이학박사 학위논문

Regulation mechanisms of itch signal transduction by sensory neuronal toll-like receptor 4

감각신경세포상의 톨유사수용체 4 의 가려움감각 조절 기전

2016 년 8 월

서울대학교 대학원

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민 현 정

Abstract

Regulation mechanisms of itch signal transduction by sensory neuronal toll-like receptor 4

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Itch, formally known as pruritus, has been defined as an unpleasant skin sensation that elicits the desire or reflex to scratch. The normal itch occurs in order to protect our body from external harmful stimuli however, excessive pruritus worsen the life quality of patient. The therapeutic strategies are limited to symptomatic treatment due to short of understanding to itch mechanism. Recent studies have indicated that Toll-like receptor 4 (TLR4) is also expressed on sensory neurons, implicating its putative role in sensory signal transmission. In this study, I suggest that TLR4 expressed on sensory neuron regulates itch sensation by modulate TRPV1 activity.

In chapter 1, data show that TLR4 on sensory neurons enhances histamine/chloroquine-induced itch signal transduction. I confirmed that TLR4 was expressed on a subpopulation of dorsal root ganglia (DRG)

sensory neurons that express TRPV1. In TLR4-knockout mice, histamine/chloroquine-induced itch responses were compromised while TLR4 activation by LPS did not directly elicit an itch response. Chloroquine receptor expression was decreased in TLR4 deficient DRG sensory neuron, while expression of histamine receptors were comparable to wild type. Histamine-induced intracellular calcium signals and inward currents were comparably reduced in TLR4-deficient sensory neurons. Reduced histamine sensitivity in the TLR4-deficient neurons was accompanied by a decrease in TRPV1 activity. Heterologous expression experiments in HEK293T cells indicated that TLR4 expression enhanced capsaicin-induced intracellular calcium signals and inward currents. Otherwise, TLR4 regulates chloroquine-induced itch sensation by enhanced expression of MrgprA3 which is known as chloroquine receptor.

In chapter 2, I revealed that direct association between TRPV1 and TLR4 through TIR domain decrease TRPV1 desensitization by dysregulation of channel expression on cell surface. I confirmed that TLR4 interact with TRPV1 through TIR domain. HEK 293T cells, transiently transfected with TIR-truncated TLR4 mutant and TRPV1, showed reduced capsaicin induced calcium signaling compared with HEK 293T cells expressing full length TLR4 and TRPV1. Although interaction between TLR4 and TRPV1 did not alter serine residue phosphorylation of TRPV1, the interaction decreased downregulation of TRPV1 after capsaicin stimuli. In consequence of increased TRPV1 expression on cell surface, capsaicin-

induced desensitization was enhanced in TLR4 KO sensory neuron.

In conclusion, TLR4 on sensory neurons enhances chloroquine/histamine-induced itch signal transduction by distinct mechanisms. TLR4 on sensory neurons enhances chloroquine-induced itch sensation by decreasing chloroquine receptor expression. However, TLR4 increases histamine-induced itch signal transduction by potentiating TRPV1 activity. The direct association between TRPV1 and TLR4 through TIR domain blocks capsaicin induced desensitization and moreover, TLR4 activation mediates TRPV1 sensitization by regulate TRPV1 trafficking to membrane.

The results suggest that TLR4 could be a novel target for the treatment of enhanced itch sensation.

Key words: Itch, Histamine, Chloroquine, Toll-like receptor 4, Transient receptor potential vanilloid 1

Student Number: 2010-30922

CONTENTS

ABSTRACT ····· i

TABLE OF CONTENTS ····· iv

LIST OF FIGURES ····· ix

BACKGROUND ····· 2

1. Ich sensation 2

1.1. Overview of Itch Sensation ····· 2

1.2. Histamine induce Itch Sensation ····· 4

2. Toll-Like Recetors 5

2.1. Overview of Toll-Like Receptor ····· 5

2.2. TLRs Expression in the Nervous System ····· 6

3. Transient receptor potential vanilloid 1 10

PURPOSE ····· 15

CHAPTER 1

TLR4 enhances histamine-mediated pruritus by potentiating TRPV1 activity

ABSTRACT ····· **17**

INTRODUCTION ····· **18**

MATERIALS AND METHODS ····· **20**

Mice 20

Behavior study 20

Primary DRG neuron culture 21

Cell culture and transfection 21

Plasmid 22

Single-cell RT-PCR 22

Immunofluorescence 23

Real-time PCR 25

Calcium Assay 26

Whole cell patch-clamp recording 27

Western blot 27

Statistical Analysis 28

RESULTS ····· **29**

TLR4 enhances histamine and chloroquine induced itch sensation 29

TLR4 enhances both HRH1 and HRH4 activation induced itch sensation 29

Sensory neurons express TLR4 30
TLR4 expression detected in various type of sensory neuron. 30
Histamine- or chloroquine- induced calcium signals are reduced in TLR4 KO sensory neuron 31
Histamine receptor expression was not affected by TLR4 expression..... 32
Capsaicin induced calcium signal and inward current were reduced in TLR4 KO sensory neuron 32
TRPA1 activity was not altered in TLR4 KO sensory neuron 33
TLR4 expression enhances TRPV1 activity 33
DISCUSSION.....53

CHAPTER 2

TLR4 enhances TRPV1 activity by direct association through TIR domain

ABSTRACT ····· **58**
INTRODUCTION ····· **60**
MATERIALS AND METHODS ····· **62**
Mice 62
Behavior study..... 62

Primary DRG neuron culture 62
Cell culture and transfection 63
Plasmid..... 64
Immunofluorescence 64
Calcium Assay..... 65
Immunoprecipitation 66
Western blot 67
Cell-surface biotinylation assay 67
Statistical Analysis..... 68
RESULTS····· 70
TRPV1 expression on TLR4 KO sensory neurons was similar to WT sensory neurons 70
Direct association between TLR4 and TRPV1 protein 70
TLR4 associates with TRPV1 through TIR domain 71
Interaction between TLR4 and TRPV1 does not alter serine residues phosphorylation of TRPV1 72
TRPV1 on TLR4 deficient sensory neuron undergoes excessive desensitization 72
TLR4 expression decreases capsaicin induced TRPV1 downregulation ... 73
Capsaicin induced acute pain attenuated in TLR4 deficient mice 74
DISCUSSION.....92
CONCLUSION····· 97

REFERENCES ····· **99**

ABSTRACT IN KOREAN ····· **117**

LIST OF FIGURES

Chapter 1

Fig. 1. TLR4 enhances histamine-induced and chloroquine-induced itch sensation ····· 35

Fig. 2. TLR4 enhances both HTMT and clobenpropit -induced itch sensation ····· 37

Fig. 3. TLR4 expressed on sensory neuron ····· 39

Fig. 4. Characterization of the TLR4-expressing sensory neurons ····· 41

Fig. 5. Histamine-induced and chloroquine-induced intracellular calcium increases were reduced in TLR4 KO neurons ····· 43

Fig. 6. Histamine receptor expression was not affected by TLR4 ····· 45

Fig. 7. Capsaicin-induced intracellular calcium signals and inward currents were compromised in TLR4 KO sensory neurons ····· 47

Fig. 8. TRPA1-mediated calcium signals were not altered in TLR4 KO neurons ····· 49

Fig. 9. TLR4 expression enhances TRPV1 activity in HEK293T cells ····· 51

Chapter 2

Fig. 1. TRPV1 expression was not changed in TLR4 KO DRG ····· 76

Fig. 2. A direct association between TLR4 and TRPV1 ····· 78

Fig. 3. TLR4 interact TRPV1 through TIR domain ····· 80

Fig. 4. Serine residues phosphorylation on TRPV1 was not affected by TLR4 expression ····· 82

Fig. 5. TLR4 regulates TRPV1 desensitization induced by capsaicin stimulus ····· 84

Fig. 6. TLR4 expression declined TRPV1 trafficking to cytosol ····· 86

Fig. 7. Capsaicin induced TRPV1 downregulation enhanced in TLR4 KO sensory neuron ····· 88

Fig. 8. Capsaicin induced acute pain attenuated in TLR4 KO mice ····· 90

Background and purpose

Background

1. Itch sensation

1. 1. Overview of Itch Sensation

Itch sensation, formally known as pruritus, has been defined as an "unpleasant skin sensation that elicits the desire or reflex to scratch" (Akiyama and Carstens 2013). Although an itch might constitute an alert system against harmful stimuli, it can worsen the living quality of patients when excessive. Indeed, patients with severe pruritus often suffer from itch-associated psychological disease such as depression or sleep deprivation.

There are numerous substances reported as a pruritogen, which refers to a substance that causes pruritus such as substance P, chloroquine, proteases, and histamine. Histamine, which is most well-known pruritogen, is released from immune cells such as mast cells and basophilic granulocytes (Shim and Oh 2008). Released histamine acts on a sensory neurons which express histamine receptor HRH1 and HRH4 (Han et al. 2006). And there are many forms of pruritus which are insensitive to antihistamine drugs and these phenomena leads to effort to elucidating the pruritogen which evoke non-histamine pruritus (Jeffry et al. 2011). In dermis of patient suffered from chronic itch, high levels of protease are detected (Steinhoff et al. 1999). These proteases are released from activated mast cell and able to activate variety types of protease receptors, including protease activated receptor 2 (PAR2) which is expressed on sensory neuron

(Reddy et al. 2008). SLIGRL, which is known as PAR2 receptor induces neuron excitation and evoke itch signal when it applied by intradermal injection (Shimada et al. 2006). 5-HT, inflammatory mediator which is released from mast cells, melanocytes and platelets, is known to evoke itch sensation through direct activation of 5-HT_7 (Morita et al. 2015). Some pruritogens are released from activated keratinocytes. Endothelin 1 is synthesized by keratinocytes which are located in normal skin and released after cutaneous injury. It is able to trigger pain sensation through binding to endothelin-A receptor, however, intrathermal injection of endothelin-1 in mice induce mixed pain and itch behavior (McQueen et al. 2007; Piovezan et al. 2000).

Once pruritogen is applied, an itch is triggered by the excitation of a subset of unmyelinated C-fibers, which express pruritogen receptors. Activated C-fibers innervate the epidermal layer of skin and transmit electrical signals to the superficial layer of the dorsal horn of the spinal cord (Schmelz et al. 1997). Signals then ascend to the thalamus through contralateral spinothalamic tracts and are eventually transmitted to various brain cortical areas including the somatosensory and cingulate cortex (Andrew and Craig 2001).

An itch was thought as a sub-modality of pain. According to this view, an itch is perceived when the intensity of noxious stimuli is too weak to induce pain. However, recent studies have proven that increasing itch intensity still induced an itch, but not pain (Handwerker et al. 1991; Ochoa

and Torebjork 1989). Moreover, it was reported that gastrin-releasing peptide (GRP) plays a key role in mediating itch sensation, but not pain, in the spinal cord. Furthermore, the induction of scratching behaviors in response to pruritogenic stimuli were attenuated in GRP receptor (GRPR) knockout mice, but pain-related responses to noxious stimuli were not affected (Sun and Chen 2007). In addition, GRPR antagonist application to the spinal cord inhibited scratching behaviors in three different itch behavior models (Sun and Chen 2007). In conclusion, these studies suggest that neuronal circuits transducing itch signals are distinct from pain.

1. 2. Histamine induce Itch Sensation

Histamine is the most well-known pruritogen that evokes an experimental itch when applied to the skin. Histamine is released from mast cells in the skin when tissues are inflamed or stimulated by allergens. Once released, the histamine-induced itch is triggered by the excitation of a subset of unmyelinated C-fiber. There are four subtypes of histamine receptors (H1R1~4). H1R1, 3, and 4 are expressed in skin-innervating sen sory neurons (Inagaki et al. 1999; Rossbach et al. 2011). Among them, only H1R1 and H1R4 cause itch sensation in mice (Rossbach et al. 2009). H1R1 is a member of the G-protein-coupled receptor (GPCR) family. It was reported that histamine treatment on H1R1-positive sensory neurons triggers intracellular calcium signals and action potentials (Han et al. 2006) and that a histamine induced intracellular calcium signal depends on PLA_2 and PLCβ3 activation (Han et al. 2006; Kim et al. 2004). Furthermore, Shim et

al. revealed that histamine-induced inward current and calcium signal were blocked by a transient receptor potential cation channel subfamily V member 1 (TRPV1) antagonist and histamine-induced scratching behavior was attenuated in TRPV1 deficient mice (Shim et al. 2007). These findings indicate that histamine induces itch sensation through TRPV1 activation.

2. Toll-Like Receptors

2.1. Overview of Toll-Like Receptor

TLRs are pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (Medzhitov 2001). TLRs are type I integral membrane glycoproteins. Considering their homology in the cytoplasmic region, TLRs are an included superfamily that have conserved a cytoplasmic domain known as Toll/IL-1R (TIR), which share three highly homologous domains known as box 1, 2 and 3 (Slack et al. 2000). The *Toll* gene was first identified in *Drosophila* and studies using *Toll* mutant flies revealed that *Toll* has antibacterial or antifungal functions (Lemaitre et al. 1996). In the mammalian, thirteen TLRs have been identified, and TLRs detect infectious agents and trigger innate immune response for external pathogens in the host organism (Kielian 2009). Among the 13 TLR2, TLR1-9 is identified in both mouse and human and TLR10 is expressed only in human, whereas, TLR 11-13 are expressed in mouse not human (De Nardo 2015). TLRs can be divided in two groups upon their subcellular localization. TLR1, 2, 4, 5, 6 and 11expressed on cell surface and recognize

PAMPs. However, TLR3, 7, 8 and 9 are expressed on intracellular vesicles such as endosomes, lysosomes and mainly recognize nucleic acids (Takeuchi and Akira 2010).

It is well known that TLRs are expressed on the cells of an innate immune system and function to sense danger or damaged signals. The ligands that activate TLRs include conserved pathogen structure and pathogen-associated molecular patterns (PAMPs) such as LPS, viral or bacterial nucleic acids, and bacterial cell wall components. In addition, TLRs also sense endogenous molecules that are released from damaged tissue or stressed cells such as heat shock proteins (HSPs), high mobility group box 1 (HMGB1), and extracellular matrix breakdown products (Beg 2002). Upon TLR activation, a series of intracellular signaling molecules are activated including mitogen-activated protein kinases (MAPKs) transcription factors such as AP-1 and NF-κB. These intracellular signaling pathways normally trigger inflammatory responses in the innate immune cells, resulting in pro-inflammatory gene expressions such as cytokines, chemokines, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). In addition, a recent study revealed that the expression and functions of TLRs are not limited to an immune cell. However, the functions of TLRs expressed on non-immune cells are not fully understood.

2. 2. TLRs Expression in the Nervous System

In mammalians, TLRs are primarily expressed in peripheral immune cells such as neutrophils, dendritic cells and macrophages (Medzhitov 2001). However, TLRs also play important roles in non-immune systems and several studies revealed that TLRs are expressed in the nervous system. In the central nervous system (CNS), microglia, which is known as a CNS resident macrophage, express a variety of TLRs like other tissue resident macrophages. Although a it is hardly detected in resting status microglia, TLR expression is rapidly increased upon the activation of cells. Previous studies revealed that TLR1-9 expression was detected in mRNA level for both humans (Olson and Miller 2004) and mice (Jack et al. 2005). Moreover, CD14, which is known as a co-receptor of TLR4, is also detected in human microglia (Rasley et al. 2002). Like other macrophages, TLRs on microglia mediate an inflammatory response. After activation, production of inflammatory mediators and phagocytosis of bacteria or amyloid fibers were significantly increased (Block et al. 2007; Chen et al. 2006; Tahara et al. 2006). Although the expression level is lower than microglia, astrocyte also express a series of TLRs. Basibsi *et al.* reported that TLR2 and 3 were expressed in primary cultured human astrocyte (Bsibsi et al. 2002). In other studies, mRNA expression of TLR 1, 3, 4, 5 and 9 were confirmed in human astrocytes (Jack et al. 2005) and TLR1-6 was confirmed in rodent primary astrocyte cultures (Bowman et al. 2003). During inflammation that occurred by traumatic injury or pathogen infection, TLR2, 3 and 4 were detected on astrocyte (Bsibsi et al. 2006; Jack et al. 2005; Park et al. 2008; Suh et al.

2007). Moreover a recent study reported that TLR2 expressed on astrocyte plays a detrimental role in intracerebral hemorrhage induced brain damage by enhancing proinflammatory gene expression (Min et al. 2015).

Neurons also express several TLRs. Prehaud et al. reported that neurons from humans express TLR3 after viral transfection (Prehaud et al. 2005). Moreover, Jackson et al. revealed that neurons in rabies or herpes simplex virus infected brain expression in TLR3 (Jackson et al. 2006). In CNS, TLR2 and 4 were detected in mouse cortical neurons *in vitro* and *in vivo* after ischemic stroke (Tang et al. 2007). In this study, TLR expression in damaged cortical neurons contributed to apoptotic neuronal cell death. Although neuronal TLRs participate in neuroinflammatory response, recent evidence suggests that TLRs is expressed on neurons engaged with neurogenesis. TLR2 is expressed on cells in the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (Okun et al. 2010; Rolls et al. 2007), and are known as neurogenesis. TLR2 and 4 are also detected in adult neuronal progenitor cells and influence proliferation and differentiation (Covacu et al. 2009; Ma et al. 2006).

It is also found that TLRs are expressed in the neurons of the PNS. Recent research revealed that TLR3, 4, 7 and 9 are expressed in the neurons of the PNS and detect PAMPs or damage associated molecular patterns (DAMPs) (Barajon et al. 2009; Due et al. 2012). These findings suggest a role for this particular receptor in sensory transmission, but the role has not been formally studied. Qui et al. revealed that stimulation of TLRs

expressed on dorsal root ganglia (DRG) neurons enhances pain sensation, which suggests that TLRs on sensory neurons are involved in not only inflammatory response but also sensory transmission (Qi et al. 2011).

