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Molecular Mechanisms of Itch Sensations

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MOLECULAR MECHANISMS OF ITCH SENSATIONS

by

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ABSTRACT OF THE DISSERTATION

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Itch is a sensation which causes scratching response to protect skin against external harmful reagents. Acute itch arises in short time period after insect bites or allergen contact. Chronic itch happens in various skin diseases such as atopic dermatitis (AD) and psoriasis. Chronic itch usually continues for over six weeks and is resistant to commonly used anti-histamine drugs. While pain has caught an early attention due to its severity, the molecular mechanism of itch has not been studied in detail. Itch and pain share common features because they are transmitted by similar neuronal circuits. However, itch is a distinct somatosensation and has opposite characteristics to pain. Itch elicits scratching but pain causes withdrawal reflex. Interestingly, minor pain induced by scratching reduces itch and suppression of pain by opioid analgesics such as morphine

elicits itch as a side effect. Despite recent advances, our understanding about itch sensory mechanisms is still primitive.

Gastrin-releasing peptide receptor (GRPR) was identified as the first itch-specific receptor in the spinal cord. GRPR cell ablation by bombesin-saporin abolished general scratching responses to pruritogens. Because GRPR mutant mice still have remained itch responses, it is possible that additional itch mediators are present in the spinal GRPR neurons. Differential screening and pharmacological or small interfering RNA (siRNA) approach identified four histamine-independent (Atp2a1, Pld3, Pacsin3 and Itpr3), three histamine-dependent (S100a8, CALR and DSP) and one both-dependent (TRPV4) itch genes. Atp2a1 and Itpr3 were selected for further characterization. Atp2a1 and Itpr3 are involved in GRPR downstream Ca^{2+} signaling pathway for histamine-independent itch.

GRPR is also expressed in peripheral tissues such as dorsal root ganglia (DRG) and skin in addition to the spinal cord. Gastrin-releasing peptide (GRP) intradermal (i.d.) injection induces dose-dependent scratching responses that are abolished in GRPR mutant mice. Peripheral (intraperitoneal (i.p.) and i.d.) application of GRPR antagonists inhibits chloroquine (CQ), GRP and allergic contact dermatitis (ACD)-induced scratching responses in mice. It is interesting to find peripheral GRPR functions for itch sensation because it will provide an alternative therapeutic route for itch by targeting peripheral GRPR activities rather than spinal cord GRPR. This also can circumvent potential side effects in central nervous system (CNS) by intrathecal (i.t.) injections.

TRPV4 was identified as a crucial itch mediator in DRG for both histamine-dependent and -independent itch by siRNA screening. TRPV4 is co-expressed with

multiple pruritic genes such as GRP, transient receptor potential vanilloid 1 (TRPV1), mas-related G-protein coupled receptor A3 (MrgprA3) and histamine receptor 1 (H1R). $TRPV4^{-/-}$ mice showed reduced scratching responses in CQ and histamine-induced pruritic models. siRNA i.t. injection efficiently reduced TRPV4 expression in DRG and decreased both types of itch consistently. i.t. application of endogenous TRPV4 agonist, 5, 6-epoxyeicosatrienoic acid (5, 6-EET), caused scratching responses that were significantly attenuated in $TRPV4^{-/-}$ mice. Interestingly, $TRPV4^{-/-}$ mice had reduced scratching responses in ACD mice model without affecting on skin inflammation. TRPV4 expression was up-regulated in ACD mice DRG suggesting that an increased TRPV4 activity may be responsible for the aggravated chronic itch. Ca^{2+} imaging with DRG culture further confirmed that TRPV4 activation is functionally involved in itch sensory transmission in peripheral neurons. TRPV4 in DRG will be a good candidate for treatment of ACD-related chronic itch.

Characterization of GRPR-mediated itch mechanisms and identification of novel itch-specific genes will provide basic understanding about itch sensory mechanism and pioneering work for future itch studies such as identifying potential itch therapeutic targets.

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ABBREVIATIONS

GRP : Gastrin-releasing peptide

GRPR : GRP receptor

NMB : Neuromedin B

NMBR : NMB receptor

GPCR : G protein-coupled receptor

PLC : Phospholipase C

IP_3 : Inositol 1, 4, 5-trophosphate

CQ : Chloroquine

i.t. : intrathecal

i.d. : intradermal

i.p. : intraperitoneal

LCM : Laser capture microdissection

siRNA : small interfering RNA

DBHQ : 2, 5-Di-tert-butylhydroquinone

TG : Thapsigargin

Atp2a1 : ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1

SERCA : sarco/endoplasmic reticulum Ca^{2+}-ATPase

Pld3 : Phospholipase D family, member 3

Pacsin3 : Protein kinase C and caseine kinase substrate in neurons 3

Itpr3 : IP3 receptor, type 3

S100a8 : Calcium-binding protein A8 (Calgranulin A)

CALR : Calreticulin

DSP : Desmoplakin

DCP : Diphenylcycloprenone

DNFB : 2, 4-dinitroflurobenzene

CD : Contact dermatitis

ICD : Irritant CD

ACD : Allergic CD

AEW : Aceton/Ether/Water

TRP : Transient receptor potential

TRPV4 : TRP vanilloid 4

5, 6-EET : 5, 6-epoxyeicosatrienoic acid

MrgprA3 : mas-related GPCR A3

H1R : Histamine receptor 1

AD : Atopic dermatitis

LN : Lymph node

SPL : Spleen

IgE : Immunoglobulin E

WBC : White blood cell

TSLP : Thymic stromal lymphopoietin

I. GENERAL INTRODUCTION

1. Itch

Itch or pruritus is defined as an unpleasant sensation that elicits desire or reflex to scratch (Rothman 1941; Stander, Steinhoff et al. 2003). Itch is a defensive sensory mechanism protecting the skin against harmful reagents and is conserved in various animal species. Acute itch is commonly elicited by skin contact with pruritic agents or allergens, while chronic itch often accompanies pathological skin conditions such as atopic dermatitis (AD) or xerosis as well as systemic disorders such as liver and kidney disease (Nojima, Carstens et al. 2003). Chronic itch lasts longer than acute itch and is often resistant to treatment with anti-histamine drugs. Thus, it has been one of the major problems in relieving patient symptoms in itch therapy. Itch is classified into four categories based on clinical aspects. First, pruritoceptive itch originates in the skin due to inflammation, dryness, or other skin damage. This type of itch information is transmitted by unmyelinated C nerve fibers. Second, neuropathic itch arises because of lesions located at the afferent pathway. Third, neurogenic itch is defined as that which originates centrally without neural pathology. Fourth, psychogenic itch is found in delusional states such as parasitophobia (Greaves and Khalifa 2004). Although recent findings have provided interesting insights into itch sensation, the molecular mechanism of itch has not been well investigated.

Pruritic information is known to be transmitted to the spinal cord via DRG. Spinal cord dorsal horn gray matter has multiple lamina layers **(Fig. 1)**. Lamina I neurons are

important components of somato-sensory circuits required for relaying nociceptive, temperature and itch signals from the periphery to the brain (Stander, Steinhoff et al. 2003). These neurons comprise the spinothalamic tract that transmits inputs from primary afferents and project to the contralateral thalamus. There is a subpopulation of spinothalamic neurons that are selectively excited by histamine and transmit pruriceptive information through a dedicated neuronal pathway. In the spinal cord of cats, histamine-induced itch sensation was found to be relayed by a subset of lamina I neurons, which were insensitive to mechanical and thermal stimuli. These lamina I neurons project to the posterior part of the ventromedial thalamic nucleus via the spinothalamic tract, which in turn projects to the dorsal insular cortex (Andrew and Craig 2001).

1.1. Histamine-dependent itch

Histamine has been studied most thoroughly among the known pruritogens. Tissue inflammation and allergen contacts induce mast cell degranulation and histamine release (Rowley and Benditt 1956). Histamine-induced itch is accompanied by a wheal, caused by the enhanced permeability of blood vessels, and a flare that is produced by an axonal reflex (Sikand, Dong et al. 2011). Histamine-induced responses are mediated by histamine receptors which are members of G protein-coupled receptors (GPCRs). Four histamine receptor subtypes (H1R-H4R) have been identified (Hill, Ganellin et al. 2002). H1R has been studied most extensively for itch sensation. H1R is coupled with Gαq proteins and activates phospholipase C (PLC) (Bakker, Timmerman et al. 2002). Activation of H1R increases calcium levels in cultured sensory neurons and this is

blocked by a PLC inhibitor, U73122 (Nicolson, Bevan et al. 2002). PLCβ3 was found to mediate histamine-induced Ca^{2+} response via H1R in sensory neurons (Han, Mancino et al. 2006). H1R signaling also stimulates phospholipase A2 (PLA2), lipoxygenase (LOX) and TRPV1 pathways resulting in neuronal excitation (Kim, Lee et al. 2004; Shim, Tak et al. 2007). Although H1R antagonists (anti-histamines) are widely used to alleviate itch symptoms, the therapeutic efficacies of anti-histamines are limited because they cannot attenuate several types of chronic itch such as AD and psoriasis. This raises questions about potential roles of the other histamine receptors or histamine-independent pathways for chronic itch.

1.2. Histamine-independent itch

Although histamine is a strong pruritic agent, it is not involved in many types of chronic itch diseases as shown by the weak therapeutic efficacy of anti-histamines (Rukwied, Lischetzki et al. 2000). Two recent studies found the existence of a histamine-independent cowage-related itch pathway in lamina I neurons of the spinal cord (Davidson, Zhang et al. 2007; Johanek, Meyer et al. 2007).

Cowhage induces robust itch-scratching responses when injected into skin. Mucunain, a cysteine protease in cowhage, was recently identified as a ligand for protease activating receptor 2 (PAR2) and 4 (PAR4) (Reddy, Iuga et al. 2008; Davidson and Giesler 2010). Cowhage-induced itch was not attenuated by anti-histamine suggesting that cowhage is a non-histaminergic pruritogen (Shelley and Arthur 1955). Whereas histamine activates C fibers that are mechanically insensitive, cowhage activates

mechanically sensitive C fibers (Schmelz, Schmidt et al. 1997; Johanek, Meyer et al. 2007; Johanek, Meyer et al. 2008; Namer, Carr et al. 2008). Unlike histamine, cowhage-mediated itch does not produce weal, flare and redness (Johanek, Meyer et al. 2007). i.d. injection of histamine induced c-Fos expression in the lamina II neurons while PAR2 agonist, SLIGRL-NH2 caused c-Fos expression in the region located dorsal to the lamina II suggesting that there are distinct spinal pathways between histamine and PAR2-mediated itch (Nakano, Andoh et al. 2008). However, a recent electrophysiological study has shown a functional overlap of both pathways (Akiyama, Carstens et al. 2009).

CQ is an anti-malaria drug that has side effects in black Africans with generalized pruritus (Osifo 1984). CQ-induced itch can not be treated with anti-histamines suggesting that CQ is a histamine-independent pruritogen (Inan and Cowan 2004; Green, Young et al. 2006). i.d. injection of CQ causes robust scratching responses in mice. Therefore, CQ provides a reliable histamine-independent itch model in mice. Recent report showed that targeted disruption of mas-related G-protein coupled receptors (Mrgprs) gene cluster reduced CQ-induced itch responses but not histamine-induced itch (Liu, Tang et al. 2009). Mrgprs genes in mouse can be grouped into several subfamilies. MrgprAs, MrgprB5, MrgprC11 and MrgprD are expressed in subsets of small diameter DRG and trigeminal ganglia neurons (Liu, Tang et al. 2009). Among the Mrgprs family, MrgprA3 was identified as the crucial receptor for CQ-induced itch. MrgprA3^{+} DRG neurons also express GRP, which is a ligand of GRPR. This suggests that activation of MrgprA3 by CQ may cause a GRP release from peripheral DRG stimulating GRPR neurons in the spinal cord for itch sensation (Liu, Tang et al. 2009). MrgprA3 homologous receptor,

human MrgprXs are also expressed in DRG suggesting that Mrgprs may have some conserved roles in sensory neurons mediating itch. The proteolytically cleaved product of pro-enkephalin A, the bovine adrenal medulla peptide 8-22 (BAM 8-22), potently activates human MrgprX1 (Lembo, Grazzini et al. 2002) and induces scratching in human by histamine-independent manner (Sikand, Dong et al. 2011).

Both itch and pain can be elicited by multiple mechanical and chemical stimuli through similar neuronal populations. Inhibition of pain pathways resulted in enhanced itch suggesting that pain has antagonistic effects on itch sensation (Lagerstrom, Rogoz et al.; D'Hoedt, Owsianik et al. 2008). Although several studies have provided critical components for itch pathways, the identity of neural circuit transmitting itch has yet to be clearly defined. Also, how itch pathways are related with pain is still unclear. Therefore, it will be important to find out molecular mechanisms of DRG and spinal cord-mediated pruritic pathways to understand overall itch sensation.

1.3. Chronic itch

Chronic itch is defined as an itch that lasts more than six weeks (Pogatzki-Zahn, Marziniak et al. 2008; Stander, Raap et al. 2011). Multiple causes can bring itchy conditions. External factors that cause a chronic itch include contact allergens and dryness. Internal factors can be allergies from food ingestion as well as infectious conditions such as psoriasis. Genetic factors also can be involved in chronic itch diseases such as AD.

1.3.1. Atopic dermatitis (AD)

AD is a pruritic inflammatory skin disease associated with a personal or familial allergic history. About 17% of people in USA have AD and its onset is increasing (Laughter, Istvan et al. 2000). The skin lesion in AD results from a defective skin barrier that induces dry itchy skin, and is aggravated by mechanical injury due to continuous scratching. This allows antigens to enter via the skin and causes immune responses. Alternatively, the inherited primary abnormality in the immune system can causes a secondary defect in skin barrier function (Oyoshi, He et al. 2009). AD has no known cure and the condition becomes worse as the itching continues. Thus, it will be important to prevent the continuous scratching in AD to relieve the chronic itch symptoms.

1.3.2. Contact dermatitis (CD)

CD is an inflammatory skin condition induced by exposure to an environmental agent. Contact allergens are non-protein chemicals named haptens with small molecular weight (Gunther, Hempel et al. 2007). There are two major types of CD, irritant CD (ICD) and allergic CD (ACD). ICD is a pro-inflammatory response due to toxic effects of reagents activating skin innate immunity. ACD requires antigen-specific acquired immunity with T cell causing skin inflammation (Nosbaum, Vocanson et al. 2009). The development of ACD has two distinct steps. *Sensitization (Induction)* phase occurs at the first contact of skin with haptens and generates antigen-specific T cells in the lymph node (LN). The sensitization step lasts for 10-15 days in man, and 5-7 days in mouse. This step has no clinical relevance. Repeated exposure to the same haptens leads to the *elicitation*

(challenge) phase. Specific T cells are activated in the dermis and trigger inflammatory process responsible for the skin lesions. This step takes 72 hours in man, and 24-48 hours in mouse (Saint-Mezard, Rosieres et al. 2004). CD is closely related with our daily life because common causes of ACD include metals (e.g., nickel), cosmetics, drugs and plants (poison ivy and oak) (Krasteva, Kehren et al. 1999).

1.3.3. Dry skin (Xerosis)

Dry skin is caused by disrupting skin barrier function in stratum corneum, reducing water-holding capacity. Xerosis is most common in elderly people because of decreases in skin vascular supply and changes in epidermal lipid composition. However, it can be caused by drugs and environmental factors. Frequent use of soaps, detergents, and irritant topicals such as alcohol and hot water can induce dry skin itch. Extremely cold dry weather during winter season and rapid shift from dry environment to humid conditions also cause skin dryness and itch (Yosipovitch 2004). The mechanism of dry skin pruritus is still unclear and effective treatments for the pruritus have not been established.

Our knowledge about chronic itch is still in its infancy. Causes and therapies of most chronic itch diseases are unknown. It is important to know what makes the itch become chronic because it will be helpful in the development of treatments. Millions of people are suffering from intractable itch currently and it is necessary to uncover molecular pathways that are involved in chronic itch. This will provide a primary clue to solve the medical problems associated with chronic itch diseases.

2. Bombesin/GRP and GRPR

2.1. Bombesin and GRP

Bombesin is a tetradecapeptide originally isolated from frog skin. It induces dose-dependent long-lasting scratching when administered intracerebroventricularly (i.c.v.) to rats (Gmerek and Cowan 1983). Pruritus induced by bombesin is not antagonized by anti-histamines or opioid antagonists (naloxone) but relieved by opioid analgesics of the benzomorphan class, which suggests an association with the opioid system (Stander, Steinhoff et al. 2003). GRP was the first identified mammalian bombesin-like peptide. The second mammalian bombesin-like peptide, neuromedin B (NMB) was purified from porcine spinal cord (Minamino, Kangawa et al. 1983). GRP and NMB have been found in birds, fish, reptiles and mammals, suggesting some conserved functions of bombesin-like peptides among the various animal species. GRP and NMB are expressed in the brain and gastrointestinal tissues, and they mediate a variety of physiological functions including thermoregulation, metabolic regulation and gastric secretion (Ganter and Pittet 2006).

GRP is expressed in a subset of small and medium-sized DRG neurons. GRP is co-localized with peptidergic markers, calcitonin gene-related peptide (CGRP) and substance P (SP), but not with isolectin B4 (IB4), a nonpeptidergic marker, and neurofilament 200 (NF200), myelinated marker showing that GRP is expressed in peptidergic unmyelinated DRG neurons. Approximately, 80% of GRP neurons co-express TRPV1, which is an important channel for detecting thermal and chemical

stimuli in DRG. In the spinal cord, GRP afferent fibers are restricted to the lamina I and II outer layers (Sun and Chen 2007).

2.2. GRPR

GRPR is a mammalian homologue of the amphibian bombesin-like peptide receptor, and it has been implicated in various physiological processes and behaviors, including food intake and fear memory (Battey and Wada 1991; Roesler, Henriques et al. 2006). GRPR is a seven trans-membrane Gαq-protein coupled receptor and activation of GRPR increases intracellular Ca^{2+} through PLC/inositol-1, 4, 5-triphosphate (IP_3) pathway (Ally, Ives et al. 2003). GRPR was identified as the first itch-mediating receptor in the spinal cord (Sun and Chen 2007). GRPR mutant mice showed significantly reduced scratching responses in compound 48/80, PAR2 agonist (SLIGRL-NH2) and CQ-induced itch models (Sun and Chen 2007). Compound 48/80 degranulates mast cells and causes histamine release (Kuraishi, Nagasawa et al. 1995). PAR2 agonist (SLIGRL-NH2) is a histamine-independent itch mediator (Steinhoff, Neisius et al. 2003; Shimada, Shimada et al. 2006). CQ causes scratching by the histamine-independent pathway because anti-histamine could not inhibit the response (Inan and Cowan 2004; Green, Young et al. 2006). The fact that histamine-induced response and pain behaviors were not changed in GRPR mutant mice (Sun, Zhao et al. 2009) strongly suggests that GRPR is a histamine-independent itch receptor and also strengthen the idea that there are itch-dedicated molecular pathways in the spinal cord.

Although the GRPR mutant mice scratch less in the itch models, the scratching

response was not completely lost. The remaining scratching behaviors of GRPR mutant mice indicate that there are additional itch genes in the spinal cord. It is very interesting and critical to identify other itch-specific molecules which will help us to uncover mechanisms underlying itch sensation.

II. Identification of novel itch genes in the mouse spinal cord GRPR neurons

; Roles of Atp2a1 and Itpr3 in GRPR-mediated itch signaling pathways

INTRODUCTION

1. Ablation of GRPR cells in the spinal cord by bombesin-saporin

GRPR mutant mice showed significantly reduced scratching behaviors caused by CQ suggesting that GRPR is a histamine-independent itch receptor. However, roles of the spinal $GRPR^+$ cells in itch transmission have not been addressed. To study GRPR expressing neuronal functions, GRPR ligand, bombesin attached to saporin toxin was used to ablate GRPR cells in the spinal cord.

Saporin is a toxic protein that is useful in biological research applications. It is a ribosome-inactivating protein (RIP) from the seeds of Saponaria officinalis (common name: soapwort). Its N-glycosidase activity inactivates the ribosomes, shutting down protein synthesis and resulting in cell death. The enzymatic activity of the saporin is highly specific. It removes a single adenine base from the ribosomal RNA in the large subunit of the ribosome, which completely blocks the ribosome to participate in protein synthesis (Stirpe, Gasperi-Campani et al. 1983). Attachment of the saporin to ligands enables the toxin to enter specific types of cells through its cell surface receptor, which allows to remove certain cell types *in vivo.* Saporin conjugates such as SP-saporin and IB4-saporin already have been used successfully to deplete spinal cord lamina I and II neurons selectively (Mantyh, Rogers et al. 1997; Vulchanova, Olson et al. 2001). After

bombesin-saporin binding, GRPR will be internalized by endocytotic pathways. The internalized saporin will inhibit protein synthesis causing cell death **(Fig. 2)**. Specific depletion of GRPR expressing neurons provided an experimental tool to allow whole genome differential screening to identify itch specific genes in the spinal cord GRPR expressing neurons.

2. Differential screening to identify novel itch genes in GRPR neurons of the spinal cord

GRPR mutant mice showed reduced histamine-independent itch behaviors and GRPR cell depletion in the spinal cord by bombesin-saporin abolished scratching responses to a range of pruritic stimuli (Sun and Chen 2007; Sun, Zhao et al. 2009). These results suggest that GRPR mediates histamine-independent itch and GRPR neurons are critical for general itch sensation. It is also important that GRPR cell-depleted mice did not change pain behaviors strongly suggesting that GRPR cells are itch-specific neurons (Sun, Zhao et al. 2009). Because GRPR mutant mice still showed scratching responses to both histamine-dependent and -independent pruritic stimuli, it is possible that other itch mediators are also present in the GRPR expressing cells. To identify novel itch genes such as additional itch receptors or GRPR downstream signaling molecules in the GRPR neurons, differential screening experiment was designed with blank- and bombesin-saporin injected mice spinal cord. For the differential screening, mice were i.t. injected with blank- and bombesin-saporin and GRPR cell ablation was confirmed by scratching responses to pruritogens, in situ hybridization (ISH) and quantitative reverse

transcription-polymerase chain reaction (QRT-PCR) **(Fig. 2)**. Superficial layers of dorsal spinal cord were dissected by laser capture microscope (LCM). Because GRPR is expressed in a small subset of lamina I neurons, dissection of the restricted regions with GRPR cells has an advantage to reduce background or misleading results from genes which have differential expression levels in the other spinal cord regions **(Fig. 3)**. DNA microarray was performed using illumina bead array. ISH, immunostaining and QRT-PCR were used to verify candidate genes. Functional studies of the candidate itch genes were performed using pharmacological and siRNA approaches. Four histamine-independent (Atp2a1, Pld3, Pacsin3 and Itpr3), three histamine-dependent (S100a8, CALR and DSP) and one both dependent (TRPV4) genes were identified. These will provide further understanding about itch signaling mechanisms and potential therapeutic targets.

RESULTS

1. Microarray data analysis

When all the three blank- and bombesin-saporin treated samples were combined together, analysis of variance (ANOVA) test using Partek Genome Suite (Partek Incorporated, St. Louis, MO) did not show candidate genes that were changed significantly in all the bombesin-saporin treated samples. Therefore, alternative data analysis strategy was used. Because the samples were not prepared at the same time and also from different bombesin-saporin injected animals, it was reasonable to compare each

group separately. With each three blank- and bombesin-saporin treated samples, nine pairings could be generated. GRPR expression level was consistently decreased in all the bombesin-saporin treated groups among the nine pairings. Fold change of gene expression and frequency were used to choose candidate genes that had reduced expression levels in the bombesin-saporin treated samples consistently. Genes that showed same decreases in at least two pairings were selected **(Table 1)**. From 2,354 genes above cut-off value (fold change: -2, frequency: 2), 123 candidate genes encoding receptors, channels and membrane proteins were selected. QRT-PCR was performed for 40 potential interesting targets and 16 genes showed significant fold decreases in bombesin-saporin treated samples **(Fig. 4)**. To confirm the expression of 16 genes in the spinal cord dorsal horn, ISH was performed. mRNA expressions of eight genes were detected in the spinal cord **(Fig. 5)**. Four genes showed expression level decreases in the bombesin-saporin treated tissues **(Fig. 6)** suggesting that they may be expressed in GRPR neurons.

2. Functional studies of candidate genes

2.1. Pharmacological approach

Although some candidate genes were expressed less in the superficial dorsal horn after GRPR cell ablation, it is still not conclusive whether they are involved in itch pathways. Because membrane proteins such as receptors and channels were chosen as primary candidates, it is convenient to carry out pharmacological studies with specific

agonists and antagonists or inhibitors to verify that they are mediating pruritus.

Among 16 candidates, five receptor-encoding genes, Glp2r, PKR1, P2rx4, Nr3c2 and Npr1, have specific agonists and Atp2a1 has specific inhibitors. Potassium channels, Kcnj15 and Kcnq2 have multiple inhibitors but they also block many other different channels. Sodium channel, Cacna1c also has inhibitors common to the others. The other candidate genes do not have specific agonists or inhibitors.

Glucagon-like peptide 2 receptor (Glp2r)

i.t. injection of glucagon-like peptide 2 (GLP2) did not cause scratches in mice. Co-injection of GLP2-GRP was also performed to examine whether GLP2 can change GRP i.t.-induced scratches but co-injection did not change GRP-induced scratching responses, either. This result suggests that Glp2r may not be involved in itch sensation.

Prokineticin receptor 1 (PKR1)

Prokineticin 1 and 2 (PK1 and PK2) peptides were injected intrathecally. Both PK1 and 2 caused grooming, biting and licking behaviors consistent with the previous report that PKR1 mediates pain (Negri, Lattanzi et al. 2006; Negri, Lattanzi et al. 2009). However, no scratching response was detected.

Purinergic receptor P2X, ligand-gated ion channel 4 (P2rx4)

P2 purinoceptor agonist, ATP was injected into mouse spinal cord to examine whether P2rx4 is involved in itch sensation. However, it did not induce scratches.

Nuclear receptor subfamily 3, group C, member 2 (Nr3c2)

Nr3c2 synthetic activator, Fludrocortisone i.t. injections did not cause scratchings. Fludrocortisone-GRP co-injection also did not show significant difference from GRP i.t.-induced scratching responses.

Natriuretic peptide receptor 1 (Npr1)

Npr1 agonist, brain natriuretic peptide (BNP) was injected intrathecally. BNP did not cause scratching responses.

ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1 (Atp2a1)

Atp2a1 inhibitors, 2, 5-Di-tert-butylhydroquinone (DBHQ) and thapsigargin (TG) were tested with several itch models. DBHQ and TG i.t. injection inhibited GRP i.t. and CQ-induced scratches without changing histamine responses, suggesting that Atp2a1 may be involved in histamine-independent itch pathways **(Figure 7)**.

2.2. siRNA screening

Most of the candidate genes do not have specific agonists or antagonists. siRNA gene knockdown approaches provide an alternative efficient method to study certain gene functions. If agonist or antagonist also affects other off-targets, it will mask the effects from the specific gene. siRNA can be used to overcome potential problems of pharmacological tests such as specificity issues. Previous pharmacological studies identified an interesting gene, Atp2a1, which is expressed on endoplasmic reticulum (ER)

membrane and regulates intracellular Ca^{2+} concentration. This suggests that GRPR functions can be mediated by ER Ca^{2+} signaling pathways. It is also consistent with the previous reports that GRPR activates PLC-IP_3 signaling pathways (Ally, Ives et al. 2003). Therefore, 30 ER Ca^{2+}-related molecules were further examined as candidates from the microarray gene list **(Table 2)**. QRT-PCR confirmed that 12 genes were reduced in bombesin-saporin treated spinal cord **(Table 3)**. Additional 11 ER Ca^{2+}-related candidate genes were also selected for siRNA screening **(Table 4)**.

2.2.1. Histamine-independent genes: Atp2a1, Pld3, Pacsin3, Itpr3

(1) Atp2a1

Atp2a1 encodes a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA1). SERCA on ER membrane plays roles to maintain internal Ca^{2+} levels that are important for multiple signaling pathways requiring intracellular Ca^{2+} responses (Berridge, Bootman et al. 2003). GRPR is a Gαq protein-coupled receptor and activates PLC/IP_3 signaling pathways (Ally, Ives et al. 2003). IP_3 produced by PLC binds to IP_3 receptor on ER membrane and release Ca^{2+} ion from ER to increase intracellular Ca^{2+} concentration. Increased Ca^{2+} levels can activates multiple downstream signaling mediators and effectors responding to extracellular stimuli. GRPR-mediated downstream signaling mechanisms for itch sensation have not been studied well previously. Therefore, Atp2a1 was chosen from the candidate genes to investigate how GRPR-mediated itch signaling is transmitted in cellular and molecular levels.

Atp2a1 siRNA showed inhibitory effects on CQ-induced itch but not on histamine model **(Figure 8a)**. This result confirms that the previous pharmacological DBHQ and TG data were specific to Atp2a1. ISH showed that Atp2a1 mRNA expression was reduced in the Atp2a1 siRNA treated spinal cord **(Fig. 8d)**. This was confirmed again by QRT-PCR with spinal cord dorsal horn total RNA **(Fig. 8c)**. i.t. application of siRNA affects both spinal cord and DRG gene expression (Kawasaki, Xu et al. 2008; Liu, Bi et al. 2010; Vergnolle, Cenac et al. 2010). Atp2a1 siRNA also reduced Atp2a1 expression in DRG **(Fig. 8c and 9h)**. Although siRNA knockdown effect was high in the spinal cord, it is also possible that Atp2a1 in DRG may have some roles for itch sensation.

Atp2a gene has three different isoforms (Atp2a1, 2, 3) and each isoform expression was examined by ISH and QRT-PCR. Atp2a1 and Atp2a2 are expressed in the spinal cord and DRG. Atp2a3 expressions were detected only in the cerebellum granular cells **(Fig. 9)**. Atp2a1 is the most abundantly expressed isoform in the spinal cord dorsal horn. Atp2a2 gene expression was found mostly in the ventral horn regions. Also, Atp2a1 is the most affected gene in the bombesin-saporin treated spinal cord suggesting that Atp2a1 is expressed in the GRPR neurons and may have functional roles in GRPR-mediated downstream signaling pathways for itch **(Fig. 9)**. To examine the possible involvement of Atp2a2 for itch, Atp2a2 siRNA experiment was also performed. Atp2a2 siRNA i.t. injection neither change CQ nor histamine itch models suggesting that Atp2a1 is the major Atp2a isoform mediating histamine-independent itch in the spinal cord **(Fig. 8b)**.

Atp2a1 was co-localized with GRPR in the spinal cord dorsal horn and DRG (88%, 84/96) **(Fig. 10)**. Atp2a1 is co-expressed with CGRP (30%, 37/122), IB4 (29%, 90/306), NF200 (26%, 51/195), MrgprA3 (5%, 22/426) and TRPV1 (31%, 36/115) in DRG **(Fig. 11)**. Co-localization of Atp2a1 with a CQ receptor, MrgprA3 and the channel involved with histamine-induced itch, TRPV1 in DRG supports potential roles of Atp2a1 in DRG for itch transmission. To examine whether Atp2a1 is involved in GRPR downstream Ca^{2+} signaling pathways, heterologous cell culture system was used. Stable GRPR expressing human embryonic kidney (HEK) cells showed intracellular Ca^{2+} increases by GRP applications **(Fig. 12a)**. Atp2a1 inhibitor, DBHQ pre-treatment releases Ca^{2+} from ER and depletes the internal Ca^{2+} store. Therefore, GRP-induced Ca^{2+} response was reduced after DBHQ treatment **(Fig. 12b)**. However, DBHQ did not abolish the GRP-induced Ca^{2+} response completely indicating that GRPR activation also causes Ca^{2+} influx from extracellular space through plasma membrane channels. GRP response in GRPR HEK cells was significantly reduced after GRPR or Atp2a1 siRNA transfection **(Fig 12 c, d)**. All these data suggest that Atp2a1 is involved in GRPR downstream Ca^{2+} signaling, which is important for CQ-induced histamine-independent itch sensation.

From 30 ER Ca^{2+}-ATPase related molecules, 7 candidate genes were further identified by siRNA screening **(Fig. 13)**.

