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GRP receptor and AMPA receptor cooperatively regulate itch-responsive neurons in the spinal dorsal horn

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Abstract

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None.

Gastrin-releasing peptide (GRP) receptor-expressing $(GRPR)^+$ neurons have a central role in the spinal transmission of itch. Because their fundamental regulatory mechanisms are not yet understood, it is important to determine how such neurons are excited and integrate itch sensations. In this study, we investigated the mechanisms for the activation of itch-responsive $GRPR^+$ neurons in the spinal dorsal horn (SDH). $GRPR^+$ neurons expressed the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) containing the GluR2 subunit. In mice, peripherally elicited histaminergic and non-histaminergic itch was prevented by intrathecal (i.t.) administration of the AMPAR antagonist NBQX, which was consistent with the fact that firing of $GRPR^+$ neurons in SDH under histaminergic and non-histaminergic itch was completely blocked by NBQX, but not by the GRPR antagonist RC-3095. Because GRP^+ neurons in SDH contain glutamate, we investigated the role of GRP^+ (GRP^+/Glu^+) neurons in regulating itch. Chemogenetic inhibition of GRP^+ neurons suppressed both histaminergic and non-histaminergic itch without affecting the mechanical pain threshold. In nonhuman primates, i.t. administration of NBQX also attenuated peripherally elicited itch without affecting the thermal pain threshold. In a mouse model of diphenylcyclopropenone (DCP)-induced contact dermatitis, GRP, GRPR, and AMPAR subunits were upregulated in SDH. DCP-induced itch was prevented by either silencing GRP^+ neurons or ablation of $GRPR^+$ neurons. Altogether, these findings demonstrate that GRP and glutamate cooperatively regulate $GRPR^+$ $AMPAR^+$ neurons in SDH, mediating itch sensation. GRP–GRPR and the glutamate–AMPAR system may play pivotal roles in the spinal transmission of itch in rodents and nonhuman primates.

Keywords

chloroquine; glutamate; histamine; nonhuman primate; pain; pruritus

1. Introduction

Itch (pruritus) is an uncomfortable sensation that evokes a desire to scratch. Given that itch is a key symptom of numerous systemic disorders, it is imperative to study the mechanisms underlying sensory processing of itch (Hay et al., 2014; Yosipovitch and Bernhard, 2013). To date, several lines of evidence have uncovered the major transmission pathway of itch. In the skin, each pruritic molecule activates its corresponding pruriceptor located on the nerve ending of a C-fiber (Bautista et al., 2014; Green and Dong, 2016). Then, transferred itch sensation is integrated and processed by a variety of interneurons in the spinal dorsal horn (SDH) (Bautista et al., 2014; Dong and Dong, 2018). However, the detailed mechanisms have yet to be determined, and there is a strong unmet need to develop novel therapeutics because standard antipruritic agents have limited effectiveness (Dong and Dong, 2018; Leung and Lowery, 2017; Yosipovitch and Bernhard, 2013).

Gastrin-releasing peptide (GRP) is a key central mediator of itch. GRP consists of bombesin family peptides (Anastasi et al., 1971; McDonald et al., 1979), and these peptides cause scratching/grooming behaviors in animals (Cowan et al., 1985; Masui et al., 1993). Notably, the GRP receptor, a G protein-coupled receptor (GPCR) (Jensen et al., 2008), is the primary regulator of itch transmission in the SDH (Sun and Chen, 2007). Intrathecal (i.t.) injection of GRP elicits robust scratching behaviors not only in rodents but also in primates (Lee and Ko,

2015; Sukhtankar and Ko, 2013; Sun and Chen, 2007). Moreover, ablation or dysfunction of $GRPR^+$ neurons in the SDH abolishes most scratching behaviors induced by several types of pruritogens in the skin (Aresh et al., 2017; Sun et al., 2009). These findings indicate that $GRPR^+$ neurons in the SDH are responsive to itch. On the other hand, knockout of either GRP or GRPR only partially decreases scratching behaviors induced by intradermal (i.d.) injection of several types of pruritogens (Wan et al., 2017; Zhao et al., 2014), which is supported by the finding that i.t. administration of a GRPR antagonist can only attenuate a part of scratching behaviors induced by such pruritogens (Akiyama et al., 2013; Kiguchi et al., 2016). Hence, neurotransmitters other than GRP may activate itch-responsive $GRPR^+$ neurons in the SDH.

Glutamate conveys pain and touch sensations (Basbaum et al., 2009; Todd, 2010). The i.t. administration of CNQX, an antagonist of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)/kainite receptor, prevents excitation of itch-related superficial neurons in the SDH and scratching behaviors caused by i.d. chloroquine or histamine (Akiyama et al., 2014). Furthermore, the activation of the neurons responding to GRP in laminae I/II of the SDH C-fiber stimulation is blocked by CNQX (Koga et al., 2011). In contrast, conditional knockout of vesicular glutamate transporter 2 (VGLUT2) in nociceptive C-fibers decreases pain but increases itch (Lagerstrom et al., 2010; Liu et al., 2010), possibly because glutamate derived from nociceptive C-fibers activates inhibitory interneurons, which suppress itch processing in the SDH. Given that GRP^+ neurons are classified as spinal excitatory neurons that contain glutamate (Haring et al., 2018), glutamate may play an important functional role in the regulation of itch-responsive neurons in the SDH. Recently, the spinal gating mechanism for itch by the repetitive activation of GRP^+ neurons resulting in the release of GRP and glutamate has been demonstrated (Pagani et al., 2019). Nevertheless, further study is needed to demonstrate how glutamate conveys the diverse peripherally elicited itch to itch-responsive neurons in the SDH. To understand the detailed role of glutamate as well as the relationship between the two itch-responsive mediators, GRP and glutamate, in this study we investigated the mechanisms underlying the activation of itch-responsive $GRPR^+$ neurons in the SDH.

2. Materials and methods

2.1. Mice

All animal experiments were approved by the Animal Research Committee of Wakayama Medical University and were carried out in accordance with the in-house guidelines for the care and use of laboratory animals of Wakayama Medical University and the ARRIVE guidelines. Male ICR (20–25 g) and C57BL/6J (5–6 weeks old) mice were purchased from SLC (Hamamatsu, Japan). R26-LSL-Gi-DREADD mice [B6N.129-$Gt(ROSA)26Sor^{tm1(CAG-CHRM4*,-mCitrine)Ute}$/J; stock #026219] (Zhu et al., 2016) and GRP-Cre (Tg) mice [B6.FVB(Cg)-Tg(Grp-cre)KH288Gsat/Mmucd; stock #037585] were purchased from The Jackson Laboratory and Mutant Mouse Resource & Research Centers (MMRRC), respectively. R26-LSL-Gi-DREADD mice and R26-LSL-tdTomato were maintained as heterozygous or homozygous genotype. For Cre-dependent expression of tdTomato or Gi-designer receptors exclusively activated by designer drugs (DREADD)

system in the *Rosa* locus in GRP-expressing cells, R26-LSL-tdTomato mice or R26-LSL-Gi-DREADD mice were crossed with GRP-Cre mice, respectively. Subsequently, male mice heterozygous for ROSA26 and GRP-Cre (transgenic) were used for the experiments. Mice were housed in plastic cages in a temperature-controlled room (23°C–24°C, 60%–70% humidity) with a 12-h dark/light cycle and provided with water and food *ad libitum*.

2.2. Drug administration in mice

Drugs were dissolved in sterile water or sterile phosphate-buffered saline (PBS) and diluted as needed. GRP (Tocris Biosciences, Bristol, UK), RC-3095 (Sigma-Aldrich, St. Louis, MO, USA), NBQX (Tocris Biosciences), bombesin-saporin (Bom-Sap; Advanced Targeting Systems, San Diego, CA, USA), and blank-Sap (Advanced Targeting Systems) were administered i.t. in a volume of 5 μl as described previously (Kiguchi et al., 2016). Under isoflurane anesthesia, mice were secured by a firm grip on the pelvic girdle, and drugs were injected by lumbar puncture between L5 and L6 vertebrae using a 30-gauge needle fitted with Hamilton microsyringe. Clozapine-N-oxide (CNO; Enzo Life Sciences, Farmingdale, NY, USA) was also administered i.t. to isoflurane-anesthetized mice, or by intraperitoneal (i.p.) injection to awake mice in a volume of 0.1 ml/10 g body weight. Chloroquine diphosphate (Sigma-Aldrich), SLIGRL-NH_2 (Tocris Biosciences), bovine adrenal medulla (BAM) 8–22 (Abcam, Cambridge, MA, USA), compound 48/80 (Sigma-Aldrich), and HTMT (Abcam) were administered i.d. in the nape of isoflurane-anesthetized mice. The drugs were administered in a volume of 100 μl using a 30-gauge needle fitted to a 1-ml syringe after having shaved the fur at the injection site. To minimize the effects of isoflurane on the scratching behaviors, exposure time was kept as short as possible, and isoflurane administration was terminated immediately after drug administration.

2.3. Scratching behaviors in mice

Mice were habituated for 60 min in plastic cages (20 × 12 × 12 cm^3) with a small amount of bedding. After the administration of each pruritic agent, the number of scratching bouts was measured in 10-min intervals for 30 min as reported previously (Kiguchi et al., 2016). One scratching bout was defined as lifting the hind paw to scratch the trunk area of body or nape regions following i.t. or i.d. injection, respectively, and then returning the paw to the floor or to the mouth for licking. Analyses were carried out in a blinded fashion.

2.4. Paw-withdrawal test in mice

To evaluate mechanical allodynia, the 50% withdrawal threshold was determined by the von Frey test as described previously (Kiguchi et al., 2018). Briefly, mice were individually placed on a 5 × 5-mm wire mesh grid floor and covered with an opaque acrylic box. After adaptation for 2–3 h, calibrated von Frey filaments (Neuroscience, Tokyo, Japan) were applied to the middle of the plantar surface of the hind paw through the bottom of the mesh floor. In the paradigm of the up-down method, testing was initiated with a 0.4-*g* force in the middle of the series (0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 *g*). Stimuli were always presented in consecutive fashion, either ascending or descending. In the absence of a paw withdrawal response to the selected force, a stronger stimulus was applied. In the presence of paw withdrawal, the next weaker stimulus was chosen. After the response threshold was first crossed (the two responses straddling the threshold), four additional stimuli were

applied. Based on the responses to the series of the von Frey filament, the 50% paw withdrawal threshold was calculated.

2.5. Contact dermatitis

Diphenylcyclopropenone (DCP; Wako, Osaka, Japan) was dissolved in acetone. For sensitization, 0.2 ml of 2% DCP were used after having shaved the fur at the back of isoflurane-anesthetized mice. Seven days after sensitization, mice were challenged with 0.2 ml of 1% DCP. The number of scratching bouts was measured for 40 min immediately after each DCP application.