Recent study has revealed that TLRs expressed in nervous system involved in pain or itch sensation. It has been reported that TLRs expressed in spinal microglia plays a critical role in neuropathic pain sensation. TLR4 expression was increased in spinal cord microglia and the expression of TLR4 is required for nerve-injury induced spinal cord microglia activation and neuropathic pain (Tanga et al. 2005; Tanga et al. 2004). TLR2 expressed in microglia also involves in neuropathic pain. After nerve injury, microglial TLR2 activation mediates Nox2 expression and ROS production and contribute to spinal cord microglia activation which results pain hypersensitivity (Kim et al. 2007; Kim et al. 2010; Lim et al. 2013).

Recently, it has been reported that Toll-like receptors (TLRs) on sensory neurons are involved in itch sensation. Studies by Liu et al. revealed that TLR3 and TLR7 on sensory neurons trigger itch sensation upon stimulation by their ligands (Liu et al. 2012; Liu et al. 2010). The activation of neuronal TLR3 by poly(IC) treatment directly results in sensory neuron excitation and induces a scratching response (Liu et al. 2012). In addition, pruritogen-induced itch sensation was impaired in TLR3 KO mice (Liu et al. 2012). Based on these findings, TLRs in the peripheral nervous system (PNS) are implicated in itch signal transduction and transmission. However, function of the sensory neuronal TLRs in pruritus is not fully understood.

3. Transient receptor potential vanilloid 1 (TRPV1)

TRPV1, also known as the capsaicin receptor and the vanilloid receptor 1, was initially tested using capsaicin without their molecular identities in the peripheral neuron. TRPV1 encoding an 838 amino acid protein (~95 kDa) has six putative transmembrane domains with a pore-forming hydrophobic region between the fifth and sixth transmembrane domains and two intracellular cytosolic tails in the N- and C-termini with three ankyrin repeats in the N-terminus (Vos et al. 2006). TRPV1 is activated not only by vanilloids such as capsaicin, but also by noxious heat (>43°C) and low pH (Caterina et al. 1997; Tominaga et al. 1998), ethanol (Caterina et al. 1997; Trevisani et al. 2002) and various lipid metabolites (Bhave et al. 2003; Caterina and Julius 2001; Chu et al. 2003; Huang et al. 2002; Hwang et al. 2000; Kwak et al. 2000; Shin et al. 2002; Zygmunt et al. 1999). As a non-selective cation channel, TRPV1 displays nearly equal permeability to Na^+, K^+, Cs^+, Li^+ and Rb^+(Caterina et al. 1997; Oh et al. 1996; Samways and Egan 2011). TRPV1 is also permeable to larger polyvalent cations including guanidinium, tetraethylammonium and NMDG (Hellwig et al. 2004; Winter et al. 1990). The permeability of TRPV1 to larger cations has been revealed in the research using local anaesthetic QX314. Co-administration of QX314 together with a TRPV1 activator can deliver QX314 intracellularly to block neuronal activity in TRPV1-expressing nerve fibers specific manner

(Binshtok et al. 2007).

TRPV1 receptors are strongly expressed in the PNS including C polymodal nociceptive and Aδ nociceptive primary afferent terminals (Guo et al. 2001; Michael and Priestley 1999; Valtschanoff et al. 2001). TRPV1 is expressed in about half of all somatic and visceral sensory neurons, with expression restricted to neurons of small to medium size in the dorsal root, trigeminal and vagal ganglia (Caterina et al. 1997; Helliwell et al. 1998). These neurons form unmyelinated or thinly myelinated nerve fibers, classified as C or Aδ fibers, that project to most organs and tissues. TRPV1 is expressed in two distinct populations of DRG neurons. One class of TRPV1-expressing neurons is nerve growth factor- sensitive and CGRP- and substance P-positive (peptidergic), and the other class contains glial cell-derived neurotrophic factor (GDNF)-sensitive IB4-binding neurons (Tominaga et al. 1998). TRPV1 in sensory neuron can detect noxious heat and convey painful signals to avoid potential tissue damage. In physiological condition, TRPV1 deficient mice showed an impaired pain nociception by acute thermal stimuli (Caterina et al. 2000; sork rDavis et al. 2000). It has long been recognised that TRPV1 is also present in the central nervous system (CNS), notably the preoptic area of the hypothalamus, where it is essential for thermoregulatory responses to avoid hyperthermia (Jancso-Gabor et al. 1970; Szolcsanyi et al. 1971). Furthermore, pharmacological studies have demonstrated capsaicin-evoked physiological or electrophysiological responses that are consistent with TRPV1 expression

in a number of brain nuclei or regions (Steenland et al. 2006). In situ hybridization has further indicated the presence of TRPV1 in several brain nuclei (Mezey et al. 2000)

Because of the importance in pain sensation and hyperalgesia, activation and potentiation mechanisms of TRPV1 are well studied. Many studies revealed that TRPV1 activation is regulated by phosphorylation. Phosphorylation by protein kinase C (PKC) activation increased channel opening, which depends on the scaffolding protein A kinase-anchoring protein (AKAP79/150). It has been proposed (Studer and McNaughton 2010) that phosphorylation enhances the affinity of capsaicin binding due to conformational alterations. PKCε sensitize TRPV1 activity through phosphorylation of Ser502 and Ser801 residues on TRPV1 (Bhave et al. 2003) and PKA dependent phosphorylation of Ser 116 and Thr 370 residues results in the reduction of TRPV1 desensitization (Mohapatra and Nau 2003). In addition, calcium/calmodulin-dependent protein kinase II (CaMKII) reportedly controls TRPV1 activity upon phosphorylation of TRPV1 at Ser 502 and Thr 704 by regulating capsaicin binding (Jung et al. 2004). Membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) induces potentiation of TRPV1 gating by binding to the proximal PIP_2-binding region of the C terminal. Binding of PIP_2 to the distal region elicits inhibition, but it is not required for PIP_2 regulation (Ufret-Vincenty et al. 2011). Inflammatory mediators released during the early stage of inflammation, such as prostaglandin (mainly prostaglandin-2), bradykinin,

nerve growth factor (NGF) and protease, are the primary contributors to pain sensitization through TRPV1. These inflammatory mediators cannot activate pain receptors directly but can potentiate the TRPV1 channel. They often activate protein kinases such as extracellular signal-regulated protein kinases (ERK), p38 and PKC through the corresponding receptors (Ji et al. 2002; Morenilla-Palao et al. 2004; Sugiura et al. 2002; Zhang et al. 2007; Zhuang et al. 2004). Activated protein kinases can phosphorylate TRPV1, leading to rapid and dynamic changes in pain sensitivity.

On the other hand, TRPV1 activity is regulated by membrane trafficking. In the nerve terminals, no endoplasmic reticulum (ER) or Golgi apparatus are present, therefore, large intracellular membrane structures play important roles in TRPV1 trafficking. In the nerve terminals, accumulated vesicles and in some cases, endosomes or lysosomes functioning as a reservoir of TRPV1, have been described. Previous study has shown that PKC activation-sensitized SNARE-dependent exocytosis and Src-dependent phosphorylation of TRPV1 are involved in inflammatory stimuli-induced rapid membrane insertion of TRPV1 from a vesicular pool (Camprubi-Robles et al. 2009).

TRPV1, like many other ion channels, is organized in a multimeric complex with other proteins that are important in regulating the location and function of the channels. Co-immunoprecipitation study by Zhang *et al*. revealed that A kinase-anchoring protein (AKAP79/150) is bind to TRPV1

channel (Zhang et al. 2008). This protein acts as scaffolding protein which can position PKA, PKC and calcineurin close to TRPV1 (Schnizler et al. 2008; Zhang et al. 2008). By the activity of enzymes which bind to AKAP79/150, interaction between AKAP79/150 and TRPV1 critically regulates TRPV1 sensitization and recovery from desensitization (Brandao et al. 2012; Zhang et al. 2008). β-Arrestin proteins, initially identified for their role in G-protein-coupled receptor desensitization also physically interact TRPV1 (Por et al. 2012). TRPV1 phosphorylation by PKA and PKC leads to increased TRPV1 and β-arrestin association and this association scaffold the phosphodiesterase PDE4D5 which desensitize TRPV1 activity (Por et al. 2012; Por et al. 2013). It was revealed that kinase bind to TRPV1 protein. After NGF stimulation, PI3 kinase can also enhances trafficking of TRPV1 to cell surface by physical protein interaction (Stein et al. 2006). Calmodulin (CaM) has binding sites in cytoplasmic domain of TRPV1 in both N-terminus and C-terminus and interaction with CaM results TRPV1 desensitization (Numazaki et al. 2003; Rosenbaum et al. 2004).

Purpose

Recent study revealed that TLRs are expressed in sensory neuron, however, the functional impact of TLRs in sensory neurons remains to be determined. Especially TLR4, which known as sensing PAMP or DAMP molecule and triggers inflammatory responses, is also expressed on sensory neurons, however, its role in sensory neuron is still unknown. In this study I tried to discover the role of sensory neuronal TLR4 in sensory signal transduction and transmission. To do this, I tried to series of experiments with the following specific aims:

1. To characterize the role of sensory neuronal TLR4 on sensory in evoking and transmitting itch sensory signal
2. To elucidate molecular mechanisms on how sensory signal TLR4 regulates itch signal transduction

The research on the above specific aims is addressed in subsequent two chapters

Chapter 1.

TLR4 enhances histamine-mediated pruritus by potentiating TRPV1 activity

Abstract

Recent studies have indicated that Toll-like receptor 4 (TLR4), a pathogen-recognition receptor that triggers inflammatory signals in innate immune cells, is also expressed on sensory neurons, implicating its putative role in sensory signal transmission. However, the possible function of sensory neuron TLR4 has not yet been formally addressed. In this regard, we investigated the role of TLR4 in itch signal transmission.

TLR4 was expressed on a subpopulation of dorsal root ganglia (DRG) sensory neurons that express TRPV1. In TLR4-knockout mice, histamine-induced itch responses were compromised while TLR4 activation by LPS did not directly elicit an itch response. Histamine-induced intracellular calcium signals and inward currents were comparably reduced in TLR4-deficient sensory neurons. Reduced histamine sensitivity in the TLR4-deficient neurons was accompanied by a decrease in TRPV1 activity. Heterologous expression experiments in HEK293T cells indicated that TLR4 expression enhanced capsaicin-induced intracellular calcium signals and inward currents.

In this study, data show that TLR4 on sensory neurons enhances histamine-induced itch signal transduction by potentiating TRPV1 activity. The results suggest that TLR4 could be a novel target for the treatment of enhanced itch sensation.

Introduction

Chronic itch is a pathological hallmark of atopic dermatitis and other infectious skin diseases. Recent studies on single nucleotide polymorphisms implicate genes encoding pattern-recognition receptors such as CD14 and toll-like receptors (TLRs) in the development and severity of atopic dermatitis (AD) (Gern et al. 2004; Niebuhr et al. 2008). TLRs are type I transmembrane receptors expressed on innate immune cells that detect pathogen infection or tissue damage. TLR activation of innate immune cells leads to expression of inflammatory mediators including cytokines, chemokines, leukotriene molecules, and histamine (Konopka et al. 2010; Varadaradjalou et al. 2003). Some of these inflammatory mediators excite sensory neurons and may trigger transmission of itch signals. Therefore, itch sensations observed during skin diseases and infections are generally attributed to sensory neuronal activation by inflammatory mediators released from innate immune cells such as mast cells or keratinocytes (Wilson et al. 2011).

Recently, some pathogen-recognition receptors including TLR3, 4, and 7 were also found to be expressed on sensory neurons (Barajon et al. 2009). These findings imply that itch signals can also be generated if these TLRs were expressed on itch-specific sensory neurons and activated by pathogen-derived molecules. This possibility is supported by a recent study showing that TLR3 activation on a subpopulation of dorsal root ganglia

(DRG) sensory neurons elicits inward currents and action potential (Liu et al. 2012). The study found that TLR3 is required for maximal pruritic effects of histamine as well as non-histaminergic pruritogens. In another study, Liu et al. found that TLR7 stimulation by imiquimod, a synthetic TLR7 agonist, directly excites sensory neurons, leading to itch-specific signal generation (Liu et al. 2010). However, the same group later reported that TLR7-mediated sensory neuron activation by microRNA elicit pain rather than itch signals (Park et al. 2014). We have also reported the pruritogenic function of imiquimod; however, this was not mediated by TLR7 (Kim et al. 2011). Thus, the pruritogenic function of TLR7 on DRG sensory neurons is controversial.

In addition to TLR3 and 7, TLR4 is reported to be expressed in certain DRG and trigeminal ganglia sensory neurons (Wadachi and Hargreaves 2006). This finding suggests a role for this receptor in sensory transmission, but this has not been formally studied. Therefore, we investigated the function of sensory neuron TLR4 in itch signal transmission. We found that TLR4 contribute to histamine-induced itch signal transduction at least partly by potentiating TRPV1 activity.

Materials and Methods

Mice

Eight-week-old C57BL6 mice were purchased from Daehan Biolink (Eumsung, Korea). TLR4 KO mice of C57BL6 background were generously provided by Dr. S. Akira (Osaka University, Japan). Mice were housed at 23 ± 2ºC with a 12-h light-dark cycle and fed food and water *ad libitum*. All surgical or experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University.

Behavior study

Scratching behavioral assays were performed as previously described (Kim et al. 2011). Mice were placed individually in transparent cages for at least 30 minutes before assays and received TLR ligands (LTA 100 μg or poly IC 100 μg), pruritogens (SLIGRL 50 μg, 5-HT 100 nmol, chloroquine (CQ) 200 μg, histamine 100 μg, HTMT 300 nmol, imiquimod (IQ) 200 μg, or clobenprorpit 100 nmol) by intradermal injection into the rostral back. Numbers of scratching bouts that included only the hind paw were analyzed at 5-minute intervals for 30 minutes. Numbers of scratching bouts were summed and compared betwenn WT and KO mice.

Primary DRG neuron culture

DRGs collected from 8-week-old mice were incubated in Hank's balanced salt solution (HBSS) (Welgene, Daegu, Korea) containing 0.33 mg/ml papain (Wothington, Lakewood, NJ, USA) and 0.65 mg/ml L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at 37°C and then HBSS containing 4 mg/ml collagenase (Roche, Mannheim, Germany) and 5 mg/ml dispase (Invitrogen, Calrsbad, CA, USA) for 10 minutes at 37°C. Samples were washed with DMEM/F12 with 10% (v/v) fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Welgene). The cell suspension was filtered through a 700-μm cell strainer and cultured in culture dish or coverslips. Dish and coverslips used to DRG culture coated with 50 μg/ml poly-D-lysine molecular weight 30,000 ~ 70,000 in DW (Sigma-Aldrich) for O/N at RT , washed and coated with 10 μg/ml laminin (Sigma-Aldrich).

Cell culture and transfection

HEK293T cells were maintained in DMEM containing 10% FBS, 100 U/ml ampicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a humidified incubator containing 5% CO_2. One day after seeded in 60mm dish, HEK293 cells were transfected using Effectene transfection reagent (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. 3 μg of plasmid DNAs were mixed with buffer EC and 8 μl of enhancer to the total volume of 150 μl and incubate at room temperature for 5 min. After

25μl of Effectene Transfection reagent was added, mixture was incubated for 10 minute at room temperature, and added onto the HEK293T cell. 30 ~ 48 h after transfection, cells were collected to further analysis.

Plasmid

The human TRPV1 was cloned into the pEGFP-N2 EcoR1/BamHI to obtain a GFP-tagged fusion TRPV1 protein at C terminal. The human TLR4 with HA-tag was obtained by PCR with proofreading Pfu DNA polymerase and two restriction digestions BamHI/NotI. PCR products were digest with restriction enzyme and ligate with pCDNA3.1.

Single-cell RT-PCR

Single-cell RT-PCR was performed as previously described (Fang et al. 2007). Briefly, single DRG neuronal cells were collected with a glass micropipette connected to micromanipulator under a microscope and transferred to a PCR tube containing RNase-free water. Digestion with DNase I was performed before reverse transcription for 1 h at 50°C. The cDNA product was used for PCR in 50 μl of PCR buffer containing 0.2 mM dNTPs, 0.2 μM outer primers, 5 μl RT product, and 0.2 μl platinum *Taq* DNA polymerase (Invitrogen). The protocol was 5 min at 95°C followed by 60 cycles of 40 s at 95°C, 40 s at 60°C, and 40 s at 72°C. Reactions were completed with 7 min of final elongation. The second round of amplification was in reaction buffer (20 μl) containing 0.2 mM dNTPs,

0.2 μM inner primers, 5 μl first-round PCR product, and 0.1 μl platinum *Taq* DNA polymerase following the same amplification procedure as the first round. The following primers were used: TLR4 outer forward 5`- ATC TGA GCT TCA ACC CCT TG-3`; TLR4 outer reverse 5`- AAT TCC CTG AAA GGC TTG GT-3`; TLR4 inner forward 5`- TCA GAA CTT CAG TGG CTG GA-3`; TLR4 inner reverse 5`- TTG ACT TGT GGA TTT TCA CG-3`; TRPV1 outer forward 5`- CAT GCT CAT TGC TCT CAT GG-3`; TRPV1 outer reverse 5`- AAC CAG GGC AAA GTT CTT CC-3`; TRPV1 inner forward 5`- CAT GGG CGA GAC TGT CAA C-3`; TRPV1 inner reverse 5`- CTG GGT CCT CGT TGA TGA TG-3`; MAP2 outer forward 5`- GAA GAG TTC CAA GGC CCA CTT-3` MAP2 outer reverse 5`- GCC TGA AAT TTG CCT TTT CC-3`; MAP2 inner forward 5`- CCT GTG CAA TTC CAG CTC AGT-3`; MAP2 inner reverse 5`- CCC CCA TGT GGC ATG AAA TAT3`.

Immunofluorescence

For DRG staining, adult mice were anesthetized with urethane and perfused with 4% paraformaldehyde and DRGs were collected. DRGs were incubated in 4% paraformaldehyde for O/N at 4°C , cryoprotected in 30% sucrose, and frozen in OCT. DRG sections (12 μm) were obtained by cryostat and mounted on collagen-coated slides. Sections were stained with mouse anti-histamine receptor H1 (Santa Cruz, Dallas, TX, USA) or anti-TRPV1 (Santa Cruz) in blocking buffer (PBS containing 5% normal serum,

2% bovine serum albumin and 0.1% Triton X-100) for O/N at 4°C then washed with PBS for three times and incubated for 1 h with Cy3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Groove, PA, USA) in blocking buffer, and coverslipped with VectaShield medium (Vector Labs, Burlingame, CA, USA).

