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Abstract

Chronic pain and itch pose ever present, steadily growing burdens to human health. Still, we have limited understanding of the mechanisms that underlie their development and persistence. Furthermore, treatments for these conditions tend to be palliative, rather than curative, leading to mixed patient outcomes. With this in mind, we used next generation sequencing to assemble a transcriptional profile of the molecular changes in skin and sensory neurons that associate with a unique, stochastic mouse model of atopic dermatitis. This model combines the genetic sensitization of a PAR2 overexpression animal with environmental challenge by house dust mite allergens. To our knowledge, this is the first profiling effort that broadened its focus beyond the skin to look at the important, itch-facilitating contribution of sensory neurons. An interesting feature of this PAR2 model is that, by virtue of its stochasticity, it may allow for the independent identification of both protective and deleterious changes. These datasets will serve as useful resources for clinicians and researchers interested in the pathogenesis and prevention of atopic dermatitis.

Among the many genetic changes detected in our analysis was brain-derived neurotrophic factor (BDNF), which is expressed by sensory neurons and has been repeatedly implicated in different pain and itch conditions. Thus, in a parallel series of studies, we investigated the neuronal expression pattern and behavioral contributions of primary afferent-derived BDNF. Contrary to previous reports, we found that BDNF expression within dorsal root ganglia predominates in large-diameter, myelinated neurons. Furthermore, we found little evidence that BDNF contributes significantly to acute or chronic pain, with one notable exception observed in the formalin test of inflammatory pain. The selective deletion of BDNF from primary sensory

neurons markedly reduced nocifensive behaviors during the second phase of the formalin test, which is thought to model tissue injury-induced post-operative pain. Surprisingly, this difference was sexually dimorphic, and only occurred in male mice. However, based on its expression pattern within sensory ganglia and its minimal apparent contribution to pain or itch, we suggest that, in the future, primary afferent-derived BDNF should be studied in the context of low-threshold mechanotransduction.

Table of Contents

List of Tables

Chapter 1

List of Figures

Chapter 1

Chapter 2

Introduction

Sensory neurons are the perceptual interface between the environment and our bodies; they convert a diverse mix of physical and chemical stimuli into electricity, the uniform currency of the nervous system. Across modalities, sensory neurons express specialized receptors in peripheral tissues and organs. Once activated, these neurons transmit electrical impulses to the spinal cord, where sensory information is integrated and relayed to the brain. Ultimately, the brain is where sensations are imbued with the sensory discriminative (what, where) and affective (emotional) qualities that transform sensation into perception. But, as with all complex and dynamic systems, one cannot understand perceptual output without first understanding the sensory input that underlies it. Primary afferent neurons are fascinating cells that initiate pleasure and pain, as well as several intractable disease states. While new technologies have revealed many features about the processing of sensory information by the central nervous system, there is still much to learn about how primary afferent neurons detect and transduce external stimuli. In particular, understanding the fundamental principles of sensory transduction in the healthy individual is critical to the analysis of changes that occur in the setting of injury.

Sensory neuron subtypes and receptors

Broadly speaking, primary afferents neurons are specialized detectors for touch, temperature, and various chemicals, including mustard oil, menthol, and capsaicin. The cell bodies of sensory neurons are located in trigeminal (TRG) and dorsal root ganglia (DRG), with axons that innervate the face and body, respectively. Sensory neurons have a unique, pseudo-unipolar morphology, meaning their peripheral (organs and tissues) and central (spinal cord) terminals are biochemically equivalent and, unlike many neurons, can both transmit and receive messages. In

addition, sensory neurons are finely tuned, and require different amounts of stimulation to be activated. Low-threshold neurons are activated by innocuous, non-painful stimuli, while high-threshold, nociceptive neurons (nociceptors) are activated by intense, often painful input. Primary afferents are further characterized by virtue of their cell diameter, degree of myelination, and electrophysiological properties (Abraira and Ginty, 2013). Aβ-neurons are large- and medium-diameter, myelinated cells that innervate both hairy and glabrous skin and conduct messages very rapidly (though slightly slower than muscle spindle Ia afferents). Aδ-neurons are medium-diameter, myelinated cells that are found primarily in hairy skin and have moderate conduction velocities. C-fibers are small diameter, unmyelinated cells that are found in both hairy and glabrous skin and have the slowest conduction velocity of all sensory afferent types. Broadly speaking, Aδ-neurons and C-fibers comprise the main classes of nociceptors, mediating fast / sharp and slow / dull pain, respectively. However, as every sensory neuron subtype contains both nociceptive and non-nociceptive cells, the determination of whether a given sensory afferent conducts pain messages must be based on the biochemical properties of that particular cell.

One of the defining biochemical properties of primary afferent neurons is the type (or types) of sensory receptors they express. This is because, critically, receptor subtypes are dedicated to detecting specific sensory modalities and intensities. Generally speaking, much more is known about nociceptors and thermoreceptors than low-threshold receptors and mechanoreceptors. All thermosensation is produced by transient receptor potential channels (TRPs), of which mammals express 28 different versions. These are grouped into six subfamilies (TRPC, TRPV, TRPM, TRPA, TRPML, and TRPP; Vriens *et al*, 2014). TRPs are activated by temperature and natural

chemical ligands, including pH, capsaicin, menthol, and eucalyptol. By contrast, due to the great difficulty of isolating and studying mechanoreceptors in heterologous expression systems, relatively little is known about the molecules that transduce touch. One exception is the well-studied family of mas related G-protein coupled receptors (Mrgprs), which includes MrgprD (Delmas *et al*, 2011). Importantly, the recent publication of a molecular structure for the mechanoreceptor Piezo1 suggests that some of the technical issues that have hindered our understanding of mechanoreception may finally be surmountable (Ge *et al*, 2015). Finally, pruritoreceptors, which detect itch, include MrgprC11, MrgprA3, and histamine receptor H1 (LaMotte *et al*, 2014). Interestingly, TRPV1, TRPA1, and TRPV4 have also been shown to play critical roles in itch transduction (Kim *et al*, 2016), although they appear to do so by regulating downstream processes or acting in heteromeric receptor complexes.

Polymodality at the level of sensory neurons and circuits

Despite the apparent specificity for a particular modality at the level of sensory receptors, ample electrophysiological and anatomical evidence suggest that primary afferent neurons often express several different receptor subtypes and can therefore be polymodal (Basbaum *et al*, 2009). As might be expected, many of these polymodal neurons can be activated by both noxious thermal and mechanical stimuli. In other situations, neurons engage different sensory modalities depending on the firing frequency generated by a particular stimulus (Sun *et al*, 2017). For example, polymodal neurons that respond to both itch and pain encode these disparate sensations as a product of their firing intensity, with more intense firing selecting for itch signaling over pain. However, these findings are contradicted by imaging studies, which suggest that most polymodal nociceptors are activated by only one type of sensory input (Emery *et al*, 2016).

Furthermore, the ablation of certain subsets of polymodal nociceptors, such as those expressing TRPV1 or MrgprD, tends to produce modality-specific behavioral defects (Braz *et al*, 2014). Together, these results imply that some sensory signals are carried via modality specific "labeled lines." However, it is currently unknown how this type of specificity could be preserved in the spinal cord, where virtually all projection neurons are polymodal (Braz *et al*, 2014). Indeed, this great convergence of sensory input nonetheless facilitates the processing of vastly different types of sensory stimuli. How this occurs is still a source of great interest and confusion.

One example of the difficulty in distinguishing labeled lines from intensity / population coding is the relationship between pain and itch. Many itch-responsive neurons are polymodal, and express nociceptor channels like TRPV1 (Ikoma *et al*, 2006). For this reason, it is perhaps not surprising that many histamine-responsive neurons respond to algogens, or pain-causing compounds. These observations support the theory that itch coding is differentiated from pain based on the intensity, pattern, or frequency of neuronal firing. On the other hand, some recent studies argue against this idea in favor of labeled-lines (Han *et al*, 2013; Han *et al*, 2014). Specifically, genetic deletion of gastrin-releasing peptide receptor (GRPR) neurons in the spinal cord, or Mrgprs in primary afferents (Liu *et al*, 2009; Liu *et al*, 2011), produces selective deficits in itch but not pain (Sun *et al*, 2007; Sun *et al*, 2009). While it is conceivable that the deletion of primary afferent receptors could influence the firing properties of a given neuron and therefore affect intensity coding, the effects seen with GRPR deletion in the spinal cord are strongly indicative of labeled itch lines. Once again, it is unclear how dedicated itch lines can be preserved after converging onto shared projection neurons in the spinal cord. Indeed, with little exception (Han *et al*, 1998), projection neurons appear to receive a convergent input from pain and itch lines.

Pain processing in the brain

As would be expected from the sensory convergence seen at the level of projection neurons, brain circuits mediating pain are shared across modalities. The brain does not have a pain center, per se, because noxious sensory input is distributed across a variety of brain regions, including somatosensory cortex, anterior cingulate cortex (ACC), insula, thalamus, and amygdala, among others (Bushnell *et al*, 2013). These distributed regions mediate unique aspects of the pain response. For example, somatosensory cortex is important for encoding the sensory discriminative features of pain, including location, intensity, and duration. By contrast, the ACC and insula are considered to be part of the emotional response to pain that generates its unpleasantness. In this regard, it is important to recognize that pain is a perception, not a sensation. This is to say, beyond detecting noxious stimuli, pain generates an affective, motivational state that promotes protective behaviors. Interestingly, the pain experience varies depending on one's attentional and motivational state. Pain, therefore, is not an objective experience, but a subjective one that is affected by primary afferent input, spinal cord integration, and brain processing.

Chronic pain and itch

The pain experience also changes in the setting of disease or injury, adding another level of complexity that can facilitate chronic and neuropathic pain states. In these conditions, debilitating pain lingers and becomes chronic, even permanent. For example, neuropathic pain persists long after the body appears to have recovered from any initial tissue injury. There are various mechanisms that lead to chronic pain, all of which are of great clinical interest. Peripheral mechanisms mostly revolve around inflammation, which causes the release of a huge

variety of chemical mediators that alter neuronal function. These chemicals include neurotransmitters, cytokines, proteases, lipids, and peptides (Basbaum *et al*, 2009). While their effects are varied, these components of the so-called "inflammatory soup" can alter gene expression in sensory neurons and increase nociceptor sensitivity, leading to elevated neuronal excitability. Most importantly, through this process of peripheral sensitization, a nociceptor that normally responds only to intense, painful stimuli, may start responding to innocuous stimuli as well. In this situation, a non-noxious stimulus will provoke pain (a condition referred to as allodynia). There are also several central mechanisms of persistent pain, often referred to collectively as central sensitization. NMDA receptor-mediated sensitization has been implicated as a spinal correlate of hippocampal long-term potentiation, which can facilitate and enhance the strength of nociceptive inputs, as well as allow innocuous stimuli to engage and activate central pain circuitry. In addition, certain pain states feature a loss of GABAergic and glycinergic tone in spinal cord interneurons, increasing the overall excitability of nociceptive circuits. Finally, spinal cord microglia are activated after nerve injury and, although the mechanism is still controversial, they can enhance nociceptive drive via the release of facilitatory signaling molecules (Basbaum *et al*, 2009).

One of the compounds implicated in microglia-mediated sensitization is the small peptide, brain-derived neurotrophic factor (BDNF). Historically, BDNF has been studied in the context of its growth factor activity, as well the as role it plays in promoting hippocampal long-term potentiation. After injury, BDNF release by microglia has been proposed to modify the excitability of spinal cord neurons, so that inhibitory neurotransmitters become excitatory, facilitating pain signaling (Coull *et al*, 2005). Interestingly, BDNF is also expressed by primary

afferent neurons (Michael *et al*, 1997; Thompson *et al*; 1999 Luo *et al*, 2001; Obata *et al*, 2006) with various studies suggesting that it regulates acute (Pezet *et al*, 2002) and chronic pain processing (Smith, 2014). As BDNF expression increases dramatically in the setting of neuropathic (Zhou *et al*, 1999; Fukuoka *et al*, 2001; Obata *et al*, 2006) and inflammatory injury (Cho *et al*, 1997; Lin *et al*, 2011), most of the research looking at BDNF in the context of pain has focused on its role in mediating persistent pain. Various studies have shown that inhibiting BDNF signaling can reduce hypersensitivity and allodynia after injury (Fukuoka *et al*, 2001; Groth *et al*, 2002; Zhao *et al*, 2006; Bao *et al*, 2014; Luo *et al*, 2016). Unfortunately, it has been difficult to accurately characterize BDNF expression within sensory ganglia, leading to some confusion about how it might mediate these different aspects of the pain experience. Specifically, anatomical studies of primary afferent-derived BDNF have been limited by the difficulty of verifying BDNF antibodies, while behavioral studies have been hindered by a lack of specific molecular and genetic tools.

As itch and pain share similar and indeed interacting circuitry, it is perhaps expected that itch can also become chronic in the setting of disease. As with persistent pain, chronic itch is precipitated by both peripheral and central mechanisms. And like persistent pain, chronic itch conditions pose an enormous, unmet clinical need (Grundmann and Ständer, 2011). In certain skin conditions with intense scratching, sensory nerve innervation is increased in pruritic skin. In addition, these nerve fibers tend to express elevated levels of itch-sensing receptors like proteinase-activated receptor 2 (PAR2). Finally, compounds such as nerve growth factor (NGF), which is upregulated in the setting of skin conditions (e.g., atopic dermatitis and psoriasis), also increase the excitability of primary afferent neurons that encode itch (Han and Dong, 2014). Together, these

observations suggest that, in the setting of chronic itch, pruritic skin is highly innervated by overly excitable pruitoreceptors. However, central sensitization also appears to be a factor in chronic itch condition; it can result from increased expression of itch-specific peptides and receptors (like GRP and GRPR) in dorsal horn interneurons, or by facilitation of spinal circuits that increase the response to itch-producing compounds. It should be noted that, in the setting of pathological itch, noxious stimuli and repetitive scratching, which normally inhibit itch, actually induce pruritus. Furthermore, in the setting of dermatitis or local inflammation, light tactile stimulation can actually produce itch in a condition known as alloknesis (LaMotte, 2007). These findings suggest that, just like pain circuits, itch wiring might be fundamentally altered in pathological settings.

PAR2 is one of many molecules implicated in chronic itch. It is expressed by both primary afferent neurons and skin keratinocytes, where expression is increased in patients with atopic dermatitis (Steinhoff *et al*, 2003). Interestingly, PAR2 has also been shown to mediate a scratch-producing circuit between skin cells and neurons. Specifically, activation of PAR2 on keratinocytes evokes the release of thymic stromal lymphopoietin (TSLP), which in turn, acts directly on C-fiber neurons to provoke scratching (Wilson *et al*, 2013). In a model of bone cancer pain, PAR2 activation also upregulates BDNF expression in the spinal dorsal horn, leading to the facilitation of nociceptive pathways and central sensitization (Bao *et al*, 2014). Therefore, one might speculate whether the activation of PAR2 in many chronic itch conditions is capable of sensitizing itch pathways to provoke chronic pruritus. However, most studies of chronic itch have focused on skin cells, even though it is primary afferents that produce the drive for pruritus.

In other words, itch, like pain, is a perception triggered by activity of the nervous system and its study and clinical management should be tailored accordingly.

Summary

In the following chapters, we address the issue of primary afferent-derived BDNF in the context of its role in generating itch and acute and chronic pain. In addition, we perform what is, to our knowledge, the first, unbiased anatomical characterization of BDNF expression in sensory neurons. In a parallel study, we conduct a detailed transcriptional analysis of nerve and skin in a unique mouse model of atopic dermatitis that allows for the isolation of both protective and deleterious genetic changes. The datasets generated by this analysis provide a fresh perspective on the myriad of genetic changes that underlie chronic itch conditions, and offer useful resources for researchers and clinicians interested in identifying novel clinical targets to improve patient outcomes.

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Chapter 1: Transcriptional profiling of skin and sensory neurons from symptomatic and asymptomatic mice in a stochastic model of atopic dermatitis

Chapter 1: Abstract

Chronic pruritus, which includes conditions like atopic dermatitis and psoriasis, poses an enormous and largely unmet clinical burden. Although the signals that trigger itch in the skin are transmitted by primary afferent neurons, genomic studies rarely focus on the neuronal underpinnings of chronic itch. In this study, we generated several transcriptional profiles of skin and primary sensory neurons in a stochastic model of atopic dermatitis produced by treating genetically sensitized mice with environmental allergens. By grouping mice based on their scratching and skin pathology, we identified three distinct phenotypes that, we propose, model different aspects of the disease. Not only did we detect increased expression of many genes that likely facilitate atopic dermatitis, we also found many significant decreases that may exert a preventative or protective role. In addition, we used qRT-PCR to validate many of the transcripts found by RNA-seq, and interestingly, found that these genes were comparably altered in a different mouse model of atopic dermatitis. These datasets will serve as useful resources for researchers and clinicians interested in the pathogenesis and prevention of atopic dermatitis, especially with regard to the role of primary sensory neurons.

