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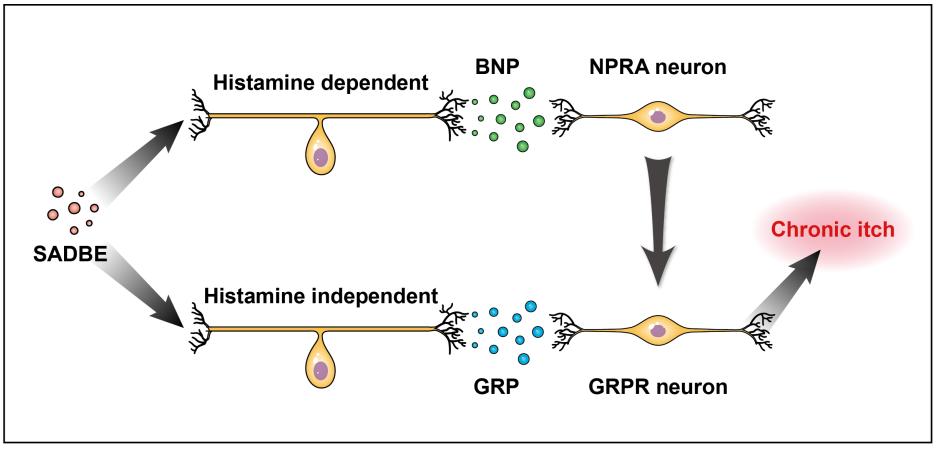
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Spinal GRPR and NPRA contribute to chronic itch in a murine model of allergic contact dermatitis.

Xueting Liu[1], De Wang[1], Yuhuan Wen[1], Liping Zeng[1], Yangyang Li[1], Tianyu Tao[1], Zhongqiu Zhao[2,3], Ailin Tao[*1]

[1]The Second Affiliated Hospital, The State Key Laboratory of Respiratory Disease, Guangdong Provincial Key Laboratory of Allergy & Clinical Immunology, Center for Immunology, Inflammation, & Immune-mediated disease, Guangzhou Medical University, Guangzhou 510260, China.

[2]Center for the Study of Itch, Department of Anesthesiology, Washington University School of Medicine.

[3]Barnes-Jewish Hospital, St. Louis, MO 63110, USA.

Corresponding author: Ailin Tao, PhD, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou 510260, China. Tel: 86-020-34153520, Fax: 86-020-34153520, E-mail: taoailin@gzhmu.edu.cn.

Abbreviations.

ACD: Allergic contact dermatitis
BNP: Brain-derived natriuretic peptide
DRG: Dorsal root ganglion
ET-1: Endothelin-1
GRP: gastrin-releasing peptide
GRPR: gastrin-releasing peptide receptor

eGFP: Enhanced green fluorescent protein
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GFP: Green fluorescent protein
H&E: Hematoxylin and eosin
NMB: Neuromedin B
NMBR: Neuromedin B receptor
NPR1: Natriuretic peptide receptor A
qPCR: Quantitative real-time PCR
SADBE: Squaric acid dibutylester
SST: somatostatin
SST2A: somatostatin receptor subtype 2A
TRPA1: Transient receptor channel potential cation channel ankyrin subtype 1
TRPV1: Transient receptor potential cation channel vanilloid subtype 1
TRPV4: Transient receptor potential vanilloid 4
TSLP: Thymic Stromal Lymphopoietin
WT: Wild-type

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Abstract

Recurrent and intractable chronic itch is a world-wide 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 (ACD) induced by squaric acid dibutylester (SADBE). We found that repeated exposure of mice to SADBE evoked persistent spontaneous scratching and significantly aberrant cutaneous and systemic immune responses lasting for weeks. SADBE induced itch requires both nonhistaminergic and histaminergic pathways, which are likely relayed by gastrin-releasing peptide receptor (GRPR) and natriuretic peptide receptor A (NPRA) in the spinal cord respectively. Employing genetic, pharmacology, RNAscope assay and cell-specific ablation

methods, we dissected a neural circuit for the 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 ACD associated chronic pruritus.

Keywords

Allergic contact dermatitis, GRPR (Gastrin-releasing peptide receptor), NPRA (Natriuretic peptide receptor A), Spinal cord

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, psoriasis) as well as a variety of non-cutaneous disorders that exacerbates skin discomfort and affects quality of life (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, gastrin releasing peptide (GRP) and natriuretic polypeptide b

(Nppb, also known as 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, gastrin-releasing peptide receptor (GRPR) and natriuretic peptide receptor A (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 remained 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 dibutylester (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., 2014, Qu et al., 2015). Unlike the chemical hapten, 2, 4-dinitrofluorobenzene (DNFB) -induced itch model that has been used for many years but the 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., 2014, Qu et al., 2015). 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 as “C57”) mice via pre-sensitization and subsequent challenges (or elicitation). We modified the protocol (Feng et

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al., 2017, LaMotte, 2016, Luo et al., 2018, Qu et al., 2014, Qu et al., 2015) 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., 2014, Qu et al., 2015). 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 K. et al., 2018, LaMotte, 2016, Luo et al., 2018, Qu et al., 2014, Qu et al., 2015, 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 as "SADBE mice"). SADBE mice exhibited elevated serum IgE levels (Figure 1c), increased spleen weight (Figure 1d), thickening of the epidermis (Figure 1e, 1g-1h), and increased infiltration of mast cells in the dermis (Figure 1f and 1i). Multiplex Luminex assay (Figure S1a) and quantitative PCR (qPCR) analysis (Figure 1j-1l and Figure S1b) revealed that the treated nape of neck skin of SADBE mice displayed significant up-regulation of the transcripts or secreted cytokines at the protein level of genes recently reported to be associated with itch (Alikhan and Maibach, 2014, Dong, 2018, Feng et al., 2017, LaMotte, 2016, Luo et al., 2018, Mishra and Hoon, 2013, Qu et al., 2014, Qu et al., 2015, Sun and Chen, 2007, Sun et al., 2009) such as those encoding various cytokines including IL-4, IL-23, IL-33, IL-27, IL-1β, TNF-α, TSLP (Figure S1a-b), *Trpm8* (Figure 1j), *Mrgprx1* (Figure 1k), *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 non-histaminergic 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 ISH (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 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* 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, the majority of *Grpr*+ cells were ablated in the cervical spinal dorsal horn of BB-sap mice (Figure 2g). The superficial dorsal horn neurochemical markers, like CGRP (Figure S2a) and PKCγ (Figure S2b) are comparable in BB-sap mice and blank-sap mice. The expression of three itch related markers, *Npr1* (Figure 4f), *Sstr2* (Figure S2c), and the dynorphin precursor, prodynorphine (*Pdyn*) (Huang J. et al., 2018, Kardon et al., 2014) (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

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(Aresh et al., 2017), the majority of *Grpr*$^+$ neurons in the spinal cord are excitatory interneurons (Figure 2h-2i).

NPRA relays SADBE-induced itch in the spinal cord

By qPCR analysis, we found that mRNA levels of *Nppb* in DRG were significantly enhanced on days 3 (Figure 3a), and mRNA levels of *Npr1* in spinal cord were significantly enhanced on days 7 and 21 (Figure 3b) after the last SADBE treatment. RNAscope analysis results also supported *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 used a neurotoxin, BNP-saporin (BNP-sap), to selectively ablate *Npr1*$^+$ neurons (Huang J. et al., 2018, 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 (Figure S3b) confirming their partial overlapping (Huang J. et al., 2018, Mishra and Hoon, 2013).We also double-stained *Npr1*$^+$ with *Vglut2* and *Vgat* and the results showed that the majority of *Npr1*$^+$ neurons expressed *Vglut2* (Figure 3h-3i).

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 spinal cord by GRPR$^+$- and NPR1$^+$-expressing neurons, we used intrathecal injections to evoke acute itch in BB-sap mice and BNP-sap mice, which have no deficits in motor activity(Huang J. et al., 2018, Mishra and Hoon, 2013, Sun et al., 2009) (Figure 4a). Firstly, when compared with the blank-sap group, scratching responses to

intrathecal BNP (Figure 4b), octreotide (an analog of somatostatin) and the KOR antagonist norbinaltorphimine (nor-BNI) (Figure S4a) were almost fully 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 J. et al., 2018, 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. Secondly, we confirmed that elimination of NPRA neurons attenuated scratching responses to intrathecal BNP (Figure 4c), but not to intrathecal GRP (Figure 4d), octreotide and nor-BNI (Figure S4b), confirming that in the spinal cord GRP, somatostatin and dynorphin act downstream (Huang J. et al., 2018, Mishra and Hoon, 2013) or independently of *Npr1*$^+$ neurons. Thirdly, double staining results further indicated that *Npr1*, *Grpr*, *Sstr2* and *Nmbr* expression largely did not overlap in the spinal cord (Figure 4e and S4c-e), which is consistent with the observation that BB-sap treatment did not significantly impact *Npr1*$^+$ (Figure 4f) and *Sstr2*$^+$ neurons (Figure S2d), and that BNP-sap mice treatment did not significantly impact *Grpr*$^+$ (Figure 4g), and *Sstr2*$^+$ neurons (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 J. et al., 2018, Mishra and Hoon, 2013, Sun et al., 2009).

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

Our evidence showed that SADBE treatment resulted in elevated levels of histamine (Figure 5a), and genes encoding histamine receptor subtype 1 (*Hrh1*) and 4 (*Hrh4*), or the enzymes critical to histamine synthesis in the nape skin and cervical DRGs (Figure 5b-5d, S5a-5b). In particular, RNAscope double staining results also supported that in DRGs of SADBE mice, 70.9% (61 of 86 cells) of *Nppb*$^+$ cells co-stained with *Hrh1*$^+$ (Figure S2B), but only few *Grp*$^+$ cells co-stained

with *Hrh1*$^{+}$ (Figure 5e) and *Nppb*$^{+}$ (Figure 5g), which are consistent with previous data (Huang J. et al., 2018, Solinski et al., 2019) and indicating 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 non-histamine 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 not affected GRP intrathecal induced acute itch (Figure 6b). We found SADBE-induced prolonged scratching was inhibited after intraperitoneal (i.p.) (Figure 6c), intrathecal (i.t.) (Figure S5c-5d) or intradermal (i.d.) (Figure S5e-5f) administration of olopatadine and JNJ7777120. The remaining SADBE induced scratching behavior in *Grpr* KO mice could be further reduced by co-injection of olopatadine and JNJ7777120 (Figure 6d). I.p. or i.t. injection of the first generation H1R antagonist diphenhydramine (Dunford et al., 2007, Ohsawa and Hirasawa, 2012) also significantly reduced prolonged itch in SADBE mice (Figure S5g-5h). As supplemental evidence, CQ-induced acute itch was significantly reduced in *Grpr* KO mice (Figure 6e) or abolished 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).