For immunocytochemistry, DRG neuronal cells or transfected HEK293T cells were seeded onto poly-D-lysine (PDL) and laminin-coated coverglass. Cells were fixed in 4% PFA in 0.1 M PBS (pH 7.4) for 15 min. After rinsing in 0.1 M PBS, cells were blocked with blocking buffer (0.1 M PBS containing 5% normal goat serum, 5% fetal bovine serum, 2% bovine serum albumin, and 0.1% Triton X-100) for 1 h at RT. Cells were incubated overnight at 4°C with mouse anti-NeuN (1:1000; Millipore, Billerica, MA, USA), rabbit anti-TLR4 (1:100; Santa Cruz), or mouse anti-HA (1:1000; Cell signaling, Danvers, MA, USA) antibody in blocking buffer. Cells were incubated for 1 h at RT with FITC- or Cy3-conjugated secondary antibody in blocking soln. and mounted with VectaShield medium.

For live cell staining, DRG neurons were seeded on PDL-coated coverglasses and incubated with 5 mg/ml Alexa Fluor 488-conjugated LPS from *Escherichia coli* O55:B5 (Molecular Probes, Eugene, OR, USA) for 1 h at 37°C. After rinsing in 0.1 M PBS, coverglasses were mounted with VectaShield medium. Images were captured using a confocal laser scanning microscopy (LSM7 PASCAL; Carl Zeiss, Jena, Germany).

Real-time RT-PCR

Real-time RT-PCR was performed using SYBR Green PCR Master Mix (ABI, Warrington, UK) as described previously (Cho et al. 2008). PCR was performed in duplicate in a total volume of 10 μl containing 10 pM primer, 4 μl cDNA, and 5 μl SYBR Green PCR Master Mix. The mRNA levels of each target gene were normalized to GAPDH mRNA. Fold-induction was calculated using the $2^{-\Delta\Delta CT}$ method as previously described (Livak and Schmittgen 2001). All real-time RT-PCR experiments were performed at least three times and are presented as mean ± SEM unless otherwise noted.

Primers for real-time RT-PCR were MrgprA3 forward: 5'-CGA CAA TGA CAC CCA CAA CAA-3'; MrgprA3 reverse: 5'-GGA AGC CAA GGA GCC AGA AC-3'; histamine receptor H1 forward: 5'-GGG AAA GGG AAA CAG TCA CA-3'; histamine receptor H1 reverse: 5'-ACT GTC GAT CCA CCA AGG TC-3'; histamine receptor H4 forward: 5'-GGA AGC TAG CCA GGT CAC T-3'; histamine receptor H4 reverse: 5'-CCG TTC TGG AAG TTG AA-3'; TRPV1 forward: 5'-AAG GCT CTA TGA TCG CAG GA-3'; TRPV1 reverse: 5'-CAG ATT GAG CAT GGC TTT GA-3'; GAPDH forward: 5'-AGG TCA TCC AGC TGA ACG-3'; GAPDH reverse: 5'-CAC CCT GTT GCT GTA GCC GTA T-3'.

Calcium assays

Calcium response in DRG neurons was measured by single-cell calcium imaging using Fura-2 AM (Invitrogen). Cells were plated on PDL-coated coverglasses and incubated overnight. Cells were incubated for 50 min at RT with 2 μM Fura-2 AM in HBSS containing 25 mM HEPES (pH 7.5) and washed with HBSS-HEPES twice before assays. A baseline reading was taken for 60 s before addition of histamine, chloroquine or capsaicin. After treatment, to test cell viability, 100 mM KCl was added. Intracellular calcium levels were measured by digital video microfluorometry with an intensified charge-coupled device camera (CasCade, Roper Scientific, Trenton, NJ, USA) coupled to a microscope and analyzed with MetaFluor software (Universal Imaging Corp., Downington, PA, USA). Fura-2 AM excitation wavelengths were selected by a Lambda DG-4 monochromator wavelength changer (Shutter Instrument, Novato, CA, USA).

To determine intracellular calcium levels in HEK293T cell population, cells were detached from plates 24 h after transfection and stained with 2 μM Fura-2 AM in Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5.0 mM HEPES, 10 mM glucose, pH 7.4) for 50 min at 37°C. Cells were then washed twice with Locke's solution and suspended at 1×10^6 cells/ml for assays. Intracellular calcium levels were monitored with dual excitation at 340 and 380 nm and emission at 500 nm by a spectrofluorophotometer (Shimadzu RF-5301-PC, Shimadzu,

Kyoto, Japan). The ratio of emission after 340 nm and 380 nm excitation (340 nm/380 nm) was used for index of intracellular calcium concentration ($[Ca^{++}]_i$). The net change in $[Ca^{++}]_i$ upon drug treatment (Δ ratio (340 nm/38nm)) was calculated by subtracting basal $[Ca^{++}]_i$ from the peak $[Ca^{++}]_i$ achieved after exposure to the drug.

Whole cell patch-clamp recording

Cells were plated on PDL-coated cover glasses and incubated in medium for at least 6 h. DRG neuron recordings were performed in HEPES buffer (10 mM HEPES, 150 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose, and 22 mM sucrose, pH 7.4). Under an upright microscope, whole-cell patch recordings were obtained from acute-isolated DRG neurons in voltage-clamp mode and switched current-clamp configuration for recordings with an Axopatch 700B (Molecular Devices, Sunnyvale, CA, USA). Pipette resistance ranged from 3 to 6 MΩ. The internal solution was 140 mM K-gluconate, 10 mM HEPES, 7 mM NaCl, 4 mM Mg-ATP, and 0.3 mM Na3-GTP, pH 7.4. Data were analyzed and plotted with pClamp (Molecular Devices).

Western blot assay

For western blots, cells were washed with PBS once and lysed with RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1mM Na_3VO_4, 1mM NaF and 1X cOmplete protease inhibitor cocktail (Roche).

Protein quantification of whole cell extract were performed using the Pierce^{TM} BCA protein assay kit ThermoFisher Scientific). Protein samples were mixed with 4X electrophoresis sample buffer (277.8 mM Tris pH6.8, 4.4% SDS, 10% 2-Mercaptoethanol, 44.4% glycerol, 0.02% bromophenol blue) and boiled for 5 minutes, separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in TBST (20 mM Tris, pH 7.4, 0.1% Tween 20, 150 mM NaCl), membranes were incubated with rabbit anti-histamine receptor H1 (1:500, Santa Cruz), or mouse anti-β-actin (1:5000, Sigma-Aldrich) antibodies. Proteins were detected with horseradish peroxidase conjugated secondary antibodies using West Save Gold western blot detection kit (Ab Frontier, Seoul, Korea). Signal was visualized by MicroChemi (DNR Bio-imaging Systems, Jerusalem, Israel).

Statistical analysis

All data are presented as mean value with SEM. Differences between groups were determined by one-way ANOVA with Tukey's multiple comparison test or Student's *t*-test. Differences were considered significant when p was less than 0.05.

Results

TLR4 enhances histamine and chloroquine induced itch sensation

I began my study by checking whether TLRs is involved in itch signal transmission triggered by the pruritogens. Compared with wild type mice, 5-HT and imiquimod-induced scratching behavior was slightly decreased in TLR2 KO mice (Fig. 1A). Scratching responses seen in TLR4 KO mice after 5-HT, SLIGRL, or imiquimod injection were comparable to responses in WT mice, while histamine-induced and chloroquine-induced scratching responses were severely compromised in TLR4 KO mice (Fig. 1C). There were no significant behavioral differences between WT and TLR3 KO mice after pruritogen treatment (Fig. 1B). Because TLR4 is known to be expressed on sensory neuron, I focused on function of TLR4 expressed on sensory neuron in histamine/chloroquine induced itch sensation.

TLR4 enhances both HRH1 and HRH4 activation induced itch sensation

Next, I test if activation of TLR4 on sensory neurons directly triggers itch sensation. LPS was introduced intradermally into the nape of mice and counted the number of scratching bouts for 30 min. In contrast to histamine, administration of up to 500 mg LPS did not significantly enhance

the scratch response over the basal level (Fig. 2A). These data indicated that TLR4 activation on sensory neurons did not induce an itch signal *per se*. Because previous data shows that histamine- or chloroquine induced itch compromised in TLR4 KO mice, itching sensation was tested with multiple dose of histamine or cholroquine. Compared with WT, itching behavior of TLR4 KO mice was attenuated in all conditions (Fig. 2B). It has been revealed that the pruritogenic histamine effect is mediated by two histamine receptors, HRH1 and HRH4 (Bell et al. 2004). To discern the receptor type involved in the TLR4 effects, I used the HRH1 agonist HTMT and the HRH4 agonist clobenpropit. Both HTMT-induced and clobenpropit-induced scratching responses were comparably reduced as histamine in TLR4 KO mice (Fig. 2C). These data show that TLR4 contributes to both HRH1- and HRH4-mediated pruritogenic histamine responses.

Sensory neurons express TLR4

I confirmed TLR4 expression in acutely isolated DRG neurons. Immunostaining detected TLR4 in a subpopulation (~21%) of DRG neurons of all sizes (Fig. 3A). LPS binding to neuronal TLR4 was confirmed by staining with fluorescently labeled LPS (Fig. 3B), with fluorescence signal detected in wild-type (WT) DRG neurons but not in TLR4-deficient neurons.

TLR4 expression detected in various type of sensory neuron

I then analyzed TLR4-expressing sensory neuronal cell types by single-cell RT-PCR. Similar to the immunostaining results, TLR4 transcripts

were detected in DRG neurons of all sizes (Fig. 4A). TLR4 was expressed in 15% of small neurons (<20 mm), 20% of medium neurons (20~40 mm), and 34% of large neurons (>40 mm) (Fig. 4B). In small neurons, all TLR4+ neurons also expressed TRPV1, while only 28% of TLR4+ medium neurons and 43% of TLR4+ large neurons were TRPV1-positive (Fig. 4A and B). Of TRPV1-positive neurons, 29, 20, and 67% cells were TLR4-positive in small-, medium-, and large-sized neurons, respectively. Studies so far indicate that histamine-induced itch signal is generated by small-sized TRPV1+ neurons (Shim et al. 2007). Thus, our data suggest that 15% of small DRG neurons that express both TRPV1 and TLR4 might be critical for histamine-induced itch transmission, although TLR4 expression was not restricted to itch-transmitting neurons.

Histamine- or chloroquine- induced calcium signals are reduced in TLR4 KO sensory neuron

Both histamine and chloroquine increase intracellular calcium concentration ($[Ca^{++}]_i$) in sensory neurons (Shim et al. 2007; Wilson et al. 2011). In primary cultured mouse DRG neurons, histamine treatment increased $[Ca^{++}]_i$ in 13% of cells, similar to previous results (Zhao et al. 2013). However, in DRG neurons cultured from TLR4 KO mice, less than 2% of cells responded to histamine treatment (Fig. 5A). The average of $[Ca^{++}]_i$ increase in the histamine-responsive subpopulation was also reduced by 30% in TLR4-deficient DRG neurons (Fig. 5B). The chloroquine-responding cell

population was also decreased in TLR4-deficient DRG cells (Fig. 5A), although the average $[Ca^{++}]_i$ increase was not altered (Fig. 5B).

Histamine receptor expression was not affected by TLR4 expression

To investigate the mechanisms of these results, I tested if TLR4 affected histamine receptor expression in sensory neurons. HRH1 level was not reduced, but was rather increased in TLR4 KO DRG neurons (Fig. 6A). The number of HRH1+ neuronal cells as measured by immunohistochemistry was not markedly altered (Fig. 6B). Although transcripts for HRH1 and HRH4 were not reduced in TLR4 KO DRGs (Fig. 6C), the expression of MrgprA3, a chloroquine receptor, was significantly reduced in TLR4 KO DRG neurons (Fig. 6D). These data suggested that the decrease in histamine-induced calcium signals was not due to a reduction in histamine receptor expression levels, although the reduced MrgprA3 expression might contribute to the decreased chloroquine-induced calcium response in TLR4-deficient sensory neurons.

Capsaicin induced calcium signal and inward current were reduced in TLR4 KO sensory neuron

Histamine-induced $[Ca^{++}]_i$ increase and action potentials in sensory neurons are mediated by TRPV1 channels (Shim et al. 2007). Since no difference was detected in histamine receptor expression between WT and

TLR4 KO DRG neurons, I tested the possibility that TLR4 affected histamine responsiveness by regulating TRPV1 activity. By measuring capsaicin-induced intracellular calcium signals in sensory neurons (Fig. 7A), I found that capsaicin treatment triggered a $[Ca^{++}]_i$ increase in 35% of WT sensory neurons. The percentage of capsaicin-responding neurons was significantly reduced in TLR4-deficient neurons. The average $[Ca^{++}]_i$ increase in the capsaicin-responding population was also reduced in the TLR4-deficient neurons compared with WT neurons (Fig. 7B). I then measured inward currents in capsaicin-treated sensory neurons by whole-cell patch clamp recording (Fig. 7C). Similar to the results on the capsaicin-induced intracellular calcium signal, the average inward current after capsaicin stimulation was significantly reduced in TLR4 KO neurons compared with WT neurons (Fig. 7C). These data showed that TRPV1 activity was affected in TLR4 KO sensory neurons.

TRPA1 activity was not altered in TLR4 KO sensory neuron

Chloroquine induces $[Ca^{++}]_i$ increase in the sensory neurons via TRPA1 (Wilson et al. 2011). When I tested for a TRPA1-induced $[Ca^{++}]_i$ increase by treating cells with AITC, a synthetic TRPA1 agonist, neither AITC-responding cell numbers nor average $[Ca^{++}]_i$ increase in AITC-responding neurons was altered in TLR4 KO sensory neurons (Fig. 8).

TLR4 expression enhances TRPV1 activity

To investigate the mechanisms underlying the TRPV1-potentiating

effects of TLR4, I adopted heterologous expression system. Capsaicin treatment of TRPV1-overexpressing HEK293T cells increased $[Ca^{++}]_i$ (Fig. 9A). When cells were co-expressed with TRPV1 and TLR4, the net $[Ca^{++}]_i$ increase rate further enhanced (0.3 vs. 0.6) (Fig. 9B). Similarly, capsaicin-induced inward currents in TRPV1-expressing HEK293T cells were greater in cells that were also expressing TLR4 (Fig. 9C and D). These data showed that TLR4 expression increased TRPV1 activity in HEK293T cells.

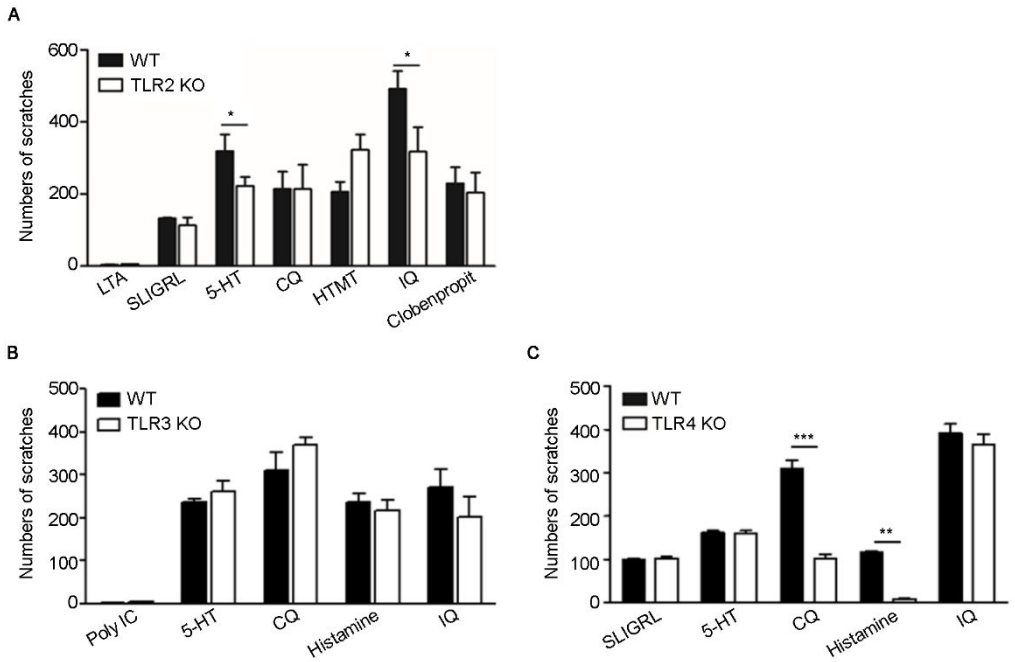
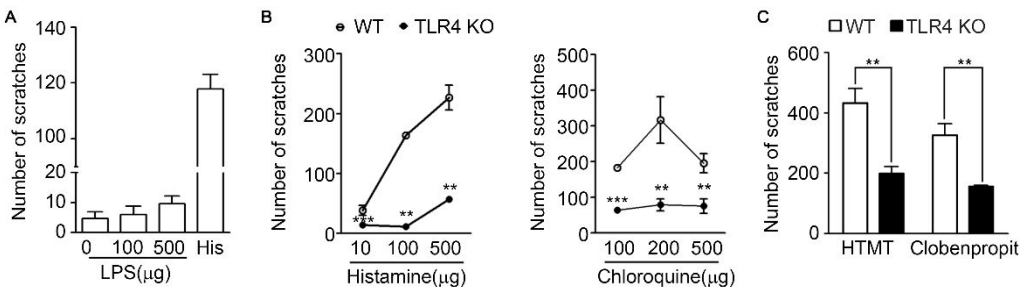

A
Numbers of scratches
600
400
200
0
WT
TLR2 KO
*
*
LTA
SLIGRL
5-HT
CQ
HTMT
IQ
Clobenpropit
B
Numbers of scratches
500
400
300
200
100
0
WT
TLR3 KO
Poly IC
5-HT
CQ
Histamine
IQ
C
Numbers of scratches
500
400
300
200
100
0
WT
TLR4 KO
***
**
SLIGRL
5-HT
CQ
Histamine
IQ

Figure 1. TLR4 enhances histamine-induced and chloroquine-induced itch sensation. **A-C**. WT, TLR2 KO, TLR3 KO or TLR4 KO mice were treated with TLR ligands (LTA 100 μg or poly IC 100 μg) or pruritogens (SLIGRL 50 μg, 5-HT 100 nmol, chloroquine (CQ) 200 μg, histamine 100 μg, HTMT 300 nmol, imiquimod (IQ) 200 μg, or clobenprorpit 100 nmol) in the nape and scratching bouts were counted for 30 min (n=6; *p<0.05, **p<0.01, *** p<0.001).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Number of scratches
140
120
100
20
10
0
0 100 500 His
LPS(μg)
B
WT TLR4 KO
Number of scratches
300
200
100
0
10 100 500
Histamine(μg)
Number of scratches
500
400
300
200
100
0
100 200 500
Chloroquine(μg)
C
WT TLR4 KO
Number of scratches
600
400
200
0
HTMT Clobenpropit

Figure 2. TLR4 enhances both HTMT and clobenpropit -induced itch sensation. **A**. LPS (100 or 500 μg) or histamine (100 μg) was introduced intradermally to WT mice under the nape. Scratching bouts were counted for 30 min (n=5). LPS at either concentration did not induce significant scratching. **B**. Histamine and chloroquine were introduced to WT and TLR4 KO mice at different doses and scratching bouts were measured (n=5; $^{**}p<0.01$, $^{***}p<0.001$). **C**. Histamine-receptor agonists HTMT (300 nmol) and clobenpropit (100 nmol), were used to treat WT and TLR4 KO mice and scratching bouts were counted (n=6; ** $p<0.01$).