(2) Phospholipase D family, member 3 (Pld3)

i.t. treatment of Pld3 siRNA inhibited CQ-induced scratches but not histamine-induced ones **(Fig. 14a)**. siRNA specificity was confirmed by ISH and QRT-PCR. Pld3

siRNA reduced Pld3 mRNA levels only in the spinal cord dorsal horn but not in the DRG **(Fig.14b-d)**. RT-PCR with LCM-dissected spinal cord dorsal horn superficial layers showed that Pld3 is the major isoform expressed in the spinal cord dorsal horn among six Pld isoforms **(Fig.14f)**. Pld3 and GRP were co-localized both in the spinal cord and DRG suggesting that Pld3 may have potential roles in GRP-mediated itch pathways **(Fig.14e)**.

(3) Protein kinase C and casein kinase substrate in neurons 3 (Pacsin3)

i.t. injection of Pacsin3 siRNA decreased CQ itch **(Fig 15a)** and this effect was from Pacsin3 knockdown in the spinal cord as well as DRG based on QRT-PCR results **(Fig. 15b)**. Pacsin3 is co-localized with GRP in the DRG **(Fig.15c)** but it is still unknown whether there is any functional relationship between Pacsin3 and GRP. Pacsin3 is known to bind and regulate TRPV4 activities (D'Hoedt, Owsianik et al. 2008). It will be interesting to study relationships between Pacsin3 and TRPV4 in DRG for itch transmission because TRPV4 was also identified as a candidate itch mediating channel from siRNA screening.

(4) IP3 receptor, type 3 (Itpr3)

There are three different Itpr genes, Itpr1, 2 and 3. Itpr3 was the most significantly reduced gene in the bombesin-saporin treated spinal cord **(Fig. 16a)**. Itpr3 siRNA reduced CQ itch due to spinal cord knockdown **(Fig.16b-d)**. Atp2a1 and Itpr3 are important in ER Ca^{2+}-mediated signaling pathways. Their functional involvement only in histamine-independent itch strongly suggests that histamine itch may have different

signaling mechanisms from histamine-independent itch mediated by GRPR **(Fig. 17)**. Consistently, there is a recent report that histamine itch is mediated by extracellular Ca^{2+} influx through TRPV1 which is independent of ER Ca^{2+} pathways (Shim, Tak et al. 2007) **(Fig. 38)**.

2.2.2. Histamine-dependent genes: S100a8, CALR, DSP

(1) Calcium-binding protein A8 (Calgranulin A; S100a8)

i.t. injection of S100a8 siRNA decreased histamine itch. However, there was no difference in CQ itch after the siRNA **(Fig. 18a)** suggesting that S100a8 is involved in histamine-dependent itch pathways. S100a8 may function in the spinal cord because the siRNA reduced spinal cord S100a8 expression **(Fig.18b)**.

(2) Calreticulin (CALR)

CALR is also a calcium binding protein in ER. CALR siRNA i.t. injection reduced histamine itch but not CQ-induced scratches **(Fig.19a)**. CALR may be involved in histamine-dependent itch pathways in the spinal cord **(Fig. 19b)**. However, CALR expression was also detected in DRG and co-expressed with GRP both in the spinal cord and DRG **(Fig. 19c, d)**. This raises a possibility that CALR may be involved in histamine-dependent pathway in GRP expressing DRG neurons.

(3) Desmoplakin (DSP)

Desmoplakin is an extracellular matrix protein. i.t. treatment of DSP siRNA decreased histamine itch **(Fig. 20a)**. The siRNA effects were resulted from spinal cord DSP knockdown **(Fig. 20b)**. Previous studies have suggested that extracellular matrix proteins are involved in Ca^{2+} influx (Schwartz, Brown et al. 1993; Bixby, Grunwald et al. 1994). Therefore, it is possible that DSP is involved in extracellular Ca^{2+}-mediated itch signaling pathways for histamine itch in the spinal cord.

2.2.3. Both-dependent gene: TRPV4

(1) TRP vanilloid 4 ion channel (TRPV4)

TRPV4 is one of TRP channels expressed in DRG. TRPV4 siRNA i.t. injection inhibited both CQ and histamine-induced scratches **(Fig. 33b)**. TRPV4 is only expressed in DRG and not in the spinal cord **(Fig. 29)**. QRT-PCR confirmed that siRNA reduced TRPV4 expression in DRG **(Fig. 33c)**. Because TRPV4 expressing DRG neurons also express GRP **(Fig. 30d-f and Fig. 32a-c)**, there is a possibility that TRPV4 may have be involved in GRP-mediated itch. Functional studies of TRPV4 in itch are described separately in the last section of this thesis.

DISCUSSION

1. Microarray

The reason that ANOVA analysis did not show any gene changed significantly in all the bombesin-saporin treated spinal cord may be sample variations. Also, the RNA amplification step can affect on the real gene expression levels. However, GRPR showed significantly reduced expression in all the bombesin-saporin treated spinal cord when compared each group separately, indicating that microarray data from the differential screening between blank- and bombesin-saporin injected mice are still valid. The primary targets are membrane proteins such as receptors and channels. There are also other candidate genes encoding signaling molecules and transcription factors. The role of dorsal horn inhibitory neurons expressing a transcription factor, *Bhlhb5* in itch has been reported recently (Ross, Mardinly et al. 2010). Loss of these neurons resulted in an enhanced pruritus. Therefore, it will be interesting to identify additional novel itch signaling molecules or transcription factors that regulate itch-specific gene expressions in the future.

2. Pharmacological approaches

Six candidate genes from differential screening were tested with pharmacological approaches. However, Atp2a1 was the only gene whose inhibitors reduced GRP- and CQ-induced itch. The other receptor or channel genes did not show significant differences in itch responses with specific agonists or antagonists. However, it is

important to find an effective dose for chemical reagents. The effects of agonists and antagonists or inhibitors were tested with i.t. injections. There are possibilities that theses candidate genes are also expressed in the peripheral tissues and mediate itch. It will be interesting to examine expressions of the candidate genes in DRG and skin and test those chemical effects by peripheral applications such as i.p. and i.d. injections.

3. siRNA screening

It is important to notice that siRNA i.t. injection affects both spinal cord and DRG gene expression (Kawasaki, Xu et al. 2008; Liu, Bi et al. 2010; Vergnolle, Cenac et al. 2010). Based on the expression patterns, the siRNA effect can be localized at either place or both. If the gene is expressed in the spinal cord or DRG only, the siRNA-mediated knockdown can identify tissue-specific roles of the candidate genes. However, when they are expressed in both tissues, it will be difficult to find out the tissue-specific gene functions. To resolve these issues, DRG and spinal cord dorsal horn cultures may be used with siRNA transfection to examine their responses to pruritogens by using Ca^{2+} imaging or electrophysiological approaches. If tissue-specific conditional mutant mice are available, behavioral studies with the mutant mice will be helpful to support siRNA results in specific tissues. siRNA efficiency also can be affected by dose, time and delivery methods. It is critical to find an efficient dose and delivery method for successful gene knockdown. Because high concentration of siRNA and vehicle (PEI) caused strong pain responses in mice, several combinations have been tested as preliminary experiments. 2.5μg siRNA per single i.t. injection provided the most efficient knockdown

without causing severe pain or toxicity problems. Single injection was not enough to knockdown gene expression consistently, therefore siRNA was injected once daily for three days.

Atp2a1 was the only target identified from the pharmacological approaches. Additional Atp2a1-related Ca^{2+} signaling molecules were selected for siRNA screenings to find more itch candidate genes. Although the other genes except Atp2a1 did not show significant changes in scratching responses with agonist or antagonist, it will be worth while to confirm them with siRNA knockdown again. Although siRNA is a useful tool to knockdown gene expressions, it may not abolish mRNA levels completely. Partial knockdown could be the potential problem of siRNA approach. Single approach seems not to be sufficient to identify gene functions in itch sensation. Combinatorial studies with siRNA and mutant mice or heterologous cell culture system will resolve the weakness of single siRNA experiment.

4. Atp2a1 and Itpr3

Atp2a1 and Itpr3 were identified as Ca^{2+} signaling molecules in GRPR downstream signaling pathway for itch. However, histamine itch receptor, H1R is also a Gαq protein-coupled receptor that release Ca^{2+} from ER upon activation. An important question is why Atp2a1 and Itpr3 are only involved in histamine-independent itch. First explanation is that H1R is not only dependent on ER Ca^{2+} but also extracellular Ca^{2+} influx through TRPV1. In this case, although one pathway is inhibited, the other pathway still can mediate the response. Another potential answer is isoform-specific signaling

pathways. Histamine-dependent and -independent itch signaling may activate intracellular Ca^{2+} response by specific isoform of signaling molecules. For example, PLCβ3 is only involved in H1R-mediated histamine itch. This also may be applied to distinguish itch from pain pathways. Each pathway can be separated by distinct signaling pathway such as intracellular and extracellular Ca^{2+} influx or balanced combination of both. Alternatively, two pathways may use similar downstream signaling pathways but certain isoform of signaling molecules can be specified for each pathway. Atp2a1 and Itpr3 have broad expression patterns in the spinal cord and DRG. Because ER Ca^{2+}-mediated responses are common signaling pathways of multiple G protein-coupled receptors (GPCRs), extensive pain behavior studies will be necessary to examine whether the candidate genes are only specific to itch.

MATERIALS AND METHODS

Laser capture microdissection (LCM) and RNA preparation

Three blank- and bombesin-saporin treated mice spinal cords were dissected and sectioned with 30μm thickness using a cryostat (Leica). Lumbar spinal cord was used for LCM because bombesin-saporin was injected into the region and it is also easier to dissect lamina I and II out from the large enlargement than the other parts of the spinal cord. Superficial layers including lamina I and II can be easily distinguished from the other regions due to its increased transparency. The tissue slides were fixed with series of ethanol as previously described (Gallup, Kawashima et al. 2005; Espina, Wulfkuhle et al.

2006). Fixed tissue slides were kept in a dry chamber with Drierite Dessicants (W.A. Hammond Drierite Co. Ltd) to reduce massive RNA degradations. The cervical part of the spinal cord was fixed with 4% paraformaldehyde (PFA) and protected with 30% sucrose in diethylprocarbonate (DEPC)-phosphate buffered saline (PBS) to verify molecular marker gene expressions. Most superficial layers of spinal cord dorsal horn were isolated using PixCell IIe LCM system (Molecular Devices) and captured with CapSure HS LCM Caps (Molecular Devices). Total RNAs were prepared from the laser captured tissues by using RNA Pico Isolation Kit (Molecular Devices). The qualities of the RNAs were assayed with Nonodrop 1000 (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray and data analysis

RNAs were amplified by WT-Ovation Pico System (NuGen) to make cDNAs. The quality of total cDNA was also measured with 2100 Bioanlyzer. Three pairs of cDNAs from blank- and bombesin-saporin treated samples were hybridized to Illumina bead mouse 6 array (version 2.0) simultaneously. Hybridized probe signals were scanned with Illumina Beadarray Reader. Raw image files were obtained with Illumina Bead Studio software. Partek Genomics Suite program was used for data analysis. The illumina raw data file was first converted to Partek file format. ANOVA statistical analysis was performed to compare fold changes with normalization. Three pairs of blank- and bombesin-saporin treated samples could generate nine comparisions. Candidate genes, which showed significantly decreased levels in the bombesin-saporin treated samples,

were selected by comparing each sample group. Multiple probe sets representing a single gene transcript were combined into one data point in order to reduce duplication of candidate genes. Ingenuity Pathways Analysis (IPA) also has been performed to find possible signaling pathways in which the candidate genes are involved.

Quantitiative reverse transcription-polymerase chain reaction (QRT-PCR)

cDNAs were synthesized from the blank- and bombesin-saporin treated spinal cord lamina I and II RNAs (from LCM dissection) using Superscript Reverse Transcriptase (RT) II (Invitrogen) and QRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and Mx3000P qPCR system (Stratagene). Primer sequences are shown in **Table. 5**

In situ hybridization (ISH) and 3,3'-Diaminobenzidine (DAB) staining

To amplify the gene of interest, the primers flanking about 1kb cDNA sequence were designed. If the gene expression patterns by ISH and probe sequences are available on the Allen Brain Atlas web site (Allen Institute for Brain Sciences: http://www.brain-map.org), the information was used to make the ISH probe. Amplified PCR fragments were sub-cloned into pBlueScript KS vector (Stratagene) and sequenced for confirmation. Once it is confirmed, the sub-cloned plasmids were used as templates for synthesizing anti-sense RNA probes by *in vitro* transcription using T7, T3 or SP6 RNA polymerases (Fisher Scientific). Digoxigenin-labeled RNA probes were made and hybridized as previously described (Birren, Lo et al. 1993). For DAB staining, slides were treated with 3%

hydrogen peroxide (H_2O_2) in PBS after ISH. Slides were incubated with Rabbit anti-GRP antibody (Immunostar, 1:1000) in PBS/1% donkey serum/0.3% Triton X-100 at 4ºC for overnight. After washing twice in PBS, slides were incubated with biotinylated secondary antibody (Jackson ImmunoResearch, 1:200) for 2 hours at RT. Slides were washed in PBS and incubated with ABC mix (mix A (1:200) and B (1:200); Vectastain Elite ABC kit) in PBS at RT for 1hour. Slides were washed in PBS and incubated with 0.02% DAB/0.00003% H_2O_2 in PBS for color development.

Pharmacological studies

(1) Agonists/antagonists

Five receptor agonists, Glucagon-like peptide 2 (GLP2, BIOMOL, 0.5-2ug), Prokineticin 1 and 2 (PK1 and PK2, Phoenix Pharmaceuticals, 0.25-0.5μmol), P2 purinoceptor agonist, ATP (Tocris, 0.5-1M), Nr3c2 synthetic activator, Fludrocortisone (Sigma, 1-5ug), Brain Natriuretic peptide (BNP, BIOMOL, 0.5-1μg) were injected into mouse spinal cord intrathecally.

(2) Inhibitors

Atp2a1 inhibitors, 2, 5-Di-tert-butylhydroquinone (DBHQ, BIOMOL, 5-10μg) and thapsigargin (TG, BIOMOL, 5-10μg) were tested with several itch models by i.t. injections.

In vivo siRNA injection

siRNA (Sigma) was dissolved in DEPC-treated PBS. ExGen 500 *in vivo* transfecting reagent (Polyethylenimine (PEI;0.18μl/1μg siRNA); Fermantas) was mixed to enhance siRNA cell penetration. siRNA solution (2.5ug siRNA-PEI mix) was injected into mice intrathecally once daily for three days as described (Kawasaki, Xu et al. 2008). After 30 min of the last injection, scratching behaviors with pruritogens were monitored for 30 min. DEPC-PBS/PEI mix was used as a control.

QRT-PCR for siRNA

Lumbar spinal cord dorsal horn and DRG were dissected out from control (DEPC-PBS/PEI) and siRNA-treated mice. Tissues were homozenized with Dounce glass homogenizer (Wheaton) and total RNA was prepared by using Trizol reagent (Invitrogen). cDNA was synthesized using Superscript First-strand synthesis system (Invitrogen) and conventional or QRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and Mx3000P qPCR system (Stratagene). Expression of target mRNA was normalized to 18S ribosomal RNA (rRNA) level.

Double fluorescence ISH and immunohistochemistry (IHC)

Two different (fluorescein and digoxygenin; Roche)-labeled probes were prepared. Fluorescein-labeled probe was purified through G50 microcolumn (Amersham-Pharmacia) and then precipitated with lithium chloride ($LiCl_2$). Two probes were added into hybridization buffer. Procedures were same as the single ISH previously described

(Birren, Lo et al. 1993). Fluorescent ISH combined with IHC was performed as described (Dong, Han et al. 2001). After ISH, Primary rabbit-GRP (Immunostar, 1:1,000) and TRPV1 (Chemicon; 1:1,000), guinea pig-CGRP (Peninsula, 1:1,000) and mouse-NF200 (Sigma, 1:1,000) antibodies were used and followed by incubating with secondary donkey anti-rabbit or mouse-IgG coupled to FITC (Jackson ImmunoResearch, 1:400). IB4-FITC (Sigma, 1:200) was used to show IB4 stainings. Slides were examined using Olympus BX51 upright microscope attached with a CCD camera.

siRNA transfection and Ca^{2+} imaging with GRPR-HEK cells.

siRNA was dissolved with RNAse-free universal buffer (20mM KCl, 6mM HEPES pH 7.5, 0.2mM $MgCl_2$) to make 20μM stock solution. The stock solution was heated at 90ºC for 2 min for denaturation and cooled down to room temperature for re-annealing. The re-annealed siRNA solution was stored at 4ºC for overnight and kept in small aliquots at -20ºC. Stable GRPR-expressing HEK cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco) and plated onto poly-ornithine-coated 12 mm diameter round glass coverslips in 24-well plate. GRPR-HEK cells were transfected with GRPR and Atp2a1 siRNA (60 pmol) by using Lipofectamine 2000 reagent (Invitrogen). After 24 hours, cell culture media was replaced with Ca^{2+} imaging buffer (140nm NaCl, 4mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM Glucose, 10mM HEPES, adjusted to pH 7.4 with NaOH). Fura-2 (Invitrogen) was diluted to 2mM stock in dimethylsulfoxide (DMSO)/20% Pluronic acid (Invitrogen). Cells were loaded with Fura-2 (2μM) diluted in the Ca^{2+} imaging buffer. The coverslips were

mounted on a Warner Instruments recording chamber (RC 26G) perfused with Ca^{2+} imaging buffer at a rate of 1ml/min. An inverted microscope (Nikon Eclipse Ti 20x objective) with Roper Cool SNAP HQ_2 digital camera was used for Fura-2 Ca^{2+} imaging after 340nm/380nm laser excitations (sampling interval 1sec, exposure time adjusted for each experiment). DBHQ (50μM) and GRP (250pM) were applied to cultures to examine Ca^{2+} responses. Nikon NIS elements version 3.1 and Graph pad prism software were used to analyze Ca^{2+} imaging data.

III. GRPR mediates itch sensation in peripheral DRG and skin

INTRODUCTION

1. Skin

Skin is a highly complex organ innervated by a variety of specialized sensory neurons sensitive to heat, cold, pressure, itch and pain (Montagu 1978). It has been an important question whether sensory neurons are the primary transduction element, or whether non-neuronal cells can act as a crucial signaling pathway, with subsequent activation of adjacent nerve terminals resulting in a perception of touch, temperature, pain, or itch. Specialized epithelial structures such as hair cells, merkel cells, and taste buds are known to play a role in sensory transduction, but recent evidence suggests that other candidates such as keratinocytes may also be primary transducers of mechanical stimuli (Lumpkin and Caterina 2007). It is highly possible that non-neuronal cells in skin may function as primary transducers of certain physical or chemical stimuli and can communicate to neighboring sensory afferents. Because itch sensation arises in skin responding to extracellular pruritogens and the signal is relayed by DRG to central nervous system (CNS), it will be necessary to understand basic molecular mechanism of itch mediated by peripheral tissues.

1.1. Keratinocytes

Keratinocytes in the epidermis proliferate from a basal layer located at the

dermal-epidermal boundary and form a well-organized stratified epithelium through a coordinated cell differentiation and migration (Lumpkin and Caterina 2007). Sensory receptors on epidermal keratinocytes have functional roles in permeability barrier homeostasis and it has been shown that TRPV1 agonists delay barrier recovery, whereas TRPV4 accelerates it (Denda, Sokabe et al. 2007). Keratinocytes also can be the primary transduction element using signal transduction such as intracellular Ca^{2+} influx to elicit a response in adjacent C-fibers (Denda, Nakatani et al. 2007). Putative keratinocyte-neuron interactions, intermediate molecules and second messenger-mediated signaling cascades have been proposed (Lumpkin and Caterina 2007).

Several evidences suggest that keratinocytes participate in the detection of physical and chemical stimuli. First, at least two populations of sensory afferent fibers have been identified within distinct epidermal layers (Zylka, Rice et al. 2005). While sensory neurons that express pro-inflammatory neuropeptides, CGRP ascend through the basal epidermal layer to terminate in the next layer, the stratum spinosum, non-peptidergic neurons that express a specific G-protein-coupled receptor, MrgprD, terminate more superficially, in the stratum granulosum. These two neuronal populations exhibit different projection patterns to the spinal cord and have distinct sensory functions (Snider and McMahon 1998). Although classical synaptic structures between keratinocytes and sensory nerve terminals have not been observed, the proximity of these cell types and close membrane apposition (Hilliges, Wang et al. 1995; Chateau and Misery 2004) will provide strong opportunity for rapid paracrine signaling. Second, keratinocytes secrete numerous chemical substances such as neurotrophins, ATP, β-

endorphin, interleukins and endothelin-1 that can modulate, activate or inhibit sensory neurons (Shu and Mendell 1999; Khodorova, Fareed et al. 2002; Koizumi, Fujishita et al. 2004; Xu, Delling et al. 2006). Interestingly, different layers of the epidermis can release certain substances with different effects. Whereas superficial keratinocytes can release anti-nociceptive molecules such as β-endorphin, stimulation of deeper epidermal keratinocytes causes a secretion of pro-nociceptive endothelin-1 (Khodorova, Navarro et al. 2003). The existence of such potentially antagonistic systems in the epidermis suggests that non-neuronal skin cells are able to distinguish and process specific signals at the upstream of the nervous system. Third, keratinocytes express various receptors that have been implicated in pain or temperature sensation. However, it is difficult to distinguish whether the receptors facilitate signaling between keratinocytes, keratinocytes and neurons or both. It will be an interesting question whether non-neuronal keratinocytes are directly involved in itch sensory signaling pathways.

1.2. Mast cells

As shown in a mouse model, mast cells and neuropeptides may play an important role in immediate hypersensitivity. During an allergic reaction, mast cells become IgE-dependently activated (type I reaction) (Ravetch and Kinet 1991). Additionally, mast cells can be IgE-independently stimulated by neuropeptides such as SP mediating an edema after being injected intracutaneously. SP also mediates the recruitment of leukocytes (Goebeler, Henseleit et al. 1994), which is partially dependent on mast cell degranulation (Yano, Wershil et al. 1989). After peptidergic stimulation, mast cells are

capable of releasing not only histamine, but several other inflammatory mediators such as IL-6, TNF-α (Ansel, Brown et al. 1993; Goodness, Albers et al. 1997), or proteases such as tryptase (Corvera, Dery et al. 1997; Molino, Barnathan et al. 1997; Steinhoff, Corvera et al. 1999; Steinhoff, Vergnolle et al. 2000) that may contribute to neurogenic inflammation and itch.

1.3. Cutaneous nerves

The axon terminals of nociceptive neurons do not possess specialized end organ structures and are referred to as free nerve endings for that reason. Cutaneous innervation consists mainly of unmyelinated fibers, accounting for around 90% of all dermal nerve fibers (Ebenezer, McArthur et al. 2007). Over 50% of the unmyelinated axons of a peripheral nerve respond not only to intense mechanical stimulation, but also to heat and noxious chemicals, and are therefore classified as polymodal nociceptors (Bessou and Perl 1969) or C-mechano-heat (CMH) nociceptors (Campbell et al. 1989). Cutaneous nerves are connected to DRG and projected to dorsal spinal cord. Therefore, it will be important to study functional roles of peripheral nerves to understand itch because they are the first transition point translating non-neuronal stimuli to neuronal activities.

2. GRP and GRPR in skin

GRP, a bombesin-like peptide was identified as one of the spinal cord dorsal horn enriched genes from dorsal vs. ventral horn differential screening (Li, Wang et al. 2006). Functional studies of GRP and GRPR have been focused in DRG and spinal cord,

respectively. However, bombesin was originally isolated from frog skin (Anastasi 1971) and most of itch sensations happen in the periphery. Also, GRP and GRPR are expressed in the peripheral tissues such as DRG and skin (Staniek, Misery et al. 1996) in addition to the spinal cord. This raised a question about GRP and GRPR roles in the peripheral region.

GRP i.d. injection caused dose-dependent scratching responses. The GRP i.d.-induced scratches were abolished in GRPR mutant mice. NMBR, a GRPR homologous receptor is also expressed in spinal cord, DRG and skin. NMB and bombesin i.d. injection caused scratching responses that were completely lost in GRPR/NMBR double mutant mice. This suggests that bombesin-related peptides and receptors have conserved functional roles for itch in peripheral tissues. GRPR antagonist i.p. and i.d. injection inhibited CQ, GRP and NMB i.d.-induced scratchings. GRPR antagonist also attenuated chronic itch in ACD model mice painted with diphenylcyclopropenone (DCP). These results strongly support that GRP and GRPR also mediate itch sensation in the peripheral regions as well as the spinal cord.

RESULTS

1. GRP and GRPR are expressed in peripheral tissues.

GRP expression was examined in spinal cord, DRG and skin **(Fig. 21)**. GRP antibody specificity was confirmed in $GRP^{-/-}$ mice DRG and skin **(Fig. 21d, f, h)**. GRP expression was detected in skin free nerve endings and was co-localized with peptidergic

CGRP^{+} fibers **(Fig. 21g-k)** consistent with the previous GRP expression results in DRG (Sun and Chen 2007). GRPR was also examined in both DRG and skin **(Fig. 22)**. GRPR cells co-express CGRP (61%), IB4 (33%) and NF200 (14%) in DRG **(Fig. 23)**. GRPR skin immunostaining showed GRPR expressions in epidermal keratinocytes and dermal mast cells **(Fig. 22e-f)** suggesting potential roles of GRPR in the periphery. GRPR^{+} nerve fibers have not been found but it requires more detail observations because DRG cells are expressing GRPR. Skin GRPR expression was further confirmed by RT-PCR with WT and GRPR mutant skin tissues **(Fig. 22g)**.

2. Peripheral GRPR is involved in itch.

GRP i.d. injection caused dose-dependent scratching responses **(Fig. 24a)**. Because GRPR positive nerves were not detected, GRP i.d. seems to activate GRPR in skin cells. GRPR mutant mice abolished GRP i.d. responses suggesting that GRP i.d.-induced itch depends on GRPR **(Fig. 24b-c)**.

NMBR, a GRPR homologous receptor, was also detected in the spinal cord, DRG and skin **(Fig. 25)**. NMB i.d. injection causes dose-dependent scratches **(Fig. 26a)** like GRP suggesting that bombesin receptor signaling mechanisms are conserved in the skin. NMB i.d.-induced scratches were significantly reduced in GRPR$^{-/-}$ mice **(Fig. 26b)** suggesting that NMB can also bind to GRPR and activate GRPR-mediated itch signaling pathways. All the bombesin-like peptide (GRP, NMB and bombesin) i.d. injection caused scratching responses but these were completely abolished in GRPR/NMBR double mutant mice **(Fig. 26c-e)** suggesting that GRPR and NMBR are the receptors mediating

peripheral bombesin peptide-related itch sensations.

3. Peripheral application of GRPR antagonists inhibits both acute and chronic itch.

GRPR specific antagonists, D-Phe-Leu-bombesin and BW-10, were applied by i.p. and i.d. injections. These antagonists inhibited CQ, GRP and NMB i.d.-induced itch **(Fig. 27a-e)** suggesting that peripheral applications of GRPR antagonists are efficient methods to inhibit GRPR-mediated itch. Interestingly, D-Phe-Leu bombesin i.p. injection partially reduced histamine-induce scratching responses **(Fig. 27f)**. It is possible that GRPR also mediates histamine itch because it is also expressed in mast cells. However, it remains a question whether peripheral GRPR activities are involved in mast cell degranulation, histamine-release or histamine receptor signaling.

To test the GRPR antagonists in chronic itch, DCP-painted ACD mouse model was used. DCP painting caused increase of skin epidermal thickness, hyperplasia and robust scratching responses **(Fig. 28a-d)**. D-Phe-Leu-bombesin i.p. injection inhibited DCP-painted mice scratches suggesting that skin GRPR is also involved in chronic itch responses **(Fig. 28e)**.

DISCUSSION

Previous studies have focused on the effect of GRP from afferent fibers based on the function of GRPR in the spinal cord. However, GRP expression both in the spinal cord and skin raises two additional possibilities for GRP-GRPR signaling mechanisms. First, although it is not known whether GRP and GRPR are expressed in the same cells

for autonomous signaling, GRP can be released from spinal cord neurons and activates closely located spinal GRPR expressing cells. Second, GRP expressions in skin free nerve endings suggest that GRP can be released from the peripheral nerves and stimulate GRPR positive skin cells such as keratinocytes and mast cells. To distinguish peripheral GRPR roles from central spinal cord GRPR, it will be necessary to use skin specific GRPR conditional mutant mice to examine itch behaviors. Activation of GRPR expressing skin cells may release other pruritogens to induced scratching responses. Ca^{2+} imaging with skin cell (keratinocytes) culture can support that GRPR in skin can be activated by peripheral GRP and mediate itch sensation. Stable fibroblast cell lines can be considered for Ca^{2+} imaging experiment, if they express GRPR endogenously.

It will also be interesting to determine whether GRP i.d.-induced itch is histamine-independent by using anti-histamine because GRPR expression was examined in dermal mast cells. D-Phe-Leu-bombesin i.d. injection inhibited histamine-induced itch suggesting that GRPR in skin also can mediate histamine-dependent pathways **(Fig. 27f)**.

While i.t. application of drugs or siRNA will be required to block spinal cord GRPR activities for itch therapy, it is inconvenient and difficult to get consistent results. Direct injection to the spinal cord may cause unwanted side effects in CNS. Therefore, identification of alternative route to inhibit itch will have great impacts on therapeutic approaches. Based on GRPR functions in peripheral tissues, i.p. and i.d. or topical applications of drugs such as GRPR antagonists can be considered to treat both acute and chronic itch.

MATERIALS AND METHODS

In situ hybridization

Lumbar spinal cord and DRG were dissected and fixed with 4% paraformaldehyde (PFA) for overnight. After washing with DEPC-PBS, tissues were protected with 20-30 % sucrose solution until they were sunk down. Tissues were embedded in OCT (Tissue-tek) and sectioned at 20μm by using cryostat (Leica). GRP, GRPR and NMBR ISH were performed as other ISH described in Chapter II.

Immunostaining

Lumbar spinal cord and DRG were processed same as in ISH procedures. Tissues were sectioned on Superfrost plus microscope slides (Fisher) or used for free-floating stainings. GRP and GRPR immunostaining in the spinal cord and DRG were performed with rabbit GRP (Immunostar, 1:1,000) and GRPR (MBL, 1:1,000) primary antibodies and followed by Cy3-conjugated anti-rabbit IgG secondary antibody (Jackson Immunoresearch, 1:400).

Skin immunostaining

Mouse neck and paw skin were dissected. Ventral paw skin was used as a glabrous skin and dorsal side of paw and neck skin was used as a hairy skin. Skin was fixed for overnight 4°C in Zamboni's fixative (2% formaldehyde, 15% [v/v] sat. picric acid, 0.1 M phosphate buffer [pH 7.3]) and frozen in OCT (Tissue-tek). Skin tissue was sectioned at 30μm and placed directly into PBS in multi-well plates for immediate staining as free-

floating sections. Tissue was washed in PBS for and then incubated on a horizontal shaker with primary antibody overnight at 4°C in incubation medium (PBS containing 1% normal donkey serum, 0.3% Triton X-100). Tissue was washed with PBS + 0.1% Triton X-100 (PBST) and incubated with secondary antibody in the same incubation medium for two hours at RT. Tissue was washed with PBST. Skin tissue was mounted on slides and observed with fluorescent microscope.

Skin RT-PCR

Dorsal neck skin was dissected from WT and GRPR mutant mice and homogenized in Trizol reagent (Invitrogen) with Dounce glass homogenizer (Wheaton). Total RNA was prepared and used to synthesize cDNA as shown in the previous chapter. Conventional RT-PCR was performed with GRPR and NMBR primers. GRPR: 5'-ACCTGAACTTGGACGTGGAC-3' and 5'-TCAGTTTGCAGCCAATTCTG-3', NMBR: 5'-ATGCCCCCCAGGTCT-3' and 5'-TCACAGTGCTATTTC-3'

Fluorescent ISH and immunostaining

DRG Fluorescent ISH was performed with the GRPR probe first and followed by immunostaining using antibodies against multiple neuronal markers such as CGRP, IB4 and NF200. Anti-rabbit CGRP (Chemicon, 1:1,000) and NF200 (Sigma, 1:1,000) primary antibodies were used. FITC-conjugated secondary rabbit antibody was used to detect the primary antibodies. IB4-FITC (Sigma, 1:200) was used to show IB4 staining.