4. 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 allergic contact dermatitis (ACD). By using a combination of neurochemical, genetic, and neurotoxin methods our work identified both GRPR and NPRA relay the chronic itch signal transmission. In SADBE induced

prolonged itch both histamine and non-histamine 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 up-regulation of genes encoding various cytokines in afflicted skin, significantly increased serum IgE, increased spleen weight, etc. In skin or DRGs, the mRNA levels of pro-inflammatory cytokines (e.g., IL-4, IL-1β, TNF-α) and a number of known itch molecules such as TRPA1, H1R, H4R, GRP, BNP, MRGPRA3, etc., 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, 2018, Kiguchi et al., 2016, Nattkemper 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. Intrathecal 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 *Grpr*$^+$, *Npr1*$^+$, and *Sstr2*$^+$ neurons overlapped very little in the dorsal spinal cord (Huang J. et al., 2018, Mishra and Hoon, 2013)(Figure 4e and S4) and almost all *Grpr*$^+$ (Aresh et al., 2017) and *Npr1*$^+$ neurons are excitatory (Figure 2h-i and 3h-i) while sst_{2A}^+ 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 and then activate *Grpr*$^+$ neurons in spinal cord (Huang J. et al., 2018, Mishra and Hoon, 2013). Since GRPR neurons act as a downstream switch, the ablating of GRPR$^+$ neurons with BB-sap resulted in a blockade of signaling from NPRA, SST_{2A}, NMBR, etc., and consequently led to itch abolishment. A variety of cell types within the skin can secret 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 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, spinally 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 1i). On the other hand, our analysis revealed a significant up-regulation of genes encoding different cytokines in the nape skin (Figure S1a-b), TRP channels, *Mrgprs*, and peptides like *Grp* and *Nppb* in the nape skin (Figure 1j-l) and cervical DRGs (S1c) indicating that the pathogenesis involved was not restricted to only a histamine dependent pathway.

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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, 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 interventions that target both histamine and non-histamine pathways could achieve better therapeutic effects for ACD. In all these intervenes 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), *Npr1* KO mice (Oliver et al., 1997), were from Dr. Zhoufeng Chen at Washington University in St Louis. *Grpr* KO mice were continuously backcrossed to C57BL/6 J background. GRPR-eGFP mice 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 SPF rooms at a 12 h 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 squaric acid dibutylester (SADBE; Sigma) was used to induce mouse model of allergic contact dermatitis (Feng et al., 2017, LaMotte, 2016, Luo et al., 2018, Qu et al., 2014, Qu et al., 2015). Neurotoxins bombesin-saporin (BB-sap) and BNP-saporin (BNP-sap) were administered to C57BL/6J mice for spinal $GRPR^+$ neurons (Sun et al., 2009) or $NPRA^+$ neurons (Huang J. et al., 2018, Mishra and Hoon, 2013, Sun et al., 2009) ablation. Detailed information of all drugs and reagents used in this study please see the Supplementary Materials and Methods.

Immunohistochemistry and RNAscope in situ hybridization (ISH) assay

Skin tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or toluidine blue (TB), or immunofluorescent staining following the standard protocols. Immunofluorescence staining and RNAscope ISH assay of DRG and spinal cord tissues were performed as described (Munanairi et al., 2018). Information for all antibodies and probes were used and imaging analysis see the Supplementary Materials and Methods.

RNA extraction and Quantitative RT- PCR (qPCR) analysis

Freshly obtained samples of mouse skin, DRG and spinal cord tissues were stored in RNAlater solutions (Qiagen) 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). cDNA was prepared using SYBR Green Fast Master Mix (Cat#A25742, Thermo Fisher). All of the above operations were performed following the manufacturer's instructions. qPCR was performed with SYBR Premix Ex Taq enzyme (TAKARA) and all samples were assayed in triplicate. NCBI primer blast was used for design the primers and in general, genomic sequences across introns were selected. The primer sequences are listed in 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 3000 g for 10 minutes at 4°C. The total IgE and different cytokines levels at different time points in mouse serum or skin were measured using an ELISA kit (Biolegend, Cat #432404) and multiplex Luminex assay kit (Invitrogen, Cat #PPX-20) following the manufacturer's instructions.

Statistical analyses

Values were presented as the mean ± standard error of 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 analysis of variance (ANOVA) followed by Bonferroni's *post hoc* tests. $P < 0.05$ was considered statistically significant.

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DATA AVAILABILITY STATEMENT

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

Conflict of interest statement

The authors declare no competing interests.

Author contributions

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

Appendix A. Supplementary data

The supplementary material for this article can be found online at

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References

Albrecht M, Dittrich AM. Expression and function of histamine and its receptors in atopic dermatitis. Molecular and cellular pediatrics 2015;2(1):16.

Alikhan A, Maibach HI. Allergic contact dermatitis. Chemical immunology and allergy 2014;100:97-100.

Aresh B, Freitag FB, Perry S, Blumel E, Lau J, Franck MCM, et al. Spinal cord interneurons expressing the gastrin-releasing peptide receptor convey itch through VGLUT2-mediated signaling. Pain 2017;158(5):945-61.

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

Dong X. Peripheral and Central Mechanisms of Itch. Neuron 2018;98(3):482-94.

Dunford PJ, Williams KN, Desai PJ, Karlsson L, McQueen D, Thurmond RL. Histamine H4 receptor antagonists are superior to traditional antihistamines in the attenuation of experimental pruritus. The Journal of allergy and clinical immunology 2007;119(1):176-83.

Feng J, Yang P, Mack MR, Dryn D, Luo J, Gong X, et al. Sensory TRP channels contribute differentially to skin inflammation and persistent itch. Nature communications 2017;8(1):980.

Hampton LL, Ladenheim EE, Akeson M, Way JM, Weber HC, Sutliff VE, et al. Loss of bombesin-induced feeding suppression in gastrin-releasing peptide receptor-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 1998;95(6):3188-92.

Journal Pre-proof

Huang J, Polgar E, Solinski HJ, Mishra SK, Tseng PY, Iwagaki N, et al. Circuit dissection of the role of somatostatin in itch and pain. Nature neuroscience 2018;21(5):707-16.

Huang K, Hu DD, Bai D, Wu ZY, Chen YY, Zhang YJ, et al. Persistent Extracellular Signal-Regulated Kinase Activation by the Histamine H4 Receptor in Spinal Neurons Underlies Chronic Itch. The Journal of investigative dermatology 2018;138(8):1843-50.

Ji RR, Chamessian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. Science 2016;354(6312):572-7.

Kardon AP, Polgar E, Hachisuka J, Snyder LM, Cameron D, Savage S, et al. Dynorphin acts as a neuromodulator to inhibit itch in the dorsal horn of the spinal cord. Neuron 2014;82(3):573-86.

Kiguchi N, Sukhtankar DD, Ding H, Tanaka K, Kishioka S, Peters CM, et al. Spinal Functions of B-Type Natriuretic Peptide, Gastrin-Releasing Peptide, and Their Cognate Receptors for Regulating Itch in Mice. The Journal of pharmacology and experimental therapeutics 2016;356(3):596-603.

LaMotte RH. Allergic Contact Dermatitis: A Model of Inflammatory Itch and Pain in Human and Mouse. Advances in experimental medicine and biology 2016;904:23-32.

Leslie TA, Greaves MW, Yosipovitch G. Current topical and systemic therapies for itch. Handbook of experimental pharmacology 2015;226:337-56.

Luo J, Feng J, Yu G, Yang P, Mack MR, Du J, et al. Transient receptor potential vanilloid 4-expressing macrophages and keratinocytes contribute differentially to allergic and nonallergic chronic itch. The Journal of allergy and clinical immunology 2018;141(2):608-19 e7.

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Mishra SK, Hoon MA. The cells and circuitry for itch responses in mice. Science 2013;340(6135):968-71.

Munanairi A, Liu XY, Barry DM, Yang Q, Yin JB, Jin H, et al. Non-canonical Opioid Signaling Inhibits Itch Transmission in the Spinal Cord of Mice. Cell reports 2018;23(3):866-77.

Nattkemper LA, Tey HL, Valdes-Rodriguez R, Lee H, Mollanazar NK, Albornoz C, et al. The Genetics of Chronic Itch: Gene Expression in the Skin of Patients with Atopic Dermatitis and Psoriasis with Severe Itch. The Journal of investigative dermatology 2018;138(6):1311-7.

Ohsawa Y, Hirasawa N. The antagonism of histamine H1 and H4 receptors ameliorates chronic allergic dermatitis via anti-pruritic and anti-inflammatory effects in NC/Nga mice. Allergy 2012;67(8):1014-22.

Oliver PM, Fox JE, Kim R, Rockman HA, Kim HS, Reddick RL, et al. Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. Proc Natl Acad Sci U S A 1997;94(26):14730-5.

Qu L, Fan N, Ma C, Wang T, Han L, Fu K, et al. Enhanced excitability of MRGPRA3- and MRGPRD-positive nociceptors in a model of inflammatory itch and pain. Brain : a journal of neurology 2014;137(Pt 4):1039-50.

Qu L, Fu K, Yang J, Shimada SG, LaMotte RH. CXCR3 chemokine receptor signaling mediates itch in experimental allergic contact dermatitis. Pain 2015;156(9):1737-46.

Sakai K, Sanders KM, Youssef MR, Yanushefski KM, Jensen L, Yosipovitch G, et al. Mouse model of imiquimod-induced psoriatic itch. Pain 2016;157(11):2536-43.

Solinski HJ, Kriegbaum MC, Tseng PY, Earnest TW, Gu X, Barik A, et al. Nppb Neurons Are Sensors of Mast Cell-Induced Itch. Cell reports 2019;26(13):3561-73 e4.

Journal Pre-proof

Journal Pre-proof

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

Sun YG, Zhao ZQ, Meng XL, Yin J, Liu XY, Chen ZF. Cellular basis of itch sensation. Science 2009;325(5947):1531-4.

Yosipovitch G, Bernhard JD. Clinical practice. Chronic pruritus. The New England journal of medicine 2013;368(17):1625-34.

Zhao ZQ, Huo FQ, Jeffry J, Hampton L, Demehri S, Kim S, et al. Chronic itch development in sensory neurons requires BRAF signaling pathways. The Journal of clinical investigation 2013;123(11):4769-80.

Zhao ZQ, Wan L, Liu XY, Huo FQ, Li H, Barry DM, et al. Cross-inhibition of NMBR and GRPR signaling maintains normal histaminergic itch transmission. The Journal of neuroscience : the official journal of the Society for Neuroscience 2014;34(37):12402-14.

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Figure Legends

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, d**) Elevated serum IgE (**c**) and increased spleen weights (**d**). (**e-i**) Representative H&E staining (**g**) immunostaining of the epidermal marker Krt14 (**h**) and toluidine blue staining (**i**) of the neck skin sections. Quantitative analysis of enhanced epidermal thickness (**e**) and increased mast cell infiltration in the dermis (**f**), n = 3-4. (**j-l)** qPCR analysis showed mRNA levels of the indicated target genes in skin, n = 5. All scale bars, 50 μm. *P < 0.05, **P < 0.01, **P < 0.001, compared to day 0.

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 (c4-c6) , n = 3. In (**a-i)**, all quantitative data as mean ± SEM. **P < 0.01, ***P < 0.001, t test. All scale bars=25 μm.