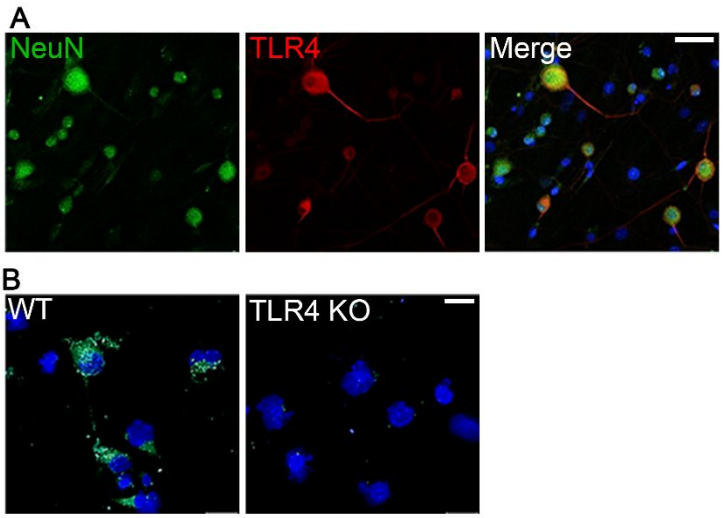
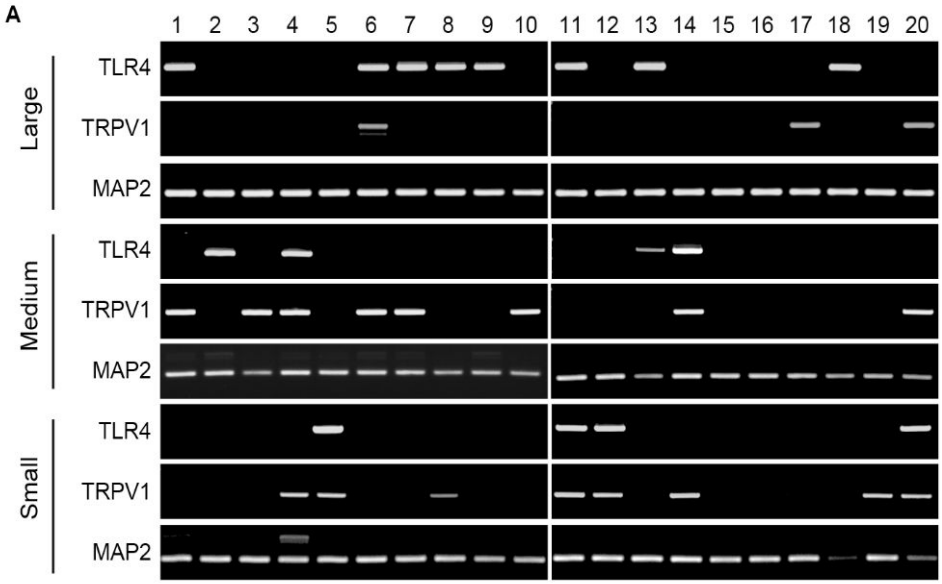

A
NeuN
TLR4
Merge
B
WT
TLR4 KO

Figure 3. TLR4 expressed on sensory neuron. **A**. Primary cultured DRG neurons were immunostained with NeuN and TLR4 antibodies. Scale bar, 50 μm. **B**. Primary sensory neurons from WT but not TLR4 KO mice were stained with Alexa488-conjugated LPS. Representative results are shown. Scale bar, 20 μm.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Large
TLR4
TRPV1
MAP2
Medium
TLR4
TRPV1
MAP2
Small
TLR4
TRPV1
MAP2

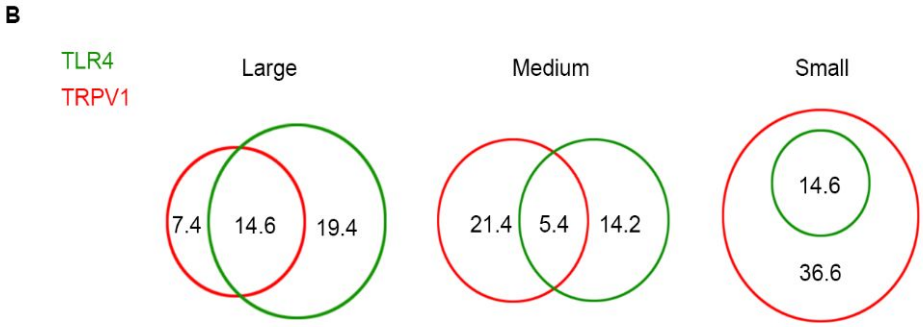

B
TLR4
TRPV1
Large
Medium
Small
7.4
14.6
19.4
21.4
5.4
14.2
14.6
36.6

Figure 4. Characterization of the TLR4-expressing sensory neurons. **A**. Expression of TLR4, TRPV1, and MAP2 measured in small (n=41), medium (n=56), and large (n=53) sensory neurons by single-cell RT-PCR. **B**. Profiles of TLR4 and TRPV1 expression in Venn diagram. The percentage of subpopulation is denoted in Venn diagram.

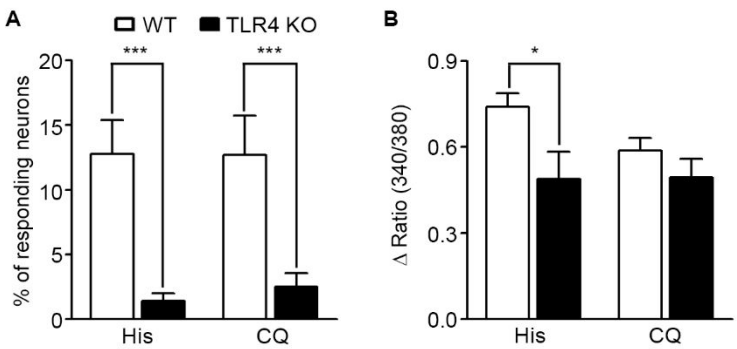

A
WT
TLR4 KO
***
***
20
15
10
5
0
% of responding neurons
His
CQ
B
*
0.9
0.6
0.3
0.0
Δ Ratio (340/380)
His
CQ

Figure 5. Histamine-induced and chloroquine-induced intracellular calcium increases were reduced in TLR4 KO neurons. **A** and **B**. DRG neurons loaded with Fura2-AM were treated with histamine (10 μg/ml) and chloroquine (CQ, 10 μg/ml) and intracellular calcium level was monitored by calcium imaging. Percentage of responding cells (**A**) and average net increase of $[Ca^{++}]_i$ (D ratio (340 nm/380 nm)) in the responding population (**B**) are in graphs (n=5, 544 cells measured; * $p<0.05$, *** $p<0.005$).

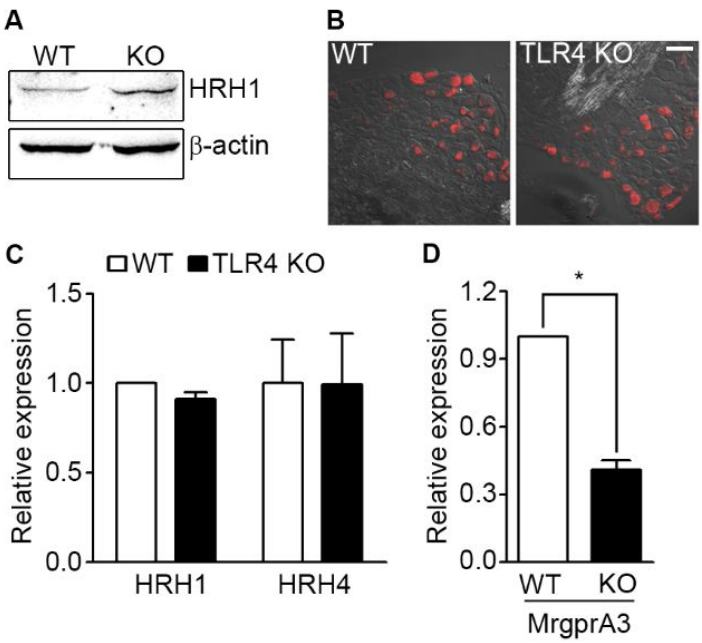

A
WT
KO
HRH1
β-actin
B
WT
TLR4 KO
C
WT
TLR4 KO
Relative expression
1.5
1.0
0.5
0.0
HRH1
HRH4
D
*
Relative expression
1.2
0.9
0.6
0.3
0.0
WT
KO
MrgprA3

Figure 6. Histamine receptor expression was not affected by TLR4. **A**. DRG neurons from WT and TLR4 KO mice were cultured and HRH1 was measured by western blot. A representative gel is shown (n=3). **B**. DRG sections from WT and TLR4 KO mice were immunostained with HRH1 antibody. Representative data are shown (n=3). Scale bar, 50 μm. HRH1-expressing neuronal percentage and size distribution were not noticeably altered in TLR4 KO DRG. **C** and **D**. The mRNA expression of histamine receptors (HRH1 and HRH4) (**C**) and MrgprA3 (**D**) were measured by real-time RT-PCR in WT and TLR4 KO sensory neurons (n=3). The MrgprA3 expression is significantly reduced in TLR4 KO neurons (*p<0.05).

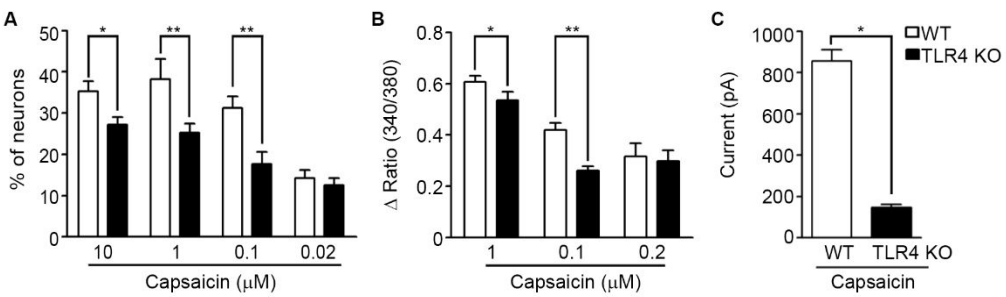

A
% of neurons
50
40
30
20
10
0
*
**
**
10
1
0.1
0.02
Capsaicin (μM)
B
Δ Ratio (340/380)
0.8
0.6
0.4
0.2
0
*
**
1
0.1
0.2
Capsaicin (μM)
C
Current (pA)
1000
800
600
400
200
0
*
WT
TLR4 KO
WT
TLR4 KO
Capsaicin

Figure 7. Capsaicin-induced intracellular calcium signals and inward currents were compromised in TLR4 KO sensory neurons. **A** and **B**. DRG neurons from WT and TLR4 KO mice were cultured and treated with capsaicin (0.02-10 μM), and intracellular calcium was monitored by calcium imaging assays. Percentage of responding cells (**A**) and average net increase of $[Ca^{++}]_i$ (Δ ratio (340 nm/380 nm)) in the responding population (**B**) are shown in graphs (n=5 with 791, 232, 335, and 215 cells measured; *p<0.05, ; **p<0.01). Numbers of capsaicin-responding cells and average calcium $[Ca^{++}]_i$ increase were compromised in TLR4 KO neurons. **C**. Capsaicin-induced inward currents measured by whole cell patch-clamp recording. Average inward current amplitude in capsaicin-responding TLR4 KO neurons (n=43) was lower than in WT neurons (n=39; *p<0.005).

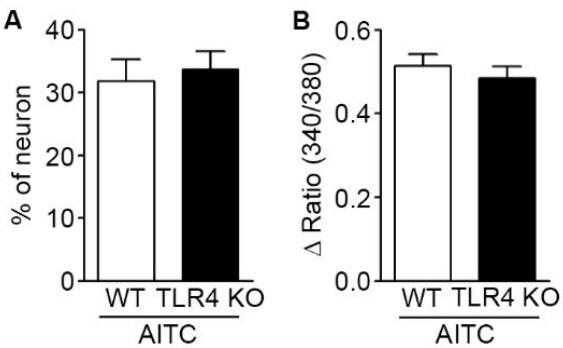

A
% of neuron
40
30
20
10
0
WT TLR4 KO
AITC
B
Δ Ratio (340/380)
0.6
0.4
0.2
0.0
WT TLR4 KO
AITC

Figure 8. TRPA1-mediated calcium signals were not altered in TLR4 KO neurons. DRG neurons from WT and TLR4 KO mice were cultured and treated with 1 μM AITC, and intracellular calcium was monitored by calcium imaging assays. Percentage of responding cells (**A**) and average net increase of $[Ca^{++}]_i$ (D ratio (340 nm/380 nm)) in the responding population (**B**) are shown in graphs (n=5).

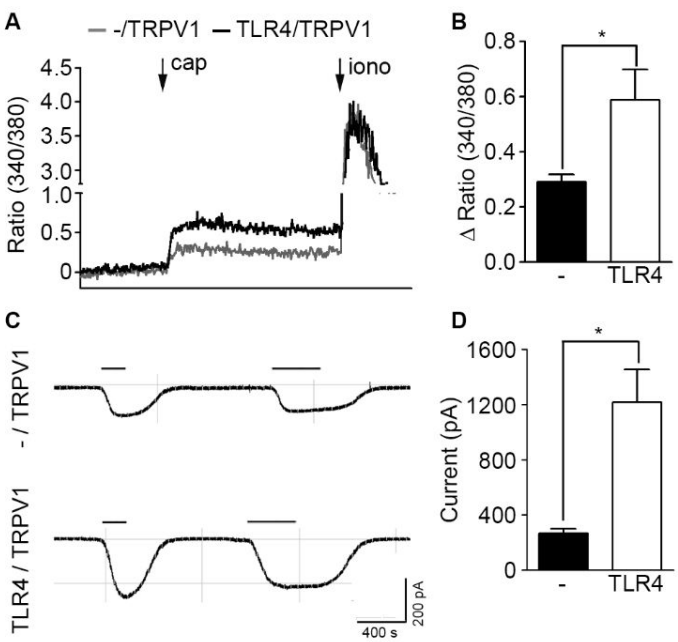

A
-/TRPV1
TLR4/TRPV1
cap
iono
Ratio (340/380)
4.5
4.0
3.5
3.0
1.0
0.5
0
B
Δ Ratio (340/380)
0.8
0.6
0.4
0.2
0.0
*
-
TLR4
C
- / TRPV1
TLR4 / TRPV1
200 pA
400 s
D
Current (pA)
1600
1200
800
400
0
*
-
TLR4

Figure 9. TLR4 expression enhances TRPV1 activity in HEK293T cells. **A**. HEK293 cells transiently overexpressing TRPV1 or TRPV1 plus TLR4 were loaded with Fura2-AM. Cells were treated with capsaicin (10 μM) followed by ionomycin (0.3 μg/ml) and intracellular calcium level was measured by population assay. Representative traces are shown. **B**. Average net increase of $[Ca^{++}]_i$ (Δ ratio (340 nm/380 nm)) is shown in a graph (n=6). **C**. Whole cell patch clamp recording was performed using HEK293 cells transiently overexpressing TRPV1 or TRPV1 plus TLR4. Representative traces of capsaicin-induce inward currents are shown. **D**. The average amplitude of the capsaicin-induced inward current in TRPV1-alone or TRPV1-plus-TLR4-expressing HEK293 cells (n=18; $*p<0.005$). The inward current in TLR4-expressing cells is six times the current of TLR4-deficient cells.

Discussion

The recent discovery of TLRs on DRG sensory neurons suggests that these pattern-recognition receptors may function in transduction or transmission of sensory signals. Studies thus far implicated TLR3 and TLR7 in itch signal transmission. In this study, I have investigated the roles of TLR4 in sensory transmission and found that TLR4 is required for optimal histamine-induced itch signal transduction through regulating TRPV1 activity. Such role of TLR4 in itch signal transduction is distinct from other TLR members. The activation of TLR3 by poly(IC) treatment directly excited sensory neurons and induced a scratch response (Liu et al. 2012). However, TLR4 stimulation by LPS did not directly excite sensory neurons or induce significant scratch response. These data suggest that, unlike TLR3, TLR4 signaling alone in sensory neurons does not elicit itch signal. Instead, histamine-induced and chloroquine-induced itch responses were severely compromised in TLR4 KO mice, suggesting TLR4 is required for optimal itch signal transmission stimulated by these pruritogens. Although a reduction in histamine-induced and chloroquine-induced itch responses were also observed in TLR3 KO mice, the mechanisms are different (Liu et al. 2012). In our study, in the absence of TLR4, histamine-induced and chloroquine-induced calcium signals in sensory neurons are severely compromised. These data suggest that the itch phenotype I observed in TLR4 KO mice came from itch signal transduction in sensory neurons. However, TLR3 is not required for pruritogen-induced itch signal

transduction in the sensory neurons. Rather, TLR3 contributes to synaptic transmission of the itch signal and central sensitization at the spinal cord level. In addition, TLR3 is expressed mainly in small-sized TRPV1+ neurons. However, TLR4 expression was not limited to small-sized neurons but was also detected in large-sized neurons. TLR4 expression was not limited to TRPV1+ neuron population either. These data suggest that, unlike TLR3, TLR4 expression is not limited to itch-specific sensory neurons. Nonetheless, the subpopulation of TRPV1+ neurons that co-express TLR4 is likely to mainly contribute to histamine-induced itch signal transduction.

