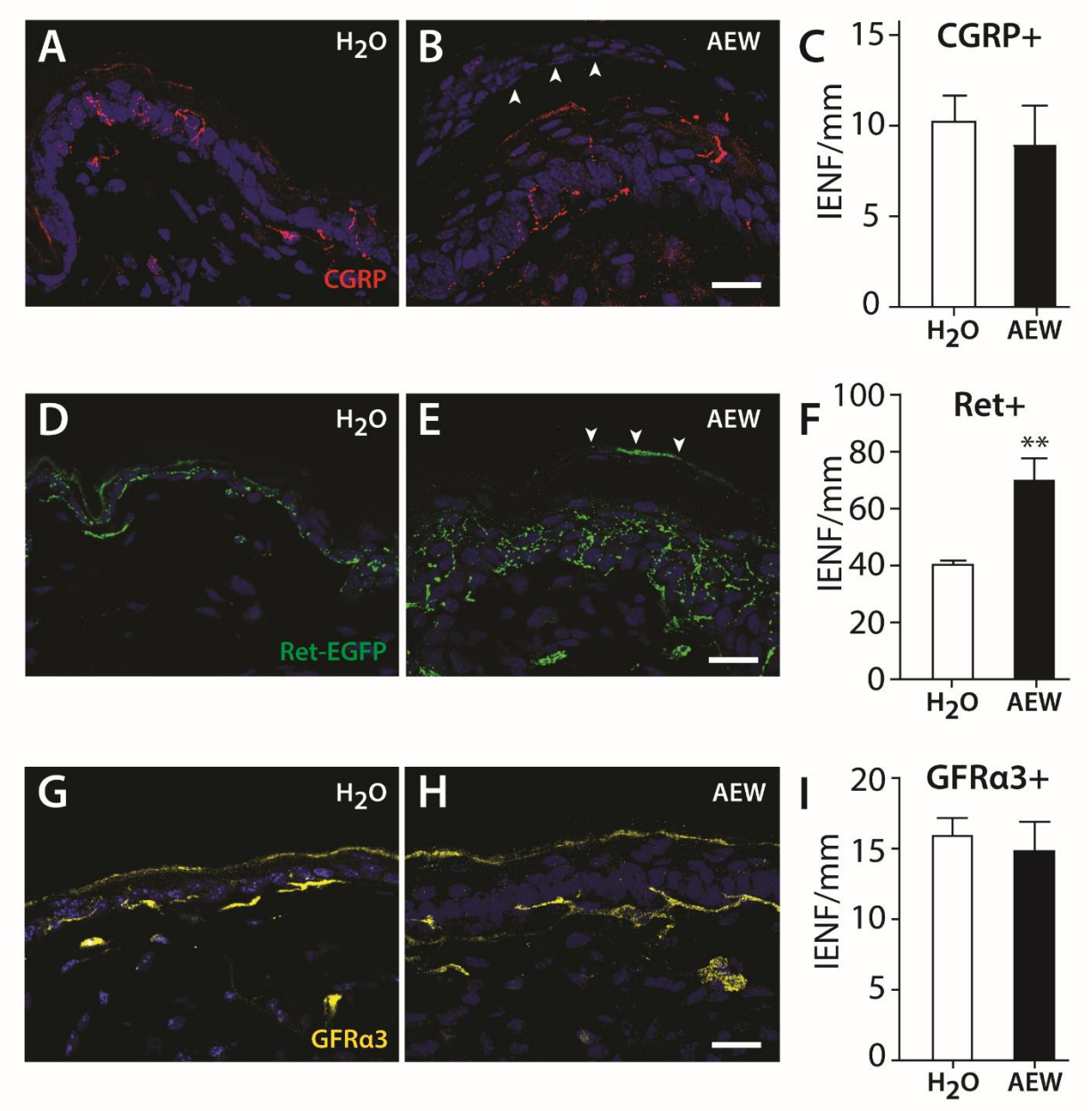

Figure 3. Hyperinnervation of Ret+, but not CGRP+ or GFRα3+ fibers after AEW treatment. **A, B)** Intraepidermal CGRP+ fibers appeared no different in the AEW and water groups. Scale bar = 20µm. Arrowheads indicate large pieces of dissociating stratum corneum which still contained nucleated keratinocytes. **C)** Quantification of CGRP+ density. **D, E)** Photomicrographs from a strain of mice in which eGFP is expressed from the Ret locus. eGFP immunostained fibers in the cheek epidermis show increased fiber density after AEW-treatment. Scale bar = 20µm. **F)** Quantification of Ret+ fiber density. **G, H)** IENFD of GFRα3+ fibers was not different between AEW and water groups. **I)** Quantification of GFRα3+ density.

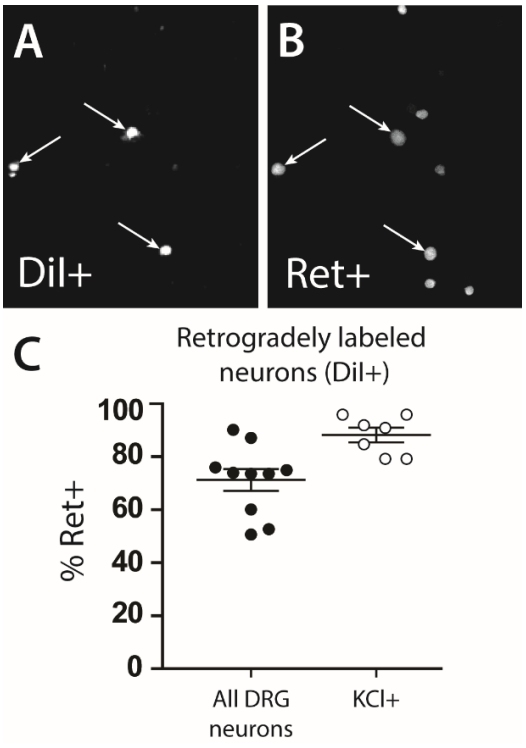

Figure 4. Retrograde labeling of trigeminal afferent fibers innervating the cheek. A, B) Two injections of DiI (10 uL each) into the cheek of an untreated Ret-EGFP mouse retrogradely labeled trigeminal ganglion neurons that were later cultured. Arrows show double-labeled neurons. **C)** 70.4 ± 4.1% of all DiI+ neurons were also Ret + (n=10 coverslips, each circle represents % per coverslip); 92.4 ± 2.7% KCl-responsive, DiI+ neurons were also Ret+ (n=5 coverslips). Total of 3 Ret-eGFP mice used.

We determined the proportion of trigeminal neurons projecting to the skin that were also Ret-positive by intradermal cheek injection of the retrograde tracer DiI into untreated Ret-EGFP mice. Trigeminal ganglia were harvested and cultured and we found that the majority of retrogradely labeled neurons were also Ret-positive (Figure 4A, B). Of all retrogradely labeled neurons, we found that 70.4 ± 4.1% were Ret-positive (Figure 4C). Additionally, when tested for physiological responses to KCl, we found that 92.4 ± 2.7% of the KCl-responsive, DiI-positive cells were also Ret-positive (Figure 4C). This increased proportion of viable Ret-positive skin-projecting neurons suggests a small loss of Ret-negative neurons in culture.

Effects of dry skin on trigeminal neuron physiology

We hypothesized that AEW treatment could alter the membrane excitability of trigeminal neurons innervating the dry skin. We specifically targeted neurons with known peripheral projections by retrogradely labeling trigeminal neurons with DiI injected into the cheek skin of C57BL/6 AEW- and water-treated mice (Figure 5A). Whole-cell patch clamp electrophysiology was then used to assess changes in membrane excitability. Rheobase was assessed using a short step and action potential threshold was determined with the first spike evoked from a ramp current (Figure 5B, C). Resting membrane potential of trigeminal neurons was not significantly

different between AEW- and water-treated mice (H_2O = -60.8 ± 2.7 mV, n=14 neurons, AEW = -57.2 ± 2.5 mV, n=15 neurons, unpaired t-test) (Figure 5D), and neither group showed spontaneous activity. There was also no significant difference in the current amplitude required to elicit an action potential between AEW- and water-treated animals (H_2O = 614.3 ± 70.4 pA, n=15; AEW = 453.3 ± 50.0 pA, n=15, unpaired t-test) (Figure 5E). Action potentials evoked by a ramp current injection showed no difference between AEW and water treatment groups in the threshold for activation (H_2O = -8.7 ± 3.7 mV, n=10; AEW = -3.8 ± 1.9 mV, n=12, unpaired t-test) (Figure 5F). Together, these data show that dry skin treatment did not produce ongoing activity or changes in membrane excitability that could be determined *in vitro*.

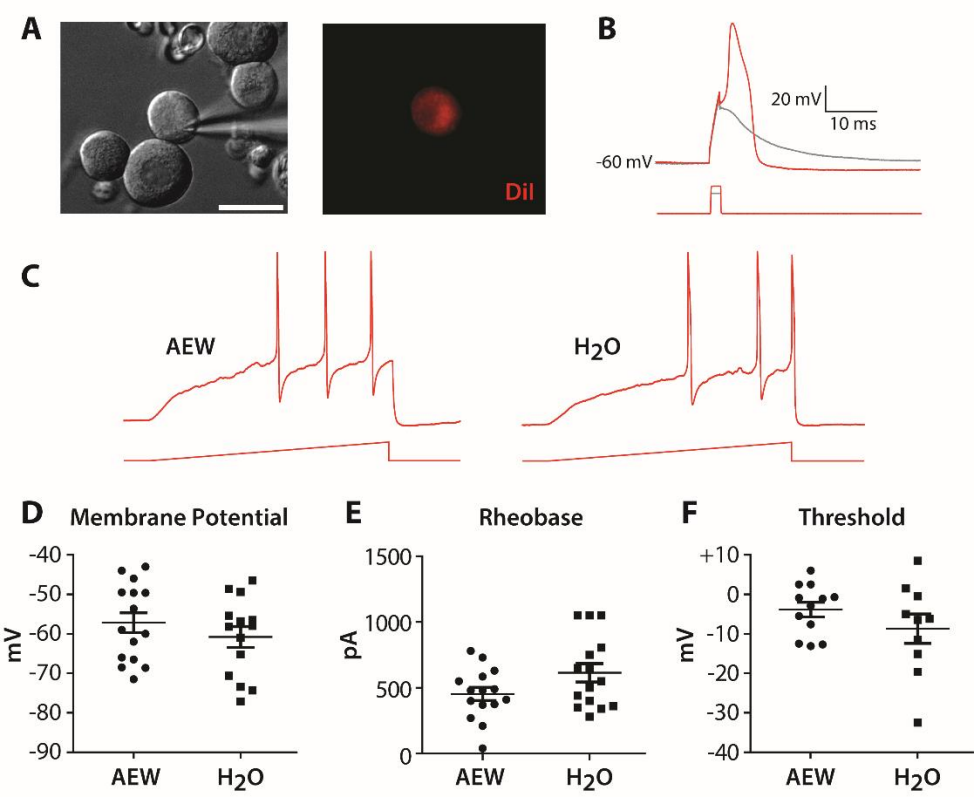

Figure 5. Electrophysiology of AEW- and water-treated trigeminal neurons. A) Photomicrograph of a patched wild type trigeminal neuron retrogradely labeled from the cheek with DiI. (Scale bar = 30µm) **B)** Example trace of a short step protocol and evoked action potential to determine rheobase. **C)** Example traces of the ramp current and evoked action potentials from AEW- and water-treated trigeminal neurons. **D)** Resting membrane potential, **E)** Rheobase, and **F)** Action potential threshold were not different between AEW- and water-treated groups.

We next determined whether AEW-induced dry skin altered the responses of pruritic receptors and pruriceptive neurons. Trigeminal ganglion neurons from AEW- and water-treated wild type C57BL/6 mice were harvested, cultured, and loaded with Fura2-AM (Figure 6A). Wilson et al., (2013) showed that TRPA1 is crucial for dry skin-induced itch and that MrgprA3 mRNA is upregulated in sensory neurons after AEW treatment. To determine whether corresponding functional changes occur in trigeminal neurons, intracellular calcium was monitored to test the responses to the TRPA1 agonist mustard oil (MO) and non-histaminergic pruritogen chloroquine (Figure 6B). We observed no significant difference between AEW- and water-treated groups in the proportion of MO-responsive neurons (H_2O = 89/1042 cells (8.54%), AEW = 107/1199 cells

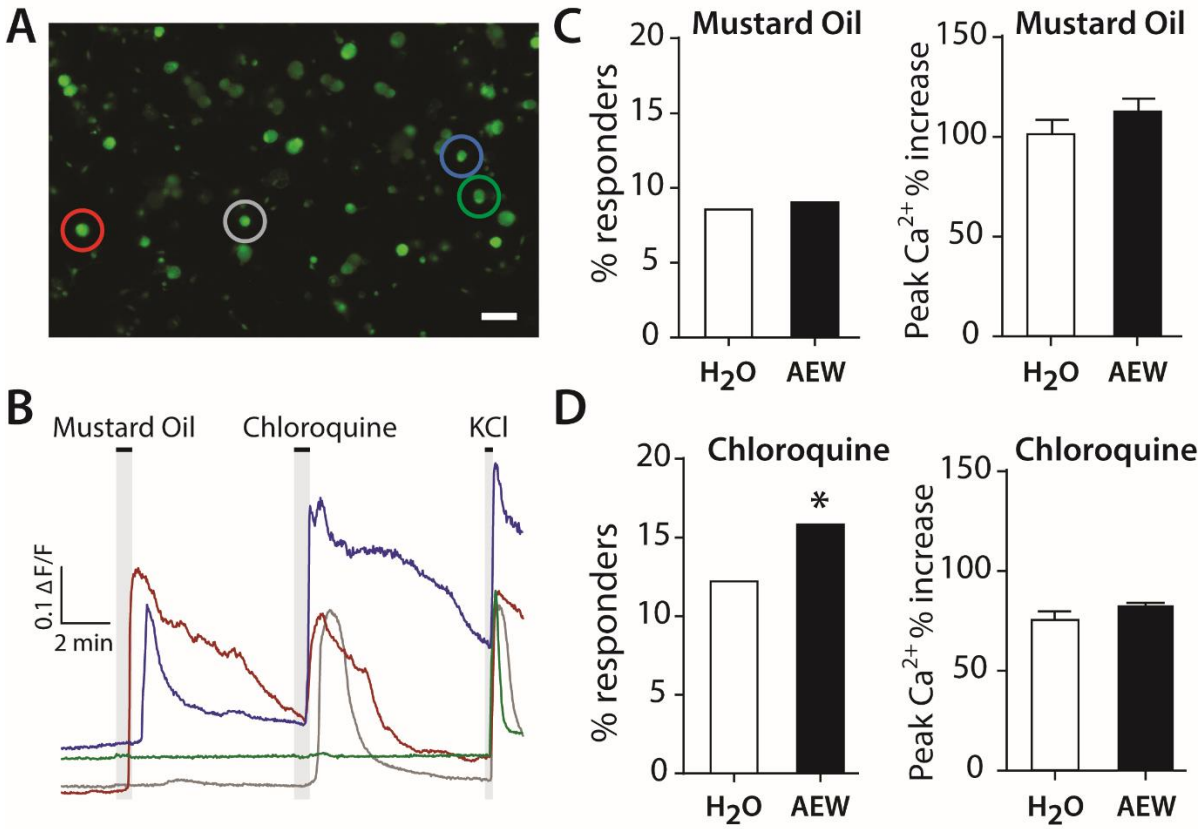

Figure 6. Increased calcium responses to chloroquine in AEW-treated trigeminal neurons in vitro. A) Image of Fura-2AM loaded trigeminal neurons with responses shown in B. (Scale bar = 50μm) **B)** Example traces of mustard oil (MO, 100 μM) and chloroquine (CQ, 1 mM) responders, and a chloroquine responder without mustard oil sensitivity. **C)** Quantification of the proportion of neurons responsive and magnitude of the response to mustard oil from AEW- and water-treated animals. **D)** AEW increased the proportion of neurons responsive to chloroquine but did not alter the magnitude of the response.

(8.92%), χ^2 test, p=0.81), or in the peak amplitude of the MO response (H_2O = 101.4 ± 7% above baseline, AEW = 112.7 ± 6% above baseline, unpaired *t*-test, p=0.24) (Figure 6C). On the other hand, the proportion of CQ-responsive neurons in AEW-treated animals was significantly increased by 27.2% (H_2O = 127/1042 cells (12.19%), AEW = 186/1199 cells (15.51%), χ^2 test, p<0.05) (Figure 6D). Peak calcium responses to CQ were not different between the two groups (H_2O = 73.4±4% above baseline, AEW = 68.7±3% above baseline, unpaired *t*-test, p=0.34).

Most MO-responsive neurons also responded to CQ regardless of treatment (H_2O = 77/89 (86.5%), AEW = 93/107 (86.9%), χ^2 test, p=1). Of the CQ-responsive neurons, most responded to MO in the water-treated group (77/127, 60.6%), while in the AEW group, half of CQ-responsive neurons responded to MO (93/186, 50.0%). Therefore, a large proportion of CQ-responsive neurons did not respond to MO. We tested whether the proportion of CQ-responsive neurons that did not respond to MO was increased in the AEW group, but this did not reach significance (χ^2 test, p=0.066).

Discussion

Dry skin pruritus is a common problem and is often associated with other dermatoses. Here we show that persistent dry skin induced both pruritus and epidermal hyperinnervation in mice. We found that Ret-positive fibers contributed to the increased fiber density, but peptidergic, CGRP-positive and GFRα3-positive fibers did not. Moreover, preventing scratching of the affected area did not prevent dry skin-induced hyperinnervation. To understand whether sensitization or ongoing activity of sensory neurons contributes to persistent dry skin pruritus, we performed *in vitro* recordings from trigeminal neurons that were determined to have innervated the treated skin. No evidence was found supporting the hypothesis that altered membrane excitability was responsible for persistent dry skin itch. On the other hand, AEW treatment produced an increase in the proportion of trigeminal neurons responsive to the histamine-independent pruritogen chloroquine, supporting the concept that the Mrgpr family of receptors is upregulated and functionally contributes to persistent dry skin itch.

In this study, AEW treatment of the cheek skin evoked scratching, but not forelimb wiping, indicating the treatment produced ongoing itch without pain. A common feature in the affected skin of patients with pruritic disease is increased epidermal innervation [9-11, 35]. Likewise, increased fiber growth in the murine epidermis after AEW treatment has been noted [12, 13]. Here, persistent AEW treatment increased total epidermal fiber density by 65%, as indicated by the pan-neuronal marker βIII-tubulin. We tested the possibility that mechanical stimulation from scratching contributed to the fiber growth. When Elizabethan collars were fitted to prevent scratching, intraepidermal innervation was still greater than in water-treated skin. The relative increase did not differ from the hyperinnervation observed in the AEW-treated animals without collars. These results demonstrate that dry skin itself is sufficient to induce hyperinnervation without the presence of scratching.