Figure 3. NPRA is required for SADBE-induced prolonged itch. (**a,b**) Increased mRNA levels of *Nppb* in DRG **(a)** and *Npr1* in spinal cord **(b)** at 3, 7, and 21 days after the last SADBE treatment detected by qPCR analysis. n = 5. (**c)** RNAscope ISH assays of *Hrh1* on frozen cervical (c2-c7) DRG sections in SADBE mice at 21 days after the last SADBE painting. (**d-f**) Impaired SADBE-induced prolonged itch in C57 SADBE mice after injection of the NPRA antagonist Anantin (2 and 10 nmol i.t.) (**d**, n = 6-8), *Npr1* KO mice (**e**, WT n = 12, KO n = 8), or

BNP-sap treated mice (**f**, Control *n* = 14, BNP-sap n = 8). (**g**) *Npr1*$^+$ neurons were significantly ablated in the cervical (c4-c6) spinal cord of BNP-sap treated mice, *n* = 4. **(h,i)** RNAscope ISH double staining for *Npr1* with *Vglut2* or *Vgat*. All scale bars = 25 μm. All bar graphs represents mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *t* test compared to vehicles.

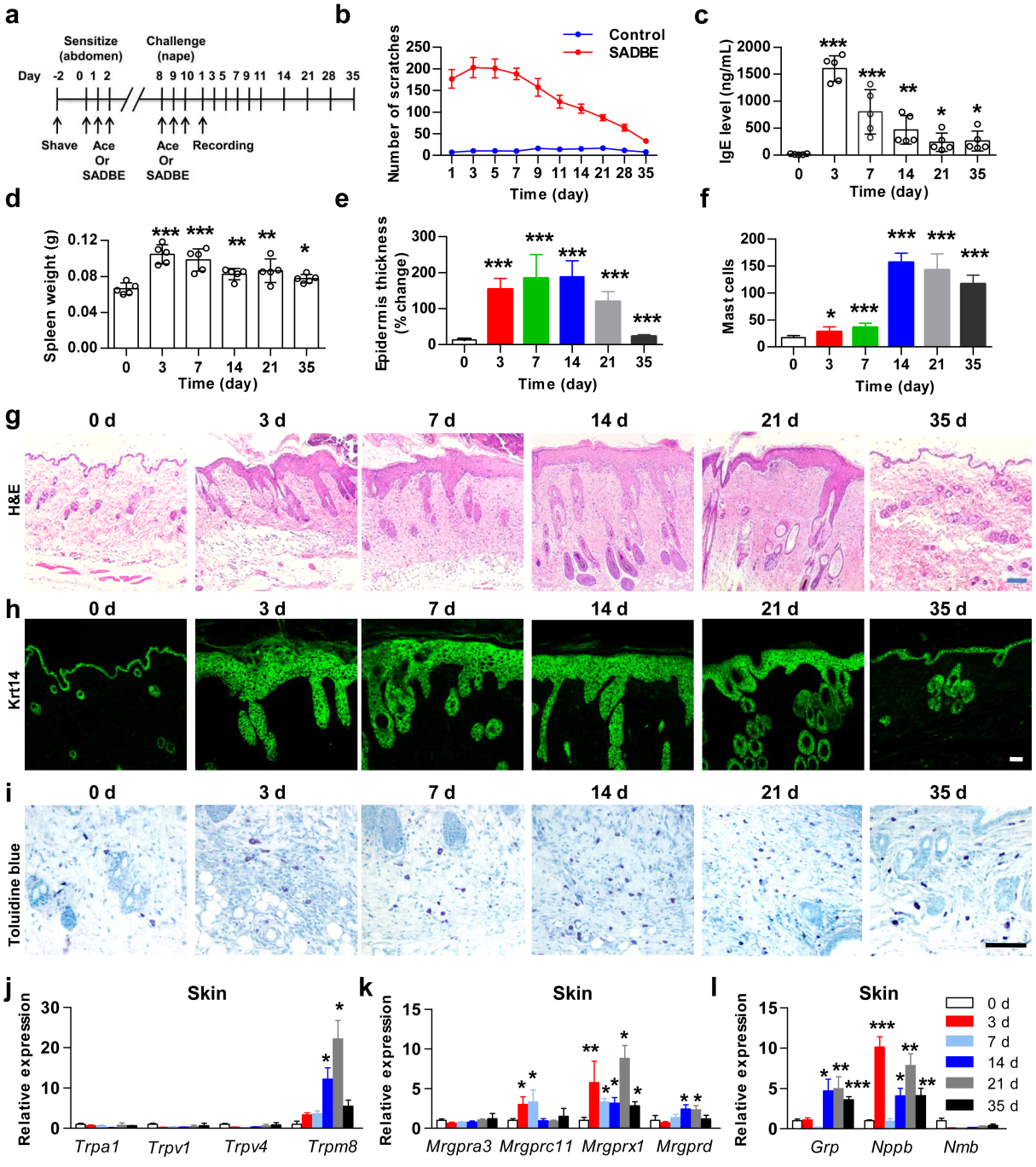
Figure 4. GRPR$^+$ neurons act downstream of NPRA$^+$ neurons in the spinal cord for itch. (**a**) Motor function assessed by the Rotarod test was comparable in mice 14 days after BB-sap (400 ng/mouse, i.t.), or the first BNP-sap (650 ng /mouse, i.t., twice) and blank-sap (650 ng/mouse, i.t.). *P* > 0.05. *n* = 8 each group. (**b-d**) i.t. injection of BNP (5 μg, *n* = 13) induced scratching in mice 14 days after bombesin-sap or blank-sap treatment. Or i.t. injection of BNP (c, 5 μg , *n* = 7, 8), GRR (d, 0.1 nmol , *n* = 8, 9) induced scratching in mice 14 days after BNP-sap and blank-sap treatment. (**e-g**) RNAscope staining showed that *Grpr*$^+$ rarely co-localized with *Npr1*$^+$ neurons in the cervical (c4-c6) spinal cord. Also *Npr1+* neurons were not significantly ablated in the BB-sap (**f**), or *Grpr*$^+$ neurons were not significantly ablated in the BB-sap (**g**) mice. All scale bars = 25 μm. *n* = 3.

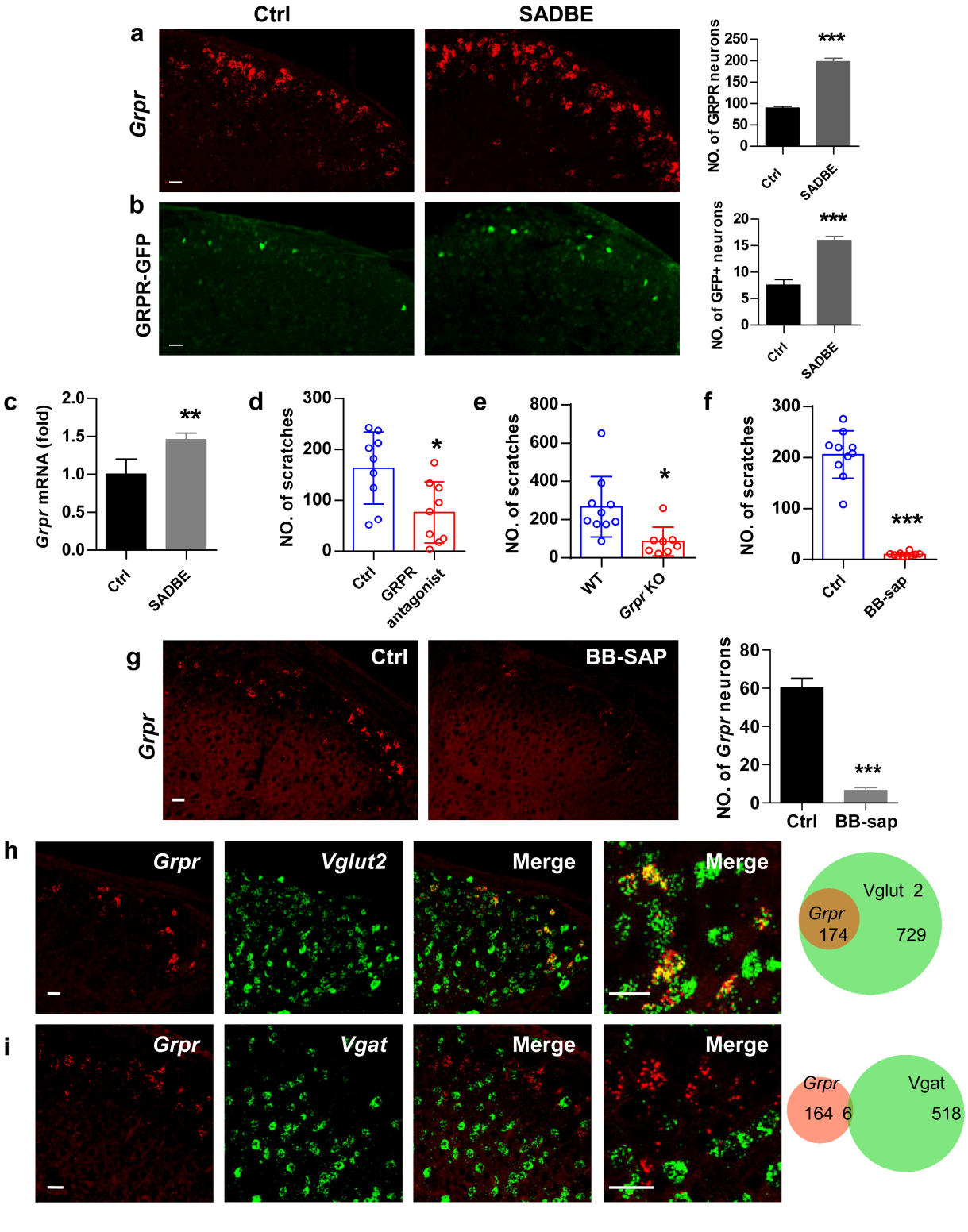
Figure 5. **Histamine dependent pathways were activated in skin and sensory neurons in SADBE mice.** (**a**) Representative immunostaining against histamine in the neck skin sections. **(b-c)** RNAscope ISH assays of *Hrh1* and *Hrh4* on nape skin paraffin (**b**) or frozen (**c**) cervical spinal (c2-c7) sections in the mice 21 days after the last SADBE painting. **(d)** RNAscope assays on frozen sections of cervical DRG (c2-c7) with *Hrh1* probes in SADBE mice at 21 days after the last SADBE painting. **(e-g)** Double RNAscope staining of *Hrh1* with *Grp* (**e**) or *Nppb* (**f**), or *Grp* with *Nppb* **(g)** on frozen cervical (c2-c7) DRG sections. Counting data is showed at the right of each panel. *n* = 3 / group. All scale bars, 50 μm.