In my study, TLR4 regulates histamine-induced and chloroquine-induced itch by distinct mechanisms. TLR4 potentiates histamine responsiveness by increasing TRPV1 basal activity. However, TLR4 does not affect TRPA1 activity. Instead, TLR4 expression increased MrgprA3 expression. Crosstalk between TLR4 and TRPV1 signals has been documented (Diogenes et al. 2011). In this study, TLR4 activation increased capsaicin-induced intracellular calcium signals and inward currents. However, we did not observe significant differences in calcium signals after LPS treatment. Our results are consistent with a report that ultrapure LPS, in contrast to standard-grade LPS, fails to induce neuronal excitability (Ochoa-Cortes et al. 2010). Instead, our data on decreased TRPV1 activity in TLR4 KO neurons indicate that TLR4 expression potentiates TRPV1 activity. It is not clear how TLR4 enhances TRPV1 activity. A recent study shows that TLR7 can enhance TRPA1 activity by direct physical interaction. This

suggests a possibility that TLR4 may also enhance TRPV1 activity by direct interaction, which needs to be tested in the future investigation.

TRPV1 is not only associated with itch signals but also involved in pain signals. In our study, we observed capsaicin-induced paw licking was also slightly reduced in TLR4 KO mice (data not shown). This result suggests that the effect of neuronal TLR4 might not be specific to itch signal, but might be involved in pain transmission as well. Of note, nerve-injury induced mechanical allodynia and hyperalgesia are severely compromised in TLR4 KO mice (Tanga et al. 2005). In this study, TLR4 involvement was interpreted mainly from the perspective of spinal cord microglia activation. Considering our data, it needs to be re-evaluated the putative contribution of sensory neuron TLR4 to the neuropathic pain development.

Itch susceptibility to pruritogens or bacterial/viral infections depends on genetic background. For instance, polymorphisms in genes for the TLR family and associated receptors are implicated in itch-associated diseases such as AD (Niebuhr et al. 2008). Our study found a putative mechanism how TLR4 expression affects itch signal transduction. In addition to polymorphisms, TLR4 expression is regulated in diseases such as bacterial infection and tissue damage. In innate immune cells, TLR4 is upregulated by various inflammatory mediators such as TNF-α and IL-1β. Whether sensory neuron TLR4 is also upregulated by these inflammatory mediators is unknown. If it is, inflammatory mediators secreted during the innate immune response in the skin might regulate itch sensitivity by

upregulating TLR4 expression in sensory neurons.

Chapter 2.

TLR4 enhances TRPV1 activity by direct association through TIR domain

Abstract

TRPV1 receptors are strongly expressed in the peripheral nervous system (PNS) including C polymodal nociceptive and Aδ nociceptive primary afferent terminals. TRPV1 in primary afferent terminals can detect noxious heat and convey painful signals to avoid potential tissue damage. Moreover, several studies indicated that TRPV1 activation is critically required in histamine induced itch sensation. In previous study, I revealed that histamine-induced itch was attenuated in TLR4 KO mice, which was due to decreased TRPV1 activity. However, underlying mechanism is still unknown, thus, I investigated the mechanisms of TLR4-mediated TRPV1 potentiation.

TRPV1 expression was not altered in TLR4 KO DRG sensory neuron. However, TLR4 expression enhance TRPV1 activity by direct association between TRPV1 and TIR on TLR4. Compared with full length TLR4 and TRPV1 overexpressed HEK 293T, TIR-deletion TLR4 mutant and TRPV1 co-expressed HEK 293T cell showed decreased protein interaction and reduced capsaicin induced calcium signaling. On the other hand, TLR4 expression reduced activation-induced downregulation of TRPV1 expression. In consequence of decreased TRPV1 expression on cell surface, capsaicin-induced desensitization was enhanced in TLR4 KO sensory neuron.

In summary, data show that direct association between TRPV1 and

TLR4 through TIR domain enhances TRPV1 activity and TLR4 expression blocks capsaicin induced desensitization by regulate TRPV1 trafficking to membrane.

Introduction

Transient receptor potential vanilloid subtype 1 (TRPV1) has been widely studied in pain mechanisms. It is expressed on subsets of nociceptive neurons and is activated by various stimuli that are perceived as painful or noxious, including capsaicin, as well as by protons, heat 42°C, and a family of endogenous-inflammation-associated lipids. Like many other channels and receptors, TRPV1 is desensitized upon continuous activation or in response to repeated exposures to capsaicin. Although the exact mechanisms for sensitization/desensitization of TRPV1 are not fully understood, it has been suggested that channel activity is regulated by phosphorylation/dephosphorylation balance and membrane trafficking. TRPV1 sensitization is often detected under inflammatory conditions, and is usually mediated by inflammatory mediators. For example, in DRG neurons prostaglandin E2 (PGE2) activates Gαs-coupled receptor to increase adenylyl cyclase production of cAMP and the subsequent activation of the cAMP-dependent kinase, PKA. PKA directly phosphorylates TRPV1 on Ser116, blocking TRPV1 desensitization and enhancing the TRPV1 response to capsaicin and other activators. Besides, recent studies revealed that LPS stimulus sensitize TRPV1 through TLR4 activation. TLR4 and TRPV1 were co-expressed in human trigeminal neurons and rat DRG sensory neurons, and moreover, TLR4 activation by pacilitaxel and LPS results TRPV1 sensitization (Diogenes et al. 2011; Li et al. 2015).

It is widely accepted that TRPV1 plays important role in pruritus,

especially in histamine induced itch sensation (Shim et al. 2007). Histamine receptors are members of the G-protein coupled receptor family, and four receptors have been discovered (Bell et al. 2004). H1R1 is a major receptor involved in the itching sensation and this receptor exerts its function through activation of TRPV1 (Kim et al. 2004). The relationship between histamine and TRPV1 is also supported by the finding that histamine-induced intracellular calcium increase was attenuated by TRPV1 antagonists in rat dorsal root ganglion (DRG) neurons (Kim et al. 2004). Mice lacking TRPV1 were impaired in scratching behavior in response to histamine injection (Shim et al. 2007). In HEK293 cells, histamine evoked inward currents only when TRPV1 and H1R were co-expressed, and not when either receptor was expressed alone (Shim et al. 2007).

In previous study, I revealed that TLR4 enhances histamine-induced pruritus through potentiating TRPV1 activity, of which the underlying mechanism is still unknown. In this study I postulated TLR4 enhances TRPV1 activity through direct protein-protein association.

Materials and Methods

Mice

Eight-week-old C57BL6 mice were purchased from Daehan Biolink (Eumsung, Korea). TLR4 KO mice of C57BL6 background were generously provided by Dr. S. Akira (Osaka University, Japan). Mice were housed at 23 ± 2ºC with a 12-h light-dark cycle and fed food and water *ad libitum*. All surgical or experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University.

Behavior study

Mice were placed in plastic cylinders on a room temperature glass surface and allowed to acclimate for approximately one hour before capsaicin application. Capsaicin was prepared as a 1.6μg solution in ethanol and was applied topically to one hind paw using a 31 G insulin syringe. Mice were observed for 5 min after capsaicin application and total duration of hind paw licking or biting was measured.

Primary DRG neuron culture

DRGs collected from 8-week-old mice were incubated in Hank's

balanced salt solution (HBSS) (Welgene, Daegu, Korea) containing 0.33 mg/ml papain (Wothington, Lakewood, NJ, USA) and 0.65 mg/ml L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at 37°C and then HBSS containing 4 mg/ml collagenase (Roche, Mannheim, Germany) and 5 mg/ml dispase (Invitrogen, Calrsbad, CA, USA) for 10 minutes at 37°C. Samples were washed with DMEM/F12 with 10% (v/v) fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Welgene). The cell suspension was filtered through a 700-μm cell strainer and cultured in poly-D-lysine (Sigma-Aldrich) and laminin-coated culture dish or glass coverslips (Sigma-Aldrich).

Cell culture and transfection

HEK293T cells were maintained in DMEM containing 10% FBS, 100 U/ml ampicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a humidified incubator containing 5% CO_2. HEK293 cells were transfected using Effectene transfection reagent (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. 3 μg of plasmid DNAs were mixed with buffer EC and 8 μl of enhancer to the the total volume of 150 μl and incubate at room temperature for 5 min. After 25μl of Effectene Transfection reagent was added, mixture was incubated for 10 minute at room temperature, and added onto the HEK293T cell. 30 ~ 48 h after transfection, cells were collected to further analysis.

Plasmid

The human TRPV1 was cloned into the pEGFP-N2 EcoR1/BamHI to obtain a GFP-tagged TRPV1 protein at C terminal. The human TLR4 with HA-tag was obtained by PCR with proofreading Pfu DNA polymerase and two restriction digestions BamHI/NotI. PCR products were digest with restriction enzyme and ligate with pCDNA3.1.

The human TLR4 deletion mutant protein with HA-tag was obtained by PCR with proofreading Pfu DNA polymerase and two restriction digestions, BamHI/NotI. PCR products were digest with restriction enzyme and ligate with pCDNA3.1. Designed deletions were verified by restriction digestion and sequencing.

Immunofluorescence

For DRG staining, adult mice were anesthetized with urethane and perfused with 4% paraformaldehyde and DRGs were collected. DRGs were incubated in 4% paraformaldehyde, cryoprotected in 30% sucrose, and frozen in OCT. DRG sections (12 μm) were obtained by cryostat and mounted on collagen-coated slides. Sections were stained with mouse anti-TRPV1 (Santa Cruz; Dallas, TX, US) in blocking soln. for O/N at 4 ºC then incubated for 1 h with Cy3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Groove, PA, USA), and coverslipped with VectaShield medium (Vector Labs, Burlingame, CA, USA).

For immunocytochemistry, transfected HEK293T cells were seeded onto poly-D-lysine (PDL)-coated coverglass. Cells were fixed in 4% PFA in 0.1 M PBS (pH 7.4) for 15 min. After rinsing in 0.1 M PBS, cells were blocked with 0.1 M PBS containing 5% normal goat serum, 5% fetal bovine serum, 2% bovine serum albumin, and 0.1% Triton X-100 for 1 h at RT. Cells were incubated overnight at 4°C with anti-HA (1:1000; Cell signaling, Danvers, MA, USA) antibody. Cells were incubated for 1 h at RT with Cy3-conjugated secondary antibody and mounted with VectaShield medium.

Calcium assays

Calcium response in DRG neurons was measured by single-cell calcium imaging using Fura-2 AM (Invitrogen). Cells were plated on PDL-coated coverglasses and incubated overnight. Cells were incubated for 50 min at RT with 2 μM Fura-2 AM in HBSS containing 25 mM HEPES (pH 7.5) and washed with HBSS-HEPES twice before assays. A baseline reading was taken for 60 s before addition of histamine, chloroquine or capsaicin. After treatment, to test cell viability, 100 mM KCl was added. Intracellular calcium levels were measured by digital video microfluorometry with an intensified charge-coupled device camera (CasCade, Roper Scientific, Trenton, NJ, USA) coupled to a microscope and analyzed with MetaFluor software (Universal Imaging Corp., Downington, PA, USA). Fura-2 AM excitation wavelengths were selected by a Lambda DG-4 monochromator wavelength changer (Shutter Instrument, Novato, CA,

USA).

To determine intracellular calcium levels in HEK293T cell population, cells were detached from plates 24 h after transfection and stained with 2 μM Fura-2 AM in Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5.0 mM HEPES, 10 mM glucose, pH 7.4) for 50 min at 37°C. Cells were then washed twice with Locke's solution and suspended at 1×10^6 cells/ml for assays. Intracellular calcium levels were monitored with dual excitation at 340 and 380 nm and emission at 500 nm by a spectrofluorophotometer (Shimadzu RF-5301-PC, Shimadzu, Kyoto, Japan). The ratio of emission after 340 nm and 380 nm excitation (340 nm/380 nm) was used for index of intracellular calcium concentration ($[Ca^{++}]_{i.}$). The net change in $[Ca^{++}]_i$ upon drug treatment (Δ ratio (340 nm/38nm)) was calculated by subtracting basal $[Ca^{++}]_i$ from the peak $[Ca^{++}]_i$ achieved after exposure to the drug.

Immunoprecipitation

For immunoprecipitation, cells were washed with PBS once and lysed with RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1mM Na_3VO_4, 1mM NaF and 1X cOmplete protease inhibitor cocktail (Roche). Protein quantification of whole cell extract were performed using the Pierce™ BCA protein assay kit ThermoFisher Scientific). Preclear the lysates containing 200 ~ 300 μg of protein with 15 μl of Protein A/G Plus-Agarose (Santa Cruz) at for 30 minute 4°C. After

preclearing, lysates were incubated with 2 μg of anti-GFP antibody (Roche) and incubated for 1 hour at 4°C, and then 20 μl of Protein A/G Plus-Agarose was added and lysates were incubated on rotating devices for O/N at 4°C. Next day, each Protein A/G Plus-Agarose pellets were washed with RIPA buffer containing protease inhibitor cocktail for three times and then 2X electrophoresis sample buffer was added and boiled to be analyzed by western blot.

Western blot assay

For western blots, cells were washed with PBS once and lysed with RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1mM Na_3VO_4, 1mM NaF and 1X cOmplete protease inhibitor cocktail (Roche). Protein quantification of whole cell extract were performed using the Pierce™ BCA protein assay kit ThermoFisher Scientific). Protein samples were mixed with 4X electrophoresis sample buffer (277.8 mM Tris pH6.8, 4.4% SDS, 10% 2-Mercaptoethanol, 44.4% glycerol, 0.02% bromophenol blue) and boiled for 5 minutes, separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in TBST (20 mM Tris, pH 7.4, 0.1% Tween 20, 150 mM NaCl), membranes were incubated with rabbit anti-TRPV1 (1:500, Santa Cruz), anti-phospho-serine (1:1000, Santa Cruz), anti-α-tubulin (1:5000, Sigma-Aldrich) or mouse anti-β-actin (1:5000, Sigma-Aldrich) antibodies. Proteins were detected with horseradish peroxidase conjugated secondary antibodies

using West Save Gold western blot detection kit (Ab Frontier, Seoul, Korea). Signal was visualized by MicroChemi (DNR Bio-imaging Systems, Jerusalem, Israel).

Cell-surface biotinylation assay

Surface biotinylation was performed on HEK 293 cells and acutely dissociated DRG neurons with Pierce™ Cell surface protein isolation kit (ThermoFisher Scientific) following manufacturer protocols. Cells were biotinylated with 500 μg/ml EZlink sulfo-NHS-LC-Biotin (Thermo, Scientific,Waltham, Massachusetts, USA) in PBS+/+ (PBS containing Ca2+ and Mg2+) solution at 4 °C for 30 min. Unreacted biotin was quenched using PBS+/+ solution that contained 0.1Mglycine for 15min. Then, cells were lysed in RIPA buffer (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, 0.1% SDS, pH 7.4, and a proteinase inhibitor mixture). A 10% volume of the lysate was saved for the determination of total protein, and the remainder was incubated with NeutrAvidin plus Ultralink beads (Thermo, Scientific, Waltham, Massachusetts, USA) overnight at 4 °C. After three washes with RIPA buffer, bound proteins were eluted with 5 × SDS loading buffer by boiling for 5 min and were analyzed by Western blot with an anti-TRPV1 antibody.

Statistical analysis

All data are presented as mean value with SEM. Differences

between groups were determined by one-way ANOVA with Tukey's multiple comparison test or Student's *t*-test. Differences were considered significant when p was less than 0.05.

Results

TRPV1 expression on TLR4 KO sensory neurons was similar to WT sensory neurons

In previous data, I revealed that TRPV1 activity was compromised in TLR4 deficient sensory neuron. However, the molecular mechanism how TLR4 expression regulates TRPV1 activity is still uncovered. I hypothesized that this finding might be due to the decreased expression of TRPV1 in TLR4 sensory neuron. To address this, TRPV1 expression was compared between WT and TLR4 deficient sensory neurons. Meanwhile, neither $TRPV1^+$ cell number nor distribution of $TRPV1^+$ neurons based on cell size was noticeably different in DRGs from TLR4 KO mice when compared to WT mice (Figs. 1A, B). Next, I tested if the expression of TLR4 affects TRPV1 expression on the sensory neuronal cytoplasmic membrane. By measuring the level of biotinylated-TRPV1 proteins at the cell membrane, the amount of membrane-expressing TRPV1 in the TLR4 KO DRG neurons was comparable to those of WT DRG neurons (Fig. 1C). Overall, these data suggested that TLR4 does not affect TRPV1 expression or membrane targeting, but rather affects TRPV1 activity in sensory neurons.

Direct association between TLR4 and TRPV1 protein

Neither TRPV1 expression nor membrane targeting were affected by TLR4 expression, therefore, I hypothesize that TLR4 modulate TRPV1

activity through direct protein interaction between TLR4 and TRPV1. To investigate this hypothesis, I adopted a heterologous overexpression system. In the HEK293T cells co-expressing TRPV1 and TLR4, the subcellular immunofluorescence intensity of TRPV1 and TLR4 was determined under confocal microscopy. Therefore, each signal matched exactly, which suggest colocalization of these two proteins (Fig. 2A). To confirm whether colocalization of these two proteins was due to direct protein interaction, immunoprecipitation was performed with lysate from TLR4/TRPV1 co-expressing cells. In these cells, immunoprecipitation of TRPV1 also pulled down TLR4 protein (Fig. 2B). These data suggest that TLR4 is physically associated with TRPV1 at the basal level.