Peripheral injection of bombesin-like peptides and GRPR antagonists

GRP, NMB and bombesin (100nmol/50μl saline) were injected intradermally. Scratching responses were examined for 30 min with 5 min intervals. GRPR antagonists, D-Phe-Leu-bombesin (Tocris, 10nmol) and BW-10 (Bachem, 10nmol) were injected intraperitoneally 30 min before the injection of bombesin peptides. D-Phe-Leu-bombesin (15nmol) was also injected intradermally 10 min before the test.

DCP painting

Diphenylcyclopropenone (DCP) was dissolved in acetone. To induce CD, mice were shaved on the back and sensitized by painting with 0.2 ml of DCP (1%) once on the skin of the neck. Seven days after the sensitization, mice were challenged by painting with 0.2 ml of DCP (0.5%) on the same place.

Skin H&E staining

Untreated control and DCP-painted skin were dissected and fixed with 4% PFA in PBS. Tissue was dehydrated by ethanol and embedded in paraffin. It was sectioned at 5μm with microtome. Section was de-paraffinized and re-hydrated with xylene, 100%, 95%, 80% ethanol and deionized water. Slides were stained with hematoxaline (Fisher) and de-stained with ethanol and water. Slides were stained with eosin (Fisher) and de-hydrated with 95%, 100% ethanol and xylene. Slides were covered with coverslip using Cytoseal-XYL mounting medium (Richard-Allen Scientific).

IV. Role of TRPV4 in itch signaling pathways

; TRPV4 mediates acute and chronic itch in peripheral DRG neurons

INTRODUCTION

1. TRP channels in itch

Transient receptor potential (TRP) ion channels are expressed in sensory nerves, keratinocytes, and certain leukocytes. TRPs are activated by temperature, pH changes, and certain toxins. Recent evidences indicate that the TRP channels are crucially involved in pruritus, making them as good candidates for future therapeutic targets in skin inflammation and itch (Steinhoff and Biro 2009).

1.1. TRPV1

TRPV1 has been studied most widely among the TRP ion channel subfamily. It is involved in noxious heat (>43ºC) sensation and mediates thermal hyperalgesia under inflammatory conditions. TRPV1 can be activated by multiple extracellular stimuli such as noxious heat, proton and capsaicin (Caterina, Schumacher et al. 1997). TRPV1 is highly expressed in peripheral DRG neurons in mammals, especially in C-fiber neurons (Cortright, Crandall et al. 2001). Several itch mediators such as histamine, bradykinin, and neurotrophins activate or sensitize TRPV1 (Steinhoff, Bienenstock et al. 2006). TRPV1 is required for histamine itch with PLCβ3 (Imamachi, Park et al. 2009). Histamine also activates TRPV1 through PLA_2 and LOX causing Ca^{2+} influxes in

sensory neurons (Shim, Tak et al. 2007). Histamine-induced Ca^{2+} response is attenuated by TRPV1 antagonists (Kim, Lee et al. 2004). In $TRPV1^{-/-}$ mice, histamine-induced scratching was attenuated suggesting that TRPV1 is a downstream effector of H1R-mediated itch signaling. Histamine evokes inward currents only when TRPV1 and H1R are co-expressed (Shim, Tak et al. 2007).

1.2. TRPV2

TRPV2 responds to noxious heat (>52ºC) and its expression was observed in broader tissues than TRPV1 (Caterina, Rosen et al. 1999; Lewinter, Skinner et al. 2004; Lewinter, Scherrer et al. 2008). Although it is unknown whether TRPV2 mediates itch signaling pathways, a recent study showed that TRPV2 is expressed in mast cells and mediates mast cell degranulation related with pain (Zhang, Spielmann et al. 2011).

1.3. TRPV3

TRPV3 is activated by innocuous and noxious heat stimuli (>33ºC). TRPV3 is expressed in mouse skin but not in DRG (Peier, Reeve et al. 2002). Although TRPV3 is co-expressed with TRPV1 in human DRG (Smith, Gunthorpe et al. 2002), major roles of TRPV3 in itch have been found in mouse skin. TRPV3 gain-of-function mutant mice caused hairless phenotype and spontaneous scratching implicating that TRPV3 may be involved in allergic dermatitis development (Yoshioka, Imura et al. 2009). This raises a possibility that other TRP channels also can mediate chronic itch.

1.4. TRPA1

TRPA1 is involved in mustard oil-and bradkinin-induced hyperalgesia. TRPA1 was originally identified as a noxious cold-activated ion channel (>17ºC) (Story, Peier et al. 2003). TRPA1 is expressed in a subset of noxious polymodal TRPV1 expressing neurons (Dhaka, Viswanath et al. 2006). TRPA1 was recently identified as a downstream mediator of MrgprA3 for CQ-induced itch in DRG (Wilson, Gerhold et al. 2011).

1.5. TRPV4

Previous siRNA screening identified TRPV4 as a potential itch mediator in DRG (Chapter II). TRPV4 was initially characterized as an osmolarity-sensitive channel. TRPV4 is involved in warm temperature sensation (>34ºC) and also activated by 4α-phorbol 12, 13-didecanoate (4αPDD), and epxoxyeicosatrienoic (EET) acids that are metabolites of anandamide and arachidonic acid (Eid and Cortright 2009). TRPV4 also mediates heat, mechanical and inflammatory pain. TRPV4 mutant mice and antisense oligodeoxynucleotide (ODN)-mediated knockdown reduced pain responses (Ding, Wang et al.; Alessandri-Haber, Dina et al. 2006; Grant, Cottrell et al. 2007). Although TRPV4 and TRPV1 share common features for pain, it is not known whether TRPV4 is involved in both pain and itch like TRPV1. TRPV4 is expressed in different types of tissues such as DRG, skin epidermal keratinocytes and mast cells (Suzuki, Watanabe et al. 2003). Therefore, it will be important to find tissue-specific TRPV4 roles in itch pathways. Combination of siRNA approaches and mutant mice studies was used to address the specific TRPV4 roles for pruritus in DRG.

In this study, TRPV4 was identified as a TRP channel that is necessary for transducing both CQ and histamine-elicited acute itch by TRPV4$^{-/-}$ and siRNA treated mice. Two different model systems for ACD and dry skin itch were used to examine whether TRPV4 mediates chronic itch in mice. Repeated application of chemical hapten, 2, 4-dinitroflurobenzene (DNFB) induces T cell-mediated hypersensitivity reaction in human skin (Garcia-Perez 1978; Perez, Narayan et al. 2004) and accompanying pruritus is resistant to anti-histamine treatment **(Fig. 34a)**. Dry skin caused by disrupting cutaneous barrier function and characterized with rough, scaly and flaky skin often accompanies with chronic pruritus (Yosipovitch 2004). Topical application of acetone/ether mixture followed by water (AEW) leads to spontaneous scratching in mice resulted from dehydration of skin stratum corneum and epidermal water loss, and dry skin itch is also mediated by histamine-independent mechanisms (Akiyama, Carstens et al.; Miyamoto, Nojima et al. 2002) **(Fig. 34b)**.

TRPV4 expression is uniquely up-regulated in DRG neurons of mice with ACD among several TRP channels examined. TRPV4$^{-/-}$ mice with ACD showed significantly attenuated pruritus during both the sensitization and elicitation phases without compromising the development of classic eczematous skin lesions and inflammatory responses. Interestingly, TRPV4$^{-/-}$ mice exhibited normal dry skin-induced pruritus **(Fig. 35)**. These data suggest that distinct signaling mechanisms underlie ACD- and dry skin-induced pruritus respectively. Hapten-elicited delayed hypersensitivity may selectively involve increased TRPV4 function, while epidermal barrier dysfunction observed in both ACD and dry skin condition does not. TRPV4 in DRG neurons may provide a novel

therapeutic target for treatment of ACD-induced chronic pruritus.

RESULTS

1. TRPV4 is expression in DRG

TRPV4 expression was examined in mouse lumbar DRG neurons with different markers. TRPV4 is expressed in approximately 19% of total DRG neurons **(Fig. 29-32)**, and its expression overlaps with CGRP (61%), IB4 (77%) and NF200 (89%) **(Fig. 30 and Fig. 31)** consistent with the previous report (Suzuki, Watanabe et al. 2003). TRPV4 is also co-expressed with 83% of cells expressing GRP, an endogenous ligand for GRPR in the spinal cord that is important for histamine-independent itch (Sun and Chen 2007; Sun, Zhao et al. 2009). **(Fig. 30d-f and Fig. 32a-c).** 87% of cells expressing MrgprA3, a receptor involved in CQ-evoked itch (Liu, Tang et al. 2009) were TRPV4 positive **(Fig. 32d-f)**. 70% of cells expressing TRPV1, which is involved in histamine-evoked itch and in multiple pruritic pathways through PLCβ3 (Shim, Tak et al. 2007; Imamachi, Park et al. 2009) overlap with TRPV4 expressions **(Fig. 30 m-o and Fig. 32g-i).** TRPV4 is completely co-localized with H1R **(Fig. 32j-l)**. This broad expression of TRPV4 in DRG suggests that TRPV4 may be involved in multiple itch signaling pathways.

2. TRPV4 is involved in acute itch

To examine whether TRPV4 is required for mediating acute pruritoceptive transmission, scratching responses of TRPV4$^{-/-}$ mice and WT littermates to i.d. injection

of histamine and CQ were observed. In mice, CQ-induced itch is likely to be mediated by MrgprA3 and its downstream TRPA1 in DRG neurons (Liu, Tang et al. 2009; Wilson, Gerhold et al. 2011), and GRPR in the spinal cord (Sun and Chen 2007). TRPV4$^{-/-}$ mice exhibited dramatic reduction of scratching behavior to both histamine and CQ injections **(Fig. 33a).** Apart from DRG neurons, TRPV4 is also expressed in skin and contributes to intercellular junction formation in keratinocytes (Sokabe, Fukumi-Tominaga et al. 2010). To identify the site of TRPV4 action responsible for histamine and CQ-evoked itch, siRNA-mediated knockdown in DRG neurons using i.t. injection was performed taking advantage of the fact that TRPV4 is not expressed in the spinal cord **(Fig. 29d)**. Mice with TRPV4 deficiency in DRG exhibited marked reduction of scratching behavior elicited by both CQ and histamine **(Fig. 33b)**. QRT-PCR confirmed that TRPV4 mRNA was significantly reduced in DRG neurons after siRNA injection, whereas TRPV1, TRPV2, TRPA1 and TRPM8 expressions were not affected **(Fig. 33c)**. Further, i.t. injection of 5, 6-EET, an endogenous TRPV4 agonist (Watanabe, Vriens et al. 2003), was used to activate TRPV4 channel in DRG neurons. Activation of TRPV4 in DRG by 5, 6-EET caused scratching responses in mice. 5, 6-EET-induced scratching behavior was significantly reduced in TRPV4$^{-/-}$ mice relative to the control WT mice **(Fig. 33e)**. Taken together, these results suggest that activation of TRPV4 in DRG neurons is required for mediating both acute histamine and CQ-induced itch.

3. TRPV4 mediates ACD-induced but not dry skin-induced pruritus

To determine whether TRPV4 has a role in chronic itch, a murine model of ACD

was employed. Repeated topical application of DNFB initiates sensitization and elicitation phases of ACD in mice (Saint-Mezard, Rosieres et al. 2004; Yamashita, Ito et al. 2010). The scratching response appeared at day 3 after the first challenge, reached a plateau by day 5. From Day 9 the number of scratching almost doubled compared to the previous day, indicating an onset of the second phase. DNFB-induced scratching was markedly attenuated in TRPV4$^{-/-}$ mice at day 3 and during the course of 14 day period examined **(Fig. 33f)**. This suggests that TRPV4 is required not only for the development but also for the maintenance of ACD-induced pruritus. Importantly, among all channels and receptors (TRPV1-2, TRPA1, TRPM8, MrgA3, and H1R) examined, TRPV4 is the only one whose expression was significantly up-regulated in DRG neurons in mice treated with DNFB **(Fig. 33d)**.

Skin pathology and inflammation were examined in these mice. Despite reduced scratching, eczematous skin lesions which are restricted to the painted area around the ears **(Fig. 36a)**, thickening of epidermis, infiltration of inflammatory mast cells and elevated serum immunoglobulin E (IgE) level were all similar between TRPV4$^{-/-}$ and WT mice painted with DNFB **(Fig. 36d-f)**. As expected, LN and spleen (SPL) sizes were all significantly increased in DNFB-painted mice suggesting that DNFB painting induces inflammatory responses. However, no differences in their size were noted between groups **(Fig. 36b)**. In addition, the numbers of neutrophils were increased in both TRPV4$^{-/-}$ and WT mice with ACD **(Fig. 36c)**. The number of white blood cells (WBC) was also increased, but the difference was not statistically significant. Thymic stromal lymphopoietin (TSLP), a marker for skin inflammation and dermatitis (Soumelis, Reche

et al. 2002; Yoo, Omori et al. 2005; Demehri, Morimoto et al. 2009) also was increased about 20 fold after DNFB treatment compared with untreated skin, but TRPV4$^{-/-}$ mice also showed similar level of increase **(Fig. 36g)**. Together, these data suggest that after initiation, scratching behavior is dependent on TRPV4 but independent of DNFB-induced skin inflammation or skin barrier dysfunction. Next, it was examined whether TRPV4 may have a role in skin dryness (xerosis)-induced itch. However, there was no significant difference in scratching behavior between TRPV4$^{-/-}$ mice and their WT littermates after AEW treatments **(Fig. 35)**. This is the first evidence that single TRP channel mediates a certain type of chronic itch selectively.

4. Functional activation of TRPV4 in DRG is required for itch transmission

Activation of TRPV4 causes intracellular Ca^{2+} increase by Ca^{2+} entry through plasma membrane, leading to cell depolarization (Nilius, Vriens et al. 2004). To determine whether TRPV4 is directly involved in DRG neuronal activation in response to pruritic stimuli, intracellular Ca^{2+} levels in DRG neurons were examined using ratiometric Ca^{2+} imaging technique. Approximately 14% of DRG neurons showed calcium spikes in response to CQ, 21% to histamine and 8% to 5, 6-EET. In contrast, TRPV4$^{-/-}$ DRG neurons exhibited significantly decreased percentage of cells that responded to CQ (6%), histamine (10%), and 5, 6-EET (1%) **(Fig. 37a)**. In addition, TRPV4 siRNA transfection effectively reduced TRPV4 expression in DRG neurons **(Fig. 37c)**, which also resulted in reduced percentage of responding cells to CQ (6%), histamine (15%) and 5, 6-EET (3%), respectively **(Fig. 37b)**. In all experiments, neuronal

health was verified with a depolarizing KCl stimulus **(Fig. 37d)**. Single cell RT-PCR confirmed that 5, 6-EET responsive cells co-express TRPV4 with GRP and TRPV1 **(Fig. 37e, f)**. These results further reinforce the idea that TRPV4 activity in DRG neurons is directly responsible for mediating pruriceptive transmission.

DISCUSSION

1. TRPV4 in acute itch

TRPV4 has emerged as the first channel important for both histamine and CQ itch. This is unique since previous studies coupled TRPV1 with histamine and TRPA1 with CQ itch respectively (Shim, Tak et al. 2007; Wilson, Gerhold et al. 2011). Interestingly, both TRPV4 and TRPA1 have similarly been implicated in inflammatory pain (Ceppa, Cattaruzza et al. 2010), suggesting that these two channels are likely to be functionally related in DRG neurons. Given that MrgprA3 and H1R are necessary for CQ- and histamine-induced itch (Han, Mancino et al. 2006; Liu, Tang et al. 2009), future studies will be needed to determine whether TRPV4 acts downstream of MrgprA3 or H1R, as well as the relationship between TRPV4 and TRPA1 or TRPV1. It will also be interesting how it relates with GRPR in the spinal cord. An endogenous TRPV4 agonist, 5, 6-EET, which induces scratching responses *in vivo* can be used to study TRPV4-related itch sensory mechanism underlying pruritus. Pacsin3 was identified as a histamine-independent itch gene from our screening. Pacsin3 can bind to TRPV4 and inhibit its function (D'Hoedt, Owsianik et al. 2008). Functional interaction between Pacsin3 and

TRPV4 for itch signaling pathways will also provide an interesting insight.

2. TRPV4 in chronic itch

The remarkable result arising from this study is that TRPV4 in DRG neurons is important for the development of ACD- but not for dry skin-induced pruritus. TRPV4 expression is uniquely up-regulated in DRG neurons of mice with ACD among several TRP channels examined. TRPV4$^{-/-}$ mice with ACD showed significantly attenuated pruritus during both the sensitization and elicitation phases without compromising the development of classic eczematous skin lesions and inflammatory responses. TRPV4$^{-/-}$ mice exhibited normal dry skin-induced pruritus. These data suggest that distinct signaling mechanisms underlie ACD- and dry skin-induced pruritus respectively. Hapten-elicited delayed hypersensitivity may selectively involve increased TRPV4 function, while epidermal barrier dysfunction observed in both ACD and dry skin condition does not.

Hapten-induced skin inflammatory response may release endogenous factors to activate TRPV4 signaling specifically. Upon encountering with external chemicals, the skin cells may release inflammatory mediators that can directly activate TRPV4 (Alessandri-Haber, Dina et al. 2006), or endogenous 5, 6-EET (Watanabe, Vriens et al. 2003). Since lack of TRPV4 neither prevents nor abolishes ACD itch, factors other than 5, 6-EET must be involved. The fact that both ACD and dry skin conditions share epidermal barrier dysfunction characterized by an altered stratum corneum, keratinization and water content indicates that additional TRPV4-independent signaling may be ascribed to itch

associated with the skin barrier dysfunction.

In human patients with chronic itch such as atopic dermatitis, scratch-itch-inflammation cycles often exacerbate the conditions by damaging the skin and increasing the inflammation (Wahlgren 1999). The present study, however, did not indicate a correlation between the intensity of scratching behavior and the severity of skin inflammation. A recent study also showed that scratching behavior does not necessarily correlate with skin inflammations (Kido, Takeuchi et al. 2010). One possibility is that a hapten or allergen is capable of initiating specific skin inflammatory response and frequent application of DNFB aggravates skin lesions regardless of scratching behavior. Alternatively, the remaining scratching bouts in TRPV4$^{-/-}$ mice are sufficient to maintain scratching-associated inflammatory components. Insofar as the relationship between scratching vs. inflammation associated with human ACD, correlative evidence is still lacking. Moreover, itch-scratch-inflammation cycles may be determined by a combination of various factors such as age of animals, type of allergens, and frequency of contacts. Hapten-induced immune response appears to follow its signature course as indicated by enhanced IgE and TSLP and is dictated by the number of times that skin is exposed to environmental substances. Results suggest that activation of TRPV4-dependent itch signaling in DRG neurons occurs at the sensitization stage, and may be maintained and potentiated in the elicitation phase by persistent skin inflammatory response. In summary, TRPV4 is a key channel important for mediating both histamine-dependent and -independent acute itch. Moreover, it is the first TRP channel that has been found to be essential for transmitting ACD-induced chronic pruritus. TRPV4 may

serve as a starting point for designing drugs that help alleviate ACD-induced pruritus. It will be important to examine whether i.t. and i.d. applications of TRPV4 specific antagonist or siRNA also can inhibit the scratching responses in ACD model because they will provide potential therapeutic approaches for ACD-related chronic itch diseases.

MATERIALS AND METHODS

Animals

C57BL/6J mice were purchased from Jackson laboratory. TRPV4 heterozygous mice (Suzuki, Mizuno et al. 2003) (RIKEN BioResource Center, Japan) were bred to generate $TRPV4^{-/-}$ mice. Both WT and $TRPV4^{-/-}$ male mice between 7 and 12 weeks were used for behavioral experiments. The animal experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Studies Committee at Washington University School of Medicine.

Double fluorescence ISH and immunohistochemistry (IHC)

Two different (fluorescein and digoxygenin, Roche)-labeled probes were prepared. Fluorescein-labeled probe was purified through G50 microcolumn (Amersham-Pharmacia) and then precipitated with lithium chloride ($LiCl_2$). Two probes were added into hybridization buffer. Procedures were same as the single ISH previously described (Birren, Lo et al. 1993). Fluorescent ISH combined with IHC was performed as described (Dong, Han et al. 2001). Slides were examined using Olympus BX51 upright microscope

attached with a CCD camera. Primary rabbit-GRP (Immunostar, 1:1,000) and TRPV1 (Neuromics; 1:1,000), guinea pig-CGRP (Peninsula, 1:1,000) and mouse-NF200 (Sigma, 1:1,000) antibodies were used and followed by incubating with secondary donkey anti-rabbit or mouse-IgG coupled to FITC (Jackson immunoresearch, 1:400). IB4-FITC (Sigma, 1:200) was used to show IB4 stainings.

Acute scratching behavior

Prior to the experiments, mice were placed in a small plastic chamber (15 × 26 × 12 cm) for 30 min to acclimate. Mice were briefly removed from the chamber and intradermally injected at the back of the neck with CQ (200μg/50μl saline; Sigma) and histamine (500μg/50μl saline; Sigma). 5, 6-EET (Enzo) was diluted into saline and injected intrathecally (150pmol/5μl; 0.1% ethanol). Hind limb scratching behavior towards the injected area was observed for 30 min with 5 min intervals. One scratch was defined as a lifting of the hind limb to the injection site and then a replacing of the limb back to the floor, regardless of how many scratching strokes take place between those two movements (Sun and Chen 2007). Mouse scratching responses were observed blindly to the treatment or genotype of the animals.

In vivo siRNA injection

TRPV4 mRNA sequence-specific siRNA (Sigma) was dissolved in DEPC-treated PBS. ExGen 500 *in vivo* transfecting reagent (Polyethylenimine (PEI; 0.18μl/1μg siRNA); Fermantas) was mixed to enhance siRNA cell penetration. siRNA solution (2.5ug

siRNA-PEI mix) was injected into mice intrathecally once daily for three days as described (Kawasaki, Xu et al. 2008). After 30 min of the last injection, scratching behaviors with pruritogens were monitored for 30 min. DEPC-PBS/PEI mix was used as a control.

QRT-PCR

Lumbar spinal cord dorsal horn and DRG were dissected out from control (DEPC-PBS/PEI) and siRNA-treated mice, and were homozenized with Dounce glass homogenizer (Wheaton) and total RNA was prepared by using Trizol reagent (Invitrogen). cDNA was synthesized using Superscript First-strand synthesis system (Invitrogen) and conventional or QRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and Mx3000P qPCR system (Stratagene). Expression of target mRNA was normalized to 18S ribosomal RNA (rRNA) level. The followings are primers used for PCR.

18sRNA, 5'-AAACGGCTACCACATCCAAG-3' and
5'-CCTCCAATGGATCCTCGTTA-3'

TRPV1, 5'-GTGATCGCCTACAGTAGCAGTG-3' and
5'-ACATGTGGAATACAGGCTGTTG-3'

TRPV2, 5'-GGTGGTTTTAGAGCCACTGAAC-3' and
5'-GTAGATGCCTGTGTGCTGAAAG-3'

TRPV4, 5'-GAGAACACCAAGTTTGTCACCA-3' and
5'-TGAATCATGATGCTGTAGGTCC-3'

TRPA1, 5’-AGTATCATCTTCGTGTTGCCCT-3’ and

5’-AGTCTCCCACTCCCATAAGACA-3’

TRPM8, 5’-ATGAATATGAGACCCGAGCAGT-3’ and

5’-GCAAAGAGGAACAGGAAGAAGA-3’

MrgA3, 5’-GGCATCACCTGGTTCCTGTTA-3’ and

5’-CCAGTGAGGCATGTCAAGTCA-3’

H1R, 5’-AGAGCATCTGAAAGAGACCCAG-3’ and

5’-GTTCTCATCCCAAGTTTCCAAG-3’

TSLP, 5’- CCAGGCTACCCTGAAACTGA-3’ and

5’- TCTGGAGATTGCATGAAGGA-3’

Allergic contact dermatitis (ACD) model

2, 4-Dinitrofluorobenzene (DNFB; Sigma) was dissolved in acetone. 50 μl of 0.15% DNFB acetone solution was applied on the back skin of neck every other day (Saint-Mezard, Rosieres et al. 2004; Yamashita, Ito et al. 2010). This was repeated for two weeks and scratch responses were measured every morning (24hrs after painting) for 30 min with 5 min intervals.

Dry skin model

Cotton soaked with acetone/diethylether (AE; 1:1) was put on the shaved area of neck for 15 sec and then distilled water was applied for 30 sec. This treatments were performed twice a day and repeated once daily for five days (Miyamoto,

Nojima et al. 2002). The number of scratch was counted after 5 min of water treatment for 30 min with 5 min intervals.

Skin histology

For hematoxylin and eosin (H&E) and toluidine blue staining using paraffin-embedded tissue sections, skin was fixed in 4% paraformaldehyde in PBS, dehydrated with ethanol and embedded in paraffin. It was sectioned at 5μm.

ELISA

Serum IgE was measured using Mouse IgE ELISA kit (Immunology Consultants Laboratory Inc., Newberg, OR)

White Blood Cell Count

Blood samples collected from the mandibular vein were used for hematological analysis with Hemavet 950 analyzer (Drew Scientific Inc., Oxford, CT), which included white blood cells (WBC) and differential counts.

DRG culture, siRNA transfection and Ca^{2+} imaging

DRG were dissected in neurobasal media (Invitrogen) and incubated with papain (30μl/2ml neurobasal; Worthington) at 37ºC for 20 min. After washing with PBS, cells were incubated with collagenase (3mg/2ml neurobasal; Worthington) at 37ºC for 20 min. After washing with PBS, cells were dissociated with flame polished glass pipette in

neurobasal media. Dissociated cells were collected through cell strainer (BD Biosciences) to remove tissue debris. Dissociated DRG cells were re-suspended with DRG culture media (2% Fetal bovine seum, 2% Horse serum, 2% B-27 supplement and 1x Glutamine in neurobasal media; Invitrogen) and plated onto poly-ornithine-coated 12 mm diameter round glass coverslips in 24-well plate. siRNA stock solution was prepared as described previously (in Chapter II). DRG cells were transfected with siRNA (60 pmol) by using Lipofectamine 2000 reagent (Invitrogen). After 24 hours, cell culture media was replaced with Ca^{2+} imaging buffer and cells were loaded with Fura-2 (2μM) diluted in the Ca^{2+} imaging buffer. Ca^{2+} imaging was performed as shown in the previous section (Chapter II). CQ (1mM), histamine (100μM) and 5, 6-EET (300pM) were applied to DRG cultures to examine Ca^{2+} responses.

Single-cell RT-PCR

Single-cell RT-PCR was performed as previously described (Park, Kim et al. 2006). DRG neurons were aspirated into a glass patch pipette with a tip diameter of 25-30μm by applying negative pressure using CellTram Vario (Eppendorf). Cells were gently put into a reaction tube containing reverse transcription reagents and incubated for 1 hr at 50°C (Superscript III, Invitrogen). The first round of PCR was preformed in 50 μl of PCR buffer containing 0.2 mM dNTPs, 0.2 μM outer primers, 5 μl cDNA and 0.2 μl platinum Taq DNA polymerase (Invitrogen). The reactions were denatured initially for 5 min at 95 °C followed by 40 cycles of 40 sec denaturation at 95 °C, 40 sec annealing at 55 °C, and 40 sec elongation at 72 °C. The reaction was completed with 7 min of final

elongation. For the second round of amplification, the reaction buffer (20 μl) contained 0.2 mM dNTPs, 0.2 μM inner primers, 5 μl of the first round PCR products and 0.1 μl platinum Taq DNA polymerase (Invitrogen). The reaction procedure was the same as the first round. A negative control was obtained from pipettes that did not harvest any cell contents, but were submerged in the bath solution. PCR products were displayed on ethidium bromide-stained 1% agarose gels.

PCR primers are as follows:

Actin (outer), 5'-TGTTACCAACTGGGACGACA-3' and
5'-TCTCAGCTGTGGTGGTGAAG-3'

Actin (inner) 5'-GATCTGGCACCACACCTTCT-3' and
5'-ACGCTCGGTCAGGATCTTC-3'

TRPV1 (outer) 5'-TGATCATCTTCACCACGGCTG-3' and
5'-CCTTGCGATGGCTGAAGTACA-3';

TRPV1 (inner) 5'-AAGGCTTGCCCCCCTATAA-3' and
5'-CACCAGCATGAACAGTGACTGT-3'

GRP (outer) 5'-CCAAGGAGCAAAACAAAACCC-3' and
5'-GCAAATTGGAGCCCTGAATCT-3'

GRP (inner) 5'-AGACTGCCTTCTGCAAACGTC-3' and
5'-AAGCCTAGCTGGAAAAAGCG-3'

Statistical analysis

Statistical analysis was performed with Student's *t*-test or two-way repeated measured

analysis of variance (ANOVA). All data were expressed as the mean ± standard error mean (SEM) and error bars represent SEM in all cases, $p < 0.05$ was considered statistically significant.

V. CONCLUSIONS AND FUTURE DIRECTIONS

Although itch was defined as an unpleasant sensation that elicits the desire to scratch by German physician Samuel Hafenreffer in 1660 (Rothman 1941), itch has been put aside because it was thought as a minor form of somatosensation relative to pain. Itch and pain are closely related each other. Mild mechanical stimulation (or pain) by scratching can inhibit itch. Nociceptive stimuli like noxious heat reduce itch while itch can be induced by antagonizing pain via opioids (Szarvas, Harmon et al. 2003). Both itch and pain can be elicited by multiple mechanical and chemical stimuli through similar neuronal populations. Although several studies found critical components for itch pathways, the identity of neural circuit transmitting itch and basic molecular mechanism have yet to be clearly defined. While the relation between itch and pain is still unclear, itch researchers have recently provided evidences suggesting that itch is a distinct sensation from pain. Currently, itch is being recognized as a medical problem, especially in chronic itch diseases such as AD. It is impossible to establish an efficient therapeutic method without understanding molecular mechanism of itch signaling pathways in the nervous system.

First, we identified novel itch-related molecules in the spinal cord and DRG by differential screening. LCM was used to dissect superficial dorsal horn including lamina I and II. This made the screening feasible with a tiny region of the spinal cord where GRPR is expressed. Another important experimental approach used in this study is *in vivo* siRNA-mediated gene knockdown in the spinal cord and DRG. i.t. injection of

siRNA affects spinal cord and DRG gene expression, therefore, siRNA provides tissue selectivity like conditional KO. One of the advantages of *in vivo* siRNA is that siRNA effect can be verified by behavioral test directly. *In vitro* siRNA transfection using cell culture may support certain gene functions in itch signaling but this will raise a question whether *in vitro* system is same as *in vivo* status. *In vivo* siRNA circumvents this potential problem of *in vitro* approaches. The primary purpose of the screening was to identify additional novel itch receptors and GRPR downstream signaling molecules, Atp2a1 and Itpr3 were found indicating that intracellular Ca^{2+} signaling is important for itch sensation. Identification of GRPR expressing neurons as itch specific cells (Sun, Zhao et al. 2009) supports the idea that there are neuronal populations for itch-dedicated pathways in the spinal cord. Functional studies of Atp2a1 and Itpr3 involved in GRPR-mediated histamine-independent pathways suggests that there are different types of signaling for distinct itch sensations in single cell. Although similar molecules can be involved in multiple pathways, isoform-specific mediators still can distinguish two different itch signaling pathways. Differential screening was performed with the spinal cord treated with bombesin-saporin but GRPR is also expressed in DRG **(Fig. 22 and 23)**. Previous study confirmed that bombesin-saporin did not change DRG primary afferent markers innervated in the spinal cord (Sun, Zhao et al. 2009). However, this still does not exclude the possibility that bombesin-saporin may affect GRPR expressing cells in DRG. The DRG may provide additional place where potentially interesting itch receptors are located. It will be worth while to study DRG gene expressions after bombesin-saporin treatment. Receptors and downstream mediators are critical components of signaling

pathways but these can be regulated by gene expression level changes. It is unclear how expressions of itch-related genes are regulated. To identify molecular mechanism of itch pathways controlled by transcriptional changes, candidate genes encoding transcription factors can be further studied. If certain itch genes like GRPR are expressed under control of specific transcription factors, this will provide a missing link between itch signaling pathways and gene expressions.