2.6. Immunohistochemistry

The lumbar dorsal root ganglia (DRG), lumbar (L4–5) or cervical (C3–5) spinal cord was collected from mice after transcardiac perfusion in PBS and fixed in 4% paraformaldehyde. Then, specimens were post-fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose at 4°C overnight. Frozen tissues embedded in freezing compound (Sakura, Tokyo, Japan) were cut longitudinally into 30-μm-thick sections with a cryostat and allowed to float in PBS. The sections were treated with PBS containing 0.3% Triton X-100 (PBST) for 1 h and then blocked with 5% normal donkey serum in 0.3% PBST at 15–25°C for 2 h. The sections were incubated with primary antibodies against GRPR (rabbit polyclonal, 1:400; MBL International, Woburn, MA, USA), GluR2 (mouse monoclonal, 1:100; Thermo Fisher Scientific, Waltham, MA, USA), GRP (rabbit polyclonal, 1:1000; ImmunoStar, Hudson, WI, USA), VGLUT2 (guinea pig, 1:400; Frontier Institute, Hokkaido, Japan), transient receptor potential cation channel subfamily V1 (TRPV1; rabbit polyclonal, 1:400; Abcam), hemagglutinin (HA) epitope-tag (mouse monoclonal, 1:250; BioLegend, San Diego, CA, USA), and c-fos (rabbit polyclonal, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. All antibodies were diluted in 1% normal donkey serum in 0.1% PBST. Subsequently, sections were rinsed in PBST, and incubated with fluorescence-conjugated secondary antibodies (1:300; Abcam) or isolectin-B4 (IB4; 1:200, Thermo Fisher Scientific) at 15–25°C for 2 h. Sections were rinsed in PBST, and then incubated with Hoechst 33342 (Thermo Fisher Scientific) at 15–25°C for 10 min. Finally, sections were air-dried on glass slides for 30 min, and coverslipped with mounting medium. Fluorescence images of SDH were detected using a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). For quantification of c-fos in each mouse, the number of positive cells in the superficial laminae of the lumbar spinal cord was the average of three randomly selected sections from one segment of each mouse before c-fos staining was viewed. All images of c-fos labeling were taken at the same time with the same camera settings, and the persons performing the counts were blinded to the treatment groups. Brightness and contrast of fluorescent micrographs were minimally processed and colorized as needed, using Adobe Photoshop.

2.7. RT-qPCR

Mice were euthanized by decapitation, and the fresh lumbar DRG, lumbar SDH (L4–5), or cervical (C3–5) SDH was collected in RNAlater solution (Thermo Fisher Scientific). The TRIzol® Plus RNA Purification Kit (Thermo Fisher Scientific) was used for the isolation of total RNA from the tissues following the manufacturer’s instructions. Briefly, tissues were

placed in a 1.5-ml RNase-free tube and homogenized with TRIzol reagent. Chloroform was added to each sample, and samples were then centrifuged at 4°C for 15 min. The aqueous phase containing RNA was transferred to a fresh tube, and RNA was isolated using a purification column. Total RNA extract was used for the synthesis of cDNA by reverse transcription as follows. Total RNA was incubated with Random Primers (Promega, Madison, WI) at 70°C for 5 min and then was cooled on ice. Samples were converted to cDNA by incubation with M-MLV Reverse Transcriptase and dNTPMix (Promega) at 37°C for 50 min. qPCR was performed using AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) by using the cDNA as the template, primers for each gene (Thermo Fisher Scientific) and SYBR® Premix Ex Taq™ II (Takara Bio, Shiga, Japan). The primer sequences are listed in Table S1. Reactions were performed under the following conditions: 3 min at 95°C, followed by 45 cycles of two steps (10 s at 95 °C and 30 s at 60°C). The fluorescence intensities were recorded, and data were normalized to β-actin (*ACTB*).

2.8. Electrophysiology

The methods used for the *in vivo* extracellular recording from the superficial SDH were similar to those described previously (Akiyama et al., 2009; Andoh et al., 2017). Briefly, adult male ICR mice (5–6 weeks old; SLC) were anesthetized with urethane (1.5 g/kg, i.p.). Urethane produces a long-lasting steady level of anesthesia that does not require administration of additional doses. A thoracolumbar laminectomy was performed exposing the level from L1 to L6, and the animal was then placed in a stereotaxic apparatus. After removing the dura and cutting arachnoid membrane to create a window large enough to let a tungsten microelectrode, the surface of spinal cord was irrigated with 95% O_2–5% CO_2-equilibrated Krebs solution (10 ml/min) containing the following (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11 glucose, and 25 $NaHCO_3$, through glass pipettes at 37 ± 1°C. Extracellular single-unit recordings of superficial SDH (lamina I and II) neurons were performed as described previously (Akiyama et al., 2009; Andoh et al., 2017). Recordings were obtained from superficial SDH neurons at a depth of 20–150 μm from the surface. Unit signals were acquired with an amplifier (EX1; Dagan corporation, Minneapolis, MN, USA). The data were digitized with an analog-to-digital converter (Digidata 1400A, Molecular Devices, Union City, CA, USA), stored on a personal computer with a data acquisition program (Clampex version 10.2; Molecular Devices), and analyzed with a special software package (Clampfit version 10.2; Molecular Devices). We searched the area on the skin where a touch (with a cotton wisp) or noxious pinch (with forceps) stimulus produced the neural response. Compound 48/80 or chloroquine were injected into the receptive area through a 30-gauge needle. GRP, RC-3095, and NBQX were perfused to the surface of the spinal cord as described previously (Funai et al., 2014; Ohashi et al., 2017). First, the responsiveness of SDH neurons to GRP application was tested. If the recorded neurons produced firing on bath applications of GRP, RC-3095 was applied to them after washing out of the applied GRP (Figure S2). Baseline was measured for 30 sec at 2 min before GRP application, and then effects of RC-3095 and/or GRP were evaluated for 30 sec at 5 min thereafter. Firing of GRP-responsive neurons was also measured for 30 sec at 10 min after i.d. administration of compound 48/80 or chloroquine, and then effects of RC-3095 and/or NBQX were also tested at 5 min after each application (Figure 2). Three to

six neurons were recorded in each mouse, and single cell recording was continued for up to 3 h depending on outcomes. Frequency (Hz) was calculated from measurements for 30 sec in all experiments.

2.9. Data analysis in mice

Data are presented as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using Student's t-test, one-way analysis of variance followed by Tukey's multiple comparison test, or two-way analysis of variance followed by Bonferroni's multiple comparison test as appropriate. Statistical significance was established at $P < 0.05$.

2.10. Nonhuman primates

All animal care and experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (Bethesda, MD, USA) and approved by the Institutional Animal Care and Use Committee of Wake Forest University (Winston-Salem, NC, USA). This study is reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010). Five adult male and female rhesus monkeys (*Macaca mulatta*), aged 9–16 years, weighing 9–13 kg, were kept at an indoor facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD, USA). Animals were individually housed in species-specific rooms with environmental controls set to maintain 21–25°C, 40–60% relative humidity, and a 12-h light-dark (light: 06:30–18:30) cycle. Their daily diet consisted of approximately 22–30 biscuits (Purina Monkey Chow; Ralston Purina Co., St. Louis, MO, USA), fresh fruit, and water *ad libitum*. Small amounts of primate treats and various cage-enrichment devices were supplied as forms of environmental enrichment.

2.11. I.t. administration in monkeys

Monkeys with i.t. catheters and subcutaneous access port were used to evaluate effects of i.t. administered test compound as described previously (Ding et al., 2015). A total volume of 1 ml saline or NBQX (1 or 3 nmol; Cayman Chemical, Ann Arbor, MI, USA) was administered through the subcutaneous access port followed by 0.35 ml of sterile saline to flush out the dead volume of the port and catheter. Drugs were administered i.t. with a 1-week inter-injection interval.

2.12. I.d. administration and itch scratching responses in monkeys

Monkeys were seated in primate chairs and both lateral sides of the upper part (i.e., the skin area over the *vastus lateralis* muscle) of hindlimbs were shaved 24 h before the experiment. The solution of the test ligand was prepared on the testing day. I.d. administration of BAM8–22 (Tocris Biosciences) was performed 30 min after i.t. administration of NBQX. The marked area was cleaned with an alcohol swab, wiping with firm pressure from the injection site outward in a circular motion and allowing skin to dry. The monkey's hindlimb was held tight by another experimenter before and during the injection. A precision guide needle (30G1/2, Becton Dickinson, Franklin Lakes, NJ) connected to a 50-μl microsyringe (Hamilton Co., Reno, NV) was placed almost flat against skin, bevel up; and then was

inserted 1/8 inch into skin. The test ligand BAM8–22 (50 nmol in 20 μl sterile water) was slowly injected and was watched for a wheal to appear. Once the injection was completed, the monkey immediately returned to his/her home cage and their potential site-specific scratching activity was recorded and scored 1–16 min after injection. A scratch was defined as one brief (<1 sec) episode of scraping contact of the forepaw or hind paw on the skin surface. Before collecting data, monkeys had been habituated with the injection procedure and experimenter for several times. As noted, the test ligand was administered i.d. with a 1-week inter-injection interval. The injection site was rotated for subsequent injections.

2.13. Tail-withdrawal assay in monkeys

The warm water tail-withdrawal assay (Ding et al., 2016) was used to evaluate thermal antinociceptive effects of NBQX. Through the positive reinforcement techniques, monkeys were trained to cooperate for the pole-and-collar transfer to a primate restraint chair. They were seated in primate restraint chairs and the lower parts of their shaved tails (~15 cm) were immersed in a thermal flask containing water maintained at 42, 46, or 50°C, which was randomly presented. Through numerous training sessions, monkeys have become adapted to this experimental setting. Water at 42°C and 46°C was used as non-noxious stimuli (i.e., no tail-withdrawal movement), and water at 50°C was used as an acute noxious stimulus (i.e., 2–3 sec tail-withdrawal latency). All tail-withdrawal latencies were measured at each temperature using a computerized timer by individuals who were blinded to the experimental conditions. If a monkey did not remove its tail within 20 sec (cutoff), the flask was removed and a maximum time of 20 sec was recorded. Test sessions began with baseline measurements at each temperature. Subsequent tail-withdrawal latencies were measured at 30 min after intrathecal administration of the test ligand.

2.14. Data analysis in monkeys

Mean values ± S.E.M. were calculated from individual data for all study endpoints. Comparisons were made for the same monkeys across all test sessions in the same experiment. Data were analyzed by either two-way analysis of variance (ANOVA) with repeated measures (tail-withdrawal data) or one-way ANOVA with repeated measures (itch data), followed by Bonferroni's multiple comparisons test. The criterion for significance for all tests was set at $P < 0.05$.

3. Results

3.1. Roles of the spinal GRP-GRPR system in acute itch

First, we determined whether GRP elicits scratching behaviors through GRPR signaling in the spinal cord. In naïve mice, compared to vehicle administration, i.t. administration of GRP (0.02–0.1 nmol) rapidly elicited scratching behaviors in a dose-dependent manner [$F(3,24) = 35.25$, $P < 0.0001$; Tukey's test, $P < 0.001$] (Fig. S1A). GRP (0.1 nmol, i.t.)-induced scratching behaviors were inhibited by pretreatment with RC-3095 (0.1 nmol, i.t.), a GRPR antagonist [$t(12) = 3.164$, $P = 0.0082$] (Fig. S1B). Next, we investigated whether i.t. administration of RC-3095 (0.1 nmol) affects peripherally elicited histaminergic and non-histaminergic itch. Scratching behaviors caused by i.d. injection of either chloroquine [$t(8) = 4.155$, $P = 0.0032$] or SLIGRL [$t(8) = 2.839$, $P = 0.0219$] (non-histaminergic pruritogens)

were partially but significantly attenuated by pretreatment with RC-3095. In contrast, RC-3095 attenuated neither compound 48/80 nor HTMT (histaminergic pruritogens, i.d.)-caused scratching behaviors (Fig. 1A). On the other hand, both non-histaminergic and histaminergic itch were markedly suppressed in mice after i.t. administration of Bom-Sap (250 ng), that can ablate $GRPR^+$ neurons [chloroquine, $t(8) = 10.79$, $P < 0.0001$; SLIGRL, $t(8) = 12.30$, $P < 0.0001$; compound 48/80, $t(8) = 7.458$, $P < 0.0001$; HTMT, $t(8) = 6.795$, $P = 0.0001$] (Fig. 1B).