TLR4 associates with TRPV1 through TIR domain

Next, to identify TRPV1 binding domain on TLR4, two types of truncation mutants of TLR4 were generated (Fig. 3A). After transfection to HEK293T cells with TRPV1 and each type of TLR4 mutants, TRPV1 was immunoprecipitated and western blot was performed whether each TLR4 co-immunoprecipitation assay was performed. Interestingly, compared with full length TLR4, mutant TLR4 without TIR domain (1-661) shows significantly decreased physical interaction with TRPV1. However, protein interaction between TRPV1 and TLR4 mutant with TIR domain (1-820) was comparable with that of TRPV1 and full length TLR4 (Fig. 3B). This data shows that TLR4 interacts with TRPV1 through the TIR domain.

Furthermore, to investigate whether physical interaction between TLR4 and TRPV1 affect to TRPV1 activation, capsaicin-induced intracellular calcium signal was measured in TRPV1/TLR4 or TRPV1/TLR4 mutant (1-661) co-expressing cells. Intracellular calcium concentration after capsaicin treatment to the TRPV1/TLR4-overexpression in HEK293T cells increased up to 1.0 (Figs. 3C, D). However, the Δratio of the TRPV1/TLR4 mutant (1-661)-overexpression cell, which was shown decreased protein interaction, was significantly decreased decreased to 0.6 (Fig. 3C, D).

Interaction between TLR4 and TRPV1 does not alter serine residue phosphorylation of TRPV1

TRPV1 undergoes a variety of protein modification, which affects protein function. Especially, serine residue phosphorylation on TRPV1 by PKA or PKC activities causes significant reduction of TRPV1 activity (Bhave and Gereau 2004). To test if TRPV1 phosphorylation is regulated through TLR4 expression, serine residue phosphorylation on TRPV1 was compared by western blot. However, serine phosphorylation of TRPV1 in TLR4 co-expression HEK 293T cells was comparable to TRPV1 overexpression HEK 293T cells (Fig. 4). This data suggest that TLR4 expression does not affect the total serine residue phosphorylation on TRPV1.

TRPV1 on TLR4 deficient sensory neuron undergoes excessive desensitization

TRPV1 activation induced by capsaicin stimuli is followed by nociceptor desensitization (Olah et al. 2001). TRPV1 desensitization is a process that depends on Ca^{2+} (Koplas et al. 1997) and other intracellular signaling pathways, which include phosphorylation or endocytosis of a receptor (Olah et al. 2001). I hypothesized that the lack of TLR4 expression in sensory neuron causes excess TRPV1 desensitization, which leads to reduced channel activity. To confirm this, capsaicin induced desensitization was compared with WT or TLR4 KO sensory neurons. Each sensory neuron was treated twice with 0.1 μM capsaicin, and intracellular Ca^{2+} increase was measured after each stimulus. To compare capsaicin induced TRPV1 desensitization of WT and TLR4 KO sensory neurons, the ΔRatio of the second peaks relative to the first peaks were calculated. Increase of intracellular calcium induced by the second application of capsaicin relative to the first application was far more reduced in TLR4 KO sensory neuron than in WT sensory neuron (Fig. 5).

TLR4 expression decreases capsaicin induced TRPV1 downregulation

TRPV1 activity is critically regulated through expression on the cell surface. Therefore, I hypothesized that attenuation in capsaicin-induced intracellular calcium increases after capsaicin treatment in TLR4 KO sensory neurons, which was due to the reduction of TRPV1 membrane expression. To prove this, TRPV1 expression on the cell membrane after

capsaicin pretreatment were confirmed with TRPV1-overexpressing HEK 293T cells and TLR4/TRPV1-co-overexpressing HEK 293T cells. In vehicle treatment cells, TRPV1 expression was detected in both cell membrane and cytosol. However, after capsacin treatment, TRPV1 expression on the cell surface was hardly detected on the cell surface of TRPV1-overexpression cells while TRPV1 expression on the membrane of TLR4/TRPV1 co-expressing cells was still strongly detected (Fig. 6).

Moreover, TRPV1 expression on cell surface was also confirmed in WT and TLR4 KO sensory neuron. After capsaicin treatment, TRPV1 expression on sensory neuron surface were decreased in both WT and TLR4 KO, however, compared with WT, TRPV1 expression on TLR4 KO sensory neuron surface was far more decreased . TRPV1 expression in WCE were also decreased in both WT and TLR4 KO neuron. TRPV1 expression in TLR4 KO sensory neuron was almost disappeared while TRPV1 expression in WT sensory neuron was still detectable (Fig. 7).

Capsaicin-induced acute pain attenuated in TLR4 deficient mice

TRPV1 plays a key role in the detection of a large array of noxious stimuli and is an essential component of the cellular signaling mechanisms in thermal hyperalgesia and pain hypersensitivity (Basbaum et al. 2009; Caterina et al. 2000; Caterina et al. 1997). As a capsaicin receptor, TRPV1 activation by capsaicin stimuli leads to nociceptive neurons and induces

pain sensation. Therefore, it is also possible that capsaicin induced pain sensation is attenuated in TLR4 deficient mice due to decreased TRPV1 activity. To confirm this, capsaicin induced acute pain response was compared between WT and TLR4 KO mice. Each of the mice received 1.6 μg of capsaicin on plantar skin of the hind paw and the licking or biting duration was analyzed for five minutes. However the licking/biting duration of WT mice was 20.05 seconds and the licking/biting duration of TLR4 KO mice decreased to 12.01 seconds (Fig. 8).

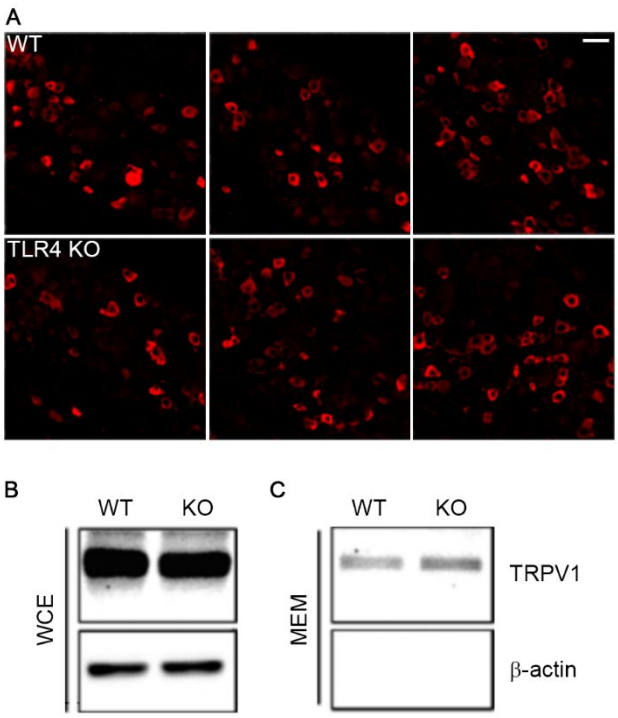
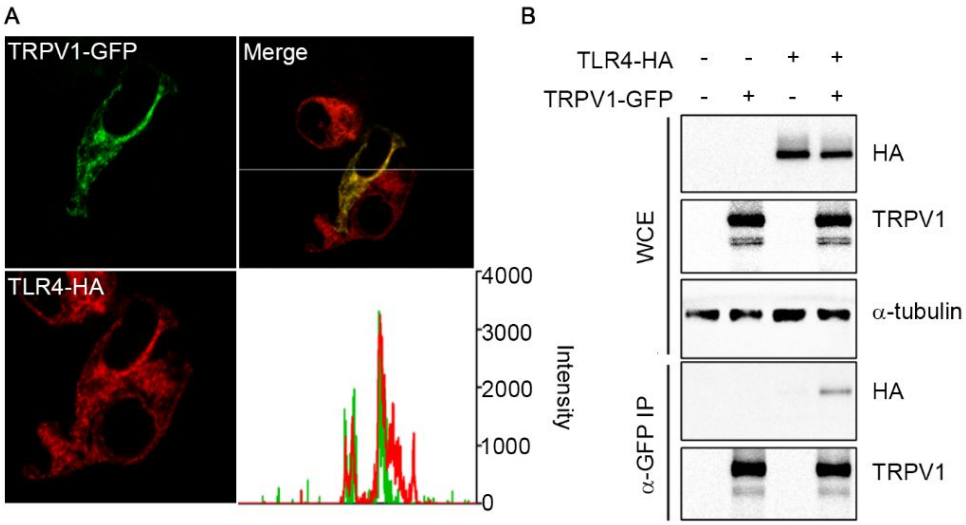

A
WT
TLR4 KO
B
WT
KO
WCE
C
WT
KO
MEM
TRPV1
β-actin

Figure 1. TRPV1 expression was not changed in TLR4 KO DRG. **A.** TRPV1 expression was measured by immunohistochemistry using WT and TLR4 KO DRG sections. Representative pictures are shown. Scale bar, 25 μm. **B.** DRG neurons from WT and TLR4 KO mice were cultured and TRPV1 was measured by western blot. A representative gel is shown (n=3). **C.** Biotinylated membrane proteins were prepared from DRG neurons from WT and TLR4 KO mice, and used for western blot assay to detect TRPV1. The amount of membrane-expressing TRPV1 in TLR4 KO DRG neurons is comparable to WT neurons.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TRPV1-GFP
Merge
TLR4-HA
4000
3000
2000
1000
0
Intensity
B
TLR4-HA - - + +
TRPV1-GFP - + - +
WCE
HA
TRPV1
α-tubulin
α-GFP IP
HA
TRPV1

Figure 2. A direct association between TLR4 and TRPV1. **A**. TRPV1-GFP and TLR4-HA were transiently overexpressed in HEK293 cells, and then staining with anti-HA antibody. The sub-cellular fluorescence intensities were analyzed under confocal microscope. The subcellular localization of TRPV1 merged with TLR4 (graph to the right). **B**. HEK293 cells were transfected with TLR4-HA, TRPV1-GFP, or TLR4-HA plus TRPV1-GFP expression vectors. Total cell extracts were immunoprecipitated with anti-GFP antibody, and then TLR4 expression was measured using anti-HA antibody. Representative gel pictures were shown (n=3).

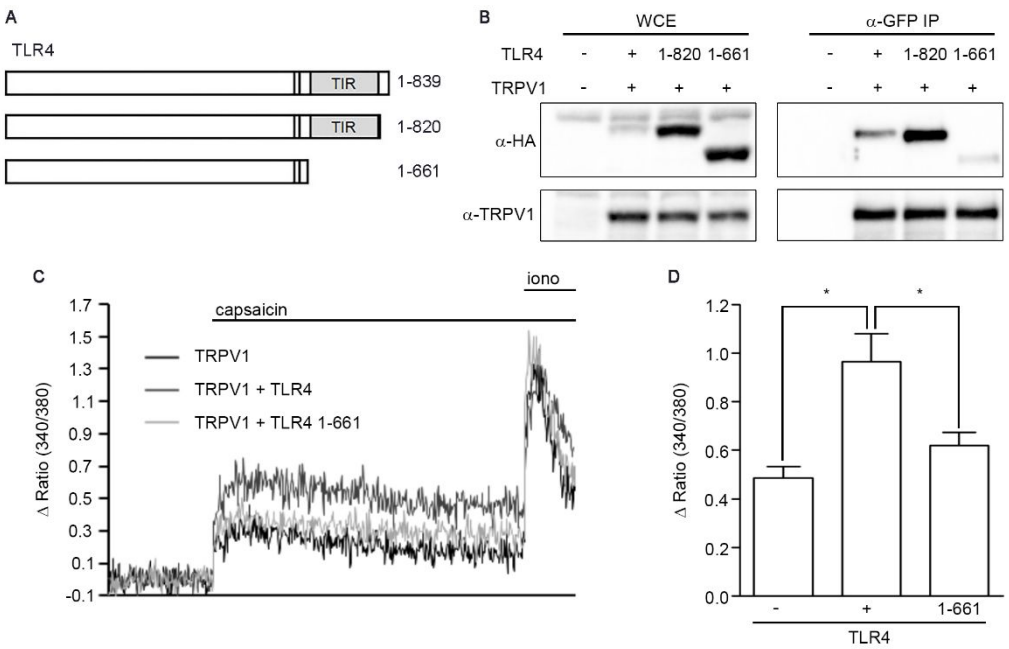
A
TLR4
TIR
1-839
TIR
1-820
1-661
B
WCE
α-GFP IP
TLR4 - + 1-820 1-661 - + 1-820 1-661
TRPV1 - + + + - + + +
α-HA
α-TRPV1
C
iono
capsaicin
TRPV1
TRPV1 + TLR4
TRPV1 + TLR4 1-661
Δ Ratio (340/380)
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
D
Δ Ratio (340/380)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
-
+
1-661
TLR4

Figure 3. TLR4 interact TRPV1 through TIR domain**. A**. Schematic diagram of the domain structure of TLR4 and the constructs used in the present study. **B.** Lysates of HEK293 cells expressing TRPV1 and TLR4 full length or truncation forms were immunoprecipitated with anti-GFP antibody. Amount of each forms of TLR4 co-immunoprecipitated with TRPV1 were measured by western blot using anti–HA antibody. **C**. HEK293T cells transiently overexpressed with TRPV1 or TRPV1 and TLR4 full length or truncated form were loaded with Fura2-AM. Cells were treated with capsaicin (10 μM) followed by ionomycin (0.3 μg/ml), and intracellular calcium level was measured by population assay. Representative traces are shown. **D**. The average increase ratio was shown in a graph (n=3, * $p<0.05$).

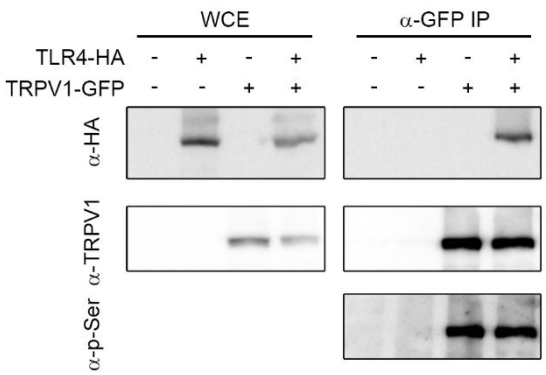
WCE
α-GFP IP
TLR4-HA - + - + - + - +
TRPV1-GFP - - + + - - + +
α-HA
α-TRPV1
α-p-Ser

Figure 4. Serine residues phosphorylation on TRPV1 was not affected by TLR4 expression. HEK293 cells were transfected with TLR4-HA, TRPV1-GFP, or TLR4-HA plus TRPV1-GFP expression vectors. Total cell extracts were immunoprecipitated with anti-GFP antibody, and then phosphorylation of serine residues on TRPV1 was measured using anti-phospho serine antibody. Representative pictures were shown.

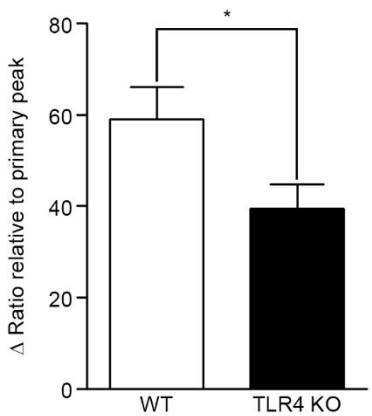
*
80
60
40
20
0
Δ Ratio relative to primary peak
WT
TLR4 KO

Figure 5. TLR4 regulates TRPV1 desensitization induced by capsaicin stimulus. TRPV1 activity was assessed via calcium imaging in sensory neuron obtained from WT or TLR4 KO mice. Following acquisition of base line, 0.1 μM of capsaicin was administered for 30 s, and cells were rinsed with normal HEPES buffer prior to second capsaicin (0.1 μM) challenge. Normalized second capsaicin response was calculated as percent of initial capsaicin response. Accumulation of Ca^{2+} was calculated from the change in $[Ca^{++}]i$ (* $p<0.05$).

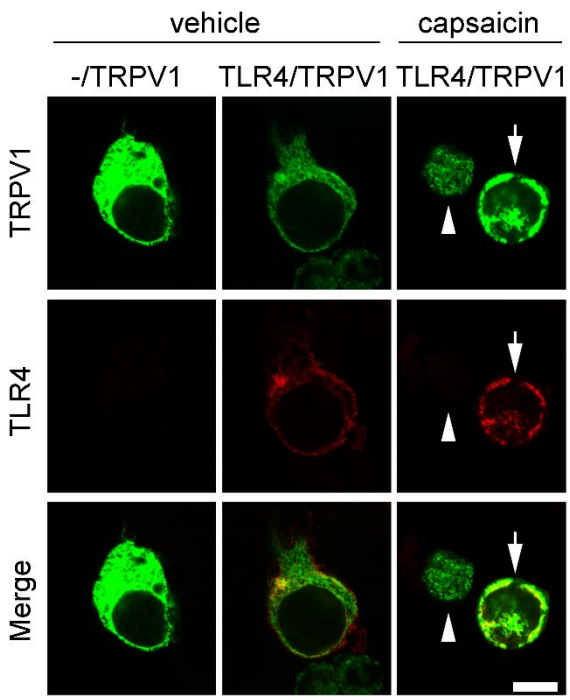
vehicle
capsaicin
-/TRPV1
TLR4/TRPV1
TLR4/TRPV1
TRPV1
TLR4
Merge

Figure 6. TLR4 expression declined TRPV1 trafficking to cytosol from membrane. HEK293T cells which transiently overexpressed TRPV1 and TLR4 were incubated with 1 μM of capsacin for 5 min. Cells were fixed with 4% paraformaldehyde and stained with anti-HA antibody to detect TLR4 expressing cells. TRPV1 expression patterns on the cells were detected under confocal microscopy Representative pictures are shown (Arrow head: $TLR4^{-}/TRPV1^{+}$ HEK293; arrow : $TLR4^{+}/TRPV1^{+}$ HEK293). Scale bar, 10 μm.