Chapter 1: Introduction

As life expectancies rise, non-fatal diseases pose an ever-increasing burden to world health. Among these diseases, skin conditions rank fourth in years lived with disability, which is a measure of the number of years individuals cope with a particular illness (Vos *et al*, 2012). Of all skin conditions, atopic dermatitis (AD; also known as eczema) ranks first in disease-adjusted life years, which accounts for how many years of life are lost due to disability (Hay *et al*, 2014). AD is a chronic, relapsing, highly pruritic skin condition for which we have only a basic mechanistic understanding (Bieber, 2008). More research into its underlying pathophysiology could dramatically reduce the social and financial cost that AD levies on patients, families, and the healthcare system (Drucker *et al*, 2017).

The diagnosis of AD is based on symptomatic hallmarks that vary with age (Watson and Kapur, 2011). These symptoms include inflamed, intensely-pruritic, and potentially lesional skin that provokes severe itch, which persists throughout the day and worsens at night. Although estimates vary, between 15 to 30% of children and 2 to 10% of adults are affected, with rates steadily growing in industrialized nations (Williams and Flohr, 2006). AD is heritable (Dold *et al*, 1992), but variation between monozygotic twins suggests that environmental factors have a strong influence on whether the disease manifests (Strachan *et al*, 2001). Interestingly, AD often precedes the development of other atopic disorders, including allergic rhinitis and asthma (Akdis *et al*, 2006), suggesting that AD involves pathological mechanisms that could be relevant for treating a variety of conditions.

Although many of the mechanistic details are still poorly understood, AD occurs when the skin barrier is impaired by chronic inflammation. It is unclear whether the inflammation originates from genetic abnormalities in the immune system, skin barrier, or both (Elias *et al*, 2008). Regardless, once the skin is inflamed and pruritic, scratching and water-loss further disrupt the skin barrier, allowing for increased penetration by high molecular weight allergens. These allergens, in turn, activate T cells, which produce cytokines (notably IL-4, IL-5, IL-12, IL-13, and interferon-γ) that feedback to exacerbate the inflammation and pruritus (Bieber, 2008). Although AD typically begins without an IgE response (Illi *et al*, 2004), dendritic cells eventually react to chronic inflammation by generating IgE antibodies against allergens (Bieber, 2007) and resident skin cells (Mothes *et al*, 2005). The response against allergens can sensitize to subsequent bouts of inflammation, while the response against resident skin skills can provoke a generalized, or "atopic" inflammation that occurs outside of areas with direct allergen contact. In rare cases, some patients suffer from a "non-allergic" form of AD, in which atopy occurs in the absence of an IgE response. However, this group is in the minority and often goes on to develop an IgE response later in life (Novak and Bieber, 2003).

Though understood to have a strong genetic component, the regulation of genes in AD is complicated and poorly understood (Barnes, 2010). AD was first thought to result from defects in a small number of genes; however, current studies suggest that AD is actually the product of reciprocal interactions between many genes (Margolis *et al*, 2014). With this genetic complexity in mind, several studies have used microarray or next generation sequencing to survey transcriptional changes in atopic human skin (Guttman-Yassky *et al*, 2009; Cole *et al*, 2014; Suárez-Fariñas *et al*, 2015; Bin and Leung, 2016). Nevertheless, these studies have all focused

on patients with active cases of AD, as it is very difficult to predict *a priori* whether genetically predisposed individuals will actually go on to develop the condition. While it is unquestionably of interest to survey the transcriptional profile of atopic skin, a better understanding of the factors that prevent genetically sensitized individuals from developing AD would provide critical information on molecular changes that might ameliorate or prevent the condition. To this end, in the present study we performed RNA-seq with a sensitized but stochastic animal model of AD, where, despite treatment with environmental allergens on a genetically predisposed background, a certain percentage of animals never developed the condition.

Animal models of AD generally fall into one of three categories: environmental, genetic or spontaneous (Jin *et al*, 2009). Environmental models rely on repeated treatment with immunological sensitizers, including ovalbumin (Spergel *et al*, 1998), house dust mite protease (Huang *et al,* 2003), haptens (Man *et al*, 2008), and food mixed with cholera toxin adjuvant (Li *et al*, 2001). Genetic models are numerous, and mostly based on the overexpression of cytokines and peptides, including IL-4 (Chan *et al*, 2001), IL-31 (Dillon *et al*, 2004), and thymic stromal lymphopoietin (TSLP; Yoo *et al*, 2005). Various enzymatic mouse knockout lines that produce an AD-like condition have also been described. Finally, the Nc/Nga inbred mouse strain spontaneously develops a condition closely resembling AD, with many of the same immunological hallmarks (Matsuda *et al*, 1997). Unquestionably, these models have led to great progress in understanding the pathophysiology of AD, but they lack the same defined, genetic defect in skin barrier function that likely predisposes many humans to the condition (Vasilopoulos *et al*, 2004; Morar *et al*, 2007; Söderhäll *et al*, 2007).

One possible link between skin barrier defects, inflammation, and pruritus is proteinase-activated receptor 2 (PAR2). Protease-activated receptors are expressed in a variety of tissues and serve many functions, including in the skin where PAR2 regulates the response to environmental allergens (Déry *et al*, 1998). Interestingly, mice that overexpress PAR2 in keratinocytes have many of the hallmarks of AD, including skin hyperplasia, lesions, and immune activation (Frateschi *et al*, 2011). Conversely, ablating PAR2 completely restores the skin barrier in a transgenic mouse model that is sensitized to barrier defects (Frateschi *et al*, 2011). In addition to maintaining skin integrity, PAR2 provides a link between skin, nerve, and immune cells. For example, upon activation by PAR2, keratinocytes release TSLP, a potent pruritogen, which acts directly on peripheral sensory nerve terminals in the skin to produce scratching (Wilson *et al*, 2013). As scratching disrupts the permeability-barrier function of the skin and generates inflammation, it is critical to the development of dermatitis (Shiratori-Hayashi *et al*, 2015). Interestingly, PAR2-TSLP signaling also contributes to the regulation of inflammation in Netherton syndrome, a genetic skin condition characterized by severe pruritus and atopic lesions (Briot *et al*, 2009). Furthermore, elevated levels of PAR2 (Steinhoff *et al*, 2003) and TSLP (Soumelis *et al*, 2002) have been detected in the skin of patients with AD. Together, these data suggest that PAR2 plays an important role in disrupting skin barrier function and regulating downstream neural and immunological processes in AD.

Given the prominent regulatory function of PAR2 in many of the processes that underlie AD, in the present report we modeled the disease using a mouse line that overexpresses PAR2 in keratinocytes (*Grhl3*$^{PAR2/+}$; Frateschi *et al*, 2011). Although overexpression of PAR2 can produce spontaneous scratching, skin lesions, and hyperplasia, which collectively resemble AD, we

observed these features in only a very small percentage of naïve *Grhl3*$^{PAR2/+}$ mice. Therefore, to provoke inflammation and immune activation in the skin, we treated the cheek of *Grhl3*$^{PAR2/+}$ mice for 9 weeks with an ointment that contains house dust mite (HDM) proteases, which activate PAR2 (Jeong *et al*, 2008). After treatment, mice were evaluated for skin health and monitored for scratching. Interestingly, slightly less than half of the mice developed an AD-like condition; a comparable number behaved normally and lacked any obvious skin defects. We performed RNA-seq on skin and trigeminal ganglia (TRG, which contain the cell bodies of primary afferent neurons) from both of these groups, as well as from untreated *Grhl3*$^{PAR2/+}$ and wild type animals as controls. To our knowledge, this is the first comprehensive RNA-seq analysis of neurons and skin in a model of AD, and the first time that an animal model has allowed for the study of a cohort that, despite being genetically and environmentally sensitized to the condition, does not develop it. This transcriptional profile provides a valuable resource for those interested in studying the molecular mechanics of AD.

Chapter 1: Results

HDM-treatment produces an AD-like phenotype in *Grhl3*$^{PAR2/+}$ mice

To induce this model of PAR2-mediated AD, *Grhl3*$^{PAR2/+}$ mice were treated twice per week for nine weeks with an extract containing concentrated HDM-proteases. We restricted treatment to one side of the face so that the untreated, contralateral tissue could be used as an internal control. Because the HDM protocol requires pretreatment with SDS to disrupt the skin barrier, we also included control groups in which wild type and *Grhl3*$^{PAR2/+}$ mice were treated with SDS followed by Vaseline. After nine weeks, 40% of the HDM-treated mice exhibited severe spontaneous scratching behavior that was restricted to the HDM-treated side (Figure 1A). All of these mice also displayed many of hallmarks of atopic skin, including erythema, dryness, edema, and excoriation on the treated side (Figure 1B). Mice with both scratching behavior and skin pathology were designed as "responders." Interestingly, 40% of the HDM-treated mice never developed any skin pathology or elevated scratching behavior, and were thus designated "non-responders." The remaining 20% of the HDM-treated mice showed elevated scratching behavior, but without skin pathology, and were designated as "pre-responders." As it was unclear whether this final group would eventually develop any of the hallmark skin pathology of AD, they were excluded from the present analysis. Importantly, none of the Vaseline-treated mice developed any elevated scratching behavior or skin pathology over 9 weeks, suggesting that the AD-phenotype requires both genetic sensitization and environmental exposure to HDM-allergens.

RNA-seq of HDM- and Vaseline-treated animals

We performed RNA-seq on ipsilateral and contralateral skin and TRGs from a variety of different treatment conditions. Samples included naïve (fully untreated) wild type mice, as well

as naïve, Vaseline-treated, HDM-treated responder and non-responder *Grhl3*$^{PAR2/+}$ mice. For all libraries, the input RNA was of high quality and the final, filtered sequencing depth was between 50-90 million reads (Table 1). Our initial focus followed an examination of genes that were differentially expressed between contralateral and ipsilateral tissues from responder and non-responder mice, where we found varying effect sizes across treatments and tissue types (Figure 2). Of particular note is that we detected only 50 differentially expressed genes in the TRGs of responder animals (Figure 2A). The majority (82%) of these changes were from increased expression, while few decreases in gene expression were observed. On the other hand, 2610 genes were altered in the TRGs of non-responder animals (Figure 2C), and interestingly, almost all of these genes (99%) were down-regulated. In skin, responder animals showed over 8000 differentially expressed genes (Figure 2B), which consisted of a heterogeneous mix of increases and decreases. Conversely, non-responder skin displayed slightly over 1000 changes (Figure 2D), but as with the TRGs from these animals, these changes tended to be decreases.

Top sequencing hits

Closer inspection of RNA-seq results showed a high degree of functional heterogeneity among the top 10 sequencing hits for each condition (Table 2). The average total magnitude of all changes from responder and non-responder datasets for skin and TRG was similar (between 1.5 and 2.6 fold, data not shown). However, the magnitude of the fold changes for the top 10 hits in each dataset varied greatly between skin and TRG. In general, top changes in the TRG were smaller, with a few notable exceptions in the non-responder group. For example, the largest change from responder TRG was 6.5 fold, while non-responder TRG had three changes that were several hundred- to almost a thousand-fold. By comparison, top changes in the skin were very

robust and almost always over 100-fold. In TRG, the top hits included secreted peptides, neurotrophic factors, G-proteins and other signaling molecules. In skin, changes included many structural proteins, protease inhibitors and antibacterial response proteins. For example, responder TRGs displayed elevated brain-derived neurotrophic factor (BDNF), which has been associated with AD in humans (Raap *et al*, 2005) and is a known mediator of PAR2-induced scratching (Wilson *et al*, 2013). In addition, responder skin had highly elevated levels of defensin beta 3 (Defb3), which is part of a host defense gene family that has been repeatedly implicated in AD (Bin and Leung, 2016).

Validation of RNA-seq results

We used qRT-PCR to validate several of the significant changes from the HDM-responder group. For 5/7 transcripts in TRG, the fold changes calculated by qPCR were not statistically different than those obtained by RNA-seq, showing good agreement between the two datasets (Figure 3A). For 2/7 transcripts, the values obtained by qPCR were statistically larger than those predicted by RNA-seq. Since qPCR is a more accurate measure of transcript abundance, this suggests that RNA-seq succeeded at detecting transcripts that changed in the setting of AD, but that the predicted magnitude of these changes needs to be confirmed by an accurate, quantitative method. We observed a similar pattern in skin samples, where 7/8 transcripts matched between qPCR and RNA-seq (Figure 3B-C). For the one transcript where there was variation between the datasets, qPCR showed that this transcript was still significantly elevated compared to baseline. To compare our results to another model of AD, we performed qPCR on lesional neck skin and cervical dorsal root ganglion (DRG) samples from IL-31 overexpression mice, which overexpress a pro-inflammatory cytokine in leukocytes and exhibit spontaneous skin and itch

phenotypes that closely resemble AD (Dillon *et al*, 2004; Cevikbas *et al*, 2017). Importantly, all PAR2 transcripts verified by qPCR also changed significantly in the IL31 animals compared to matched tissue from non-lesional control animals. Even more impressively, these changes were statistically similar to those exhibited by HDM-responders. Together, these data suggest that the RNA-seq datasets are accurate and likely relevant to multiple models of AD.

Chapter 1: Discussion

Understanding the mechanisms that underlie AD is an important step in developing new approaches to management. In the present study, we treated *Grhl3*$^{PAR2/+}$ mice with HDM allergens, which produces a model that has many of the classic hallmarks of AD, including intense pruritus, immune activation (Frateschi *et al*, 2011), erythema, dryness, edema, and excoriation. By restricting the HDM-treatment to one side of the face, we were able to use contralateral tissue as an internal control for transcript expression levels. Interestingly and somewhat unexpectedly, the development of the AD phenotype was stochastic, with only some mice developing scratching behavior and skin damage. For this reason, after nine weeks of treatment, we classified the animals into three distinct groups based on scratching behavior and skin phenotype: non-responders, pre-responders, and responders. We then used RNA-seq analysis of skin and TRG samples from responder and non-responder animals to identify genes that associate with AD.

The datasets for responders and non-responders feature an abundance of differentially expressed transcripts, many of which have previously been implicated in AD. For example, we found changes in Ilr4a (Namkung *et al*, 2011; Tanaka *et al*, 2012; Miyake *et al*, 2013; Hussein *et al*, 2014; Gharagozlou *et al*, 2015; Hussein *et al*, 2016; Narożna *et al*, 2016), Il13r (Hussein *et al*, 2011; Namkung *et al*, 2011), Tlr2 (Oh *et al*, 2009; Potaczek *et al*, 2011), IL18 (Ibrahim *et al*, 2012; Trzeciak *et al*, 2016), TNFα (Behniafard *et al*, 2012; Babić *et al*, 2016), and Hrnr (Knüppel *et al, 2012;* TrzeciakI *et al*, 2016). To verify the RNA-seq results, we submitted several transcriptional changes to analysis by qRT-PCR. In most cases (12/15), the magnitude of change was comparable between RNA-seq and qPCR. For the few instances in which qPCR data

differed from RNA-seq, qPCR showed that these transcripts were nonetheless significantly elevated in ipsilateral tissue compared to contralateral control. Thus, it appears that the transcriptional profiles generated by RNA-seq more accurately predict the presence of differential expression patterns rather than their exact magnitude. We appreciate that, a priori, one cannot determine the extent to which a particular change contributes to the pathogenesis of atopic dermatitis. However, the profiles that we defined are unquestionably accurate and provide an extensive and very useful resource for future studies of the molecular changes that occur in AD.