3.2. Expression of AMPAR in spinal $GRPR^+$ neurons

To identify the glutamate receptor that regulates $GRPR^+$ neurons in the SDH following C-fiber activation, we evaluated the mRNA expression level of AMPAR by reverse transcription quantitative polymerase chain reaction (RT-qPCR). In the SDH, at 2 weeks after Bom-Sap treatment (250 ng, i.t.), mRNA expression levels of *GRPR* [$t(11) = 16.43$, $P < 0.0001$], but not *NK1R* (substance P receptor), were markedly downregulated in comparison with blank-Sap treatment. Notably, the mRNA expression level of AMPAR subunit *GluR2* [$t(11) = 2.875$, $P = 0.0151$] was significantly decreased in Bom-Sap-treated mice. *GluR1* was also slightly decreased, but no significant change was observed (Fig. 1C). By immunohistochemistry (IHC), we observed co-localization of GluR2 and GRPR in the SDH of naïve mice (93.4 ± 1.6% of $GRPR^+$ neurons expressed GluR2; 44.7 ± 3.4% of $GluR2^+$ neurons expressed GRPR), supporting that $GRPR^+$ neurons also express AMPAR (Fig. 1D). To investigate the contribution of AMPAR to peripherally elicited itch, AMPAR antagonist NBQX was i.t. administered to mice. Scratching behaviors elicited by i.d. administration of non-histaminergic (chloroquine [$F(2,12) = 13.02$, $P = 0.0010$; Tukey's test, $P < 0.01$] and SLIGRL [$t(8) = 2.715$, $P = 0.0264$]) or histaminergic (compound 48/80 [$F(2,12) = 8.940$, $P = 0.0042$; Tukey's test, $P < 0.01$] or HTMT [$t(8) = 4.010$, $P = 0.0039$]) pruritogens were attenuated by the pretreatment with NBQX (0.1–0.3 nmol) (Fig. 1E).

3.3. Firing of spinal $GRPR^+$ neurons through AMPAR

We further examined whether the glutamate–AMPAR system directly activates $GRPR^+$ neurons in the transmission of histaminergic and non-histaminergic itch in an electrophysiological study. If the recorded neurons produced firing by bath applications of GRP, we regarded those as GRP-responsive neurons. As expected, bath applications of GRP (500 nM) increased spontaneous firing of some SDH neurons [total, $t(98) = 3.600$, $P = 0.0005$; positive, $t(22) = 8.239$, $P < 0.0001$] (Fig. 2A–B, S2A–B), and GRP-induced firing was completely blocked by RC-3095 (3 μM), indicating that GRP can activate such neurons through GRPR signaling [$F(4,20) = 14.52$, $P < 0.0001$; Tukey's test, $P < 0.001$] (Fig. S2A–B). Moreover, we checked the location of GRP-responsive and nonresponsive neurons in the SDH. Compared to GRP-nonresponsive neurons, the majority of GRP-responsive neurons were located mainly on the superficial layer of the SDH (Fig. 2C). Importantly, i.d. administration of histaminergic (compound 48/80; Fig. 2D, E) or non-histaminergic (chloroquine; Fig. 2F, G) pruritogens elicited firing in the majority of GRP-responsive neurons. Both compound 48/80- and chloroquine-evoked firing were slightly inhibited by application of RC-3095 to the surface of the spinal cord, and combined application of RC-3095 and NBQX (20 μM) completely blocked compound 48/80- [$F(3,16) = 17.43$, $P <$

0.0001; Tukey's test, $P < 0.001$] and chloroquine- [$F(3,20) = 11.49$, $P = 0.0001$; Tukey's test, $P < 0.001$] evoked firing (Fig. 2D–G).

3.4. Localization of GRP$^+$ neurons in the SDH

Subsequently, we demonstrated the expression of GRP in the SDH. By RT-qPCR, we showed that the mRNA expression level of *GRP* in the SDH was markedly greater than that in the DRG [$t(6) = 14.42$, $P < 0.0001$] (Fig. S3A). To evaluate the anatomical relationship between GRP and glutamate, protein expression of GRP in the SDH was visualized by IHC. GRP was mainly localized on the surface area of the SDH and was partially co-localized with VGLUT2, a marker of glutamatergic neurons, but not IB4, which labels non-peptidergic C-fibers. These results suggested that some GRP$^+$ neurons may also release glutamate as a co-neurotransmitter (Fig. S3B). For Cre-dependent expression of tdTomato in GRP$^+$ neurons, R26-LSL-tdTomato mice were crossed with GRP-Cre mice (Fig. 3A). tdTomato was clearly expressed in the SDH of GRP-Cre/R26-LSL-tdTomato mice (19.8 ± 0.6% neurons expressed tdTomato), but not R26-LSL-tdTomato (heterozygous) mice (Fig. 3B, S4A), indicating that the cell bodies of GRP$^+$ neurons are located in the SDH. On the other hand, tdTomato expression in the DRG of GRP-Cre/R26-LSL-tdTomato mice was markedly lower than that in SDH (Fig. S4B).

3.5. Effects of chemogenetic silencing of spinal GRP$^+$ neurons

To reveal the functional significance of GRP$^+$ (GRP$^+$/Glu$^+$) neurons in itch transmission in the SDH, we evaluated the effects of chemogenetic silencing of such neurons on peripherally elicited itch using the Cre-dependent DREADD system. For Cre-dependent expression of Gi-DREADD in GRP$^+$ neurons, R26-LSL-Gi-DREADD mice were crossed with GRP-Cre mice (Fig. 3C). HA-tag was highly expressed in the SDH of GRP-Cre/R26-Gi-DREADD mice compared to R26-LSL-Gi-DREADD (heterozygous) mice (Fig. 3D). Moreover, in GRP-Cre/R26-Gi-DREADD mice, HA-tag overlapped with GRP, and partially co-localized with VGLUT2, but not IB4 or TRPV1, indicating that the expression of Gi-DREADD was restricted to GRP$^+$ neurons in the SDH (Fig. 3E).

3.6. Roles of spinal GRP$^+$ neurons in acute itch

Since i.p. administration of CNO at higher doses (≥1.0 mg/kg) attenuated chloroquine- [$F(4,20) = 15.31$, $P < 0.0001$; Tukey's test, $P < 0.001$] or compound 48/80- [$F(2,12) = 27.35$, $P < 0.0001$; Tukey's test, $P < 0.001$] induced scratching behaviors in wild-type mice (Fig. S5), 0.3 mg/kg of CNO was used for selective activation of the GRP-Gi-DREADD system. In GRP-Gi-DREADD mice, i.p. administration of CNO significantly suppressed chloroquine- [$t(12) = 2.936$, $P = 0.0125$], SLIGRL- [$t(12) = 2.473$, $P = 0.0293$], BAM8–22- [$t(11) = 2.363$, $P = 0.0376$], and HTMT- [$t(12) = 2.263$, $P = 0.0430$] induced scratching behaviors (Fig. 4A), while i.t. administration of CNO (3 nmol) suppressed chloroquine- [$t(10) = 2.627$, $P = 0.0253$] and compound 48/80- [$t(10) = 2.342$, $P = 0.0412$] induced itch (Fig. 4B). No suppressive effects of systemic CNO on chloroquine-or compound 48/80-induced scratching behaviors were observed in R26 heterozygous mice (Fig. S6). To confirm the suppressive effects of the GRP-Gi-DREADD system on peripherally elicited itch, the expression of c-fos, an indicator of neural excitation, in the SDH was evaluated by IHC. The number of c-fos$^+$ cells was increased in the ipsilateral SDH following i.d. chloroquine

treatment in comparison with that in the contralateral SDH, and attenuated by i.p. CNO treatment, consistent with the behavioral study [$F(3,20) = 89.25$, $P < 0.0001$; Tukey's test, $P < 0.001$] (Fig. 4C). On the other hand, i.p. CNO administration did not affect mechanical pain thresholds in either R26 heterozygous or GRP-Gi-DREADD mice (Fig. S7).

3.7. Roles of AMPA receptor in acute itch in monkeys

In addition, we obtained proof-of-concept evidence that the glutamate–AMPAR system regulates acute itch in the SDH of rhesus monkeys. A previous study has demonstrated that BAM8–22 elicits itch sensations in humans (Sikand et al., 2011). Therefore, we chose BAM8–22 as a pruritogen to test the inhibitory effects of the AMPAR antagonist, NBQX. Consistent with findings in mice, i.t. administration of NBQX (1, 3 nmol) dose-dependently attenuated scratching responses elicited by i.d. administration of BAM8–22 (50 nmol) in monkeys [$F(2,12) = 3.986$, $P = 0.0471$; Tukey's test, $P < 0.05$] (Fig. 5A). There was no difference in tail-withdrawal latencies to 42, 46, and 50°C between i.t. NBQX (3 nmol) and vehicle treatment, indicating that NBQX did not affect the thermal nociceptive threshold in monkeys (Fig. 5B).

3.8. GRP-GRPR and glutamate-AMPAR systems in pathological itch

Finally, to validate the roles of the GRP–GRPR and glutamate–AMPAR systems in pathological itch, we induced contact dermatitis by application of DCP. Repeated application of DCP elicited scratching behaviors in a time-dependent manner, compared to acetone control [$F(1,30) = 25.93$, $P < 0.0001$; Bonferroni's test, $P < 0.001$] (Fig. 6A, B). In the SDH, on days 7 and 14 after DCP application, the mRNA expression levels of *GRP* [$F(1,31) = 60.70$, $P < 0.0001$; Bonferroni's test, $P < 0.001$] and *GRPR* [$F(1,31) = 20.03$, $P < 0.0001$; Bonferroni's test, $P < 0.01$] were significantly increased, consistent with scratching behaviors (Fig. 6C). The upregulation of GRP and GRPR in the SDH after DCP application was confirmed by IHC (Fig. 6D). Moreover, the mRNA expression levels of *GluR1* [$t(11) = 2.324$, $P = 0.0403$] and *GluR2* [$t(11) = 2.681$, $P = 0.0214$] in the SDH were upregulated on day 14 after DCP application (Fig. 6E). Like acute itch, DCP-induced pathological itch was also suppressed by either silencing of GRP^+ neurons using GRP-Gi-DREADD [$t(14) = 2.243$, $P = 0.0416$] or ablation of $GRPR^+$ $AMPAR^+$ neurons after Bom-Sap treatment [$t(15) = 2.890$, $P = 0.0112$] (Fig. 6F–H).

