Spinal GRPR and NPRA Contribute to Chronic Itch in a Murine Model of Allergic Contact Dermatitis

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Recurrent and intractable chronic itch is a worldwide problem, but mechanisms, especially the neural mechanisms, underlying chronic itch still remain unclear. In this study, we investigated the peripheral and spinal mechanisms responsible for prolonged itch in a mouse model of allergic contact dermatitis induced by squaric acid dibutyester. We found that repeated exposure of mice to squaric acid dibutyester evoked persistent spontaneous scratching and significantly aberrant cutaneous and systemic immune responses lasting for weeks. Squaric acid dibutyester—induced itch requires both nonhistaminergic and histaminergic pathways, which are likely relayed by GRPR and NPRA in the spinal cord, respectively. Employing genetic, pharmacologic, RNAscope assay, and cell-specific ablation methods, we dissected a neural circuit for prolonged itch formed as Grpr⁺ neurons act downstream of Npr1⁺ neurons in the spinal cord. Taken together, our data suggested that targeting GRPR and NPRA may provide effective treatments for allergic contact dermatitis–associated chronic pruritus.


INTRODUCTION

Itch is an unpleasant sensation accompanied by scratching behavior or a desire to scratch. Even though acute itching is a protective reaction of the body to stimuli from either the internal or external environment, chronic itch is an important manifestation of dermatologic diseases (e.g., allergic dermatitis, atopic eczema, and psoriasis), as well as a variety of noncutaneous disorders, that exacerbates skin discomfort and affects quality of life (Dong and Dong, 2018; Nattkemper et al., 2018; Yosipovitch and Bernhard, 2013). The lack of understanding of the pathogenesis of persistent and recurrent itch hampers the emergence of effective cures.

In many cases, chronic itch reflects the resiliency of the pruriception system in a manner similar to pain that is prolonged even after healing of the injured tissues. In chronic pain settings, significant neural plasticity of the central neurons and circuits occurs and is considered responsible for long-lasting and persistent pain hypersensitivity, even when peripheral inflammation and nerve injuries no longer exist (Basbaum et al., 2009; Ji et al., 2016). Currently, there are at least two peptides, GRP and NPPB (also known as brain-derived natriuretic peptide, or BNP), that are claimed to be itch-specific neuropeptides (Mishra and Hoon, 2013; Sun and Chen, 2007). The cells expressing receptors for these two neuropeptides, GRPR and NPRA (or NPR1), respectively, have been identified as itch-specific neurons in the spinal cord (Mishra and Hoon, 2013; Sun et al., 2009). However, whether and how they play a certain role in persistent and recurrent itch remains unclear.

Allergic contact dermatitis (ACD) is one of the most common skin diseases worldwide, and patients may report prolonged itch that lasts weeks after the original allergens or chemicals have been cleared (Alikhan and Maibach, 2014; LaMotte, 2016). Squaric acid dibutyester (SADBE), a small molecule hapten, was previously used as a medicine for alopecia areata and recently has been used as a popular ACD inducer in mice (Feng et al., 2017; LaMotte, 2016; Luo et al., 2018; Qu et al., 2015, 2014). Unlike the chemical hapten DNFB-induced itch model that has been used for many years but whose exact working mechanism is still unclear, remarkable progress has been achieved recently in understanding the peripheral mechanisms of SADBE-induced chronic itch (Feng et al., 2017; LaMotte, 2016; Luo et al., 2018; Qu et al., 2015, 2014). Therefore, in this study, we employed this animal model to investigate whether GRPR and NPRA are critical molecules and circuits in the spinal cord for persistent and prolonged itch associated with ACD.

RESULTS

SADBE challenges lead to prolonged itch and increased inflammation

In this study, SADBE was used as an ACD inducer in C57BL/6j (referred to as C57) mice via presensitization and subsequent challenges (or elicitation). We modified the protocol
Figure 1. Analysis of skin and immune system in mice with SADBE-induced prolonged itch. (a) Schematic experimental protocol. (b) Spontaneous scratching until 5 weeks after last SADBE treatment (n = 7). (c) Elevated serum IgE and (d) increased spleen weights. (e) Quantitative analysis of enhanced epidermal thickness and (f) increased mast cell infiltration in the dermis, n = 3-4. (g) Representative H&E staining, (h) immunostaining of the epidermal marker KRT14, and (i) toluidine blue staining of the neck skin sections. (j–l) qPCR analysis showed mRNA levels of the indicated target genes in skin, n = 5. *P < 0.05, **P < 0.01, ***P < 0.001, compared with day 0. SADBE, squaric acid dibutylester. Bar = 50 μm.
Figure 2. GRPR is essential for SADBE-induced prolonged itch. (a–c) Increased Grpr levels 7 days after the last SADBE painting in C57 mice detected by RNAscope ISH (a, n = 3) or qPCR (c, n = 5) or in GRPR-eGFP mice detected with anti-GFP antibody (b, n = 3). (d–f) SADBE-induced prolonged itch in C57 mice decreased after i.t. GRPR antagonist (d, n = 9) or in Grpr KO (e, WT n = 11, KO n = 8) or C57 mice after BB-sap (400 ng, i.t.) (f, n = 10). (g) RNAscope ISH assay showed abolishment of Grpr+ neurons in BB-sap mice (n = 4). (h, i) Double RNAscope ISH of Grpr+ with Vglut2+ or Vgat+ in cervical spinal cord.
(Feng et al., 2017; LaMotte, 2016; Luo et al., 2018; Qu et al., 2015, 2014) by challenging the nape of neck skin instead of the ear skin in mice and observed the scratching behavior up to 5 weeks (Figure 1a). As expected, SADBE initiated sensitization and elicitation phases in mice that manifested as spontaneous scratching behavior (Feng et al., 2017; LaMotte, 2016; Luo et al., 2018; Qu et al., 2015, 2014). Surprisingly, SADBE induced a very prolonged itch with a peak time approximately 3–7 days after the last SADBE painting and lasted about 5 weeks (Figure 1b), which had never been reported before (Feng et al., 2017; Huang et al., 2018b; LaMotte, 2016; Luo et al., 2018; Qu et al., 2015, 2014; Zhao et al., 2013).