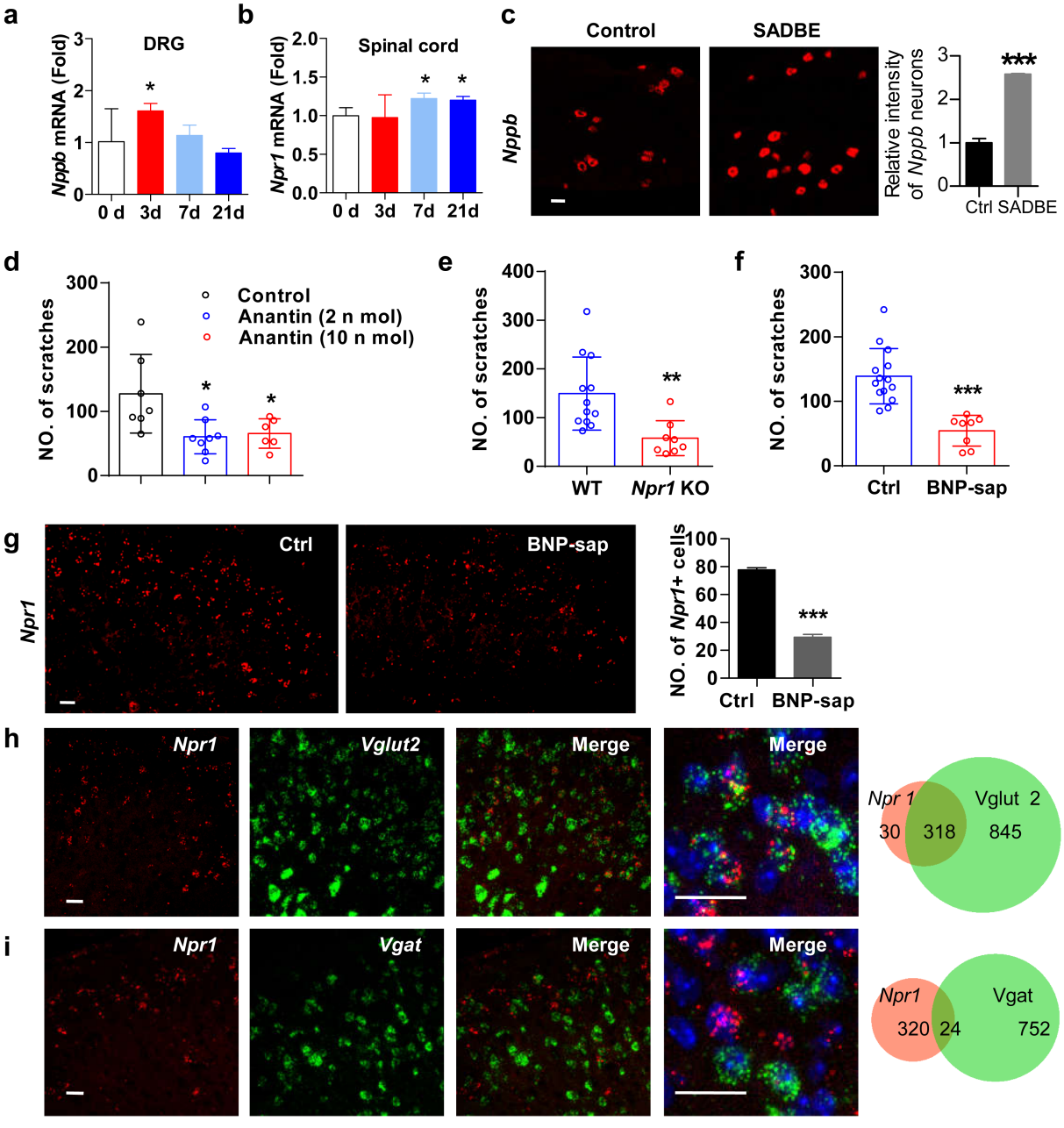
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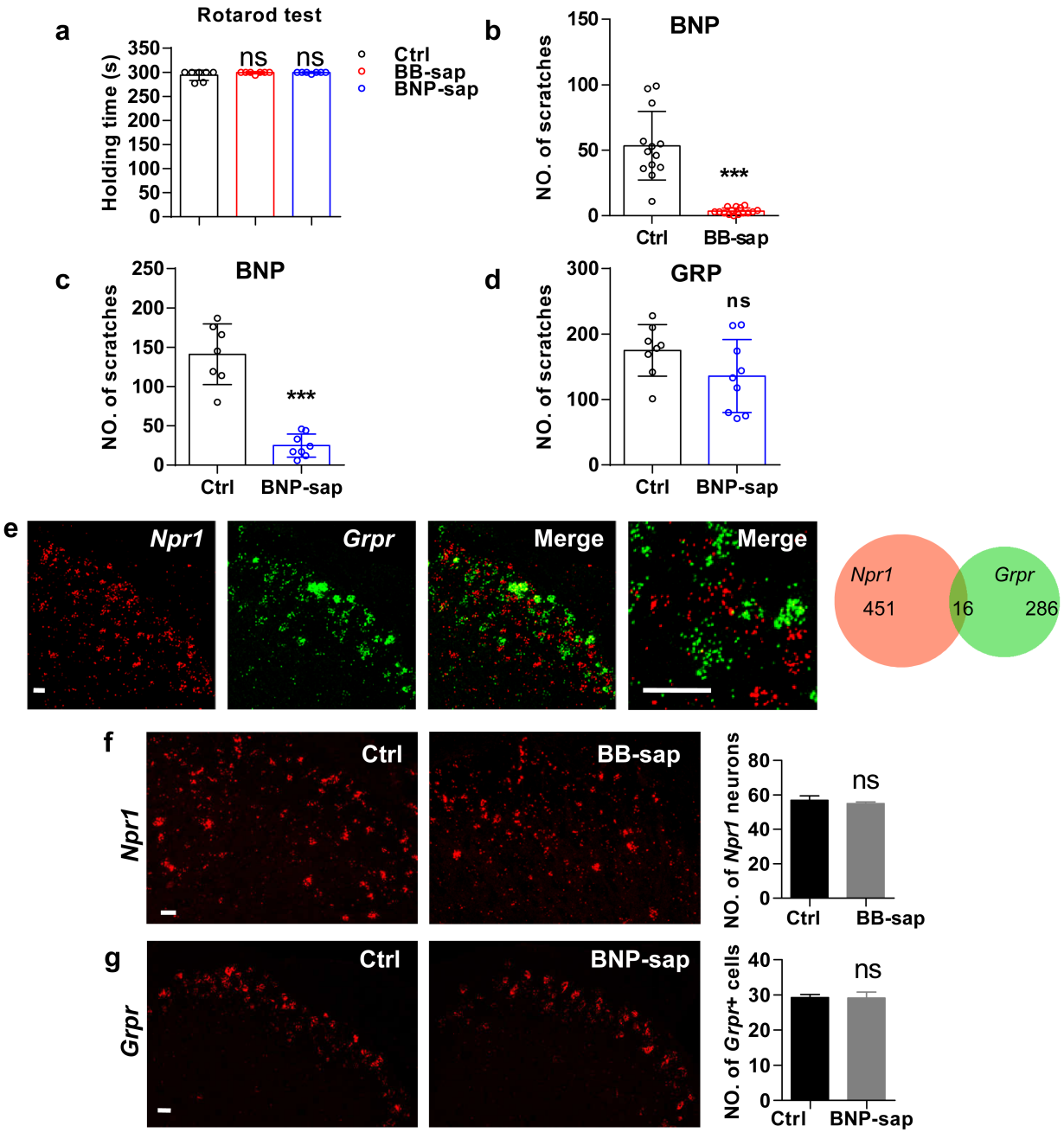
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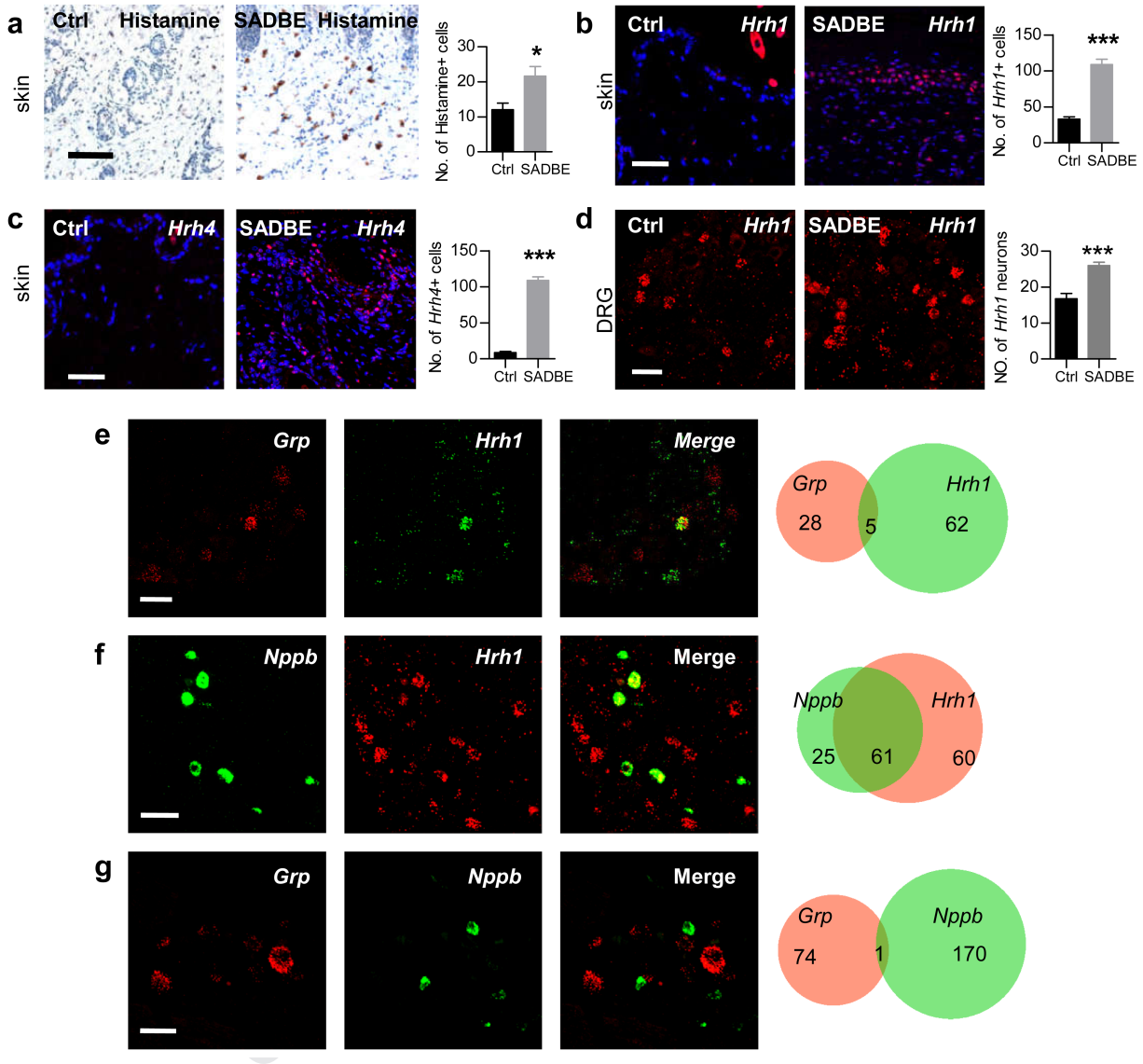
Figure 6. BNP/NPRA and GRP/GRPR convey histamine-dependent and -independent components of SADBE-induced prolonged itch respectively. (a and **b)** Acute itch in C57 mice induced by histamine (500 μg, i.d.. **a**) but not by GRP (0.1 nmol, i.t., **b**) was significantly reduced after treatment with H1R antagonist olopatadine (i.p. 10 mg/kg) and H4R antagonist JNJ7777120 (i.p. 30 mg/kg) or their co-injection . **(c)** Chronic itch induced by SADBE in C57 mice, or *Grpr* KO mice was significantly reduced by treated with olopatadine and JNJ7777120 or their co-injection. **(e-h)** Acute itch induced by choloroquine (CQ, 200 μg, i.d.) in *Grpr* KO mice **(e)**, bombesin-sap treated mice **(f)**, or induced by histamine (500 μg, i.d.) in *Npr1* KO mice **(g)**, BNP-sap treatment mice **(h)** respectively. **(i-j)** 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, BNP) and spinal interneurons expressing neuropeptide receptors (GRPR, NPRA). Histamine-dependent component relies on BNP and its receptor NPRA, and histamine-independent component relies on GRP and its receptor GRPR (**i**). Dorsal hon interneurons, to a large extent, do not co-express these two receptors but after activation of $NPRA^+$ (also others like SST_{2A}^+ and $NMBR^+$ neurons etc) neurons, itch information is transmitted by $GRPR^+$ neurons to the spinal projection neurons then to the brain, thereby forming or maintaining the prolonged itch (**j**).