4. Discussion

Histamine has long been considered a typical mediator of itch through H1 and H4 receptors (Green and Dong, 2016; Simons and Simons, 2011). Compound 48/80 and HTMT are often used for evaluating histaminergic itch, even though compound 48/80 also drives histamine receptor-independent mechanisms (Meixiong et al., 2019). On the other hand, non-histaminergic pruritogens such as chloroquine and SLIGRL elicit itch through their corresponding pruriceptors (Mas-related GPCRs and protease-activated receptors, respectively) (Liu et al., 2009; Liu et al., 2011). Once C-fibers are activated by diverse pruriceptors and downstream signaling such as TRPV1 and TRPA1 (Bautista et al., 2014; Dong and Dong, 2018), itch information drives GRP–GRPR system activation in the SDH. Despite the functional significance of GRP in eliciting itch (Akiyama et al., 2013; Mishra

and Hoon, 2013; Nattkemper et al., 2013; Sukhtankar and Ko, 2013; Sun and Chen, 2007), it remains unclear whether the primary or the spinal neurons are the main source of itch-mediating GRP. A genetic study using GRP promoter-driven expression of fluorescent proteins revealed that GRP^+ neurons are present abundantly in the SDH (Gutierrez-Mecinas et al., 2014), where they receive monosynaptic input from primary neurons (Sun et al., 2017). Even if the expression levels of GRP are higher in the SDH than in the DRG (Fleming et al., 2012; Solorzano et al., 2015), GRP expression in the DRG has been shown by IHC (Barry et al., 2016; Kiguchi et al., 2016; Takanami et al., 2014). However, recent studies using RNA sequencing at single-cell resolution reported that the *GRP* gene product is synthetized in the SDH but not in primary sensory neurons (Goswami et al., 2014; Haring et al., 2018; Usoskin et al., 2015). We also found that GRP-tdTomato expression was mainly observed in the SDH, whereas it was not clear in the DRG, suggesting that the cell bodies of GRP^+ neurons, at least, are located in the SDH. Notably, it is important to mention that GRP is certainly released from the surface of the SDH.

An *in vivo* electrophysiological study revealed that $GRPR^+$ neurons that responded to GRP application were distributed mainly within the superficial layer of the SDH, and the majority of those $GRPR^+$ neurons were depolarized by i.d. administration of either chloroquine or compound 48/80. Furthermore, activation of $GRPR^+$ neurons by peripherally elicited itch was not affected by RC-3095, while it was completely blocked by a combination of RC-3095 and NBQX, indicating that glutamate may be a main activator for $GRPR^+$ neurons under physiological conditions. Consistent with this observation, it was also recently reported that GluR2 is highly expressed on $GRPR^+$ neurons (Freitag et al., 2019), indicating a critical role of AMPAR in the activation of $GRPR^+$ neurons. Although behavioral experiments demonstrated that i.t.-administered RC-3095 (0.1 nmol) partially but significantly attenuated non-histaminergic itch, the concentration of GRP released by itch stimuli could not induce depolarization of $GRPR^+$ neurons in the SDH. As higher doses of RC-3095 ($\geq$ 0.3 nmol, i.t.) caused motor dysfunction, 0.1 nmol was chosen as the maximum dose for evaluating GRPR-specific roles in scratching behaviors (Kiguchi et al., 2016; Sukhtankar and Ko, 2013). Optogenetic burst stimulation of $GRPR^+$ neurons in the SDH opens the gate for itch (Pagani et al., 2019), and transmitter-dependent complicated mechanisms may underlie the activation of $GRPR^+$ neurons, ultimately resulting in itch. Given that GRP and glutamate can activate $GRPR^+$ neurons, the distribution and relationship between GRP and glutamate are important to understand the excitation pattern of such neurons. Our result that GRP expression was partially overlapped with VGLUT2 is also supported by other reports using fluorescently labelled GRP^+ neurons controlled by a GRP promoter (Gutierrez-Mecinas et al., 2014; Sun et al., 2017) and single-cell RNA sequences (Haring et al., 2018). GRP expression did not overlap with that of IB4 or TRPV1, indicating that GRP^+ neurons act as interneurons that receive the itch sensation from nociceptive C-fibers and then convey it to $GRPR^+$ neurons in the SDH. As primary sensory neurons also release glutamate, i.t.-administered NBQX might also block the synaptic transmission between primary neurons and GRP^+ neurons. Nevertheless, accumulating evidence demonstrates that GRP^+ neurons also release glutamate as a co-neurotransmitter to activate $GRPR^+$ $AMPAR^+$ neurons in the SDH.

In order to present direct evidence of the role of GRP^+ (GRP^+/Glu^+) neurons in the spinal transmission of itch, using the Gi-DREADD system we determined the effects of chemogenetic silencing of GRP^+ neurons on peripherally elicited itch. Exogenous administration of CNO can only induce canonical Gi pathway signaling leading to neuronal silencing, which has been helpful for demonstrating cell-specific functions (Roth, 2016; Wess et al., 2013). We demonstrated that HA expression, reflecting hM4Di expression, overlapped with that of endogenous GRP, and is partially co-localized with VGLUT2, confirming that HA-hM4Di expression was restricted to GRP^+ neurons. It is noteworthy that recent reports show that the metabolite of CNO, clozapine, reduces locomotor activity, and higher doses of CNO might have off-target effects (Gomez et al., 2017). Likewise, systemic administration of CNO (≥1.0 mg/kg) attenuated scratching behaviors elicited by chloroquine or compound 48/80 in wild-type mice, whereas i.t. CNO (3 and 10 nmol) did not affect scratching behaviors. Thus, we used 0.3 mg/kg (i.p.) or 3 nmol (i.t.) as suitable doses for evaluating GRP^+ neuron-specific functions. Compared to the effects of i.t. RC-3095, induction of the GRP–Gi-DREADD system attenuated not only non-histaminergic itch but also histaminergic itch. These results suggested that glutamate derived from GRP^+ neurons contributes to transmission of itch from C-fibers to itch-responsive $GRPR^+$ $AMPAR^+$ neurons in the SDH. Furthermore, behavioral outcomes were also supported by the finding that GRP-Gi-DREADD attenuated c-fos expression in the SDH after i.d. chloroquine. On the other hand, a previous study reported that GRP^+ cells seldom express c-fos in the SDH following i.d. chloroquine (Bell et al., 2016). It remains a possibility that glutamate released from SDH neurons lacking GRP contributes to the depolarization of $GRPR^+$ $AMPAR^+$ neurons in the SDH under physiological conditions. This possibility might explain why Bom-Sap treatment suppressed peripherally elicited itch to a greater extent than chemogenetic silencing of GRP^+ neurons did.

Despite scientific advances in knowledge on the physiology of itch, the spinal mechanisms for pathological itch are poorly understood. The mRNA expression levels of *GRP*, *GRPR*, and AMPAR subunits (*GluR1* and *GluR2*) in the SDH were significantly upregulated in parallel with scratching behaviors after DCP application, indicating that these two systems might be critical for pathological itch by contact dermatitis. Previous studies reported that GRP expression and the number of $GRPR^+$ cells in the SDH were increased in rodents and primates under abnormal itch state (Kagami et al., 2013; Nattkemper et al., 2013; Zhao et al., 2013). Nevertheless, due to diversity of pathological itch, further analyses for each disease are required for understanding spinal transmission of pathological itch. A key question that still needs to be answered is how such phenomena in the SDH are maintained through the skin or neurological events. While the upregulation of GRPR and AMPAR subunits in the SDH after DCP application suggest enhancement of two ligand–receptor systems, chemogenetic silencing of GRP^+ neurons or ablation of $GRPR^+$ $AMPAR^+$ neurons suppressed DCP-induced itch. These findings indicate that the GRP–GRPR and glutamate–AMPAR systems play a pivotal role in not only physiological but also pathological itch.

Since glutamate is a well-characterized neurotransmitter for pain (Basbaum et al., 2009; Todd, 2010), it is interesting that it discriminates between pain and itch in the SDH. Here, we found that selective inhibition of GRP^+ neurons by Gi-DREADD attenuated peripherally elicited itch, but did not affect mechanical pain thresholds in mice. Recently, it was reported

that selective activation of GRP^+ neurons by Gq-DREADD results only in itch-related behaviors (Albisetti et al., 2019). Nevertheless, a previous report demonstrated that selective excitation of spinal GRP^+ neurons elicited both itch- and pain-related behaviors in an intensity-dependent manner (Sun et al., 2017), supporting the notion that GRP^+ neurons, but not GRP itself, may regulate not only itch but also pain in rodents. Based on pharmacological assays, i.t. administration of GRP has been shown to elicit scratching behaviors without affecting pain threshold in primates (Lee and Ko, 2015). Therefore, unlike glutamate, GRP may act mainly in itch. Moreover, GRPR knockout mice and Bom-Sap-treated mice showed normal response to different pain stimuli (Sun and Chen, 2007; Sun et al., 2009). Electrophysiological experiments revealed that GRP-responsive $GRPR^+$ $AMPAR^+$ neurons were mainly distributed more at the surface of the SDH in comparison with neurons that did not respond to GRP, being consistent with the expressions of GRP and GRPR by IHC. These lines of evidence suggest that itch-responsive neurons and pain neurons may exhibit different properties modulated by responses to GRP and their locations, and that they also respond to glutamate derived from itch-related input from upstream neurons.

Given the anatomical and functional differences of the ligand-receptor systems between rodents and primates (Phillips et al., 2014), it is important to determine if the findings obtained in mice can be translated to primates. To address such concerns, we have established several experimental settings and techniques for evaluating *in vivo* pharmacological profiles of ligands in nonhuman primates (Ding et al., 2016; Ding et al., 2015; Ding et al., 2018). BAM8–22 has been shown to elicit itch sensation in humans (Sikand et al., 2011). We found that i.d. BAM8–22 elicited scratching responses in nonhuman primates within a short period of time (i.e., first 15 min after application), which is the same duration of action as reported in humans (Sikand et al., 2011). Pretreatment with i.t. NBQX dose-dependently attenuated i.d. BAM8–22-induced scratching responses. Even though AMPAR largely contributes to the transmission of pain in the SDH of rodents (Basbaum et al., 2009; Todd, 2010), the dose of NBQX used here did not affect the thermal pain threshold of primates. If there are intensity-dependent differences between pain and itch, the amount of neurotransmitter release might also be associated with nociceptive modalities. Hence, it is hypothesized that lower doses of NBQX could block the activation of AMPAR conveying itch but not pain, supporting the notion that the glutamate–AMPAR system also plays a critical role in the spinal transmission of itch in primates.