We then focused on the cellular and molecular changes in the immune and nervous systems of mice with SADBE-induced prolonged itch (referred to as SADBE mice). SADBE mice exhibited elevated serum IgE levels (Figure 1c), increased spleen weight (Figure 1d), thickening of the epidermis (Figure 1e, g, and h), and increased infiltration of mast cells in the dermis (Figure 1f and i). Multiplex Luminex assay (Supplementary Figure S1a) and qPCR analysis (Figure 1j–l and Supplementary Figure S1b) revealed that the treated nape of neck skin of SADBE mice displayed significant upregulation of the transcripts or secreted cytoketons at the protein level of genes recently reported to be associated with itch (Alikan and Maibach, 2014; Dong and Dong, 2018; Feng et al., 2017; LaMotte, 2016; Luo et al., 2018; Mishra and Hoon, 2013; Qu et al., 2015, 2014; Sun and Chen, 2007; Sun et al., 2009), such as those encoding various cytoketons including IL-4, IL-23, IL-33, IL-27, IL-1β, TNF-α, and TSLP (Supplementary Figure S1a and b); Trpm8 (Figure 1j); Mrgps1 (Figure 1k); and Grp and Nppb (Figure 1l) in the afflicted skin area.

**GRPR relays SADBE-induced persistent itch in the spinal cord**

GRPR is an itch-specific mediator that has been proposed primarily for relaying nonhistaminergic itch in the spinal cord (Sun and Chen, 2007) and continues to be the focus of numerous itch-related studies. On day 7 after the last SADBE painting, the cervical spinal Grpr mRNA level was significantly increased in SADBE mice detected by both RNAscope in situ hybridization (Figure 2a) and qPCR (Figure 2c). Similarly, anti-GFP antibody staining also showed that GFP+ cells were increased in the GRPR-eGFP mice (Zhao et al., 2013) 7 days after the last SADBE painting (Figure 2b).

To further elucidate the role of GRPR in SADBE-induced prolonged itch, we used either pharmacological and genetic blockade of GRPR signaling (Sun and Chen, 2007) or the neurotoxin bombesin-saporin (BB-sap) to selectively ablate GRPR+ neurons (Sun et al., 2009). The robust spontaneous scratching in SADBE mice on day 7 was significantly decreased by intrathecal (i.t.) injection of the GRPR antagonist deamino-Phe19,D-Ala24,D-Pro26-D-Phe27-GRP (Zhao et al., 2013) (Figure 2d) or was remarkably reduced in Grpr knockout (KO) mice (Figure 2e) and, notably, was abolished in BB-sap mice (Figure 2f). Together, results from these different approaches demonstrate that GRPR in the spinal cord is essential for SADBE-induced prolonged itch.

We further examined the molecular expression of various markers in their spinal cords. As predicted, most Grpr+ cells were ablated in the cervical spinal dorsal horn of BB-sap mice (Figure 2g). The superficial dorsal horn neurochemical markers, like CGRP (Supplementary Figure S2a) and PCKγ (Supplementary Figure S2b) are comparable in BB-sap mice and blank-saporin (blank-sap) mice. The expression of two itch-related markers, Sstr2 (Supplementary Figure S2c), and the dynorphin precursor prodynorphine (Pdyn) (Huang et al., 2018a; Kardon et al., 2014) (Supplementary Figure S2d), were comparable in BB-sap mice and blank-sap mice. Thus, these results confirm that the Grpr+ neurons were selectively ablated. We double-stained Grpr+ neurons with Vglut2 (a glutamatergic neuronal marker) and Vgat (an inhibitory neuronal marker) in SADBE mice. Consistent with previous data (Aresh et al., 2017), most Grpr+ neurons in the spinal cord are excitatory interneurons (Figure 2h and i).