Second, previous studies with GRPR (Sun and Chen 2007) only focused on the spinal cord. In this study, we found peripheral GRPR roles in itch. However, there are still several questions to be addressed. Compared with i.t. injection (1 nmol), GRP i.d. injection requires much higher dose (100 nmoles) **(Fig. 24a)**. GRP may be easily degraded in the skin or can not diffuse well. It is uncertain whether GRP activates $GRPR^{+}$ free nerves, epidermal keratinocytes or mast cells. Because we don't know the exact mechanism of peripheral GRP-GRPR activation for itch, it will be necessary to study peripheral GRPR roles using tissue-specific conditional KO mice. Peripheral GRPR is also important in chronic itch **(Fig. 28)**. DCP-painted mice model was used to represent human contact dermatitis. In addition to acute itch induced by CQ and histamine, it is important to understand chronic itch pathways to establish an efficient therapeutic method. This will translate basic molecular mechanism of itch to clinical relevance in human.

Third, TRPV4 was identified from siRNA screening. One of the candidate genes from differential screening with bombesin-saporin treated spinal cord was Pacsin3. Although TRPV4 is not expressed in the spinal cord, Pacsin3 was known to bind TRPV4

and regulates TRPV4 activity (D'Hoedt, Owsianik et al. 2008). Because Pacsin3 and TRPV4 are expressed in DRG, it is possible that both can be involved in itch sensory pathways. Functional studies of TRPV4 by siRNA and KO mice confirmed that TRPV4 is an important TRP channel in DRG for both acute and chronic itch. It is interesting that TRPV4 mediates both histamine-dependent and -independent itch in that the other TRP channels such as TRPV1 and TRPA1 are involved in either type of itch. i.t. injection of an endogenous TRPV4 agonist, 5, 6-EET caused scratching behaviors in mice **(Fig. 33e)**. 5, 6-EET also induced intracellular Ca^{2+} increase in DRG neurons **(Fig. 37d, e)**. Although Ca^{2+} response is one indicator of cellular activities, it does not always represent neuronal activation such as action potential firings. Therefore, electrophysiological studies with TRPV4 agonist and antagonist in normal and chronic itch mice DRG will be required to verify neuronal TRPV4 functions for itch. Another important finding is that an increased TRPV4 expression in DRG is responsible for aggravated itch/scratching responses in ACD mice model **(Fig. 33d)**. This broad involvement of TRPV4 in itch makes it more interesting itch therapeutic target. To examine relationships between TRPV4 and other TRP channels such as TRPA1 and TRPV1, siRNA and KO mice studies can be combined. GRP and GRPR are critical itch mediators in DRG and spinal cord. It will be also an interesting question how TRPV4 is related with GRP and GRPR-mediated pathways. These studies will help understand itch signaling pathways in peripheral DRG neurons in detail.

Itch is well conserved in vertebrates. It has long been considered as a basic sensory or defensive mechanism to protect our body from external harmful reagents.

However, it is now being recognized as a medical problem in clinical field. Itch is closely related with our daily life. Bug (mosquito) bite, contact allergen such as pollens, plants (poison ivy), chemicals and metals (nickel) cause human itching. Climate condition like dryness affect skin status and induce itchness. Patients with chronic itch disease such as AD and psoriasis, immune system defects, and systemic liver disease are suffering from intractable itch symptoms. Several therapeutic approaches such as dialysis for renal failure, cancer therapy and pain drugs have itch as side effects and this reduces their effectiveness. Recent studies have identified critical components of itch signaling pathways but our knowledge about detail mechanism of pruritus is still infancy. The results in this thesis work will further advance our knowledge about itch sensory mechanism and provide therapeutic insights for itch-related human diseases.

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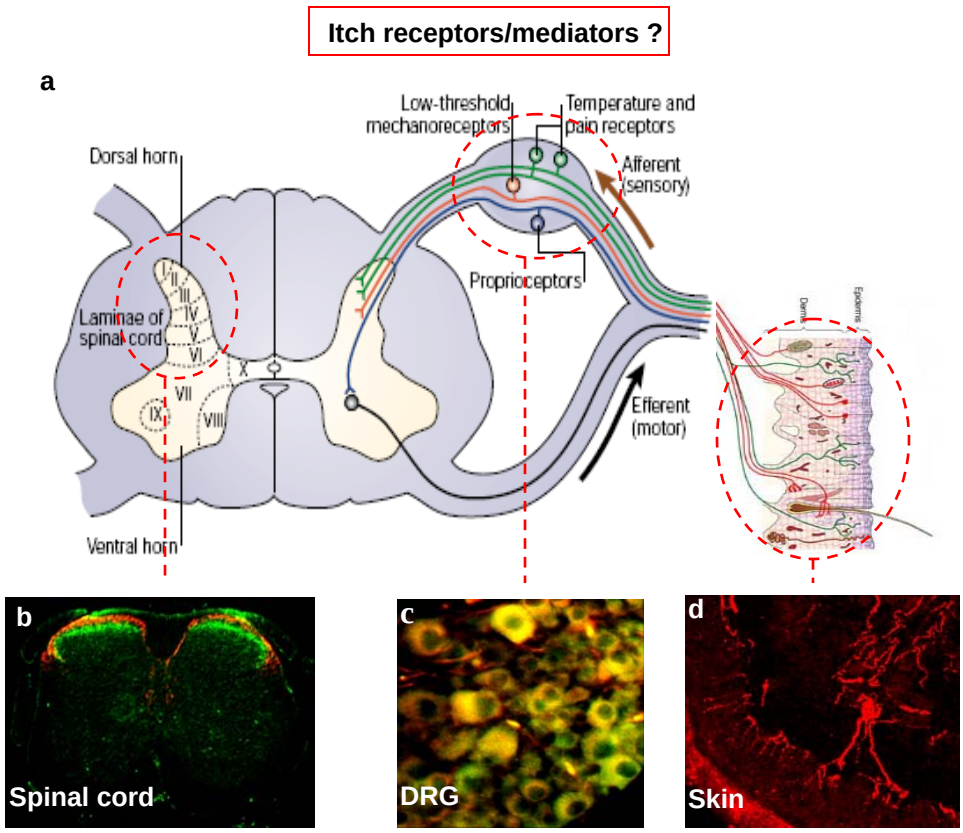


Figure 1. Neuronal circuits for somatosensations.

(a) Skin receives external sensory inputs through peripheral nerves. Sensory information is relayed through DRG and spinal cord. DRG has distinct cell populations that express specific receptors recognizing various stimuli. Spinal cord dorsal horn has multiple laminar layers. Superficial dorsal horn is an important place to transmit the somatosensory signals to brain producing proper responses to external environment. Therefore, it will be interesting to identify Itch-specific molecules in these neuronal circuits. Modified from (Patapoutian, Peier et al. 2003). (b) Spinal cord dorsal horn lamina I and II are shown by SP (red) and IB4 (green) immunostaining. (c) Different populations of DRG neurons expressing TRPV4 (red), GRP (green) and both (yellow). (d) CGRP+ free nerve endings are innervated into skin epidermis as shown by CGRP immunostaining (Red). SP : Substance P, IB4 : Isolectin B4, CGRP : Calcitonin gene-related peptide

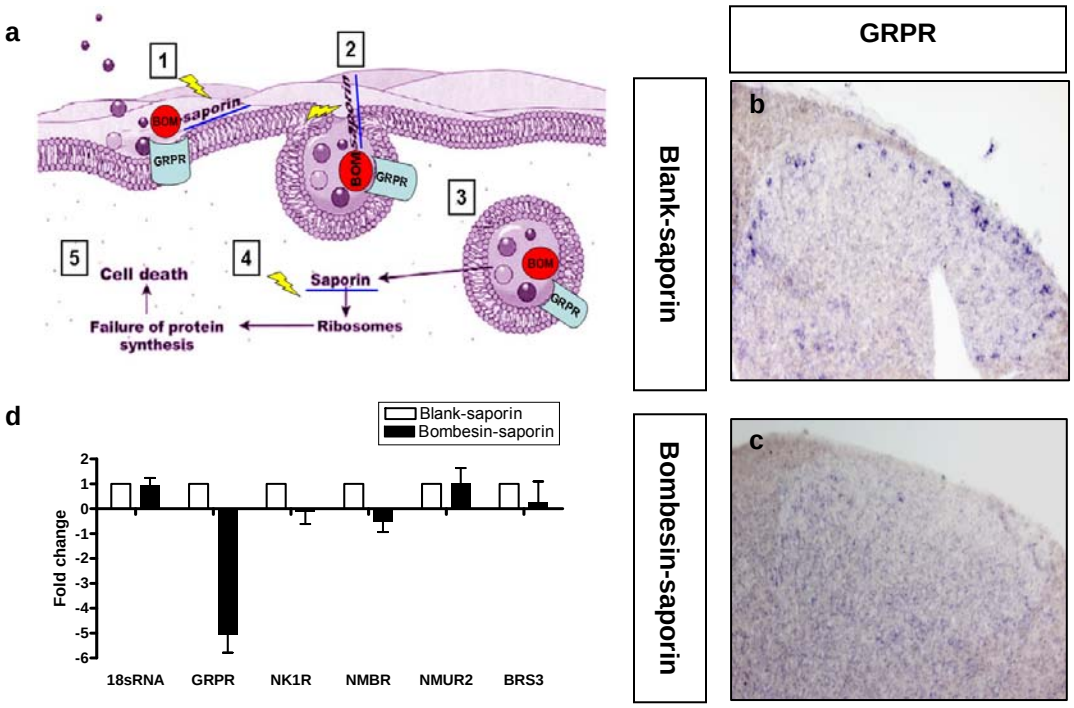


Figure 2. GRPR cell ablation by bombesin-saporin.

(a) Bombesin-saporin kills GRPR cells by inhibiting protein translation. 1. Bombesin-saporin binds to GRPR, 2. Bombesin-saporin bound GRPR is internalized into cytosol by endocytosis. 3. Saporin is released by proteolysis. 4. Saporin inhibits protein translation by targeting ribosomal RNAs. 5. Failure of protein synthesis causes cell death. BOM : Bombesin. Modified from (Wiley 2000) (b, c) In situ hybridization shows that GRPR expressing cells were ablated by bombesin-saporin treatment. (d) QRT-PCR confirmed that bombesin-saporin treatment selectively reduced GRPR expression without affecting other superficial dorsal horn marker genes (Student t-test, $p<0.05$, n=9). NK1R : Tachykinin receptor 1 , NMBR : Neuromedin B receptor, NMUR2 : Neuromedin U receptor 2, BRS3 : Bombesin-like receptor 3

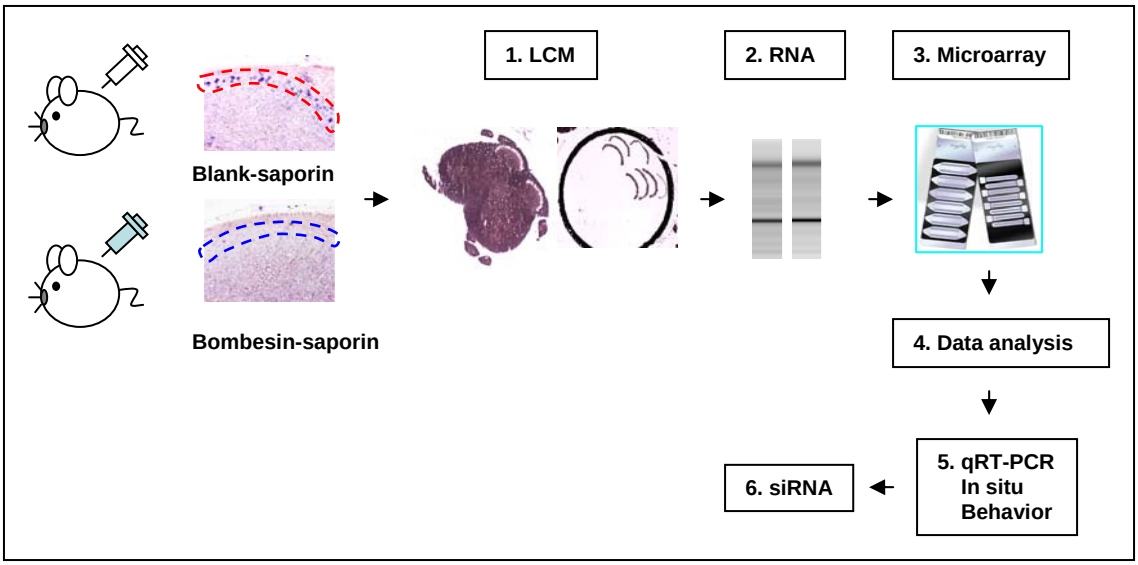


Figure 3. Differential screening to identify itch genes in GRPR neurons.

1. Blank- and bombesin-saporin treated spinal cord dorsal horn superficial layers were dissected using laser capture microscope (LCM). 2. Total RNA was extracted and amplified. RNA integrity was measured by Bio-analyzer. 3. cDNA was synthesized and hybridized with illumine bead array. 4. Microarray data were analyzed with Partek Genome Suite and Ingenuity Pathway Analysis (IPA). 5. Candidate genes were verified by QRT-PCR, in situ hybridization (ISH) and behavioral tests. 6. siRNA studies further confirmed the roles of candidate genes in itch sensation.

Table 1. Microarray candidate genes that have reduced expressions in bombesin-saporin treated spinal cord.

SYMBOL	Gene	Fold Change	Frequency
Grpr	Gastrin releasing peptide receptor	- 6	9
Glp2r	Glucagon-like peptide 2 receptor	- 5	9
V1rf1	Vomeronasal 1 receptor, F1	- 4	9
Fgfr3	Fibroblast growth factor receptor 3	- 4	9
Kcne1	Potassium voltage-gated channel, Isk-related subfamily, member 1	- 4	9
Taar7e	Trace amine-associated receptor 7E	- 4	9
Fpr-rs6	Formyl peptide receptor, related sequence 6	- 3	9
Tnfrsf7	Tumor necrosis factor receptor superfamily, member 7	- 3	9
Tas2r119	Taste receptor, type 2, member 119	- 3	9
Xlkd1	Lymphatic vessel endothelial hyaluronan receptor	- 3	9
Grm1	Glutamate receptor, metabotropic 1	- 3	9
Ptger4	Prostaglandin E receptor 4	- 2	9
Tlr5	Toll-like receptor 5	- 6	8
Cd244	CD244 natural killer cell receptor 2B4	- 5	8
Kcnj15	Potassium inwardly-rectifying channel, subfamily J, member 15	- 4	8
Kcng1	Potassium voltage-gated channel, subfamily G, member 1	- 3	8
V2r15	Vomeronasal 2, receptor 30	- 3	8
Il27ra	Interleukin 27 receptor, alpha	- 3	8
Kcnq2	Potassium voltage-gated channel, subfamily Q, member 2	- 3	8
Klra9	Killer cell lectin-like receptor subfamily A, member 9	- 3	8
C5r1	Complement component 5a receptor 1	- 2	8
Gabt4	Solute carrier family 6 (neurotransmitter transporter, GABA), member 11	- 2	8
Gpr18	G protein-coupled receptor 18	- 11	7
Gpr133	G protein-coupled receptor 133	- 7	7
Esrrb	Estrogen related receptor, beta	- 5	7
Gpr35	G protein-coupled receptor 35	- 4	7
GPR106	LGR8, GPCR, relaxin/insulin-like family peptide receptor 2	- 3	7

Gpr31c	G protein-coupled receptor 31, D17Leh66c region	- 3	7
Npy5r	Neuropeptide Y receptor Y5	- 2	7
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	- 10	6
Mrgprb4	MAS-related GPR, member B4	- 10	6
Ret	ret proto-oncogene	- 9	6
Gpr73	Prokineticin receptor 1 (Prokr1, PKR1)	- 7	6
Mrgpra3	MAS-related GPR, member A3	- 6	6
P2rx4	Purinergic receptor P2X, ligand-gated ion channel 4	- 5	6
Mrgpra2	MAS-related GPR, member A2	- 5	6
Accn3	Amiloride-sensitive cation channel 3	- 4	6
Sorcs1	VPS10 domain receptor protein SORCS 1	- 4	6
Edg7	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 7	- 3	6
Mrgpra7	MAS-related GPR, member A7	- 3	6
Chrm2	Cholinergic receptor, muscarinic 2, cardiac	- 3	6
Cacna1b	Calcium channel, voltage-dependent, N type, alpha 1B subunit	- 3	6
Cacna1c	Calcium channel, voltage-dependent, L type, alpha 1C subunit	- 3	6
Il1r2	Interleukin 1 receptor, type II	- 2	6
Lbr	Lamin B receptor	- 2	6
Itpr3	Inositol 1,4,5-triphosphate receptor 3	- 2	6
Anktm1	Transient receptor potential cation channel, subfamily A, member 1	- 2	6
Scn4b	Sodium channel, type IV, beta	- 2	6
Sstr4	Somatostatin receptor 4	- 2	6
Trpc6	Transient receptor potential cation channel, subfamily C, member 6	- 2	6
Paqr3	Progestin and adipoQ receptor family member III	- 2	6
Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	- 4	5
Ptgdr	Prostaglandin D receptor	- 2	5
Il17rb	Interleukin 17 receptor B	- 2	5

Nr3c2	Nuclear receptor subfamily 3, group C, member 2	- 7	4
Tlr6	Toll-like receptor 6	- 5	4
V1rl1	Vomeronasal 1 receptor, L1	- 4	4
V1rh10	Vomeronasal 1 receptor, H10	- 3	4
Fgfr4	Fibroblast growth factor receptor 4	- 2	4
Tas2r105	Taste receptor, type 2, member 105	- 10	3
Sstr2	Somatostatin receptor 2	- 10	3
Cacnb1	Calcium channel, voltage-dependent, beta 1 subunit	- 9	3
Oprd1	Opioid receptor, delta 1 (DOR)	- 9	3
Celsr2	Cadherin, EGF LAG seven-pass G-type receptor 2	- 8	3
Oprm1	Opioid receptor, mu 1 (MOR)	- 8	3
Trik	Potassium channel, subfamily K, member 18	- 6	3
5-Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	- 6	3
Pparg	Peroxisome proliferator activated receptor gamma	- 6	3
Mrc2	Mannose receptor, C type 2	- 5	3
Clic6	Chloride intracellular channel 6	- 5	2
P2ry10	Purinergic receptor P2Y, G-protein coupled 10	- 5	3
Il18r1	Interleukin 18 receptor 1	- 4	3
Tas2r136	Taste receptor, type 2, member 136	- 4	3
Scnn1b	Sodium channel, nonvoltage-gated 1 beta	- 4	3
Mrgprx1	MAS-related GPR, member X1	- 4	3
Adora3	Adenosine A3 receptor	- 4	3
Htr7	5-hydroxytryptamine (serotonin) receptor 7	- 4	3
Ppyr1	Pancreatic polypeptide receptor 1	- 4	3
V1ri9	Vomeronasal 1 receptor, I9	- 3	3
Gfra3	Glial cell line derived neurotrophic factor family receptor alpha 3	- 3	3
Trpm1	Transient receptor potential cation channel, subfamily M, member 1	- 3	3
Mc2r	Melanocortin 2 receptor	- 3	3
Klra12	Killer cell lectin-like receptor subfamily A, member 12	- 3	3
V1rh1	Vomeronasal 1 receptor, H1	- 3	3
V2r15	Vomeronasal 2, receptor 30	- 3	3
Igf1r	Insulin-like growth factor I receptor	- 3	3
P2ry2	Purinergic receptor P2Y, G-protein coupled 2	- 3	3
Catsper3	Cation channel, sperm associated 3	- 3	3
V1rd13	Vomeronasal 1 receptor, D13	- 3	3
Il7r	Interleukin 7 receptor	- 3	3
V1rc33	Vomeronasal 1 receptor, C33	- 3	3
Nptxr	Neuronal pentraxin receptor	- 2	3
Npr1	Natriuretic peptide receptor 1	- 2	3
Kcna10	Potassium voltage-gated channel, shaker-related subfamily, member 10	- 2	3
Kcnc3	Potassium voltage gated channel, Shaw-related subfamily, member 3	- 2	3
Tas2r110	Taste receptor, type 2, member 110	- 2	3
V1rc23	Vomeronasal 1 receptor, C23	- 2	3
Avpr1b	Arginine vasopressin receptor 1B	- 2	3
Kcnf1	Potassium voltage-gated channel, subfamily F, member 1	- 2	3
V1rc21	Vomeronasal 1 receptor, C21	- 2	3
Ildr1	Immunoglobulin-like domain containing receptor 1	- 2	3
Klri1	Killer cell lectin-like receptor family I member 1	- 2	3
Mc3r	Melanocortin 3 receptor	- 2	3
Chrng	Cholinergic receptor, nicotinic, gamma polypeptide	- 12	2
Tas2r126	Taste receptor, type 2, member 126	- 6	2
Kcnk4	Potassium channel, subfamily K, member 4	- 5	2

Esrrg	**Estrogen-related receptor gamma**	**- 5**	**2**
V1rh9	**Vomeronasal 1 receptor, H9**	**- 5**	**2**
Calcr	**Calcitonin receptor, transcript variant a**	**- 5**	**2**
V2r10	**Vomeronasal 2, receptor 89**	**- 5**	**2**
Gabrp	**Gamma-aminobutyric acid (GABA-A) receptor, pi**	**- 4**	**2**
Folr4	**Folate receptor 4 (delta) (Folr4), transcript variant 2**	**- 3**	**2**
Kcnk4	**Potassium channel, subfamily K, member 4**	**- 3**	**2**
V2r1b	**Vomeronasal 2, receptor 26**	**- 3**	**2**
Ptger2	**Prostaglandin E receptor 2 (subtype EP2)**	**- 3**	**2**
Cntfr	**Ciliary neurotrophic factor receptor**	**- 3**	**2**
Mass1	**G protein-coupled receptor 98**	**- 2**	**2**
Tlr9	**Toll-like receptor 9**	**- 2**	**2**
Cnga2	**Cyclic nucleotide gated channel alpha 2**	**- 2**	**2**
Ncr1	**Natural cytotoxicity triggering receptor 1**	**- 2**	**2**
Gpr43	**Free fatty acid receptor 2 (Ffar2)**	**- 2**	**2**
Tnfrsf22	**Tumor necrosis factor receptor superfamily, member 22**	**- 2**	**2**
Mrgpra8	**MAS-related GPR, member A8**	**- 2**	**2**

Candidate genes were selected if their fold change (negative) and frequency are higher than 2. 123 genes encoding receptor, channel and membrane protein were considered as primary targets. Gray: not reduced in QRT-PCR, Yellow: reduced in QRT-PCR,

Blue: not confirmed by agonists or antagonists, Red: confirmed by agonists or antagonists

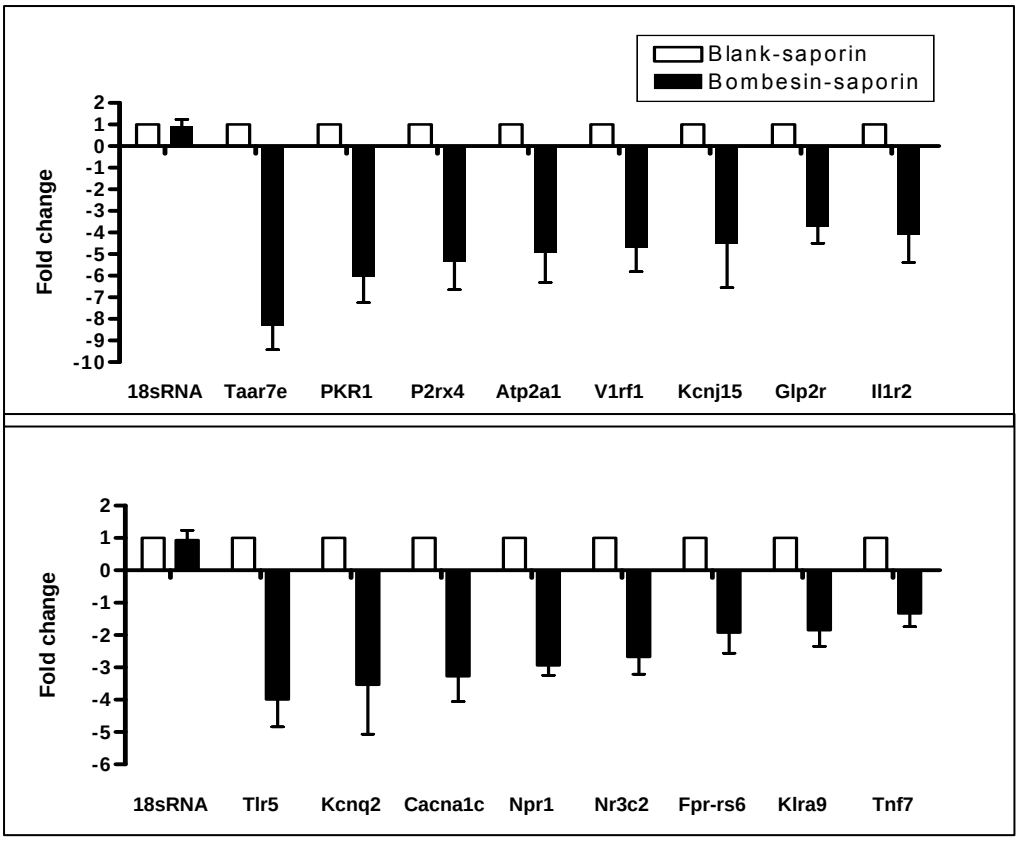


Figure 4. QRT-PCR showed that 16 genes have reduced expression levels in bombesin-saporin treated samples. QRT-PCR was performed with total RNA from superficial layers of dorsal spinal cord dissected by LCM. 18sRNA was used as a reference to calculate relative fold change between control (blank-saporin) and bombesin-saporin treated samples. Student t-test ($p<0.05$, N=9)

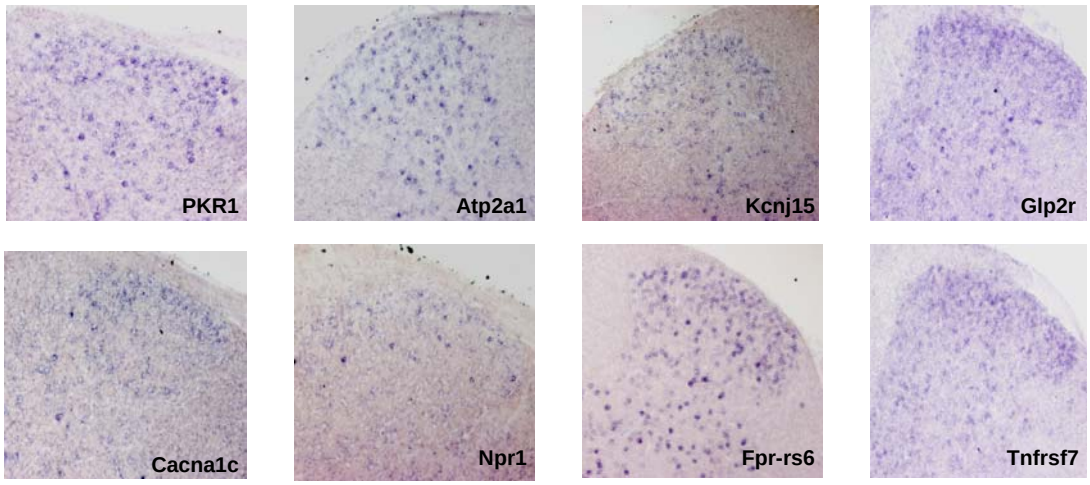


Figure 5. ISH showed that 8 candidate genes are expressed in the spinal cord dorsal horn. ISH was performed for 16 genes confirmed by QRT-PCR. Half of them showed specific ISH signals in the spinal cord.

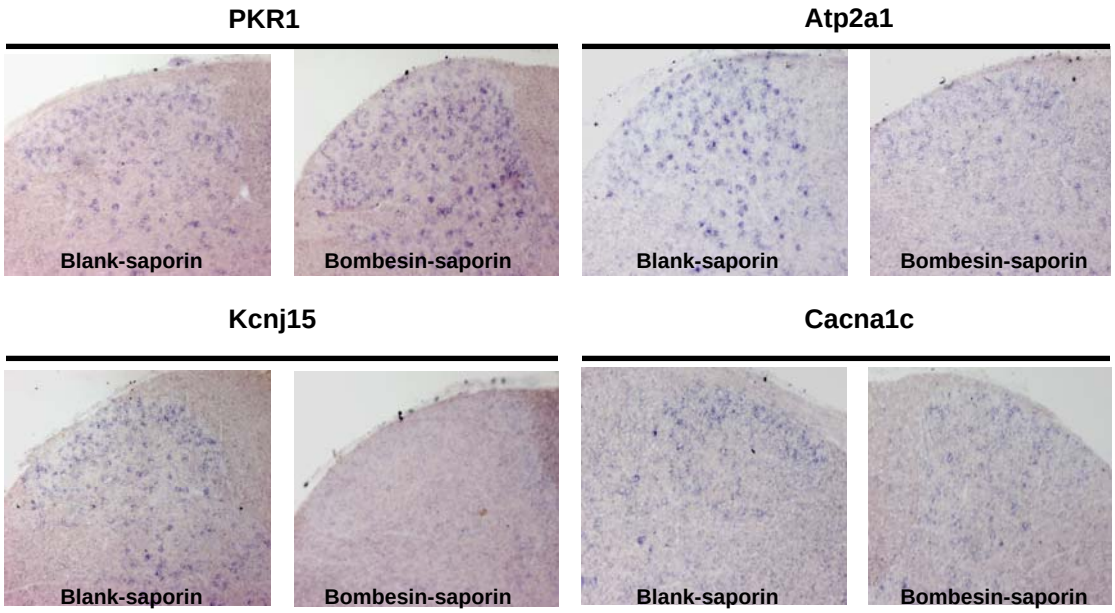


Figure 6. ISH showed that expressions of 4 candidate genes were decreased in the bombesin-saporin treated spinal cord. Cervical spinal cord from blank- and bombesin-saporin treated spinal cord was used for ISH to compare candidate gene expressions between them.

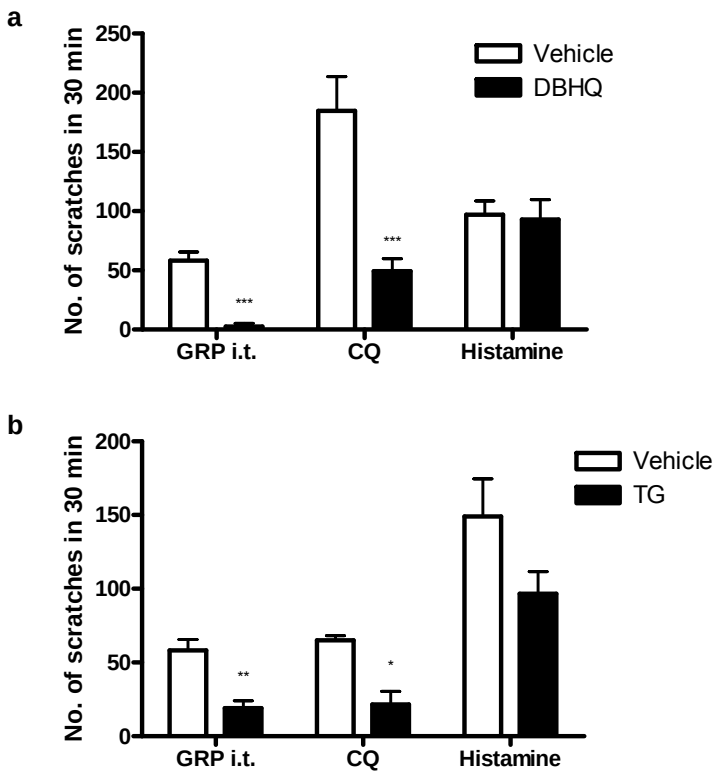


Figure 7. Atp2a1 inhibitor, DBHQ and TG, i.t. injections inhibited histamine-independent itch. DBHQ (10μg, vehicle: 5%DMSO) and TG (5μg, vehicle: saline) were injected intrathecally 10 min before GRP (0.5nmol, i.t.), CQ (200μg, i.d.) and histamine (1mg, i.d.) treatments. Student t-test (*p<0.05, **p<0.01, ***p<0.001) was used for statistical analysis (n=8). DBHQ: 2, 5-Di-tert-butylhydroquinone, TG: Thapsigargin, CQ: Chloroquine, i.t.: intrathecal injection, i.d.: intradermal injection

Table 2. Microarray candidate genes that may be involved in Ca2+ signaling.