In conclusion, we clearly demonstrated that AMPAR was co-expressed with GRPR on the itch-responsive neurons in the SDH, and GRP and glutamate cooperatively regulated $GRPR^+$ $AMPAR^+$ neurons, mediating itch sensation. Glutamate derived from GRP^+ neurons played pivotal roles in the spinal transmission of not only peripherally elicited non-histaminergic and histaminergic itch under physiological conditions, but also pathological itch by contact dermatitis. Given that GRP–GRPR and glutamate–AMPAR systems are key components for regulating itch-responsive neurons, it is imperative to know how these systems are modulated under a variety of pathological conditions. Importantly, we also confirmed the functional significance of the glutamate–AMPAR system in the spinal transmission of itch in nonhuman primates. These lines of evidence provide a great opportunity to uncover the comprehensive regulatory systems of itch.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Bom-Sap	bombesin-saporin
CNO	clozapine-*N*-oxide
DCP	diphenylcyclopropenone
DREADD	designer receptors exclusively activated by designer drugs
DRG	dorsal root ganglia
GRP	gastrin-releasing peptide
GRPR	gastrin-releasing peptide receptor
IHC	immunohistochemistry
SDH	spinal dorsal horn
TRPV1	transient receptor potential cation channel subfamily V1
VGLUT2	vesicular glutamate transporter 2

References

Akiyama T, Merrill AW, Carstens MI, Carstens E, 2009 Activation of superficial dorsal horn neurons in the mouse by a PAR-2 agonist and 5-HT: potential role in itch. J Neurosci 29, 6691–6699. [PubMed: 19458238]

Akiyama T, Tominaga M, Davoodi A, Nagamine M, Blansit K, Horwitz A, Carstens MI, Carstens E, 2013 Roles for substance P and gastrin-releasing peptide as neurotransmitters released by primary afferent pruriceptors. J Neurophysiol 109, 742–748. [PubMed: 23155177]

Akiyama T, Tominaga M, Takamori K, Carstens MI, Carstens E, 2014 Roles of glutamate, substance P, and gastrin-releasing peptide as spinal neurotransmitters of histaminergic and nonhistaminergic itch. Pain 155, 80–92. [PubMed: 24041961]

Albisetti GW, Pagani M, Platonova E, Hosli L, Johannssen HC, Fritschy JM, Wildner H, Zeilhofer HU, 2019 Dorsal Horn Gastrin-Releasing Peptide Expressing Neurons Transmit Spinal Itch But Not Pain Signals. J Neurosci 39, 2238–2250. [PubMed: 30655357]

Anastasi A, Erspamer V, Bucci M, 1971 Isolation and structure of bombesin and alytesin, 2 analogous active peptides from the skin of the European amphibians Bombina and Alytes. Experientia 27, 166–167. [PubMed: 5544731]

Andoh T, Uta D, Kato M, Toume K, Komatsu K, Kuraishi Y, 2017 Prophylactic Administration of Aucubin Inhibits Paclitaxel-Induced Mechanical Allodynia via the Inhibition of Endoplasmic Reticulum Stress in Peripheral Schwann Cells. Biol Pharm Bull 40, 473–478. [PubMed: 28381802]

Aresh B, Freitag FB, Perry S, Blumel E, Lau J, Franck MCM, Lagerstrom MC, 2017 Spinal cord interneurons expressing the gastrin-releasing peptide receptor convey itch through VGLUT2-mediated signaling. Pain 158, 945–961. [PubMed: 28157737]

Barry DM, Li H, Liu XY, Shen KF, Liu XT, Wu ZY, Munanairi A, Chen XJ, Yin J, Sun YG, Li YQ, Chen ZF, 2016 Critical evaluation of the expression of gastrin-releasing peptide in dorsal root ganglia and spinal cord. Mol Pain 12.

Basbaum AI, Bautista DM, Scherrer G, Julius D, 2009 Cellular and molecular mechanisms of pain. Cell 139, 267–284. [PubMed: 19837031]

Bautista DM, Wilson SR, Hoon MA, 2014 Why we scratch an itch: the molecules, cells and circuits of itch. Nat Neurosci 17, 175–182. [PubMed: 24473265]

Bell AM, Gutierrez-Mecinas M, Polgar E, Todd AJ, 2016 Spinal neurons that contain gastrin-releasing peptide seldom express Fos or phosphorylate extracellular signal-regulated kinases in response to intradermal chloroquine. Mol Pain 12.

Cowan A, Khunawat P, Zhu XZ, Gmerek DE, 1985 Effects of bombesin on behavior. Life Sci 37, 135–145. [PubMed: 2409426]

Ding H, Czoty PW, Kiguchi N, Cami-Kobeci G, Sukhtankar DD, Nader MA, Husbands SM, Ko MC, 2016 A novel orvinol analog, BU08028, as a safe opioid analgesic without abuse liability in primates. Proc Natl Acad Sci U S A 113, E5511–5518. [PubMed: 27573832]

Ding H, Hayashida K, Suto T, Sukhtankar DD, Kimura M, Mendenhall V, Ko MC, 2015 Supraspinal actions of nociceptin/orphanin FQ, morphine and substance P in regulating pain and itch in non-human primates. Br J Pharmacol 172, 3302–3312. [PubMed: 25752320]

Ding H, Kiguchi N, Yasuda D, Daga PR, Polgar WE, Lu JJ, Czoty PW, Kishioka S, Zaveri NT, Ko MC, 2018 A bifunctional nociceptin and mu opioid receptor agonist is analgesic without opioid side effects in nonhuman primates. Sci Transl Med 10.

Dong X, Dong X, 2018 Peripheral and Central Mechanisms of Itch. Neuron 98, 482–494. [PubMed: 29723501]

Fleming MS, Ramos D, Han SB, Zhao J, Son YJ, Luo W, 2012 The majority of dorsal spinal cord gastrin releasing peptide is synthesized locally whereas neuromedin B is highly expressed in pain- and itch-sensing somatosensory neurons. Mol Pain 8, 52. [PubMed: 22776446]

Freitag FB, Ahemaiti A, Jakobsson JET, Weman HM, Lagerstrom MC, 2019 Spinal gastrin releasing peptide receptor expressing interneurons are controlled by local phasic and tonic inhibition. Sci Rep 9, 16573. [PubMed: 31719558]

Funai Y, Pickering AE, Uta D, Nishikawa K, Mori T, Asada A, Imoto K, Furue H, 2014 Systemic dexmedetomidine augments inhibitory synaptic transmission in the superficial dorsal horn through activation of descending noradrenergic control: an in vivo patch-clamp analysis of analgesic mechanisms. Pain 155, 617–628. [PubMed: 24355412]

Gomez JL, Bonaventura J, Lesniak W, Mathews WB, Sysa-Shah P, Rodriguez LA, Ellis RJ, Richie CT, Harvey BK, Dannals RF, Pomper MG, Bonci A, Michaelides M, 2017 Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. Science 357, 503–507. [PubMed: 28774929]

Goswami SC, Thierry-Mieg D, Thierry-Mieg J, Mishra S, Hoon MA, Mannes AJ, Iadarola MJ, 2014 Itch-associated peptides: RNA-Seq and bioinformatic analysis of natriuretic precursor peptide B and gastrin releasing peptide in dorsal root and trigeminal ganglia, and the spinal cord. Mol Pain 10, 44. [PubMed: 25123163]

Green D, Dong X, 2016 The cell biology of acute itch. J Cell Biol 213, 155–161. [PubMed: 27114499]

Gutierrez-Mecinas M, Watanabe M, Todd AJ, 2014 Expression of gastrin-releasing peptide by excitatory interneurons in the mouse superficial dorsal horn. Mol Pain 10, 79. [PubMed: 25496164]

Haring M, Zeisel A, Hochgerner H, Rinwa P, Jakobsson JET, Lonnerberg P, La Manno G, Sharma N, Borgius L, Kiehn O, Lagerstrom MC, Linnarsson S, Ernfors P, 2018 Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types. Nat Neurosci 21, 869–880. [PubMed: 29686262]

Hay RJ, Johns NE, Williams HC, Bolliger IW, Dellavalle RP, Margolis DJ, Marks R, Naldi L, Weinstock MA, Wulf SK, Michaud C, C JLM, Naghavi M, 2014 The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions. J Invest Dermatol 134, 1527–1534. [PubMed: 24166134]

Jensen RT, Battey JF, Spindel ER, Benya RV, 2008 International Union of Pharmacology. LXVIII. Mammalian bombesin receptors: nomenclature, distribution, pharmacology, signaling, and functions in normal and disease states. Pharmacol Rev 60, 1–42. [PubMed: 18055507]

Kagami S, Sugaya M, Suga H, Morimura S, Kai H, Ohmatsu H, Fujita H, Tsunemi Y, Sato S, 2013 Serum gastrin-releasing peptide levels correlate with pruritus in patients with atopic dermatitis. J Invest Dermatol 133, 1673–1675. [PubMed: 23353988]

Kiguchi N, Kobayashi D, Saika F, Matsuzaki S, Kishioka S, 2018 Inhibition of peripheral macrophages by nicotinic acetylcholine receptor agonists suppresses spinal microglial activation and neuropathic pain in mice with peripheral nerve injury. J Neuroinflammation 15, 96. [PubMed: 29587798]

Kiguchi N, Sukhtankar DD, Ding H, Tanaka K, Kishioka S, Peters CM, Ko MC, 2016 Spinal Functions of B-Type Natriuretic Peptide, Gastrin-Releasing Peptide, and Their Cognate Receptors for Regulating Itch in Mice. J Pharmacol Exp Ther 356, 596–603. [PubMed: 26669425]

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG, Group NCRRGW, 2010 Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160, 1577–1579. [PubMed: 20649561]

Koga K, Chen T, Li XY, Descalzi G, Ling J, Gu J, Zhuo M, 2011 Glutamate acts as a neurotransmitter for gastrin releasing peptide-sensitive and insensitive itch-related synaptic transmission in mammalian spinal cord. Mol Pain 7, 47. [PubMed: 21699733]

Lagerstrom MC, Rogoz K, Abrahamsen B, Persson E, Reinius B, Nordenankar K, Olund C, Smith C, Mendez JA, Chen ZF, Wood JN, Wallen-Mackenzie A, Kullander K, 2010 VGLUT2-dependent sensory neurons in the TRPV1 population regulate pain and itch. Neuron 68, 529–542. [PubMed: 21040852]

Lee H, Ko MC, 2015 Distinct functions of opioid-related peptides and gastrin-releasing peptide in regulating itch and pain in the spinal cord of primates. Sci Rep 5, 11676. [PubMed: 26119696]

Leung B, Lowery D, 2017 Patent watch: The evolving intellectual property landscape for pruritus therapies. Nat Rev Drug Discov 16, 670. [PubMed: 28883664]

Liu Q, Tang Z, Surdenikova L, Kim S, Patel KN, Kim A, Ru F, Guan Y, Weng HJ, Geng Y, Undem BJ, Kollarik M, Chen ZF, Anderson DJ, Dong X, 2009 Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. Cell 139, 1353–1365. [PubMed: 20004959]

Liu Q, Weng HJ, Patel KN, Tang Z, Bai H, Steinhoff M, Dong X, 2011 The distinct roles of two GPCRs, MrgprC11 and PAR2, in itch and hyperalgesia. Sci Signal 4, ra45.

Liu Y, Abdel Samad O, Zhang L, Duan B, Tong Q, Lopes C, Ji RR, Lowell BB, Ma Q, 2010 VGLUT2-dependent glutamate release from nociceptors is required to sense pain and suppress itch. Neuron 68, 543–556. [PubMed: 21040853]

Masui A, Kato N, Itoshima T, Tsunashima K, Nakajima T, Yanaihara N, 1993 Scratching behavior induced by bombesin-related peptides. Comparison of bombesin, gastrin-releasing peptide and phyllolitorins. Eur J Pharmacol 238, 297–301. [PubMed: 7691621]

McDonald TJ, Jornvall H, Nilsson G, Vagne M, Ghatei M, Bloom SR, Mutt V, 1979 Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. Biochem Biophys Res Commun 90, 227–233. [PubMed: 496973]

Meixiong J, Anderson M, Limjunyawong N, Sabbagh MF, Hu E, Mack MR, Oetjen LK, Wang F, Kim BS, Dong X, 2019 Activation of Mast-Cell-Expressed Mas-Related G-Protein-Coupled Receptors Drives Non-histaminergic Itch. Immunity 50, 1163–1171 e1165. [PubMed: 31027996]

Mishra SK, Hoon MA, 2013 The cells and circuitry for itch responses in mice. Science 340, 968–971. [PubMed: 23704570]

Nattkemper LA, Zhao ZQ, Nichols AJ, Papoiu ADP, Shively CA, Chen ZF, Yosipovitch G, 2013 Overexpression of the gastrin-releasing peptide in cutaneous nerve fibers and its receptor in the spinal cord in primates with chronic itch. J Invest Dermatol 133, 2489–2492. [PubMed: 23558404]

Ohashi N, Uta D, Sasaki M, Ohashi M, Kamiya Y, Kohno T, 2017 Acetaminophen Metabolite N-Acylphenolamine Induces Analgesia via Transient Receptor Potential Vanilloid 1 Receptors Expressed on the Primary Afferent Terminals of C-fibers in the Spinal Dorsal Horn. Anesthesiology 127, 355–371. [PubMed: 28542001]

Pagani M, Albisetti GW, Sivakumar N, Wildner H, Santello M, Johannssen HC, Zeilhofer HU, 2019 How Gastrin-Releasing Peptide Opens the Spinal Gate for Itch. Neuron.