**NPRA relays SADBE-induced itch in the spinal cord**

By qPCR analysis, we found that mRNA levels of Nppb in the dorsal root ganglion (DRG) were significantly enhanced on day 3 (Figure 3a), and mRNA levels of Npr1 in the spinal cord were significantly enhanced on days 7 and 21 (Figure 3b) after the last SADBE treatment. RNAscope analysis results also supported that Nppb was upregulated in the DRGs of SADBE mice (Figure 3c). SADBE-induced prolonged scratching was significantly attenuated by i.t. injection of the NPRA antagonist anantin (2 and 10 nmol) (Figure 3d) and was also significantly decreased in both Npr1 KO mice (Figure 3e) and after using a neurotoxin, BNP-saporin (BNP-sap), to selectively ablate Npr1+ neurons (Huang et al., 2018a; Mishra and Hoon, 2013) (Figure 3f). To learn more about the neuronal circuitry involved, we also examined expression of various markers in the cervical spinal cord of BNP-sap mice treated with SADBE. Consequently, about 63% of Npr1+ neurons (Figure 3g) were ablated. A small proportion of Pdyn+ neurons were also ablated (Supplementary Figure S3b), confirming their partial overlapping (Huang et al., 2018a; Mishra and Hoon, 2013). We also double-stained Npr1+ with Vglut2 and Vgat and the results showed that most Npr1+ neurons expressed Vglut2 (Figure 3h and i).

**GRPR+ - and NPR1+ -expressing neurons in the spinal cord are two distinctive populations for itch signal processing**

To examine how itch signal is processed in the spinal cord by GRPR+ - and NPR1+ -expressing neurons, we used i.t. injections to evoke acute itch in BB-sap mice and BNP-sap mice, which have no deficits in motor activity (Huang et al., 2018a; Mishra and Hoon, 2013; Sun et al., 2009) (Figure 4a). First, when compared with the blank-sap group, scratching responses to i.t. BNP (Figure 4b), octreotide (an analog of somatostatin), and the KOR antagonist norbinaltorphimine (Supplementary Figure S4a) were almost fully

(c4–c6), n = 3. All quantitative data are mean ± SEM. **P < 0.01, ***P < 0.001, t-test. BB-sap, bombesin-saporin; Ctrl, control; ISH, in situ hybridization; i.t., intrathecal; KO, knockout; SADBE, squaric acid dibutylester; WT, wild type. Bar = 25 μm.
abolished in BB-sap mice. Treatment of BB-sap mice abolished the scratching behavior in all acute and chronic itch models that we and other labs have tested so far (Huang et al., 2018a; Mishra and Hoon, 2013; Sun et al., 2009; Zhao et al., 2013), supporting that spinal cord GRPR-expressing neurons function as itch labeled line cells. Second, we confirmed that elimination of NPRA neurons attenuated scratching responses to i.t. BNP (Figure 4c) but not to i.t. GRP (Figure 4d), octreotide, and norbinaltorphimine (Supplementary Figure S4b), confirming that, in the spinal
cord, GRP, somatostatin, and dynorphin act downstream of Npr1 neurons. Third, double staining results further indicated that Npr1, Grpr, Sstr2, and Nmbr expression largely did not overlap in the spinal cord (Figure 4e and Supplementary Figure S4c), which is consistent with the observation that BB-sap treatment did not significantly impact Npr1+ neurons (Figure 4f) and Sstr2+ neurons (Supplementary Figure S2d), and that BB-sap mice treatment did not significantly impact Grpr+ (Figure 4g) and Sstr2+ neurons (Supplementary Figure S3a). Thus, although these subpopulations in the spinal cord are relatively independent, they form a neural circuit in the spinal cord to convey itch sensation (Huang et al., 2018a; Mishra and Hoon, 2013; Sun et al., 2009).

Both histaminergic and nonhistaminergic pathways contribute to SADBE-induced prolonged itch

Our evidence showed that SADBE treatment resulted in elevated levels of histamine (Figure 5a), and genes encoding HRH1 and HRH4 or the enzymes critical to histamine synthesis in the nape skin and cervical DRGs (Figure 5b–d and Supplementary Figure S5a and b). In particular, RNA-scope double staining results also supported that in DRGs of SADBE mice, 70.9% (61 of 86 cells) of Nppb+ cells cosedimented with Hrh1+ (Supplementary Figure S2b), but only a
few $\text{Grp}^+$ cells costained with $\text{Hrh1}^+$ (Figure 5e) and $\text{Nppb}^+$ (Figure 5g), which are consistent with previous data (Huang et al., 2018a; Solinski et al., 2019) and indicate that the BNP/NPRA pathway is histamine-responsive (Solinski et al., 2019) and important to SADBE-induced prolonged itch.