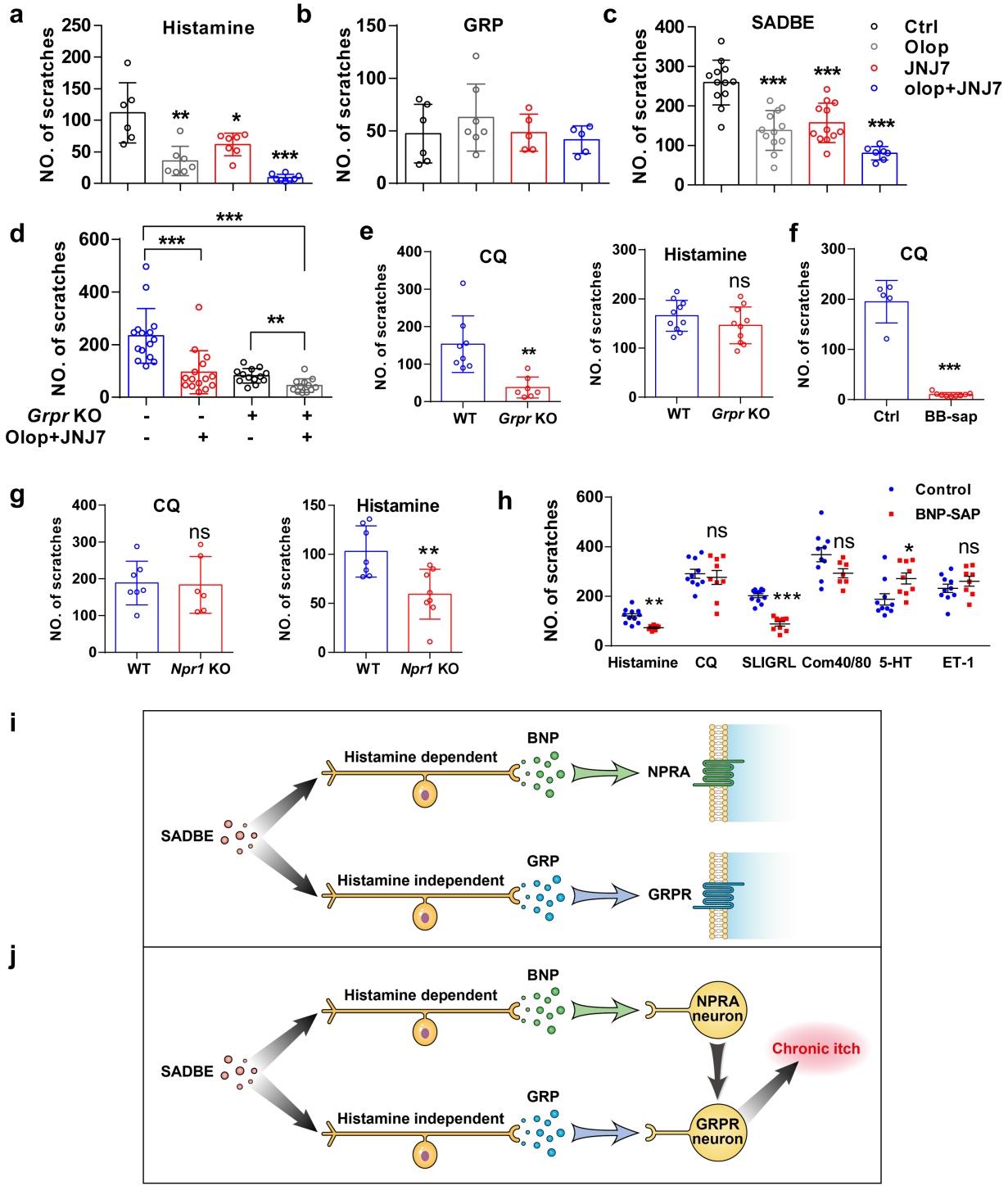

a
Sensitize (abdomen)
Challenge (nape)
Day -2 0 1 2 8 9 10 1 3 5 7 9 11 14 21 28 35
Shave
Ace Or SADBE
Ace Or SADBE
Recording
b
Control
SADBE
Number of scratches
Time (day)
c
IgE level (ng/mL)
Time (day)
d
Spleen weight (g)
Time (day)
e
Epidermis thickness (% change)
Time (day)
f
Mast cells
Time (day)
g
0 d
3 d
7 d
14 d
21 d
35 d
H&E
h
Krt14
i
Toluidine blue
j
Skin
Relative expression
Trpa1
Trpv1
Trpv4
Trpm8
k
Skin
Relative expression
Mrgpra3
Mrgprc11
Mrgprx1
Mrgprd
l
Skin
Relative expression
Grp
Nppb
Nmb

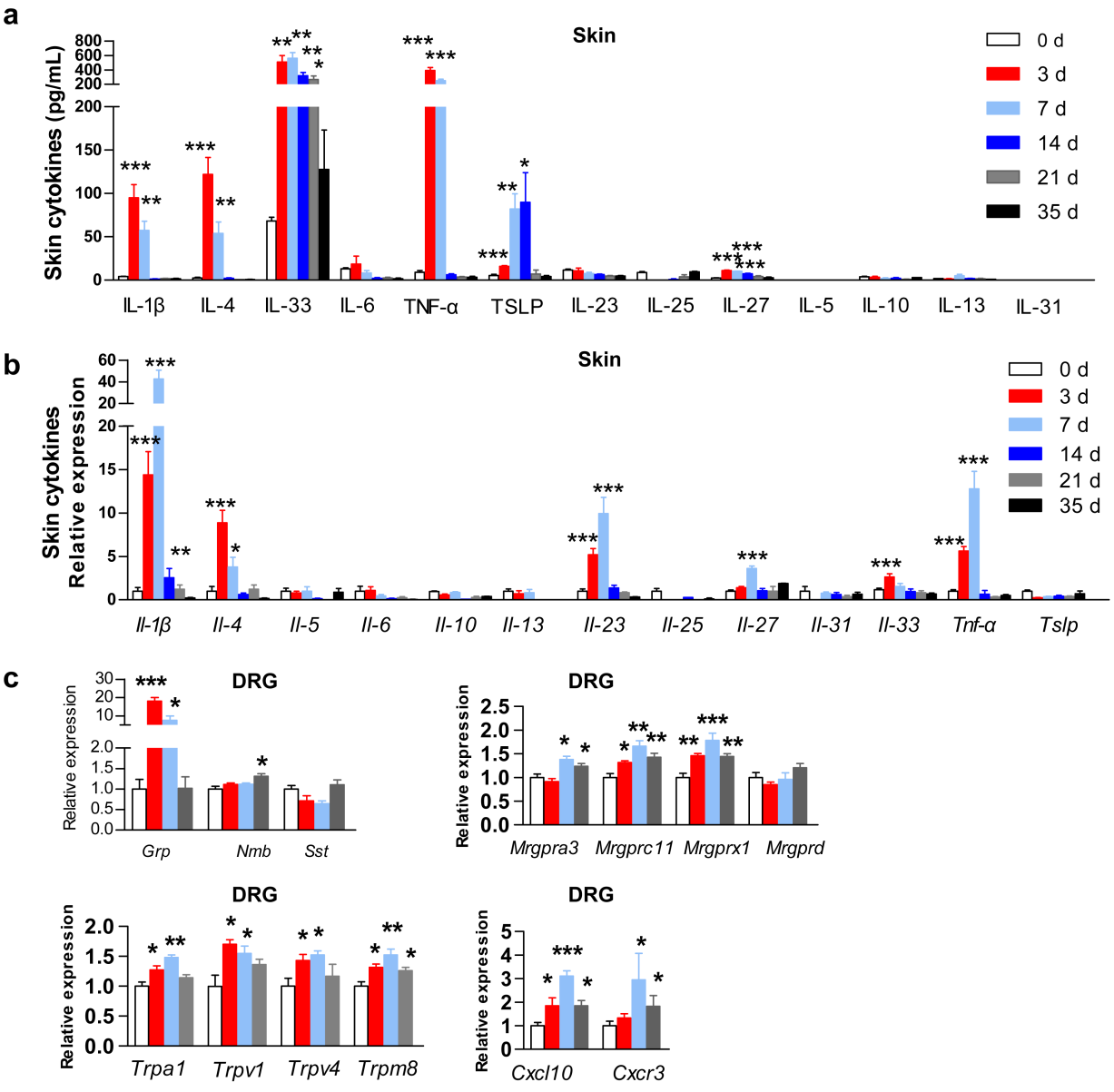

a
Ctrl
SADBE
Grpr
NO. of GRPR neurons
***
Ctrl
SADBE
b
GRPR-GFP
NO. of GFP+ neurons
***
Ctrl
SADBE
c
Grpr mRNA (fold)
**
Ctrl
SADBE
d
NO. of scratches
*
Ctrl
GRPR antagonist
e
NO. of scratches
*
WT
Grpr KO
f
NO. of scratches
***
Ctrl
BB-sap
g
Ctrl
BB-SAP
Grpr
NO. of Grpr neurons
***
Ctrl
BB-sap
h
Grpr
Vglut2
Merge
Merge
Vglut 2
Grpr
174
729
i
Grpr
Vgat
Merge
Merge
Grpr
Vgat
164 6
518

