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A central role for R7bp in the regulation of itch sensation

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Abstract

Itch is a protective sensation producing a desire to scratch. Pathologic itch can be a chronic symptom of illnesses such as uremia, cholestatic liver disease, neuropathies and dermatitis, however current therapeutic options are limited. Many types of cell surface receptors, including those present on cells in the skin, on sensory neurons and on neurons in the spinal cord, have been implicated in itch signaling. The role of G protein signaling in the regulation of pruriception is poorly understood. We identify here two G protein signaling components whose mutation impairs itch sensation. R7bp (a.k.a. Rgs7bp) is a palmitoylated membrane anchoring protein expressed in neurons that facilitates Gαi/o -directed GTPase activating protein activity mediated by the Gβ5/R7-RGS complex. Knockout of R7bp diminishes scratching responses to multiple cutaneously applied and intrathecally-administered pruritogens in mice. Knock-in to mice of a GTPase activating protein-insensitive mutant of Gαo (*Gnao1* G184S/+) produces a similar pruriceptive phenotype. The pruriceptive defect in *R7bp* knockout mice was rescued in double knockout mice also lacking *Oprk1*, encoding the G protein-coupled kappa-opioid receptor whose activation is known to inhibit itch sensation. In a model of atopic dermatitis (eczema), *R7bp* knockout mice showed diminished scratching behavior and enhanced sensitivity to kappa opioid agonists. Taken together, our results indicate that R7bp is a key regulator of itch sensation and

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suggest the potential targeting of R7bp-dependent GTPase activating protein activity as a novel therapeutic strategy for pathological itch.

Introduction

Itch is an irritating sensation that typically alerts us to the presence on our skin's surface of potentially harmful agents, such as fungi, parasites, insects, and certain alkaloids. The itch sensation triggers a desire to scratch. Like pain, itch is a protective sensation that promotes behavior to minimize damage to the host from harmful environmental factors. Itch detection begins in the skin, is conveyed by afferent sensory neurons that project to the dorsal horn of the spinal cord, and traverses at least two spinal interneurons before reaching spinal projection neurons that transmit the signal to higher brain centers resulting in conscious pruriception [4; 6; 9; 12; 28]. In contrast to acute itch, pathologic itch can be a chronic and often-debilitating manifestation of systemic and organ-specific illness[46].

Many types of receptors, present on cells in the skin, on sensory neurons and on neurons in the spinal cord, have been implicated in itch signaling. Such receptors include both G protein-coupled receptors (GPCR) and non-GPCR.

Regulator of G protein signaling (RGS) proteins negatively regulate GPCR signaling in many cell types [31]. RGS proteins act as GTPase activating proteins (GAPs) for G protein α subunits, accelerating the intrinsic hydrolysis rate of Gα-bound GTP to rapidly terminate G protein signaling. The R7-RGS subfamily of RGS proteins (RGS6, RGS7, RGS9, or RGS11) share similar domain architecture, including a G protein-γ like (GGL) domain that enables heterodimerization with Gβ5 (Fig. 1A)[3; 33; 38]. Gβ5 and R7-RGS proteins can be found in a heterotrimeric complex with R7-binding protein (R7BP, a.k.a. RGS7BP), a palmitoylated membrane-anchoring protein (Fig. 1A) (see [3; 16] for reviews). The regulatory effects of the Gβ5/R7-RGS complex on GPCR signaling and its Gαi/o-directed GAP activity are greatly enhanced by R7BP palmitoylation and membrane anchoring [7; 30]. A body of *in vitro* evidence suggests that the GAP activity of Gβ5/R7-RGS-containing complexes is specific for pertussis toxin-sensitive Gα subunits (Gαo and/or Gαi) [11; 30; 34; 35]. The physiology of the endogenous Gβ5/R7-RGS/R7BP complex within signaling circuits of the nervous system *in vivo* however is poorly understood.