In addition to hyperinnervation, histological studies of patients with atopic dermatitis or psoriasis indicate that itch severity correlates positively with nerve growth factor (NGF) in the skin

and the NGF-receptor TrkA in nerve fibers [9, 35-38]. Increased epidermal fibers and expression of NGF have been observed in a mouse model of atopic dermatitis [39, 40], and in mice with acute acetone-induced skin barrier dysfunction [12, 13]. While the specific contribution of hyperinnervation to itch sensation is not clear, these observations suggest the idea that peptidergic, TrkA-positive fibers may be important regulators of atopic and dry skin pruritus. The present study shows that repeated AEW treatment resulted in persistent dry skin itch, but we observed no increase of the CGRP-positive or GFRα3-positive fibers which likely express TrkA [34]. This may be due to differences between the biology underlying human atopic dermatitis and mouse models of acute dry skin. Our data do not rule out the possibility of functional contributions to dry skin itch from the CGRP-positive or GFRα3-positive subset of fibers or other peptidergic fibers, and it should be noted that fiber sprouting is not a prerequisite for sensory neurons to signal itch.

A majority of the fibers innervating the epidermis are non-peptidergic and express the receptor tyrosine kinase for the GDNF family of neurotrophic factor ligands, Ret, rather than TrkA [24, 41]. GDNF release from skin keratinocytes and fibroblasts was recently acknowledged to play an important role in sensory neurite outgrowth *in vitro* with implications for pruritus [42]. Artemin, which activates Ret and GFRα3, is increased in human atopic skin and artemin-treated mice displayed increased sprouting of peripheral nerve fibers and itch-like behaviors [43]. Likewise, an increase in GFRα3 immunostained fibers was found in artemin over-expressing mice [25, 26]. However, in the AEW model of dry skin no sprouting of GFRα3-positive fibers was observed. Neurturin is another potentially interesting GDNF family ligand but its role in pruritus is currently unknown. The Mrgpr family of histamine-independent receptors has been shown to be selectively localized to Ret-positive DRG neurons and is expressed in epidermal nerve terminals [17, 18, 41, 44, 45]. Our results show that AEW treated skin resulted in a significant increase in Ret-positive fibers penetrating into the epidermis. This suggests that Ret-positive, non-peptidergic fibers could play a role in itch induced by dry skin.

The functional mechanisms engaged by pruriceptive sensory neurons to produce itch in dry skin conditions remain unresolved, but are thought to be independent of histamine signaling. Anti-histamines are generally not effective for treating chronic itch, including itch from dry skin [2, 46]. Furthermore, mast cell-deficient mice exhibit normal scratching after AEW treatment, indicating that factors released from mast cells are unlikely to generate the itch from dry skin [3]. Recent studies have pointed to the involvement of specific non-histaminergic mechanisms for dry skin itch. For example, ablation of the chloroquine-activated MrgprA3-expressing subset of sensory neurons drastically reduced AEW-induced scratching [17]. Also, in vivo recordings from spinal dorsal horn neurons showed enhanced responses to non-histaminergic pruritogens after AEW treatment, but not to histamine [47]. Taken together, these studies support the idea that dry skin pruritus signals through a non-histaminergic pathway.

Several signaling pathways for histamine-independent itch have now been identified. Scratching in mice deficient for TRPA1 was greatly reduced after chloroquine or AEW treatment, suggesting an important role for TRPA1 in non-histaminergic itch [7, 19]. Moreover, AEW treatment increased the message for MrgprA3 in both skin and sensory neurons [7]. MrgprA3, which functionally couples to TRPA1, is present on Ret-positive neurons, suggesting that dry skin itch involves non-peptidergic fibers [17]. Although TRPA1 was initially shown to be expressed in peptidergic sensory neurons [48], recent data have demonstrated robust modulation of TRPA1 function in Ret-positive neurons and TRPA1 expression in non-peptidergic IB4-positive fibers that innervate the epidermis [25, 26, 49-52]. While the hyperinnervation of Ret-positive neurons is consistent with a potential role in dry skin itch, functional studies are necessary to determine whether changes in neural sensitization or receptor expression occur after AEW treatment.

To gain insight into the functional changes of potential pruriceptive neurons exposed to dry skin, we hypothesized that locally released inflammatory mediators or neurotrophic factors act directly on pruriceptive sensory neurons to induce sensitization. Peripheral sensitization could explain the hyperknesis (heightened itch) and alloknesis (itch produced by a non-itchy stimulus)

commonly associated with pruritic diseases. To target neurons that directly innervated the treated skin, we performed whole-cell recordings from trigeminal ganglion neurons retrogradely labeled from the cheek skin of animals exposed to AEW or water treatment. With this strategy, we found no evidence for sensitization of trigeminal neurons in the AEW versus water-treated groups. It is possible that culture conditions reset differences that may have existed *in vivo* and future studies to examine the excitability of sensory fibers may yield different results. It is also possible that dry skin is associated with changes in excitability that are more pronounced at the fiber terminals in the skin and these did not translate into our *in vitro* studies of sensory neuron cell bodies. On the other hand, previous studies have shown that manipulations *in vivo* have produced altered sensory neuron physiology *in vitro* [53-55].

To test whether changes occur in the expression of functional pruritic receptors, we examined receptor-mediated responses to mustard oil and chloroquine in cultured trigeminal neurons from AEW- and water-treated animals. Interestingly, we found that AEW treatment expanded the population of sensory neurons with functional responses to chloroquine. In contrast, we found that responses to mustard oil were no different between AEW- and water-treated animals. These results mirror data showing an increase in mRNA for MrgprA3, but not TRPA1, in AEW-treated animals [7]. Overall, we found that ~10 percent of trigeminal neurons responded to mustard oil, which is consistent with the expression of TRPA1 in sensory ganglion in several reports [56-58]. While other studies have shown as many as 25% of sensory neurons are TRPA1-positive, TRPA1 expression can vary by innervation target, exposure to growth factors, and changes in other experimental methods [21, 48, 51]. Interestingly, many of the neurons that responded to chloroquine after AEW showed no mustard oil responses at all, suggesting that the MrgprA3 receptor may couple to other channels in addition to TRPA1. In favor of this idea, MrgprA3 was recently found to modulate TRPM8, TRPC, and TRPV1 [59]. Additionally, TRPV3-deficient mice exhibited greatly reduced scratching after AEW treatment compared to water-

treated controls, indicating that other mechanisms in addition to TRPA1 may account for AEW-induced scratching [14].

In summary, our results show that dry skin-induced pruritus is associated with non-peptidergic fiber growth into the epidermis and an expanded population of sensory neurons responsive to the non-histaminergic pruritogen chloroquine. Although sprouting of peptidergic, TrkA-positive fibers is observed in the skin of patients with atopic dermatitis and other dermatoses, our results suggest that non-peptidergic fibers may also play a role in chronic itch related to xerosis and compromised barrier integrity. Dry skin-induced itch, which particularly affects the older population and often co-exists with other dermatoses, may be improved by topical treatment directed at prevention of Ret-positive neural sprouting in the epidermis.

Author Contributions

All authors contributed to the design of the study and edited the manuscript. MVV, SD, and VKS applied AEW treatment and performed behavior experiments. MVV and SD completed histology experiments. MVV performed calcium imaging. SD conducted electrophysiology experiments.

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Chapter 4

Neurotrophic factors selectively modulate pruritogen-evoked itch

This chapter is adapted from the following manuscript:
Valtcheva MV, Golden JP, Sheahan TD, Pullen MY, Vogt SK, Jain S, Davidson S, Gereau RW. Neurotrophic factors selectively modulate pruritogen responses in mouse but not human sensory neurons. 2016. In Preparation.

Abstract

Neurotrophic factors such as nerve growth factor (NGF) and the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs) regulate innervation and directly sensitize nociceptors, but little is known about their role in itch. Studies of skin samples from patients with chronic itch conditions such as atopic dermatitis and psoriasis demonstrate increased intraepidermal nerve fiber density, increased artemin, and increased NGF and its receptor TrkA. We tested the hypothesis that neurotrophic factors (NTFs) can modulate pruritogen-induced itch. We investigated whether NGF and the GFLs GDNF, neurturin, and artemin can directly induce or modulate histaminergic and non-histaminergic itch. Intradermal injection of NTFs into the cheek skin of mice did not induce spontaneous scratching or wiping behavior, indicating that NTFs alone do not induce pain or itch. Acute pretreatment with NGF selectively potentiated histamine-induced scratching. On the other hand, artemin enhanced chloroquine-evoked itch, but reduced histamine-induced scratching. We studied dissociated trigeminal ganglion neurons from Ret-eGFP reporter mice to examine the effects of NGF and artemin on calcium responses to pruritogens and transient receptor potential (TRP) channel agonists. The vast majority of histamine- and chloroquine-responsive neurons were Ret-positive. Overnight incubation with NGF significantly increased the number of histamine responders. Both acute and overnight treatment with artemin increased the proportion of chloroquine-responsive neurons. We also found that *TrkA*^{F592A} mutation resulted in altered TrkA signaling that produced an analgesic phenotype, but had no effect on histamine- or chloroquine-induced itch. Our findings indicate that NGF and artemin have select roles in the regulation of pruritogen-evoked itch. Additionally, TrkA signaling may not play a prominent role in the maintenance of pruriceptors in adulthood. These findings support the role of NTFs as regulators of histaminergic and histamine-independent itch.

Introduction

Itch, or pruritus, is mediated primarily by small diameter primary afferents that innervate the skin and mucosa [1]. Pruriceptive nerve endings exist in close proximity to keratinocytes, dermal fibroblasts, and immune cells in the skin, which can release pruritogens, pro-inflammatory mediators, and neurotrophic factors (NTFs) that directly modulate primary afferent responses and receptor expression [1-7]. NGF and the GDNF family ligands (GFLs) regulate the development, morphology, target innervation, and physiology of peptidergic and non-peptidergic sensory neurons [8-12]. Previous studies indicate that a large proportion of neurons expressing pruritogen receptors also express TrkA and Ret, the tyrosine kinase receptors activated by NGF and GFLs, respectively [13-16]. NGF and the GFLs directly sensitize the transient receptor potential (TRP) channels TRPV1 and TRPA1, and can increase their expression in primary afferents [17-21]. Histamine-evoked itch and sensory neuron responses are dependent on TRPV1 [20, 22, 23]. On the other hand, itch evoked by chloroquine is functionally dependent on TRPA1 [19, 21, 22]. These findings suggest that neurotrophic growth factors may play an important role in regulating normal and pathological itch.

Pruritic skin diseases are marked by epidermal hyperplasia and increased inflammatory cell infiltration, which contribute to increased local levels of inflammation and NTFs. Studies of patients with atopic dermatitis and psoriasis have demonstrated that the severity of itch correlates with increased levels of nerve growth factor (NGF) in the skin and its receptor TrkA in epidermal nerve fibers [24-27]. A recent study also found that artemin, a glial cell line-derived neurotrophic factor (GDNF) family ligand, is increased in the skin of patients with atopic dermatitis [28]. In addition, intradermal injection of NGF in human subjects enhanced itch produced by the non-histaminergic pruritogen cowhage [29], while intravenous administration of artemin for the treatment of sciatica resulted in frequent reports of pruritus [30, 31], further supporting a role for NTFs in the modulation or generation of itch.

Neurotrophic factors are critical in the control of epidermal innervation, and epidermal

hyperinnervation is another common feature of pruritic skin diseases [27, 32, 33]. Indeed, cultured keratinocytes from patients with atopic dermatitis increased local NGF levels relative to healthy keratinocytes and produced greater axon growth in co-cultures with porcine sensory neurons [34]. TrkA inhibition in a mouse model of atopic dermatitis reduced both dermatitis-associated hyperinnervation and scratching behavior [35]. In addition, we recently found that the acetone-ether-water model of dry skin itch is associated with epidermal hyperinnervation by Ret-positive fibers [36], suggesting a role for both NGF and the GDNF family ligands (GFLs) in the regulation of epidermal innervation in the context of itch.

In this study we investigated the roles of the major peripheral NTFs in regulating the responses of sensory neurons to histamine and the histamine-independent pruritogen chloroquine. Using a TrkA receptor mutant, we further investigated whether endogenous growth factor signaling contributes to normal itch sensation and functional pruritogen receptor expression in trigeminal ganglion neurons.

Materials and Methods

Animals

All experiments were conducted in accordance with the National Institutes of Health guidelines and received the approval of the Animal Care and Use Committee of Washington University in St. Louis, School of Medicine. Adult (8-12 week old) male littermate mice were housed on a 12 hour light-dark cycle and allowed *ad libitum* access to food and water. The effects of nerve growth factor (NGF) and the GDNF family ligands GDNF, neurturin, and artemin on spontaneous and pruritogen-induced scratching behavior were tested in C57BL/6 (Jackson lab) male littermates. Heterozygous Ret-EGFP reporter animals were used to test the effects of neurotrophic factors on pruritogen responses *in vitro* [10, 37]. Homozygous *TrkA*^{F592A} mutants (a generous gift from Dr. Wenqin Luo) and wild type littermates were obtained from heterozygous breeding pairs [38].

Males aged 8 to 12 weeks were used for all behavior experiments. Males and females were used for calcium imaging and immunohistochemistry experiments.

Pruritogen-induced scratching behavior

Because of the well-established role of NTFs in pain sensitization, the cheek assay was used to differentiate between itch- and pain-specific effects of NTFs on behavioral responses to pruritogens [39]. At least one day prior to the start of behavior experiments, the cheek skin was shaved with electric clippers. On the day of experiment, animals were placed in individual plexiglass observation chambers and allowed to acclimate in the presence of white noise for 2 hours. Using gentle restraint, animals were treated with a growth factor (0.2µg/10µl 0.9% normal saline solution) or vehicle (0.9% normal saline solution) by direct intradermal injection into the cheek skin using a 29½ gauge needle and 3/10cc insulin syringe. Neurotrophic growth factors used were: Nerve Growth Factor (Harlan), and GDNF, neurturin, artemin (R&D Systems). Behavioral responses were recorded immediately following injection using a video camera, and scratching and wiping behavior was scored at a later time by observers blinded to treatment. A single bout of scratching was defined as any number of individual scratch events separated by licking or biting of the hind-limb, holding the limb motionless, or putting the limb down on the surface. Wiping behavior indicating pain was defined as a rostrally-directed movement of the ipsilateral forelimb across the cheek starting from the ear [39, 40]. One hour after pre-treatment, animals were injected into the cheek skin with a pruritogen (histamine (1mg/10µl, Sigma) or chloroquine (200µg/10µl, Sigma)). Pruritogen-induced scratching and wiping responses were video recorded for 30 minutes immediately after injection and scored at a later time by observers blinded to pre-treatment. The number of scratch bouts from both treatment and vehicle groups were normalized to the vehicle mean for the day. For TrkA mutant experiments, 8-12 week old $TrkA^{F592A}$ males and their wild type littermates were injected with histamine (500µg/10µl) or

chloroquine (200µg/10µl). Behavior was recorded and scored at a later time by experimenters that were blinded to mouse genotype.

Primary trigeminal ganglion cultures and calcium imaging

Experiments were performed using 8-12 week old Ret-EGFP reporter mice (C57BL/6) obtained from Dr. Sanjay Jain [37]. For TrkA mutant experiments, 8-12 week old homozygous *TrkA*^{F592A} mutants and their wild type littermates were used. Animals were sacrificed by decapitation and both trigeminal ganglia (TG) were carefully removed and cut into several pieces. TG were incubated in papain (15U/ml) (Worthington, Lakewood, NJ) in Hank's buffered saline solution without Ca^{2+} or Mg^{2+} and with 10 mM HEPES at 37°C, 5% CO_2 for 20 minutes. TG were washed and then incubated in collagenase (1.5 mg/ml) for 20 minutes. TG were triturated with a fire-polished Pasteur pipette, passed through a 40µm filter to separate large debris, and the dissociated cells were plated on poly-D-lysine and collagen-coated glass coverslips. Cells were cultured overnight in Neurobasal A medium supplemented with B27, 100U/mL penicillin/streptomycin, 2 mM Glutamax, and 5% heat-inactivated fetal bovine serum (Gibco). No additional growth factors were added to the media unless otherwise stated for specific experiments. All calcium imaging recordings were performed within 24 hours of plating. For experiments using Ret-EGFP animals, two animals were sacrificed per experiment and all four trigeminal ganglia were pooled prior to plating the cells. Alternating coverslips were treated with NTF (100ng/ml) or vehicle. At least two experiments (n=4 animals) were used per study. For TrkA mutant experiments, TG from one mutant and one age-matched wild type littermate were cultured simultaneously, but maintained separately.

The protocol for calcium imaging was adapted from our previous studies [41-44]. TG neurons from Ret-EGFP mice were incubated for 45 minutes in 3 µg/mL (3µM) of the cell-permeant ratiometric calcium indicator Fura-2 AM (Molecular Probes) and then incubated in external solution (in mM): 130 NaCl, 5 K, 2 $CaCl_2$, 1 $MgCl_2$, 30 Glucose, 10 HEPES for a 20-

minute de-esterification prior to recording. Coverslips were positioned in a recording chamber and continuously perfused with external solution at room temperature. Cells were viewed using an inverted microscope (Olympus Optical, Tokyo, Japan) and images were captured with a Hamamatsu Orca camera. Regions of interest encompassing all Fura-2-loaded cells were identified *a priori* and the ratio of fluorescence emission at an alternating excitation wavelength of 357 and 380 nm was recorded with SimplePCI Software. The experimental protocol consisted of a 2-minute baseline measurement followed by bath application of the following stimuli (concentration, application duration, source): histamine (100 μM, 30 seconds, Sigma, St. Louis), chloroquine (1mM, 30 seconds, Sigma, St. Louis), capsaicin (200nM, 20 seconds, Sigma, St. Louis), mustard oil (100μM, 30 seconds, Sigma, St. Louis). Each stimulus application was followed by a wash with external solution for at least 8 minutes prior to subsequent stimulus applications. All experiments were concluded with a 10 second application of 50mM KCl to identify live neurons. Peak responses were determined by calculating the absolute increase in 357/380nm emission ratio above baseline (the average fluorescence measured in the 30 seconds immediately prior to stimulus application). A change from baseline ≥10% was set as the threshold for a response. Cells that did not respond to KCl were excluded from physiological analysis. Prior to recording, an image of EGFP-expressing neurons was captured. During data analysis, the EGFP image was overlayed with the regions of interest from the recording to identify which histamine- and chloroquine-responsive neurons were Ret-positive.

Heat, Mechanical, and Cold Thresholds

All behavioral experiments were completed between 8 AM and 6 PM. All animals were individually tested in clear plexigalss observation boxes after a 2-hour acclimation period in the presence of white noise. Experimenter was blinded to mouse genotype. Heat withdrawal latency was determined using the Hargreaves assay (Model 390 Series 8, IITC Life Science Inc.) performed on a glass plate maintained at 30°C [45]. Paw withdrawal latencies were determined

using a radiant heat stimulus set at 15% active intensity and 3% inactive intensity. Stimulus cut-off was set to 20 seconds to avoid tissue injury. Five independent measurements were obtained for each paw, and consecutive trials on the same paw were separated by at least 15 minutes.