An interesting characteristic of the PAR2 model is that, as for human AD, there is stochasticity in disease formation, where variability occurs even on a genetically sensitized background (Strachan *et al*, 2001). After HDM treatment, 40% of mice fell into the responder category (scratching with skin abnormalities), 40% were non-responders (neither scratching nor skin abnormalities), and 20% were pre-responders (scratching in the absence of skin abnormalities). Both skin and TRG from non-responder animals displayed very large numbers of differentially expressed genes. Most provocative is our finding that of the 2610 genes that changed in non-responder TRGs, 99% had decreased expression levels compared to contralateral tissue. To our knowledge, this type of extreme asymmetry has not been previously reported in sequencing profiles of skin diseases and suggests that the absence of an atopic phenotype in non-responder animals may have resulted from active, compensatory regulation by cells in the TRG. Gene ontology analysis revealed that many of the down-regulated genes affect T- and B-cell activation, chemokine-mediated inflammation, and histamine, serotonin, interleukin, and toll-receptor signaling. Perhaps unsurprisingly, all of these genes have been implicated in itch-

relevant pathways. An alternative hypothesis to protective down-regulation is that the disease simply develops more slowly in non-responder animals, and that the observed changes represent a nascent stage of atopic dermatitis. However, as none of the genes implicated in early, pre-symptomatic atopic dermatitis were altered in the non-responder group, we do not favor this hypothesis (Prescott *et al*, 2008; Belderbos *et al*, 2012). One final caveat is that although primary afferent neurons comprise a major cell type in the TRG, sensory ganglia also contain satellite cells and resident macrophages cells that could influence RNA-seq results.

Based on our results, we suggest that the pre-responder condition, which was not sequenced in this study, might serve as the best model for those interested in the early stages of atopic dermatitis, before the skin barrier is broken down and persistent immune activation occurs. Crucially, molecular changes that precede skin barrier destruction are probably free of compensatory healing processes that intervene after the skin barrier is damaged in responder animals. These changes complicate the transcriptional profiles of responder animals, which appear to include a mix of deleterious and restorative genes. Conversely, one of the valuable attributes of the responder group is that healing processes can themselves become damaging and pathogenic in the setting of chronic inflammation or atopic dermatitis, further reinforcing the disease (Segre, 2006). For example, skin barrier disruption provokes the release of TNF-α, IFN-γ, IL-1, and GM-CSF, which stimulate both keratinocyte proliferation and local inflammation. Thus, one of the most unique aspects of the PAR2 model is that it can provide insight not only into the development and persistence of AD, but into its prevention as well.

Beyond representing different stages of skin disease, these datasets are unique in that they contain analyses of sensory ganglia. It is critical that we gain a greater understanding of the neuronal changes that underlie AD for several reasons. First, scratching is produced by neuronal signaling and required for the development and maintenance of dermatitis (Shiratori-Hayashi *et al*, 2015). Indeed, keratinocytes respond to PAR2-activation by releasing TSLP directly onto neurons to produce itch (Wilson *et al*, 2013). Second, DRG neurons regulate the function of immune cells in the spinal cord and periphery to modulate inflammation (Pinho-Ribeiro *et al,* 2017). These observations support the view that skin, nerve, and immune cells have an active, pathological dialogue in the setting of AD, and that breaking this communication at any level might be sufficient to ameliorate the disease. Finally, as pruritus is one of the most intense and debilitating symptoms of AD, there is high clinical demand for therapeutic options that specifically target itch (Hong *et al*, 2011; Yarbrough *et al*, 2013). While many studies have evaluated the transcriptional profile of atopic skin, none have used RNA-seq or microarray to study DRG neurons associated with itch (Morita *et al*, 2015; Liu *et al*, 2016; Stantcheva *et al*, 2016). To our knowledge, this report is the first to generate a transcriptional profile of sensory ganglia in a model of AD. By publishing these profiles, it is our intention to offer clinically relevant datasets that will engage researchers and clinicians interested in understanding, treating, and preventing this disease.

Chapter 1: Methods

Animals

Animal experiments were approved by the UCSF Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory animals. *Ghrl3*$^{PAR2/+}$ mice were generously provided by Dr. Shaun Coughlin at UCSF.

House dust mice (HDM)-treatment

The right cheek of each mouse was shaved with an electric razor prior to every application of HDM ointment. We first applied a detergent (SDS, 4%) to the exposed cheek under isoflurane (2%), after which the mice were returned to their home cages for 2 hours. The mice were briefly reanesthetized with isoflurane, and HDM extract (Biostir AD; Biostir, Kobe, Japan) was then generously applied to the exposed cheek. The extract was applied twice per week for 9 weeks. For vehicle control, we treated *Ghrl3*$^{PAR2/+}$ mice with SDS followed by Vaseline in place of HDM-extract. For genetic control, we used naïve *Ghrl3*$^{PAR2/+}$ or wild type C57BL/6J mice from The Jackson Laboratory.

Scratch quantification and responder designation

To document spontaneous scratching, we habituated mice for 1 hour in plastic cylinders and then recorded video of scratching behavior for the next 30 minutes. Hindpaw scratching was quantified as the total number of discrete scratching bouts throughout the recording. Animals were deemed "responders" when they showed: 1) A significant increase in scratching behavior compared to their own behavior at baseline; 2) Some combination of skin erythema, dryness, edema, and excoriation. HDM-treated mice that showed neither elevated scratching nor skin

pathology were designated non-responders, while those that demonstrated significantly elevated scratching without skin pathology were designated pre-responders and excluded from this analysis.

RNA-seq

Mice were anesthetized and transcardially perfused with saline (10 mL). Skin and trigeminal ganglia both ipsilateral and contralateral to the HDM-treated cheek were removed. As intact skin samples are difficult to homogenize, this tissue was flash frozen and cut into 50 μm sections on a cryostat prior to RNA extraction. All samples were then homogenized in TRIzol (Ambion) and purified using the PureLink RNA Mini Kit (Ambion) with on-column DNAse treatment. Sample quality was measured using the RNA 6000 Pico Kit (Agilent); skin and TRG samples had average RINs of 9.4 and 8.6, respectively. cDNA libraries were prepared using the Ovation Mouse RNA-Seq System (Nugen). Sequencing was performed on the Illumina HiSeq 2500 system in rapid run, paired-end, 2x100bp mode. Galaxy was used to generate FPKM (fragments per kilobase of exon per million reads mapped) values for each library, and differential expression analysis was conducted using the Cufflinks Suite. Only concordant alignments with Q > 20 were used to calculate differential expression.

RNA preparation from IL31-overexpression animals

Lesional neck skin and DRG samples from IL31 mice were prepared using the same RNA extraction protocol as for RNA-seq. Due to the topographic nature of spontaneous IL31 lesions, skin was removed from the nape of the neck and corresponding cervical DRGs. As lesions were bilateral, neck skin and cervical DRGs from wild type littermates were used as controls.

qPCR confirmation of RNA-Seq hits

RNA was prepared as described above. cDNA was generated using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). mRNA levels were quantified with the Bio-Rad CFX Connect System using PowerUp SYBR Green Master Mix (Applied Biosystems). Melting curves were generated to ensure the specificity of all primers. Transcripts were normalized to actin expression.

Primers

The primers used for qRT-PCR and *Ghrl3*$^{PAR2/+}$ genotyping were as follows:

Allele	**Forward Primer (5' – 3')**	**Reverse Primer (5' – 3')**
9130204L05Rik	GGGTGGCTCTTCTCCTTTGTA	AAAGGTGGGCAGAACTGCTT
Actb	GCCTTCCTTCTTGGGTATGGAA	CAGCTCAGTAACAGTCCGCC
Angptl2	CAGGAGAGAAGAGGCTTTCAGT	TTCATGTTGCGGCTCTCCTT
Bdnf	GACGACATCACTGGCTGACA	ATTGCGAGTTCCAGTGCCTT
Cma1	CACGGAGTGCATACCACACT	GAACCTTCTGGAAGCTCAGGG
Defb8	ATTTCTCCTGGTGCTGCTGTG	GCAGCATTTGAAAGGAGATCC
Ghrl3$^{PAR2/+}$	CACCCCCTCAGCTAAGAAGGAA	CTGGGTTTCCAATCTGCCAATAAG
Il1b	TGCCACCTTTTGACAGTGATG	AAGGTCCACGGGAAAGACAC
Il4ra	TTACTATACACACGCCGAGCC	ATGCCAGGACCCTTCTCTCT
Klk7	GGGGTGCTGGTGGACAAATA	GAGGGAAAGGTCACGTCTGG
Nptx2	AATAGGGCCTCTCCCTCGTT	CGGGGGAAATACTCGATGGG
Npy1r	CGTTCCCTGCTAGGCATCAT	AGGGACCTGTTTTGCCACTT
Ptgds2	CACTCTATCACTGGCACCCC	TTGGCACATTTCTTCCCCCA
Spink12	AGCAGGTGCCTTTCTGCTTT	AGAATGCACAGCGGTTTTGG
Tmem79	AGCTCCTTTCCGGAGATCCT	CAAGGAGCCCGAGTACGATG
Trpa1	CTCCATGGGATGACCCCTCT	AGAACCACTTCCTTGCGCTT
Vgf	CATCGCTCATACTCCAGCCA	GGGCTCTCCAGATTGACTCG

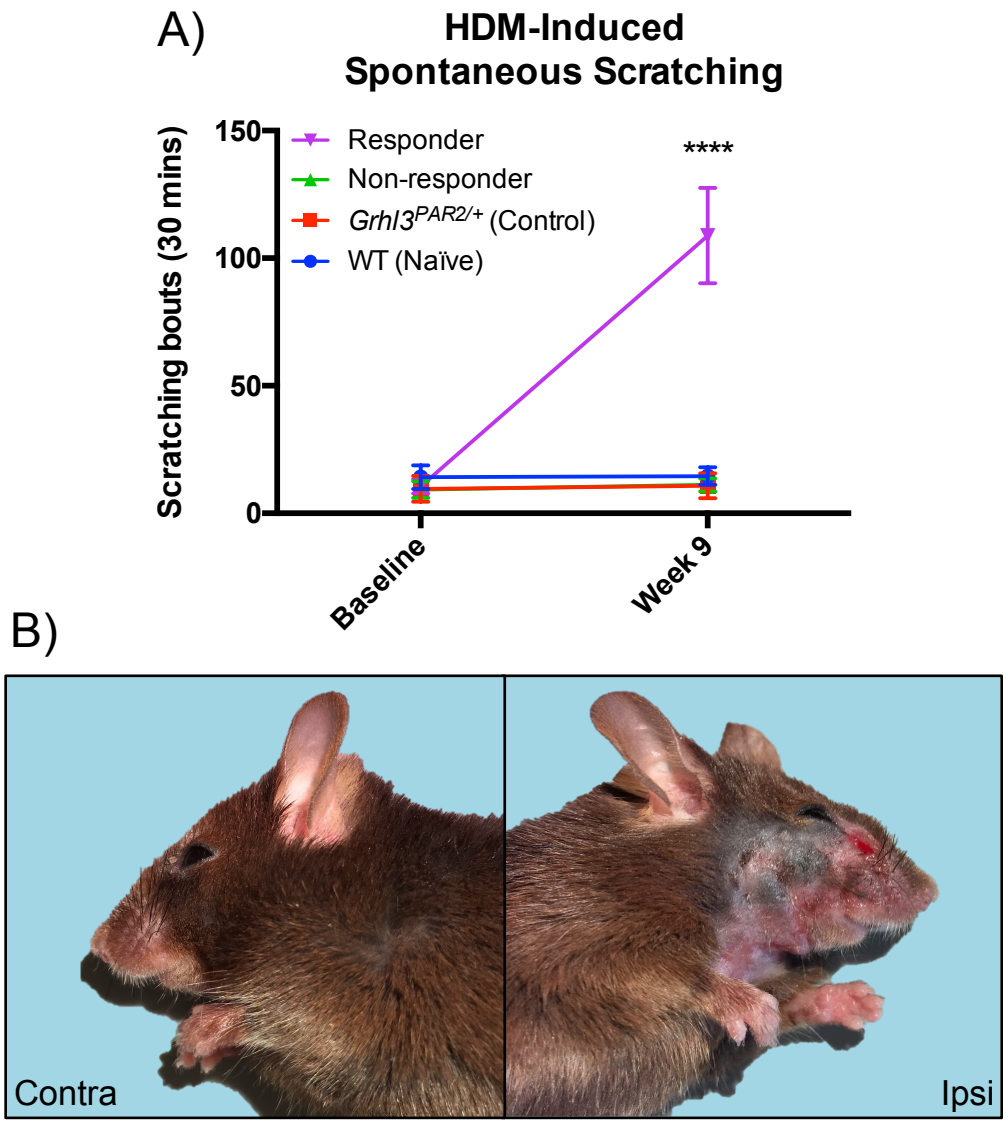
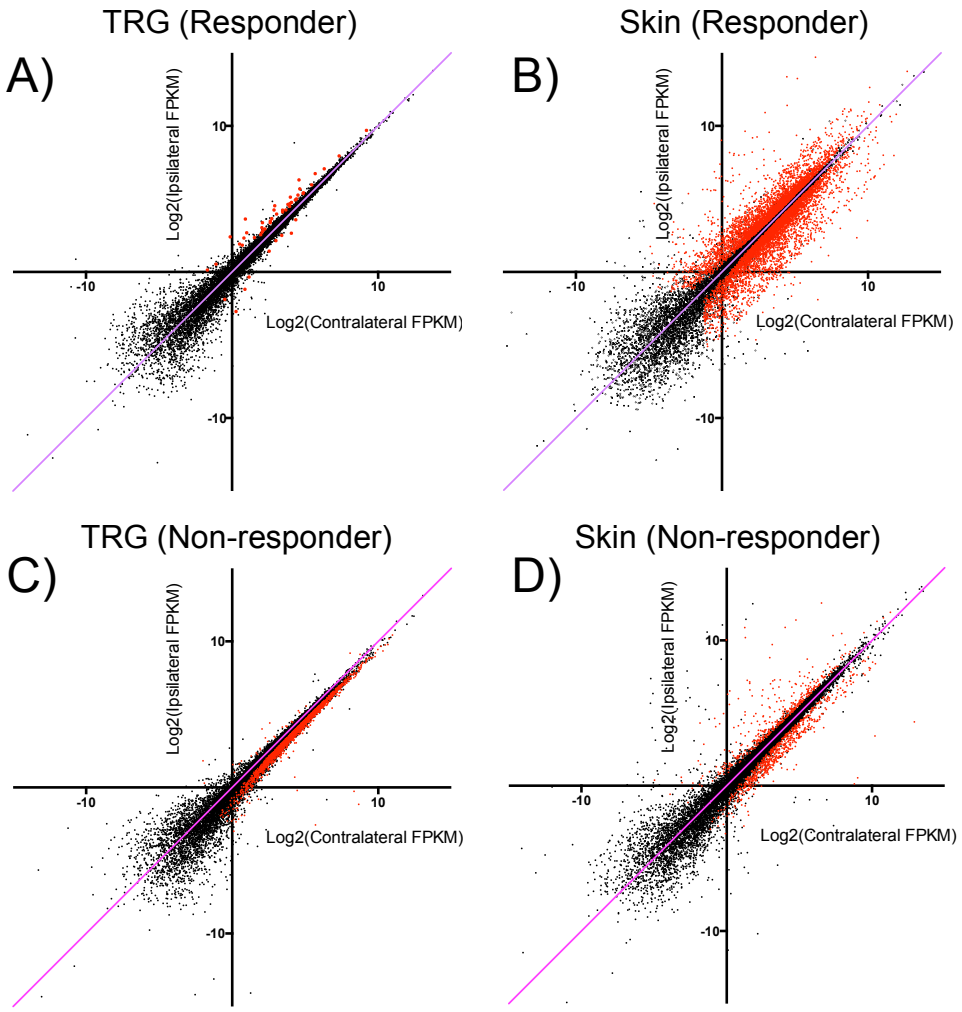


Figure 1. HDM-treatment produces a stochastic, atopic dermatitis-like phenotype in *Grhl3*$^{PAR2/+}$ mice. (A) After HDM treatment, mice were classified into groups based on their level of spontaneous scratching and skin phenotype. Responder mice showed highly elevated, spontaneous scratching by the end of 9 weeks of treatment. No other group showed a significant change in scratching over the treatment period. (B) The ipsilateral cheek of responder mice exhibited many of the classic hallmarks of atopic dermatitis, including erythema, dryness, edema, and excoriation. Contralateral skin appeared normal. Data are means ± SEM. Statistical

significance was determined by repeated measures two-way ANOVA with Bonferroni correction for multiple comparisons; ****$p < .0001$ compared to all other groups. N = 5 for naïve and control mice; N = 7 for responder / non-responder mice.