Finally, we again performed behavior tests to assess whether SADBE-induced prolonged itch is affected by targeting histamine or nonhistamine pathways. Targeting histamine pathways by using the selective H1R antagonist olopatadine and H4R antagonist JNJ7777120 (Dunford et al., 2007; Ohsawa and Hirasawa, 2012) significantly attenuated histamine-induced (Figure 6a) but did not affect i.t. GRP-induced acute itch (Figure 6b). We found that SADBE-induced prolonged scratching was inhibited after intraperitoneal (Figure 6c), i.t. (Supplementary Figure S5c and d), or intradermal (Supplementary Figure S5e and f) administration of olopatadine and JNJ7777120. The remaining SADBE-induced scratching behavior in $\text{Grpr}^{-/-}$ mice could be further reduced by coinjection of olopatadine and JNJ7777120 (Figure 6d). Intraperitoneal or i.t. injection of the
Figure 6. BNP/NPRA and GRP/GRPR convey histamine-dependent and -independent components of SADBE-induced prolonged itch, respectively. (a) Acute itch in C57 mice induced by histamine (500 μg, i.d.) but not by (b) GRP (0.1 nmol, i.t.) was significantly reduced after treatment with H1R antagonist olopatadine (i.p. 10 mg/kg) and H4R antagonist JNJ7777120 (i.p. 30 mg/kg) or their coinjection. (c) Chronic itch induced by SADBE in C57 mice or (d) Grpr KO mice was significantly reduced by olopatadine and JNJ7777120 or their coinjection. (e–h) Acute itch induced by CQ (200 μg, i.d.) and histamine in (e) Grpr KO mice, (f) BB-sap treated mice, (g) Npr1 KO mice, or histamine, CQ, SLIGRL-NH2 (100 μg, i.d.), compound 48/80 (100 μg, i.d.), 5-HT (10 μg, i.d.), and ET-1 (50 ng, i.d.) in (h) BNP-sap treatment mice, respectively. (i) Schematic diagram of proposed model of the itch microcircuit in SADBE-induced prolonged itch. Repeated exposure of SADBE results in the release of a variety of inflammatory mediators, which act at least directly or indirectly through two distinctive itch peptides (GRP and BNP) and spinal interneurons expressing neuropeptide receptors (GRPR and NPRA). The histamine-dependent component relies on BNP and...
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First-generation H1R antagonist diphenhydramine (Dunford et al., 2007; Ohsawa and Hirasawa, 2012) also significantly reduced prolonged itch in SADBE mice (Supplementary Figure S5g and h). As supplemental evidence, chloroquine-induced acute itch was significantly reduced in Grpr KO mice (Figure 6e) or abolished in BB-sap mice (Sun et al., 2009) (Figure 6f), whereas histamine-induced acute itch was significantly attenuated in Npr1 KO mice (Figure 6g) and BNP-sap mice (Figure 6h).

**DISCUSSION**

In this study, we have delineated the important role of spinal GRPR and NPRA in the central sensitization and prolonged pruritus associated with experimental ACD. By using a combination of neurochemical, genetic, and neurotoxin methods, our work identified that both GRPR and NPRA relay the chronic itch signal transmission. In SADBE-induced prolonged itch, both histamine and nonhistamine signaling pathways are activated, which are mainly processed by BNP/NPRA and GRP/GRPR, respectively (Figure 6i), with a unique spinal neurocircuit, as Grpr+ neurons act as a downstream switch of Npr1+ neurons (Figure 6j).

SADBE-induced aberrant responses in the immune system and activation of neurons and thus complicated neuro-immune interactions mediating the disease progression. Our data indicated that SADBE-induced ACD resulted in significant upregulation of genes encoding various cytokines in afflicted skin, significantly increased serum IgE, and increased spleen weight. In skin or DRGs, the mRNA levels of proinflammatory cytokines (e.g., IL-4, IL-1β, and TNF-α) and a number of known itch molecules such as TRPA1, H1R, H4R, GRP, BNP, and Mrgprs were significantly elevated. Some cytokines or itch mediators, namely IL-4, GRP, BNP, and H4R, were still expressed at high levels in skin or DRGs 3 weeks after the last SADBE painting. As recent data suggested, IL-4, IL-5, IL-31, and IL-33 have receptors, expressed in both mouse and human sensory neurons, that facilitate local depolarization and the action potential by which chronic itch was evoked and maintained (Dong and Dong, 2018; Kiguchi et al., 2016; Nattemper et al., 2018). In all, epidermal hyperplasia, dermal inflammation, mast cell infiltration, and upregulation of cytokines and itch mediators make up the innate and adaptive immune system responses induced by SADBE, and multifaceted interactions between the nervous and immune systems may account for the induction and maintenance of prolonged itch.

Our study also provided several lines of evidence for proposing a spinal circuit for BNP/NPRA and GRP/GRPR in SADBE-induced prolonged itch. The expression of GRPR and NPRA was increased in the spinal cord of SADBE mice. I.t. injection of GRPR and NPRA antagonists alleviated SADBE-induced prolonged itch. Consistently, SADBE-induced prolonged itch was significantly inhibited in mice after genetic blockade of GRPR or NPRA or selective ablation of Grpr+ or Npr1+ neurons. Finally, our cell population characterization data supported that Grpr+, Npr1+, and Sstr2+ neurons overlapped very little in the dorsal spinal cord (Huang et al., 2018a; Mishra and Hoon, 2013) (Figure 4e and Supplementary Figure S4) and almost all Grpr+ (Aresh et al., 2017) and Npr1+ neurons are excitatory (Figures 2h and i and 3h and i) whereas Sst2a+ neurons are inhibitory, as previously reported (Kardon et al., 2014). Together, the staining results and mouse behavioral data are consistent with the notion that BNP activates Npr1+ neurons, which then activate Grpr+ neurons in the spinal cord (Huang et al., 2018a; Mishra and Hoon, 2013). Because GRPR neurons act as a downstream switch, the ablating of GRPR+ neurons with BB-sap resulted in a blockade of signaling from NPRA, SST2A+ and NMBR and consequently led to itch abolishment.