To determine the 50% withdrawal threshold to mechanical stimuli, von Frey filaments were applied using the up-down method [46]. Calibrated von Frey filaments (North Coast Medical) were pressed against the hind paw skin between the anterior and posterior footpads until the filament just bent. A response was counted when a mouse lifted its hind paw away from the applied filament. Three trials were performed to determine the average withdrawal threshold (g) for each animal.

Cold sensitivity was determined using the cold plantar assay [47]. Animals were acclimated on top of a ¼ inch glass plate. Finely crushed dry ice was tightly packed into a modified 10 ml syringe to make a cold probe 1 cm in diameter. The cold probe was then applied to the glass beneath the hind paw and withdrawal latency was measured with a stopwatch. Three trials were conducted on each paw and consecutive trials to the same paw were separated by at least 15 minutes. Stimulus cut-off of 20 seconds was used to prevent tissue damage.

Immunohistochemistry

TrkA^{F592A} homozygous mice and wild type littermates were deeply anesthetized (ketamine-xylazine-acepromazine: 38-1.92-0.38 mg/mL; 2.7 mL/kg), footpad skin was removed, and animals were perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde solution. Footpads were immersion-fixed in Zamboni's fixative overnight, rinsed in PBS, and cryoprotected in 30% sucrose. L4 DRG were dissected and cryoprotected in 30% sucrose. Tissue was embedded in optimal cutting temperature (OCT) compound and flash frozen on dry ice. Sections were cut on a cryostat at a width of 18µm (DRG) and 30 µm (footpad skin), and collected on glass slides. For immunostaining, tissue was post-fixed on the slide in 4% PFA for 5 minutes, washed with TBS, and blocked using 1% BSA and 0.05% TritonX-100. Sections were then

incubated in primary antibody diluted in Tyramide Signal Amplification (TSA) reagent overnight at 4°C in a humidified chamber. Antibodies and dilutions used: rabbit anti-βIII tubulin (1:1000, Covance), goat anti-CGRP (1:350-1000, AbD Serotec), Alexa Fluor 488/555 donkey anti-rabbit (1:200-400, Invitrogen), Alexa Fluor 488 donkey anti-goat (1:200-350, Invitrogen). Slides were sealed with ProLong Gold with DAPI (Molecular Probes).

Intraepidermal fiber density of the footpad skin was determined using previously described methods [10, 48]. MetaMorph Software (Molecular Devices, Sunnyvale, CA) was used to measure the length of the dermal-epidermal border. In each examined section, labeled fibers crossing the dermal-epidermal border were counted on an upright epifluorescent microscope (Nikon 80i; CoolSnapES camera). For each animal, six sections separated by >90µm were analyzed by a blinded experimenter and the mean fiber density per animal was calculated. Representative images of fibers stained with the above-described methods were obtained using a Leica SPE Confocal microscope. Images were collected across the z-plane at 1µm width and maximum projection images were generated using ImageJ software.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.04 (2014). For comparisons of scratching behavior, unpaired t-test was used to compare the total scratch bouts, wipes, or normalized scratch bouts. For calcium imaging experiments, differences between the proportion of responders in each treated group were determined using a χ^2 test, where each cell counted as an individual observation. Peak calcium increase in response to stimuli was analyzed using unpaired t-test. For all statistical analyses, significance was defined at $p<0.05$. Data are presented as mean ± S.E.M or overall proportion of responders (%).

Results

Neurotrophic factors selectively modulate scratching responses to pruritogens

Acute administration of neurotrophic factors can directly sensitize noxious responses to heat, cold, mechanical, and chemical stimuli [18, 49, 50]. To determine if neurotrophic factors can also acutely potentiate scratching responses to pruritogens, NGF, GDNF, neurturin (NRTN), or artemin (ARTN) were injected into the cheek skin of wild type mice. Scratching and wiping behavior was recorded immediately after growth factor injection to determine the direct effects of neurotrophic factors on spontaneous pain- and itch-specific behaviors. There were no significant differences in scratching or wiping behavior between the vehicle- and neurotrophic factor-treated groups, indicating that acutely administered NGF, GDNF, neurturin, or artemin alone do not directly induce pain or itch (Figure 1). To determine the effects of neurotrophic factors on histamine-dependent itch, one hour after NTF treatment all animals were injected with histamine

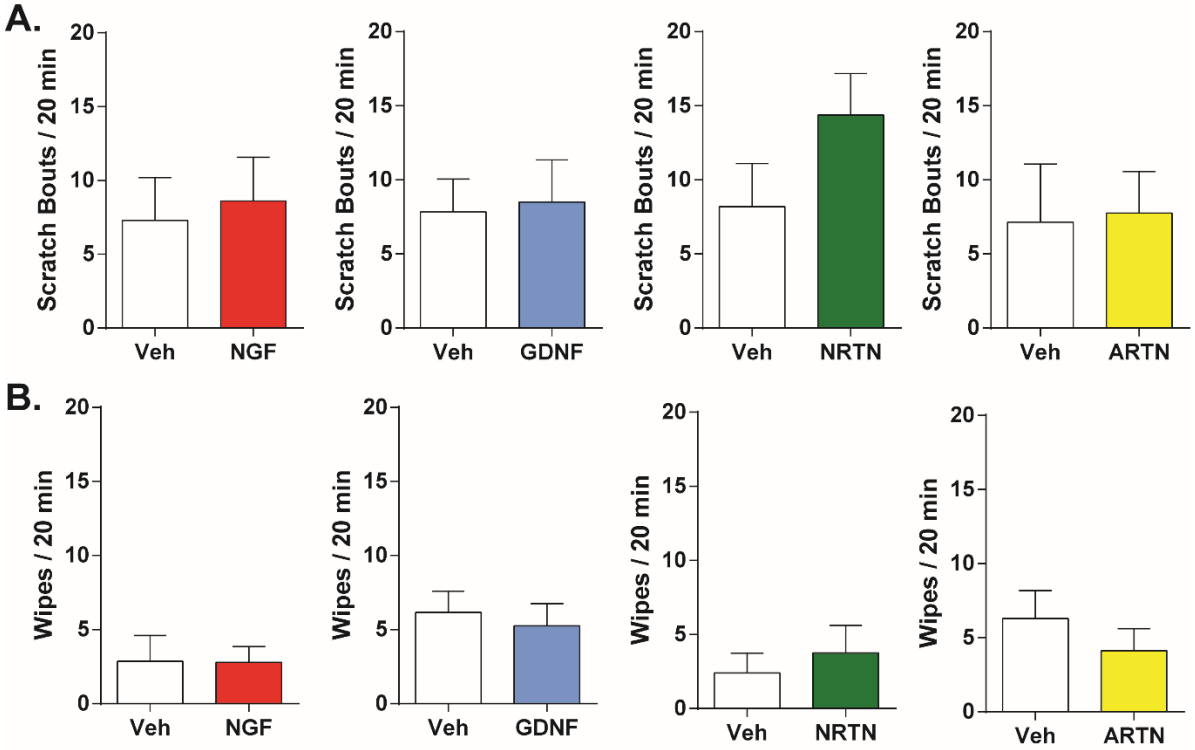

Figure 1. Neurotrophic factors do not induce spontaneous itch or pain. A. Scratch bouts measured immediately after intradermal injection with neurotrophic factor (0.2µg/10µl) or vehicle (10µl 0.9% NaCl) (N=6-8 animals/group, t-test, p=NS). **B.** Wipes were measured immediately after NTF or vehicle injection.

into the cheek skin. NGF pre-treatment significantly potentiated histamine-induced scratching behavior (Figure 2A), but did not affect wiping. GDNF and neurturin administration did not affect histamine-induced scratching or wiping (Figure 2B, C). Pre-treatment with artemin significantly reduced histamine-induced scratching (Figure 2D), but did not affect wiping behavior. The effects of neurotrophic growth factors on histamine-independent itch were determined using the pruritogen chloroquine. One-hour pre-treatment with NGF, GDNF, and neurturin did not affect scratching or wiping behavior induced by chloroquine (Figure 2E-G). On the other hand, artemin

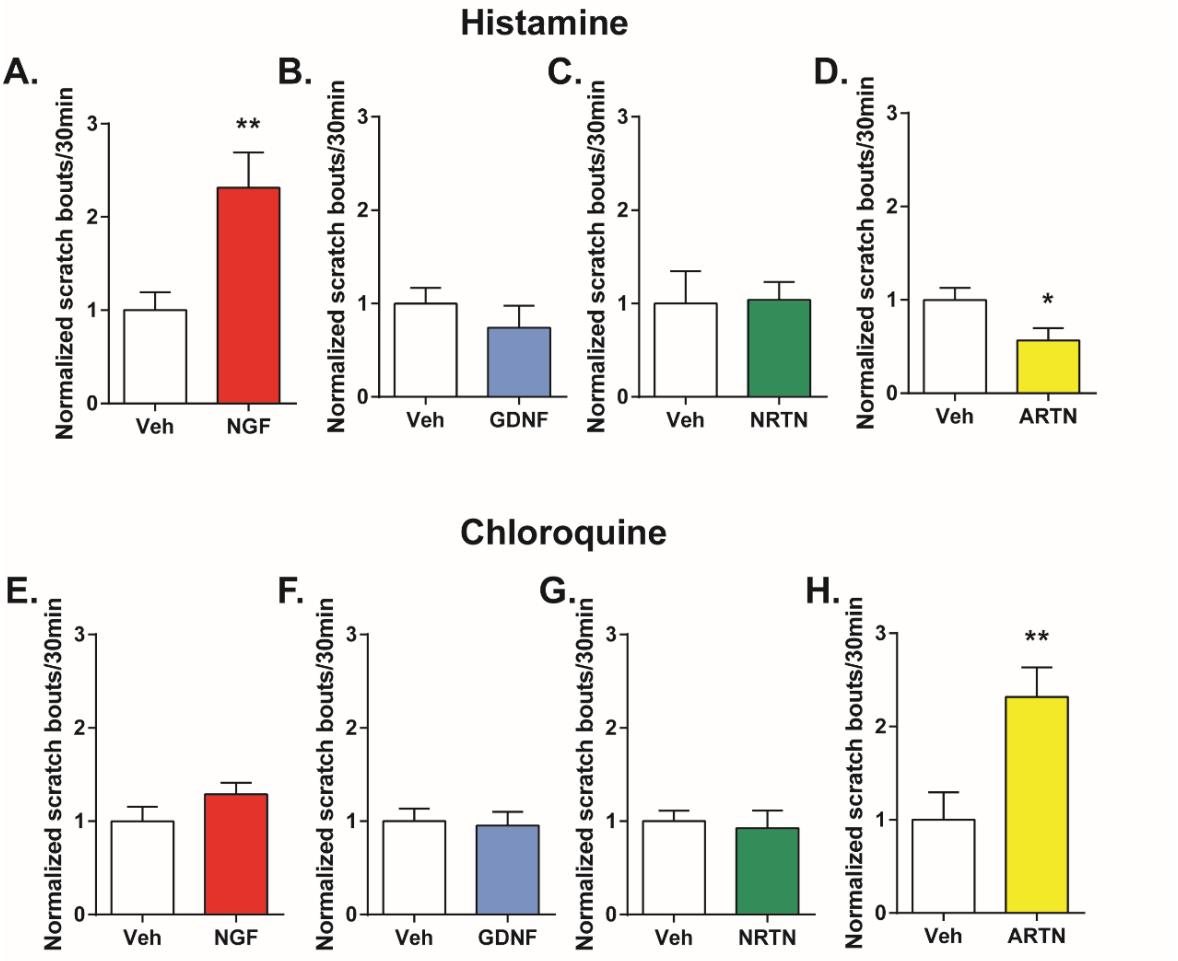

Figure 2. Neurotrophic factors modulate scratching responses to pruritogens.
A. Histamine-induced scratching was potentiated by NGF pre-treatment. **B-C.** GDNF and neurturin had no effect on histamine responses. **D.** Artemin pre-treatment reduced the total scratch bouts evoked by histamine. **E-G.** Chloroquine-induced scratching was not changed by NGF, GDNF, or neurturin. **H.** Artemin significantly potentiated chloroquine-evoked scratching responses. (N=7-10 per group, t test, *p<0.05, **p<0.01)

pre-treatment significantly potentiated chloroquine-induced scratching responses, with no effect on wiping behavior (Figure 2 H). These findings indicate that acutely applied NGF and artemin selectively modulate histaminergic and histamine-independent itch.

NGF and artemin directly modulate sensory neuron responses to pruritogens

To further investigate the mechanisms by which NGF and artemin potentiate itch, we used ratiometric calcium imaging to study trigeminal sensory neuron responses to pruritogens in the presence of these growth factors. A large proportion of chloroquine-responsive neurons express MrgprA3 and represent a specific subset of primary afferents that express both the peptidergic marker CGRP and the nonpeptidergic markers IB4 [13]. We utilized Ret-EGFP reporter mice to better characterize Ret expression in the subsets of functionally-identified histamine- and chloroquine-responsive neurons (Figure 3A-B).

The effects of NGF on histamine responses were measured after acute 1-hour incubation or overnight incubation in NGF (100ng/ml) or vehicle. Because TRPV1 is activated downstream of histamine receptor activation and plays a key role in histaminergic itch, responses to capsaicin were also measured (Figure 3C). Of histamine-responsive neurons in the vehicle group, 80.3% were Ret-positive (n=71 cells, Figure 3D). Acute, 1-hour pretreatment with NGF did not change the total number of histamine-responsive neurons (N=78/482 vehicle cells (15.4%) vs. N=99/555 NGF-treated cells (17.8%), χ^2 test, not significant) or the peak calcium responses to histamine (Figure 3E). Similarly, 1-hour NGF did not change the proportion of capsaicin-responsive neurons (N=53/193 (27.5%) vehicle neurons vs N=68/226 (30.1%) NGF-treated neurons, χ^2 test, not significant). Overnight incubation with NGF resulted in a significant increase in the proportion of histamine-responsive neurons (N=43/477 (9.1%) vehicle neurons vs. N=89/620 (14.4%) NGF-treated neurons, χ^2 test, $p<0.01$) (Figure 3F). There was no effect on peak calcium responses to histamine. Overnight NGF treatment did not change the overall proportion of

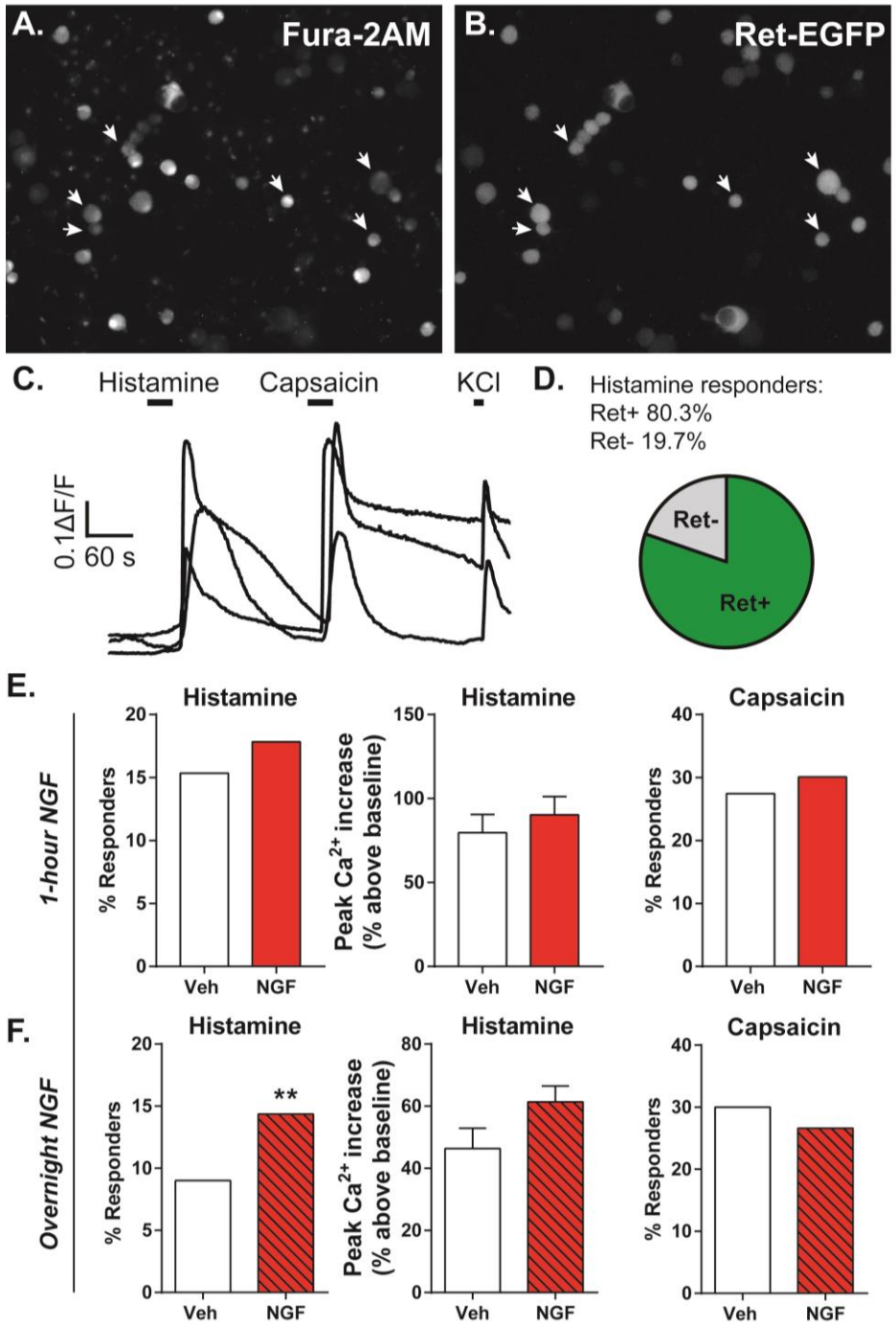

Figure 3. NGF increases the proportion of histamine-responsive neurons. A, B. Representative images of Fura-2-labeled and Ret-EGFP-positive TG neurons. Arrows indicate cells labeled by both markers. **C.** Representative traces of neurons treated tested for histamine and capsaicin responses. **D.** Of histamine-responsive neurons (n=71), 80.3% were Ret+. **E.** 1-hour pre-treatment with NGF did not affect the number or peak of histamine responses, or the proportion of capsaicin responsive neurons. **F**. Overnight pre-treatment with NGF increased the proportion of histamine-responsive neurons, but did not affect peak histamine responses or total capsaicin responsive neurons (χ^2 test, **p<0.01).

capsaicin-responsive neurons. Neither treatment changed the proportion of histamine-responsive neurons that also responded to capsaicin (1-hour: 96.9% veh vs. 92.7% NGF; overnight: 90.1% veh vs. 87.6% NGF). These results indicate that acutely applied NGF does not affect the number of histamine-responsive neurons *in vitro*. However, overnight exposure to NGF increased the proportion of histamine-sensitive neurons, suggesting an expansion of histamine receptor expression in previously histamine-unresponsive neurons.