Sequencing	Average	Total	Aligned	Alignment	Filtered	Filtered	Tissue	Ipsi / Contra	N =
Dataset Type	RIN	Reads	Reads	Rate (%)	Reads	Alignment Rate (%)	Skin / TRG	to HDM treatment	# of mice
WT									
Naïve	9.1	1.04E+08	8.22E+07	79	6.42E+07	62	Skin	Contra	4
	9.5	1.29E+08	1.02E+08	79	7.89E+07	61	Skin	Ipsi	4
	8.3	1.13E+08	9.24E+07	82	6.54E+07	58	TRG	Contra	4
	8.6	1.07E+08	8.68E+07	81	6.35E+07	60	TRG	Ipsi	4
***Grhl3*$^{PAR2/+}$**									
Naïve	9.3	1.17E+08	8.68E+07	75	6.49E+07	56	Skin	Contra	4
	9.6	1.13E+08	8.44E+07	75	6.17E+07	56	Skin	Ipsi	4
	8.6	1.08E+08	8.36E+07	77	6.27E+07	58	TRG	Contra	4
	8.8	1.11E+08	8.24E+07	74	5.91E+07	54	TRG	Ipsi	4
Control	9.5	1.28E+08	9.53E+07	74	7.39E+07	58	Skin	Contra	4
	9.7	1.26E+08	9.89E+07	78	7.83E+07	62	Skin	Ipsi	4
	8.5	1.27E+08	8.92E+07	71	6.66E+07	53	TRG	Contra	4
	8.5	8.28E+07	6.64E+07	80	4.98E+07	60	TRG	Ipsi	4
HDM / Non-Responder	9.2	1.49E+08	1.22E+08	82	9.09E+07	61	Skin	Contra	4
	9.6	1.29E+08	1.04E+08	81	7.92E+07	61	Skin	Ipsi	4
	8.7	1.30E+08	9.56E+07	75	7.35E+07	57	TRG	Contra	4
	8.9	1.06E+08	7.71E+07	72	6.05E+07	56	TRG	Ipsi	4
HDM / Responder	9.5	1.43E+08	1.03E+08	72	7.96E+07	55	Skin	Contra	4
	9.7	1.38E+08	1.06E+08	77	8.21E+07	59	Skin	Ipsi	4
	8.7	1.43E+08	9.89E+07	69	7.60E+07	53	TRG	Contra	5
	8.8	1.39E+08	9.61E+07	69	7.49E+07	54	TRG	Ipsi	5

Table 1. Sequencing characteristics of RNA-seq libraries from skin and sensory ganglia suggest dataset quality and depth are satisfactory. For each condition, 4-5 replicates were used with an average RNA integrity number (RIN) between 8.3 and 9.7, indicating that input RNA was intact and of high quality. From each library, we obtained about 83 – 143 million reads, which were aligned to the genome with success rates of 69 - 82%. These aligned reads were filtered using a minimum quality score of 20, for an inferred base call accuracy of 99%. After alignment and filtering, each library contained, on average, between 50 - 90 million reads, suggesting that sequencing depth was sufficient to proceed with differential expression analysis.



E)

Phenotype	Tissue	Significant Changes	Increases	Decreases
Responder	TRG	50	41 **(82%)**	9 **(18%)**
	Skin	8041	3849 **(48%)**	4192 **(52%)**
Non-responder	Skin	1031	393 **(38%)**	638 **(62%)**
	TRG	2610	27 **(1%)**	2583 **(99%)**

Figure 2. Profiles of differentially expressed genes vary dramatically across tissue type and treatment. We focused our analysis on ipsilateral and contralateral tissue in responder and non-responder mice. Each plot features 23,991 genes from the mouse genome, with significant changes in red and non-significant changes in black. Changes that fall above the purple line are increased in ipsilateral tissue compared to contralateral. Changes that fall below the line are

enriched on the contralateral side compared to ipsilateral. (A) In responder animals, trigeminal ganglia (TRG) had only 50 significant changes, most of which (82%) were increased on the ipsilateral side. (B) By contrast, responder skin featured 8041 changes, which were evenly divided between increases and decreases, relative to the contralateral TRG. (C) Non-responder TRGs had 2610 significant changes, of which 99% were decreases. (D) Skin from responder mice showed 1031 significant changes, with the predominance (62%) of decreases in ipsilateral tissue. (E) Summary of TRG and skin profiles from responder and non-responder animals.

Transcript	FPKM (Contra)	FPKM (Ipsi)	Fold Change	Function
Responders (TRG)				
Fabp4	1.89135	12.3499	6.5	Fatty-acid binding
Nts	7.57747	23.2002	3.1	Neuropeptide
Fam210b	7.33383	18.9986	2.6	Unknown
Tmem173	4.25659	10.3951	2.4	Innate immune response
Bdnf	15.9545	34.8155	2.2	Neurotrophin
Nptx2	25.0992	51.444	2.0	Synapse formation
Vgf	51.9446	106.169	2.0	Unknown
Angptl2	6.6193	12.6089	1.9	Signaling molecule
Pkib	8.33584	15.6147	1.9	Kinase inhibitor
Gbp2	16.7169	6.30706	-2.7	G-protein
Non-responders (TRG)				
Cga	4.1175	16.086	3.9	Peptide hormone
Nts	5.97485	21.3892	3.6	Neuropeptide
Lipf	407.077	0.449405	-905.8	Lipase
Bpifb1	325.851	0.834844	-390.3	Innate immune response
Dmbt1	52.2314	0.169178	-308.7	Unknown
Lyz1	21.9805	0.865029	-25.4	Antibacterial
Ltf	19.5029	1.62549	-12.0	Broad activity
Cd52	14.39	4.18488	-3.4	Unknown
Ndufb4	18.3726	5.55978	-3.3	Reductase
2900055J20Rik	10.2327	3.46964	-2.9	Unknown
Responders (Skin)				
Tmprss11bnl	0.0402542	25.6928	638.3	Broad activity
Defb3	0.973096	517.587	531.9	Antibacterial response
S100a9	21.7893	10514	482.5	Broad activity
S100a8	84.6742	26671.9	315.0	Broad activity
BC100530	35.8696	9685.53	270.0	Protease inhibitor
Krt6b	2.99661	797.645	266.2	Keratin
Stfa1	7.77771	1935.2	248.8	Protease inhibitor
Mmp13	0.203346	41.1207	202.2	Peptidase
Lce3b	1.43981	289.64	201.2	Stratum corneum
Gm1553	95.0026	0.26977	-352.2	Unknown
Non-responders (Skin)				
Gm5938	0.982573	1050.28	1068.9	Unknown
Scgb1b2	2.36297	1887.84	798.9	Androgen-binding
Scgb2b24	2.42997	1173.06	482.7	Androgen-binding
S100a9	16.3242	1898.15	116.3	Broad activity
BC100530	27.2024	2713.43	99.7	Protease inhibitor
Stfa3	8.76019	837.604	95.6	Protease inhibitor
Chia1	439.954	1.09296	-402.5	Glycosidase
Smr3a	7710.7	21.0697	-366.0	Unknown
Dcpp1	516.155	1.45733	-354.2	Unknown
Amy1	1178.19	10.7562	-109.5	Amylase

Table 2. Top 10 sequencing hits for skin and sensory ganglia in responder and non-responder animals. For each condition, increases on the ipsilateral side are highlighted in blue; decreases are highlighted in red. In general, fold changes were smaller in trigeminal ganglia (TRG) than skin, with several notable exceptions in non-responder TRGs. These genes come from a variety of functional classes, including peptides, G-proteins, hormones, proteases, immune markers, and structural proteins.

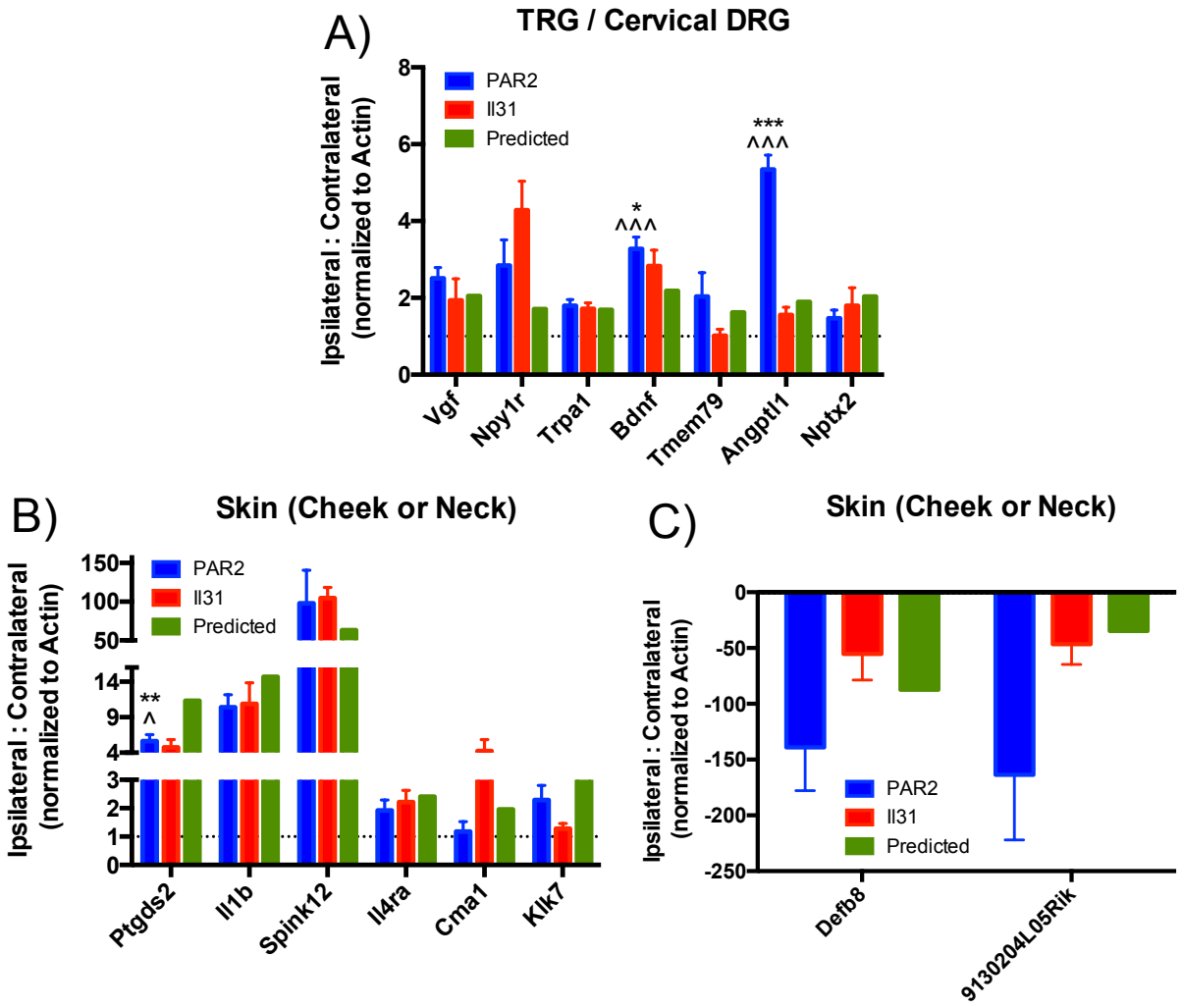


Figure 3. qRT-PCR validation of significant changes detected by differential expression analysis. (A) In trigeminal ganglia (TRG), 2/7 transcripts differed from their predicted values. Nevertheless, these transcripts were significantly elevated compared to baseline. (B-C) In cheek skin, only 1/8 changes differed significantly from the value predicted by RNA-seq. As in the TRG, this transcript was also significantly elevated compared to baseline. Changes were also compared to those found in tissue obtained from IL-31 overexpression mice. Data are means ± SEM. Statistical significance was determined by one sample t-test; *p < .05, **p < .01, ***p < .001 vs. predicted value; ^p < .05, ^^^p < .001 vs 1 (baseline). In 6 Par2 animals, we quantified Vgf, Npy1r, Trpa1, and BDNF; N = 5 for Tmem79, Angptl1, and Nptx2; N = 4 for transcripts

illustrated in B and C. For IL-31 animals, N = 5 for Tmem79, Angptl1, and Nptx2; N = 4 for Vgf, Npy1r, Trpa1, and Bdnf; N = 5 and 4 for transcripts illustrated in B and C, respectively. Cheek skin and TRGs were used for PAR2 mice. Neck skin and cervical dorsal root ganglia (DRG) were used for IL31 mice.

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Chapter 2: Anatomical and functional characterization of primary afferent-derived BDNF suggest a limited contribution to pain and itch

Chapter 2: Abstract

Brain-derived neurotrophic factor (BDNF) plays critical roles in neuronal growth and development, and is an essential component of learning and memory. Nonetheless, BDNF is also expressed by primary afferent sensory neurons in the peripheral nervous system. Anatomical and functional studies of primary afferent-derived BDNF have been limited by the unavailability of appropriate molecular tools. Here, we use targeted, inducible molecular approaches to unambiguously characterize the anatomical distribution of primary afferent BDNF and its contribution to a variety of pain and itch behaviors. Contrary to prior reports, we found that BDNF is expressed primarily by large-diameter primary afferents, with almost half of BDNF-expressing neurons not counterstaining with any of the commonly used biochemical markers for sensory neurons. In addition, primary afferent-specific deletion of BDNF produced few itch or acute and chronic pain behaviors, beyond a male-specific decrease in responsiveness during the second phase of the formalin test. The anatomical distribution of BDNF suggests that, going forward, low-threshold mechanical and motor behaviors should be used to probe the behavioral contribution of primary afferent-derived BDNF.

Chapter 2: Introduction

Neurotrophins promote growth and survival across a variety of neuronal subtypes in the central and peripheral nervous system. The neurotrophin BDNF is a critical contributor to neuronal growth and development, synaptic transmission, neurogenesis, learning and memory, and disease (Binder and Scharfmann, 2004). Though widely synthesized in the central nervous system (Conner *et al*, 1997), BDNF is also expressed in sensory neurons of the dorsal root (DRG; Barakat-Walter, 1996) and trigeminal ganglion (TRG; Wetmore and Olson, 1995), where studies suggest that BDNF regulates acute (Pezet and Malcangio, 2002) and chronic pain processing (Smith, 2014). However, the precise mechanism of action is not well understood and behavioral studies have been limited by several factors. First, the BDNF null mutation is lethal (Ernfors *et al*, 1994); second, ANA-12, a specific antagonist for TrkB, the BDNF receptor, has anxiolytic and antidepressant properties (Cazorla *et al*, 2011) that interfere with pain studies; third, TrkB binds to neurotrophins other than BDNF, so TrkB-scavengers are inherently non-specific; and fourth, because of the lethality of the null mutation, it is very difficult to verify the specificity of BDNF antibodies (Dieni *et al*, 2012). Thus, the anatomical and functional characterization of BDNF in sensory ganglia is incomplete, largely because of the problematic nature of verifying BDNF antibodies and in situ probes.

Several studies have reported that BDNF is expressed by a population of small to medium-diameter sensory neurons in the DRG, where it overlaps primarily with calcitonin gene-related peptide (CGRP), a marker of small, peptidergic nociceptors (Michael *et al*, 1997; Thompson *et al*, 1999; Luo *et al* 2001; Obata *et al*, 2006). Once synthesized, BDNF undergoes anterograde transport to spinal cord laminae I-II and the periphery (Michael *et al*, 1997). Within primary

afferent neurons, BDNF is packaged into large, dense-core vesicles (Michael *et al*, 1997; Luo *et* al, 2001) and secreted in response to electrical stimulation (Lever *et al*, 2001), potassium, and cyclophosphamide, which produces peripheral inflammation (Qiao *et al*, 2015). Interestingly, BDNF expression changes dramatically in the setting of both neuropathic (Zhou et al, 1999; Fukuoka *et al*, 2001; Obata *et al*, 2006) and inflammatory injury (Cho *et al*, 1997; Lin *et al*, 2011). For example, in the setting of chronic inflammatory pain, studies have reported an increase in the number of BDNF-expressing neurons in the DRG, with de novo expression in large cells and decreased expression in small ones (Cho *et al*, 1997; Zhou et al, 1999; Fukuoka *et al*, 2001; Obata *et al*, 2006). BDNF is also expressed heterogeneously throughout neurons in both the dorsal and ventral spinal cord (Gomez-Pinilla *et al*, 2004), which complicates attempts at understanding the specific functions of primary afferent-derived BDNF.