A variety of cell types within the skin can secrete histamine and, in turn, histamine can act on a large variety of cell types in the skin and neuronal tissues in cases of allergy, inflammation, and itch because its receptors are widely expressed (Albrecht and Dittrich, 2015). However, chronic itch is generally believed to be histamine-independent and resistant to antihistamines. We found that SADBE treatment significantly increased the expression of histamine and their synthases in skin and the expression of Hrh1 and Hrh4 in the skin and DRGs. When given peripherally, spinal, or systemically, selective H1R and H4R antagonists significantly attenuated SADBE-induced long-term itching. Mast cells, as one of the main sources of histamine release in the skin, were significantly increased in the dermis after SADBE treatment (Figure 1f and i). In contrast, our analysis revealed a significant upregulation of genes encoding different cytokines in the nape skin (Supplementary Figure S1a and b), TRP channels, Mrgprs, and peptides such as Grp and Nppb in the nape skin (Figure 1j–l) and cervical DRGs (Supplementary Figure S1c), indicating that the pathogenesis involved was not restricted to only a histamine-dependent pathway.

In all, our findings demonstrated that both peripheral and central sensitization occurs in chronic itch settings. In SADBE mice, peripheral sensitization may be initiated by aberrant cutaneous immune responses and promoted by the crosstalk between the immune system and sensory neurons. In the spinal cord, central sensitization takes place using a complicated network that includes the primary afferents, spinal cord neurons and glia, and even descending fibers (Basbaum et al., 2009; Dong and Dong, 2018; Ji et al., 2016). Combined strategies for refractory itch are needed that target both peripheral and central sensitization and, related to our work more specifically, either directly or indirectly target BNP/NPRA or GRP/GRPR systems. One of the priorities of our work in clinical research is to develop more specific, small molecule inhibitors with fewer side effects that would target either BNP/NPRA or GRP/GRPR systems. Our work also suggests that combined its receptor NPRA, and the histamine-independent component relies on GRP and its receptor GRPR. (j) Dorsal horn interneurons, to a large extent, do not co-express these two receptors, but after activation of NPRA+ (also others like SST2A+ and NMBR+ neurons) neurons, itch information is transmitted by GRPR+ neurons to the spinal projection neurons then to the brain, thereby forming or maintaining the prolonged itch. BB-sap, bombesin-saporin; BNP-sap, brain-derived natriuretic peptide—saporin; CQ, chloroquine; ET-1, endothelin-1; 5-HT, serotonin; i.d., intradermal; i.p., intraperitoneal; i.t., intrathecal; KO, knockout; SADBE, squaric acid dibutylester.
interventions that target both histamine and nonhistamine pathways could achieve better therapeutic effects for ACD. In all these, interventions could reduce the use of immunosuppressants or steroids, which are still common treatments for chronic itch (Leslie et al., 2015).

**MATERIALS AND METHODS**

**Mice**

C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Breeding breeders of Grpr KO mice (Hampton et al., 1998; Zhao et al., 2013), GRPR-eGFP mice (Zhao et al., 2013), and Npr1 KO mice (Oliver et al., 1997) were from Dr. Zhoufeng Chen at Washington University in St. Louis, Missouri. Grpr KO mice were continuously backcrossed to C57BL/6J background. GRPR-eGFP mice were continuously backcrossed to CD1 background. All mice used for behavior tests were genotyped and allocated to experimental groups or control groups. Animals were housed in specific pathogen-free rooms at a 12-hour light-dark cycle with suitable relative humidity (55 ± 5%) and temperature (22 ± 2 °C). All animal experiments were performed under protocols approved by the Animal Studies Committee of Guangzhou Medical University.

**Itch behavioral tests**

Acute and chronic behaviors were performed as previously described (Sun and Chen, 2007; Zhao et al., 2014). The contact sensitizer SADBE (Sigma, St. Louis, MO) was used to induce the mouse model of ACD (Feng et al., 2017; LaMotte, 2016; Luo et al., 2018; Qu et al., 2015, 2014). Neurotoxins BB-sap and BNP-sap were administered to C57BL/6J mice for spinal GRPR+ neuron (Sun et al., 2009) or NPARA+ neuron (Huang et al., 2018a; Mishra and Hoon, 2013; Sun et al., 2009) ablation. For detailed information of all drugs and reagents used in this study, please see the Supplementary Materials and Methods.

**Immunohistochemistry and RNAscope in situ hybridization assay**

Skin tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with H&E or toluidine blue or immunofluorescent staining following the standard protocols. Skin tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with H&E or toluidine blue or immunofluorescent staining following the standard protocols. Immunofluorescence staining and RNAscope in situ hybridization assay of DRG and spinal cord tissues were performed as described previously (Munanaire et al., 2018). For information about all antibodies and probes used and imaging analysis, see the Supplementary Materials and Methods.

**RNA extraction and qPCR analysis**

Freshly obtained samples of mouse skin, DRG, and spinal cord tissues were stored in RNA later solutions (Qiagen, Hilden, Germany) in a refrigerator at −80 °C. To extract whole tissue RNA, the samples were homogenized using a homogenizer and processed using the RNeasy Mini Kit (Qiagen). After total RNA extraction, samples were treated with DNase (Turbo DNA Free Kit, Invitrogen, Carlsbad, CA). cDNA was prepared using cDNA was prepared using PrimeScript RT Master Mix (Cat#RR036A, Takara, Kusatsu, Japan). All of these processes were performed following the manufacturer’s instructions. qPCR was performed with SYBR Premix Ex Taq enzyme (Takara, Kusatsu, Japan), and all samples were assayed in triplicate. NCBI primer blast was used to design the primers and in general, genomic sequences across introns were selected. The primer sequences are listed in Supplementary Table S1.