To determine whether artemin directly modulates sensory neuron responses to the non-histaminergic pruritogen chloroquine, dissociated TG neurons from RetEGFP reporter animals were tested. Responses to mustard oil were also tested to determine if TRPA1 modulation is another possible mechanism for the potentiation of chloroquine-induced itch (Figure 4A). In the vehicle-treated group, 83.0% of the total chloroquine-responsive neurons were Ret-positive (Figure 4B). Acute pre-treatment with

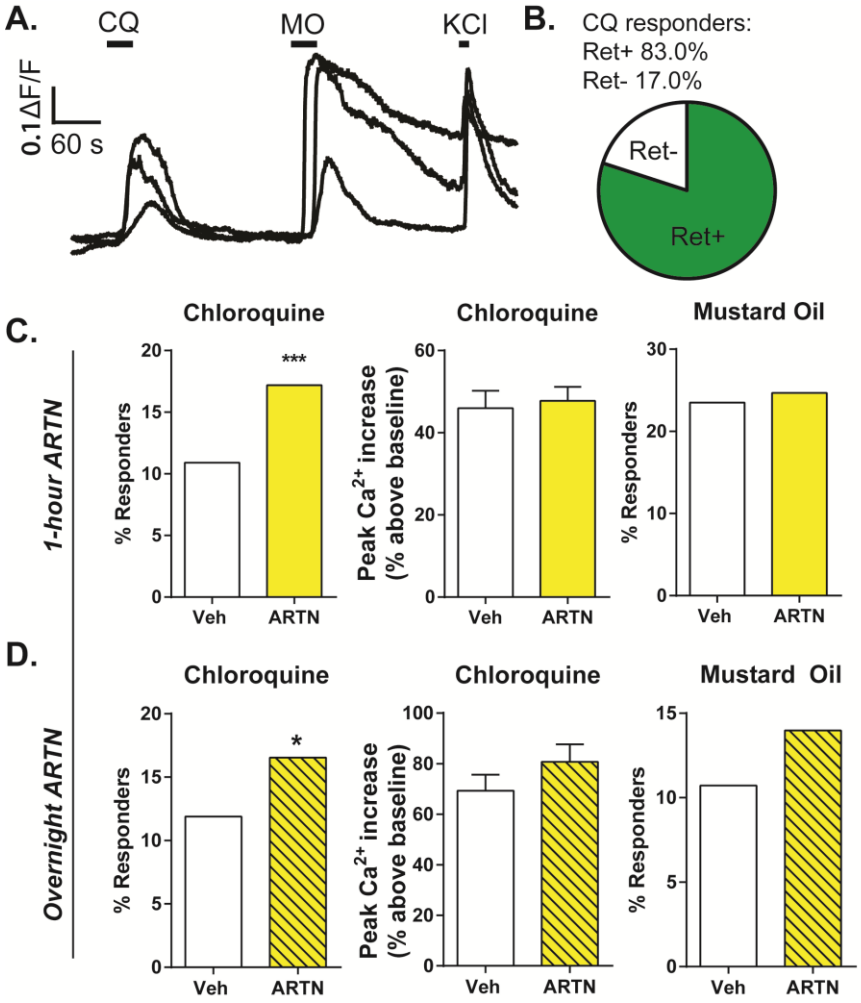

Figure 4. Artemin increases the proportion of CQ-responsive neurons. A. Representative traces of neurons that were treated tested for chloroquine (CQ) and mustard oil (MO) responses. **B.** 83% of chloroquine-responsive neurons were Ret-positive (n=93). **C, D.** Both 1 hour (**C**) and overnight (**D**) pretreatment with artemin (100ng/ml) significantly increased the proportion of chloroquine-responsive neurons (χ^2 test, *p<0.05, ***p<0.001).

artemin for 1 hour resulted in a significant increase in the proportion of chloroquine-responsive neurons from 10.9% in the vehicle group (N=104/956 cells) to 17.2% in the artemin-treated group (N=131/762 cells, χ^2 test, $p<0.001$) (Figure 4C). Artemin pre-treatment did not affect peak calcium responses to chloroquine or the proportion of mustard oil-sensitive neurons (24.7% of vehicle- vs 23.5% of artemin-treated neurons, χ^2 test, not significant). Overnight pre-treatment with artemin likewise increased the proportion of chloroquine-responsive neurons (N=51/429 vehicle neurons (11.9%) vs. N=84/508 artemin-treated neurons (16.5%), χ^2 test, $p<0.05$), but did not change peak responses to chloroquine or the proportion of mustard oil-responsive neurons (Figure 4D). Neither treatment changed the proportion of chloroquine responders that also responded to mustard oil (1-hour: 72.1% vs. 71.8%; overnight: 66.7% vs. 63.1%). These findings indicate that acute and prolonged artemin pre-treatments significantly increase the proportion of chloroquine-responsive neurons *in vitro*, which may contribute to the potentiation of chloroquine-induced itch behavior.

TrkA^{F592A} mutation increases nociceptive detection thresholds but does not affect pruritogen-induced scratching

To investigate whether endogenous NGF-TrkA signaling plays a role in regulating itch sensation and pruriceptor physiology, we obtained *TrkA^{F592A}* mutant animals. These animals have a phenylalanine (F) to alanine (A) mutation in the ATP-binding pocket of TrkA that renders the receptor sensitive to pharmacological inhibition by the small molecule kinase inhibitor 1NMPP1 [38]. We tested untreated homozygous mutants and their wild type littermates to confirm normal baseline pain and itch sensation. *TrkA^{F592A}* mutants did not differ from their wild type littermates in body mass (n=4-5 per genotype, Figure 5A). However, homozygous *TrkA^{F592A}* mutants with no pharmacological treatment had significantly increased baseline heat withdrawal latencies compared to their wild type littermates (N=7 per genotype, unpaired t test, *$p<0.05$) (Figure 5B). *TrkA^{F592A}* mutants also demonstrated decreased sensitivity to mechanical and cold stimuli (Figure

5C-D). These results suggest that even without the use of pharmacological inhibitors, the *TrkA*^{F592A} mutation has effects on endogenous TrkA function. Despite the observed effects on heat, mechanical, and cold detection thresholds, histamine- and chloroquine-induced scratching behavior was not different between *TrkA*^{F592A} mutants and their wild type littermates (Figure 5E-F). These results indicate that the *TrkA*^{F592A} mutation alters baseline nociceptive behaviors but does not affect pruritogen-evoked itch.

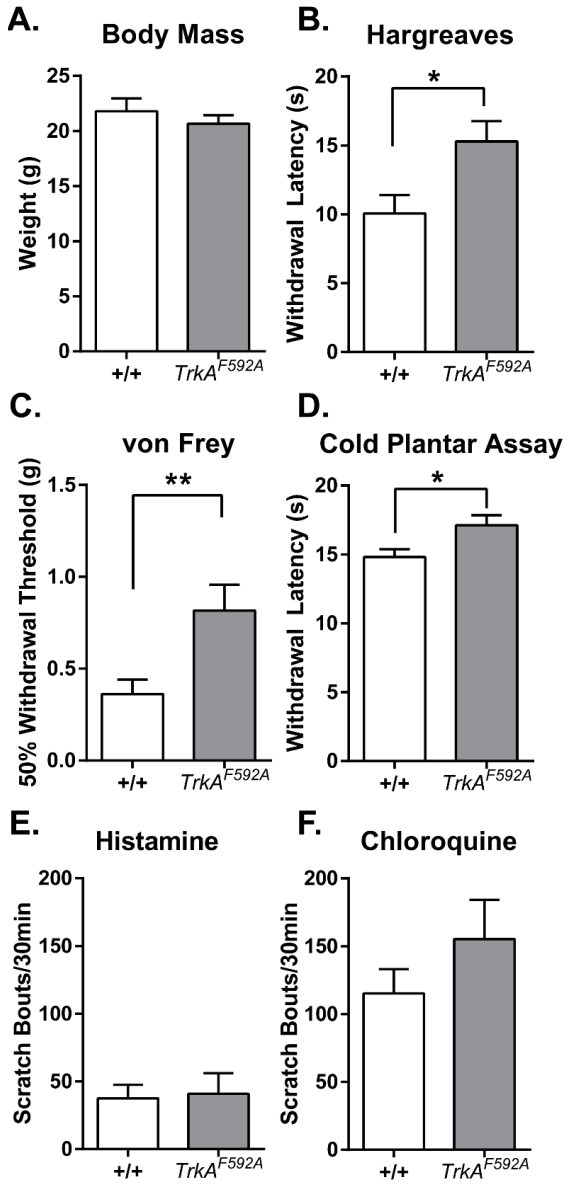

Figure 5. *TrkA*^{F592A} mutation increased baseline heat, mechanical, and cold thresholds, but did not affect histamine- and CQ-evoked scratching responses. Mutants demonstrate normal body weight (**A**), but decreased nociceptive responses (**B-D**). Histamine- and chloroquine-induced itch was not different between genotypes (**E-F**). (n=7 per genotype, unpaired t test, *p<0.05, **p<0.01).

NGF-TrkA signaling regulates sensory neuron survival during development and maintains target innervation and receptor expression in adulthood. The observed changes in pain detection thresholds may be due to loss of intraepidermal nerve fiber density or sensory neuron loss during development. To determine whether the *TrkA*^{F592A} mutation results in a hypomorphic receptor, we used immunohistochemistry to quantify skin innervation. There was a significant decrease in βIII-tubulin immunoreactive epidermal nerve fibers in the footpad skin of *TrkA*^{F592A} mutants when compared to wild type littermates, indicating a loss of total intraepidermal fiber density (Figure 6A-C). CGRP-positive fibers

were also significantly decreased in the mutant group, indicating a substantial loss of peptidergic innervation (Figure 6D-F).

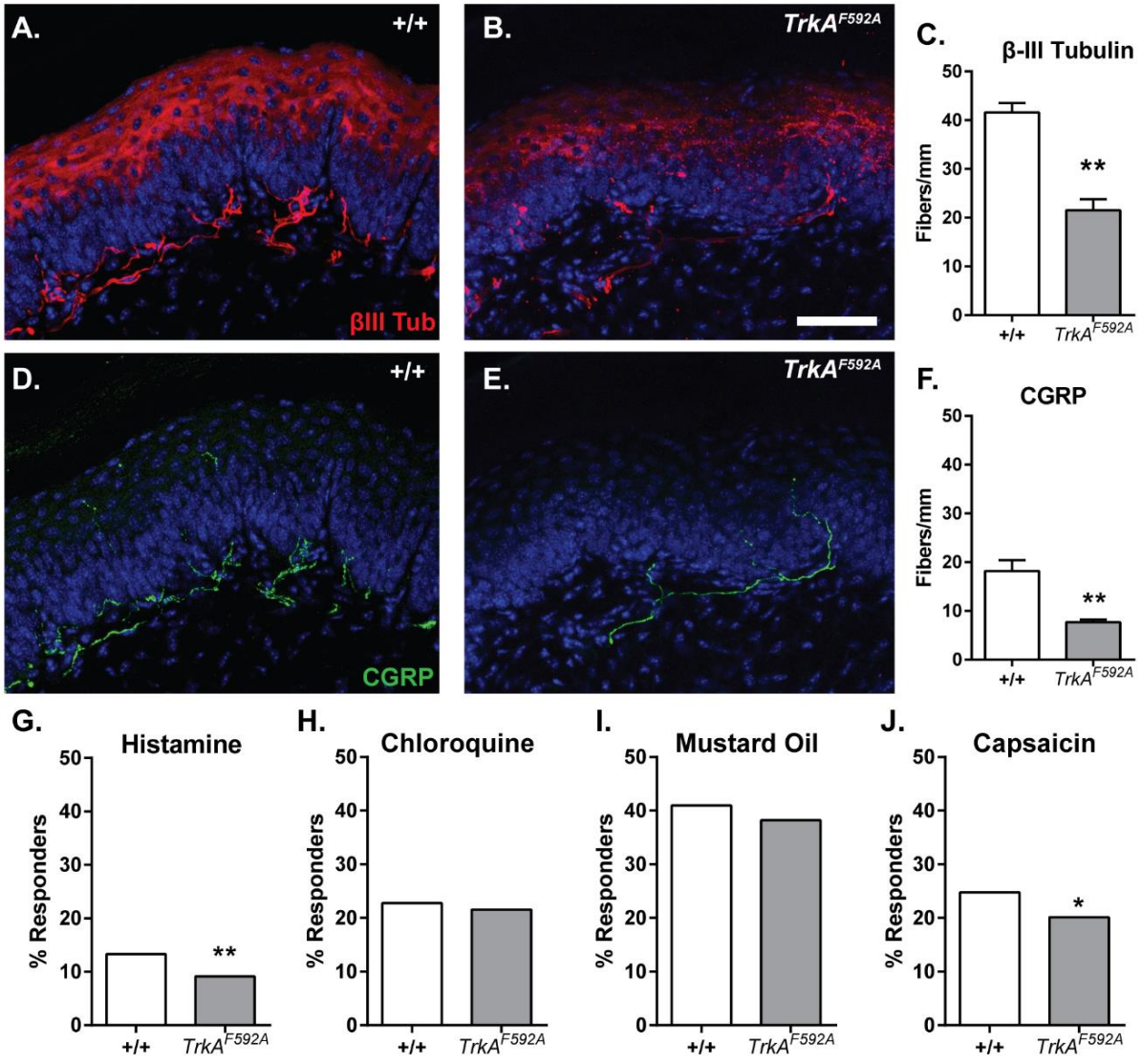

Figure 6. Altered TrkA signaling results in peptidergic intraepidermal fiber loss, but minor changes in sensory neuron responses to pruritoges and algogens. Representative images of βIII-tubulin-labeled intraepidermal fibers in wild type (**A**) and mutant (**B**) footpad skin. **C.** *TrkA*^{F592A} mutants had a significant reduction on total intraepidermal fiber density (n=3-4 animals per group, t test, **p<0.01). **D-E.** Representative images of CGRP+ fibers in wild type (**D**) and mutant (**E**), demonstrate the significant reduction in peptidergic fibers (**F,** n=3-4 animals per group, t test, **p<0.01). Calcium imaging studies show a reduction in the proportion of histamine (**G**) and capsaicin (**J**) responders, but no effect on chloroquine- or mustard oil-responsive neurons (**H-I**) (χ^2 test, *p<0.05, **p<0.01).

To determine whether altered NGF-TrkA signaling in the *TrkA*^{F592A} mutant contributes to changes in pruriceptor and nociceptor subpopulations, we tested trigeminal neuron responses to the pruritogens histamine and chloroquine, and the algogens mustard oil and capsaicin. Despite the lack of behavioral effects on histamine-induced scratching, our data indicate a significant decrease in the overall proportion of histamine-responsive neurons from 13.3% to 9.1% in mutant animals (N=145/1089 +/+ neurons; N=78/853 *TrkA*^{F592A} neurons, χ^2 test, $p<0.01$) (Figure 6G). There were no differences in the proportion of chloroquine- and mustard oil-responsive neurons (Figure 6H-I). *TrkA*^{F592A} mutation resulted in a small but significant decrease in the proportion of capsaicin-responsive neurons (N=270/819 (24.8%) wild type neurons versus N=172/681 (20.2%) *TrkA*^{F592A} neurons, χ^2 test, $p<0.05$) (Figure 6J). These findings indicate the *TrkA*^{F592A} mutation likely alters endogenous TrkA signaling, resulting in a substantial loss of peripheral target innervation. Population studies using calcium imaging suggest that histamine- and capsaicin-responsive subpopulations depend on TrkA activity, while chloroquine- and mustard oil-responsive neurons may be maintained via TrkA-independent mechanisms.

Discussion

Chronic pruritus is a tremendous clinical problem associated with a number of dermatological diseases, including atopic dermatitis and psoriasis. Lesional skin in pruritic conditions exhibits increased levels of growth factors and epidermal innervation [24-27], suggesting that neurotrophic growth factors contribute to pruriceptor fiber growth and excitability. Here we investigated the direct effects of NGF and the GDNF family ligands on pruritogen-evoked itch behaviors and sensory neuron responses *in vitro.* Our data demonstrate distinct roles for NGF and artemin in the modulation of histamine- and chloroquine-evoked itch, respectively. Using *TrkA*^{F592A} mutants, we tested the role of endogenous TrkA signaling in the maintenance of pruriceptors in adulthood, and determined that pruriceptive neurons are likely supported by TrkA-independent mechanisms.

The role of NGF in nociceptor plasticity has been extensively studied [51], yet little is known about the direct effects of NGF on pruriceptor physiology. Increased serum and skin levels of NGF in atopic dermatitis, prurigo nodularis, and psoriasis, suggest a role for NGF-TrkA signaling in the induction or maintenance of chronic pruritus [24-26, 52, 53]. Several studies have demonstrated acute application of NGF induces thermal and mechanical hyperalgesia in both rodents and humans [50, 51, 54-60]. One study tested the effects of a single intradermal injection of NGF on pruritogen-induced itch in healthy human volunteers, and determined that there was a potentiation of cowhage-induced itch, but not histamine-induced itch [29]. These results contrast with our finding that acutely applied NGF selectively potentiated histaminergic itch in mice. One factor that may contribute to this difference is the timeline of treatment. In the human study, investigators tested pruritogen responses at days 7 and 21 after NGF treatment, in order to coincide with previously identified peak of thermal and mechanical hyperalgesia, respectively. The 1-hour time point applied in our study likely restricts the effects of NGF to local receptor expression or sensitization at the fiber terminals, but it does not provide enough time for broad transcriptional or fiber growth changes to take place in trigeminal neurons [61]. The effects of

NGF treatment in humans one hour after intradermal injection remain unknown, but our findings suggest that acute sensitization of histamine-responsive fibers could occur.

To investigate the direct effects of NGF on pruriceptive neurons and identify a potential mechanism for the sensitization observed *in vivo*, we measured dissociated trigeminal neuron calcium responses to histamine after treatment with NGF. Overnight incubation with NGF resulted in a significant increase in the number of histamine-responsive neurons, but acute 1-hour treatment had no effect. The lack of acute effect observed in culture suggests that NGF-induced sensitization of histamine-evoked itch may be dependent on NGF activation of local inflammatory cells such as mast cells to release mediators that can directly activate or sensitize afferent fibers innervating the skin [62, 63]. The expansion of the histamine-responsive proportion of neurons with prolonged overnight treatment suggests that NGF is a transcriptional regulator of histamine receptors in neurons and can induce expression in neurons that were previously histamine-insensitive. Unexpectedly, NGF pre-treatment did not alter the proportion of capsaicin-responsive neurons, despite its well-known contribution to TRPV1 expression and sensitization. In summary, our data indicate that NGF increases histamine receptor expression in sensory neurons, suggesting that NGF may be a transcriptional regulator for receptors involved in itch, as well as pain.