Of course, as with all signaling molecules, biological function also depends on the expression pattern of the receptor for BDNF. Among the neurotrophin receptors, BDNF interacts with the p75 neurotrophin receptor ($p75^{NTR}$), a low-affinity receptor that binds all neurotrophins, and TrkB, a high-affinity receptor that is selective for BDNF and neurotrophin-4/5 (NT-4/5; Patapoutian and Reichardt, 2001). In the DRG, $p75^{NTR}$ is expressed in both neurons and satellite cells (Zhou *et al*, 1996), while high levels of TrkB have been detected in small- to medium-diameter DRG neurons (Watson *et al*, 1999; Lin *et al*, 2011). Within the spinal cord, $p75^{NTR}$ is expressed in superficial layers, with more diffuse staining in deeper laminae (King *et al*, 2000). TrkB is expressed fairly broadly among all neurons in the spinal cord (King *et al*, 2000; Liebl *et al*, 2001), while glia express TrkB to a much lesser extent (Zhou *et al*, 1993; Yan *et al*, 1997). In the periphery, nerve terminals (Liang and Johansson, 1998; Li *et al*, 2011), skin (Calavia *et al*,

2010), and immune cells express both $p75^{NTR}$ and TrkB (Besser and Wank, 1999; Fischer *et al*, 2008), suggesting that release of BDNF from primary afferents could have a variety of functional consequences beyond direct engagement of pain circuitry in the spinal cord. Finally, TrkB expression has been shown to increase in the setting of inflammation (Lee *et al*, 1999), suggesting that BDNF might engage de novo signaling pathways after injury.

Mechanistically, BDNF binding to TrkB leads to dimerization and autophosphorylation of the receptor, which activates intracellular signaling cascades that include mitogen-activated protein kinase (MAPK), phospholipase C-γ (PLC-γ) and phosphatidylinositol-3 kinase (PI3-K; Reichardt, 2006; Garraway & Huie, 2016). A truncated form of TrkB also exists in the central and peripheral nervous system, where it appears to serve a dominant negative or opposing function to that of full-length TrkB (Pezet *et al*, 2002). As BDNF can activate multiple downstream signaling cascades, discovering TrkB in a particular cell is not sufficient to explain TrkB's function upon activation. Furthermore, TrkB is widely distributed throughout multiple cell types in the spinal cord, which complicates attempts at defining the effects of BDNF release by primary afferents. For example, TrkB is expressed in projection neurons of the spinothalamic tract (Slack *et al*, 2005) as well as interneurons of the substantia gelatinosa (Salio *et al*, 2005), two cell types with distinct roles in pain processing. Physiologically, spinal BDNF is capable of producing NDMA-dependent facilitation (Garraway *et al*, 2003) and sensitization (Thompson *et al*, 1999; Shu *et al*, 1999; Groth and Aanonsen, 2002; Cao *et al*, 2012), similar to the role it plays in the hippocampus to generate learning and memory. Additionally, BDNF can reduce $GABA_A$-mediated inhibition of presynaptic terminals in the spinal cord, contributing to behavioral

hypersensitivity (Chen *et al*, 2014). Together, present data suggest that BDNF facilitates pain processing in the spinal cord.

Beyond anatomy and electrophysiology, several behavioral studies proposed that BDNF profoundly influences the processing and expression of pain messages, which in turn impacts nocifensive behaviors. Some groups reported modest, pronociceptive effects on heat thresholds after subcutaneous injection of BDNF (Thompson *et al*, 1999); others found robust decreases in thermal (Shu *et al*, 1999; Groth and Aanonsen, 2002) and mechanical thresholds (Coull *et al*, 2005) when BDNF was injected intrathecally. Conversely, intrathecal injection of a TrkB-scavenger dramatically reduced thermal hyperalgesia and mechanical allodynia in models of bone-cancer (Bao *et al*, 2014) and inflammatory pain (Groth and Aanonsen, 2002). Inhibiting BDNF signaling with an intrathecal antibody also decreased thermal hyperalgesia in the spinal nerve ligation model of neuropathic pain (Fukuoka *et al*, 2001). A recent study suggests that a monoclonal antibody to proBDNF, the precursor to mature BDNF, can attenuate various types of inflammatory and surgical pain (Luo *et al*, 2016). Consistent with these findings, Zhao *et al* (2006) reported that Nav1.8-Cre-dependent deletion of BDNF from a subpopulation of primary sensory neurons in female mice reduced behavior in the second-phase of the formalin test, a model of inflammatory, post-surgical pain (Zhao *et al*, 2006). However, the same mice were hypersensitive in the hot plate test of thermal pain, contradicting studies that have revealed a pronociceptive effect of BDNF on thermal and mechanical thresholds in acute and neuropathic pain. Critically, though NaV1.8 is also expressed by non-nociceptive neurons in the DRG, it only labels a small percentage of low-threshold afferents (Shields *et al*, 2012; Benn *et al*, 2001; Amaya *et al*, 2000), which are important for generating allodynia after nerve injury.

Furthermore, anatomical (Damarjian *et al*, 2004; Lu *et al* , 2015) and behavioral studies (Seal *et al*, 2009) suggest that NaV1.8 is also expressed in vagal afferents (Blasius *et al*, 2011) and the brain (Gautron *et al*, 2011), which complicates attempts to use NaV1.8-Cre to generate nociceptor-specific knockouts.

In addition to neurons, microglia have also been suggested to release BDNF in the spinal cord to generate allodynia in the setting of nerve injury (Coull *et al*, 2005). Specifically, ATP release in the setting of injury is thought to stimulate spinal microglia, which release BDNF onto spinal lamina I neurons, causing a shift in their transmembrane anion gradient that results in GABA-mediated hyperexcitation. However, though the behavioral evidence for this phenomenon is very convincing, groups attempting to locate BDNF in microglia have found extremely low levels in the brain (Parkhurst *et al*, 2013; Bennett *et al*, 2016) and nothing in the spinal cord, even in the setting of nerve injury (Denk *et al*, 2016). By comparison, the amount of BDNF expression in primary afferents neurons is much higher, and interestingly, these neurons terminate in lamina I of the spinal cord (Michael *et al*, 1997), suggesting that they are capable of engaging the same circuitry that has been implicated in microglia studies. Conversely, behavioral experiments that used BDNF antibodies and TrkB-scavengers could have generated their behavioral effects by interfering with BDNF signaling from microglia, rather than neurons. Resolving these discrepancies will require the use of more accurate genetic tools to investigate the distribution of BDNF in primary afferents and the role BDNF plays in producing pain behavior.

Clearly there is a lack of consensus as to the source of primary afferent-derived BDNF and the extent to which BDNF contributes to the processing of acute and chronic pain messages. This

controversy exists in part because anatomical studies have been unable to validate the specificity of their molecular tools. Given the importance of Aβ-LTMRs in generating allodynia after nerve injury (Seal *et al*, 2009), establishing the true expression pattern of BDNF in the DRG is of great importance. In addition, behavioral studies have been unable to selectively interfere with BDNF signaling from primary afferents. For example, TrkB inhibitors also block signaling by NT-4/5, which, like BDNF, is expressed in the spinal cord (Scarisbrick *et al*, 1999) and DRG (Heppenstall and Lewin, 2001). Furthermore, the NaV1.8 knockout study did not eliminate BDNF from all DRG neurons, including some that have been implicated in allodynia after nerve injury (Seal *et al*, 2009). BDNF signaling is also sexually dimorphic (Liu *et al*, 2012) and critical for DRG development (Valdés-Sánchez *et al*, 2010), suggesting that the results of the knockout study should be revisited using a conditional deletion in both males and females. In the present study, we used an inducible, knock-in reporter mouse to unambiguously characterize the expression of BDNF in adult DRG neurons. We also generated an inducible, sensory-afferent specific deletion of BDNF from adult male and female mice, and examined the effects of this deletion in a battery of behavioral tests that measured responsiveness to pruritogens and acute and chronic pain.

Chapter 2: Results

Anatomical characterization of BDNF-lacZ animals

Previous attempts to characterize the expression of BDNF in primary afferent neurons have relied on immunohistochemical or in situ hybridization methods (Cho *et al*, 1997; Michael *et al*, 1997; Thompson *et al*, 1999; Zhou et al, 1999; Fukuoka *et al*, 2001; Luo *et al* 2001; Obata *et al*, 2006; Lin *et al*, 2011). However, BDNF antibodies are known to be problematic, and in situ studies, although more reliable, were not comprehensive. Specifically, there were no attempts to double label sections so that the subclasses of neurons that express BDNF could be determined. Because of the importance of relating expression patterns to sensory neuron subpopulations, here we took advantage of a knock-in reporter animal that expresses Cre-inducible βGal under control of the BDNF promoter. By crossing this animal with a tamoxifen-inducible Advillin-CreERT2, we were able to selectively label primary afferent neurons in the adult dorsal root ganglion. This BDNF-LacZ animal has been used previously to characterize BDNF expression in brain (Gorski *et al*, 2003), but to our knowledge this is the first analysis of primary sensory neurons.

Our investigation focused on DRGs L4-5, as these levels correspond to the plantar surface of the rear hindpaw, where most of our behavioral experiments were conducted. Figure 1 illustrates that the intensity of βGal expression within DRG neurons varied considerably. Immunolabeling in high-expressing cells completely filled both the nucleus and cytoplasm. Medium-expressing cells were characterized by punctate staining of the nucleus and the cytoplasm. Low-expressing cells had punctate staining in the cytoplasm and little to no nuclear labeling (Figure 1A). For the purposes of this investigation, all cells with βGal-staining were treated equally, regardless of staining intensity. Cell counts showed that the total number of βGal-positive cells per DRG was

about 200 out of approximately 11,000 neurons, with similar numbers in male and female mice (Figure 1B).

We next characterized βGal-positive cells with a panel of markers that distinguish subpopulations of neurons in the DRG (Figure 1C). Almost half of the βGal-neurons (40%) co-expressed NF200 (Figure 1D), a marker of large- and medium-diameter, myelinated fibers, while 19% expressed peripherin, a marker of most C-fibers (Figure 1E). A small percentage of BDNF immunoreactive neurons (14%) co-expressed TRPV1 (Figure 1F) or CGRP (12%, Figure 1G), markers of small, nociceptive fibers. Few neurons were IB4-binding (7%, Figure 1H), another marker of small, unmyelinated afferents. Finally, we rarely detected βGal-positive neurons that co-expressed tyrosine hydroxylase (TH; 0.5%, Figure 1I), a marker of small, unmyelinated, low-threshold mechanoreceptive afferents. It is worth noting that TH expression is higher in levels of DRG that correspond to hairy skin (Li *et al*, 2011), though in this study our TH antibody staining was adequate in L4-5. Note that because the βGal reporter animal is not a fusion construct between LacZ and BDNF, the βGal pattern defines the neurons that express BDNF, rather than the intracellular staining pattern of BDNF itself.

Conditional, primary-afferent-driven deletion of BDNF

Since BDNF is known to play an important role in DRG development (Valdés-Sánchez *et al*, 2010), we took advantage of an inducible Advillin-CreERT2 that is expressed in nearly all primary afferent neurons (Lau *et al*, 2011). Crossing these mice with a Floxed-BDNF line generated a conditional knockout (cKO) for use in behavioral and anatomical studies. qPCR showed that BDNF-cKO mice had almost completion deletion of BDNF from DRG and

trigeminal ganglion (TRG) neurons (96% and 91%, respectively; Figure 2A). Surprisingly, BDNF knockdown was found to occur in the spinal cord as well (43%). However, BDNF ELISA clearly demonstrated that levels of BDNF protein were unchanged in tamoxifen-treated spinal cord, while in TRG they dropped below the detection limit of the analysis (Figure 2B). We also crossed the Advillin-CreERT2 line with Cre-dependent tdTomato reporter animals. After tamoxifen treatment, we only detected tdTomato in primary afferent terminals in spinal dorsal horn (Figure 2C). Finally, no βGal staining was observed in the spinal cords of BDNF-LacZ reporter animals (Figure 2D). Together, these results suggest that Advillin-CreERT2 is insufficient to drive recombination in spinal cord cells, and that the decreased signal observed with qPCR represents a depletion of BDNF message from primary afferent terminals. Indeed, other studies have found that BDNF is actively transported into both the spinal cord and periphery (Michael *et al*, 1997).

Baseline tests of pain and itch

We assessed BDNF-cKO mice using behavioral tasks that tested for nociception and pruritoception. Importantly, the conditional knockout mice responded normally on the rotarod test (Figure 3A), which demonstrates that there were no significant motor abnormalities that would compromise tests that assayed hindlimb withdrawal. In addition, mice responded normally in the Von Frey test, which is a measure of mechanical reflexes (Figure 3B). BDNF-cKO and vehicle-treated control mice did not differ in nociceptive tests of thermal sensation, including the hotplate (Figure 4A) and Hargreaves tests (Figure 4C) for heat, and the acetone test for cold responsiveness (Figure 4E). Chemonociception in response to hindpaw injection of capsaicin was also unchanged in the cKO mice (Figure 4D). Interestingly, BDNF-cKO mice showed a

sexually dimorphic phenotype in the tail immersion test of heat pain at 49° C, where male knockout mice had increased latencies compared to all other groups (Figure 4B). Responsiveness to most pruritogens did not differ (Figure 5A), with the exception of histamine (Figure 5B), for which we recorded increased scratching in female control mice that disappeared after tamoxifen treatment.

Sexually dimorphic, reduced responsiveness in the formalin test of inflammatory pain

The formalin test is a model of persistent inflammation that is considered a reliable correlate of post-operative pain. A previous report showed that NaV1.8-driven deletion of primary-afferent BDNF produces female specific insensitivity in the second phase of the formalin test (Zhao *et al*, 2006). However, NaV1.8 does not label all nociceptors in the DRG, and in fact labels some low-thresholds neurons as well (Shields *et al*, 2012). As advillin-expression is even broader than that of NaV1.8, we were surprised to find that female cKO animals responded normally in all phases of the formalin test (Figure 6A). In contrast, we found that male animals showed dramatic reductions in their responses during the second phase of the test, which lasts from about 15-50 minutes after formalin injection (Figure 6B).

Sensitization of nociceptive circuits in the spinal cord occurs after hindpaw formalin and this manifests as secondary hyperalgesia, in which there is mechanical allodynia of tissue near the inflamed hindpaw. In many respects this secondary hyperalgesia is also a consequence of formalin-induced damage to peripheral afferent terminals in the skin. To determine whether loss of the second phase resulted in decreased post-formalin mechanical allodynia, we examined Von Frey mechanical thresholds in male animals one day after the formalin injection. Interestingly,

the BDNF-cKO mice displayed the same degree of mechanical allodynia as control mice (Figure 6C). In addition, the levels of spinal Fos, a marker of neuronal activity (Figure 7A-B), and primary afferent-ATF3 (Figure 7C-D), a marker of neuronal injury, were also similar between control and BDNF-cKO groups.

Tests of neuropathic and chronic inflammatory pain

Previous studies reported that BDNF is upregulated in DRG neurons after both peripheral nerve injury (Zhou *et al*, 1999; Fukuoka *et al*, 2001; Obata *et al*, 2006) and chronic inflammation (Cho *et al*, 1997; Lin *et al*, 2011). Furthermore, BDNF has been shown to be acutely pronociceptive (Thompson *et al*, 1999; Shu *et al*, 1999; Coull *et al*, 2005), and inhibiting TrkB signaling reduces hyperalgesia and allodynia in multiple models of chronic pain (Fukuoka *et al*, 2001; Bao *et al*, 2014). Therefore, we expected BDNF-cKO animals to recapitulate these findings in various models of chronic neuropathic and inflammatory pain. Surprisingly, these animals developed normal mechanical allodynia in the spared-nerve injury model of neuropathic pain (Figure 8A). In addition, knockout animals had both mechanical allodynia and thermal hypersensitivity in the Paclitaxel model of chemotherapy-induced neuropathic pain (Figure 8B-C). It is of note that female control mice developed significantly greater thermal hypersensitivity than any other group. While the amount of hypersensitivity decreased somewhat after female mice were treated with tamoxifen, this decrease was not statistically significant. Finally, BDNF-cKO mice developed normal allodynia and hypersensitivity after injection of chronic Freund's adjuvant (CFA, Figure 8D-E), which generates a model of chronic inflammation and nerve injury. Together, these results suggest that BDNF does not play a role in the development of neuropathic

or chronic inflammatory pain, although a role in the long-term maintenance of these conditions cannot be eliminated.