Phillips KA, Bales KL, Capitanio JP, Conley A, Czoty PW, t Hart BA, Hopkins WD, Hu SL, Miller LA, Nader MA, Nathanielsz PW, Rogers J, Shively CA, Voytko ML, 2014 Why primate models matter. Am J Primatol 76, 801–827. [PubMed: 24723482]

Roth BL, 2016 DREADDs for Neuroscientists. Neuron 89, 683–694. [PubMed: 26889809]

Sikand P, Dong X, LaMotte RH, 2011 BAM8–22 peptide produces itch and nociceptive sensations in humans independent of histamine release. J Neurosci 31, 7563–7567. [PubMed: 21593341]

Simons FE, Simons KJ, 2011 Histamine and H1-antihistamines: celebrating a century of progress. J Allergy Clin Immunol 128, 1139–1150 e1134. [PubMed: 22035879]

Solorzano C, Villafuerte D, Meda K, Cevikbas F, Braz J, Sharif-Naeini R, Juarez-Salinas D, Llewellyn-Smith IJ, Guan Z, Basbaum AI, 2015 Primary afferent and spinal cord expression of gastrin-releasing peptide: message, protein, and antibody concerns. J Neurosci 35, 648–657. [PubMed: 25589759]

Sukhtankar DD, Ko MC, 2013 Physiological function of gastrin-releasing peptide and neuromedin B receptors in regulating itch scratching behavior in the spinal cord of mice. PLoS One 8, e67422.

Sun S, Xu Q, Guo C, Guan Y, Liu Q, Dong X, 2017 Leaky Gate Model: Intensity-Dependent Coding of Pain and Itch in the Spinal Cord. Neuron 93, 840–853 e845. [PubMed: 28231466]

Sun YG, Chen ZF, 2007 A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature 448, 700–703. [PubMed: 17653196]

Sun YG, Zhao ZQ, Meng XL, Yin J, Liu XY, Chen ZF, 2009 Cellular basis of itch sensation. Science 325, 1531–1534. [PubMed: 19661382]

Takanami K, Sakamoto H, Matsuda KI, Satoh K, Tanida T, Yamada S, Inoue K, Oti T, Sakamoto T, Kawata M, 2014 Distribution of gastrin-releasing peptide in the rat trigeminal and spinal somatosensory systems. J Comp Neurol 522, 1858–1873. [PubMed: 24254931]

Todd AJ, 2010 Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci 11, 823–836. [PubMed: 21068766]

Usoskin D, Furlan A, Islam S, Abdo H, Lonnerberg P, Lou D, Hjerling-Leffler J, Haeggstrom J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P, 2015 Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci 18, 145–153. [PubMed: 25420068]

Wan L, Jin H, Liu XY, Jeffry J, Barry DM, Shen KF, Peng JH, Liu XT, Jin JH, Sun Y, Kim R, Meng QT, Mo P, Yin J, Tao A, Bardoni R, Chen ZF, 2017 Distinct roles of NMB and GRP in itch transmission. Sci Rep 7, 15466. [PubMed: 29133874]

Wess J, Nakajima K, Jain S, 2013 Novel designer receptors to probe GPCR signaling and physiology. Trends Pharmacol Sci 34, 385–392. [PubMed: 23769625]

Yosipovitch G, Bernhard JD, 2013 Clinical practice. Chronic pruritus. N Engl J Med 368, 1625–1634. [PubMed: 23614588]

Zhao ZQ, Huo FQ, Jeffry J, Hampton L, Demehri S, Kim S, Liu XY, Barry DM, Wan L, Liu ZC, Li H, Turkoz A, Ma K, Cornelius LA, Kopan R, Battey JF Jr., Zhong J, Chen ZF, 2013 Chronic itch development in sensory neurons requires BRAF signaling pathways. J Clin Invest 123, 4769–4780. [PubMed: 24216512]

Zhao ZQ, Wan L, Liu XY, Huo FQ, Li H, Barry DM, Krieger S, Kim S, Liu ZC, Xu J, Rogers BE, Li YQ, Chen ZF, 2014 Cross-inhibition of NMBR and GRPR signaling maintains normal histaminergic itch transmission. J Neurosci 34, 12402–12414. [PubMed: 25209280]

Zhu H, Aryal DK, Olsen RH, Urban DJ, Swearingen A, Forbes S, Roth BL, Hochgeschwender U, 2016 Cre-dependent DREADD (Designer Receptors Exclusively Activated by Designer Drugs) mice. Genesis 54, 439–446. [PubMed: 27194399]

Highlight

- Firing of $GRPR^+$ neurons in the dorsal horn was blocked by AMPAR antagonist.
- Peripherally elicited itch was attenuated by AMPAR antagonist in mice and monkeys.
- Peripherally elicited itch was attenuated by silencing of GRP^+/Glu^+ neurons.
- GRP-GRPR and glutamate-AMPAR systems may be enhanced under contact dermatitis.

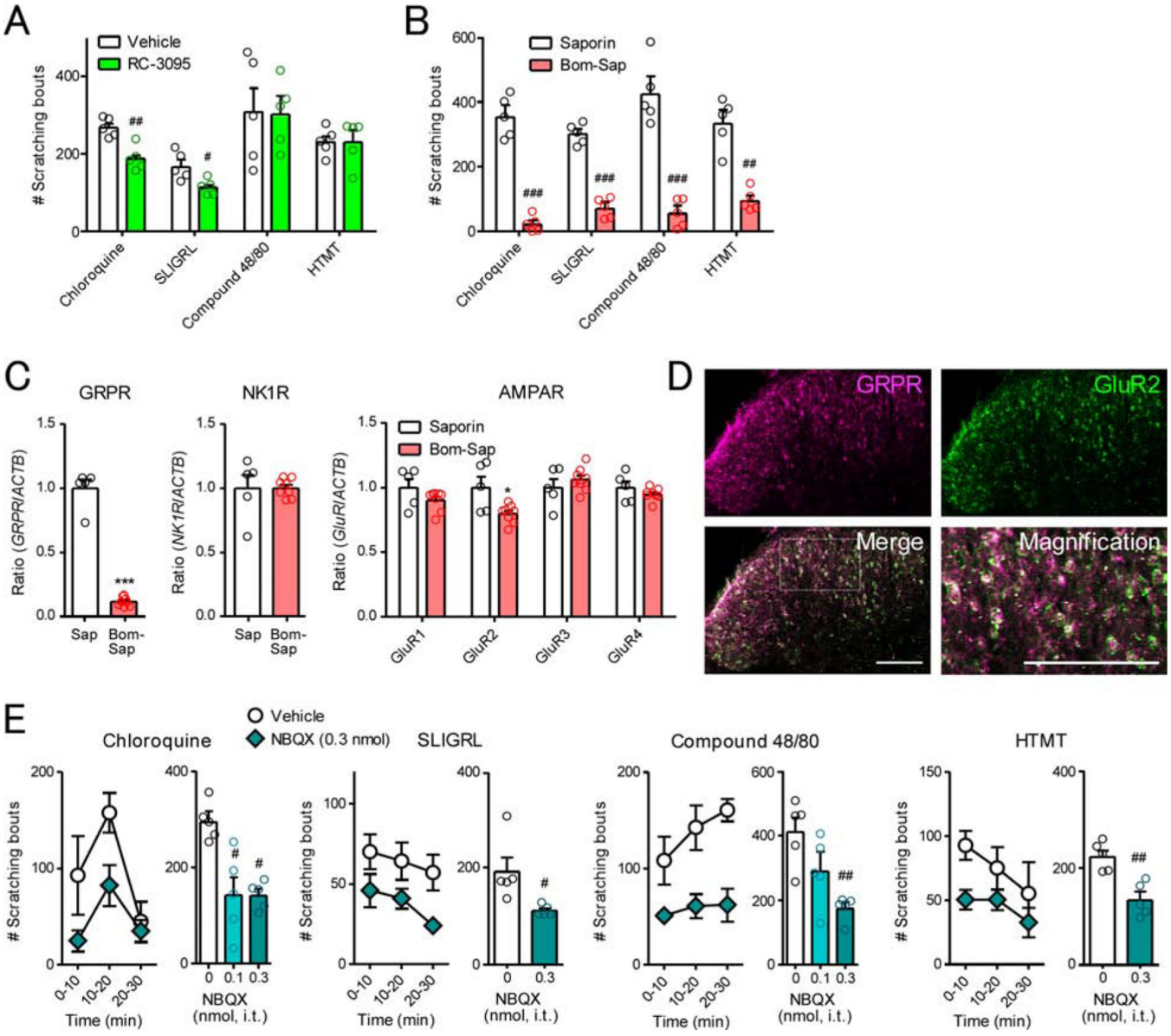

Fig. 1.

Localization of AMPAR on GRPR$^+$ neurons in the spinal dorsal horn (SDH) and itch regulation. (A) GRPR antagonist, RC-3095 (0.1 nmol) or vehicle was intrathecally (i.t.) administered 10 min prior to intradermal (i.d.) injection of pruritogens. (B) Bombesin-saporin (Bom-Sap, 250 ng), GRPR$^+$ cell-targeting toxin, or blank-saporin (Sap, 250 ng) was administered i.t. 2 weeks before i.d. injection of pruritogens. (A, B, E) Non-histaminergic pruritogens [chloroquine (300 nmol) and SLIGRL (100 nmol)] and histaminergic pruritogens [compound 48/80 (300 nmol) and HTMT (300 nmol)] were used. Scratching bouts were observed immediately after i.d. administration up to 30 min. (A, B) Total number of scratching bouts for 30 min are shown. n = 5. $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ vs vehicle or Sap. (C) The mRNA expression levels of *GRPR*, *NK1R*, and AMPAR subunits (*GluR1–4*) in the lumbar SDH at 2 weeks after i.t. administration of Bom-Sap (250 ng) or blank-Sap (250 ng) were evaluated by RT-qPCR. n = 5–8. $^{***}P < 0.001$, $^{*}P < 0.05$ vs Sap.

(D) Protein expression of GRPR and GluR2 in the lumbar SDH were visualized by immunohistochemistry in naïve mice. Representative micrographs from four mice are shown. Scale bars = 100 μm. (E) AMPAR antagonist, NBQX (0.1, 0.3 nmol), or vehicle was i.t. administered 10 min prior to i.d. injection of pruritogens. Time course in 10-min intervals (left) and total number of scratching bouts for 30 min are shown (right). n = 5. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs vehicle. (A–C, E) Each value represents the mean ± S.E.M.