**ELISA and multiplex Luminex assay**

Serum samples were taken from mice at different time points after the last SADBE painting followed by centrifugation at 3,000g for 10 minutes at 4 °C. The total IgE and different cytokine levels at different time points in mouse serum or skin were measured using an ELISA kit (Biolegend, San Diego, CA, Cat #432404) and multiplex Luminex assay kit (Invitrogen, Cat #PPX-20) following the manufacturer’s instructions.

**Statistical analyses**

Values were presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 5 (GraphPad, San Diego, CA). Unpaired t-test was used to analyze statistical comparisons between two groups. Multiple comparisons were compared by one-way ANOVA followed by Bonferroni’s post hoc tests. P < 0.05 was considered statistically significant.

**Data availability statement**

No dataset was created related to this article. Data are available upon request.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: XL, ZZ, AT; Funding Acquisition: AT; Investigation: XL, DW, YW, LZ, YL, TT; Methodology: XL, DW, LZ; Supervision: AT; Writing - Original Draft Preparation: XL, ZZ, AT; Writing - Review and Editing: XL, ZZ, AT.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.01.016.
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**SUPPLEMENTARY MATERIALS AND METHODS**

**Drugs and reagents**

GRP18-27 (Cat# H-3120), brain-derived natriuretic peptide (BNP, Cat# H-7558), SLIGRL-NH2 (Cat# H-5078), and GRPR antagonist deamino-Phe19,D-Ala24,D-Pro26-D-Phe27-GRP (Cat# H-2756) were from Bachem (King of Prussia, PA). Histamine (Cat# H7250), chloroquine (Cat# C6628), squaric acid dibutylester (SADBE, Cat# 339792), compound 48/80 (Cat# C2313), serotonin (Cat# H9523), histamine H1R antagonist olopatadine (Cat# O0391), diphenhydramine (Cat# D3630), and H4R antagonist NJ7777120 (Cat# J3770) were purchased from Sigma (St. Louis, MO). Bombesin-saporin (Cat# IT-40-25), BNP-saporin (Cat# IT-69-25) and blank-saporin (Cat# IT-21-25) were made by Advanced Targeting Systems (San Diego, CA). Endothelin (Cat# 1160), octreotide (Cat# 1818 ), Npr1 antagonist anantin (Cat# A2278), A967079 (Cat# 4716), and norbinaltorphimine (Cat# 0347) were purchased from Tocris (Minneapolis, MN). Doses of drugs and injection routes are indicated in figure legends.

**Acute scratching behavior tests**

Male mice (8–12 weeks old) were used for behavioral testing unless otherwise stated. All mice were acclimated to the environment for at least one day before the experiment. Scratching behavior was video recorded from a side angle (HDR-CX190 camera, Sony). The video was then viewed by persons who were unaware of the treatments or mouse genotypes to quantify mouse scratching behavior.

Itch behaviors were performed as previously described (Sun and Chen, 2007; Zhao et al., 2014). Mice were transferred to plastic chambers (10 x 10.5 x 15 cm) 30 minutes before the experiment, from which they were quickly removed for drug injection. The injection volume was 5 µl for intrathecal (i.t.) administration into the subarachnoid space or 50 µl intradermal injection into the skin of one side of the nape of neck. After the injection, the mice were quickly placed back into the chambers. The scratching behavior was identified by lifting the hind limbs to the neck (intradermal) or trunk (i.t.) injection sites and the returning of the hind limbs back. The scratches to the side of the intradermal injection site were counted for the intradural test and scratches to both sides of the lower limbs were counted for the i.t. test. The dosages used are listed below (exceptions will be mentioned in the figure legends).

For intradermal injection, histamine (500 µg), chloroquine (200 µg), endothelin-1 (50 ng), serotonin (10 µg), compound 48/80 (100 µg), and SLIGRL-NH2 (100 µg) were dissolved in sterile saline and administered in a volume of 50 µl per mouse.

For intrathecal injection, NPPB (5 µg), GRP (0.5 nmol), GRPR antagonist deamino-Phe19,D-Ala24,D-Pro26-D-Phe27-GRP (0.5 nmol), Npr1 antagonist anantin (2 and 10 nmol), kappa-opiod receptor antagonist norbinaltorphimine (75 µg), and the somatostatin-receptor agonist octreotide (100 ng) were all prepared in sterile saline and administered in a volume of 5 µl per mouse.

For the antihistamine pharmacological experiments, a first-generation antihistamine, diphenhydramine (10 mg/kg), or the H1R antagonist olopatadine (10 mg/kg) and H4R antagonist NJ7777120 (30 mg/kg) were tested using intraperitoneal injections 30 minutes before the start of behavior recording. Specific doses are listed in the figure legends.

**Assessment of motor function**

As previously described (Sun et al., 2009), a rotating system for accelerated treadmills was used to assess coordinate motor activity and general motor function of the mice. The animals were tested three times at 15-minute intervals, and the average latency of each drop of the animals from the rotating rod was averaged as the result.