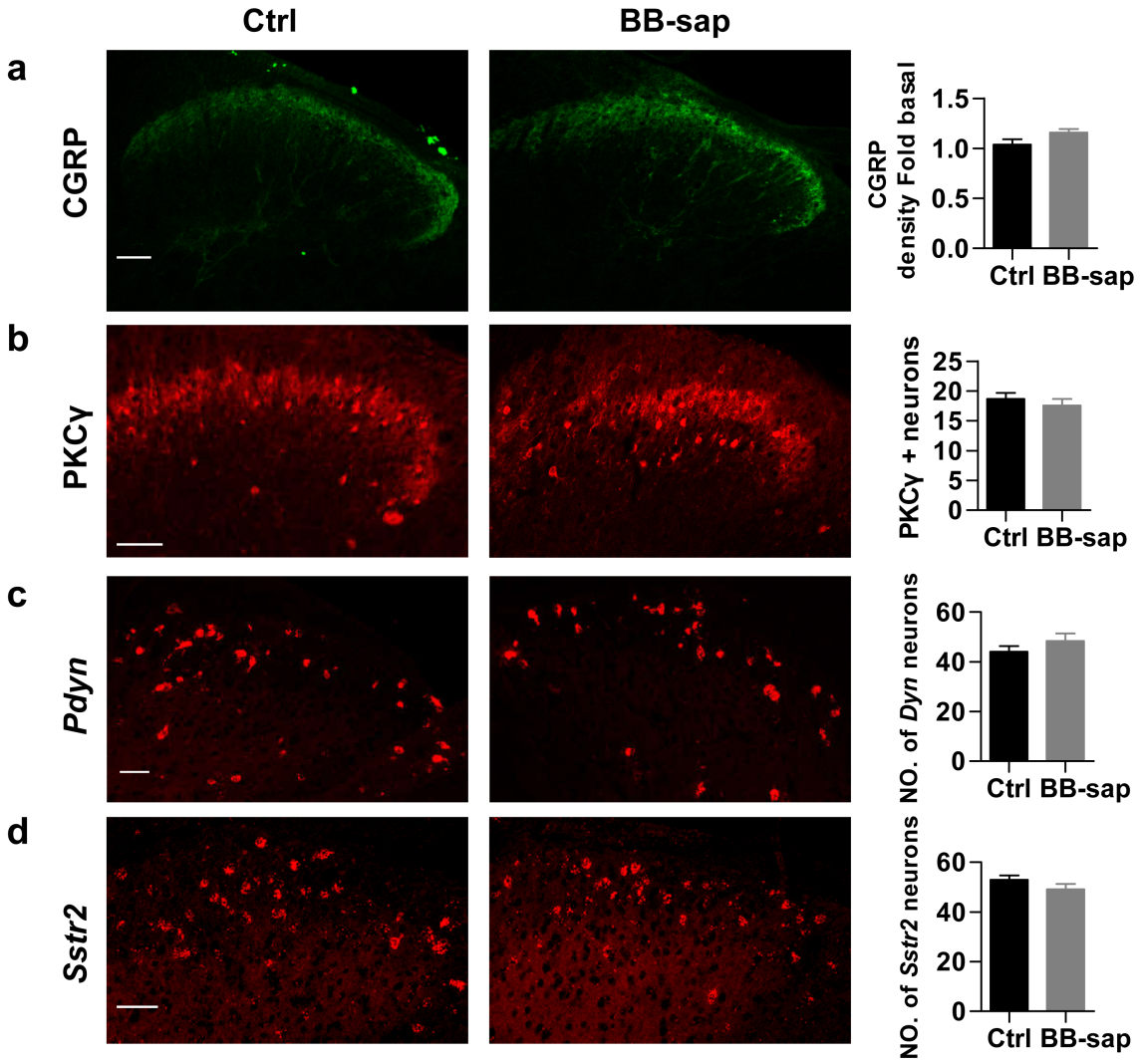

a
DRG
Nppb mRNA (Fold)
0 d
3d
7d
21d
b
Spinal cord
Npr1 mRNA (Fold)
c
Control
SADBE
Nppb
Relative intensity of Nppb neurons
Ctrl SADBE
d
NO. of scratches
Control
Anantin (2 n mol)
Anantin (10 n mol)
e
NO. of scratches
WT
Npr1 KO
f
NO. of scratches
Ctrl
BNP-sap
g
Npr1
Ctrl
BNP-sap
NO. of Npr1+ cells
h
Npr1
Vglut2
Merge
Npr 1
Vglut 2
30
318
845
i
Npr1
Vgat
Merge
Npr1
Vgat
320
24
752

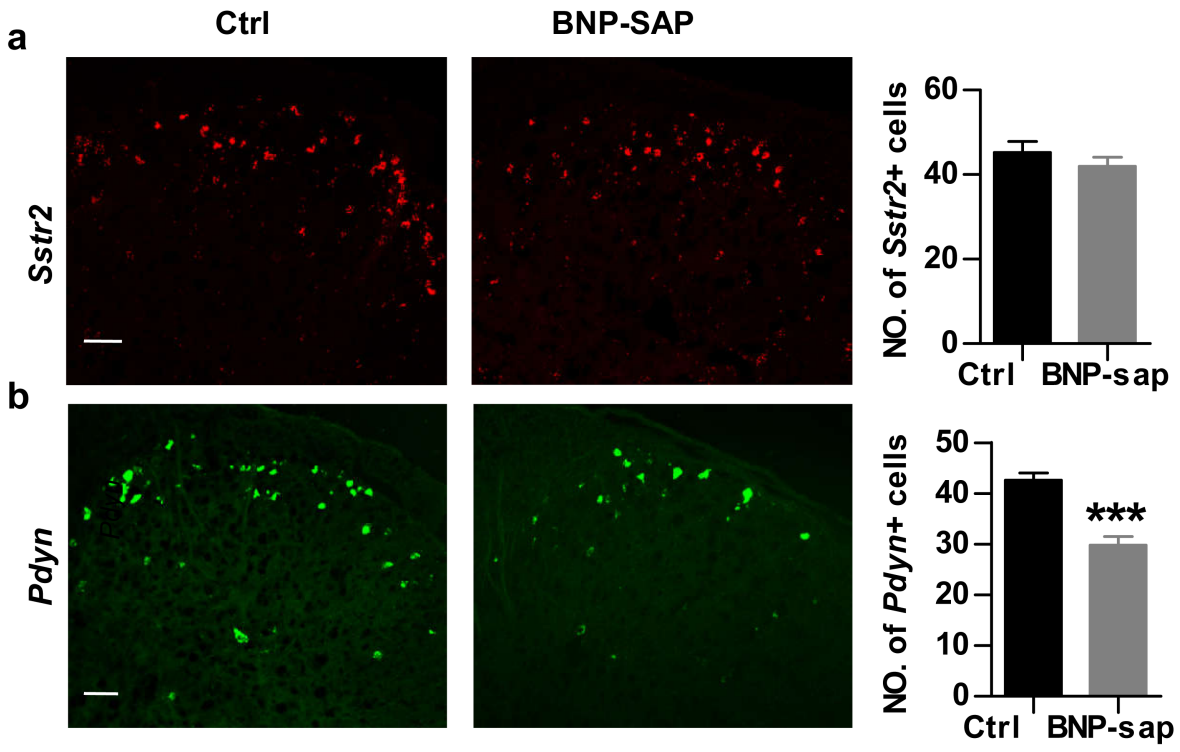

a
Rotarod test
Holding time (s)
350
300
250
200
150
100
50
0
ns
ns
Ctrl
BB-sap
BNP-sap
b
BNP
NO. of scratches
150
100
50
0
***
Ctrl
BB-sap
c
BNP
NO. of scratches
250
200
150
100
50
0
***
Ctrl
BNP-sap
d
GRP
NO. of scratches
300
200
100
0
ns
Ctrl
BNP-sap
e
Npr1
Grpr
Merge
Merge
Npr1
451
16
Grpr
286
f
Ctrl
BB-sap
Npr1
NO. of Npr1 neurons
80
60
40
20
0
ns
Ctrl
BB-sap
g
Ctrl
BNP-sap
Grpr
NO. of Grpr+ cells
40
30
20
10
0
ns
Ctrl
BNP-sap


a
Ctrl
Histamine
SADBE
Histamine
skin
No. of Histamine+ cells
30
20
10
0
*
Ctrl
SADBE
b
Ctrl
Hrh1
SADBE
Hrh1
skin
No. of Hrh1+ cells
150
100
50
0
***
Ctrl
SADBE
c
Ctrl
Hrh4
SADBE
Hrh4
skin
No. of Hrh4+ cells
150
100
50
0
***
Ctrl
SADBE
d
Ctrl
Hrh1
SADBE
Hrh1
DRG
NO. of Hrh1 neurons
30
20
10
0
***
Ctrl
SADBE
e
Grp
Hrh1
Merge
Grp
28
5
Hrh1
62
f
Nppb
Hrh1
Merge
Nppb
25
61
Hrh1
60
g
Grp
Nppb
Merge
Grp
74
1
Nppb
170