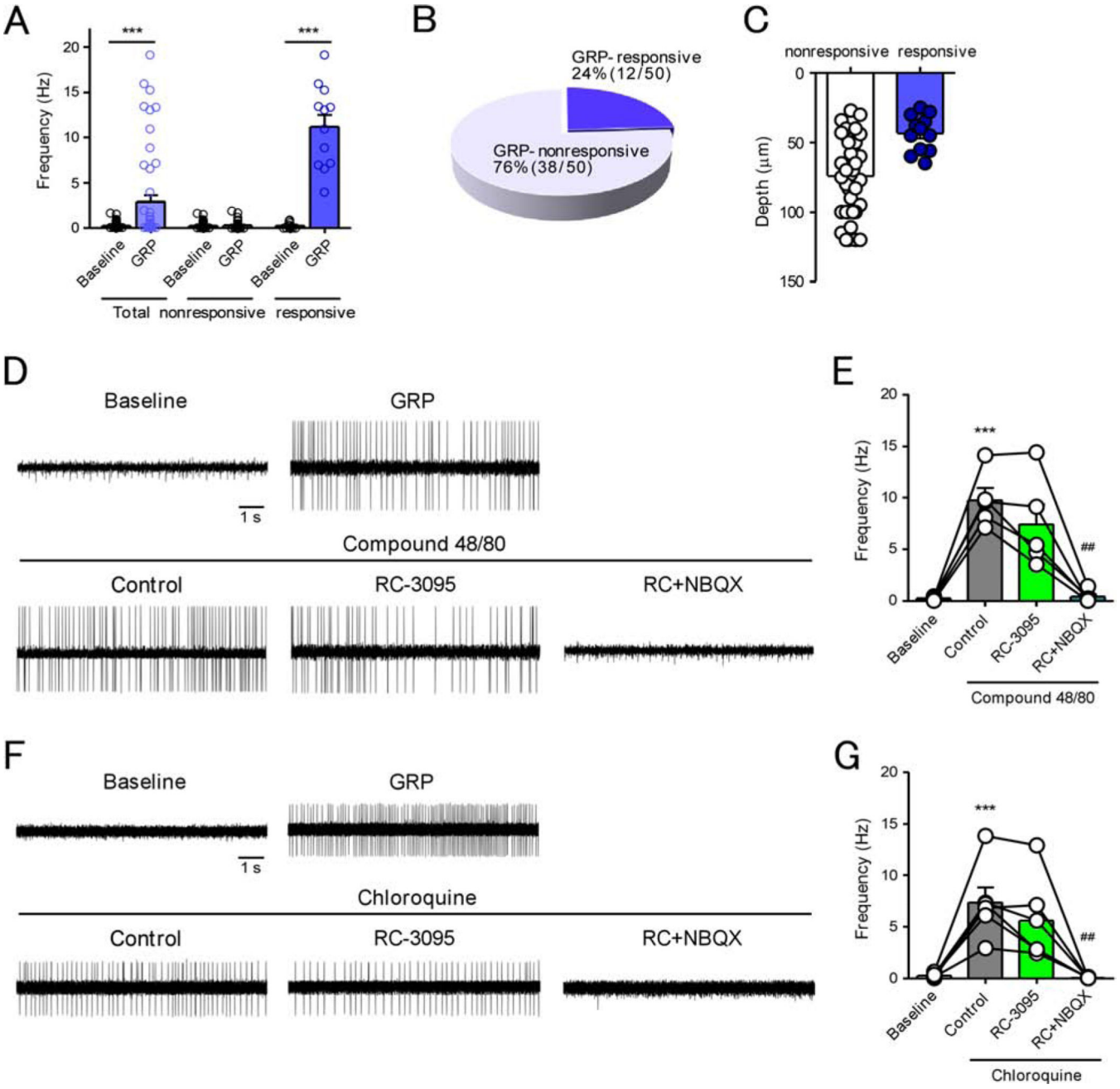

Fig. 2.

AMPAR is critical for peripherally evoked firing of GRPR$^+$ neurons in the SDH. Spontaneous firing in the absence and presence of GRP (500 nM) in Krebs solution obtained from lumbar SDH neurons using *in vivo* extracellular recording. (A) Responsiveness of GRP application on spontaneous firing from 50 SDH neurons. (B) Percentage of GRP-responsive neurons. (C) Summary of recording depth on GRP-responsive and nonresponsive neurons. (A–C) n = 12–50. ***P < 0.001 vs baseline. Representative traces (D, F) and summaries (E, G) showing the effects of RC-3095 (3 μM) and NBQX (20 μM) on compound 48/80-(300 nmol) or chloroquine- (300 nmol) evoked firing of GRP-responsive SDH neurons. n = 5–6.

***P < 0.001 vs baseline. ##P < 0.01 vs control. (A–C, E, G) Each value represents the mean ± S.E.M.

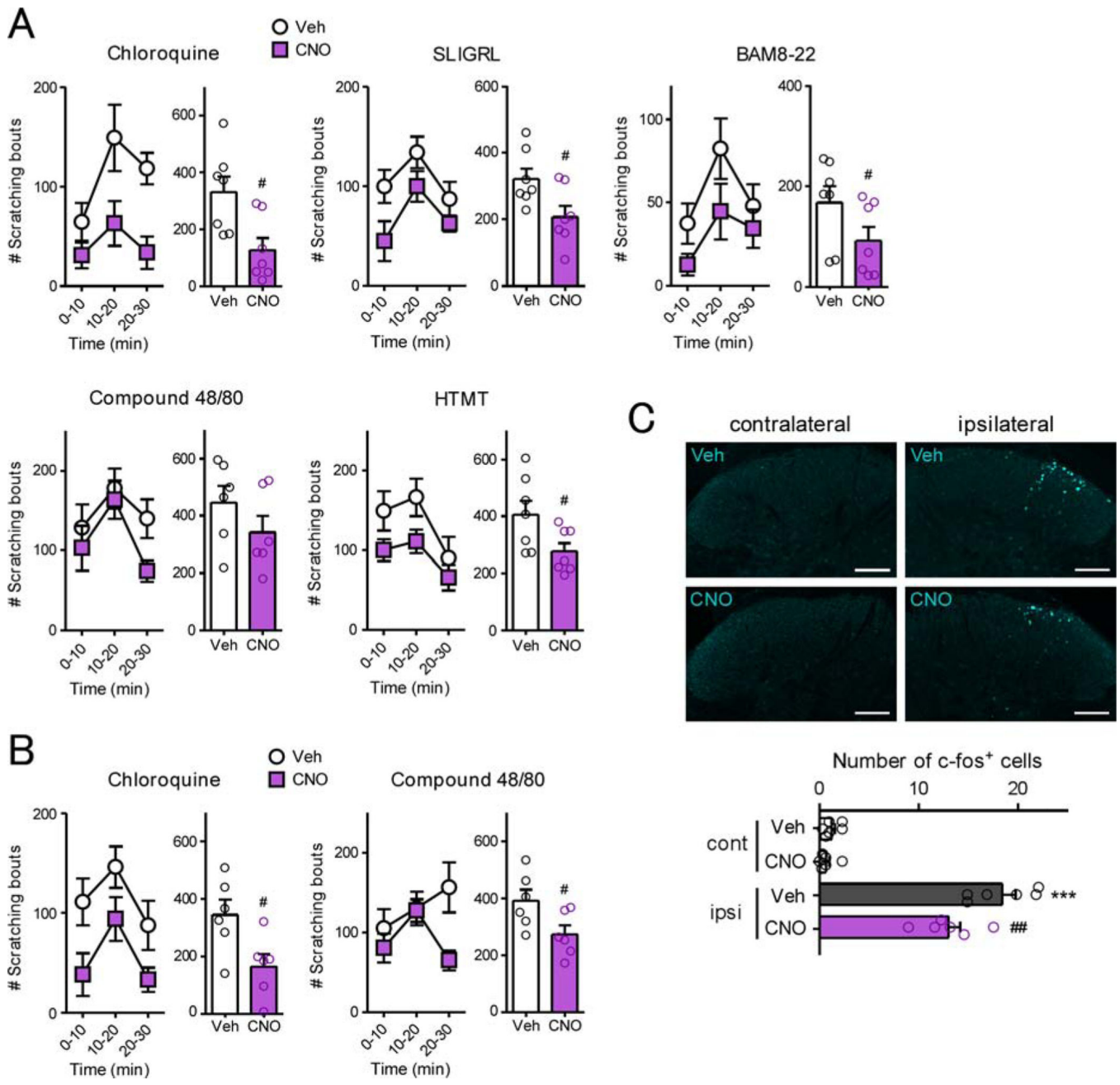

Fig. 3.

Expression of the Cre-dependent fluorescence and Gi-DREADD system on GRP^{+} neurons in the SDH. (A) R26-LSL-tdTomato mice were crossed with GRP-Cre mice for generation of GRP-tdTomato mice. (B) Cre-dependent expression of tdTomato in the SDH of GRP-tdTomato mice but not ROSA26 heterozygous mice. The protein expression of GRP was visualized by immunohistochemistry. (C) R26-LSL-Gi-DREADD mice were crossed with GRP-Cre mice for generation of GRP-Gi-DREADD mice. (D) The Cre-dependent expression of HA-tagged hM4Di in the SDH of GRP-Gi-DREADD mice but not of ROSA26 heterozygous mice was visualized by immunohistochemistry. (E) Double immunostaining of HA, GRP, TRPV1, VGLUT2, or IB4-labeled non-peptidergic C-fibers in

the SDH of GRP-Gi-DREADD mice. Representative micrographs from four mice are shown. Scale bars = 100 μm.

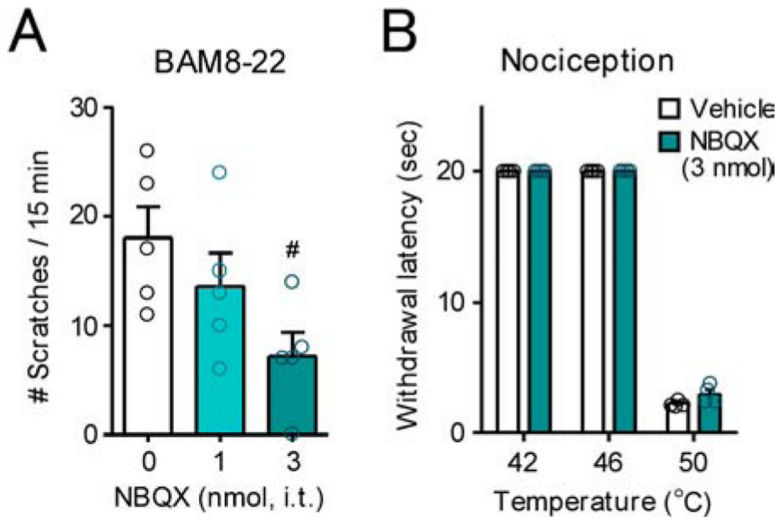

Fig. 4.