Chapter 2: Discussion

In this study, we characterized the expression of primary afferent-derived BDNF, as well as its contribution to the processing of pain and itch-inducing messages. In distinct contrast to previous reports (Michael *et al*, 1997; Thompson *et al*, 1999; Luo *et al*, 2001; Obata *et al*, 2006), we found that BDNF is expressed primarily by large, myelinated neurons, with a much smaller percentage of neurons co-expressing the peptidergic marker CGRP. We went on to delete BDNF by using a tamoxifen-inducible, primary afferent-specific Cre. This approach avoided the inevitable concern that deletion of BDNF during embryonic development could produce changes that complicate the interpretation of BDNF phenotypes in the adult. Although we investigated the behavioral consequences of our deletion with an array of nociceptive tests, we were ultimately unable to make a definitive determination of the function of primary afferent BDNF. However, we found that male knockout animals developed phenotypes in the tail immersion and formalin tests, whereas female mice only developed a phenotype in response to histamine. Perhaps most surprisingly, despite ample evidence showing that BDNF expression changes dynamically in the setting of nerve injury (Lin *et al*, 2011; Cho *et al*, 1997; Zhou *et al*, 1999; Fukuoka *et al*, 2001; Obata *et* al, 2006), BDNF knockout animals developed normal mechanical allodynia and thermal hypersensitivity across several models of neuropathic and chronic inflammatory pain.

A major distinction between our anatomical analysis and previous studies of BDNF expression in sensory neurons is that we did not rely on BDNF antibodies or in situ probes. Since the BDNF null mutation is lethal (Ernfors *et al*, 1994), it is difficult to verify BDNF antibody and probe specificity for immunohistochemistry. The expression analysis performed in this study obviated this concern by using a Cre-dependent, knock-in reporter construct that drove βGal expression

selectively in adult animals (Gorski *et al*, 2003). By double labeling with biochemical markers for different DRG neuron subtypes, we found, contrary to previous studies (Michael *et al*, 1997; Thompson *et al*, 1999; Luo *et al*, 2001; Obata *et al*, 2006), that BDNF expression predominates in large, myelinated neurons. The large neurons also exhibited greater staining intensity than did βGal neurons with a smaller diameter. As the endogenous BDNF promoter drives βGal-expression in this reporter mouse, staining intensity likely correlates with the amount of BDNF transcribed in a given cell. Based on this finding, we suggest that myelinated neurons synthesize more BDNF than do other sensory neurons in the DRG.

Another surprising aspect of BDNF expression in the DRG is that, despite using a comprehensive panel of antibodies to co-stain neurons, we were unable to biochemically characterize almost half of BDNF-expressing neurons. As most TRPV1- and CGRP-positive and IB4-binding afferents also express peripherin, and as there is little overlap between NF200 and peripherin, at a minimum, we were only able to characterize about 60% of the BDNF population when factoring in NF200 (40% overlap) and peripherin staining (20% overlap). One possibility is that the population of uncharacterized neurons expresses TRPM8, a marker of small, cold-responsive cells that are largely non-overlapping with peripherin, TRPV1, CGRP, and IB4 (Dhaka *et al*, 2008; Takashima *et al*, 2010). It is also possible that BDNF labels a biochemically distinct subset of DRG neurons that does not co-express the commonly studied cell type markers. In this way, it would be similar to TH, which marks a biochemically unique subset of primary afferent neurons (Li *et al*, 2011).

Establishing the biochemical character of DRG neurons is important because it speaks directly to their function in both health and disease. The predominance of BDNF expression in large, non-nociceptive cells suggests a possible contribution of BDNF to innocuous mechanotransduction (Abraira and Ginty, 2013). Unfortunately, by definition, low-threshold signaling does not produce a defensive behavioral response; therefore, it is very challenging to study in animal models. On the other hand, there is electrophysiological evidence that large, myelinated neurons require BDNF in postnatal life to generate normal mechanotransduction (Carroll *et al*, 1998). Specifically, although myelinated neurons can survive in the absence of BDNF, studies indicate that their mechanical sensitivity to tactile stimuli is dramatically reduced. Reconciling these findings will require innocuous behavioral tasks that specifically engage large, myelinated neurons, rather than small, unmyelinated neurons, which showed little BDNF expression in our analysis. One possibility would be to use a texture discrimination task to evaluate the role BDNF plays in sensory processing (Maricich *et al*, 2012). When performed with the paws, this task engages Merkel cells, which are innervated exclusively by large, myelinated afferents.

Although we did not investigate low-threshold mechanotransduction, we did conduct a comprehensive array of tests to measure acute and chronic pain. Contrary to numerous behavioral reports that relied on peripheral application of BDNF or intrathecal administration of BDNF- or TrkB-scavengers (Shu *et al*, 1999; Thompson *et al*, 1999; Fukuoka *et al*, 2001; Groth *et al*, 2002; Coull *et al*, 2005; Bao *et al*, 2014), we found few pain or itch phenotypes when we deleted BDNF selectively from primary afferent neurons. Of particular note are the interesting discrepancies between our findings and those of Zhao *et al* (2006), who deleted BDNF from sensory neurons using an NaV1.8-Cre. The authors reported hotplate hypersensitivity and

formalin hyposensitivity selectively in female mice after BDNF deletion. In comparison, after deletion we found tail immersion and formalin hyposensitivity selectively in male mice, and decreased responses to histamine selectively in female mice. However, there is an important difference in the genetic approach used by these two studies. The BDNF deletion generated by Zhao *et al* was developmental and designed to target primarily small, nociceptive afferents (Amaya *et al*, 2000; Benn *et al*, 2001; Shields *et al*, 2012). Importantly, there is evidence that some neurons in the brain are NaV1.8-expressing, and that this expression might even be greater during development (Damarjian *et al*, 2004; Blasius *et al*, 2011; Gautron *et al*, 2011; Lu *et al*, 2015). It is difficult to envision how this particular animal could exhibit unique behaviors not seen in our broader version of the same knockout, unless developmental issues are at play. Indeed, as BDNF serves an essential role in DRG development (Valdés-Sánchez *et al*, 2010), even though Zhao *et al* observed no neuronal loss in their animals, it is possible that connectivity of some DRG neurons might have been fundamentally altered in the absence of BDNF.

Contrary to Zhao *et al*, we found a sexually dimorphic effect of BDNF knockout on the responsiveness to formalin, where the only deficit occurred in male mice. There is precedent for sexual dimorphism in BDNF signaling, as TrkB expression is regulated by androgens (Liu *et al*, 2012) and genetic deletion of microglial BDNF has been shown to produce pain phenotypes selectively in males (Sorge *et al*, 2015). The formalin phenotype in our study was restricted to the second phase of formalin responses. Canonically, activity in the first phase of the formalin test is thought to drive central sensitization (Latremoliere and Woolf, 2009) in the spinal cord and thereby catalyze the second phase (Tjølsen *et al*, 1992). However, the second phase of the formalin test persists even after the first phase has been blocked with opioids (Taylor *et al*, 1997)

or after the great majority of C-fibers, thought to be responsible for the first phase, are eliminated (Shields *et al*, 2010). In addition, the release of inflammatory mediators, which affect the second phase of behavior, is reduced after opioid treatment (Malmberg *et al*, 1995). Interestingly, despite no outward pain behavior and reduced inflammatory signaling, opioids are not sufficient to completely eliminate formalin-driven central sensitization (Buerkle *et al*, 1998). BDNF has been shown to produce sensitization and facilitation of laminae I-II neurons in the spinal cord (Kerr *et al*, 1999; Garraway *et al*, 2003), as well as to phosphorylate spinal NMDA receptors (Slack *et al*, 2002; Slack *et al*, 2004; Liu *et al*, 2015). These observations, when viewed in light of our results, suggest that BDNF may be necessary for spinal sensitization in the formalin model for male, but not female mice. As the formalin test is thought to model post-surgical pain, this finding could have particular clinical relevance.

Lastly, beyond its role in modulating pain processing, BDNF also promotes axonal regrowth and functional recovery after nerve injury (Lindsay *et al*, 1988; Takemura *et al*, 2012; Zheng *et al*, 2016). However, differences in behavior between BDNF-deficient and wild type animals, when measured by rotarod and toe spreading reflex, have not been reported until at least several weeks after injury (Takemura *et al*, 2012; Zheng *et al*, 2016), which is beyond the scope of our present study. It would be interesting to revisit BDNF conditional knockout animals in the context of recovery and nerve regrowth, rather than pain, in models of nerve injury. As with any investigation into the relevance of BDNF expression by myelinated afferents, studies looking at recovery will require nuanced measures of low threshold sensory processing.

Chapter 2: Methods

Animals

Animal experiments were approved by the UCSF Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory animals. *Bdnf*$^{fl/fl}$ and Ai14 (tdTomato) reporter mice were purchased from the Jackson Laboratory. *Bdnf*$^{LacZ/+}$ reporter mice were provided by Dr. Zachary Knight at UCSF. Advillin-CreERT2 mice were kindly provided by Dr. Ardem Patapoutian at the Scripps Institute.

Tamoxifen treatment

Tamoxifen (Sigma) was dissolved in corn oil by vigorous vortexing for 30 minutes. Cre was induced by injecting animals I.P. with 150 mg / kg daily for 5 consecutive days, as described previously (Lau *et al*, 2011).

Behavioral analyses

For all behavioral tests, animals were first habituated for 1 hour in a Plexiglas cylinder. The experimenter was always blind to whether the animal received tamoxifen. All mechanical (Von Frey), thermal (Hargreaves, tail immersion, hotplate, acetone) and ambulatory (rotarod) tests were conducted as described previously (Martinez-Caro and Laird, 2000; Wang *et al*, 2013). For the capsaicin test, capsaicin (Sigma) was first dissolved in 100% ethanol (3.0 mg / mL). This stock was mixed 1:1:8 with Tween-80 (Sigma) and saline, and injected into the hindpaw (10 µL), with behavior then video recorded for the next 5 minutes. Behavior was scored as the total duration of hindpaw licking. For the acetone test, behavior was quantified as the total duration of rear paw lifting, licking or shaking over 5 consecutive, 30-second trials.

Pruritogen-evoked scratching

At least 24 hours prior to testing, mice were shaved at the nape of the neck under isoflurane anesthesia. The following pruritogens were dissolved in saline and injected (50 μL, s.c.) into the neck: chloroquine (Sigma, 100 μg), endothelin-1 (Sigma, 25 ng), histamine (Sigma 500 μg), SLIGRL (Sigma 100 μg), and TSLP (Sigma 2.5 μg). The mice were video recorded, and from these videos we counted the total number of discrete scratching bouts that occurred during the first 30 minutes after the injection.

Neuropathic and inflammatory pain models

Chronic Freund's Adjuvant (CFA)

The CFA model of chronic inflammation was induced as described previously (Malberg *et al*, 2003). Briefly, CFA (Sigma) was diluted 1:1 with saline and vortexed for 30 minutes. When fully suspended, we injected 20 μL of CFA into one hindpaw. Heat and mechanical withdrawal thresholds were measured 3 days later using the Hargreaves and Von Frey tests, respectively. All animals had been measured prior to injection, so data were analyzed as a drop in threshold compared to baseline.

Formalin

The formalin test was conducted as described previously (Shields *et* al 2010). Briefly, intraplantar formalin was injected into one hindpaw (10 μL, 2% in saline) and nocifensive behavior was video recorded over the following hour. The cumulative duration of all behaviors (licking, lifting, shaking, biting) was measured in 5-minute bins.

For immunohistochemistry, animals were perfused 1.5-2 hours after injection to stain for Fos in the spinal cord, and 48 hours after injection to stain for ATF3 in the DRG. See immunohistochemistry for the list of antibodies and concentrations used.

Paclitaxel

Paclitaxel (Sigma) was diluted in Kolliphor EL (Sigma, 1:1 with 100% ethanol) to a final concentration of 6.0 mg / mL. Aliquots of 20 µL were stored at -20 °C until use, when they were diluted 15x with saline. Mice were injected IP with 4.0 mg / kg, once per day, for five consecutive days. Heat and mechanical thresholds were measured at 12 and 13 days, respectively, after the final injection.

Spared-Nerve Injury (SNI)

SNI was conducted as described previously (Shields *et al*, 2003). All behavior and immunohistochemistry were conducted one week after injury.

Immunohistochemistry

Tissue was immunostained as described previously (Braz *et al*, 2012). Antibodies used included ATF3 (rabbit, 1:2k, Santa Cruz Biotechnology), Fos (rabbit, 1:5k, Oncogene), Iba1 (rabbit, 1:1k, Wako), NeuN (mouse, 1:5k, Sigma), βGal (chicken, 1:10k, Abcam), TRPV1 (guinea pig, 1:5k, generous gift of the David Julius lab), NF200 (mouse, 1:20k, Sigma), CGRP (mouse, 1:10k, Sigma), tyrosine hydroxylase (rabbit, 1:5k, Millipore), biotinylated IB4 (goat, 1:500, Vector Labs). Fluorescent secondary antibodies were used at a 1:1k dilution, while streptavidin-conjugated fluorophore was used at 1:5k.

BDNF ELISA

Advillin-CreERT2::*Bdnf*$^{fl/fl}$ mice were injected with tamoxifen or corn oil as described above. Two weeks after the final injection, spinal cords (5 mm of lumbar enlargement) and TRGs were homogenized in 20x volume lysis buffer (pH 7.0) containing Tris-HCl (100 mM), NaCl (1 M), EDTA-Na^2 (4 mM), bovine serum albumin (2%), Triton X-100 (2%), sodium azide (0.1%), and cOmplete ULTRA protease inhibitor cocktail (Roche). Homogenates were centrifuged at 14k x g for 30 minutes at 4 °C. Spinal cord supernatants were diluted 10x and TRG supernatants diluted 5x. All supernatants were immediately assayed with the Human BDNF SimpleStep ELISA Kit (Abcam). Absorbance was measured at 450 nm with a Biotek H4 Plate Reader.

Quantitative PCR

Tissue was homogenized in TRIzol (Ambion) and RNA was purified using the PureLink RNA Mini Kit with on-column DNAse treatment (Ambion). cDNA was prepared with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). mRNA levels were quantified with the Bio-Rad CFX Connect System using PowerUp SYBR Green Master Mix (Applied Biosystems). All transcripts were normalized to actin.

Genotyping

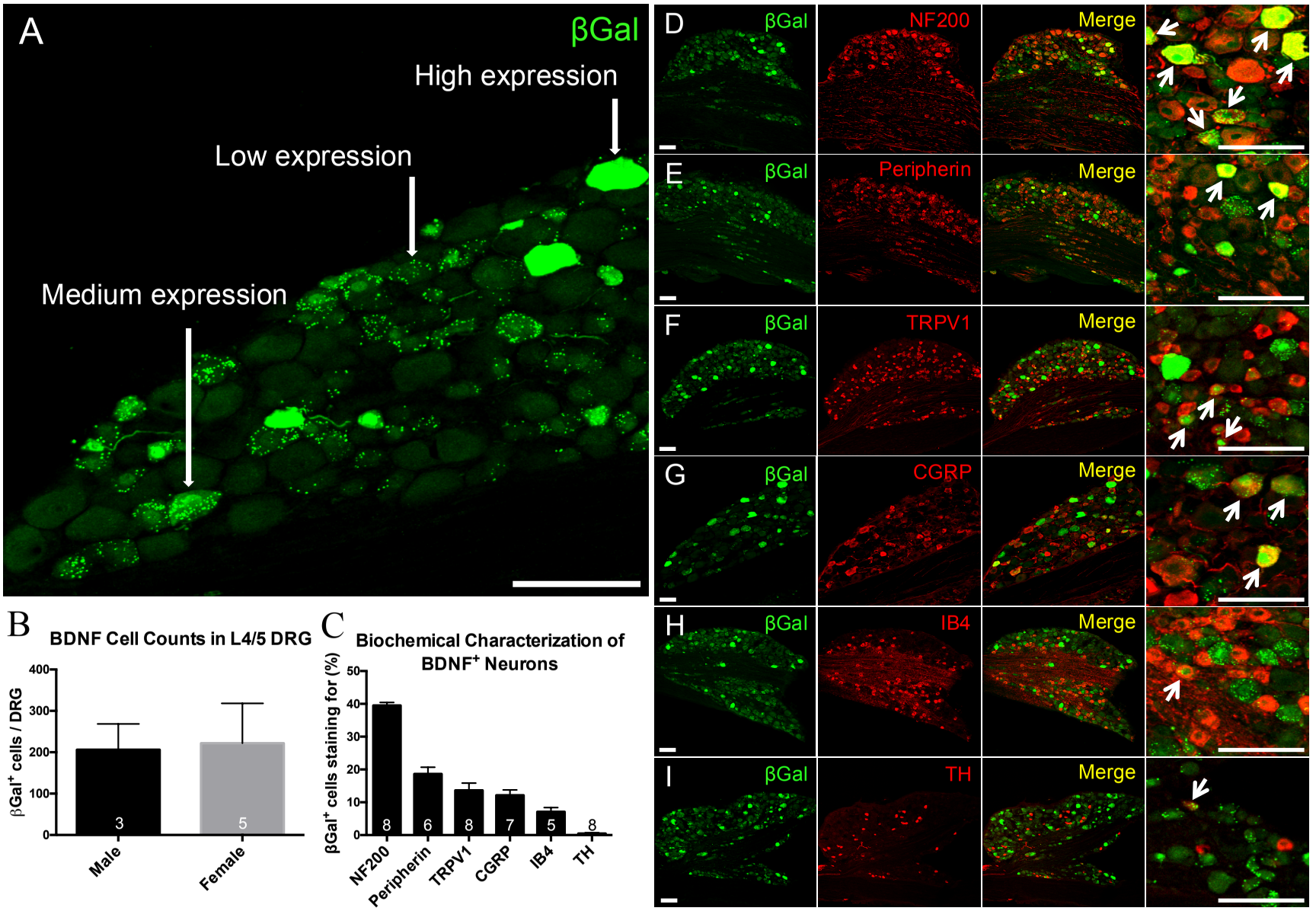
All genotyping parameters followed the instructions listed on the Jackson Laboratories website. The primer sets used included: *Bdnf*$^{LacZ/+}$ (GGTCTGAAATTACAAGCAGATGG and TGTCCGTGGACGTTTACTTCT); Advillin-CreERT2 (GCGGTCTGGCAGTAAAAACTATC and GTGAAACAGCATTGCTGTCACTT); *Bdnf*$^{fl/fl}$ (TGTGATTGTGTTTCTGGTGAC and GCCTTCATGCAACCGAAGTATG).