Studies have demonstrated that GFLs can also induce hyperalgesia by direct sensitization of nociceptors and modulation of nociceptor phenotypes. Acutely, all three GFLs sensitize TRPV1 to produce thermal hyperalgesia. Inflammation and neuropathy models demonstrate that artemin and its co-receptor GFRα3 are upregulated and mediate cold hyperalgesia [49, 64] . On a more chronic scale, overexpression studies show that artemin increases both the expression of TRPV1 and TRPA1 and intraepidermal fiber innervation, which contribute to increased sensitivity to heat, cold, and chemical stimuli [17, 65].

We determined the effects of the GNDF family ligands (GFLs) GDNF, neurturin, and artemin on pruritogen-evoked itch. Our findings indicate that GNDF and neurturin do not affect

pruritogen-evoked itch, while artemin potentiates chloroquine-induced itch but suppresses histamine responses. To activate the receptor tyrosine kinase Ret, each GFL requires a specific co-receptor known as the GDNF receptor α (GFRα1-4), conferring GFL-cell type specificity [8]. MrgprA3-positive neurons represent a subset of the chloroquine-responsive population, and molecular studies using reporter mice demonstrate that this group of cells expresses both the peptidergic marker CGRP, and the non-peptidergic marker Ret [13, 66]. Our calcium imaging studies in Ret-EGFP reporter mice indicate the vast majority of functionally identified chloroquine-sensitive neurons are indeed Ret-positive. The artemin co-receptor GFRα3 is also found in sensory neurons that express Ret, TrkA, and peptidergic markers such as TRPV1 and CGRP [49, 65, 67], suggesting that MrgprA3 and GFRα3 may colocalize to the same population. Furthermore, artemin was previously demonstrated to enhance warmth-induced "itch-like" grooming responses in mice [28], and clinical trials testing artemin as a potential treatment for sciatica found the most commonly reported adverse effect (occurring in more than 40% of participants) was pruritus [30, 31]. These findings support the idea that artemin can acutely sensitize itch responses to non-histaminergic pruritogens. Furthermore, our calcium imaging studies demonstrate that artemin increased the proportion of chloroquine-responsive neurons in culture, indicating artemin may also be a transcriptional regulator of chloroquine receptor expression.

To determine the role of endogenous TrkA signaling in the maintenance of pruriceptors in adulthood, we used the *TrkA*^{F592A} mutant. Despite no reports of a detrimental effect on neuronal survival in the early postnatal period [38], we found that the *TrkA*^{F592A} mutation resulted in significant intraepidermal fiber loss and increased pain detection thresholds in adulthood. These findings are consistent with the analgesic phenotypes of NGF/TrkA knock-out animals [68, 69], suggesting that this mutation alters normal TrkA signaling and likely results in a hypomorphic receptor. Interestingly, pruritogen-evoked itch and sensory neuron responses in these animals remained largely intact. *In vitro* studies of sensory neurons demonstrate a small but significant

reduction in the proportion of histamine- and capsaicin-responsive neurons. Prior studies have demonstrated the proportion of histamine-responsive neurons can range between approximately 10-15%, which may explain why this reduction did not result in robust *in vivo* effects. These findings suggest that endogenous NGF-TrkA signaling may not be required for the maintenance of pruriceptive fiber innervation and functional pruritogen receptor expression in adult sensory neurons. However, further characterization of sensory neuron loss and receptor activation is need to clarify the extent functional loss in these mutants.

In summary, we tested the hypothesis that neurotrophic factors regulate pruritogen-induced itch and sensory neuron responses. We found NGF and artemin have distinct roles in the sensitization of histaminergic and histamine-independent itch. In addition, NGF and artemin regulate pruritogen receptor expression in vitro, suggesting a potential mechanism for itch sensitization in pruritic skin diseases where high levels of growth factors can be found in the skin. Furthermore, a mutation in the high-affinity NGF receptor TrkA confirms its involvement in nociceptor maintenance, but indicates it may not be required for normal itch responses. Neurotrophic growth factors represent potential clinically relevant mediators of itch and further studies should explore their role in chronic and inflammatory itch mechanisms.

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Chapter 5

Functional studies of human sensory neuron responses to pruritogens

This chapter is adapted from the following manuscripts:

Valtcheva MV*, Copits BA*, Davidson S, Sheahan TD, Pullen MY, McCall JG, Dikranian K, Gereau RW. Surgical extraction of dorsal root ganglia from organ donors and preparation of primary human sensory neuron cultures. *Nature Protocols*. 2016. In Press.

Valtcheva MV, Golden JP, Sheahan TD, Pullen MY, Vogt SK, Jain S, Davidson S, Gereau RW. Neurotrophic factors selectively modulate pruritogen responses in mouse but not human sensory neurons. 2016. In Preparation.

Abstract

Primary cultures of rodent sensory neurons are widely used to investigate the cellular and molecular mechanisms involved in pain and itch. The translation of preclinical findings into effective patient therapies may be greatly improved by direct validation in human tissues. We developed an approach to extract and culture human sensory neurons in collaboration with a local organ procurement organization. Here we describe the surgical procedure for extraction of human dorsal root ganglia (hDRG) and the culture techniques used to prepare viable adult human sensory neurons for functional studies. Dissociated sensory neurons can be maintained in culture for more than 10 days, and are amenable to electrophysiological recording and calcium imaging. We further applied this approach to determine the effects of growth factors on human pruriceptor populations. Our findings indicate that NGF and artemin did not change the proportion of pruriceptive neurons, suggesting different mechanisms may be involved in the sensitization of mouse versus human sensory neurons. The approach we have developed can be applied at any institution with access to organ donors that consent to tissue donation for research and provides an invaluable resource for improving translational research.

Introduction

Preclinical studies of pain, itch, nerve injury, regeneration, and axonal transport rely heavily on the use of primary cultures of dissociated rodent sensory neurons. Many candidate molecular targets and genes have been identified using this approach, yet few of these findings have directly translated into effective and safe clinical treatments [1-5]. Several notable failures in translation have raised questions about possible differences in fundamental biological mechanisms between humans and rodents [6-12]. For example, NK1 receptor antagonists failed to provide significant analgesia in humans, despite strong preclinical effects in rodents [6]. In addition, $GABA_A$ receptor-mediated currents in cultured human sensory neurons were recently found to exhibit distinct biophysical features from those in rodents, demonstrating the importance of preclinical validation between species [13-16].

Recent advances in induced pluripotent stem cell (iPSC) methods have produced nociceptor-like cells derived from human fibroblasts that express TRP channels and can be sensitized by inflammatory mediators such as PGE_2 [17, 18]. Unlike sensory neurons obtained from organ donors, such methods are not restricted by donor availability because they do not rely on post-mortem tissue collection and can be further strengthened by a living donor's developing medical history. However, the iPSC approach is still significantly limited by low nociceptor conversion rates (<5%) and the use of varying transcription factor combinations to produce nociceptor-like populations. While both approaches can be informative, direct studies in human sensory neurons may more accurately recapitulate the complex overlapping sensory neuron subpopulations and downstream signaling cascades involved in sensory processing.

Cultured human sensory neurons can serve as a valuable tool in functional and genetic studies to validate the numerous molecular targets identified in animal models. By using techniques such as patch-clamp electrophysiology, calcium imaging, immunohistochemistry, gene knock-down, and viral gene transfer, the fundamental physiological properties of human sensory neurons can be more accurately characterized. These approaches could lead to the

discovery of novel target receptors or channels and allow for in-depth characterization of pharmacological agents in native human cells. Furthermore, the potential of novel therapeutic interventions involving gene therapies, and optogenetic and chemogenetic manipulations of cellular activity can be verified directly in human sensory neurons. The protocol we developed allows us to obtain DRG from multiple spinal levels, giving us the opportunity to culture from half of the ganglia, while preserving the remaining tissue for RNA sequencing and immunohistochemical analysis. This permits parallel functional and gene expression studies from the same donor with the ability to correlate observations with patient medical record information [14, 16, 19]. Future studies employing a combination of these approaches can help identify human biomarkers for chronic pain and itch.

Recent studies in the field of itch have identified the Mas-related G protein-coupled receptor (Mrgpr) subtypes A3 and C11 as key mediators of acute and chronic itch in mice [20-22]. However, these receptors are not evolutionarily conserved in humans, which instead have four MRGPRX receptors (MRGPRX1-4). Studies using heterologous systems have determined that the human MRGPRX1 receptor can be activated by both MrgprA3 and MrgprC11 agonists, although key differences in the pharmacological and signaling profiles of these receptors indicate distinct molecular mechanisms in mice versus humans [20-24]. In this study, we demonstrate the pruriceptive subpopulations of human sensory neurons that respond to histamine and chloroquine, and their functional overlap with TRP channels. In our prior studies we determined that NGF and artemin increased the proportion of histamine- and chlroquine-responsive neurons in mouse. We now test the effects of these growth factors in human sensory neurons and use RNA sequencing to determine the expression of pruritogen receptors, TRP channels, and neurotrophic growth factor receptors in whole human DRG.

Materials and Methods

Access to human donor tissue

We established a collaboration with Mid-America Transplant, a local organ procurement organization. Extraction procedures were approved by Mid-America Transplant and appropriate regulatory documentation and agreements were obtained, including IRB waiver from the Human Research Protection Office at Washington University in St. Louis and material transfer agreement. Mid-America Transplant obtained documentation of donor or family consent to tissue donation for research and provided access to all medical records available at procurement. Since establishing this approach at our institution, we have had access to donor tissue approximately 1-2 times per month. Once a donor becomes available and consent has been obtained for tissue donation to research, we are contacted by Mid-America Transplant staff, generally 3-8 hours prior to organ procurement.

Surgical extraction of human dorsal root ganglia (hDRG)

Sterile operating room procedures were followed throughout tissue extraction in order to not interfere with further tissue procurement after DRG have been removed. Once the transplant surgical team completed the extraction of all organs to be used for transplantation (e.g. liver, kidneys, heart), all remaining viscera were either retracted or removed. The lumbar spinal column was exposed by removing the abdominal vasculature, including the abdominal aorta, inferior vena cava, and lymphatics, and remaining connective tissue located anterior to the spine in the retroperitoneal space. The *psoas major* and *psoas minor* muscles were cut and retracted or removed from their origin to expose the lateral aspects of the spinal column and the roots of the lumbar plexus. Using a mallet and straight osteotome, L1 and L5 vertebral bodies were transected, stopping at the spinal canal (Figure 1a). With the use of a mallet and curved osteotomes, the pedicles were transected bilaterally between L1 and L5, while staying close to

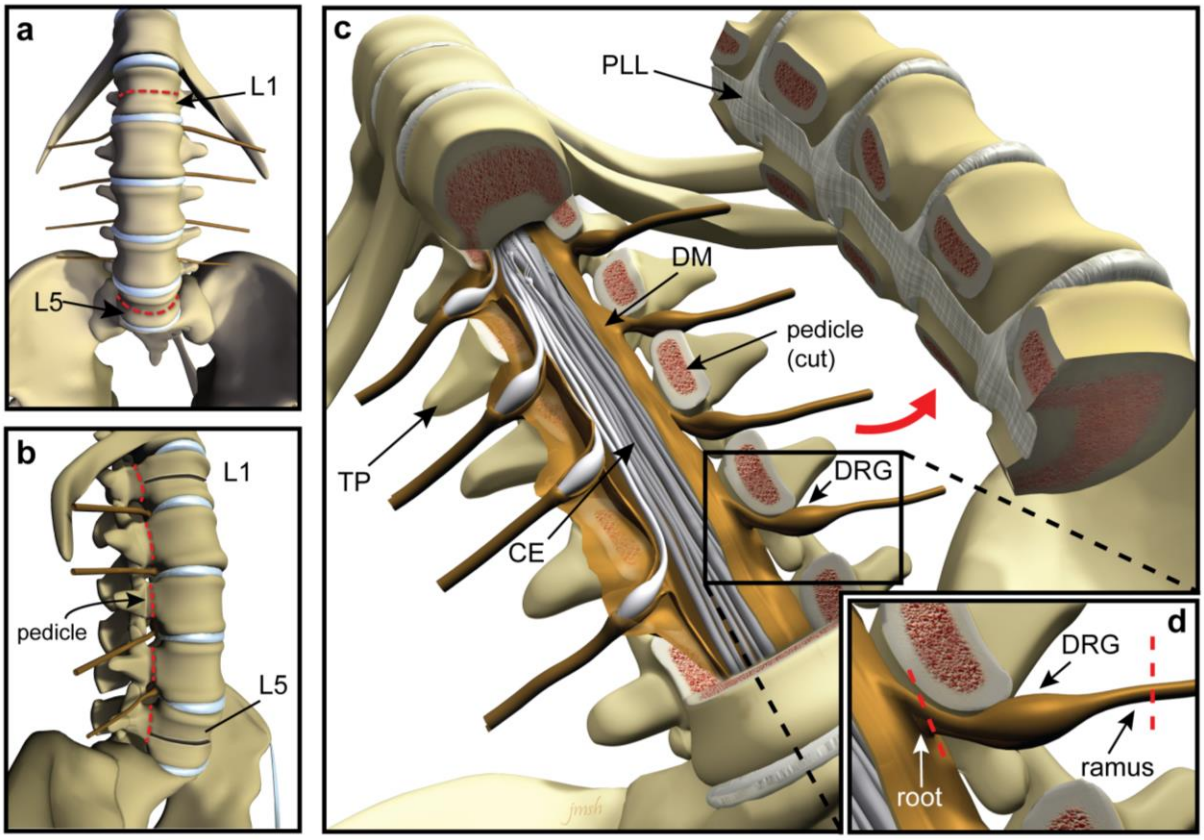

Figure 1. Surgical extraction of hDRG using ventral approach. (**a-b**) Illustration depicting the ventral (**a**) and lateral (**b**) views of the spinal column with lumbar vertebral bodies L1 and L5 indicated by arrows. Red dashed lines indicate the location of bone transection. After the spinal column is visualized, lumbar vertebrae can be correctly identified by counting up from L5, which is located directly above the sacrum (**a,b**). Using a straight osteotome and surgical mallet, the L1 and L5 vertebral bodies are transected, stopping at the spinal canal (red dashed lines in panel **a**, black lines indicate transected bone in panel **b**). Using a curved osteotome and mallet or autopsy saw, the pedicles of each vertebrae are transected bilaterally between L1 and L5 (red dashed lines in panel **b**). (**c**) Illustration showing the anterior portion of the vertebral column removed to expose the spinal canal where the cauda equina and DRG are located (TP: transverse process, CE: cauda equina, PLL: posterior longitudinal ligament, DM: dura mater, DRG: dorsal root ganglion). (**d**) Each DRG is dissected away from surrounding bone and connective tissue and the nerve roots and rami are cut to completely free each ganglion (red dashed lines).

the posterior edge of the vertebral bodies, but anterior to the spinal canal (Figure 1b). The entire anterior portion of the vertebral column between L1 and L5 was manually removed by lifting carefully to minimize tearing of structures in the intervertebral foramina and simultaneously cutting any dural attachments to the posterior longitudinal ligament (Figure 1c). This approach ensured that the transverse processes, laminae, and spinous processes remain in place. It is essential to leave the dorsal portion of the vertebral column intact to maintain the integrity of the body for

further tissue procurement and funeral service purposes. When devising our approach, this was a major concern for Mid-America Transplant.

DRG were visualized by a gentle tug on the dura mater and each ganglion was dissected away from surrounding connective tissue and bone using DeBakey pick-ups and dissecting scissors. The spinal roots and rami of peripheral nerves were then cut to completely free the DRG (Fig. 1d). Each DRG was fully submerged in ice-cold *N*-methyl-D-glucamine artificial cerebrospinal fluid solution (NMDG-aCSF) in air-tight 50 ml conical tubes and maintained on ice for transport. NMDG-based aCSF solutions reduce neuronal permeability to sodium and calcium, reduce oxidative stress, and are very beneficial for producing adult rodent brain and spinal cord slices for electrophysiological recordings [25-28]. DRG tissue from human organ donors was extracted as soon as possible to minimize post-mortem interval. We were generally able to extract DRG 1.5-3 hours post-mortem, immediately after the surgical resection of organs for transplantation. However, we have obtained viable neuron cultures from tissues extracted up to 4 hours post-mortem.

Cleaning and dissection of DRG

Upon return to the laboratory, each individual DRG was placed in a 60 mm petri dish filled with ice cold NMDG-aCSF and maintained on ice for the duration of cleaning and dissection. Using large spring scissors and forceps, any remaining fat and connective tissue surrounding DRG were removed (Figure 2a). With the aid of a dissection microscope, forceps and large spring scissors were used to identify the edge of the dura along the severed ends of the central and peripheral nerve processes. The dura was then cut longitudinally from the peripheral to the central endings. The dura was removed by continually pulling away from the ganglion and cutting along the edge where the dura meets the DRG and nerves (Figure 2b). The remaining central and peripheral nerve processes were trimmed with a scalpel, so that only the ganglion body remained

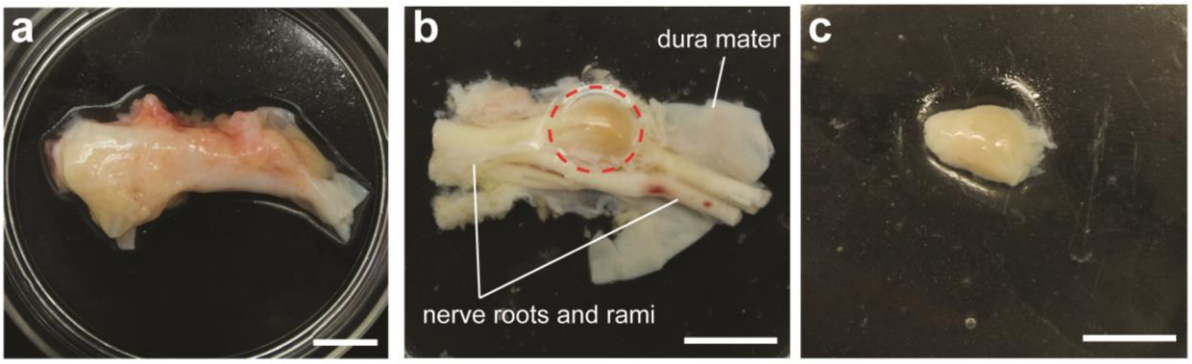

Figure 2. Cleaning of hDRG prior to dissociation. (**a**) Extracted ganglia are first cleaned of all visible fat and connective tissue. (**b**) Dura mater is then removed to expose underlying ganglion and nerve roots and rami. (**c**) Nerve roots are trimmed so that only the ganglion remains for dissociation. Scale bars represent 1 cm.

(Figure 2c). One ganglion from each spinal level was used for culture, while the corresponding contralateral DRG was saved for immunohistochemistry and RNA sequencing studies, to allow for direct functional comparisons. Once the contralateral ganglion was cleaned, it was cut into small pieces and part of the tissue was immediately frozen in RNAlater for RNA sequencing or RT-PCR, while the rest of the DRG was post-fixed in 4% paraformaldehyde solution for intact ganglion histology.