Chemogenetic silencing of GRP^{+} neurons in the SDH prevents peripherally elicited itch. Clozapine-*N*-oxide (CNO) was intraperitoneally (A, C; i.p., 0.3 mg/kg) or i.t. (B; 3 nmol) administered 40 min prior to i.d. injection of pruritogens in GRP-Gi-DREADD mice. Non-histaminergic pruritogens [chloroquine (300 nmol), SLIGRL (100 nmol), and BAM8–22 (50 nmol)] and histaminergic pruritogens [compound 48/80 (300 nmol) and HTMT (300 nmol)] were used. Scratching bouts were observed immediately after i.d. administration up to 30 min. Time course in 10-min intervals (left) and total number of scratching bouts for 30 min are shown (right). n = 6–7. $^{\#}P < 0.05$ vs vehicle. (C) Chloroquine (300 nmol) was i.d. administered to the right calf of GRP-Gi-DREADD mice, and the protein expression of c-fos in the lumbar SDH at 2 h after administration was visualized by immunohistochemistry. Representative micrographs and mean number of c-fos^{+} cells in each group are shown. n = 6. ***$P < 0.001$ vs contralateral/vehicle. $^{\#}P < 0.05$ vs ipsilateral/vehicle. (A–C) Each value represents the mean ± S.E.M.

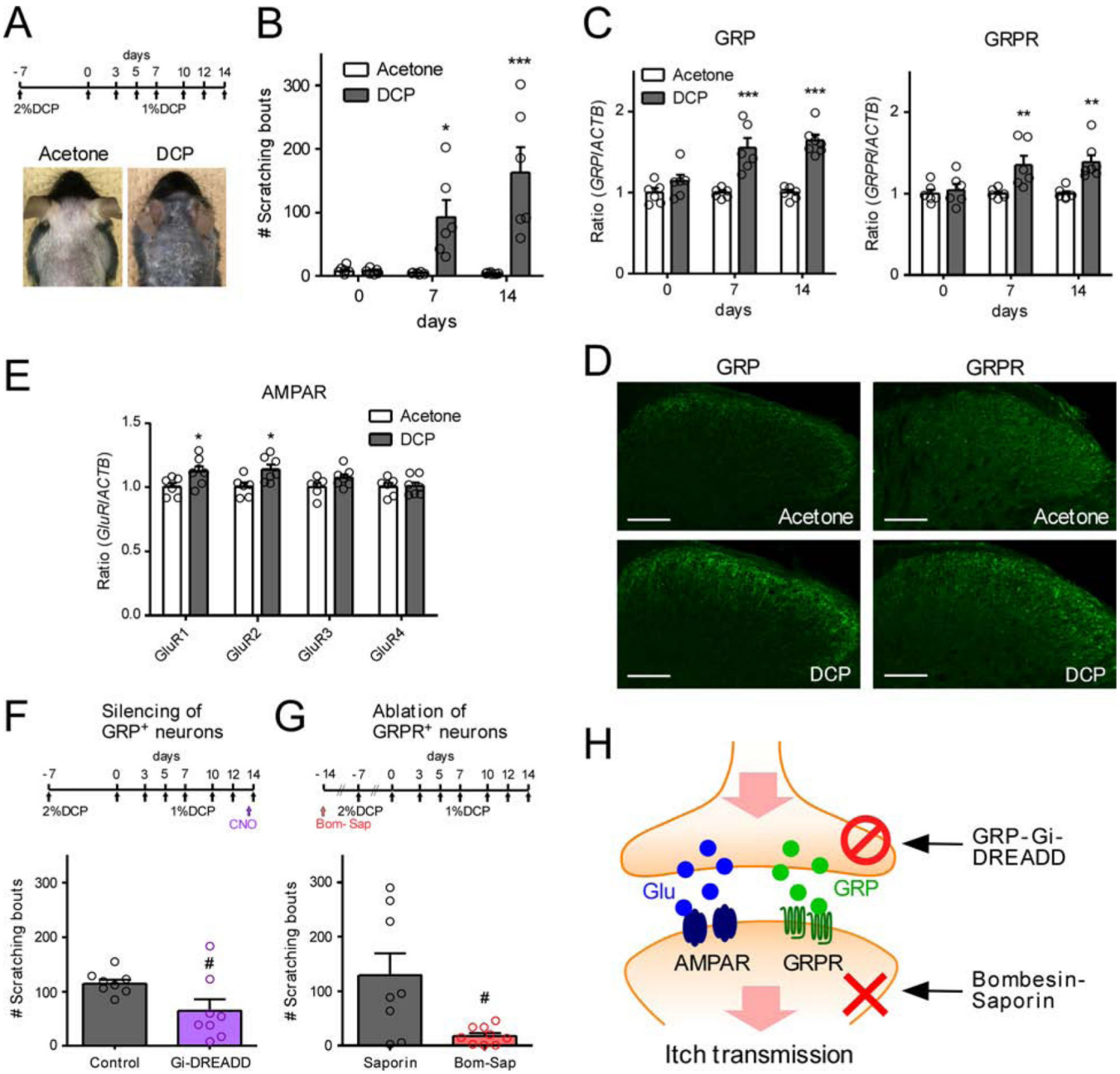

Fig. 5.

Behavioral effects of intrathecal administration of AMPAR antagonist NBQX in monkeys. (A) Effects on peripherally elicited itch. NBQX (1, 3 nmol) was i.t. administered 30 min prior to i.d. administration of BAM8–22 (50 nmol). Scratching was recorded 1 min after i.d. administration up to 16 min. Total number of injection site-specific scratching for 1–16 min is shown. Each value represents the mean ± S.E.M. n = 5. *P < 0.05 vs vehicle. (B) Effects on thermal nociception. Temperature-response curves were measured by using the tail-withdrawal latency at 30 min after i.t. administration of NBQX (3 nmol). Each value represents the mean ± S.E.M. n = 4.

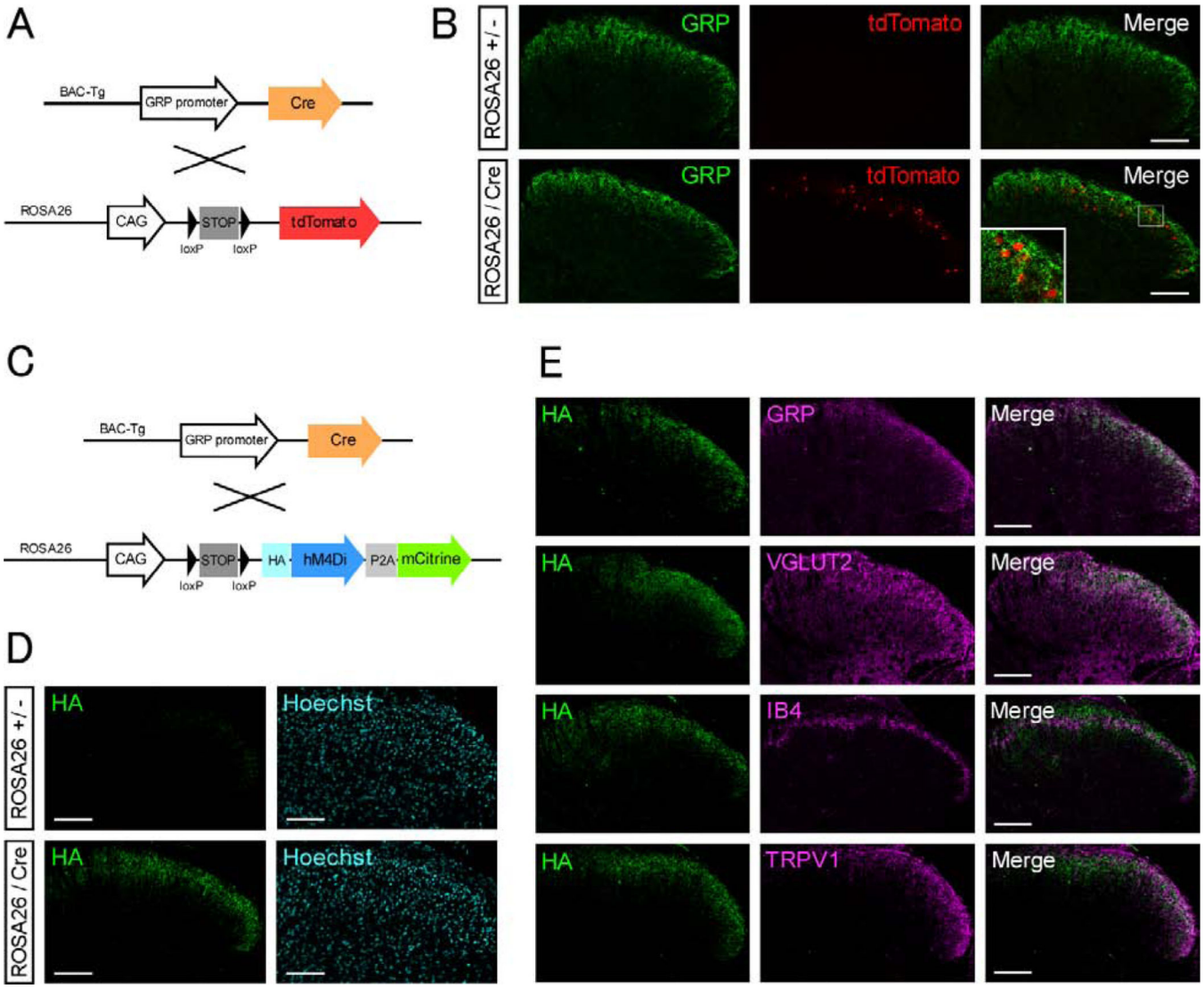

Fig. 6.

Roles of GRP-GRPR and glutamate-AMPAR systems in pathological itch by contact dermatitis. (A) Schedule of the diphenylcyclopropenone (DCP) application and photo of the acetone- or DCP-treated mice on day 7. (B, F, G) Scratching bouts were observed immediately after DCP application up to 40 min, and the total number of scratching bouts are shown. (B) Time course of DCP-induced scratching behavior. The mRNA expression levels of *GRP* and *GRPR* (C) on indicated days and AMPAR subunits (*GluR1–4*; E) on day 14 in the cervical SDH were evaluated by RT-qPCR. n = 6. ***P < 0.001, **P < 0.01, *P < 0.05 vs acetone. (D) Protein expression of GRR and GRPR in the cervical SDH on day 14 were visualized by immunohistochemistry. Representative micrographs from four mice are shown. Scale bars = 100 μm. (F) Inhibition of DCP-induced scratching behavior by CNO administration (0.3 mg.kg, i.p., 30 min prior to DCP) in GRP-Gi-DREADD mice. (G) Prevention of DCP-induced scratching behavior in mice after Bom-Sap treatment (250 ng, i.t., 7 days before experiment). n = 8–9. $^{\#}P < 0.05$ vs control or saporin. E–G) Each value represents the mean ± S.E.M. (H) Schematic spinal transmission of itch. Under

physiological and pathological condition, GRP and glutamate released from SDH neurons activate itch-responsive spinal neurons through GRPR and/or AMPAR.

Table 1.

Primer sequences for RT-qPCR

Gene	Forward (5' to 3')	Reverse (5' to 3')
ACTB	CAGCTGAGAGGGAAATCGTG	TCTCCAGGGAGGAAGAGGAT
GRP	GGAAGAAGCTGCAAGGGATT	GATCCCAAGTAGGCTGGAGA
GRPR	CCCTGCAGTTTATGGGCTTA	GAACAGGTTTGGCACGTTTC
NK1R	CTGATCTCTTCCCCAACACC	ACCACGATGACCGTATAGGC
GluR1	ACAGGAACATGCGGCTTTTA	TCTCAAAGCTGTCGCTGATG
GluR2	TGCTATCAATGTGGGGAACA	TCGCAGTCAAGGATTACACG
GluR3	GAAGCAGCAGTGCAAAACAA	TCCTGCCTTCTGTCCATTTC
GluR4	TTGTGAGTGTTGGGAAGCAC	TGACATTTGCTCCTCCATGT