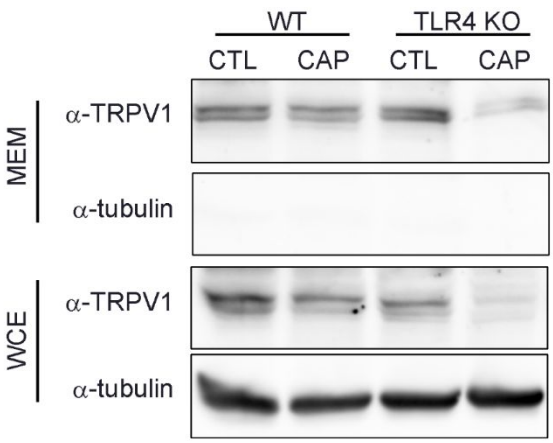
WT
TLR4 KO
CTL
CAP
CTL
CAP
MEM
α-TRPV1
α-tubulin
WCE
α-TRPV1
α-tubulin

Figure 7. Capsaicin induced TRPV1 downregulation enhanced in TLR4 KO sensory neuron. DRG neurons from WT and TLR4 KO mice were cultured and 0.1 μM of capsaicin was treated for 5 minutes. Protein expressed on sensory neuron surface was biotinylated and protein fractions of plasma membrane were purified by NeutrAvidin plus Ultralink beads. TRPV1 expressed on cell surface or WCE was measured by western blot.

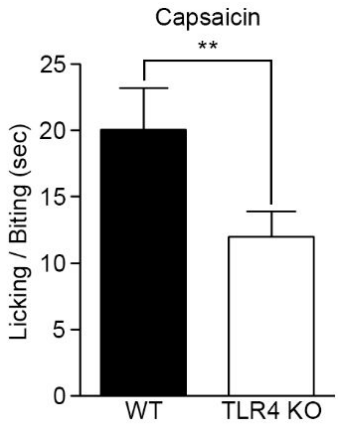
Capsaicin
**
25
20
15
10
5
0
Licking / Biting (sec)
WT
TLR4 KO

Figure 8. Capsaicin induced acute pain attenuated in TLR4 KO mice

To evaluate capsaicin induced acute pain, WT or TLR4 KO mice received 1.6 μg of capsaicin by intraplantar injection on right hind paw. Licking/biting time of right hind paw measured for 5 minutes from just after mice received capsaicin (** p<.0.01).

Discussion

In previous study I suggest that TLR4 expression on sensory neurons potentiates histamine- and chloroquine-induced itch signal transduction. As such, I sought to investigate underlying mechanism how TLR4 regulate TRPV1 activation.

TLR4 is well characterized for its role as receptor in recognition of exogenous molecules celled as PAMP and also DAMP (Akira et al. 2001). In this study I provide first evidence that TLR4 in sensory neuron functions as channel activator through direct association. I suggest a novel association between TLR4 and TRPV1 heterologous transfected HEK cell and moreover, data suggest that interaction between two proteins enhances TRPV1 activity.

In present study, data suggest that TIR domain in TLR4 serves as binding site of TRPV1. The TIR domain of TLR4 serves as homodimerization domain and binding site for other cytosolic adaptor proteins containing the TIR domain such as MyD88, Mal, TIR domain containing adapter inducing IFN-β-related adapter molecule (Akira et al. 2001). These proteins are involved in TLR-mediated regulation of NF-κB activity and immune responses. Although TRPV1 does not contain the TIR domain, immunoprecipitation data revealed that TRPV1 interact with TIR domain on TLR4. Moreover, compared with full length TLR4/TRPV1 co-expressing cell, TIR domain truncated mutant TLR4/TRPV1 overexpressing

cell shows reduced capsaicin induced intracellular calcium increase. These results show that interaction between TLR4 and TRPV1 thorough TIR domain contributes to TRPV1 sensitization.

However, it is still unclear that how TLR4 expression enhances TRPV1 activity. As shown in previous data, capsaicin induced calcium signaling and inward currents of TRPV1 over-expressing HEK293T cell were significantly increased by TLR4 expression without any exogenous stimulus. Recent studies showed that TRPV1 sensitization/desensitization are regulated by protein-protein interaction such as β-arrestin or AKAP79/150. As scaffolding protein, AKAP79/150 contains binding sites for PKA, PKC and calcineurin which are known to regulate TRPV1 activity by protein phosphorylation (Schnizler et al. 2008). Recent studies revealed that β-arrestin, initially identified for their role in G-protein-coupled receptor2 desensitization, controls TRPV1 phosphorylation status and results desensitization of channel by promote multiprotein complex formation (Por et al. 2013). To modulate TRPV1 function, direct association is critical for both AKAP 79/150 and β-arrestin, and this suggests that there is possibility that TLR4 increase TRPV1 activity through regulating binding activity of AKAP75/150 or β-arrestin to TRPV1.

Several studies revealed that TRPV1 phosphorylation mediated by PKA or PKC leads to channel sensitization (Bhave et al. 2002; Premkumar and Ahern 2000). Phosphorylation of serine or threonine residues located in N or C terminal of receptor modulate channel activity. The serine-116, 502

and 800 residues play a critical role in PKA/PKC-dependent rescuing from desensitization (Bhave et al. 2003; Bhave et al. 2002; Mohapatra and Nau 2003). Therefore I explored the possibility if TLR4 regulate TRPV1 activation through serine residue phosphorylation. As shown in data, serine phosphorylation was not altered by TLR4 expression. However, it has been suggested that not only serine residues but also threonine residues on TRPV1 are phosphorylated by PKA or PKC and results potentiating TRPV1 activity (Bhave et al. 2003; Bhave et al. 2002; Numazaki et al. 2003). Therefore, it is possible that TRPV1 activity modulation by TLR4 interaction is achieved by phosphorylation of other amino acids such as threonine.

As a transmembrane receptor, TRPV1 function at plasma membrane and potentiating channel function is critically determined by increasing its trafficking at the cell surface. TRPV1 trafficking to cell surface is a potential mechanism underlying proinflammatory mediator-induced heat hyperalgesia (Ji et al. 2002; Zhang et al. 2005). Previous study revealed that PKC activation-sensitized SNARE-dependent endocytosis and Src-dependent phosphorylation of TRPV1 are involved in inflammatory stimuli-induced rapid membrane insertion of TRPV1s from vesicular pool (Camprubi-Robles et al. 2009). In this study, although TRPV1 expression on surface was not affected by TLR4 expression in sensory neurons, TLR4 expression in sensory neuron decreases TRPV1 desensitization by reducing downregulation of TRPV1. Therefore, one of the next key question

concentrated on elucidating underlying mechanism of TRPV1 trafficking modulation caused by TLR4 expression.

Although its functions and activities are well studied in sensory neuron, it is discovered that TRPV1 expressed on not only sensory neuron but also other cells. Recent study revealed that TRPV1 expressed on helper T cell plays important role in activation and proinflammatory properties of cells (Bertin et al. 2014). During inflammatory response, intracellular calcium increase results activation and functional modulation in variety immune cells and, because of calcium permeability with TRPV1, its activation is able to modulate the immune response. As shown in data in this study to understand itch mechanism mainly focused on interaction between TLR4 and TRPV1 expressed in sensory neuron. In the aspects that TLR4 participates in many inflammatory diseases accompanied with pathological pruritus, extended function of TRPV1 regulated by TLR4 expression might cause enhanced itch sensation by increasing immune cell activation and inflammatory mediator secretion related with pruritus.

Collectively, the results in this study revealed that two distinct TRPV1 activity regulation mechanisms of TLR4. First, TLR4 direct associate with TRPV1 by TIR domain and potentiate TRPV1 activity. Second, TLR4 decrease capsaicin induced TRPV1 desensitization by downregulation of TRPV1 expression. These finding expand our knowledge about the mechanisms of TRPV1 sensitization and suggest new target for treating enhanced itch sensation.

Conclusion

In this study, I revealed that TLR4 regulates itch sensation induced by histamine or chloroquine however, the underlying mechanisms are distinct.

In chapter 1. behavior data revealed that histamine or chloroquine – induced pruritus was decreased in TLR4 KO mice. This result suggests that these two sensations regulated by TLR4 expression. Decreased chloroquine induced itch sensation was due to decreased chloroquine receptor, MrgprA3, expression. However, expression of histamine receptors, HRH1 and HRH4, was not altered in TLR4 KO sensory neuron. TLR4 expression on sensory neuron enhances histamine responsiveness by potentiating TRPV1 activity while TLR4 does not affect TRPA1 activity.

In chapter 2. I revealed the novel interaction between TRPV1 and TLR4 which results enhanced TRPV1 activity. The direct association between TRPV1 and TLR4 through TIR domain was confirmed by Immunoprecipitation. Moreover, Calcium assay revealed that the interaction between TRPV1 and TLR4 increases the channel activity of TRPV1. On the other hand, TLR4 expression also blocked capsaicin induced desensitization. After TRPV1 activation by capsaicin, TRPV1 expression in both membrane fraction and whole cell extract were far more decreased in TLR4 KO sensory neuron.

The results suggest that TLR4 could be a novel target for the

treatment of enhanced itch sensation.

References

Akira S, Takeda K, Kaisho T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2(8):675-80.

Akiyama T, Carstens E. 2013. Neural processing of itch. Neuroscience 250:697-714.

Andrew D, Craig AD. 2001. Spinothalamic lamina I neurons selectively sensitive to histamine: a central neural pathway for itch. Nat Neurosci 4(1):72-7.

Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, Rumio C. 2009. Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. J Histochem Cytochem 57(11):1013-23.

Basbaum AI, Bautista DM, Scherrer G, Julius D. 2009. Cellular and molecular mechanisms of pain. Cell 139(2):267-84.

Beg AA. 2002. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol 23(11):509-12.

Bell JK, McQueen DS, Rees JL. 2004. Involvement of histamine H4 and H1 receptors in scratching induced by histamine receptor agonists in Balb C mice. Br J Pharmacol 142(2):374-80.

Bertin S, Aoki-Nonaka Y, de Jong PR, Nohara LL, Xu H, Stanwood SR, Srikanth S, Lee J, To K, Abramson L and others. 2014. The ion channel TRPV1 regulates the activation and proinflammatory properties of CD4(+) T cells. Nat Immunol 15(11):1055-63.

Bhave G, Gereau RWt. 2004. Posttranslational mechanisms of peripheral sensitization. J Neurobiol 61(1):88-106.

Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt. 2003. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proc Natl Acad Sci U S A 100(21):12480-5.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt. 2002. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 35(4):721-31.

Binshtok AM, Bean BP, Woolf CJ. 2007. Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. Nature 449(7162):607-10.

Block ML, Zecca L, Hong JS. 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8(1):57-69.

Bowman CC, Rasley A, Tranguch SL, Marriott I. 2003. Cultured astrocytes express toll-like receptors for bacterial products. Glia 43(3):281-91.

Brandao KE, Dell'Acqua ML, Levinson SR. 2012. A-kinase anchoring protein 150 expression in a specific subset of TRPV1- and CaV 1.2-positive nociceptive rat dorsal root ganglion neurons. J Comp Neurol 520(1):81-99.

Bsibsi M, Persoon-Deen C, Verwer RW, Meeuwsen S, Ravid R, Van Noort JM. 2006. Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. Glia 53(7):688-95.

Bsibsi M, Ravid R, Gveric D, van Noort JM. 2002. Broad expression of

Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol 61(11):1013-21.

Camprubi-Robles M, Planells-Cases R, Ferrer-Montiel A. 2009. Differential contribution of SNARE-dependent exocytosis to inflammatory potentiation of TRPV1 in nociceptors. FASEB J 23(11):3722-33.

Caterina MJ, Julius D. 2001. The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci 24:487-517.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D. 2000. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288(5464):306-13.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389(6653):816-24.

Chen K, Iribarren P, Hu J, Chen J, Gong W, Cho EH, Lockett S, Dunlop NM, Wang JM. 2006. Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid beta peptide. J Biol Chem 281(6):3651-9.

Cho IH, Hong J, Suh EC, Kim JH, Lee H, Lee JE, Lee S, Kim CH, Kim DW, Jo EK and others. 2008. Role of microglial IKKbeta in kainic acid-induced hippocampal neuronal cell death. Brain 131(Pt 11):3019-33.

Chu CJ, Huang SM, De Petrocellis L, Bisogno T, Ewing SA, Miller JD, Zipkin RE, Daddario N, Appendino G, Di Marzo V and others. 2003. N-

oleoyldopamine, a novel endogenous capsaicin-like lipid that produces hyperalgesia. J Biol Chem 278(16):13633-9.

Covacu R, Arvidsson L, Andersson A, Khademi M, Erlandsson-Harris H, Harris RA, Svensson MA, Olsson T, Brundin L. 2009. TLR activation induces TNF-alpha production from adult neural stem/progenitor cells. J Immunol 182(11):6889-95.

De Nardo D. 2015. Toll-like receptors: Activation, signalling and transcriptional modulation. Cytokine 74(2):181-9.

Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM. 2011. LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. J Dent Res 90(6):759-64.

Due MR, Piekarz AD, Wilson N, Feldman P, Ripsch MS, Chavez S, Yin H, Khanna R, White FA. 2012. Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. J Neuroinflammation 9:200.

Fang Z, Park CK, Li HY, Kim HY, Park SH, Jung SJ, Kim JS, Monteil A, Oh SB, Miller RJ. 2007. Molecular basis of Ca(v)2.3 calcium channels in rat nociceptive neurons. J Biol Chem 282(7):4757-64.

Gern JE, Reardon CL, Hoffjan S, Nicolae D, Li Z, Roberg KA, Neaville WA, Carlson-Dakes K, Adler K, Hamilton R and others. 2004. Effects of dog ownership and genotype on immune development and atopy in infancy. J Allergy Clin Immunol 113(2):307-14.

Guo A, Simone DA, Stone LS, Fairbanks CA, Wang J, Elde R. 2001.

Developmental shift of vanilloid receptor 1 (VR1) terminals into deeper regions of the superficial dorsal horn: correlation with a shift from TrkA to Ret expression by dorsal root ganglion neurons. Eur J Neurosci 14(2):293-304.

Han SK, Mancino V, Simon MI. 2006. Phospholipase Cbeta 3 mediates the scratching response activated by the histamine H1 receptor on C-fiber nociceptive neurons. Neuron 52(4):691-703.

Handwerker HO, Forster C, Kirchhoff C. 1991. Discharge patterns of human C-fibers induced by itching and burning stimuli. J Neurophysiol 66(1):307-15.

Helliwell RJ, McLatchie LM, Clarke M, Winter J, Bevan S, McIntyre P. 1998. Capsaicin sensitivity is associated with the expression of the vanilloid (capsaicin) receptor (VR1) mRNA in adult rat sensory ganglia. Neurosci Lett 250(3):177-80.

Hellwig N, Plant TD, Janson W, Schafer M, Schultz G, Schaefer M. 2004. TRPV1 acts as proton channel to induce acidification in nociceptive neurons. J Biol Chem 279(33):34553-61.

Huang SM, Bisogno T, Trevisani M, Al-Hayani A, De Petrocellis L, Fezza F, Tognetto M, Petros TJ, Krey JF, Chu CJ and others. 2002. An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. Proc Natl Acad Sci U S A 99(12):8400-5.

Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D and others. 2000. Direct activation of capsaicin receptors by

products of lipoxygenases: endogenous capsaicin-like substances. Proc Natl Acad Sci U S A 97(11):6155-60.

Inagaki N, Nakamura N, Nagao M, Musoh K, Kawasaki H, Nagai H. 1999. Participation of histamine H1 and H2 receptors in passive cutaneous anaphylaxis-induced scratching behavior in ICR mice. Eur J Pharmacol 367(2-3):361-71.

Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A, Antel JP. 2005. TLR signaling tailors innate immune responses in human microglia and astrocytes. Journal of Immunology 175(7):4320-30.

Jackson AC, Rossiter JP, Lafon M. 2006. Expression of Toll-like receptor 3 in the human cerebellar cortex in rabies, herpes simplex encephalitis, and other neurological diseases. J Neurovirol 12(3):229-34.

Jancso-Gabor A, Szolcsanyi J, Jancso N. 1970. Stimulation and desensitization of the hypothalamic heat-sensitive structures by capsaicin in rats. J Physiol 208(2):449-59.

Jeffry J, Kim S, Chen ZF. 2011. Itch signaling in the nervous system. Physiology (Bethesda) 26(4):286-92.

Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. 2002. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron 36(1):57-68.

Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H, Oh U. 2004. Phosphorylation of vanilloid receptor 1 by Ca2+/calmodulin-dependent kinase II regulates its vanilloid binding. J Biol Chem 279(8):7048-54.

Kielian T. 2009. Overview of toll-like receptors in the CNS. Curr Top Microbiol Immunol 336:1-14.

Kim BM, Lee SH, Shim WS, Oh U. 2004. Histamine-induced Ca(2+) influx via the PLA(2)/lipoxygenase/TRPV1 pathway in rat sensory neurons. Neurosci Lett 361(1-3):159-62.

Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, Choi SY, Park K, Kim JS, Akira S and others. 2007. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J Biol Chem 282(20):14975-83.

Kim D, You B, Jo EK, Han SK, Simon MI, Lee SJ. 2010. NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. Proc Natl Acad Sci U S A 107(33):14851-6.

Kim SJ, Park GH, Kim D, Lee J, Min H, Wall E, Lee CJ, Simon MI, Lee SJ, Han SK. 2011. Analysis of cellular and behavioral responses to imiquimod reveals a unique itch pathway in transient receptor potential vanilloid 1 (TRPV1)-expressing neurons. Proc Natl Acad Sci U S A 108(8):3371-6.

Konopka L, Wierzbicki M, Brzezinska-Blaszczyk E. 2010. Lipopolysaccharide from Porphyromonas gingivalis stimulates rat mast cells to cysteinyl leukotriene generation and upregulates Toll-like receptor -2 and -4 expression. Int J Immunopathol Pharmacol 23(3):803-10.

Koplas PA, Rosenberg RL, Oxford GS. 1997. The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. J

Neurosci 17(10):3525-37.