Dissociation and culturing of DRG

Each DRG to be used for culture was minced with a scalpel as finely as possible into pieces no larger than 3 mm in diameter. All tissue fragments were placed into a 14 ml snap-cap tube containing 3 ml of pre-warmed (37°C) papain/NMDG-aCSF solution (1.5U/ml) and incubated for 1 hour at 37°C and 5% CO_2. The tube was gently agitated every 20 min to mix tissue with solution. The tissue was washed carefully 3 times with 3 ml of fresh NMDG-aCSF, warmed to 37°C. The tissue was then incubated in 3 ml of NMDG-aCSF-collagenase (15mg/ml) solution for 1 hour at 37°C and 5% CO_2, while agitating gently every 20 min to mix the tissue with the solution. Tissue was carefully washed 3 times with 3 ml of fresh NMDG-aCSF warmed to 37°C. The remainder of NMDG-aCSF solution was then removed and 2 ml of pre-warmed DRG medium were added. DRG tissue was then triturated gently through the fire-polished tip of a sterile glass

Pasteur pipette until the solution became cloudy and little resistance remained (approximately 10-20 times). The solution of dissociated neurons was carefully passed through a 100 µm sterile filter into a 50 ml conical tube. The filtrate was centrifuged at room temperature for 4 min at 180*g*. The supernatant containing debris was carefully removed and the pellet was re-suspended in 1 ml warmed DRG medium by pipetting up and down several times. Cell suspension was centrifuged again for 3 min at 180*g*. The supernatant was removed and the pellet was re-suspended in 1 ml warmed DRG medium by mixing gently. At this point, the cell suspension was counted using a hemocytometer and diluted to desired concentration. The cell suspension was plated onto poly-D-lysine and collagen-coated glass coverslips for a final plating density of approximately 500 cells/coverslip. The cell-plated coverslips were incubated at 37°C, 5% CO_2 for 1-1.5 hours to allow neurons to adhere to the glass and then 900 µl warm DRG medium was added. Dissociated hDRG cultures were maintained at 37°C, 5% CO_2 in a humidified chamber until use. Half of the culture medium was replaced with fresh warmed medium every 3-4 days.

Reagents

Poly-D-lysine (PDK) solution was made by dissolving 5 mg PDK in 50 ml sterile water, aliquoted into 1 ml (0.1 mg/ml) per tube, and stored at -20°C. Rat tail collagen (Sigma) was dissolved in sterile water for a final concentration of 1 mg/ml and stored at 4°C up to 2-3 months. PDK/collagen solution was made at a final concentration of 0.01 mg/ml PDK and 0.2 mg/ml collagen. Papain solution was made by adding 0.5 µl of 0.5 M NaOH to 1 mg/50µl L-cysteine and then combined with 45U papain in 3 ml of NMDG-aCSF. Solution was made fresh and warmed to 37°C immediately prior to use. DRG medium was Neurobasal A media supplemented with B27, 100U/mL penicillin/streptomycin, 2 mM Glutamax, and 5% fetal bovine serum (Gibco). For electrophysiology experiments, neurotrophic factor (NTF)-treated neurons were plated in the presence of 25ng/ml human β-nerve growth factor (hNGF) (Cell Signaling Technology) and recombinant human glial cell line-derived neurotrophic factor (hGDNF) (Peprotech).

NMDG-artificial cerebrospinal fluid (aCSF) solution: The reagents listed in Table 1 were combined in 450 ml Milli-Q H_2O in the order listed. pH was adjusted to 7.4 with NMDG/HCl and osmolarity was adjusted to 300-310 mOsm with H_2O or sucrose [25, 26]. NMDG-aCSF solution was always made fresh and bubbled with carbogen gas (95% O_2, 5% CO_2) for at least 15 min prior to use to achieve stable pH and saturate with oxygen.

Table 1. Reagents used to prepare NMDG-aCSF.

Reagent	**Final Concentration (mM)**	**MW or Concentration of stock**	**500 ml**
NMDG	93	195.2	**9.08 g**
HCl		12.1 N	**3.5 ml**
KCl	2.5	2.5 M	**500 µl**
NaH_2PO_4	1.25	1.25 M	**500 µl**
$NaHCO_3$	30	84.0	**1.26 g**
HEPES	20	2 M	**5 ml**
Glucose	25	2.5 M	**5 ml**
Ascorbic acid	5	176.1	**0.44 g**
Thiourea	2	76.1	**0.08 g**
Na^+pyruvate	3	110.0	**0.17 g**
$MgSO_4$	10	2 M	**2.5 ml**
$CaCl_2$	0.5	1 M	**250 µl**
N-acetylcysteine	12	163.2	**0.98 g**

Immunocytochemistry

Coverslips of dissociated neurons were fixed in 4% paraformaldehyde/ 4% sucrose. Antibodies used include: Rabbit anti-TRPV1 (1:800), custom-made serum directed against the TRPV1 C-terminus peptide CLKPEDAEVFKDSMVPGEK, specificity confirmed in TRPV1-/- mice [29, 30]; Mouse anti-βIII-tubulin (1:2000) – EMD Millipore (cat. no. 05-559), species reactivity (hm/bv/rat/ms), Antibody Registry ID: AB_309804; Guinea pig anti-GFAP (1:1000) – Synaptic Systems (cat. no. 173 004), species reactivity (hm/rat/ms/ck), Antibody Registry ID: AB_1064116;. Mouse anti-synapsin 1 (1:2000) – Synaptic Systems (cat. no. 106 001), species reactivity (hm/ms/rat/avian/zebrafish), Antibody Registry ID: AB_887805; Guinea pig anti-tau (1:2000) – Synaptic Systems (cat. no. 314 004), species reactivity (hm/ms/rat), Antibody Registry ID: AB_1547385. Secondary antibodies used were donkey Alexa Fluor 555/647 (1:2000).

Patch-clamp Electrophysiology

Fire polished, filamented glass electrodes were pulled using a P-97 horizontal puller (Sutter Instrument Company) with open tip resistances ranging from 2.0–4.5 MΩ. The internal pipette solution contained (in mM): 120 K+ gluconate, 5 NaCl, 2 MgCl2, 0.1 CaCl2, 10 HEPES, 1.1 EGTA, 4 Na2ATP, 0.4 Na2GTP, 15 sodium phosphocreatine; pH adjusted to 7.3 using KOH, osmolarity 291 mOsm. For the duration of recording, cells were continuously perfused with external solution at room temperature containing (in mM): 145 NaCl, 3 KCl, 2.5 CaCl2, 1.2 MgCl2, 10 HEPES, 7 glucose, adjusted to pH 7.4 with NaOH. Neurons were recorded with an EPC10 amplifier (HEKA Instruments) and Patchmaster software (HEKA Instruments). After acquiring gigaseal and break-in, neurons were given at least 2 minutes to stabilize and then a series of protocols to determine membrane excitability were performed. Action potentials were evoked in current clamp mode using a series of increasing 1 second ramp current injections. The first action potential of a train was used to determine threshold, defined as the voltage at which the first derivative of the membrane potential increased by 10 V/s. Rheobase was established from the step or ramp current pulse at which the first action potential was triggered.

Calcium imaging

The protocol for calcium imaging was the same as our previous studies in mouse [29-33]. Neurons were incubated for 45 minutes in 3 µg/mL (3µM) of the cell-permeant ratiometric calcium indicator Fura-2 AM (Molecular Probes) and then incubated in external solution (in mM): 130 NaCl, 5 K, 2 $CaCl_2$, 1 $MgCl_2$, 30 Glucose, 10 HEPES for a 20-minute de-esterification prior to recording. Coverslips were positioned in a recording chamber and continuously perfused with external solution. Recordings took place at room temperature. Cells were viewed under an inverted microscope (Olympus Optical, Tokyo, Japan) and images were captured with a Hamamatsu Orca camera. Regions of interest encompassing all Fura-loaded cells were identified a priori and the ratio of fluorescence emission at an alternating excitation wavelength of 357 and

380 nm was recorded with SimplePCI Software. The experimental protocol consisted of a 2-minute baseline followed by 30 second bath application of 100 μM histamine (Sigma, St. Louis), 8 minutes of external solution wash, 30 seconds of 1mM chloroquine (CQ, Sigma, St. Louis), 8 minutes external solution wash, 30 seconds of 100 μM mustard oil (MO, Sigma, St. Louis), 8 minutes of external solution, 20 seconds of 200nM capsaicin (Sigma, St. Louis), 4-8 minutes of external solution, and 10 seconds of high KCl (50mM). Peak responses were determined by calculating the absolute increase in Fura-2 signal above baseline immediately prior to each stimulus. A change from resting level of ≥10% was set as the threshold for a response to a bath applied stimulus. Cells unresponsive to capsaicin or high K+ were excluded from physiological analysis. Calcium imaging recordings were performed on the 5^{th}-7^{th} days *in vitro* (DIV) to allow glial cells to peel off neurons and expose the plasma membrane for reliable Fura-2 signal visualization. For NTF treatment, cells from the same donor were treated with growth factor (100ng/ml human nerve growth factor, Peprotech; 100ng/ml human artemin, R&D Systems) or vehicle the evening prior to recording. The overall proportion of responders were determined for each donor and used for statistical comparisons (unpaired t test). Post-hoc measurements of cell diameter of responsive neurons were made in ImageJ using images obtained immediately prior to calcium imaging recording in SimplePCI.

RNA sequencing of whole human DRG

RNA and library preparation: Fresh DRG tissue from organ donors was cut into several sections and frozen at -80°C in RNAlater (Ambion) immediately after extraction. For RNA preparation, DRGs were thawed, cut into small pieces, and put in TRIzol reagent (Life Technologies Inc). Tissue was then homogenized using an electric homogenizer and total RNA was isolated per the manufacturer's instructions. Only samples with RNA integrity number (RIN) of > 7 were used for further analysis. Library preparation was done at the Genome Technology Access Center at Washington University (GTAC). Briefly, 30 ng of total RNA were reverse transcribed and amplified

using the SMARTer Ultra low input RNA for Illumina Sequencing –HV (Clontech). After cDNA preparation and shearing using an ultra-sonicator (Covaris), the libraries were prepared using VeraSeq Ultra DNA Polymerase for 12 cycles. The forward primer used was: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT. The reverse primer contains the unique reverse complement of the respective index nucleotides. The sequence of the reverse primer where "N" denotes the reverse complement of 7 nucleotides of the index is CAAGCAGAAGACGGCATACGAGATNNNNNNNGTGACTGG-AGTTCAGACGTGTGCTCTTCCGA. The purified cDNA yield was 16.5 ng/µl in 30µl.

Nextgeneration sequencing and analysis: Single end sequencing was done on the Illumina HiSeq2500 platform following the manufacturer's protocol at the GTAC. The raw demultiplexed RNA-seq reads were aligned with STAR version 2.0.4b (https://github.com/alexdobin/STAR/releases) (GRCh37 assembly). Low base quality reads and adaptors were clipped. All known genes with raw counts were enumerated to the matching gtf file from the same reference build from Ensembl with Subread:featureCounts (version 1.4.5) (http://sourceforge.net/projects/subread/) using the Ensembl gene ID as the key. Reads per kilobase of transcript per million mapped reads (RPKM) values were generated in R using raw counts. We assessed sequencing performance for total number of aligned reads, uniquely aligned reads, number of genes and transcripts detected, and found less than 1% of ribosomal fraction and Spearman correlation of >0.9 between samples.

Results

Establishing primary cultures of human dorsal root ganglia

We developed a collaboration with the local organ procurement organization Mid-America Transplant to gain access to human dorsal root ganglia from deceased organ donors that have consented to tissue donation for research. After devising a surgical approach that provides a short post-mortem interval and minimal interference with further tissue procurement (Figure 1), we were able to obtain DRG from a number of donors of both sexes and varying ages (Table 2). We established a culturing protocol which follows our prior published studies of mouse sensory neurons very closely, utilizing the same coverslip coating solution, digestive enzymes, and culture medium [30-32, 34-37]. However, we incorporated a number of key modifications: a specifically designed preservation solution for transport, new instructions for tissue preparation, changes to enzyme digestion duration, and amount of time in culture prior to functional recordings [30-32, 34-37]. Our studies using hDRG show that these adaptations maintain neuronal viability in transport and address several issues that arise from dissociation of substantially larger tissues with more abundant connective tissue than rodent DRG [19].

Table 2. Donor demographics. (COD: cause of death, CVA: cerebrovascular accident).

Donor #	Age	Sex	Race/Ethnicity	COD
1	27	F	White	Head trauma
2	30	F	White	CVA/Stroke
3	31	F	White	Head trauma
4	44	F	Middle Eastern	CVA/Stroke
5	58	F	White	CVA/Stroke
6	10	M	White	Head trauma
7	21	M	White	CVA/Stroke
8	22	M	Black	Anoxia
9	41	M	White	Head trauma
10	44	M	White	CVA/Stroke
11	47	M	White	Head trauma

The total number of coverslips obtained for functional studies depended on the number of ganglia cultured and cell density needed for the particular study. We found that one DRG can produce approximately 10-12 coverslips plated at a density of 500 cells/coverslip. However, this

can vary based on several factors, including DRG size (L1 is considerably smaller than L2-4), degree of dissociation, and general health of the tissue. Younger donors tended to have DRG that were easier to dissociate and neuron number and responses in culture were generally better. The density of 500 cells/coverslip provides an optimal number of cells for calcium imaging studies (~20-30 cells per viewing field at a 10x magnification) without excessive debris. Future studies can adjust the plating density to fit investigator needs. For example, plating at a lower density of 200 cells/coverslip was suitable for electrophysiological recordings and immunocytochemical studies, but did not provide robust numbers for calcium imaging studies.

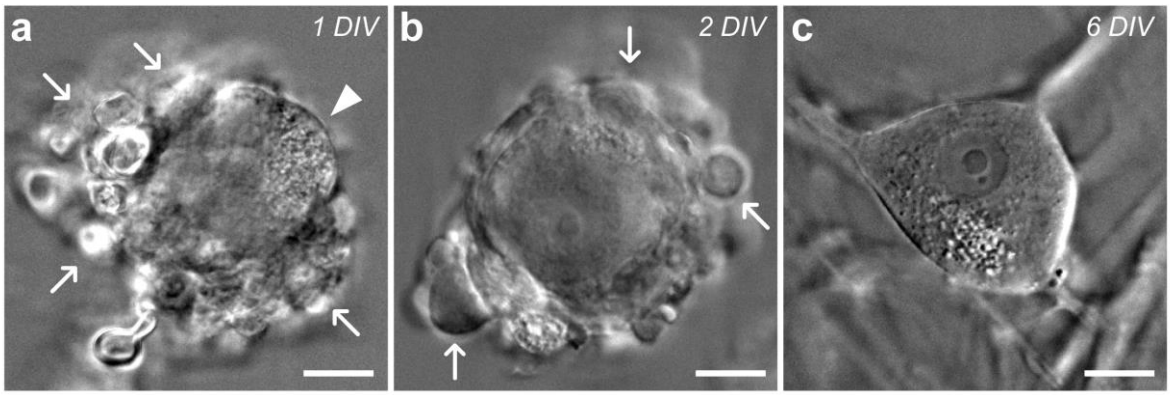

Figure 3. Dissociated hDRG neurons over time *in vitro*. Infra-red differential interference contrast microscopy (IR-DIC) images of cultured human sensory neurons. (a) Initially, most dissociated neurons are encased in glial cells (white arrows) after 1 day *in vitro* (DIV), but can be identified by partially visible plasma membrane (white arrowhead). (b) As time in culture progresses, glia (white arrows) continue to peel off and adhere to the coverslip, exposing more of the plasma membrane. (c) After 6 DIV, the plasma membrane of most neurons is completely exposed, leaving them amenable to patch-clamp recordings and calcium imaging studies. Scale bars represent 20 μm.

Appearance of neurons in culture

While most rodent studies can be completed within 24 hours of cell plating, human sensory neurons were often tightly encased by satellite glia until the third or fourth day *in vitro* (DIV). During these first days in culture, glial cells peeled off allowing membrane access for patch pipettes or direct fluorescence visualization (Figure 3). More vigorous dissociation protocols can yield neurons free of supporting glia, but the health and survival of these neurons may be reduced [16]. The neurons produced with our extraction and dissociation protocol could be maintained in culture

for more than 10 days. Whole ganglia and dissociated neurons can be studied using validated antibodies and standard immunocytochemical techniques to characterize cell morphology and the localization and expression of ion channels, receptors, and intracellular proteins (Figure 4) [28, 30]. In addition, the coverslips used for functional studies by patch-clamp [16, 36-38] and calcium imaging [31, 36] can be fixed immediately after the experiment to allow for immunocytochemical studies of the same functionally-characterized neurons.

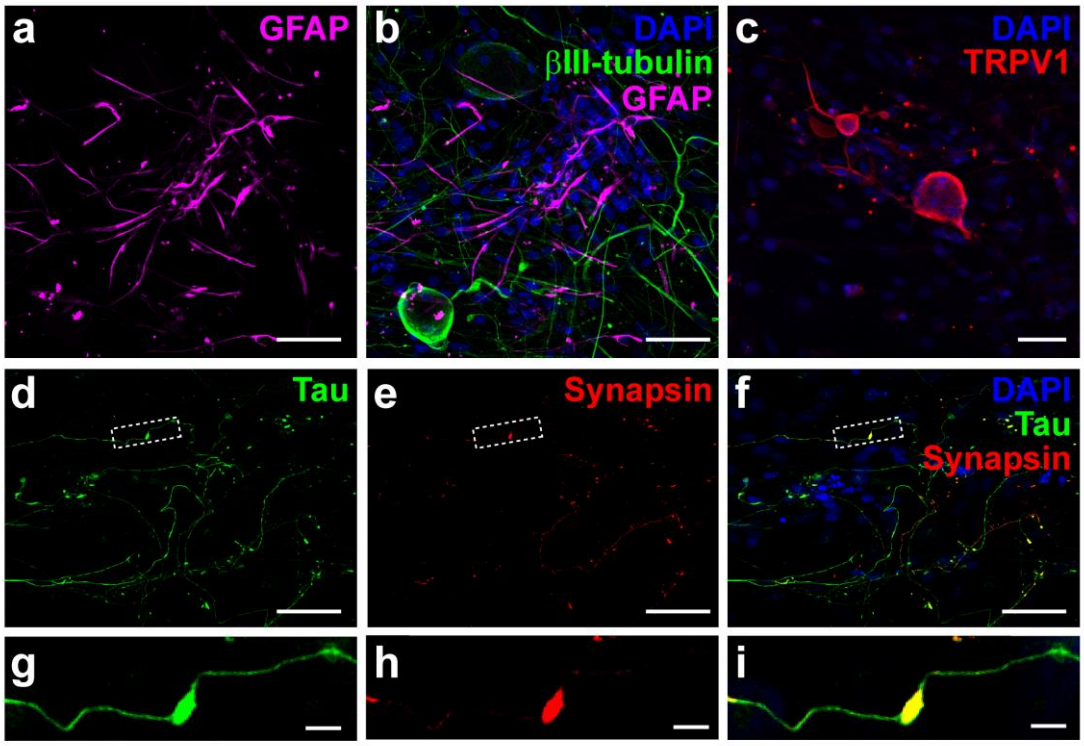

Figure 4. Immunocytochemical analysis of cultured hDRG neurons. Collapsed confocal micrographs of immunolabeled neurons using standard techniques. (**a**) $GFAP^+$ cells (purple) - presumably satellite glia that initially encase dissociated hDRG neurons – that have migrated onto the coverslip after 8 DIV. (**b**) Merged image with neurons immunolabeled for the cytoskeletal protein βIII-tubulin (green) and nuclei labeled with DAPI (blue). (**c**) TRPV1 immunofluorescence (red), a common nociceptive marker in rodent DRG, was observed in subsets of cultured human sensory neurons. Nuclei are labeled with DAPI (blue). (**d-f**) Fluorescence images of cultured hDRG demonstrating extensive axonal process growth and branching marked by tau immunoreactivity (green) and a component of the synaptic vesicle release machinery, synapsin (red). Merged images are shown in (**f**) with DAPI-labeled nuclei (blue). (**g-i**) Cropped sections from the boxed areas in (**d-f**), showing an *en passant*-type presynaptic enlargement (synapsin, red) that formed along a sensory axon (tau, green). Scale bars represent 50 µm for panels (**a-f**), and 5 µm for the panels shown in (**g-i**).