**Mouse model of allergic contact dermatitis**

The contact sensitizer SADBE (Sigma) was used to induce allergic contact dermatitis in mice. Once a day for three consecutive days, 20 µl of 1% SADBE (dissolved in acetone) was applied to the shaved abdomen skin of mice. Five days later, the SADBE treatment group was challenged with 20 µl of 1% SADBE topically applied to the shaved nape skin once daily for three consecutive days, whereas acetone alone was used as a vehicle control.

Spontaneous scratching behavior with the hind paws was quantified by recording the number of scratches for 60 minutes. The behavioral results of the first day refer to observations 24 hours after the last SADBE application. To obtain a time course of SADBE-induced chronic itch, scratching was consistently observed at 10 AM on days 1, 3, 5, 7, 9, 11, 14, 21, 28, and 35 after the last SADBE painting. Also, at 3, 7, 14, 21, and 35 days after the last SADBE painting, samples of the back skin after SADBE treatment and their controls (painted with acetone only) for a comprehensive analysis including histology, ELISA, immunostaining, qPCR, and RNAscope analysis as shown in Results.

**Histology and immunostaining of skin or spinal cord tissue**

Skin tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned in 5-µm sections on a microtome. The sections were stained with H&E- or toluidine blue—stained sections were photographed with a light microscope. The thickness of epidermis was measured, the number of mast cells was counted using Image software, and the mean of at least 10 randomly selected sections was calculated for each mouse.

For immunofluorescent staining, skin paraffin sections (5 µm) were deparaffinized and then incubated in a blocking solution containing 3% goat serum and 0.1% Triton X-100 in PBS at room temperature for 2 hours. Specimens were then incubated with primary antibodies in blocking solution at 4 °C overnight. A mouse anti-Krt14 antibody (cytokeratin 14; 1:300, Cat No. Ab7800, Abcam, Cambridge, United Kingdom) was used for labeling keratinocytes. FITC-conjugated avidin (1:1,000, Cat No. 434411; Thermo Fisher Scientific) was used for labeling keratinocytes. FITC-conjugated avidin (1:1,000, Cat No. 434411; Thermo Fisher Scientific) was used for labeling keratinocytes. FITC-conjugated avidin (1:1,000, Cat No. 434411; Thermo Fisher Scientific) was used for labeling keratinocytes. FITC-conjugated avidin (1:1,000, Cat No. 434411; Thermo Fisher Scientific) was used for labeling keratinocytes.
Alexa Fluor 488 (Cat No. A11001, Thermo Fisher Scientific) was used for staining for 2 hours at room temperature. DAPI (Invitrogen) was used for staining of nuclei. After washing, tissue sections were mounted using Fluoromount-G and imaged after drying.

Immunofluorescence staining of spinal cord tissues was performed as described (Zhao et al., 2007). Briefly, following the standard protocol, mice were anesthetized and perfused, and spinal cord tissues were immediately removed, post-fixed in 4% paraformaldehyde overnight at 4 °C, and cryoprotected in 25% sucrose. Spinal cord sections (20 μm) were obtained via a cryostat and were incubated in blocking solution for 2 hours at room temperature. The sections were then incubated with primary antibodies overnight at 4 °C; washed three times in 0.1% Triton X-100 in PBS, and incubated with the secondary antibodies for 2 hours at room temperature. After washing, the sections were mounted on slides with Fluoromount-G (Southern Biotech, Birmingham, AL) and coverslipped. The following antibodies were used: mouse anti-CGRP (1:3,000; Cat No. AB1971, Abcam), rabbit anti-PKCγ (1:500; Cat No. sc-211, Santa Cruz Biotechnology, Dallas, TX); GFP (1:500; Cat No. ab13970, Abcam), goat anti-rabbit Alexa 488 conjugated (Cat No. A11008, Thermo Fisher Scientific), goat anti-mouse Alexa 488 conjugated (Cat No. A11001, Thermo Fisher Scientific), goat anti-rabbit Alexa 568 conjugated (Cat No. A11011, Thermo Fisher Scientific), goat anti-mouse Alexa 568 conjugated (Cat No. A11004, Thermo Fisher Scientific). All imaging and analysis were performed on a Nikon C2+ Confocal Microscope. At least three mice/group and 10 cervical spinal cord (c4–c6) sections from each mouse were included for statistical comparison.