SYMBOL	Gene	Fold Change	Frequency
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	- 6	10
Pip5k1c	Phosphatidylinositol-4-phosphate 5-kinase	- 6	9
Grpr	Gastrin-releasing peptide receptor	- 6	9
Rgr3	Regulator of G-protein signaling 13	- 2	9
Pla2g1b	Phospholipase A2, group IB, pancreas	- 17	8
Pik3c2g	Phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide	- 5	8
Arhgap19	Rho GTPase activating protein 19	- 4	8
Pld3	Phospholipase D family, member 3	- 3	8
Pacsin3	Protein kinase C and casein kinase substrate in neurons 3	- 3	8
Gps1	G protein pathway suppressor 1	- 2	8
Itpkb	Inositol 1,4,5-trisphosphate 3-kinase B	- 4	7
Arfgap1	ADP-ribosylation factor GTPase activating protein 1	- 3	7
Rohn	Rho family GTPase 2	- 3	7
Cabp4	Calcium binding protein 4	- 4	6
Pde6d	Phosphodiesterase 6D, cGMP-specific, rod, delta	- 4	6
Pla2g5	Phospholipase A2, group V	- 3	6
Pitpnc1	Phosphatidylinositol transfer protein, cytoplasmic 1	- 2	6
Prkcm	Protein kinase C, mu	- 2	6
Rgs4	Regulator of G-protein signaling 4	- 2	6
Rgs11	Regulator of G-protein signaling 11	- 2	4
S100a8	S100 calcium binding protein A8 (calgranulin A)	- 4	3
Racgap1	Rac GTPase-activating protein 1	- 4	3
Gnat3	Guanine nucleotide binding protein, alpha transducing 3	- 2	3
Rdgb2	Phosphatidylinositol transfer protein, membrane-associated 2	- 2	3
Pde6g	Phosphodiesterase 6G, cGMP-specific, rod, gamma	- 2	3
Pla2g3	Phospholipase A2, group III	- 2	3

Pla2g4d	**Phospholipase A2, group IVD**	**- 10**	**2**
Pik3r2	**Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)**	**- 3**	**2**
Arhgap8	**Rho GTPase activating protein 8**	**- 3**	**2**
Plcz1	**Phospholipase C, zeta 1**	**- 2**	**2**
Rgs3	**Regulator of G-protein signaling 3**	**- 2**	**2**

30 candidate genes were selected for potential GRPR downstream Ca2+ signaling molecules

Table 3. QRT-PCR further selected 11 Ca2+ signaling candidate genes that showed reduced expressions in bombesin-saporin treated spinal cord.

SYMBOL	Gene	Fold Change	Frequency
Atp2a1	**ATPase, Ca++ transporting, cardiac muscle, fast twitch 1**	**- 6**	**10**
Pip5k1c	**Phosphatidylinositol-4-phosphate 5-kinase**	**- 6**	**9**
Pik3c2g	**Phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide**	**- 5**	**8**
Pld3	**Phospholipase D family, member 3**	**- 3**	**8**
Pacsin3	**Protein kinase C and casein kinase substrate in neurons 3**	**- 3**	**8**
Itpkb	**Inositol 1,4,5-trisphosphate 3-kinase B**	**- 4**	**7**
Prkcm	**Protein kinase C, mu**	**- 2**	**6**
Itpr3	**Inositol 1,4,5-triphosphate receptor 3**	**- 2**	**6**
S100a8	**S100 calcium binding protein A8 (calgranulin A)**	**- 4**	**3**
Pik3r2	**Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)**	**- 3**	**2**
Plcz1	**Phospholipase C, zeta 1**	**- 2**	**2**
Itpr3	**Inositol 1,4,5-triphosphate receptor 3**	**- 2**	**6**

Table 4. 11 ER-Ca2+ related genes for siRNA screening.

PLB	**Phospholamban**
SLN	**Sarcolipin**
Calnexin	**Calnexin**
CALR	**Calreticulin**
Erp57	**ER oxidoreductase**
DSP	**Desmoplakin**
Pik3r2	**Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)**
Arhgap8	**Rho GTPase activating protein 8**
Plcz1	**Phospholipase C, zeta 1**
Rgs3	**Regulator of G-protein signaling 3**
TRPV4	**Transient receptor potential (TRP) vanilloid 4 ion channel**

Target genes were decided by QRT-PCR results and functional relations with ER-Ca2+ signaling pathways.

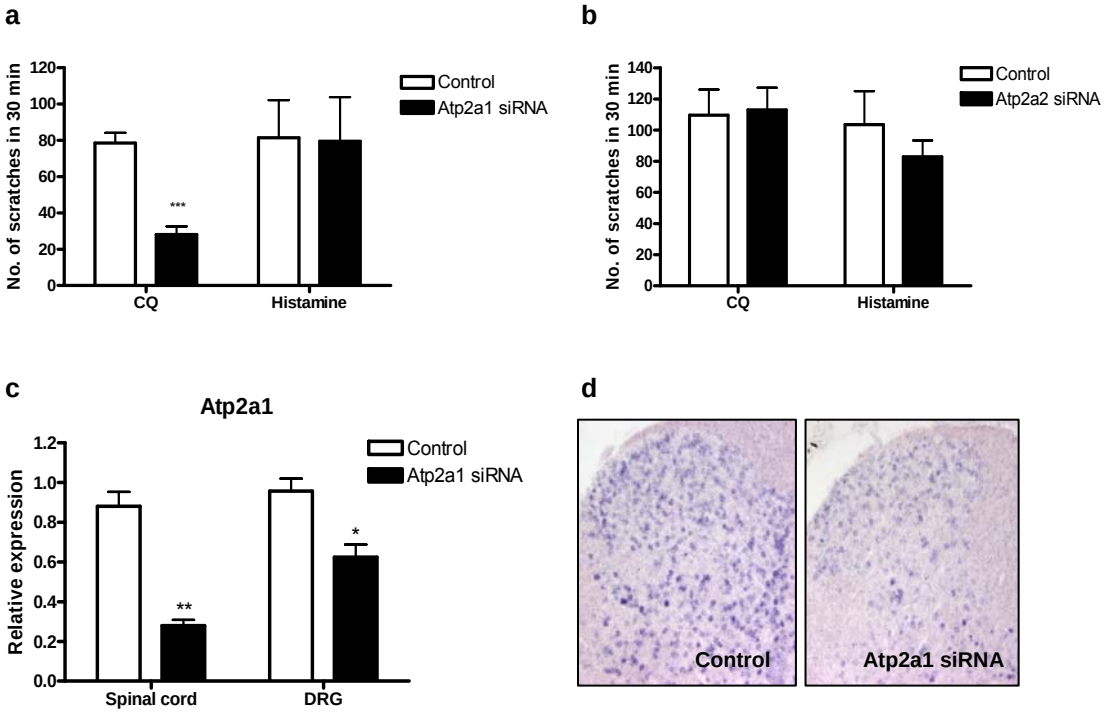


Figure 8. Atp2a1 siRNA i.t. injection decreased CQ-induced scratching.

(a) Atp2a1 siRNA treated mice showed reduced CQ-induced scratches but not histamine-itch. (b) Another isoform of Atp2a gene, Atp2a2 siRNA did not change both CQ and histamine-induced scratches suggesting that the Atp2a1 siRNA effect is specific to Atp2a1. siRNA effect was confirmed by QRT-PCR and ISH. (c) QRT-PCR showed that Atp2a1 siRNA decreased spinal cord Atp2a1 expressions more significantly than DRG. (d) ISH confirmed that Atp2a1 expressions were reduced in the spinal cord dorsal horn.

Control: PEI/DEPC-PBS, Student t-test (*p<0.05, **p<0.01, ***<0.001, n=9)

PEI : Polyethylenimine, DEPC : Diethylprocarbonate**,** PBS : phosphate-buffered saline

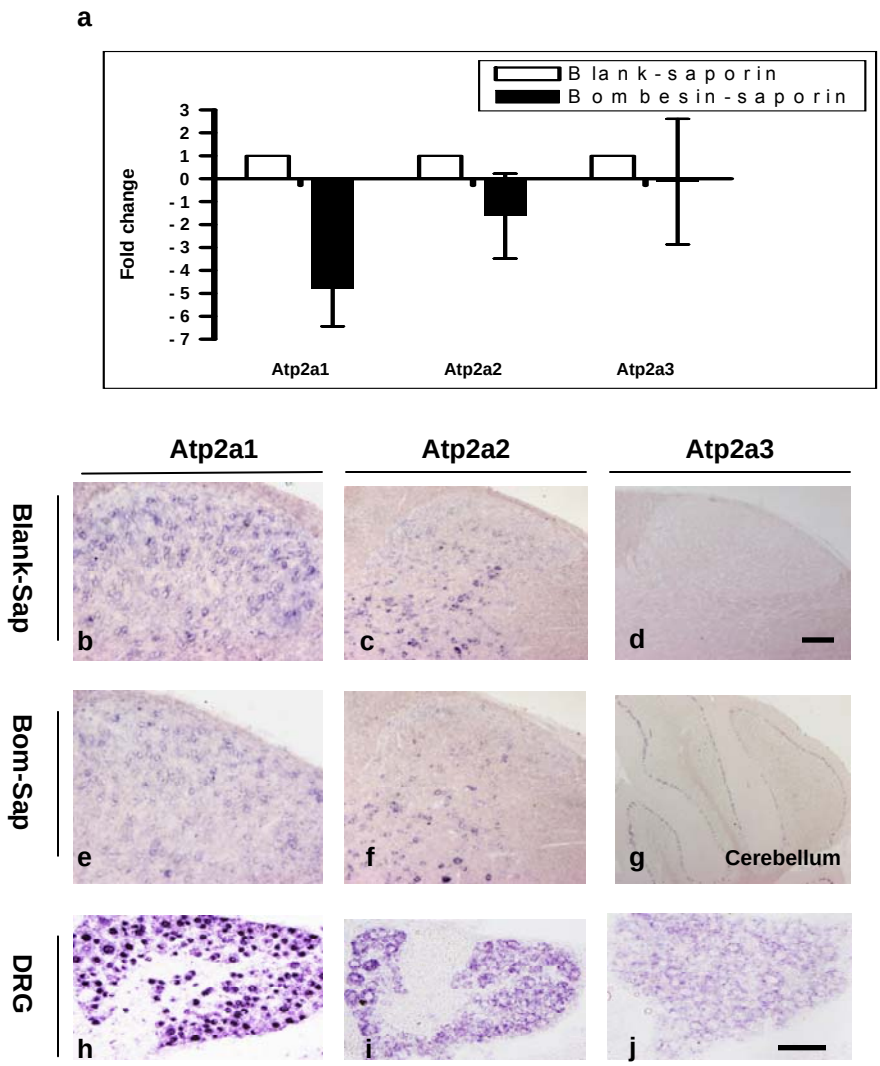


Figure 9. Atp2a gene has three different isoforms. (a) QRT-PCR showed that Atp2a1 expression level was significantly decreased in bombesin-saporin treated spinal cord but Atp2a2 expression was not affected (n=9). (b-c) Atp2a1 and Atp2a2 are expressed in the spinal cord. While Atp2a1 was strongly expressed in the superficial dorsal horn, Atp2a2 expression was examined in deep dorsal horn and ventral horn. (d) Atp2a3 was not detected in the spinal cord. (e-f) Bombesin-saporin treatment decreased Atp2a1 expressions in the superficial dorsal horn without affecting Atp2a2. (g) Atp2a3 probe detected Atp2a3 expressions in the cerebellum granular cell layers. (h-j) ISH showed that Atp2a1 and Atp2a2 are also expressed in DRG. Atp2a3 is not expressed in DRG. Scale bars, 100μm (b-f); 50μm (h-j)

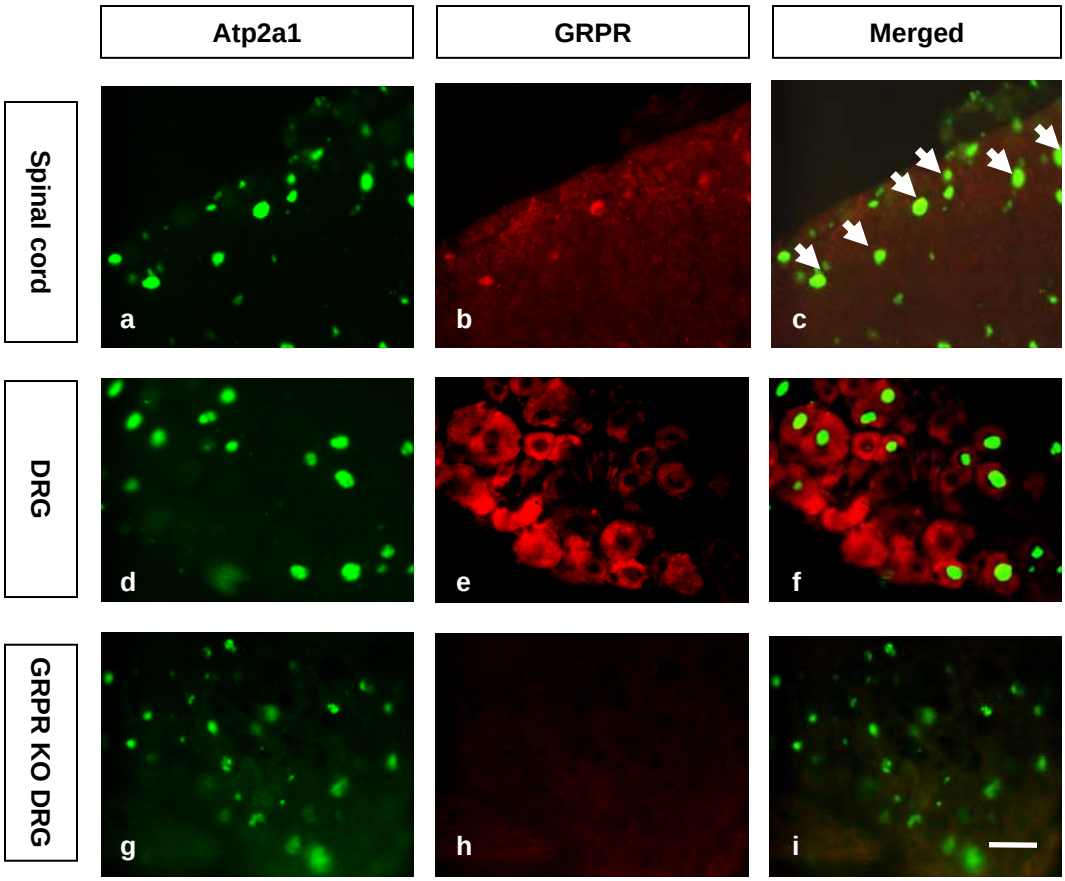
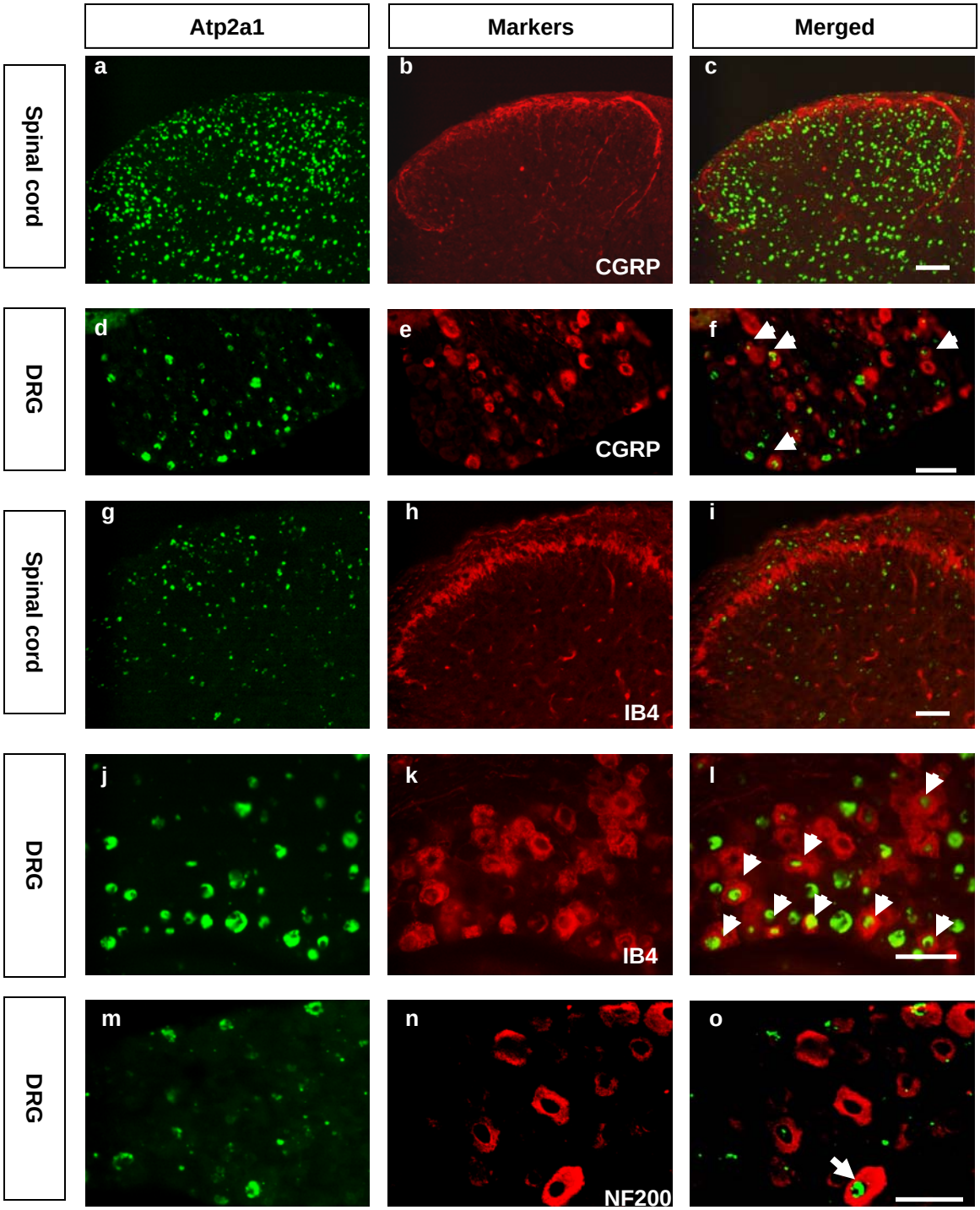


Figure 10. Atp2a1 is co-localized with GRPR in the spinal cord and DRG.

(a-c) spinal cord GRPR neurons co-express Atp2a1. (d-f) Most GRPR expressing neurons in DRG are co-localized with Atp2a1(88%, 84/96). (g-i) Specificity of GRPR staining in DRG was confirmed with GRPR mutant DRG.

(a-c) Atp2a1 fluorescent ISH (FISH)-GRPR immunostaining,

(d-i) Atp2a1-GRPR double FISH, Scale bar, 50μm


Atp2a1
Markers
Merged
Spinal cord
DRG
Spinal cord
DRG
DRG
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
CGRP
CGRP
IB4
IB4
NF200

(Continued)

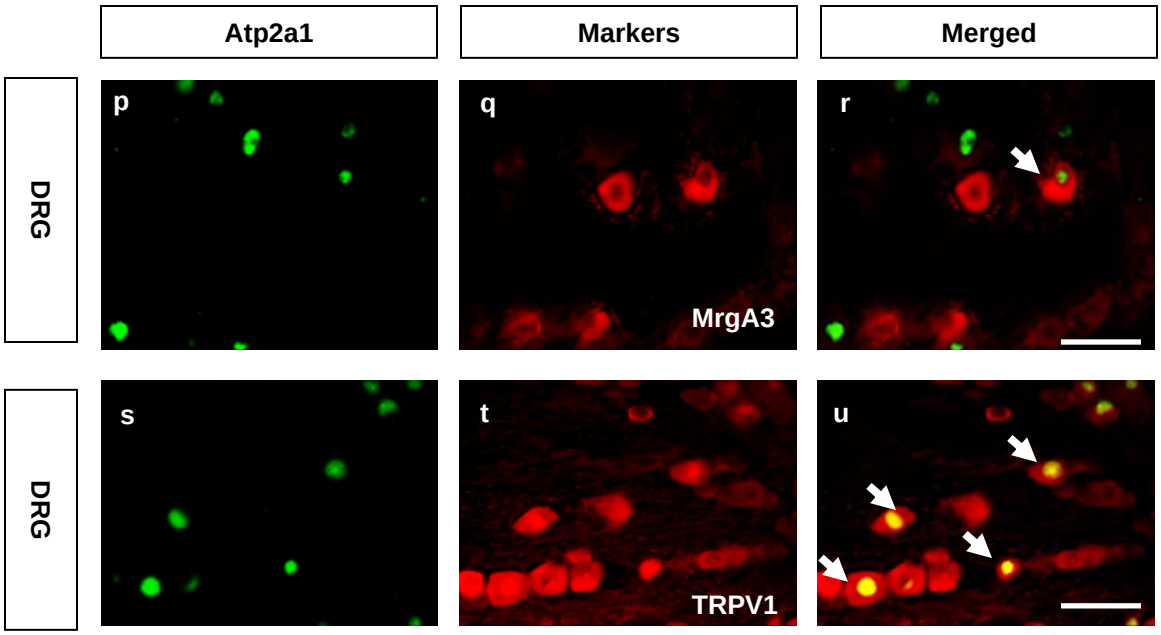


Figure 11. Expression and characterization of Atp2a1 cells in the spinal cord and DRG.

Atp2a1 is broadly expressed in the spinal cord dorsal horn (a-c, g-i). Atp2a1 is co-expressed with CGRP (d-f, 30%, 37/122), IB4 (j-l, 29%, 90/306), NF200 (m-o, 26%, 51/195), MrgprA3 (p-r, 5%, 22/426) and TRPV1 (s-u, 31%, 36/115) in DRG.

Green: Atp2a1 FISH, Red: immunostaining of marker genes except MrgA3 FISH.

Scale bar, 100μm (a-c, g-i), 50μm (d-f, j-u)

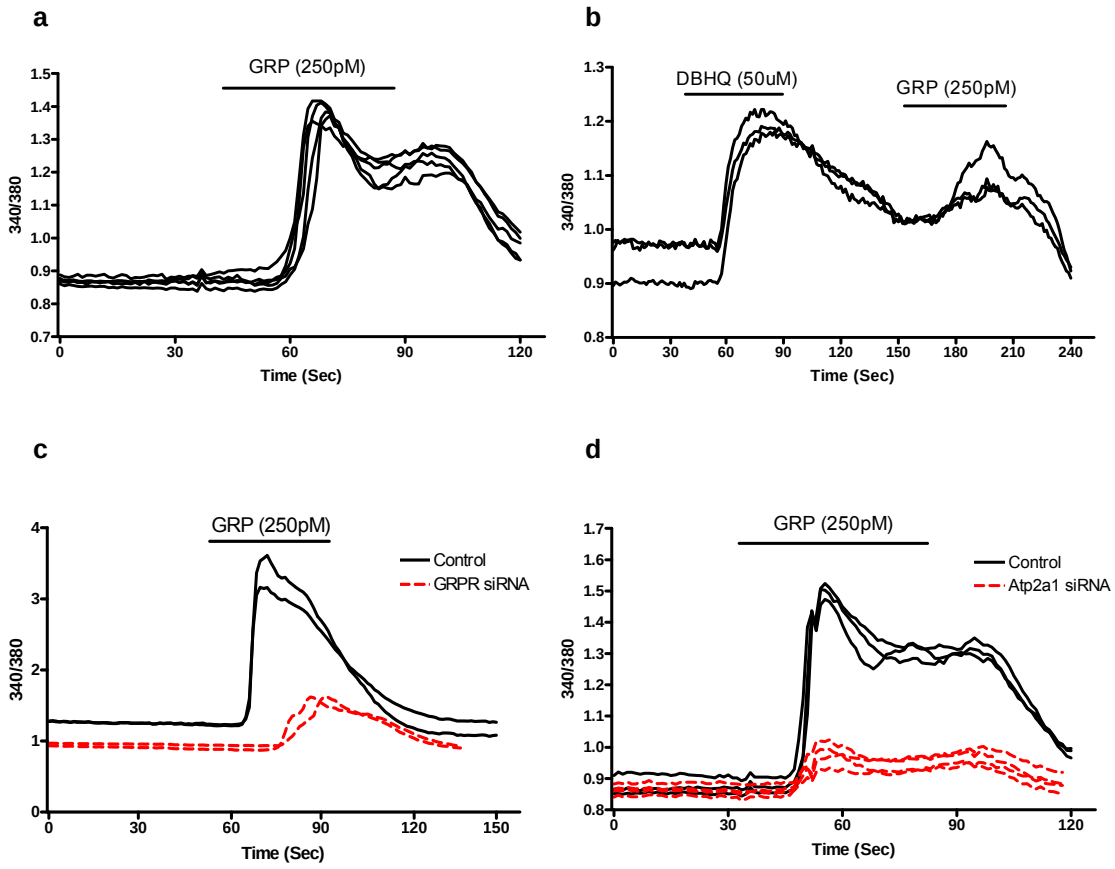


Figure 12. Atp2a1 is involved in GRPR mediated Ca^{2+} signaling pathway.

(a) GRPR-HEK cells cause Ca^{2+} responses with GRP (250pM). (b) DBHQ (50μM) induces a Ca^{2+} leak from ER by inhibiting Atp2a1 function. Pre-treatment with DBHQ reduced the GRP-induced Ca^{2+} responses. (c) GRPR siRNA (60pmol) transfection reduced the GRP Ca^{2+} responses. (d) Atp2a1 knockdown by Atp2a1 siRNA (60pmol) also attenuated the GRP Ca^{2+} responses.

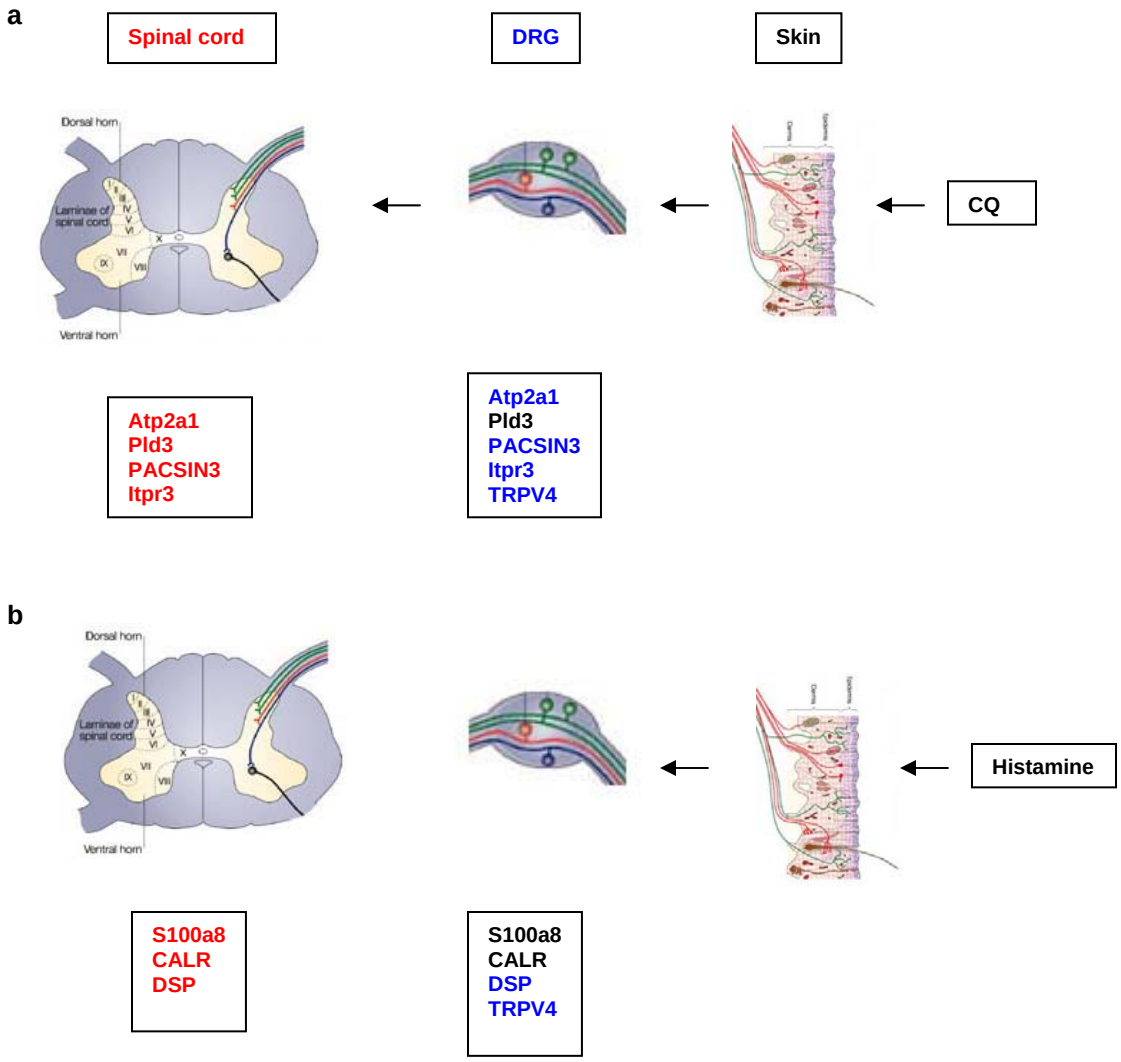


Figure 13. siRNA screening identified novel itch candidate genes.

(a) 4 histamine-independent genes (Atp2a1, Pld3, Pacsin3, Itpr3) and (b) 3 histamine-dependent genes (S100a8, CALR, DSP) were identified. TRPV4 is a both-dependent gene in DRG.

CQ and histamine were used to verify whether the candidate genes are involved in histamine-independent or -dependent itch pathways after siRNA i.t. injections.

Red: itch genes in the spinal cord, Blue: itch genes in DRG

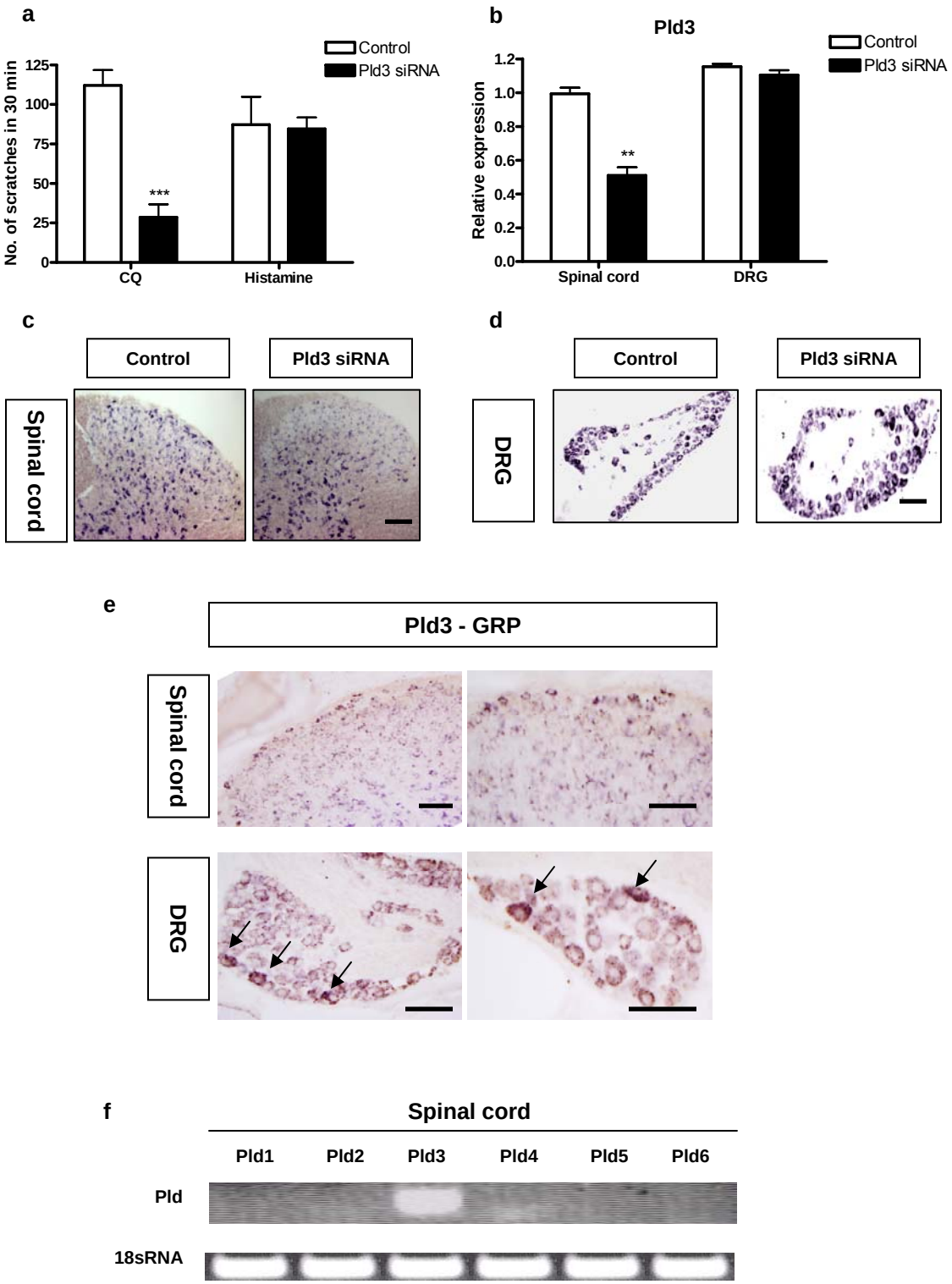

a
Control
Pld3 siRNA
No. of scratches in 30 min
125
100
75
50
25
0
***
CQ
Histamine
b
Pld3
Control
Pld3 siRNA
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Spinal cord
DRG
c
Control
Pld3 siRNA
Spinal cord
d
Control
Pld3 siRNA
DRG
e
Pld3 - GRP
Spinal cord
DRG
f
Spinal cord
Pld1
Pld2
Pld3
Pld4
Pld5
Pld6
Pld
18sRNA

Figure 14. Pld3 is a histamine-independent itch gene in the spinal cord.