Electrophysiological properties of hDRG neurons cultured in the presence or absence of growth factors

Prior studies using primary cultures of hDRG neurons have applied human nerve growth factor (hNGF, 25 ng/mL), and human glial cell line-derived neurotrophic factor (hGDNF, 25 ng/mL) in the culture medium [16]. However, growth factors can directly modulate neuronal excitability and receptor expression, thus confounding any future studies on the effects of growth factors on pruriceptor sensitization. To determine if culturing with or without growth factors significantly impacted sensory neuron physiology, we used patch-clamp electrophysiology techniques to measure the basic excitability and membrane properties of the neurons in our cultures. We found that cells cultured without growth factors survive for more than 10 days without measurable effects on membrane properties. Gigaohm seals were obtained from >95% of selected neurons. We were careful to only patch neurons with a clearly visible soma devoid of satellite glial cells and exhibited a “smooth” membrane (Figure 3c). Whole-cell recordings were successful from >95% of these neurons, which included stable access resistance and a resting membrane potential hyperpolarized over -40 mV. We found that neurons could be cultured in the absence or presence of the growth factors NGF and GDNF, and that chronic treatment did not affect cellular excitability, resting membrane potential, or soma size (Figure 5). The ability to maintain these neurons in culture in the absence of growth factors allows for future studies to determine the acute effects of growth factors on receptor expression and neuronal sensitization.

Effects of NGF and artemin on human pruriceptors

Very little is currently known about the human pruriceptive sensory neuron populations and whether they can be sensitized by neurotrophic factors. To investigate the direct effects of neurotrophic factors on dissociated hDRG neurons, we measured calcium responses to the pruritogens histamine and chloroquine, the TRPA1 agonist mustard oil, and the TRPV1 agonist

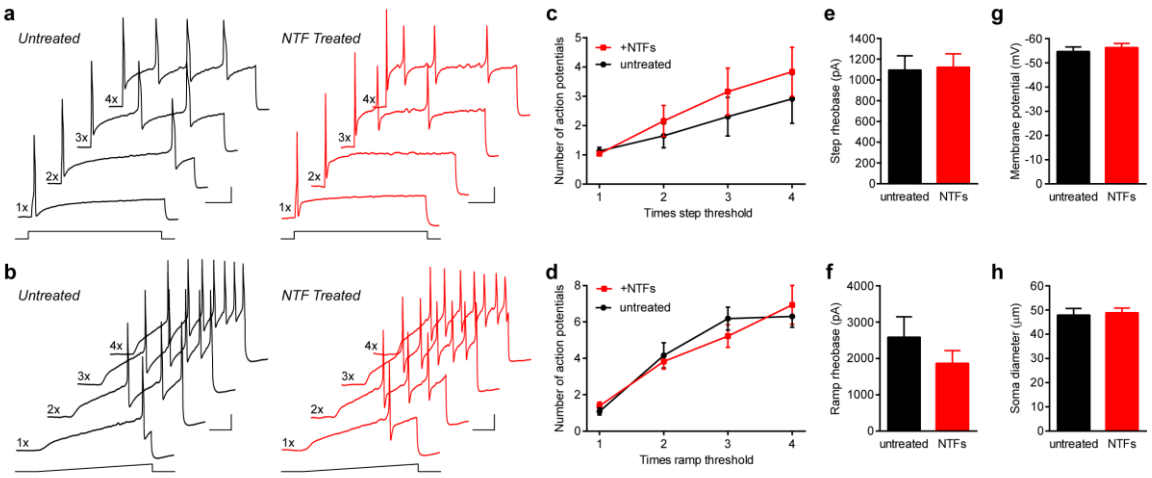

Figure 5. Long-term culturing with neurotrophic factors does not alter hDRG excitability.
Human sensory neurons were grown in the absence (black traces) or presence (red traces) of the neurotrophic factors NGF and GDNF (NTFs, 25ng/ml each, added to wells chosen randomly from all spinal levels). Recordings were performed between 4-9 DIV and cells were excluded from further analysis if the resting membrane potential was more depolarized than -40 mV. (**a,b**) Voltage traces illustrating action potential firing to 1 s depolarizing step (**a**) or ramp (**b**) current injections of 1-4 times current threshold (rheobase) of neurons grown in the absence (black traces) or presence of NTFs (red traces). Scale bars are 20 mV and 200 ms. (**c,d**) Summary plots of the number of action potentials elicited in neurons in response to both step (**c**) and ramp (**d**) stimuli, which were not significantly different between the two culturing conditions (two-tailed Mann-Whitney test, P values range from 0.143 to 0.964). (**e,f**) Quantification of step (**e**) or ramp (**f**) current threshold to elicit an action potential. No differences were found between the two groups (two-tailed Mann-Whitney test, P=0.738 for panel e, and P=0.361 for panel f). (**g**) Summary graph of the resting membrane potential for cells cultured in the absence or presence of NTFs, which were not significantly different (two-tailed unpaired t-test; P=0.657). (**h**) Average soma diameters of neurons in both culturing conditions, which were not significantly different (two-tailed unpaired t-test; P=0.775). This suggests that the preferential survival of sub-populations of sensory neurons with different cell diameters was not influenced by either culturing condition. 19-30 cells were used for quantification per condition from four donors (4-30 cells for each measurement per donor), and all data are represented by the mean ± s.e.m of cells pooled across all donors.

capsaicin. Prior to recording, neurons were maintained in culture without growth factors for 5-7 days. Due to the precious nature of human tissue, we focused our studies on the growth factors we previously determined had effects on mouse pruriceptors: NGF and artemin. Once free of glial cells, neurons were treated with either a growth factor or vehicle overnight. On the next day, neurons were incubated with the fluorescent calcium indicator Fura-2AM and calcium responses

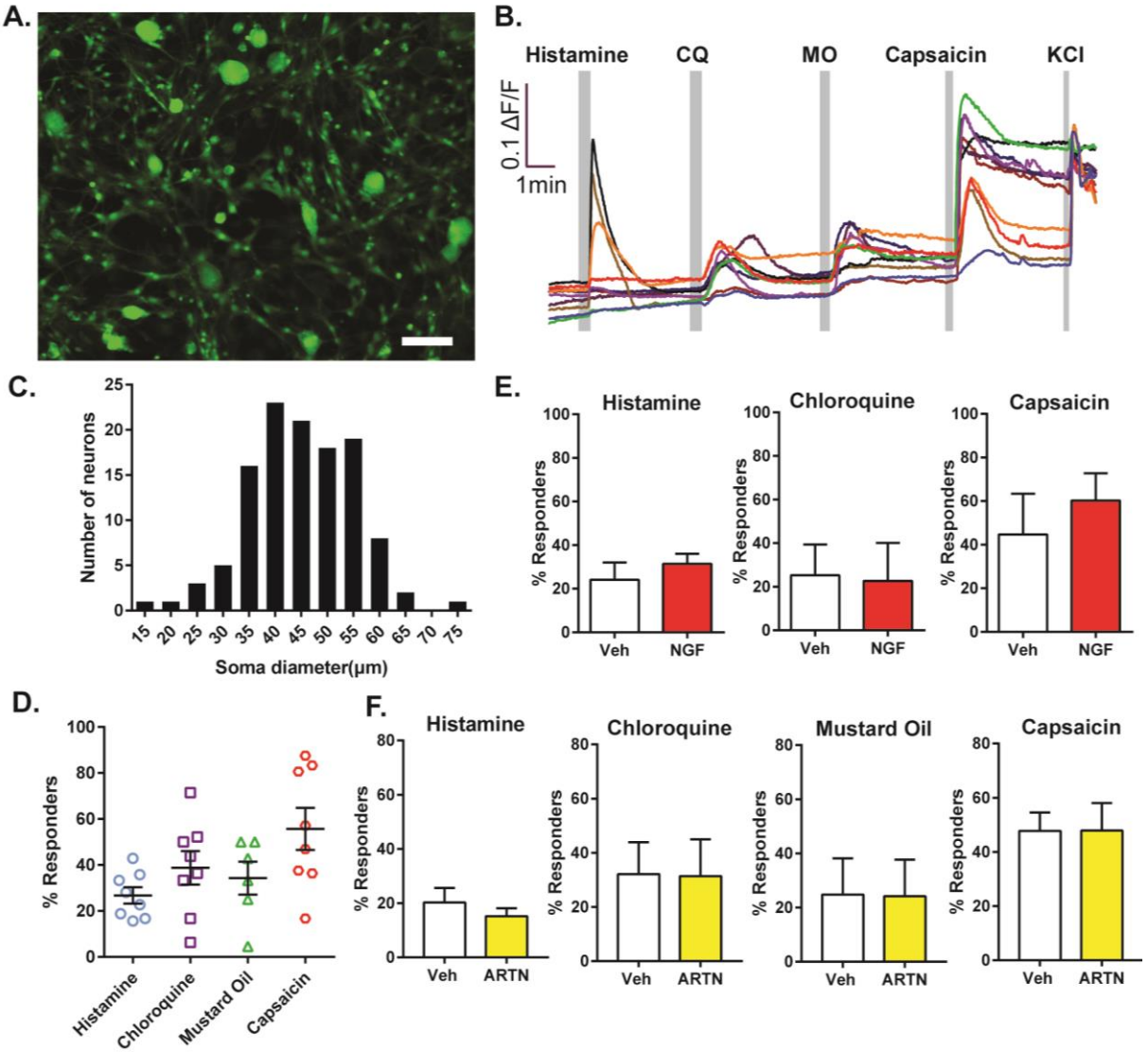

Figure 6. Effects of NTFs on human sensory neuron responses to pruritogens. A. Calcium responses of dissociated hDRG neurons were measured using Fura-2AM (scale bar=100μm). **B.** Representative traces of calcium responses to the indicated bath-applied stimuli. **C.** Frequency histogram of cell diameter of responsive neurons. **D.** Distribution of stimulus-responsive neurons in control groups across all donors. Each data point represents an individual donor. **E.** Overnight treatment with NGF (100ng/ml) did not change the proportion of histamine-, chloroquine-, or capsaicin-responsive hDRG neurons (N=3 donors, 9-86 cells/donor). **F.** Pretreatment with artemin (ARTN, 100ng/ml) did not affect the overall proportion of responsive neurons (N=3 donors, 10-42 cells/donor, unpaired t test).

to bath applied stimuli were recorded (Figure 6A-B). The average soma diameter of vehicle-treated neurons in culture was 45 μm (± 9.9 μm SD, minimum: 16.8 μm, maximum 72.6 μm, n=118 neurons) (Figure 6C).

We determined the relative distribution of pruritogen- and algogen-responsive neurons in our donor pool by calculating the proportion of vehicle-treated cells that responded to a given stimulus for each individual hDRG donor (n=6-8 donors) (Figure 6D). To asses if human pruritogen receptors colocalize with TRP channels, we determined the proportion of neurons that responded to more than one stimulus (Table 3). Our data demonstrate that 87% of all histamine-responsive neurons also responded to capsaicin, indicating that most histamine-sensitive neurons are also TRPV1-positive. More than half of histamine responders also responded to chloroquine, while only a third of chloroquine-sensitive neurons responded to histamine. and more than half also responded to chloroquine (Table 1). Chloroquine-responsive neurons were largely TRPV1-positive (74%), but more than half also responded to mustard oil. Most TRPA1-positive neurons responded to chloroquine, but few also responded to histamine or capsaicin. Overnight pre-treatment with NGF or artemin did not change the proportion of neurons that responded to histamine, chloroquine, mustard oil, or capsaicin (Figure 6E-F). These data indicate that the expression of human pruritogen receptors was not significantly affected by growth factor treatment, suggesting that the mechanisms behind NTF regulation of itch-sensitive neurons differ between mouse and human.

Table 3. Functionally overlapping subpopulations of human sensory neurons.

		% Responding to another stimulus			
		Histamine	CQ	MO	Capsaicin
Total responsive neurons	Histamine+		56.00%	27.74%	87.27%
	CQ+	36.84%		53.85%	73.68%
	MO+	15.63%	65.62%		36.84%
	Capsaicin+	40.00%	51.85%	24.14%	

RNA sequencing was used to quantify transcripts encoding pruritogen receptors, TRP channels, and neurotrophic factor receptors in whole human DRG. Transcriptome data were obtained from 5 donors, and confirm the presence of histamine receptor 1 (HRH1) and 2 (HRH2) transcripts, but none of the subtypes 3 and 4 (HRH3, HRH4) (Figure 7). Sequencing data further demonstrate the presence of MRGPRX1, the putative ortholog of the mouse MrgprA3/C11

pruritogen receptors. Interestingly, there was also a considerable amount of MRGPRX3 transcripts detected, however, very little is known currently about the physiological role of MRGPRX3. Despite the high proportion of capsaicin-responsive neurons identified in our functional studies, TRPV1 transcript levels in whole DRG tissue were surprisingly low. TRPA1 RNA was clearly detectable, though levels were not as high as the pruritogen receptor levels. Transcripts for the neurotrophic factor receptors TrkA (NTRK1) and Ret were detected, in addition to the GFRα 1-3 co-receptors. Consistent with prior mouse studies which demonstrate no role for persephin-GFRα4 signaling in sensory neurons, RNA sequencing found no GFRα4 transcripts in human DRG.

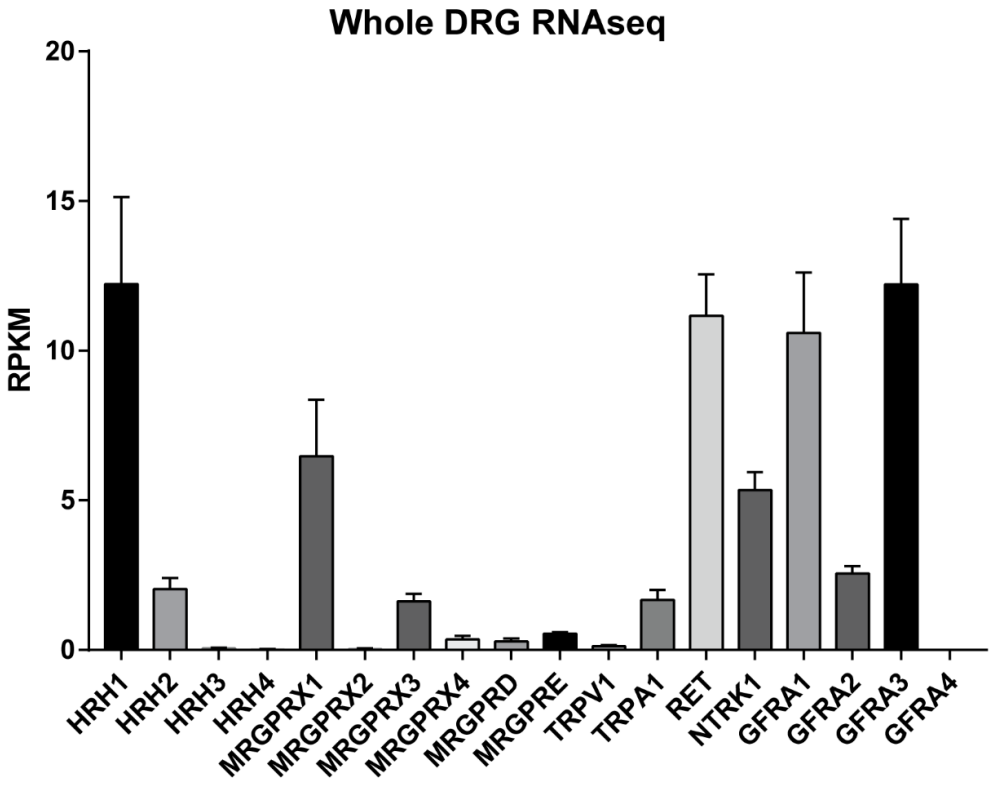

Figure 7. Expression of pruritogen receptors, TRP channels, and growth factor receptor transcripts in hDRG. RPKM values were averaged across individual donors (N=5). Data are presented as mean ± s.e.m.

Discussion

Several recent studies have identified species differences and validated molecular mechanisms using human sensory neurons, but few have provided a detailed description of extraction or culturing methodologies [13-16]. Here, we provide a detailed surgical extraction protocol for post-mortem access to human dorsal root ganglia (hDRG) immediately after organ procurement from consented donors. We demonstrate a culturing protocol that yields primary neurons that are amenable to a variety of functional approaches. In addition, we demonstrate that maintaining adult sensory neurons in culture without growth factors did not have a significant effect on their basic electrophysiological properties. We then demonstrate the distribution of pruriceptive and nociceptive populations in human DRG did not change in the presence of NGF or artemin. Future applications of this protocol have the potential to significantly impact our understanding of human neurophysiology and provide a platform for better translational studies of itch and pain mechanisms.

The extraction protocol we present here addresses several key issues that organ procurement organizations (OPOs) are likely to be concerned with when negotiating a research collaboration. The ventral surgical approach through the abdominal cavity eliminates the need to move the donor during the procedure and preserves structural integrity of the body, thus significantly reducing post-mortem interval for DRG retrieval and resulting in minimal interference with other surgical procedures. Maintaining a sterile intraoperative field during the extraction allows subsequent tissue procurement of bone and skin, ensuring maximal utilization of donor tissues by the OPO. Importantly, this approach maintains the overall integrity of the donor body for funeral purposes, allowing access to all who consent to tissue donation for research without being restricted to donors who opt for cremation.