**RNAscope in situ hybridization assay**

RNAscope in situ hybridization assay was performed as described previously (Munanairi et al., 2018; Wang et al., 2012). Briefly, slides were incubated with hydrogen peroxide for 10 minutes, washed, mildly boiled in target retrieval reagents for 15 minutes, washed, and dried, and hydrophobic barriers were added around the sections. Protease III/Plus Reagent was applied for 15 minutes, washed, and sections were incubated with target probes for 2 hours. The following probes consisting of 20 ZF oligonucleotides and obtained from Advanced Cell Diagnostics were used: *Hrh1* (Cat No. 318771, target region bases 549–1,453; accession number NM_001252642.2), *Hrh4* (Cat No. 528021, target region bases 246–1,224; accession No. NM_153087.2), *Nppb* (Cat No. 425021, target region bases 4–777; accession No. NM_008726.5), *Gpr* (Cat No. 317861, target region bases 22–825; accession No. NM_175012.2), *Npr1* (Cat No. 484531, target region bases 941–1,882; accession No. NM_008727.5), *Grpr* (Cat No. 317871, target region bases 463–1,596; accession No. NM_008177.2), *Nmb* (Cat No. 406461, target region bases 25–1,131; accession No. NM_008703.2), *Pdyn* (Cat No. 318771, target region bases 33–700; accession No. NM_018863.3), *Sstr2* (Cat No. 437681, target region bases 2–1,159; accession No. NM_001042606.2), *Vglut2* (Slc17a6, Cat No. 319171, target region bases 1,986–2,998; accession No. NM_080853.3), and *Vgat* (Slc32a1, Cat No. 319191, target region bases 894–2,037; accession No. NM_009508.2). Slides were counterstained with DAPI (Molecular Probes, Eugene, OR) and coverslips were mounted with Fluoromount-G (Southern Biotech). Sections were subsequently imaged on a Nikon C2+ confocal microscope (Nikon Instruments, Inc.) in three channels with a ×20 objective lens. Cells were considered positive if three punctate dots were present in the nucleus and/or cytoplasm. For colocalization studies, dots associated with single DAPI stained nuclei were counted as copositive. Cell counting was performed by a person who was blinded to the experimental design.

**Bombesin-saporin treatment**

The mice were given a single i.t. injection of bombesin-saporin (400 ng/mouse) or blank-saporin (400 ng/mouse). These mice were used for behavioral experiments 14–21 days after toxin injection.

**BNP-saporin treatment**

Mice were treated with twice by i.t. injection of BNP-saporin (650 ng/mouse) or blank-saporin (650 ng/mouse) on day 1 and day 8. These mice were used for behavioral experiments 14–21 days after the second BNP-saporin injection.

**SUPPLEMENTARY REFERENCES**


Supplementary Figure S1. Strong upregulation of cytokines and itch mediators in nape skin and cervical DRG from SADBE mice. (a) The secreted cytokines at the protein levels in nape skin from SADBE mice analyzed by multiplex Luminex assay. (b, c) qPCR analysis showing mRNA levels of the indicated target genes in skin and DRG. *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Bonferroni’s test, all compared with day 0. DRG, dorsal root ganglion; SADBE, squaric acid dibutylester.
Supplementary Figure S2. Molecular expression in cervical spinal cord of BB-sap mice. (a) The number of CGRP⁺ and (b) PKCy⁺ cells detected by immunostaining or (c) Pdyn⁺ and (d) Sstr2⁺ cells detected by RNAscope ISH were comparable in BB-sap mice and blank-sap mice. Bar figures in (a–d, right) as the quantitative analysis data represented mean ± SEM. Student’s t-test, n = 4 mice/group. BB-sap, bombesin-saporin; blank-sap, blank-saporin; ISH, in situ hybridization. Bar = 50 μm.

Supplementary Figure S3. Molecular expression in cervical spinal cord of BNP-sap mice. (a, b) Pdyn⁺ (b) but not Sstr2⁺ (a) cells were significantly ablated in the cervical spinal cord of the BNP-sap group compared with that of the blank-sap group (***P < 0.001). Student’s t-test, n = 4 mice/group. Blank-sap, blank-saporin; BNP-sap, brain-derived natriuretic peptide–saporin; Ctrl, control. Bar = 50 μm.
Supplementary Figure S4. Characterization of SST2A⁺, NPRA⁺, and GRPR⁺ neurons in the spinal cord for itch. (a) Intrathecal injection of octreotide (100 ng, n = 11, 7) or nor-BNI (75 μg, n = 5) induced scratching that decreased in mice after BB-sap. (b) Intrathecal injection of octreotide (100 ng, n = 9), and nor-BNI (75 μg, n = 8, 9) induced scratching that decreased in mice after BNP-sap. All data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus the blank-sap, unpaired t-test. (c) Double RNAscope staining showed Sstr2⁺ rarely colocalized with Grpr⁺, and Npr1⁺ rarely colocalized with (d) Sstr2⁺, (e) Nmbr⁺, and (f) Dyn⁺ cells in the superficial dorsal horn. Bar = 25 μm. n = 3. BB-sap, bombesin-saporin; blank-sap, blank-saporin; BNP-sap, brain-derived natriuretic peptide-saporin; nor-BNI, norbinaltorphimine.
Supplementary Figure S5. SADBE-induced prolonged itch was driven by histamine-dependent systems. (a) qPCR analysis showing increased mRNA levels of *Hrh1* and *Hrh4* in skin and (b) cervical (c2–c7) DRG in mice with SADBE-induced chronic itch. For day 0, 3, 7, and 21 in (a, b), n = 5. (c–f) Itch behavior tests in C57 mice 7 days after the last SADBE painting and treated with the selective H1R antagonist olopatadine (i.p. 10 mg/kg) and H4R antagonist JNJ7777120 either intrathecally (i.t. 3 and 10 μg) (c, d, n=7), or intradermally (i.d. 0.3 and 0.9 μmol) (e, f, n=7). (g, h) Prolonged itch 7 days after the last SADBE painting was significantly attenuated by diphenhydramine (g, 10 mg/kg, i.p.; h, 0.3 and 0.9 μg, i.t.). DRG, dorsal root ganglion; i.d., intradermal; i.p., intraperitoneal; i.t., intrathecal; SADBE, squaric acid dibutylester.
### Supplementary Table S1. Primers Used for qPCR

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