Kwak J, Wang MH, Hwang SW, Kim TY, Lee SY, Oh U. 2000. Intracellular ATP increases capsaicin-activated channel activity by interacting with nucleotide-binding domains. J Neurosci 20(22):8298-304.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86(6):973-83.

Li Y, Adamek P, Zhang H, Tatsui CE, Rhines LD, Mrozkova P, Li Q, Kosturakis AK, Cassidy RM, Harrison DS and others. 2015. The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4. J Neurosci 35(39):13487-500.

Lim H, Kim D, Lee SJ. 2013. Toll-like receptor 2 mediates peripheral nerve injury-induced NADPH oxidase 2 expression in spinal cord microglia. J Biol Chem 288(11):7572-9.

Liu T, Berta T, Xu ZZ, Park CK, Zhang L, Lu N, Liu Q, Liu Y, Gao YJ, Liu YC and others. 2012. TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice. J Clin Invest 122(6):2195-207.

Liu T, Xu ZZ, Park CK, Berta T, Ji RR. 2010. Toll-like receptor 7 mediates pruritus. Nat Neurosci 13(12):1460-2.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402-8.

Ma Y, Li J, Chiu I, Wang Y, Sloane JA, Lu J, Kosaras B, Sidman RL, Volpe JJ, Vartanian T. 2006. Toll-like receptor 8 functions as a negative regulator of neurite outgrowth and inducer of neuronal apoptosis. J Cell Biol 175(2):209-15.

McQueen DS, Noble MA, Bond SM. 2007. Endothelin-1 activates ETA receptors to cause reflex scratching in BALB/c mice. Br J Pharmacol 151(2):278-84.

Medzhitov R. 2001. Toll-like receptors and innate immunity. Nat Rev Immunol 1(2):135-45.

Mezey E, Toth ZE, Cortright DN, Arzubi MK, Krause JE, Elde R, Guo A, Blumberg PM, Szallasi A. 2000. Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. Proc Natl Acad Sci U S A 97(7):3655-60.

Michael GJ, Priestley JV. 1999. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J Neurosci 19(5):1844-54.

Min H, Hong J, Cho IH, Jang YH, Lee H, Kim D, Yu SW, Lee S, Lee SJ. 2015. TLR2-induced astrocyte MMP9 activation compromises the blood brain barrier and exacerbates intracerebral hemorrhage in animal models. Mol Brain 8:23.

Mohapatra DP, Nau C. 2003. Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent

protein kinase pathway. J Biol Chem 278(50):50080-90.

Morenilla-Palao C, Planells-Cases R, Garcia-Sanz N, Ferrer-Montiel A. 2004. Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. J Biol Chem 279(24):25665-72.

Morita T, McClain SP, Batia LM, Pellegrino M, Wilson SR, Kienzler MA, Lyman K, Olsen AS, Wong JF, Stucky CL and others. 2015. HTR7 Mediates Serotonergic Acute and Chronic Itch. Neuron 87(1):124-38.

Niebuhr M, Langnickel J, Draing C, Renz H, Kapp A, Werfel T. 2008. Dysregulation of toll-like receptor-2 (TLR-2)-induced effects in monocytes from patients with atopic dermatitis: impact of the TLR-2 R753Q polymorphism. Allergy 63(6):728-34.

Numazaki M, Tominaga T, Takeuchi K, Murayama N, Toyooka H, Tominaga M. 2003. Structural determinant of TRPV1 desensitization interacts with calmodulin. Proc Natl Acad Sci U S A 100(13):8002-6.

Ochoa-Cortes F, Ramos-Lomas T, Miranda-Morales M, Spreadbury I, Ibeakanma C, Barajas-Lopez C, Vanner S. 2010. Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. Am J Physiol Gastrointest Liver Physiol 299(3):G723-32.

Ochoa J, Torebjork E. 1989. Sensations evoked by intraneural microstimulation of C nociceptor fibres in human skin nerves. J Physiol 415:583-99.

Oh U, Hwang SW, Kim D. 1996. Capsaicin activates a nonselective cation channel in cultured neonatal rat dorsal root ganglion neurons. J Neurosci

16(5):1659-67.

Okun E, Griffioen KJ, Son TG, Lee JH, Roberts NJ, Mughal MR, Hutchison E, Cheng A, Arumugam TV, Lathia JD and others. 2010. TLR2 activation inhibits embryonic neural progenitor cell proliferation. J Neurochem 114(2):462-74.

Olah Z, Szabo T, Karai L, Hough C, Fields RD, Caudle RM, Blumberg PM, Iadarola MJ. 2001. Ligand-induced dynamic membrane changes and cell deletion conferred by vanilloid receptor 1. J Biol Chem 276(14):11021-30.

Olson JK, Miller SD. 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 173(6):3916-24.

Park C, Cho IH, Kim D, Jo EK, Choi SY, Oh SB, Park K, Kim JS, Lee SJ. 2008. Toll-like receptor 2 contributes to glial cell activation and heme oxygenase-1 expression in traumatic brain injury. Neurosci Lett 431(2):123-8.

Park CK, Xu ZZ, Berta T, Han Q, Chen G, Liu XJ, Ji RR. 2014. Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1. Neuron 82(1):47-54.

Piovezan AP, D'Orleans-Juste P, Souza GE, Rae GA. 2000. Endothelin-1-induced ET(A) receptor-mediated nociception, hyperalgesia and oedema in the mouse hind-paw: modulation by simultaneous ET(B) receptor activation. Br J Pharmacol 129(5):961-8.

Por ED, Bierbower SM, Berg KA, Gomez R, Akopian AN, Wetsel WC,

Jeske NA. 2012. beta-Arrestin-2 desensitizes the transient receptor potential vanilloid 1 (TRPV1) channel. J Biol Chem 287(44):37552-63.

Por ED, Gomez R, Akopian AN, Jeske NA. 2013. Phosphorylation regulates TRPV1 association with beta-arrestin-2. Biochem J 451(1):101-9.

Prehaud C, Megret F, Lafage M, Lafon M. 2005. Virus infection switches TLR-3-positive human neurons to become strong producers of beta interferon. J Virol 79(20):12893-904.

Premkumar LS, Ahern GP. 2000. Induction of vanilloid receptor channel activity by protein kinase C. Nature 408(6815):985-90.

Qi J, Buzas K, Fan H, Cohen JI, Wang K, Mont E, Klinman D, Oppenheim JJ, Howard OM. 2011. Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. J Immunol 186(11):6417-26.

Rasley A, Anguita J, Marriott I. 2002. Borrelia burgdorferi induces inflammatory mediator production by murine microglia. J Neuroimmunol 130(1-2):22-31.

Reddy VB, Iuga AO, Shimada SG, LaMotte RH, Lerner EA. 2008. Cowhage-evoked itch is mediated by a novel cysteine protease: a ligand of protease-activated receptors. J Neurosci 28(17):4331-5.

Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, Schwartz M. 2007. Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol 9(9):1081-8.

Rosenbaum T, Gordon-Shaag A, Munari M, Gordon SE. 2004. Ca2+/calmodulin modulates TRPV1 activation by capsaicin. J Gen Physiol

123(1):53-62.

Rossbach K, Nassenstein C, Gschwandtner M, Schnell D, Sander K, Seifert R, Stark H, Kietzmann M, Baumer W. 2011. Histamine H1, H3 and H4 receptors are involved in pruritus. Neuroscience 190:89-102.

Rossbach K, Wendorff S, Sander K, Stark H, Gutzmer R, Werfel T, Kietzmann M, Baumer W. 2009. Histamine H4 receptor antagonism reduces hapten-induced scratching behaviour but not inflammation. Exp Dermatol 18(1):57-63.

Samways DS, Egan TM. 2011. Calcium-dependent decrease in the single-channel conductance of TRPV1. Pflugers Arch 462(5):681-91.

Schmelz M, Schmidt R, Bickel A, Handwerker HO, Torebjork HE. 1997. Specific C-receptors for itch in human skin. J Neurosci 17(20):8003-8.

Schnizler K, Shutov LP, Van Kanegan MJ, Merrill MA, Nichols B, McKnight GS, Strack S, Hell JW, Usachev YM. 2008. Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. J Neurosci 28(19):4904-17.

Shim WS, Oh U. 2008. Histamine-induced itch and its relationship with pain. Mol Pain 4:29.

Shim WS, Tak MH, Lee MH, Kim M, Kim M, Koo JY, Lee CH, Kim M, Oh U. 2007. TRPV1 mediates histamine-induced itching via the activation of phospholipase A2 and 12-lipoxygenase. J Neurosci 27(9):2331-7.

Shimada SG, Shimada KA, Collins JG. 2006. Scratching behavior in mice induced by the proteinase-activated receptor-2 agonist, SLIGRL-NH2. Eur J

Pharmacol 530(3):281-3.

Shin J, Cho H, Hwang SW, Jung J, Shin CY, Lee SY, Kim SH, Lee MG, Choi YH, Kim J and others. 2002. Bradykinin-12-lipoxygenase-VR1 signaling pathway for inflammatory hyperalgesia. Proc Natl Acad Sci U S A 99(15):10150-5.

Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, Dower SK. 2000. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. J Biol Chem 275(7):4670-8.

sork rDavis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K and others. 2000. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405(6783):183-7.

Steenland HW, Ko SW, Wu LJ, Zhuo M. 2006. Hot receptors in the brain. Mol Pain 2:34.

Stein AT, Ufret-Vincenty CA, Hua L, Santana LF, Gordon SE. 2006. Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. J Gen Physiol 128(5):509-22.

Steinhoff M, Corvera CU, Thoma MS, Kong W, McAlpine BE, Caughey GH, Ansel JC, Bunnett NW. 1999. Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. Exp Dermatol 8(4):282-94.

Studer M, McNaughton PA. 2010. Modulation of single-channel properties

of TRPV1 by phosphorylation. J Physiol 588(Pt 19):3743-56.

Sugiura T, Tominaga M, Katsuya H, Mizumura K. 2002. Bradykinin lowers the threshold temperature for heat activation of vanilloid receptor 1. J Neurophysiol 88(1):544-8.

Suh HS, Zhao ML, Rivieccio M, Choi S, Connolly E, Zhao Y, Takikawa O, Brosnan CF, Lee SC. 2007. Astrocyte indoleamine 2,3-dioxygenase is induced by the TLR3 ligand poly(I:C): mechanism of induction and role in antiviral response. J Virol 81(18):9838-50.

Sun YG, Chen ZF. 2007. A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature 448(7154):700-3.

Szolcsanyi J, Joo F, Jancso-Gabor A. 1971. Mitochondrial changes in preoptic neurons after capsaicin desensitization of the hypothalamic thermodetectors in rats. Nature 229(5280):116-7.

Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K. 2006. Role of toll-like receptor signalling in Abeta uptake and clearance. Brain 129(Pt 11):3006-19.

Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. Cell 140(6):805-20.

Tang SC, Arumugam TV, Xu X, Cheng A, Mughal MR, Jo DG, Lathia JD, Siler DA, Chigurupati S, Ouyang X and others. 2007. Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. Proc Natl Acad Sci U S A 104(34):13798-803.

Tanga FY, Nutile-McMenemy N, DeLeo JA. 2005. The CNS role of Toll-

like receptor 4 in innate neuroimmunity and painful neuropathy. Proc Natl Acad Sci U S A 102(16):5856-61.

Tanga FY, Raghavendra V, DeLeo JA. 2004. Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem Int 45(2-3):397-407.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D. 1998. The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21(3):531-43.

Trevisani M, Smart D, Gunthorpe MJ, Tognetto M, Barbieri M, Campi B, Amadesi S, Gray J, Jerman JC, Brough SJ and others. 2002. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. Nat Neurosci 5(6):546-51.

Ufret-Vincenty CA, Klein RM, Hua L, Angueyra J, Gordon SE. 2011. Localization of the PIP2 sensor of TRPV1 ion channels. J Biol Chem 286(11):9688-98.

Valtschanoff JG, Rustioni A, Guo A, Hwang SJ. 2001. Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. J Comp Neurol 436(2):225-35.

Varadaradjalou S, Feger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, Arock M. 2003. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. Eur J Immunol 33(4):899-906.

Vos MH, Neelands TR, McDonald HA, Choi W, Kroeger PE, Puttfarcken PS, Faltynek CR, Moreland RB, Han P. 2006. TRPV1b overexpression

negatively regulates TRPV1 responsiveness to capsaicin, heat and low pH in HEK293 cells. J Neurochem 99(4):1088-102.

Wadachi R, Hargreaves KM. 2006. Trigeminal nociceptors express TLR-4 and CD14: a mechanism for pain due to infection. J Dent Res 85(1):49-53.

Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, Bautista DM. 2011. TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. Nat Neurosci 14(5):595-602.

Winter J, Dray A, Wood JN, Yeats JC, Bevan S. 1990. Cellular mechanism of action of resiniferatoxin: a potent sensory neuron excitotoxin. Brain Res 520(1-2):131-40.

Zhang H, Cang CL, Kawasaki Y, Liang LL, Zhang YQ, Ji RR, Zhao ZQ. 2007. Neurokinin-1 receptor enhances TRPV1 activity in primary sensory neurons via PKCepsilon: a novel pathway for heat hyperalgesia. J Neurosci 27(44):12067-77.

Zhang X, Huang J, McNaughton PA. 2005. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J 24(24):4211-23.

Zhang X, Li L, McNaughton PA. 2008. Proinflammatory mediators modulate the heat-activated ion channel TRPV1 via the scaffolding protein AKAP79/150. Neuron 59(3):450-61.

Zhao ZQ, Huo FQ, Jeffry J, Hampton L, Demehri S, Kim S, Liu XY, Barry DM, Wan L, Liu ZC and others. 2013. Chronic itch development in sensory

neurons requires BRAF signaling pathways. J Clin Invest 123(11):4769-80.

Zhuang ZY, Xu H, Clapham DE, Ji RR. 2004. Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. J Neurosci 24(38):8300-9.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, Julius D, Hogestatt ED. 1999. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400(6743):452-7.

국문초록

감각신경세포상의 톨유사수용체 4 의 가려움감각 조절 기전

가려움 또는 소양감이라고 알려진 이 감각은 긁는 반응을 유도시키는 불쾌한 자극으로 정의된다. 일반적으로 가려움은 해로운 외부자극에 대항하여 신체를 보호하기 위한 기작으로 일어나나, 과도한 소양감은 환자의 삶의 질을 떨어트린다. 이의 치료방법은 대부분 대증요법에 의존하고 있는데 이는 가려움에 대한 이해가 아직 부족한데 기인한다. 최근 연구에 의하면 선천성 면역세포 상에서 발현, 감염원을 인지하여 이들 세포에서 면역반응을 일으키는 것으로 알려진 톨유사수용체4가 감각신경세포상에서도 발현하는 것이 보고된 바 있다. 이에 이 수용기가 감각신경전달에도 중요한 역할을 할 것으로 미루어 짐작할 수 있다. 위 연구에서 본인은 감각신경세포상에서 발현하는 톨유사수용체4가 TRPV1의 활성화 조절을 통하여 가려움 감각신경 전달을 조절함을 밝히고자 한다.

1장에서는 신경세포상의 톨유사수용체4가 히스타민 또는 클로로퀸 유래 가려움 감각을 강화시킴을 확인하였다. 우선 톨유사수용체4가 TRPV1을 발현하는 척수후절근 신경세포에서 발현함을 확인하였다. 또한 톨유사수용체4의 리간드인 LPS 처리에 의한 소양감은 일어나지 않으나 톨유사수용체4 결핍 생쥐에서

클로로퀸 또는 히스타민 유래 가려움이 감소함을 밝혔다. 이에 각각 클로로퀸과 히스타민 수용기의 발현량을 감각신경세포에서 비교해본 결과 톨유사수용체4 결핍 생쥐에서 감소된 클로로퀸 수용기의 발현 감소를 확인하였으나 히스타민 수용기의 발현은 차이가 없음을 확인하였다.한편 히스타민 유래 가려움의 감소는 히스타민에 의한 세포내 칼슘 농도 증가가 톨유사수용체4 결핍 신경세포에서 감소하고 또한 이것이 TRPV1활성화 감소에 의한 것임을 확인하였다. 그리고 HEK293T세포에 TRPV1을 과 발현 시킨 후 이의 활성화에 의한 세포 내 칼슘 증가를 측정한 결과 톨유사수용체4가 함께 과 발현된 경우 캡사이신에 의한 칼슘 신호 및 inward current가 감소함을 관찰하였다.

2장에서는 톨유사수용체4가 TRPV1과 TIR 도메인을 통해 상호결합 함으로서 의 세포 내 이입을 감소시켜 탈 감작을 줄임을 밝혔다. 우선 톨유사수용체4가 TIR도메인을 통해 TRPV1과 상호작용을 함을 확인하였다. 또한 TRPV1을 TIR도메인 결핍 톨유사수용체4 돌연변이와 과 발현 시킬 경우 자연형 톨유사수용체4와 과 발현되었을 때와 비교해서 활성화가 감소함을 세포 내 칼슘 증가 측정을 통해 확인하였다. 두 단백질간의 결합이 TRPV1상의 세린 인산화를 변화시키지는 않으나, 캡사이신 자극 이후 채널 단백질의 세포막상 발현 감소를 완화시킴을

확인할 수 있었다. 이에 따라 톨유사수용체4 결핍 감각신경세포에서 캡사이신자극 유래 탈 감작이 자연형 감각신경세포와 비교하여 증가함을 확인할 수 있었다.

결론적으로 본 연구에서 신경세포에 발현하는 톨유사수용체4가 각기 다른 기작을 통해 클로로퀸 또는 히스타민 유래 가려움 감각신호 전달을 조절함을 밝혔다. 클로로퀸 유래 가려움 감각의 경우 톨유사수용체4에 의한 수용기 발현 증가로 인하여 증가함을 확인하였다. 한편 히스타민유래 가려움 감각의 경우 톨유사수용체4가 TRPV1과 TIR도메인을 통해 결합하여 탈 감작 기작을 감소시킴으로써 이를 증가시킴을 확인하였다.

이 연구 결과는 톨유사수용체4가 증대된 가려움증의 치료 타겟이 될 수 있음을 제시하고 있다.

주요어: 가려움, 히스타민, 클로로퀸, 톨유사수용체 4, TRPV1

학번: 2010-30922