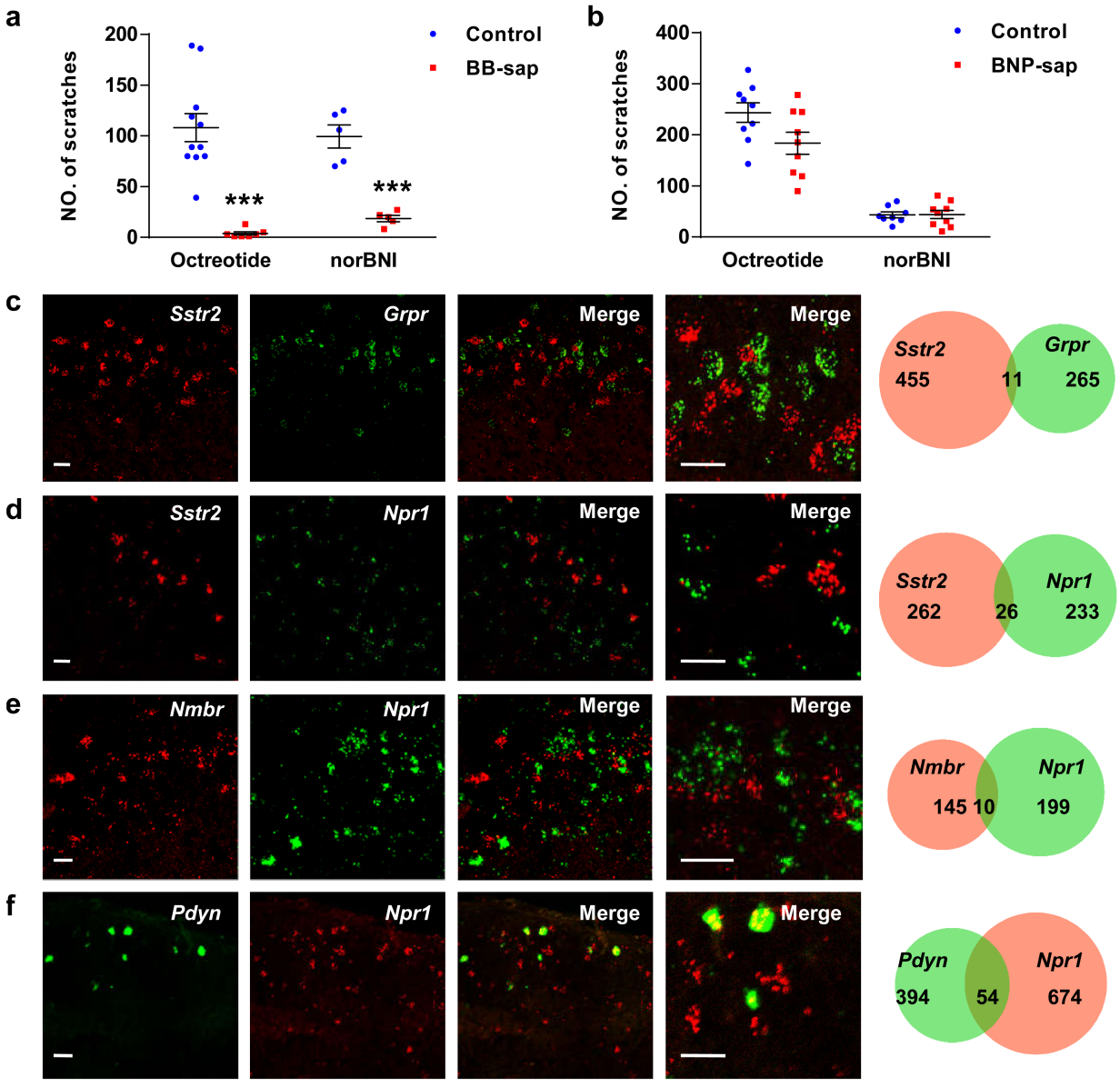

a
Histamine
NO. of scratches
250
200
150
100
50
0
**
*
***
b
GRP
NO. of scratches
150
100
50
0
c
SADBE
NO. of scratches
400
300
200
100
0
***
***
***
Ctrl
Olop
JNJ7
olop+JNJ7
d
NO. of scratches
600
400
200
0
***
***
**
Grpr KO
Olop+JNJ7
e
CQ
NO. of scratches
400
300
200
100
0
**
WT
Grpr KO
Histamine
NO. of scratches
300
200
100
0
ns
WT
Grpr KO
f
CQ
NO. of scratches
300
200
100
0
***
Ctrl
BB-sap
g
CQ
NO. of scratches
400
300
200
100
0
ns
WT
Npr1 KO
Histamine
NO. of scratches
150
100
50
0
**
WT
Npr1 KO
h
Control
BNP-SAP
NO. of scratches
600
400
200
0
**
ns
***
ns
*
ns
Histamine
CQ
SLIGRL
Com40/80
5-HT
ET-1
i
SADBE
Histamine dependent
BNP
NPRA
Histamine independent
GRP
GRPR
j
SADBE
Histamine dependent
BNP
NPRA neuron
Histamine independent
GRP
GRPR neuron
Chronic itch

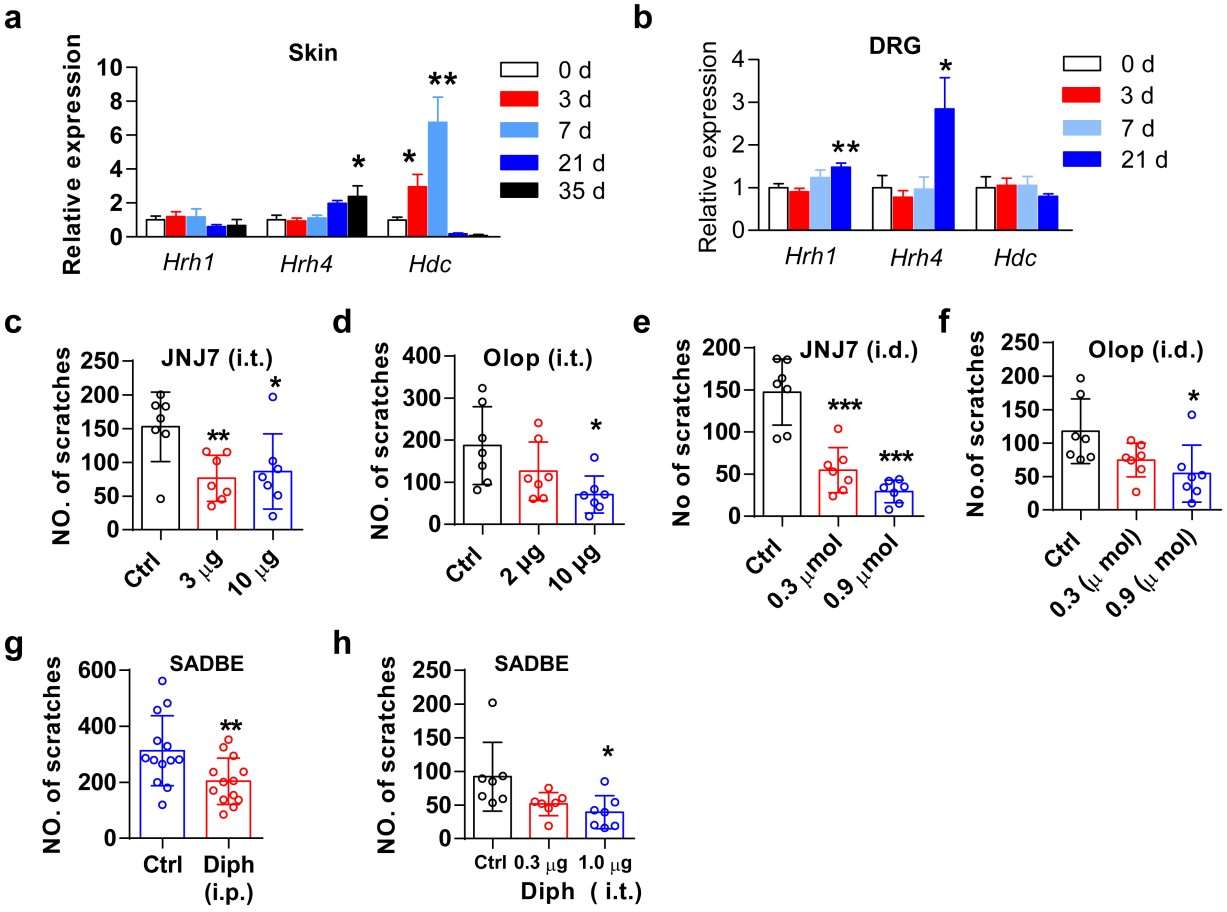

a
Skin
Skin cytokines (pg/mL)
0 d
3 d
7 d
14 d
21 d
35 d
IL-1β
IL-4
IL-33
IL-6
TNF-α
TSLP
IL-23
IL-25
IL-27
IL-5
IL-10
IL-13
IL-31
b
Skin
Skin cytokines
Relative expression
Il-1β
Il-4
Il-5
Il-6
Il-10
Il-13
Il-23
Il-25
Il-27
Il-31
Il-33
Tnf-α
Tslp
c
DRG
Relative expression
Grp
Nmb
Sst
Mrgpra3
Mrgprc11
Mrgprx1
Mrgprd
Trpa1
Trpv1
Trpv4
Trpm8
Cxcl10
Cxcr3


Ctrl
BB-sap
a
CGRP
CGRP density Fold basal
1.5
1.0
0.5
0.0
Ctrl BB-sap
b
PKCγ
PKCγ + neurons
25
20
15
10
5
0
Ctrl BB-sap
c
Pdyn
NO. of Dyn neurons
60
40
20
0
Ctrl BB-sap
d
Sstr2
NO. of Sstr2 neurons
60
40
20
0
Ctrl BB-sap



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a
NO. of scratches
200
150
100
50
0
Control
BB-sap
***
***
Octreotide
norBNI
b
NO. of scratches
400
300
200
100
0
Control
BNP-sap
Octreotide
norBNI
c
Sstr2
Grpr
Merge
Merge
Sstr2
455
11
Grpr
265
d
Sstr2
Npr1
Merge
Merge
Sstr2
262
26
Npr1
233
e
Nmbr
Npr1
Merge
Merge
Nmbr
145
10
Npr1
199
f
Pdyn
Npr1
Merge
Merge
Pdyn
394
54
Npr1
674


a
Skin
Relative expression
0 d
3 d
7 d
21 d
35 d
Hrh1
Hrh4
Hdc
b
DRG
Relative expression
0 d
3 d
7 d
21 d
Hrh1
Hrh4
Hdc
c
JNJ7 (i.t.)
NO. of scratches
Ctrl
3 μg
10 μg
d
Olop (i.t.)
NO. of scratches
Ctrl
2 μg
10 μg
e
JNJ7 (i.d.)
No of scratches
Ctrl
0.3 μmol
0.9 μmol
f
Olop (i.d.)
No.of scratches
Ctrl
0.3 (μ mol)
0.9 (μ mol)
g
SADBE
NO. of scratches
Ctrl
Diph (i.p.)
h
SADBE
NO. of scratches
Ctrl
0.3 μg
1.0 μg
Diph (i.t.)

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Supplemental Information for

Spinal GRPR and NPRA contribute to chronic itch in a murine model of allergic contact dermatitis.

Xueting Liu[1], De Wang[1], Yuhuan Wen[1], Liping Zeng[1], Yangyang Li[1], Tianyu Tao[1], Zhongqiu Zhao[2,3], Ailin Tao[*1]

[1]The Second Affiliated Hospital, The State Key Laboratory of Respiratory Disease, Guangdong Provincial Key Laboratory of Allergy & Clinical Immunology, Center for Immunology, Inflammation, & Immune-mediated disease, Guangzhou Medical University, Guangzhou 510260, China.

[2]Center for the Study of Itch, Department of Anesthesiology, Washington University School of Medicine.

[3]Barnes-Jewish Hospital, St. Louis, MO 63110, USA.

Corresponding author: Ailin Tao, PhD, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou 510260, China. Tel: 86-020-34153520, Fax: 86-020-34153520, E-mail: taoailin@gzhmu.edu.cn.

Inventory of supplementary materials submitted:

I. **Supplementary figures and legends**

II. **Table S1**

III. **Supplemental Methods and References**

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Supplementary figure legends

Figure S1. Strong up-regulation 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 to day 0.

Figure S2. Molecular expression in cervical spinal cord of BB-sap mice mice. (**a**-**d**) The number of $CGRP^+$ (**a**) and $PKC\gamma^+$ (**b**) cells detected by immunostaining, or *Prodyn*+(**d**), *Sstr2*+ (**d**) 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. All scale bars, 50 μm.

Figure S3. Molecular expression in cervical spinal cord of BNP-sap mice. $Pdyn^+$(**b**) but not $Sstr2^+$(**a**) cells were significantly ablated in the cervical spinal cord of the BNP-sap group compared to that of the blank-sap group (*** *P* < 0.001). Scale bars = 50 μm. Student's *t*-test, *n* = 4 mice / group. All scale bars, 50 μm.