Recent publications indicate that it may also be possible to culture human sensory neurons in the presence of other types of culture media [13, 14, 16]. Investigators may decide to incorporate the same enzymatic digestion, plating protocol, or culture media they have previously

used in animal model studies if the goal is to make direct comparisons to those prior studies. In such cases, this protocol can provide a positive control to confirm the viability of neurons as investigators adopt the extraction and dissection procedures in their own laboratories. Notable differences between our protocol and those previously described include the specific types of enzymes used (papain versus trypsin and DNase [13], or proprietary enzyme mixture [14, 16]) and the duration of enzymatic digestion (up to 3.5 hours in some studies). The type of culture medium and use of neurotrophic factors has also varied between studies, with several utilizing DMEM/F12-based media and all studies supplementing with varying concentrations of NGF and/or GDNF. The successful culturing of human cells in a variety of different culture media and following different digestion methodologies suggests that individual labs may be able to adapt this protocol. Producing viable cultures of adult human sensory neurons using conditions similar to those used in previous studies of rodent neurons should reduce sources of variation and allow for more direct comparison between rodent and human sensory neurons.

Previous studies demonstrate that NGF and artemin are increased in the lesional skin of patients with pruritic diseases [39-44]. In addition, in chapter 4, we demonstrated that NGF and artemin can increase the proportion of histamine- and chlorquine-sensitive neurons. To determine if the same mechanism exists in human sensory neurons, we tested neuronal calcium responses to pruritogens and algogens after incubation with NGF or artemin. Our data demonstrate that overnight pre-treatment with either NTF did not alter the proportion of histamine- or chloroquine-responsive neurons. There was also no change in the proportion of capsaicin and mustard oil responders. Several factors may contribute to the observed differences between mouse and human responses to NTF treatment. The time spent in culture is longer in human experiments because it was necessary to wait for the glial cells to expose the plasma membrane of neurons to allow reliable calcium imaging recordings. Time in culture can be associated with transcriptional changes that could be a major contributor to the observed differences between mouse and human neurons. Future studies can address this by recording responses in mouse neurons that were

maintained in culture for longer. Alternatively, our results may simply indicate that human and mouse pruriceptive neurons are not modulated by NGF and artemin via the same mechanisms.

Sequencing data from whole DRG tissue obtained from the same donors that provided neurons for functional studies confirm the presence of pruritogen and neurotrophic growth factor receptors. The detection of MRGPRX1 transcripts in hDRG and direct activation of sensory neurons by chloroquine strongly suggest a functional role for MRGPRX1 in human sensory neuron physiology. Despite activation by the same group of agonists, MRGPRX1 is not a true ortholog of the mouse MrgprA3 or MrgprC11 receptors. In heterologous systems, these receptors may engage different downstream mechanisms such as activation of different TRP channels (TRPV1 versus TRPA1) [23]. These fundamental differences in receptor signaling may contribute to the differences we observed in NTF responses of mouse versus human chloroquine-sensitive neurons.

To our knowledge, this study is the first to apply ratiometric calcium imaging in dissociated human DRG cultures to characterize the human subpopulations that respond to pruritogens. The proportion of pruritogen- and algogen-responsive sensory neurons varied across donors, with histamine- and chloroquine-responsive neurons encompassing a larger proportion than what is generally reported in rodents. Most pruriceptive neurons responded to capsaicin, suggesting the possibility of a functional link between pruriceptor GPCR signaling and TRPV1. Future studies utilizing pharmacological interventions such as QX-314 and TRPV1 antagonists could directly test this hypothesis. The specific culturing protocol we have described can produce live adult sensory neurons that are amenable to physiological and biochemical measures and are similar to those studied in our previous publications [16, 19]. Dissociated cultures of human sensory neurons are a valuable resource that enables the precise manipulation of external factors to study neuronal physiology specific to humans and can strengthen future studies of itch and pain mechanisms.

Author Contributions

M.V.V., S.D., B.A.C., and K.D. developed the surgical approach. M.V.V., S.D., B.A.C., and T.D.S. optimized existing rodent culturing protocol to establish viable hDRG cultures. M.V.V., T.D.S., M.Y.P. and B.A.C. performed extractions and generated cultures for studies. B.A.C. and M.V.V. performed immunocytochemistry. B.A.C., M.Y.P., and J.G.M. collected and analyzed electrophysiology data. M.V.V. performed calcium imaging experiments and RNA extraction for RNA sequencing. RNAseq data was obtained in collaboration with Sanjay Jain and GTAC. All authors contributed to writing the Nature Protocol manuscript.

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Chapter 6

Conclusions and Future Directions

The work presented in this dissertation applies a multifaceted approach to address key questions about the molecular mechanisms that underlie the neurobiology of acute and chronic itch. In chapter 2, we utilized global genetic knock outs and acute pharmacological inhibition to characterize the contribution of PKCδ to acute histamine-induced itch. We applied the AEW model of dry skin itch to investigate the anatomical and functional changes associated with persistent itch in chapter 3. Our work identified the specific subset of Ret-positive fibers as likely contributors to dry skin-induced pruritus. In chapter 4 we investigated the effects of neurotrophic factors on pruriceptive responses, and found distinct roles for NGF and artemin the sensitization of histamine-dependent and -independent itch. Finally, in chapter 5 we demonstrated a novel method for the extraction and successful culturing of human sensory neurons, allowing us to perform the first studies to functionally characterize the pruriceptive subpopulations in humans.

PKCδ as an intracellular mediator of itch

In chapter 2, we investigated the role of the specific PKC isoform PKCδ in acute pruritogen-induced itch. Our studies indicate that PKCδ contributes to histamine-evoked scratching behavior, but may be dispensable for non-histaminergic itch induced by the pruritogens chloroquine and β-alanine. In the peripheral nervous system, PKCδ expression was restricted to small diameter sensory neurons, and was found in both peptidergic and nonpeptidergic neurons. Physiological studies of cultured adult DRG using genetic and pharmacological tools demonstrate that PKCδ mediates histamine-induced responses of sensory neurons. In addition, PKCδ may act downstream of the histamine receptor to modulate TRPV1 activity, as there was a significant reduction in capsaicin responses after histamine application. We conclude that PKCδ is involved in the sensory neuron responses that mediate acute histaminergic itch.

Several questions remain about the precise role of PKCδ in histamine-induced itch. First, it is not clear whether PKCδ is engaged directly downstream of histamine receptor signaling or if it plays a more constant modulatory role in modulating or engaging downstream effector proteins

such as TRPV1. Future studies could determine if PKCδ is engaged downstream of histamine receptor activation by studying PKCδ translocation to the membrane. This assay has been validated previously, and can be further strengthened by the use of a PKCδ-KO-validated antibody [1]. The lack of reliable histamine receptor antibody availability can be overcome by combining calcium imaging measurements to identify histamine-responsive neurons with immunocytochemistry to probe for PKCδ translocation at specific time points after histamine activation.

Previous findings from our lab showed that PKCδ deletion had no effect on acute pain, but significantly reduced CFA-induced inflammatory heat hyperalgesia, suggesting a potential function for PKCδ in nociceptor sensitization [2]. The expression of PKCδ in half of TRPV1-positive neurons certainly suggests that it could be a modulator of TRPV1 expression or function. In addition, PKCδ may be involved downstream of PGE_2, NGF, and IL-6 signaling [3-6]. Indeed, NGF-induced ERK phosphorylation was inhibited by the PKCδ pharmacological inhibitor rottlerin [5]. PKCδ has also been implicated in sickle cell-associated pain, paclitaxel-induced hyperalgesia, and NGF-TrkA induced axon growth in osteoarthritis [7-9]. Future studies could investigate whether there is indeed a functional link between PKCδ and inflammation-induced sensitization of TRPV1. Furthermore, PKCδ deletion could be tested in the context of inflammatory itch models such as dry skin and allergic contact dermatitis.

Our studies demonstrate that despite a significant reduction in histamine-evoked itch, histamine responses were not completely inhibited, indicating that a substantial number of histamine-responsive neurons remain functional. This suggests other PKCδ-independent mechanisms also contribute to the signaling of histaminergic itch. These findings support the idea that the histamine-responsive population of sensory neurons is a heterogeneous group of cells, and distinct mechanisms may mediate downstream activation of histamine-dependent signaling cascades. This may be further reflected by the involvement of several histamine receptor subtypes, including H4R, whose role in histamine-induced itch is not clearly defined yet.

PKC activation is a key step in numerous signaling cascades, and several PKC isoforms have been identified in sensory neurons. However, isoform-specific effects on itch and pain remain poorly understood. A number of recent studies demonstrate inflammatory pain is mediated by PKCε, but this is one of the first studies to address the role of a specific PKC isoform in itch [10-19]. Ultimately, because of their extensive expression throughout the body, pharmacological inhibition of PKC isoforms may not provide an optimal approach to treating chronic pain or itch. However, by further dissecting the specific pathways associated with these kinases, we may be able to identify a downstream target that is more specific and amenable to clinical translation.

Pruriceptor changes associated with models of chronic itch

The work presented in chapter 3 demonstrates the specific anatomical and functional changes that are associated with a model of dry skin itch. Dry skin treatment resulted in a significant increase in scratching, but not wiping behavior, indicating that it elicits an itch-specific phenotype. The resulting intraepidermal hyperinnervation was independent of scratching, and included mostly Ret-positive non-peptidergic fibers. Interestingly, there were no changes in basic membrane excitability properties of trigeminal neurons that innervated the treated skin. However, there was an increase in the proportion of chloroquine-responsive trigeminal neurons, indicating de novo expression of pruritogen receptors resulting in an expansion of the pruritic subpopulation of sensory neurons.

The acetone-ether-water (AEW) model is widely used as a model of chronic itch, because it shares several key characteristics with pruritic skin conditions. The skin changes observed in this model include epidermal hyperplasia, parakeratosis, and spongiosis, which are also frequently observed in the lesional skin of patients with atopic dermatitis and psoriasis. Barrier dysfunction is another key feature of this model that is also associated with pruritic skin diseases [20]. However, like xerosis in humans, the dry skin model is not marked by inflammatory infiltration, which is a common feature in most chronic itch conditions. A major question remains

regarding whether Ret+ fibers are also mediators of inflammatory itch. Inflammatory models of chronic itch include ovalbumin hypersensitivity resembling allergic itch, MC903/Vitamin D3 analog treatment which results in an atopic dermatitis-like phenotype, and trinitrochlorobenzene-induced contact dermatitis [21]. Future studies can utilize existing reporter lines such as RetEGFP or MrgprA3-TdTomato, to examine changes in pruritogen receptor expression and physiology under different types of inflammatory itch. These models may also allow a more chronic time scale to be applied, in order to determine the effects of long-term itch (>1-2 weeks) on pruriceptor plasticity and innervation. Identifying the distinct mechanisms or subpopulations of pruriceptors that mediate distinct types of itch could greatly aid in the development of more specific treatments by minimizing the side effects that frequently result from systemic immunomodulatory treatments.

The “itch-scratch cycle” in chronic pruritic conditions is a vicious loop that results in further inflammation and damage to the skin, thereby eliciting even more itch, anxiety, and frustration in patients. The use of Elizabethan collars allowed us to block the pro-inflammatory effects of scratching in order to determine the intrinsic changes that result from the induction of dry skin. Interestingly, epidermal hyperplasia and hyperinnervation still occurred, despite lack of mechanical stimulation and injury at the skin. This suggests that transepidermal water loss directly contributes to the pathological changes observed in the dry skin model, and it is these epidermal changes that likely drive the anatomical and functional effects associated with dry skin itch.

Neurotrophic factors and their effects on itch

In chapter 4, we examined the direct effects of neurotrophic factors on itch sensation and pruriceptor physiology. We found that acutely administered neurotrophic factors do not induce spontaneous itch or pain. Nerve growth factor (NGF) pretreatment selectively potentiated histamine-induced itch, but did not affect chloroquine-evoked scratching responses. On the other hand, artemin pretreatment potentiated chloroquine-induced itch, but appeared to inhibit histamine responses. Our data suggest distinct roles for NGF and artemin in the modulation of

histamine-dependent and histamine-independent itch. To further examine the mechanisms by which these growth factors sensitized pruritogen-induced itch, we tested the effects of NGF and artemin on histamine- and chloroquine- induced calcium responses. Acute 1-hour pre-treatment with NGF did not affect the proportion of histamine-responsive neurons. On the other hand, prolonged overnight incubation significantly increased the proportion of histamine responders, indicating that NGF likely induces de novo expression of histamine receptors in sensory neurons. Artemin increased the proportion of chloroquine-responsive neurons after both acute and overnight pre-treatment. These results indicate that neurotrophic growth factors can sensitize itch responses by upregulating the expression of pruritogen receptors via different mechanisms. Further studies are needed to determine the effects of artemin on histamine responses. Neurotrophic growth factors have long been studied as modulators of pain and several findings indicate NGF and artemin can directly sensitize TRPV1 function and upregulate its expression. Future studies can further dissect the downstream pathways underlying NGF- and artemin-induced sensitization of itch [22]. A better understanding of NTF modulation of pruriceptive responses will ultimately further our understanding of the distinct neurbiological mechanisms that mediate itch versus pain.

A second major concept addressed in the studies presented in Chapter 4 is the role of endogenous growth factor signaling in the maintenance of pruriceptors. In characterizing the *TrkA*^{F592A} mutant, we determined that this single point mutation most likely results in aberrant TrkA receptor function. *TrkA*^{F592A} mutants share several characteristics with TrkA/NGF knock-out phenotypes, including significant hypoalgesia and profound loss of peptidergic intraepidermal fiber innervation [23-26]. However, unlike the full loss of NGF/TrkA signaling, *TrkA*^{F592A} animals survived well into adulthood and appeared to be less severely affected. This suggests that the *TrkA*^{F592A} receptor is most likely hypomorphic, with enough residual function to support the survival of some sensory neurons. Perhaps the most interesting finding from this study is the lack of physiological effect on pruritogen-evoked scratching. In addition, calcium imaging studies

demonstrate histamine- and chloroquine-sensitive neurons are still present in *TrkA*^{F592A} mutants. These results suggest that pruriceptor innervation and receptor expression may not be fully dependent on TrkA/NGF signaling. Further studies are needed to fully characterize which sensory populations are lost in order to begin to understand the loss of function in the *TrkA*^{F592A} receptor.

This study raises another interesting question regarding the dual expression of neurotrophic factor receptors in itch-sensitive neurons. If TrkA signaling is not important for the maintenance of pruriceptive neurons, the next obvious candidate is the receptor tyrosine kinase Ret, which regulates non-peptidergic neurons. Most Mrgpr family of receptors express Ret [27], and the MrgprA3-positive subset of pruriceptors is marked by the expression of both TrkA and Ret [28, 29]. Future studies can conditionally delete Ret in sensory neurons either acutely or during development [30], by taking advantage of the numerous available Cre-recombinase lines. A more specific role in pruriceptor function could be addressed by deleting Ret in MrgprA3-positive neurons under the control of MrgprA3-Cre [28]. Furthermore, Ret deletion could be combined with TrkA deletion (*TrkA*^{F592A} animals also harbor LoxP sites at the TrkA locus), to avoid neurotrophic factor receptor redundancy or compensation. The GFRα-GFL complex associates with Ret to induce phosphorylation of key Ret tyrosine residues, which then serve as docking sites for adaptor proteins such as Src, PLCγ, and Shc to activate downstream signaling cascades including the PI3K and MAPK [31]. Specific Ret tyrosine mutants could be studied to delineate whether specific downstream signaling mechanisms are involved in the maintenance versus sensitization of pruriceptor populations [32].

The work presented in chapter 4 addressed the effects of acutely applied neurotrophic growth factors on histamine- and chloroquine-induced itch. However, as mentioned previously, there are a number of other acute and chronic itch models that can be applied in order to study the role of neurotrophic factors and their receptors in the development or maintenance of chronic pruritus [21]. Understanding the effects of neurotrophic factors on neurophysiology is becoming an even more pressing issue as clinical trials using growth factors or growth factor-modulating

compounds are well underway for the treatment of a number of diseases, including sciatica, osteoarthritis, and Parkinson's disease [33-38]. It is critical that we understand the wide range of effects of neurotrophic factors in order to inform future studies of the potential for clinically relevant adverse effects of new therapies.

Human sensory neurons: a key step toward translation

In chapter 5, we described a protocol we developed for the surgical extraction of human dorsal root ganglia (hDRG) from deceased organ donors. By applying a few key modifications to our mouse sensory neuron culturing protocol, we were able to dissociate and successfully culture adult human sensory neurons. These neurons could be maintained in culture for over 10 days and were amenable to several approaches including immunocytochemistry, RNA sequencing, and even patch-clamp electrophysiology and calcium imaging. The work on this project contributes tremendously to the field of pain and itch, by providing investigators with the means to generate human primary sensory neuron cultures. Many research institutions are located within or affiliated with a local medical center, where organ donation and transplantation takes place on a frequent basis. By providing a clear road map for the extraction process, other researchers could establish their own collaborations with local organ procurement organizations that will grant them access to donor tissue donated for research. Expanding preclinical access to human tissues has the potential to greatly improve the translation of preclinical findings into clinically effective treatments. Furthermore, the validation of key findings in target tissues will save tremendous amounts time and money.

Once we established that culturing without growth factors did not affect the basic electrophysiological properties of human neurons, we proceeded to determine whether we could observe the same effects of NGF and artemin on pruritogen-induced responses *in vitro.* Very few studies have been able to study functional responses of human sensory neurons, and to our knowledge, this is one of the first to characterize the human pruriceptive populations of cultured

hDRG [39-46]. Our findings demonstrate the proportion of histamine-, chloroquine-, mustard oil-, and capsaicin-responsive neurons and characterize the degree of overlap between these functional receptors. The application of NGF and artemin did not change the overall proportion of responsive human neurons, which contrasts with our observations in mouse neurons in chapter 4. It is possible that the mechanisms of pruriceptor sensitization by neurotrophic factors are fundamentally different between species. However, it is also possible that there are inherent differences between the cultures due to the absolute age of human versus mouse neurons or the difference in time *in vitro* prior to recording secondary to the significant glial encapsulation of human neurons.

The protocol we have established for extraction and culturing of human DRG has opened the door to a number of exciting clinically relevant future studies and has resulted in several collaborations with research groups across the country. The extraction procedure itself has allowed us to also obtain spinal cord and intervertebral disc tissue. Future studies can begin to address key questions about the sensitization of nociceptors by intervertebral disc tissue, which can secrete pro-inflammatory cytokines and growth factors that are implicated in chronic low back pain. Spinal cord tissue can be used to investigate the sensory neuron inputs into the dorsal horn while serving as a model for studying synaptic transmission in humans.

We have only begun to characterize the physiology of human sensory neurons. Future studies can start to validate or discover mechanisms behind sensory neuron sensitization by direct treatment with growth factors or other clinically relevant inflammatory compounds [46]. Co-cultures using peripheral tissues such as keratinocytes can be used to build *in vitro* models of disease states to investigate the interactions between sensory neurons and their target tissues. Furthermore, advances in RNA sequencing technologies could allow for the full genomic characterization of the heterogeneous subsets of sensory neurons in human and determine how they differ from model organisms [29]. Transcriptome sequencing techniques can be combined with functional approaches such as calcium imaging and patch-clamp electrophysiology to identify

the transcriptional profiles of functionally-identified subsets of neurons. In conclusion, access to human neurons provides a vast number of opportunities to study normal and pathological neural processing, and will be an invaluable tool for the validation and generation of novel treatments.

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Publications

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