Imaging methods

All images were taken on an LSM 700 confocal microscope (Zeiss) equipped with 405-nm (5-mW fiber output), 488-nm (10-mW fiber output), 555-nm (10-mW fiber output), and 639-nm (5-mW fiber output) diode lasers using a 20x Plan-Apochromat (20x/0.8) objective (Zeiss). Image acquisition was done with ZEN 2010 (Zeiss), and image dimensions were 1,024 × 1,024 pixels with an image depth of 12 bits. Two times averaging was applied during image acquisition. Laser power and gain were adjusted to avoid saturation of single pixels. Adjustment of brightness/contrast and changing of artificial colors (LUT) were done in Fiji/ImageJ. The same imaging parameters and adjustments were used for all images within an experiment.

Cell Counting

For cell counting of DRG neurons, we collected 12-μm cryosections of the L4/5 DRG from at least three animals per group. The sections were directly mounted on Superfrost microslides. To avoid double counting of the same cell, we mounted, immunostained, and counted neurons in every fifth section of each ganglion. ATF3-immunoreactive neurons were identified using the particle analyzer function in ImageJ. To quantify the percentage of βGal-immunoreactive DRG neurons that co-expressed NF200, peripherin, CGRP, TRPV1, IB4, and TH, we counted at least 100 βGal-positive neurons for each mouse, and calculated the percentage of double-labeled neurons (βGal / neuronal marker). To analyze nerve injury-induced microglia responses in the dorsal spinal cord, we quantified the total Iba1 intensity in the dorsal horn of the spinal cord using the 3 most intensely stained sections from each mouse. At least 3 mice per condition were analyzed.


A
βGal
High expression
Low expression
Medium expression
B
BDNF Cell Counts in L4/5 DRG
βGal+ cells / DRG
400
300
200
100
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3
5
Male
Female
C
Biochemical Characterization of BDNF+ Neurons
βGal+ cells staining for (%)
50
40
30
20
10
0
8
6
8
7
5
8
NF200
Peripherin
TRPV1
CGRP
IB4
TH
D
βGal
NF200
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βGal
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Figure 1. BDNF expression in the DRG using a BDNF-LacZ reporter. (A) βGal expression was found in a heterogeneous population of primary afferent neurons. Levels of expression varied, with some cells completely filled by βGal (high expression), others with punctate nuclear and cytoplasmic staining (medium expression), and some with only punctate cytoplasmic staining (low expression). (B) The number of βGal-positive cells was comparable in DRGs from male and female mice. (C) By counterstaining with a panel of biochemical markers, we determined that almost 40% of BDNF-positive neurons expressed NF200 (D), a marker of sensory neurons with myelinated axons. Approximately 20% of BDNF cells expressed peripherin (E), a marker of most small-diameter, unmyelinated afferents. Similarly, about 14% of BDNF-positive neurons expressed TRPV1 (F) and 12% expressed CGRP (G), markers of small-diameter, peptidergic nociceptors. IB4 (H), a marker of unmyelinated, non-peptidergic nociceptors, was expressed in about 7% of BDNF neurons. Finally, there was almost no overlap (0.5%) with (I) tyrosine hydroxylase (TH), a marker of small-diameter, low-threshold, mechanosensitive afferents. Data are means ± SEM. Statistical significance was determined by Student's t-test. Arrows in magnified merged images indicate cells with overlap between βGal and other markers. Numbers of animals are indicated in histograms. Scale bars = 100 μm.

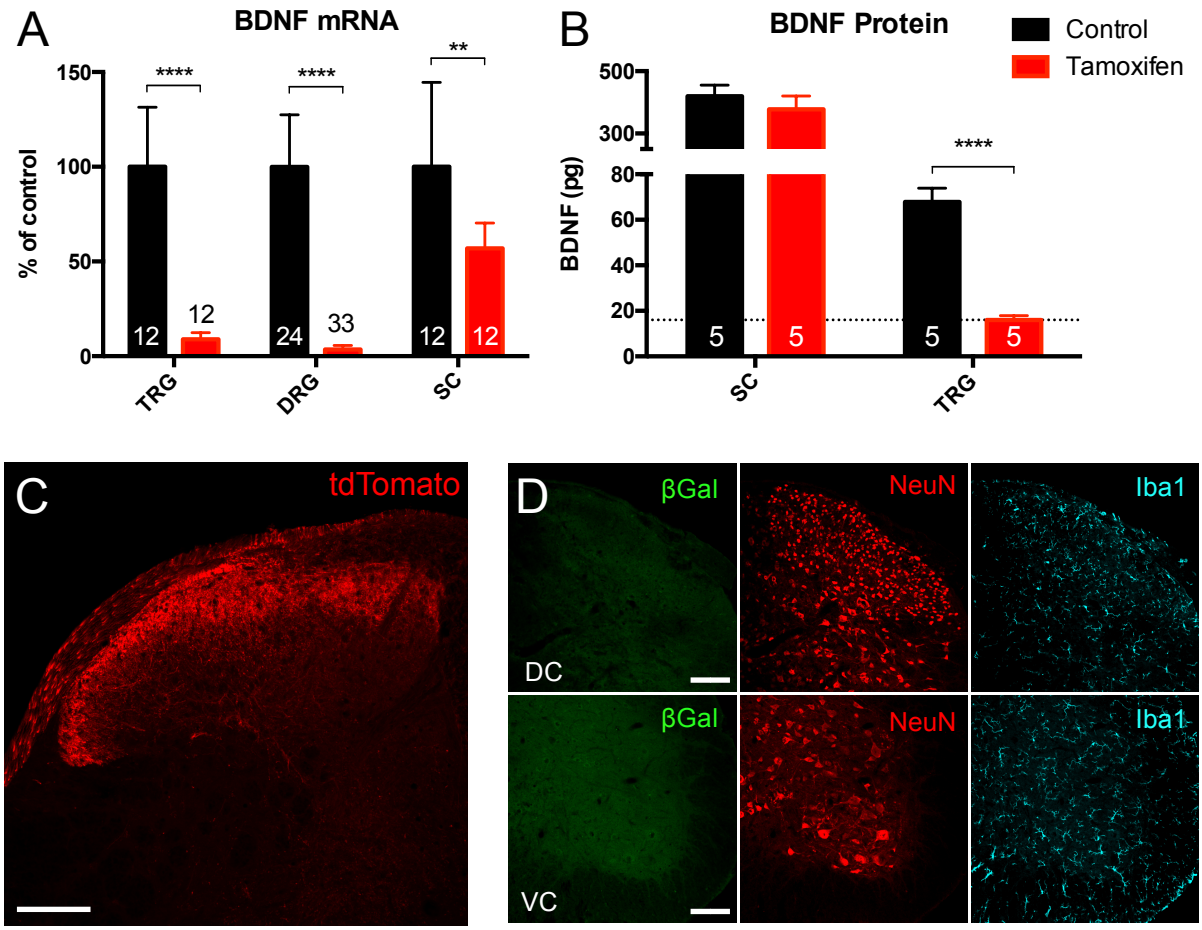

Figure 2. Primary afferent-specific deletion of BDNF using Advillin-CreERT2 mice. (A) qRT-PCR showed highly significant depletion of BDNF in TRG (91%) and DRG (97%) after tamoxifen treatment, compared to control animals treated with vehicle. Surprisingly, the lumbar enlargement of the spinal cord (SC) also showed a significant decrease in BDNF mRNA (43%). (B) However, ELISA showed that BDNF peptide was only reduced in primary sensory ganglia (TRG), not in the spinal cord. The dotted line indicates the detection limit of the ELISA, with values below this line statistically indistinguishable from zero. (C) Consistent with this finding, after crossing Advillin-CreERT2 animals with a tdTomato reporter line and treating with tamoxifen, we only detected tdTomato immunoreactivity in primary afferent terminals in the

spinal cord dorsal horn. (D) Furthermore, using the BDNF-LacZ reporter, we did not detect β-gal staining in the dorsal (DC) or ventral (VC) spinal cord. Neuronal (NeuN) and microglial (Iba1) staining in the spinal cord was intact in these animals. Together, these results suggest that Advillin-CreERT2 does not drive Cre expression in the spinal cord. Data are means ± SEM. Statistical significance was determined by multiple t-tests with Bonferroni correction for multiple comparisons; **$p < .01$, ****$p < .0001$. Numbers of animals are indicated in histograms. Scale bars = 100 μm.

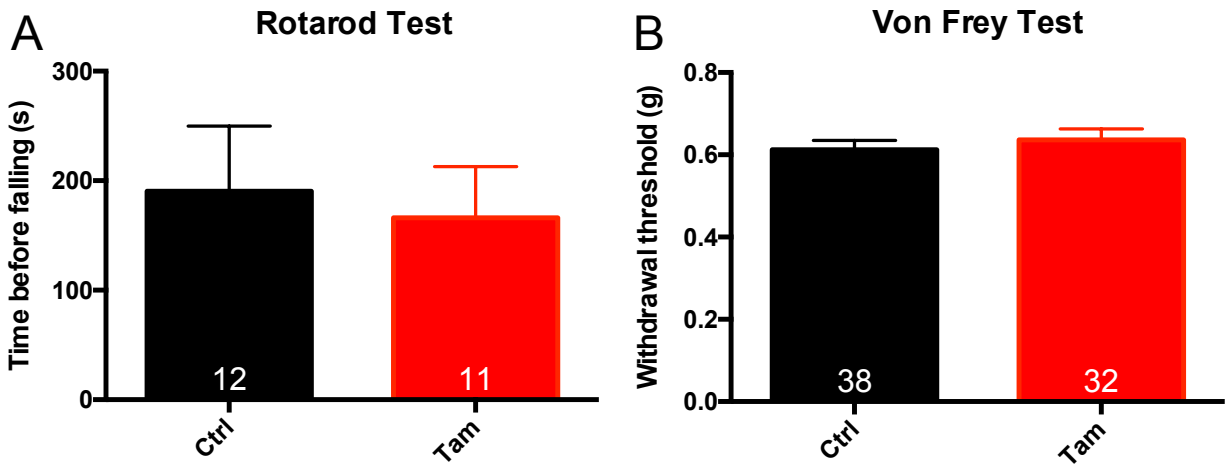

Figure 3. Baseline tests of motor and mechanical responsiveness after sensory neuron deletion of BDNF. BDNF cKO mice (Tam) displayed normal behavior compared to vehicle-injected controls (Ctrl) on the (A) rotarod test of motor behavior and the (B) Von Frey test of mechanical thresholds. Data are means ± SEM. Statistical significance was determined by Student's t-test. Numbers of animals are indicated on histograms.

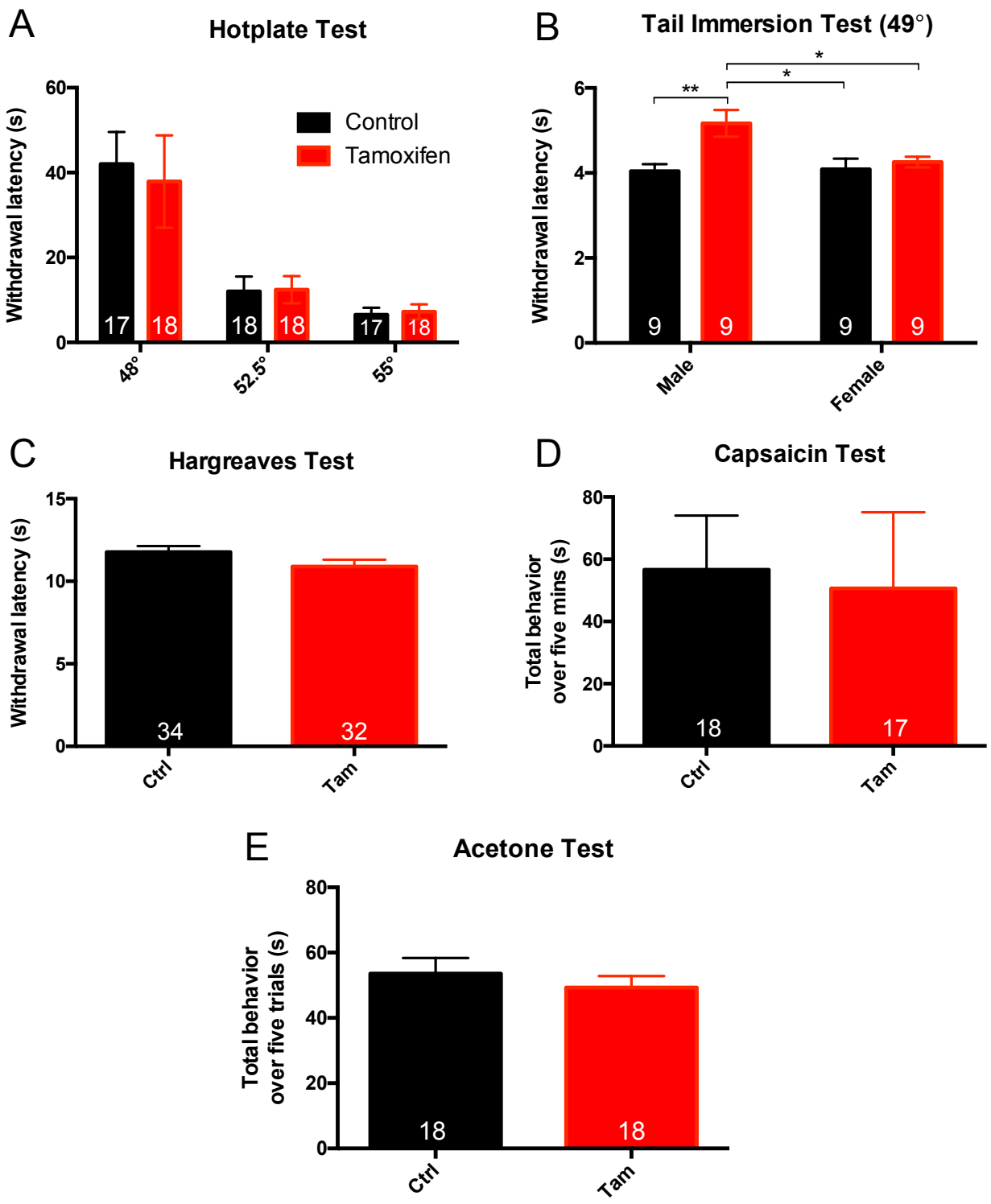

Figure 4. Baseline tests of thermal responsiveness after sensory neuron deletion of BDNF.

(A) BDNF cKO mice responded normally on the hotplate test. (B) Male knockout mice showed a

significant hyposensitivity in the tail immersion test compared to all other groups. However, all knockout mice responded normally in the Hargreaves (C), capsaicin (D), and acetone tests (E). Data are means ± SEM. For (A) statistical significance was determined by multiple t-tests with Bonferroni correction for multiple comparisons. For (B), statistical significance was determined by two-way ANOVA with Bonferroni correction for multiple comparisons; *$p < .05$, **$p < .01$. For (C-E), statistical significance was determined by Student's t-test. Numbers of animals are indicated on histograms.