(a) Pld3 siRNA i.t. injection inhibited CQ-induced itch without changing histamine responses (***p<0.001, n=4-9). (b-d) siRNA effect was examined by QRT-PCR and ISH. Pld3 siRNA decreased Pld3 mRNA levels only in the spinal cord (**p<0.01, n=3). ISH also confirmed the decreased level of Pld3 only in the spinal cord. (e) Pld3 expressing spinal neurons overlap with GRP^+ afferent fibers. $Pld3^+$ DRG cells co-express GRP. GRP immunostaining with 3, 3'-Diaminobenzidine (DAB) was performed after Pld3 ISH. Arrows represent Pld3-GRP co-localizations. Blue: ISH, Brown: GRP (f) RT-PCR with LCM dissected spinal cord RNA showed that Pld3 is the major Pld isoform expressed in the superficial dorsal horn. Weak expressions of Pld2 and Pld4 were also detected. Student t-test was used for statistical analysis. *p<0.05 was considered as significantly different. PEI/DEPC-PBS was used as a control for siRNA i.t. injection. Scale bar, 100μm (c, e, spinal cord), 50μm (d, e, DRG)

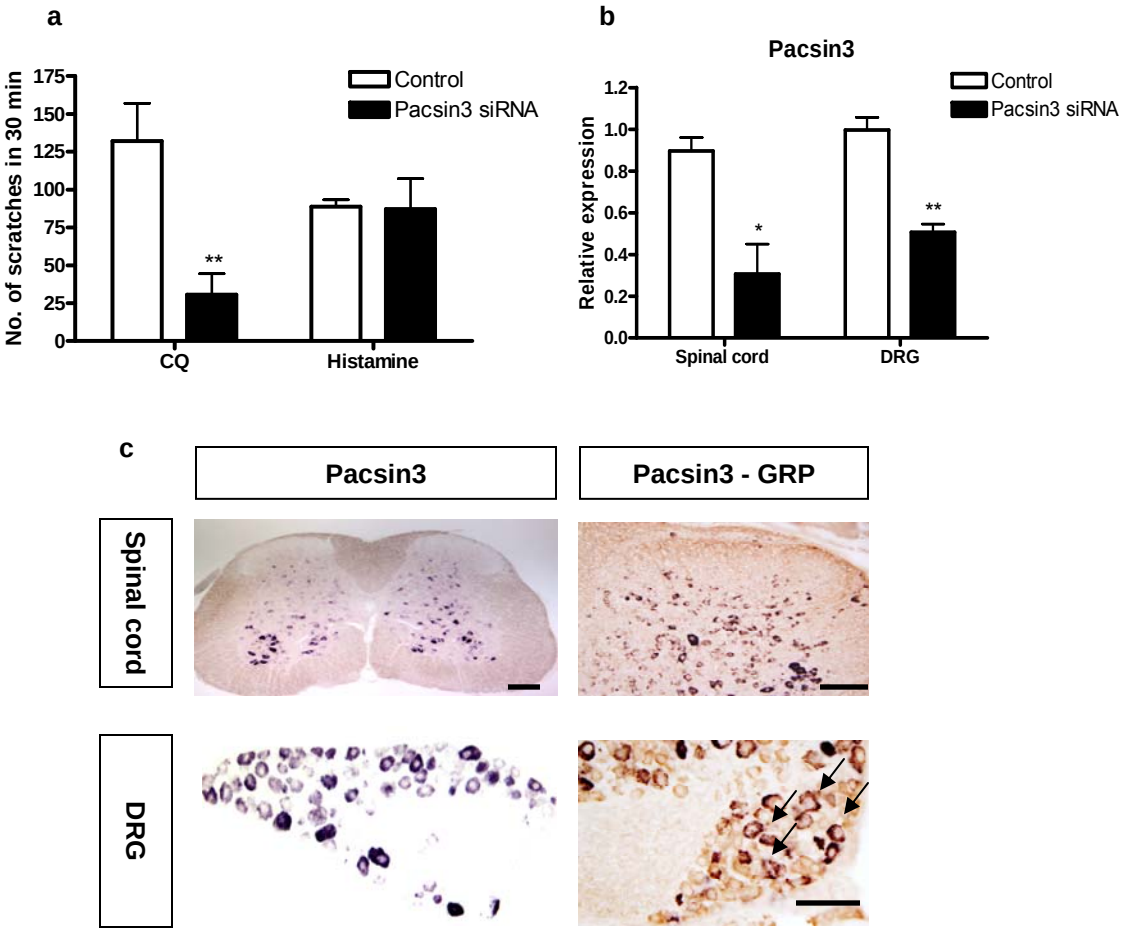


Figure 15. Pacsin 3 is a histamine-independent itch gene both in the spinal cord and DRG.

(a) Pacsin3 siRNA i.t. injection decreased CQ-induced itch (**P<0.01, n=5). (b) Pacsin3 siRNA reduced Pacsin3 expressions both in the spinal cord and DRG (*p<0.05, **p<0.01, n=3).

(c) ISH showed that Pacsin3 is not strongly expressed in the superficial dorsal horn.

Few Pacsin3^{+} cells were observed in the superficial dorsal horn. DRG has strong Pacsin3 expressions in a subset of neuronal populations. Double staining showed that few Pacsin3^{+} cells overlap with GRP^{+} afferent fibers in the dorsal horn. Pacsin3^{+} DRG cells co-express GRP.

Scale bar, 100μm (c, spinal cord), 50μm (c, DRG)

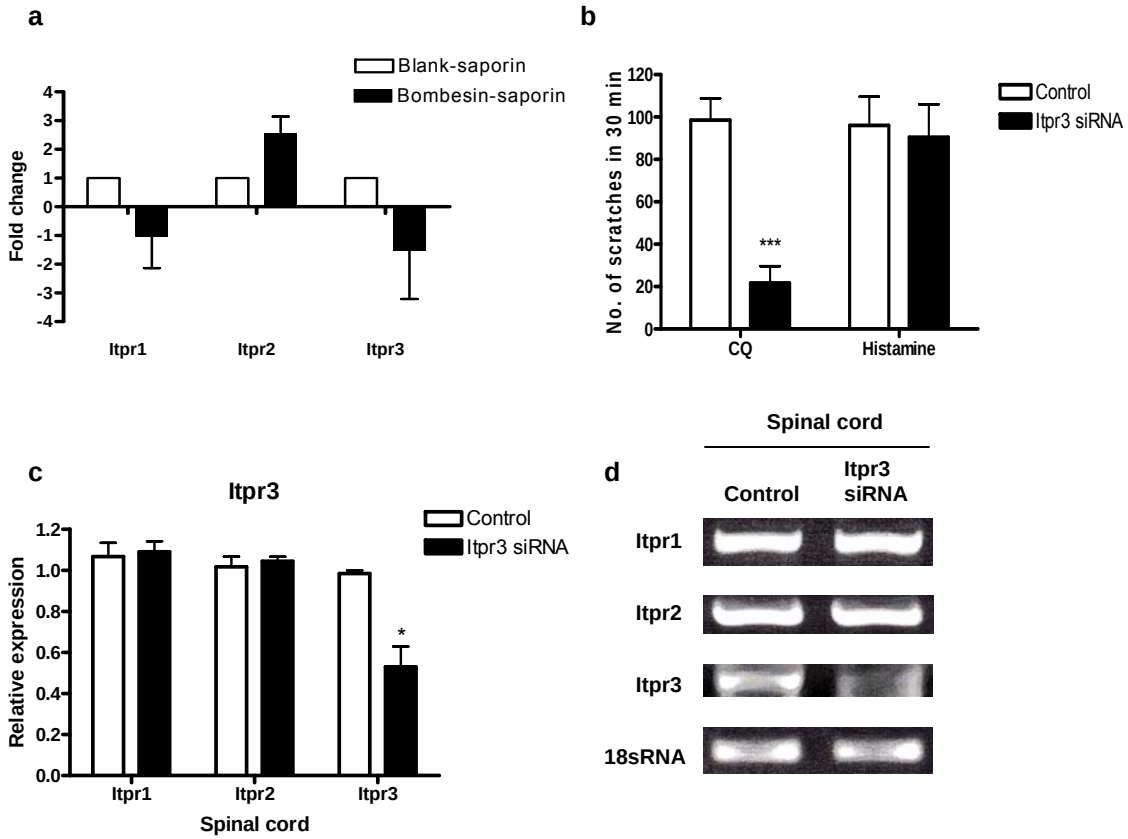


Figure 16. Itpr3 is a histamine-independent itch gene in the spinal cord.

(a) QRT-PCR showed that Itpr3 was the most significantly reduced gene among Itpr genes in the bombesin-saporin treated spinal cord suggesting that Itpr3 is co-expressed in GRPR+ cells. (b) Itpr3 siRNA reduced CQ-induced scratches (***$p<0.001$, n=5). (c-d) QRT-PCR confirmed that Itpr3 siRNA specifically decreased Itpr3 expression in the spinal cord not changing the other two isoforms, Itpr1 and 2 (*$p<0.05$, n=3).

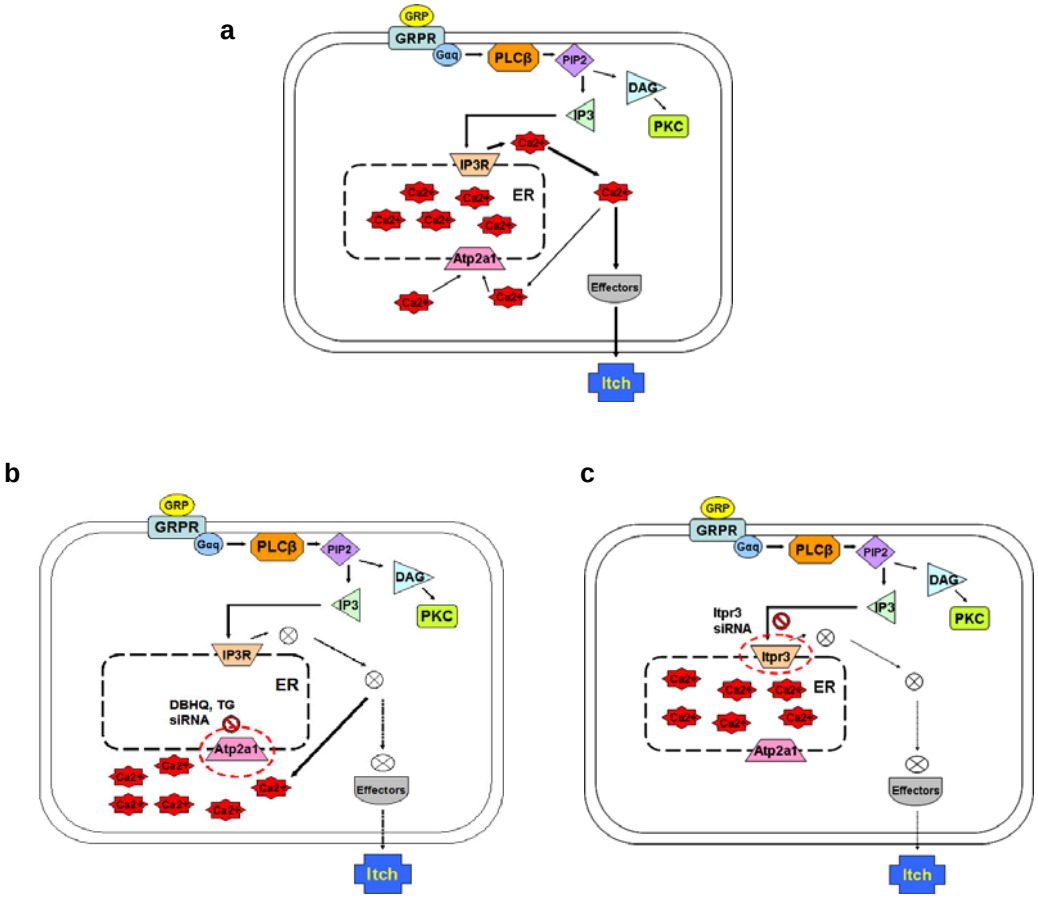


Figure 17. Atp2a1 and Itpr3 are involved in GRPR downstream Ca^{2+} signaling pathway for itch. (a) GRP binds to GRPR and activates PLC/IP_3 signaling pathways. IP_3 binds to IP_3 receptor on ER membrane and release Ca^{2+} to intracellular space. Increased Ca^{2+} can activate multiple downstream effector molecules mediating itch transmission. Atp2a1 refills ER with Ca^{2+} by active transport using ATP and maintains Ca^{2+} concentration gradient. This is an important process to make the cell ready to respond to extracellular stimuli. (b) Atp2a1 inhibition by DBHQ or siRNA impairs the active Ca^{2+} transport and depletes ER Ca^{2+} store resulting in reduced histamine-independent itch mediated by GRPR. (c) Itpr3 siRNA inhibits IP_3 receptor directly and blocks Ca^{2+} release by IP_3. Atp2a1 and Itpr3 are crucial downstream signaling molecules for GRPR itch signaling pathways.

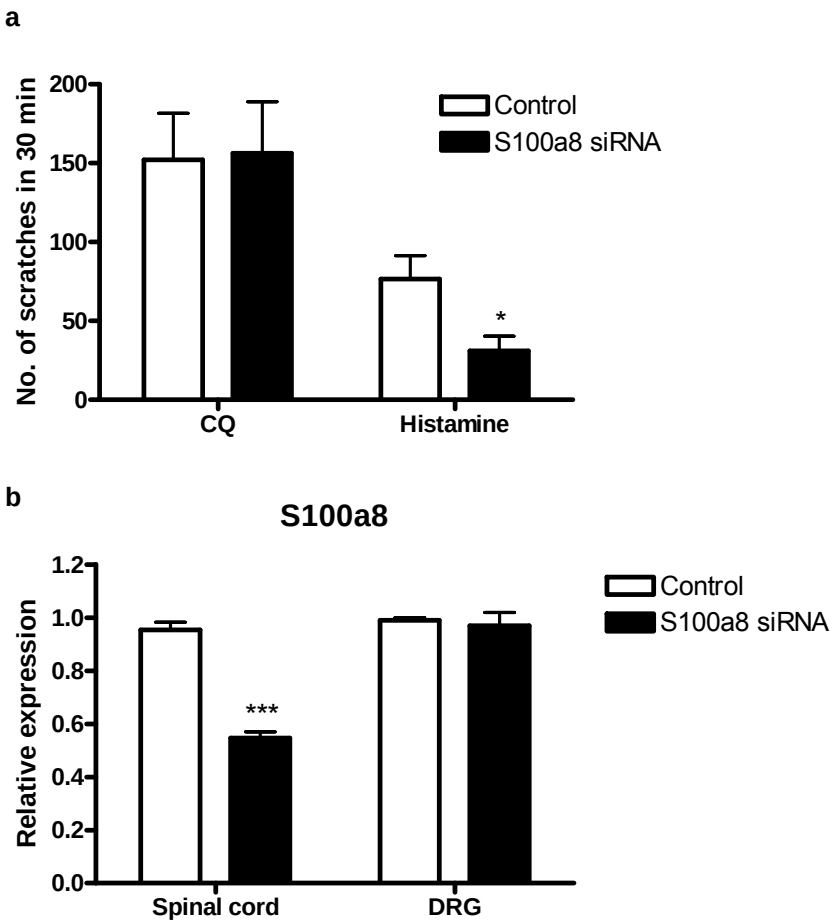


Figure 18. S100a8 is a histamine-dependent itch gene in the spinal cord.

(a) S100a8 siRNA i.t. injection decreased histamine-induced scratches only (*$p<0.05$, n=6).

(b) siRNA reduced S100a8 expression in the spinal cord suggesting that S100a8 is involved in histamine itch in the spinal cord (***$p<0.001$, n=3).

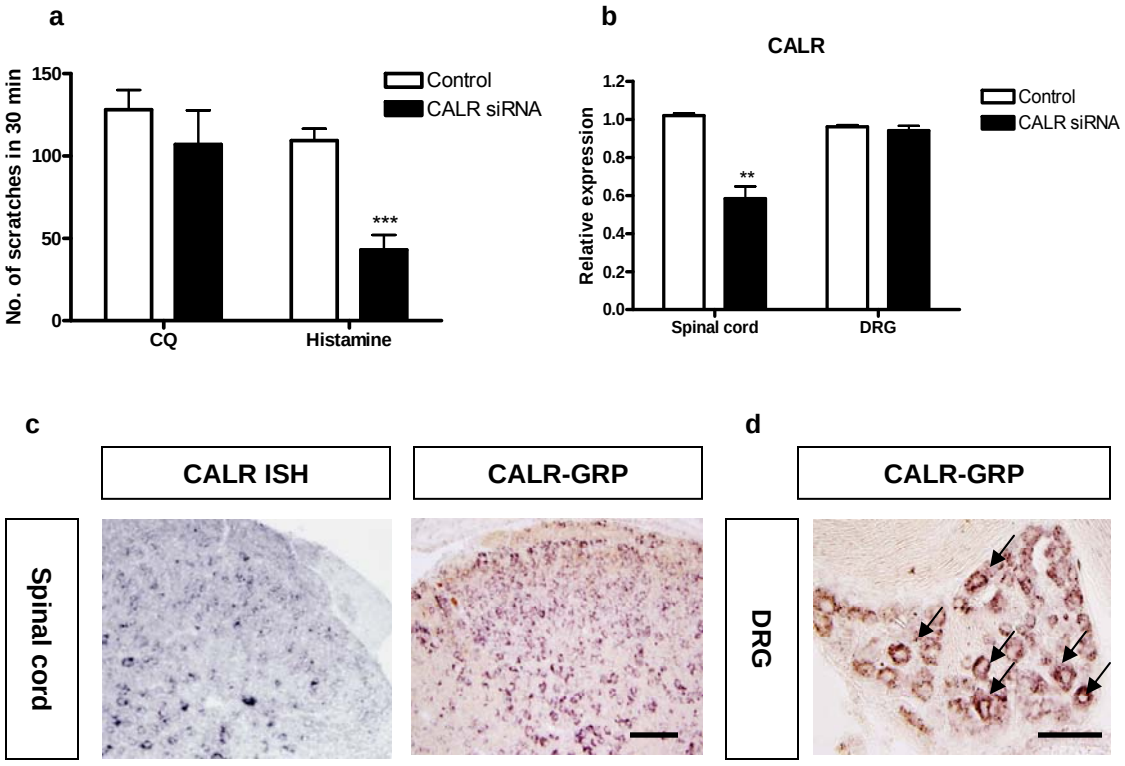


Figure 19. CALR is a histamine-dependent itch gene in the spinal cord.

(a) CALR siRNA reduced histamine-induced itch (***p<0.001, n=5). (b) CALR siRNA reduced mRNA levels only in the spinal cord (**p<0.01, n=3). (c) ISH showed spinal cord CALR expressions overlapped with GRP stainings. (d) $CALR^{+}$ neurons co-express GRP in DRG. Scale bars, 100μm (c), 50μm (d)

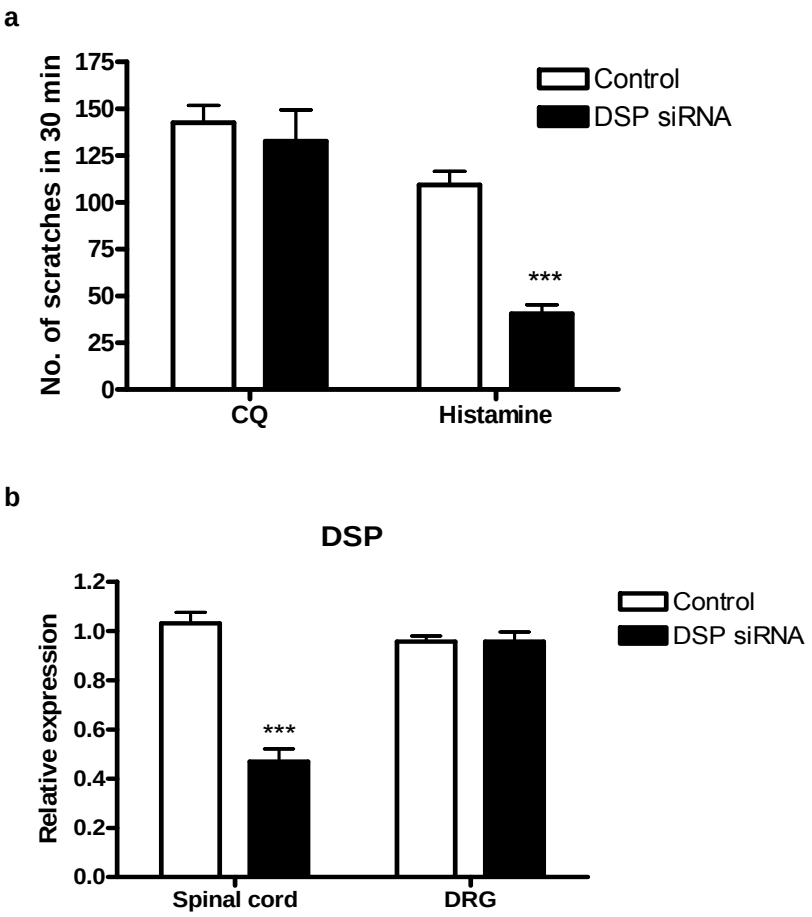


Figure 20. DSP is a histamine-dependent itch gene in the spinal cord.

(a) DSP siRNA decreased histamine-induced scratches (***p<0.001, n=5). (b) DSP siRNA reduced mRNA levels in the spinal cord (***p<0.001, n=3).

Table 5. QRT-PCR primer sequences

Gene	Primers
18sRNA	5' AAACGGCTACCACATCCAAG 3' CCTCCAATGGATCCTCGTTA
Actin	5' GGCTGTATTCCCCTCCATCG 3' CCAGTTGGTAACAATGCCATGT
GAPDH	5' AGGTCGGTGTGAACGGATTTG 3' TGTAGACCATGTAGTTGAGGTCA
GRPR	5' GGCAGAATTGGCTGCAAACTG 3' ACAATGGCTTTGTACCTGTCAG
NMBR	5' CTATCGTGAACCCCATGGAC 3' ATGCATGCTGTGAAACTGCT
BRS3	5' CTTTCGGAGACCTGTTACTTCTG 3' AGCACTTTACAACCGACCTTTC
NK1R	5' CTCCACCAACACTTCTGAGTC 3' TCACCACTGTATTGAATGCAGC
NMUR2	5' CAAGCGCAGTGACCTATCCC 3' GGCATACCTAAGAGCAGGACC
Taar7e	5' CAGGGCATCAGACAGCTACA 3' ATACACATACGTGGGCGTGA
PKR1	5' GGCTCTGGGTGAGAATACCA 3' CTTGGCAGCAAAGAAAGTCC
P2rx4	5' CTCGGGTCCTTCCTGTTCG 3' GTTTCCTGGTAGCCCTTTTCC
Atp2a1	5' TGTTTGTCCTATTTCGGGGTG 3' AATCCGCACAAGCAGGTCTTC
V1rf1	5' GGTATCACAGACTGTTGCTGG 3'CATTGCTTGTGGAACACCTTTAC
Kcnj15	5' ACCCCGAGTCATGTCAAAGAG 3' GCTTGTATCGCCACTTCATGT
Glp2r	5' AGGAGACAGTTCAGAAGTGGG 3' GCCAGCACACGTACTTATCAA
Il1r2	5' CCACTGTGAGCAAATGTCTGT 3' GCGCCCTTATACCACTGTATC
Tlr5	5' GCAGGATCATGGCATGTCAAC 3' ATCTGGGTGAGGTTACAGCCT
Kcnq2	5' TACGGGCCAGAGGAACTACG 3' CAGTCGCAGGACCAGTGAG
Cacna1c	5' ATGAAAACACGAGGATGTACGTT 3' ACTGACGGTAGAGATGGTTGC
Npr1	5' GCTTGTGCTCTATGCAGATCG 3' TCGACGAACTCCTGGTGATTTA
Nr3c2	5' CAGACCTTGGAGCGTTCTTC 3' GCAAAATCCCAGACCGACTA
Fpr-rs6	5' CCAAGATAGGCAGAAGAGGTTTT 3' TGAAGGAGGCCAGAGTGCTT
Klra9	5' AACTGCAGCAACATGCAAAG 3' CTGTGTCCCGTGAGGAATCT
Tnfrsf7	5' TCTATAGCCAGCGGTCATCC 3' AACAAGATTGCACCCAGGAC

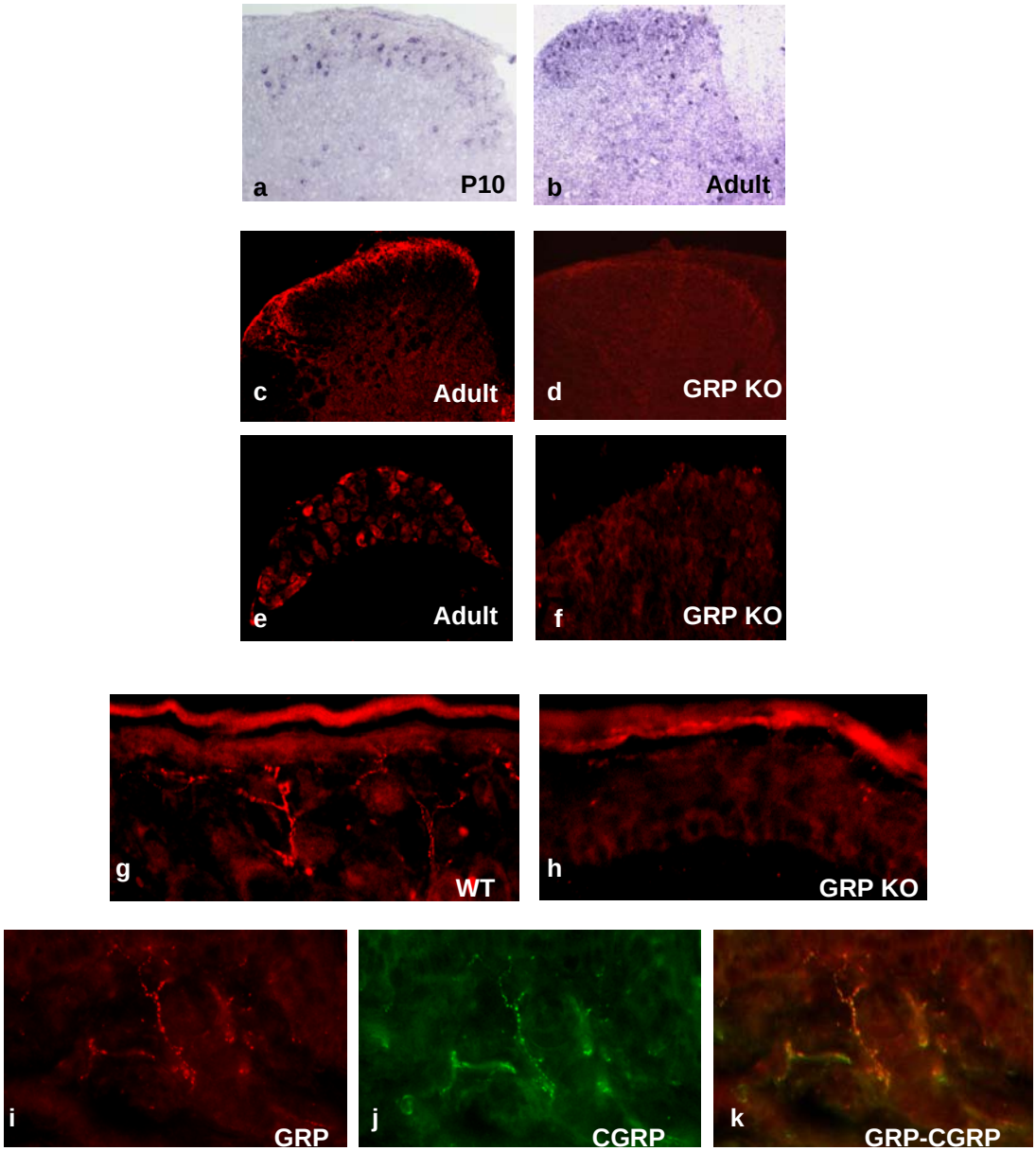


Figure 21. GRP is expressed in the spinal cord, DRG and skin. (a, b) ISH showed that GRP is expressed in neurons of spinal cord dorsal horn. (c, d) GRP is expressed in afferent fivers in lamina I. (e, f) GRP is expressed in a small subset of DRG neurons. (g, h) GRP^{+} free nerve endings are innervated into skin. (i, k) GRP^{+} nerve fibers are co-localized with CGRP suggesting that they are peptidergic nerves. Scale bars, 100μm (a-d), 50μm (e-f)

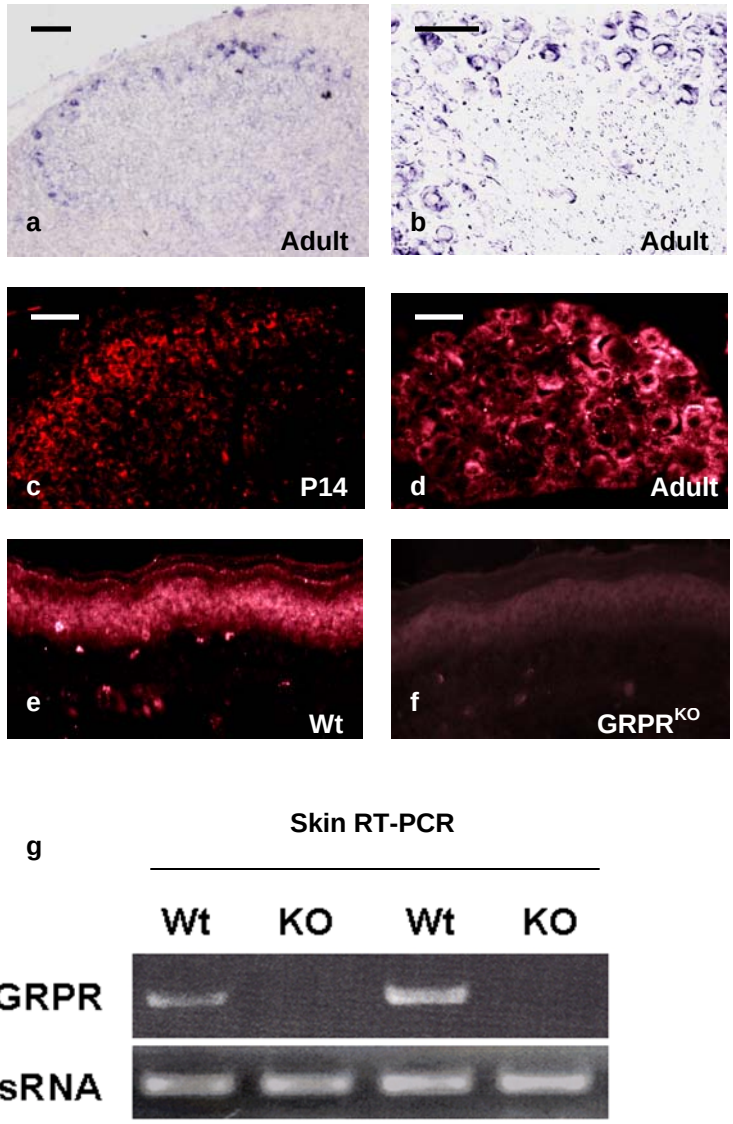


Figure 22. GRPR is expressed in the spinal cord, DRG and skin. (a, b) ISH showed that GRPR is expressed in spinal cord laminar I and DRG neurons. (c, d) Immunostaining also detected GRPR expressions both in the spinal cord and DRG. (e, f) GRPR is expressed in skin keratinocytes and dermal mast cells. (g) RT-PCR with skin total RNA confirmed that GRPR is expressed in wild type (Wt) but not in GRPR KO skin.