Figure S4. Characterization of SST_{2A}^+, $NPRA^+$ and $GRPR^+$ neurons in the spinal cord for itch. (a) Intrathecal injection of octreotide (100 ng, *n* = 11, 7), or nor-binaltorphimine (nor-BNI, 75 μg, *n* = 5) induced scratching decreased in mice after bombesin-sap. (**b**) Intrathecal injection of octreotide (100 ng, *n* =9), and nor-BNI (75 μg, *n* = 8, 9) induced scratching decreased in mice after BNP-sap. In (**a**,**b)** all data represent means ± SEM. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, versus the blank-sap, unpaired *t* test. (**c**-**f**) Double RNAscope staining showed $Sstr2^+$ rarely co-localized

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with *Grpr*$^{+}$(**c**), and *Npr1*$^{+}$ rarely co-localized with *Sstr2*$^{+}$(**d**) , *Nmbr*$^{+}$ (**e**) and *Dyn*$^{+}$(**f**) cells in the superficial dorsal horn. Scale bars 25 μm. *n* = 3.

Figure S5. SADBE-induced prolonged itch was driven by histamine dependent systems. (**a**, **b**) qPCR analysis showing increased mRNA levels of *Hrh1* and *Hrh4* in skin (**a**) and cervical (c2-c7) DRGs (**b**) 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 (10 mg/kg, i.p.) (**g**), (0.3 and 0.9 μg, i.t.) (**h**).

Table S1. Primers used for real-time quantitative PCR

Target	**Forward primer (’5 to 3’)**	**Reverse primer (’5 to 3’)**
Mrgpra3	GAATGGGAGAAAGCAACACCAGT	GTCATTGTCGAGGCTGAGGTGT
Mrgprd	TTTGCGGAAGGACAGATTGG	TGCTGTCAAGAGTGGAGTTCA
Mrgprc11	ACTCTCTGCTACGGATCATTGA	TGATTGCTGCATTGCCTAAGATA
Mrgprx1	ACAAGTCAGCTCCTCAACTCC	GTGTCGTGGGATGAGATGGTT
Trpa1	ATGGGATGACCCCTCTCCAC	AAAGCAGTCCAGCCATTGTG
Trpv1	ACCACGGCTGCTTACTATCG	CGGAAATAGTCCCCAACGGT
Trpv4	TGGAGGAGAAAGGTCGTGGA	GTCCATCTAGGTCCGCAGTG
Trpm8	AAACATCCCCTTCCCCTTCG	GTTGTCGTTGGCTTTCGTGT

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Gapdh	CCCAGCAAGGACACTGAGCAA	TTATGGGGGTCTGGGATGGAAA
Actb	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA
Grp	TGGGCTGTGGGACACTTAAT	GCTTCTAGGAGGTCCAGCAAA
Nppb	GTCAGTCGTTTGGGCTGTAAC	AGACCCAGGCAGAGTCAGAA
Grpr	TGATTCAGAGTGCCTACAATCTTC	CTTCCGGGATTCGATCTG
Npr1	TGGAGACACAGTCAACACAGC	CGAAGACAAGTGGATCCTGAG
Tslp	AAGCCAGCTTGTCTCCTGAA	TGTGCCATTTCCTGAGTACC
Il-1β	CACTCATTGTGGCTGTGGAGAAG	TTGTTCATCTCGGAGCCTGTAG
Il-4	TGTAGGGCTTCCAAGGTGCT	TGATGCTCTTTAGGCTTTCCAG
Il-5	GACAAGCAATGAGACGATGAGG	CGTCTCTCCTCGCCACACTTCT
Il-6	TGTATGAACAACGATGATGCACTT	CTCTGGCTTTGTCTTTCTTGTTATC
Il-10	GCGCTGTCATCGATTTCTCC	CACACTGCAGGTGTTTTAGCTT
Il-13	CAACATCACACAAGACCAGACTCC	GGTATCGGGGAGGCTGGAG
Il-25	CCCAGCAAAGAGCAAGAACC	ACCCGATTCAAGTCCCTGTC
Il-27	TGTCCACAGCTTTGCTGAATC	CGAAGTGTGGTAGCGAGGAA
Il-33	AGACCAGGTGCTACTACGCT	ACGTCACCCCTTTGAAGCTC
Il-31	CCACACAGGAACAACGAAGCCT	CCCGGTCCAGGCTGAAACACG
Il-23	GGTTGTGACCCACAAGGACT	AGGGAGGTGTGAAGTTGCTC
Tnf-α	AGAGGCACTCCCCCAAAAGAT	GGGTCTGGGCCATAGAACTGAT
Hrh1	AGAGGCTTCGCTCACATTCC	CTTCCGCTCTCGGTTCAAGT
Hrh4	GGCTATTTCTGACTTCCTCGTG	TGCAGATTCCACTTCCAAAA
Cxcr3	AGAATCATCCTGGTCTGAGACAA	AAGATAGGGCATGGCAGC
Cxcl10	CCCACGTGTTGAGATCATTG	CACTGGGTAAAGGGGAGTGA
Hdc	CGTGAATACTACCGAGCTAGAG	ACTCGTTCAATGTCCCCAAAG

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Supplementary Materials and Methods

Drugs and reagents

GRP_{18-27} (Cat# H-3120), BNP (Cat# H-7558), SLIGRL-NH2 (Cat# H-5078), 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 (CQ, Cat# C6628), squaric acid dibutylester (SADBE, Cat# 339792), compound 48/80 (Cat# C2313), serotonin (5-HT, Cat# H9523), histamine H1R antagonist olopatadine (Cat# O0391), diphenhydramine (Cat# D3630), and H4R antagonist JNJ7777120 (Cat# J3770) were purchased from Sigma (St. Louis, MO). Bombesin-saporin (BB-sap, Cat# IT-40-25), BNP-saporin (BNP-sap (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 (nor-BNI, 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 in order to quantify mouse scratching behavior.

Itch behaviors were performed as previously described (Sun and Chen, 2007, Zhao et al., 2014). 30 minutes prior to the experiment, mice were transferred to plastic

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chambers (10 x 10.5 x 15 cm) 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 (i.d.) 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 (i.d.) or trunk (i.t.) injection sites and the returning of the hind limbs back. The scratches to the side of i.d. injection site were counted for the i.d. 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), 5-HT (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 nM), Npr1 antagonist anantin (2 and 10 nmol), kappa-opioid receptor antagonist norbinaltorphimine (nor-BNI, 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 JNJ7777120 (30mg/kg), were tested using intraperitoneal (i.p.) injections 30 minutes before the start of behavior recording. Specific doses are listed in the figure legends.

Assessment of motor function

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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 3 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 squaric acid dibutylester (SADBE; Sigma) was used to induce contact allergic dermatitis in mice. 20 μL of 1% SADBE (dissolved in acetone) was applied to the shaved abdomen skin of mice once a day for three consecutive days. Five days later, the SADBE treatment group was challenged with 20 μL 1% SADBE topically applied to the shaved nape skin once daily for 3 consecutive days, while 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. In order 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 application, blood, spleen, cervical (c2-c7) DRG and spinal cord tissues were collected from the SADBE-treated mice 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

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Skin tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned in 5 µm sections on a microtome. The sections were stained with hematoxylin and eosin (H&E) or toluidine blue (TB) following the standard protocols. HE and TB stained sections were photographed with a light microscope. The thickness of epidermis was measured and the number of mast cells were counted using Image J 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 (PBS-T) 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) was used for labeling keratinocytes. FITC-conjugated avidin (1:1,000, Cat No. 434411; Thermo Scientific) was used for labeling mast cells. A rabbit anti-histamine antibody (1:500, Cat No. 22939, ImmunoStar Inc) was used. Secondary goat anti-rabbit antibody conjugated to Alexa Fluor 568 (Cat No. A11011, Thermo Fisher) and goat anti-mouse antibody conjugated to Alexa Fluor 488 (Cat No. A11001, Thermo Fisher) 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, spinal cord tissues were immediately removed, post-fixed in 4% paraformaldehyde overnight at 4°C and cryoprotected in 25% sucrose. 20 µm spinal cord sections were obtained via a cryostat and were incubated in blocking solution for

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2 hours at room temperature. The sections were then incubated with primary antibodies overnight at 4°C, washed three times in PBS-T 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) and coverslipped. The following antibodies were used: mouse anti-CGRP (1:3000; Cat No. AB1971, Abcam), rabbit anti-PKCγ (1:500; Cat No. sc-211, Santa Cruz); GFP (1:500; Cat No. ab13970, Abcam), goat anti-rabbit Alexa 488 conjugated (Cat No. A11008, Thermo Fisher), goat anti-mouse Alexa 488 conjugated (Cat No. A11001, Thermo Fisher), goat anti-rabbit Alexa 568 conjugated (Cat No. A11011, Thermo Fisher), goat anti-mouse Alexa 568 conjugated (Cat No. A11004, Thermo Fisher). All imaging and analysis were performed on a Nikon C2+ Confocal Microscope. At least three mice/group and 10 cervical spinal cord (c4-6) sections from each mouse were included for statistical comparison.

RNAscope *in situ* hybridization (ISH) assay

RNAscope ISH assay was performed as described (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, 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 ZZ oligonucleotides and obtained from Advanced Cell Diagnostics were used: *Hrh1* (Cat No. 318771, target region bases 549 - 1453; accession number NM_001252642.2), *Hrh4* (Cat No. 528021, target region bases 246 - 1224; accession No. NM_153087.2), *Nppb* (Cat No. 425021, target region bases 4 - 777; accession No. NM_008726.5), *Grp* (Cat No. 317861, target region bases 22 - 825; accession No. NM_175012.2), *Npr1* (Cat No. 484531, target region

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bases 941 - 1882; accession No. NM_008727.5), *Grpr* (Cat No. 317871, target region bases 463-1596; accession No. NM_008177.2), and *Nmbr* (Cat No. 406461, target region bases 25 - 1131; 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 - 1159; accession No. NM_001042606.2), *Vglut2* (*Slc17a6*, Cat No. 319171, target region bases 1986 - 2998; accession No. NM_080853.3), *Vgat* (*Slc32a1*, Cat No. 319191, target region bases 894 - 2037; accession No. NM_009508.2). Slides were counterstained with DAPI (Molecular Probes) 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 20X objective lens. Cells were considered positive if three punctate dots were present in the nucleus and/or cytoplasm. For co-localization studies, dots associated with single DAPI stained nuclei were counted as co-positive. 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 (BB-sap, 400 ng /mouse) or blank-saporin (blank-sap, 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 (BNP-sap, 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-sap injection.

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References

Munanairi A, Liu XY, Barry DM, Yang Q, Yin JB, Jin H, et al. Non-canonical Opioid Signaling Inhibits Itch Transmission in the Spinal Cord of Mice. Cell reports 2018;23(3):866-77.

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

Sun YG, Zhao ZQ, Meng XL, Yin J, Liu XY, Chen ZF. Cellular basis of itch sensation. Science 2009;325(5947):1531-4.

Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn 2012;14(1):22-9.

Zhao ZQ, Chiechio S, Sun YG, Zhang KH, Zhao CS, Scott M, et al. Mice lacking central serotonergic neurons show enhanced inflammatory pain and an impaired analgesic response to antidepressant drugs. J Neurosci 2007;27(22):6045-53.

Zhao ZQ, Wan L, Liu XY, Huo FQ, Li H, Barry DM, et al. Cross-inhibition of NMBR and GRPR signaling maintains normal histaminergic itch transmission. The Journal of neuroscience : the official journal of the Society for Neuroscience 2014;34(37):12402-14.

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