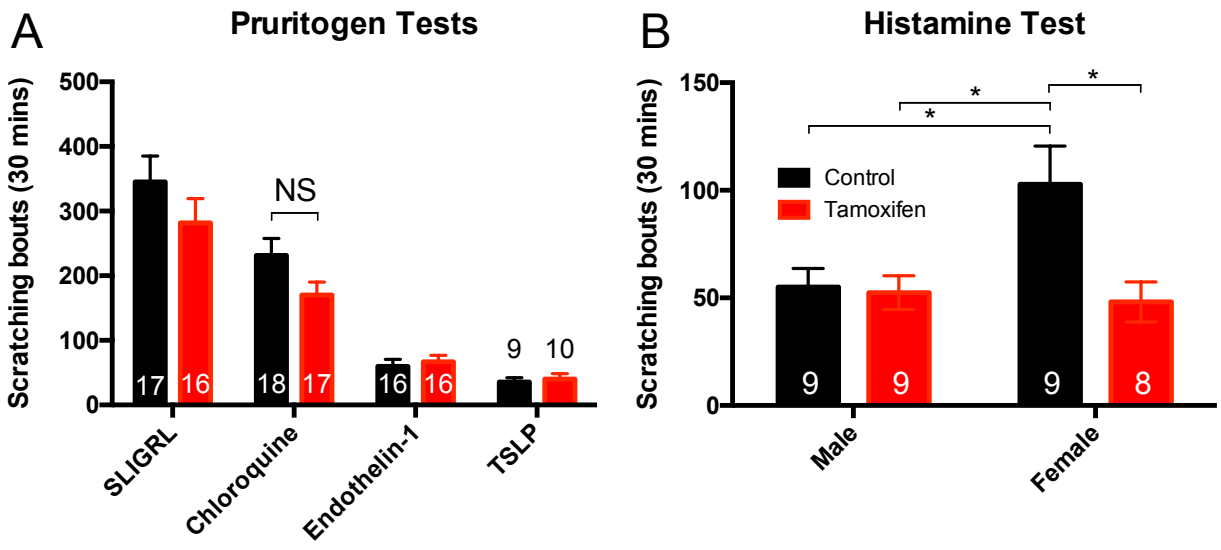

Figure 5. Baseline tests of pruritogen responsiveness after sensory neuron deletion of BDNF. (A) BDNF cKO mice scratched normally in response to nape of the neck injections of pruritogens, including SLIGRL, chloroquine, endothelin-1 and TSLP. (B) Female mice showed statistically elevated scratching to histamine compared to vehicle-injected control animals, but these responses disappeared after tamoxifen treatment. Data are means ± SEM. For (A), statistical significance was determined by multiple t-tests with Bonferroni correction for multiple comparisons. For (B), statistical significance was determined by two-way ANOVA with Bonferroni correction for multiple comparisons; *$p < .05$. Numbers of animals are indicated on histograms.

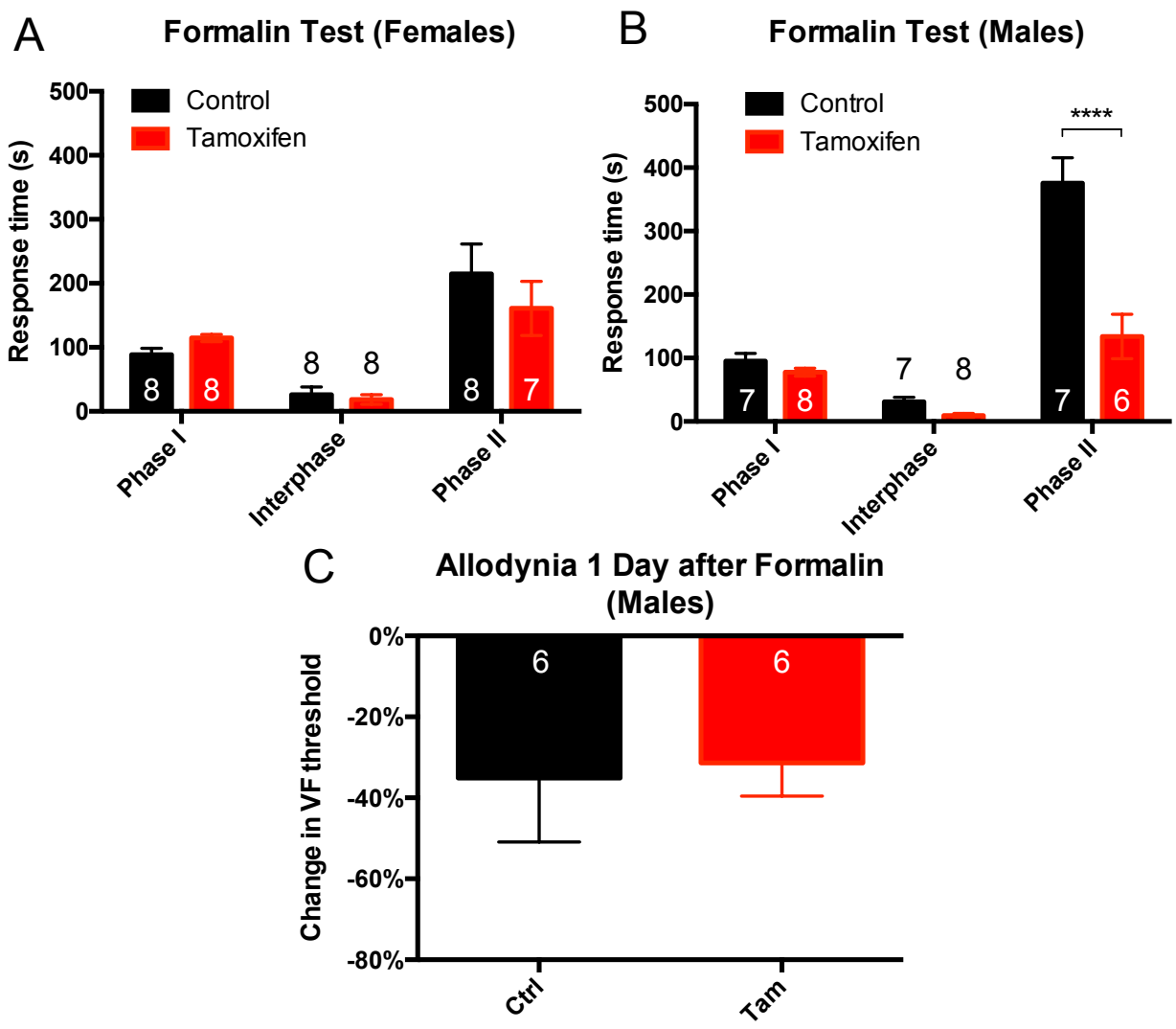

Figure 6. Sexually dimorphic formalin responses after sensory neuron deletion of BDNF. (A) Female BDNF cKO mice did not respond differently from vehicle-injected controls during any of the three phases of the formalin test**.** (B) Male BDNF cKO mice showed a large, highly significant decrease in nocifensive behavior during the second phase of the formalin test. (C) Despite the dramatic reduction in behavior during a phase of the formalin test typically thought to represent central sensitization in the spinal cord, control and cKO male mice developed comparable mechanical allodynia 24 hours after the formalin injection. Data are means ± SEM.

For (A-B), statistical significance was determined by multiple t-tests with Bonferroni correction for multiple comparisons; ****$p < .0001$. For (C), statistical significance was determined by Student's t-test. Numbers of animals are indicated on histograms.

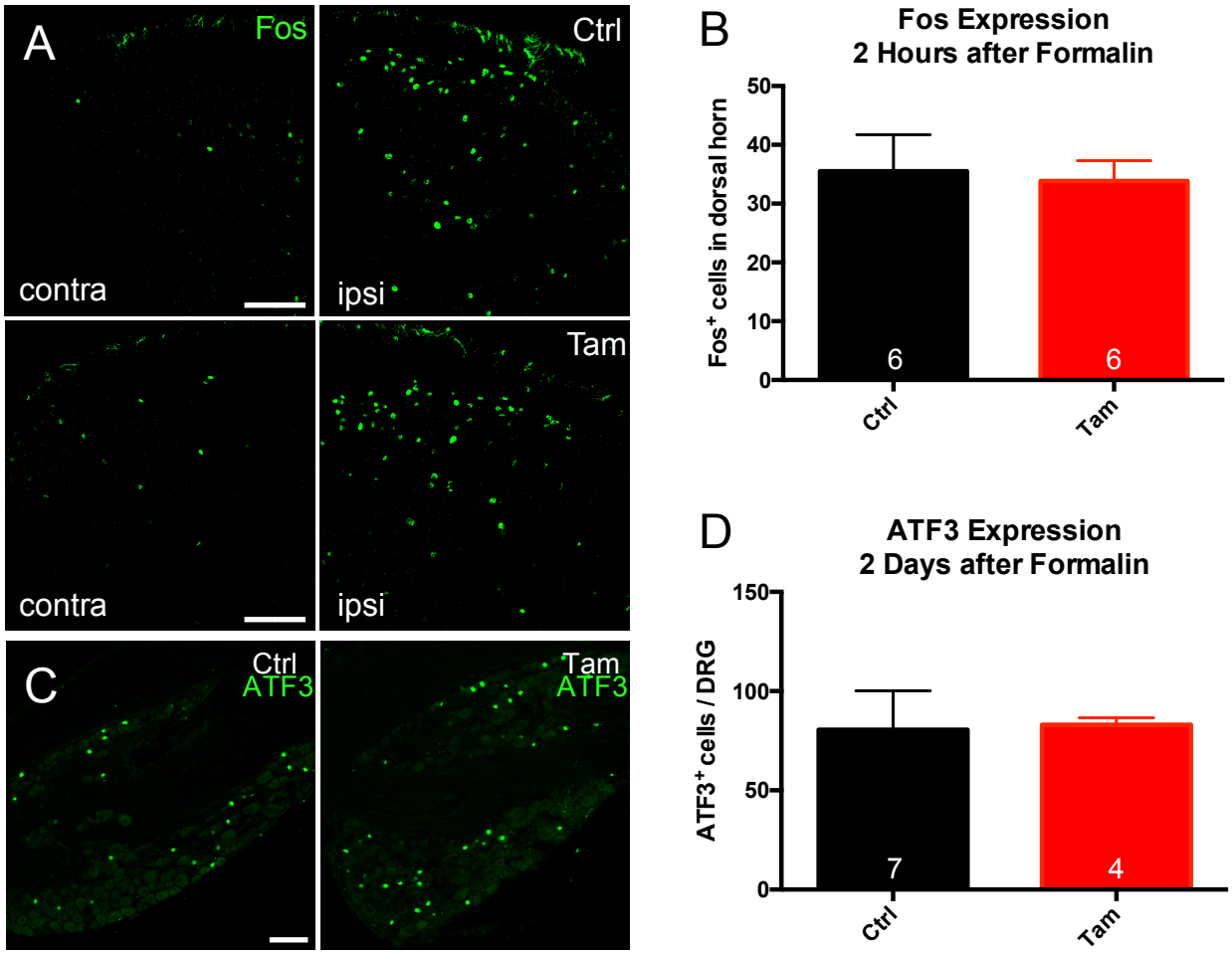

Figure 7. Formalin-induced Fos and ATF3 expression male BDNF-cKO mice. (A) Two hours after hindpaw formalin, tamoxifen- and vehicle-treated mice showed elevated Fos staining in the dorsal horn ipsilateral to the injected paw. (B) However, the number of Fos^+ cells did not differ between the two groups. (C) Both groups also showed elevated ATF3 expression, a marker of injured peripheral afferents, in L4/5 DRGs 2 days after the formalin injection. (D) However, the number of $ATF3^+$ neurons was comparable in tamoxifen- and vehicle-treated animals. Data are means ± SEM. Statistical significance was determined by Student's t-test. Numbers of animals are indicated on histograms. For (A) scale bars = 50 μm. For (C), scale bar = 100 μm.

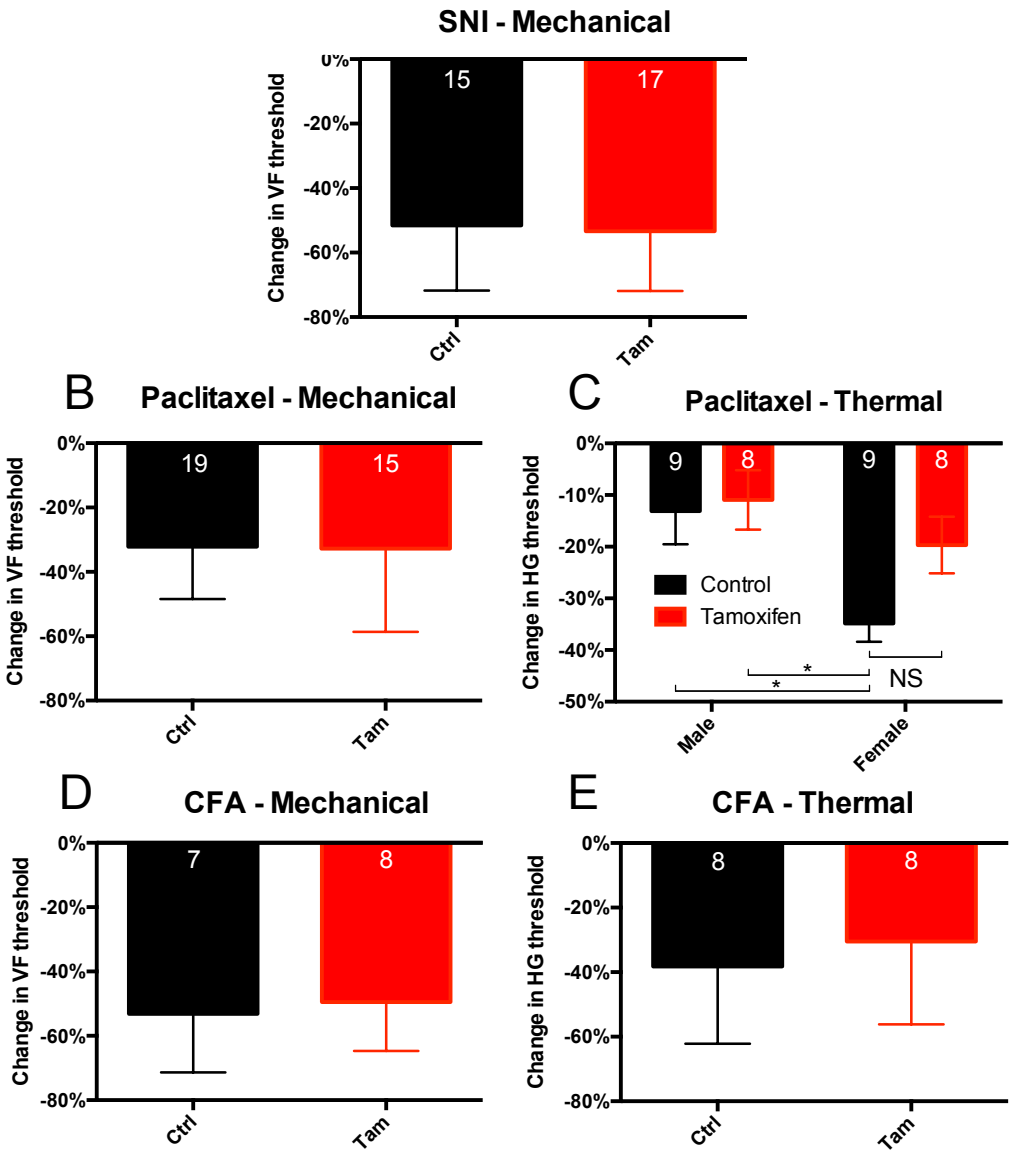

Figure 8. Tests of chronic inflammatory and neuropathic pain after sensory neuron deletion of BDNF. (A) BDNF-cKO animals developed normal mechanical allodynia one week after SNI and (B) 13 days after a course of paclitaxel (measured by Von Frey; VF). (C) Unexpectedly, female control mice developed greater thermal (heat) hypersensitivity 12 days after paclitaxel compared to male mice (measured by Hargreaves; HG). While this hypersensitivity decreased in BDNF cKO mice, the difference was not statistically significant (p

> .05). (D) Mechanical allodynia and (E) thermal hypersensitivity also developed normally in BDNF cKO mice 3 days after hindpaw injection of CFA, which creates a model of chronic inflammation. Data are means ± SEM. For (A, B, D-E), statistical significance was determined by Student's t-test. For (C), statistical significance was determined by two-way ANOVA with Bonferroni correction for multiple comparisons; *p < .05. Numbers of animals are indicated on histograms.

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