Scale bars, 100μm (a, c), 50μm (b, d)

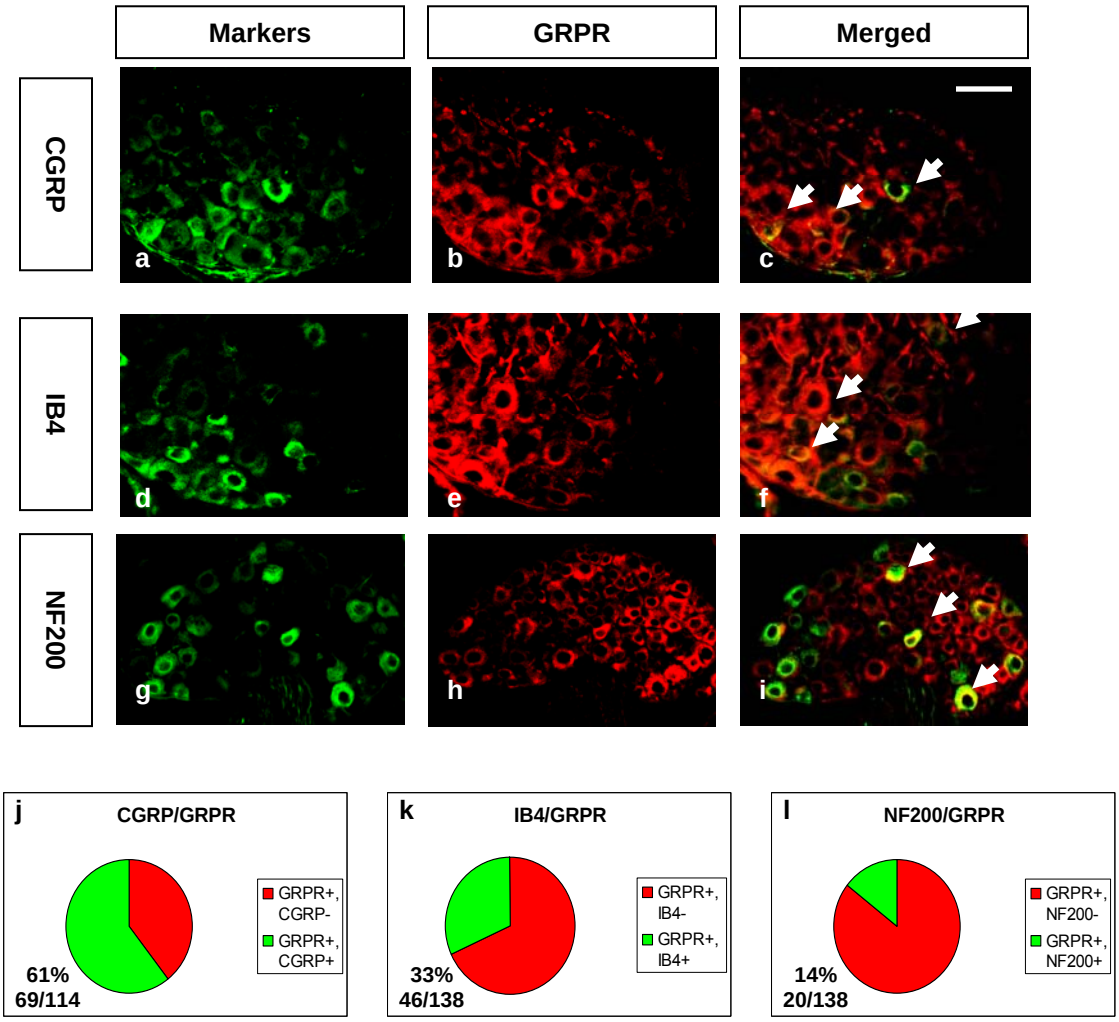


Figure 23. GRPR is co-expressed with CGRP, IB4 and NF200 in DRG.

(a-c and j) Majority of GRPR+ cells (61%, 69/114) co-express CGRP. (d-f and k) 33% (46/138) of GRPR+ cells are IB4+. (g-i and l) 14% (20/138) of GRPR+ cells overlap with NF200.

White arrow : co-localization, Scale bar 50μm

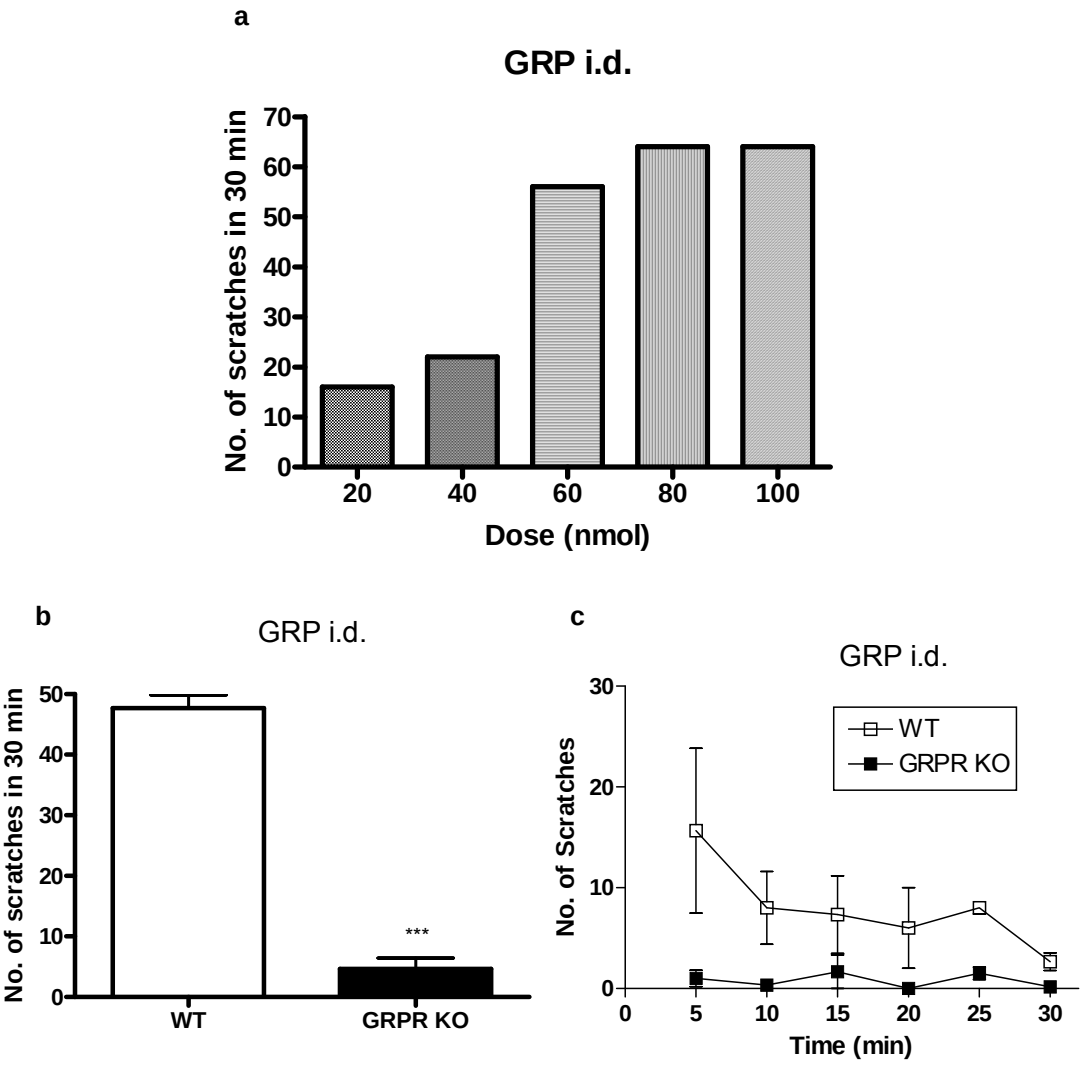


Figure 24. GRP i.d. injection causes itch in mice. (a) GRP induces dose-dependent scratching responses by i.d. injection. (b, c) GRP i.d.-induced scratching was abolished in GRPR KO mice (***p<0.001, n=6).

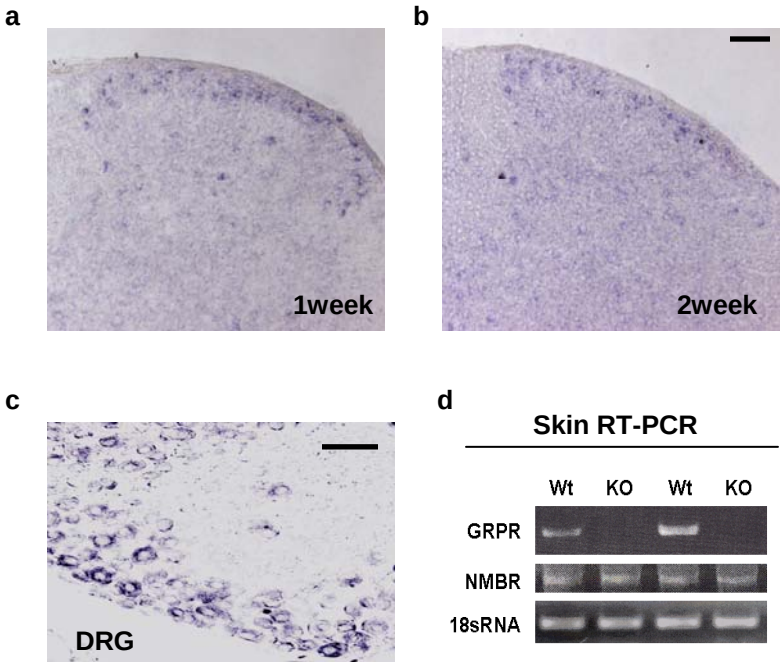


Figure 25. NMBR, a GRPR homologous receptor, is also expressed in the spinal cord, DRG and skin. (a, b) ISH showed NMBR expression in juvenile (1-2 weeks) spinal cord dorsal horn. (c) NMBR is expressed in DRG neurons. (d) NMBR expression was observed in skin by RT-PCR. Wt : Wild type, KO : GRPR KO, Scale bar, 100μm (a, b), 50μm (c)

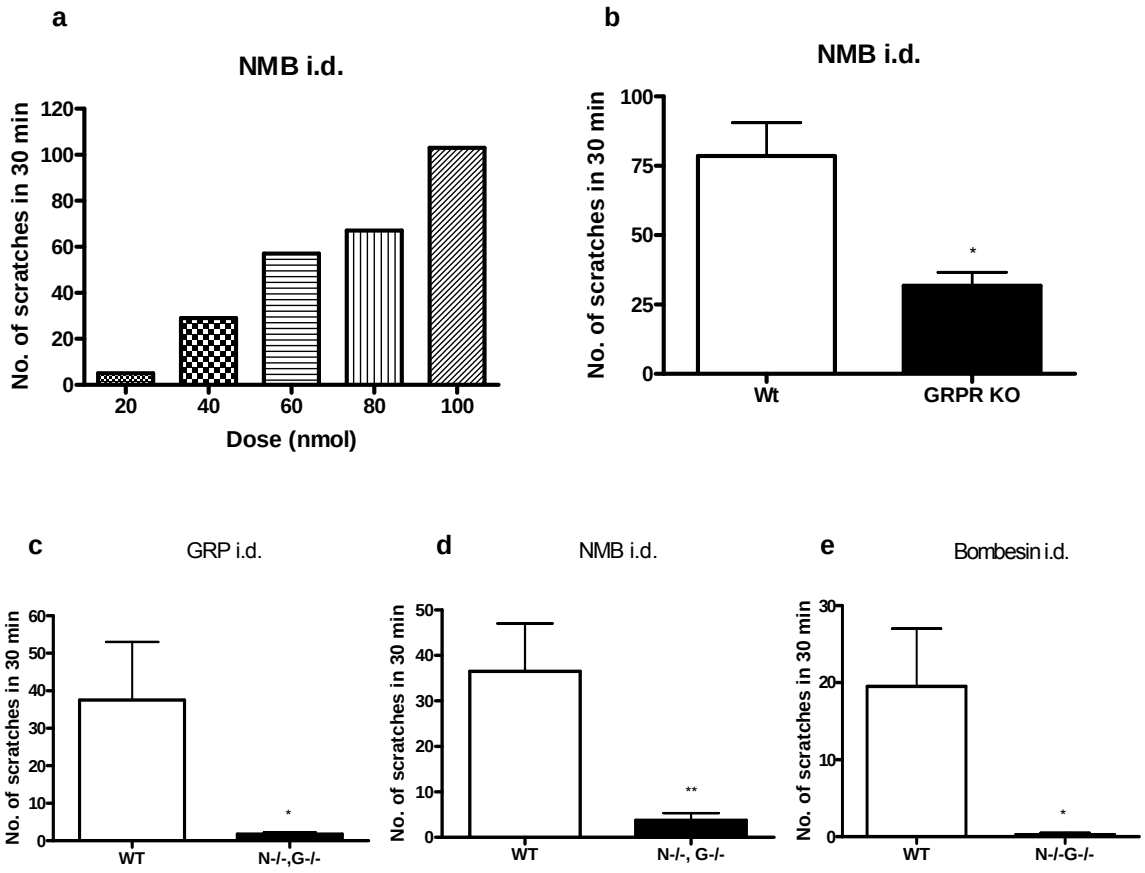


Figure 26. Bombesin-like peptide i.d. injection causes itch mediated by peripheral GRPR and NMBR. (a) NMB, a GRP homologous peptide induces dose-dependent scratching by i.d. injection. (b) NMB i.d.-induced scratching was reduced significantly in $GRPR^{-/-}$ mice suggesting that NMB i.d. response is partially dependent of GRPR (*$p<0.05$, n=4). (c-e) All the bombesin-like peptides, GRP, NMB and bombesin i.d. injections causes scratching responses that are abolished in $NMBR/GRPR^{-/-}$ ($N^{-/-}$, $G^{-/-}$) mice (*$p<0.05$, **$p<0.01$, n=4). Scratching responses induced by peripheral bombesin-like peptides are mediated by GRPR and NMBR.

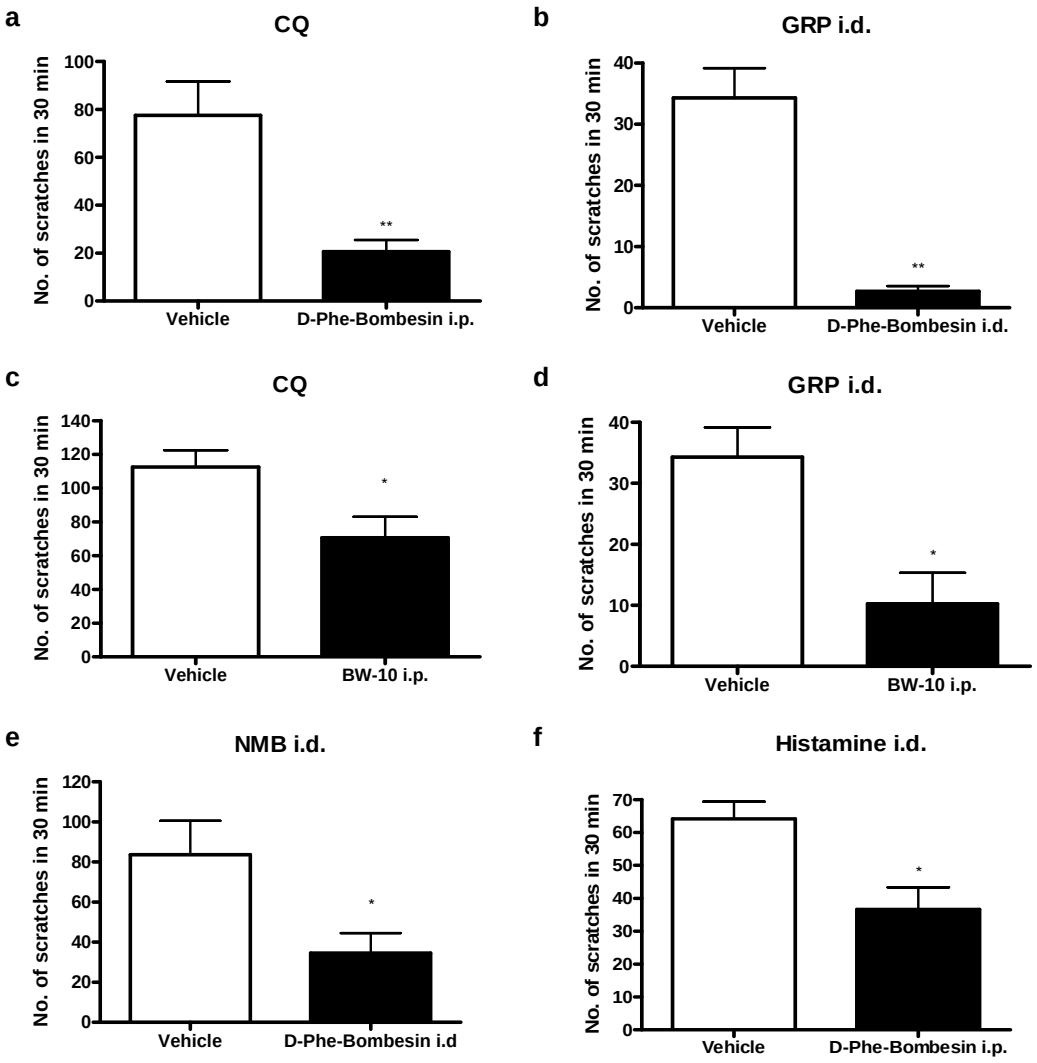


Figure 27. Peripheral applications of GRPR antagonists inhibit CQ and GRP i.d.-induced scratches. (a) D-Phe-Leu-bombesin (10nmol) i.p. injection inhibited CQ-induced itch (**p<0.01, n=4-6). (b) D-Phe-Leu-bombesin (15nmol) i.d. injection abolished GRP (100nmol) i.d. -induced scratches (*p<0.01, n=6). (c) BW-10 (10nmol) i.p. injection reduced CQ-induced itch significantly (*p<0.05, n=6). (d) BW-10 (10nmol) i.p. injection decreased GRP i.d.-induced scratches (*p<0.05, n=6). (e) NMB i.d.-induced scratches were reduced after D-Phe-Leu -Bombesin i.d. injection (*p<0.05, n=6). (f) Histamine itch was also decreased by D-Phe-Leu -Bombesin (10nmol) i.p. injection (*p<0.05, n=8). i.p. and i.d. injections were performed 30min and 10min before the test, respectively. Saline was used as a vehicle.

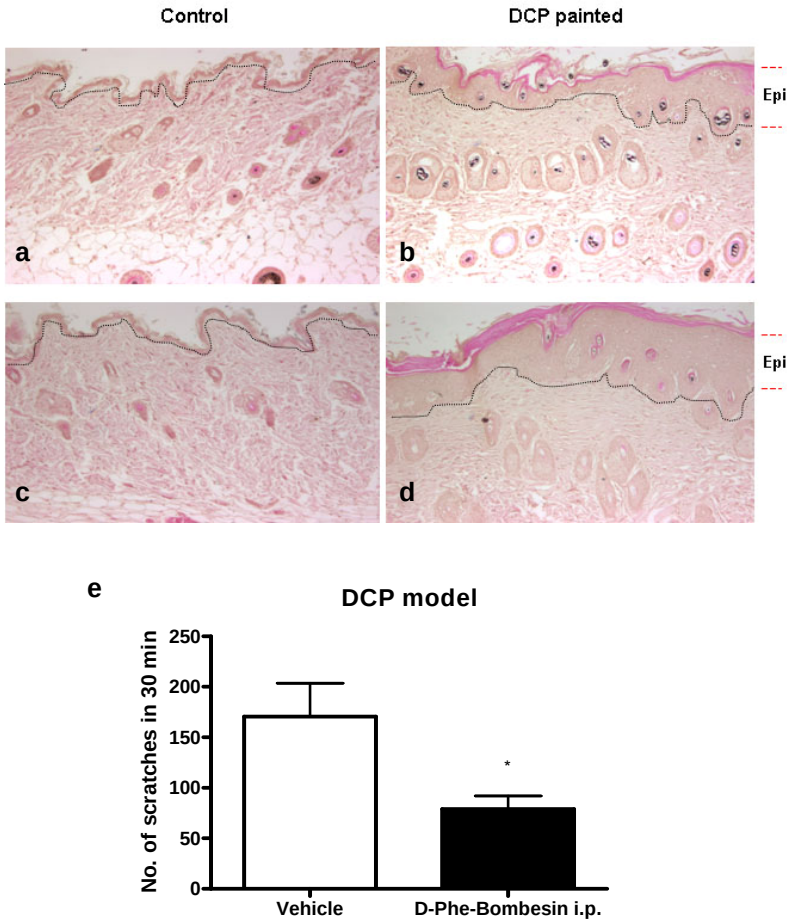


Figure 28. GRPR antagonist, D-Phe-Leu-bombesin attenuated scratching responses significantly in DCP-painted ACD mice model. 1% DCP was painted for sensitization. 0.5% DCP was applied again after 7 days to induce ACD. (a) DCP painting causes increased epidermal thickness, skin inflammation and robust scratching. (b) D-Phe-Leu-bombesin i.p. injection decreased DCP-painted mice scratches. At 14 day, D-Phe-Leu-bombesin (15nmol) was i.p. injected 30min before counting scratches (*$p<0.05$, $n=9$). Epi: epidermis

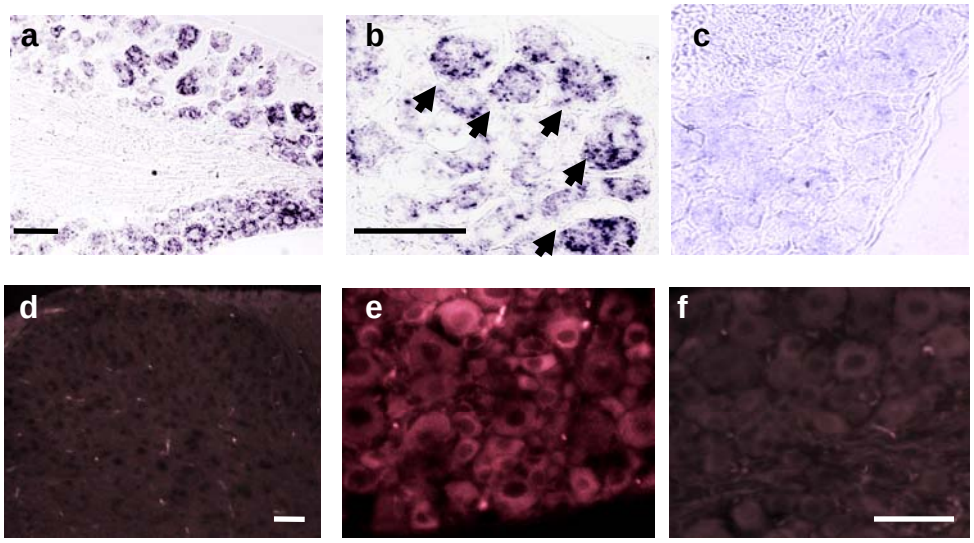


Figure 29. Specific TRPV4 expression in WT DRG but not in the spinal cord and TRPV4$^{-/-}$ DRG. (a) TRPV4 expression in WT DRG. Higher magnification of DRG shown in (b). (d) TRPV4 is not expressed in the spinal cord. TRPV4 staining was absent in TRPV4$^{-/-}$ DRG (c and f). Scale bar 50μm (a, b, f), 100μm (d)

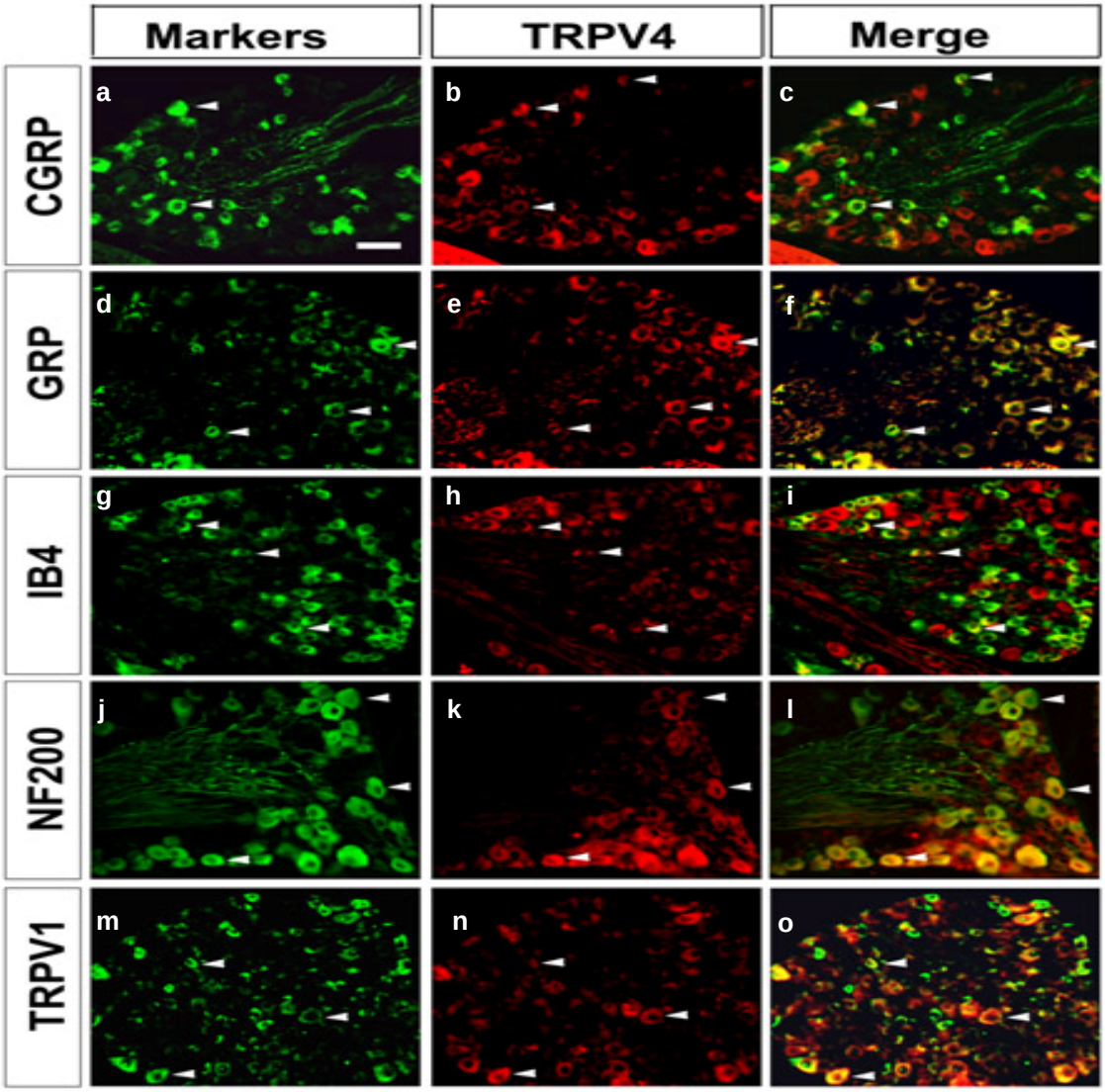


Figure 30. Expression of TRPV4 in DRG neurons. TRPV4 is expressed in about 19% (375/1,565) of mouse lumbar DRG neurons. TRPV4 is co-localized with CGRP (61%, 139/227) (a-c), GRP (83%, 427/516) (d-f), IB4 (77%, 162/211) (g-i), NF200 (89%, 224/252) (j-l) and TRPV1 (70%, 196/282) (m-o) Green : Immunostaining of marker genes, Red : TRPV4 fluorescence ISH. White arrowheads indicate double-stained cells.

CGRP: calcitonin gene-related peptide, IB4: isolectin B4, NF200: neurofiliament 200,

Scale bar 50μm

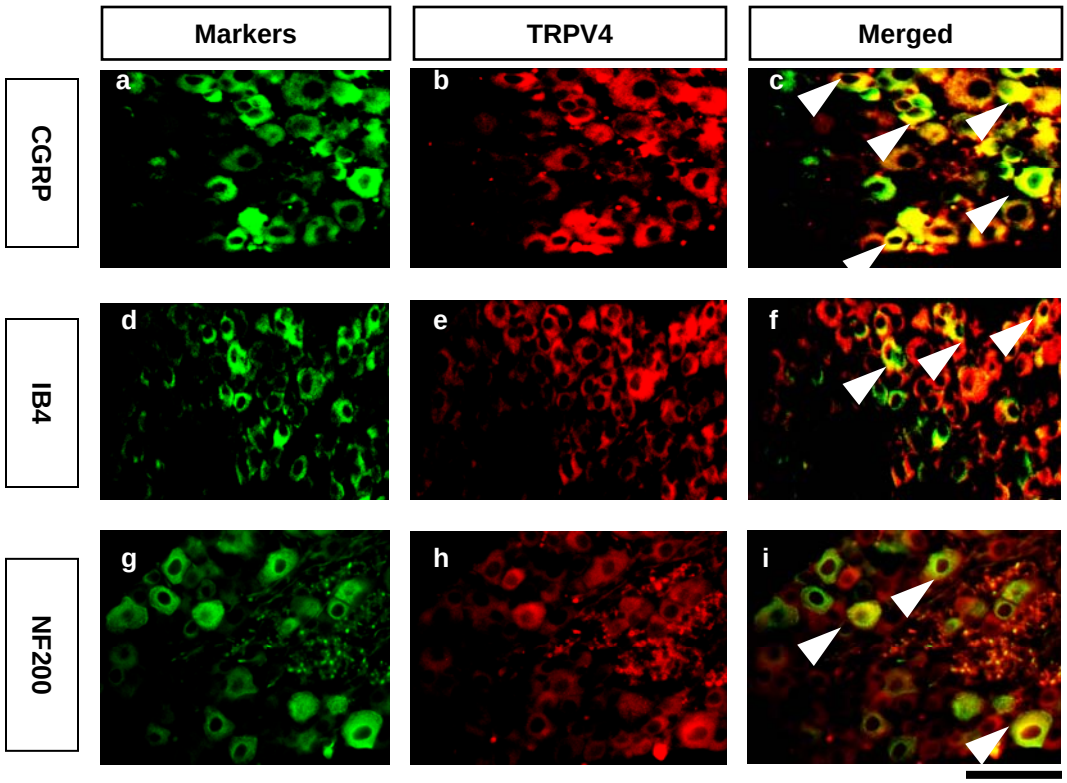


Figure 31. TRPV4 is expressed in both large and small DRG neurons.

TRPV4 is co-expressed with CGRP (61%, 139/227) (a-c), IB4 (77%, 162/211) (d-f) and NF200 (89%, 224/252) (g-i). Green: Immunostaining of various markers, Red: TRPV4 FISH signal, White arrowheads: co-localization, Scale bar 50μm

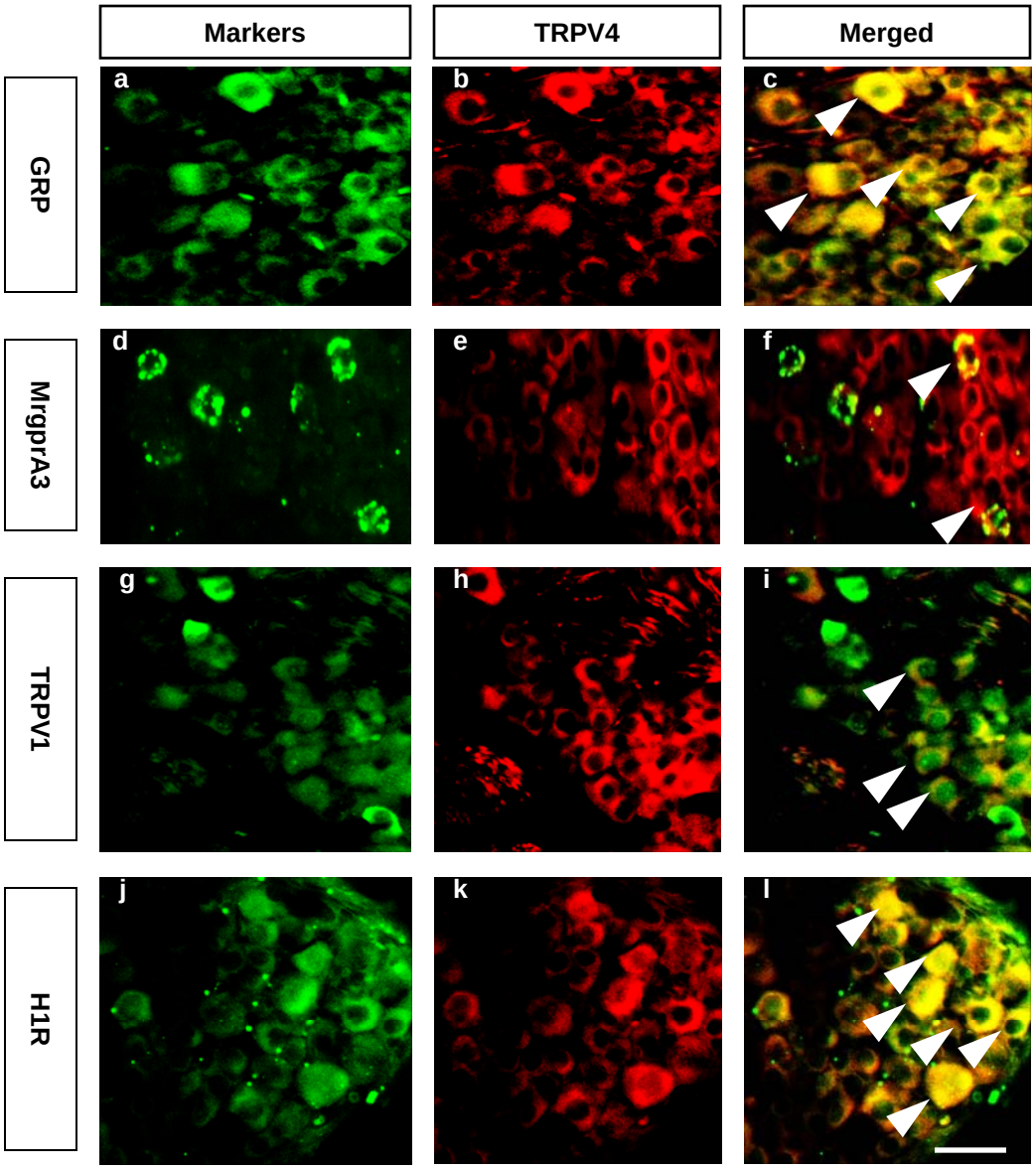


Figure 32. TRPV4 is co-expressed in histamine-independent and -dependent pruritic genes in DRG. TRPV4 is co-localized with GRP (83%, 427/516) (a-c),MrgprA3 (87%, 62/71) (d-f) and H1R (100%, 78/78) (g-i), TRPV1 (70%, 196/282) (j-l). White arrowheads : co-localization, Green : immunostaining of pruritic markers except MrgprA3 FISH, Red : TRPV4 FISH, Scale bar 50μm

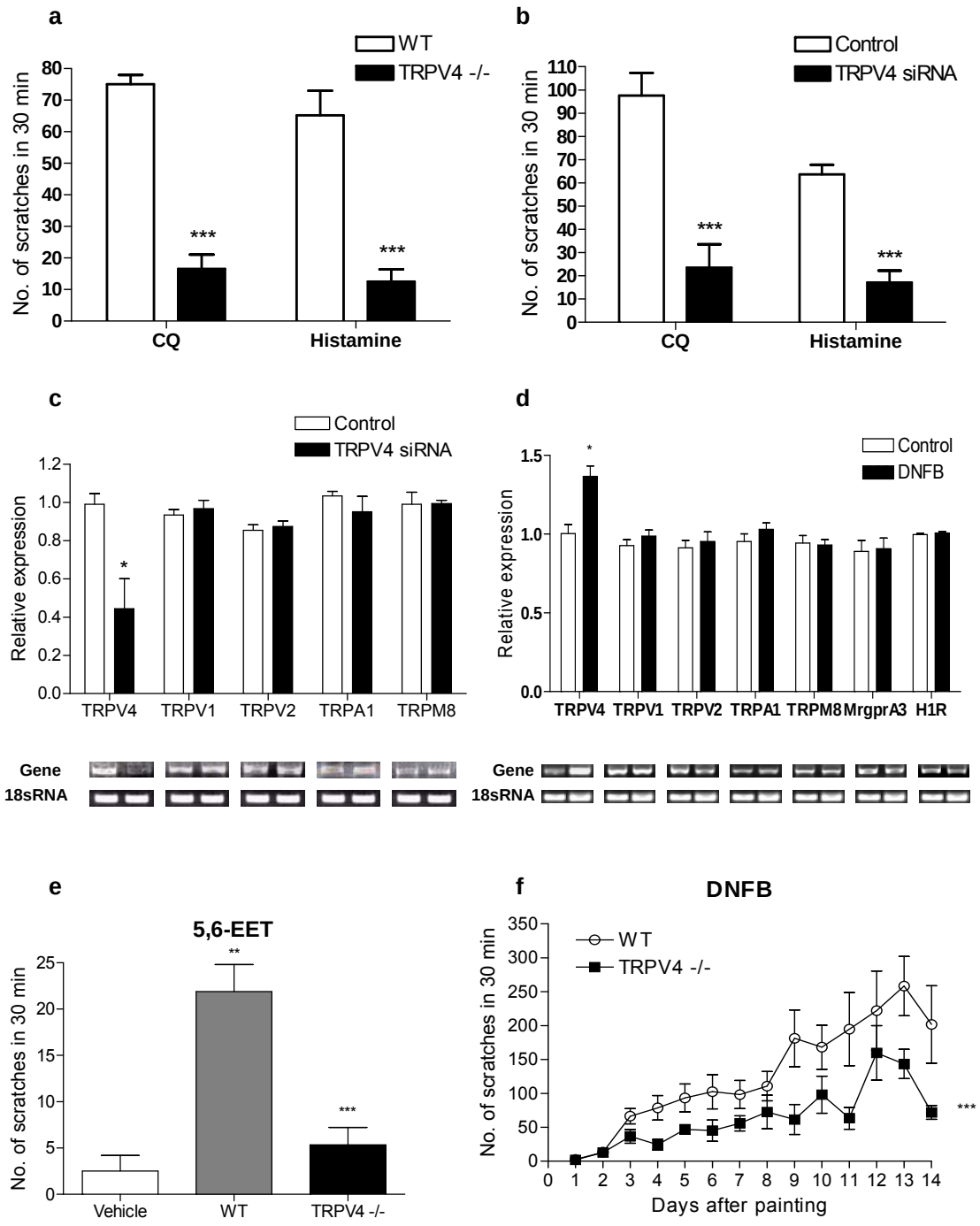

a
WT
TRPV4 -/-
No. of scratches in 30 min
CQ
Histamine
b
Control
TRPV4 siRNA
No. of scratches in 30 min
CQ
Histamine
c
Control
TRPV4 siRNA
Relative expression
TRPV4
TRPV1
TRPV2
TRPA1
TRPM8
Gene
18sRNA
d
Control
DNFB
Relative expression
TRPV4
TRPV1
TRPV2
TRPA1
TRPM8
MrgprA3
H1R
Gene
18sRNA
e
5,6-EET
No. of scratches in 30 min
Vehicle
WT
TRPV4 -/-
f
DNFB
WT
TRPV4 -/-
No. of scratches in 30 min
Days after painting

Figure 33. Acute and contact dermatitis-induced chronic itch were reduced in TRPV4 deficient mice. (a) Acute scratching behaviors induced by intradermal injection of CQ (200μg/50μl) and histamine (500μg/50μl) were reduced in TRPV4$^{-/-}$ mice relative to WT littermate controls ($p<0.001$; n=6). (b) TRPV4 siRNA knockdown mice also have defects in both CQ and histamine-induced itch compared to control mice injected with DEPC-PBS/PEI ($p<0.001$, n=6-10). (c) qRT-PCR showed that TRPV4 expression was reduced after i.t. injection of siRNA without changing TRPV1, TRPV2, TRPA1 and TRPM8 in DRG. (d) TRPV4 expression in DRG was up-regulated after DNFB treatment without changing other TRP channels, MrgprA3 and H1R ($p<0.05$, n=3). (e) i.t. administration of endogenous TRPV4 agonist, 5, 6-EET (150pmol/5μl) caused scratching responses compared to WT littermate controls injected with vehicle (0.1% ethanol) ($p<0.01$, n=6). Scratching behaviors by 5, 6-EET were significantly reduced in TRPV4$^{-/-}$ mice versus control WT littermates ($p<0.001$, n=6). (f) Scratching behavior evoked by DNFB-painting was attenuated in TRPV4$^{-/-}$ mice compared to WT controls ($p<0.01$, n=7). All data are mean ± standard error mean (s. e. m.). Error bars represent s. e. m. Student's *t*-test (a-d) and Two-way ANOVA (e) were used to analyze data significance,

CQ: chloroquine, WT: wild type, DEPC-PBS: diethylprocarbonate-phosphate buffered saline, PEI: polyethylenimine

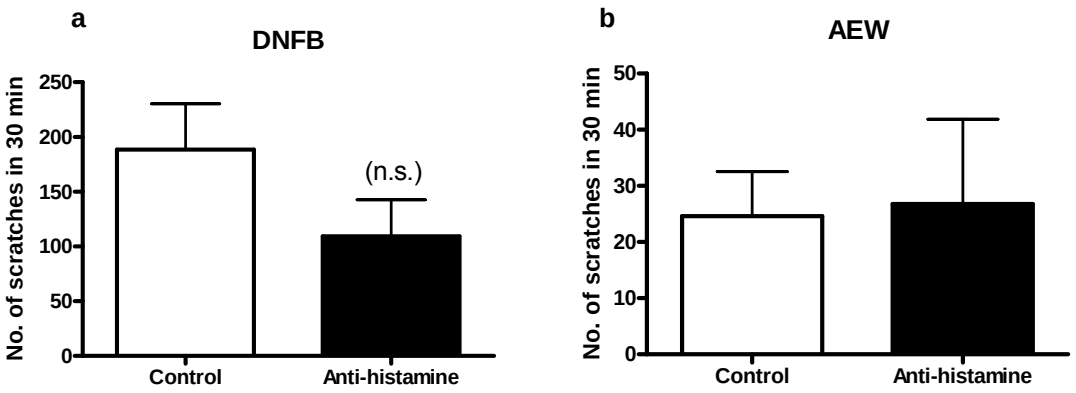


Figure 34. DNFB and AEW-induced scratching behaviors were not inhibited by anti-histamine. Saline (control) and 10mg/kg antihistamine (chlorpheniramine) in 10ml saline were injected intraperitoneally 30 min before the test. (a) DNFB model (n.s., not significant, p=0.1655, n=7) (b) AEW model (n=4-5)

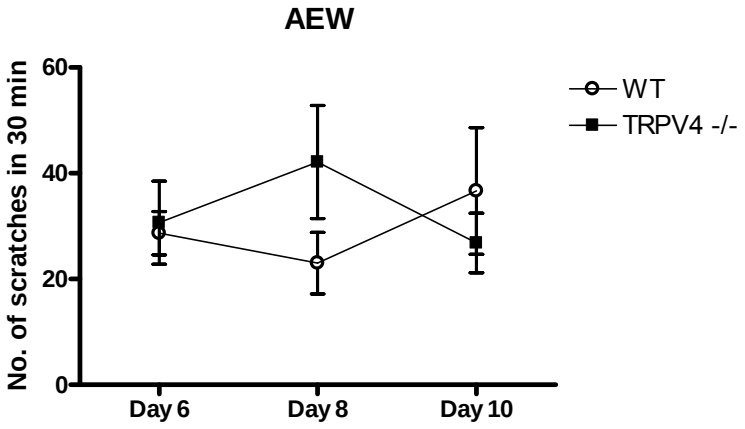


Figure 35. Dry skin itch is not affected in TRPV4$^{-/-}$ mice.

AEW-induced dry skin itch is not affected in TRPV4$^{-/-}$ mice compared to WT littermate controls (n=7).

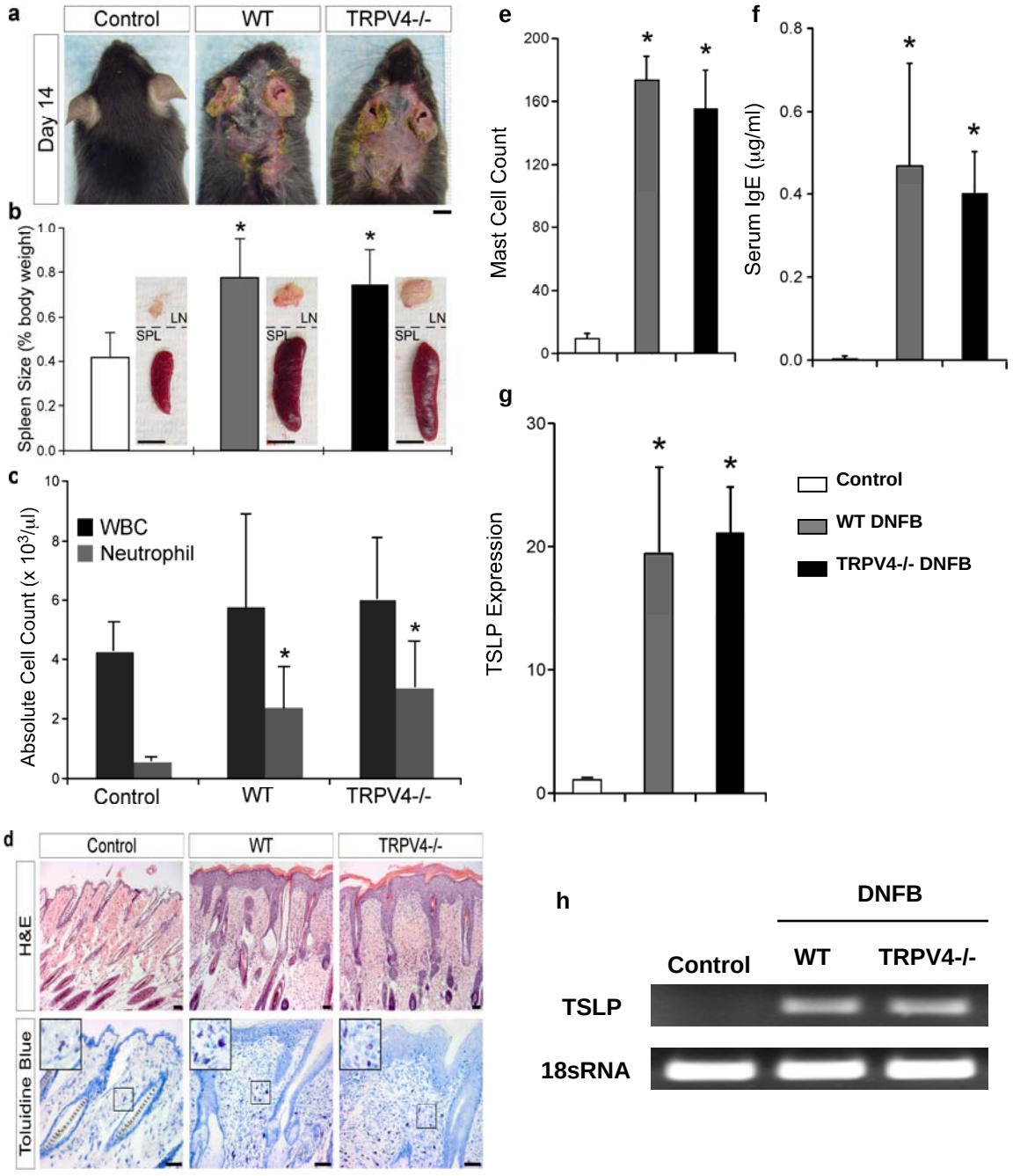

a
Control
WT
TRPV4-/-
Day 14
b
Spleen Size (% body weight)
LN
SPL
c
Absolute Cell Count (x 10³/μl)
WBC
Neutrophil
Control
WT
TRPV4-/-
d
Control
WT
TRPV4-/-
H&E
Toluidine Blue
e
Mast Cell Count
f
Serum IgE (μg/ml)
g
TSLP Expression
Control
WT DNFB
TRPV4-/- DNFB
h
DNFB
Control
WT
TRPV4-/-
TSLP
18sRNA

Figure 36. Repeated DNFB treatment caused an identical allergic contact dermatitis in WT and TRPV4$^{-/-}$ mice. (a) Macroscopic picture of DNFB-treated areas on the dorsal skin of the WT and TRPV4$^{-/-}$ mice shows a similar dermatitis in these animals including the extent of erythema (scale bar: 5mm). (b, c) The systemic manifestations of allergic dermatitis are equally present in DNFB-treated WT and TRPV4$^{-/-}$ mice as highlighted by (b) their enlarged spleen and lymph nodes (% of body weight), and (c) significantly increased blood neutrophil counts (Absolute cell counts ($\times 10^3$/μl), LN: lymph Node, SPL: spleen, WBC: white blood cell, scale bars: 5mm). (d) H&E and Toluidine blue staining of the DNFB-treated skin demonstrates a similar degree of epidermal hyperplasia, hyperkeratosis, acanthosis, dermal inflammation and mast cell infiltration (insets) in WT and TRPV4$^{-/-}$ animals. (scale bar: 50μm). (e) The quantitative analysis confirms that the extent of mast cell infiltration in the dermis of WT and TRPV4$^{-/-}$ mice is comparable. The bar graphs show the average number of mast cells in 9 random 200X microscope fields. (f) Serum IgE levels (μg/ml) are highly elevated and are comparable in DNFB-treated WT and TRPV4$^{-/-}$ mice. (g, h) TSLP, a hallmark of allergic skin disease, is equally overexpressed in DNFB-treated WT and TRPV4$^{-/-}$ skin as determined by its mRNA levels in the skin. Representative pictures are presented; n=6 for each group; $p<0.01$ compared to the control (i.e. Control, WT and TRPV4$^{-/-}$ cohort). Student's *t*-test (b-c, e-g), H&E: Hematoxylin and eosin, IgE: immunoglobulin E, TSLP: thymic stromal lymphopoietin

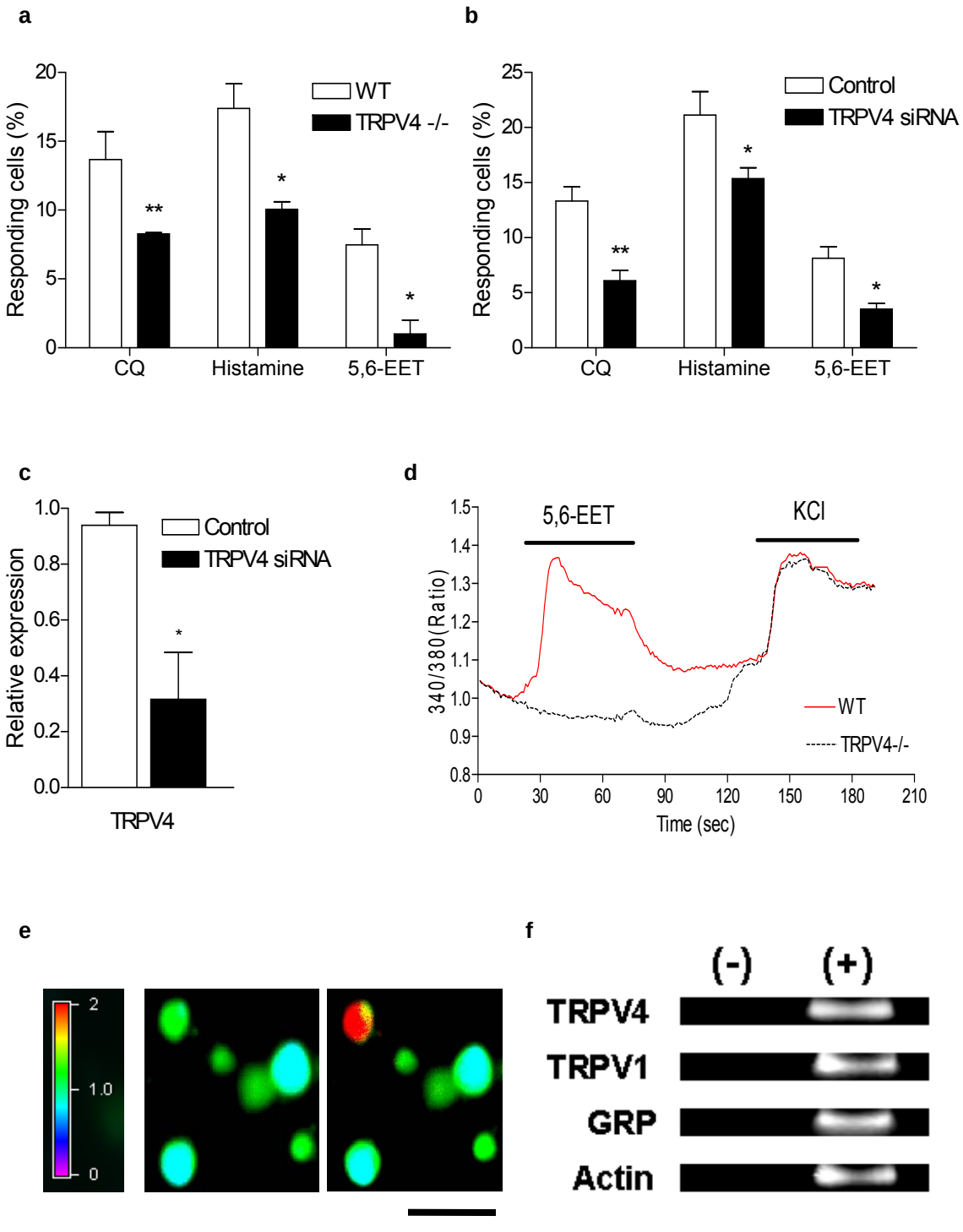

a
Responding cells (%)
WT
TRPV4 -/-
CQ
Histamine
5,6-EET
b
Responding cells (%)
Control
TRPV4 siRNA
CQ
Histamine
5,6-EET
c
Relative expression
Control
TRPV4 siRNA
TRPV4
d
5,6-EET
KCl
340/380(Ratio)
WT
TRPV4-/-
Time (sec)
e
f
(-)
(+)
TRPV4
TRPV1
GRP
Actin

Figure 37. TRPV4 is involved in neuronal activation of DRG by CQ, histamine and 5, 6-EET. DRG neurons increase intracellular Ca^{2+} responding to CQ (14%, 37/269), histamine (21%, 57/269) and 5, 6-EET (8%, 22/269). (a) The numbers of CQ (6%, 15/243), histamine (10%, 24/243) and 5, 6-EET (1%, 3/243) responsive DRG cells were reduced in $TRPV4^{-/-}$ mice DRG (**p<0.01 and *p<0.05, n=6). (b) The responding cell populations to CQ (6%, 11/177), histamine (15%, 26/177) and 5, 6-EET (3%, 6/177) were reduced in the TRPV4 siRNA-transfected DRG culture consistently with the $TRPV4^{-/-}$ DRG (**p<0.01 and *p<0.05, n=6). (c) TRPV4 expression was reduced in DRG cells after TRPV4 siRNA transfection (p<0.05, n=3). (d, e) 5, 6-EET caused DRG Ca^{2+} responses in WT DRG neurons but not in the $TRPV4^{-/-}$ DRG. White arrow indicates 5, 6-EET responding cells with increased intracellular Ca^{2+}. (f) Single cell RT-PCR showed that 5, 6-EET responding cells express TRPV1 and GRP.

Student's *t*-test (a-c), Scale bar 50μm

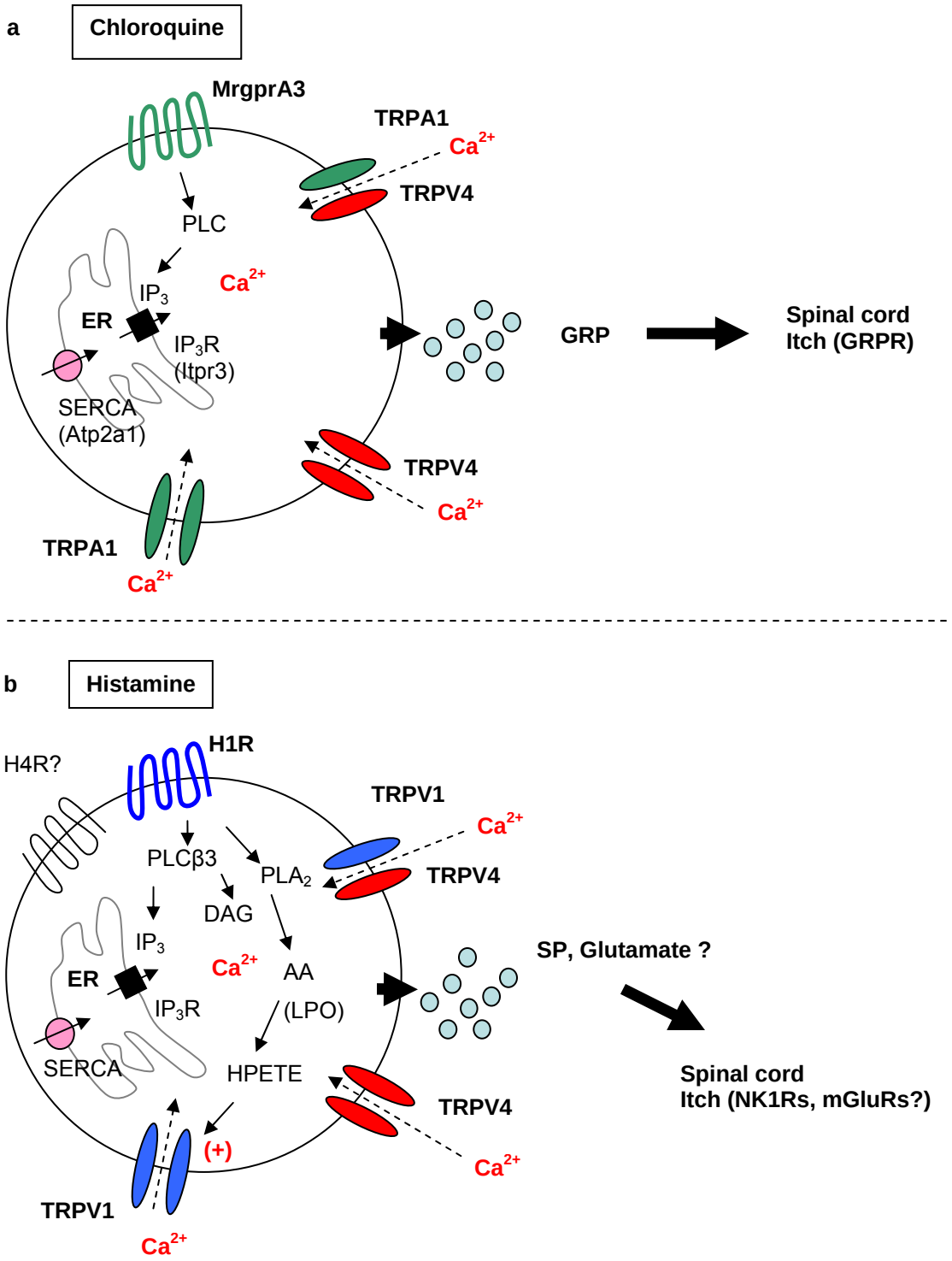

a
Chloroquine
MrgprA3
TRPA1
Ca2+
TRPV4
PLC
Ca2+
IP3
ER
IP3R
(Itpr3)
SERCA
(Atp2a1)
GRP
Spinal cord
Itch (GRPR)
TRPV4
Ca2+
TRPA1
Ca2+
b
Histamine
H4R?
H1R
TRPV1
Ca2+
PLCβ3
PLA2
TRPV4
DAG
IP3
Ca2+
AA
ER
IP3R
(LPO)
SERCA
HPETE
SP, Glutamate ?
Spinal cord
Itch (NK1Rs, mGluRs?)
TRPV4
Ca2+
(+)
TRPV1
Ca2+

Figure 38. Potential mechanism of TRPV4-mediated itch pathways. (a) CQ activates MrgprA3 and downstream PLC/IP_3 Ca^{2+} signaling pathway. IP_3R and SERCA have critical roles for intracellular Ca^{2+} increase. TRPA1 and TRPV4 are involved in CQ-induced itch pathway. TRPA1 and TRPV4 may form either homo- or heteromers and increase intracellular Ca^{2+} through membrane Ca^{2+} influx. Activation of DRG cells will cause a GRP release to stimulate GRPR in the spinal cord to transmit itch sensory information. (b) Histamine activates H1R in DRG. H4R has been reported to be involved in histamine signaling pathways recently (Rossbach, Nassenstein, et. al. 2011). However, detail characterization of H4R-mediated itch pathways in DRG have not been done. H1R has multiple downstream signaling pathways. (1) H1R is a GPCR and has PLCβ3/IP_3/Ca^{2+} pathway. (2) DAG, the product of PLCβ3-mediated membrane lipid hydrolysis, directly activates TRPV1 (Woo, Jung, et. al. 2008). (3) PLA_2-AA-HPETE pathway is also involved in TRPV1 activation for histamine itch (Kim, Lee et al. 2004). TRPV4 may also form heteromers with TRPV1 and regulate histamine itch signaling pathways. Activation of histamine-dependent signaling will release neuropeptides such as SP and glutamate. Spinal cord NK1Rs or mGluRs can be involved in central itch transmissions. AA : Arachidonic acid, HPETE : 12-Hydroperoxy-5,8,10,14-eicosatetraenoic acid

CURRICULUM VITAE

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EDUCATION

2011 Ph.D. **Developmental Biology,** Washington University in St. Louis, MO, U.S.A.

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AWARDS

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2005 **Rita Levi-Montalcini Award** (for the best graduate student poster)
The Annual Developmental Biology Program Retreat

PUBLICATION

Qin Liu, Zongxiang Tang, Lenka Surdenikova, **Seungil Kim**, Kush N. Patel, Andrew Kim, Fei Ru, Yun Guan, Hao-Jui Weng, Yixun Geng, Bradley J. Undem, Marian Kollarik, Zhou-Feng Chen, David J. Anderson, and Xinzhong Dong (2009) Sensory neuron-specific GPCRs Mrgprs are itch receptors mediating chloroquine-induced pruritus. ***Cell*** 139(7): 1353-65

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Joe Jeffery, **Seungil Kim** and Zhou-Feng Chen (2011) Molecular mechanism of itch signaling in the nervous system. ***Physiology*** (In press)

Seungil Kim, Shadmehr Demehri, Zhong-Qui Zhao, Ahu Turkoz, Joseph Jeffry, Raphael Kopan and Zhou-Feng Chen (2011) TRPV4 mediates allergic contact dermatitis-induced but not dry skin-induced pruritus. (In preparation)

ABSTRACTS

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East Coast Worm Meeting, Yale University, U.S.A.

2003 Prashant Raghavan, Jungsoon Lee, **Seung-il Kim**, Byung-jae Park, and Tae Ho Shin. "CeUBC9 is required for the repression of MEP-1 function by PIE-1" The 14th International C. elegans Meeting, University of California, Los Angeles, U.S.A.

1998 Sequence Analysis of the *Bombyx* Fibroin gene promoters
The 53th conference of the Korean Association of Biological Sciences, The Genetics Society of Korea.

INVENTION

2010 **Invention Disclosure.** "Blocking pruritus by targeting GRPR in the skin by systemic injection of gastrin-releasing peptide receptor (GRPR) antagonists." Washington University Office of Technology Management. WU OTM Case No. 010306

2010 **Invention Disclosure.** "Identification of genes that may be explored to block in the spinal cord and sensory neurons." Washington University Office of Technology Management WU OTM Case No. 010422

2011 **Invention Disclosure.** "TRPV4 as a therapeutic target to treat allergic contact dermatitis (ACD)-induced itch." Washington University Office of Technology Management WU OTM Case No. 011469