We herein identify R7bp as a previously unsuspected critical regulator of acute and chronic pruriception *in vivo*, whose function to promote Gαi/o-directed GAP activity may represent a novel target for the therapy of pathological itch.

Materials and Methods

Mouse husbandry and genotyping

Mice were housed and treated in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and maintained in a specific-pathogen-free facility, with 4–5 animals per cage in a temperature-controlled room with a 12-hour light/dark cycle and access to food and water *ad libitum*. Age-matched female mice from littermate cohorts, 3 to 8 months of age, were used for all the experiments. Mice with a

heterozygous germline deletion of exons 1 and 2 of *R7bp* in a C57BL/6 background were a generous gift of Dr. Kirill A. Martemyanov, University of Minnesota (currently at the Department of Neuroscience, Scripps Florida) [2]. Mice heterozygous for *R7bp* deletion were bred to generate wild type and *R7bp* homozygous KO pups for testing. Kappa-opioid receptor (*Oprk1*) knockout mice (B6.129S2-Oprk1^{tm1Kff}/J; Stock Number 007558) were obtained from The Jackson Laboratory (Bar Harbor, Maine). The generation and characterization of mice harboring a heterozygous gain-of-function knock-in mutation in *Gnao1* that prevents Gαo turnoff by regulators of G protein signaling proteins (*Gnao1* G184S/+) were previously described [8; 18]. For this study *Gnao1* G184S/+ heterozygotes in a C57BL/6 background were employed. Mouse genotyping was performed by QPCR analysis of genomic DNA extracted from mouse tails using the DirectPCR (tail) solution (Viagen, Cat# 102-T) according to manufacturer's instructions. The *R7bp* knock out allele was identified employing the primer pair, Fwd: 5′-CTG CAA GCC AGT AGT GCC AGT CCC-3′, and Rev: 5′-GGA ACT TCG CTA GAC TAG TAC GCG T-3′. The wild type *R7bp* allele was identified employing the primer pair, Fwd: 5′-TCC AAG AGT TCA ACA CGC AAG TGG-3′, and Rev: 5′-GGC CAT TTC ACA GCC TTT GGT TCT-3′. The *Oprk1* knock out allele was identified employing the primer pair, Fwd: 5′-AGG GGA TTT CAA CCT GTC TG-3′, and Rev: 5′-CTC CAG ACT GCC TTG GGA AAA-3′. The wild type *Oprk1* allele was identified employing the primer pair, Fwd: 5′-AGG GGA TTT CAA CCT GTC TG-3′, and Rev: 5′-CCA CAC TGC CAT TAC TGT CG-3′.

Behavioral testing

For all behavioral testing the evaluator was blind to the genotype of the mouse being tested. Age-matched female mice from littermate cohorts, 3 to 8 months of age, were used for all the experiments. All behavioral testing was performed during the light cycle (daylight hours). Animal experiments were conducted according to NIH guidelines using protocols approved by the Animal Care and Use Committee of the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases.

Motor coordination testing

Motor coordination testing on the accelerating rotarod and open field-testing for locomotor activity were performed as previously described [47].

Testing for nociception

The hot plate test for thermal analgesia was performed as described by Terzi *et al* [41]. Thermal nociception using the tail immersion assay was assayed as previously described by Sun *et al* [40]. The von Frey test for mechanical pain was performed as described by Sun and Chen [39] using a set of calibrated von Frey filaments. Each filament was applied 5 consecutive times and the smallest filament that evoked reflexive flinches of the paw on 3 of the 5 trials was taken as the paw withdrawal threshold. Mechanical nociception was estimated by the Randall-Selitto assay utilizing an electronic algesimeter (IITC 2500 Digital Paw Pressure Meter, IITC Life Science, Woodland Hills, CA) as previously described [37]. The nocifensive response to the activation of specific nociceptors in the trigeminal sensory ganglia that innervates the cornea was assessed by the eye wipe assay, using drops (50 μl) of

dilute capsaicin (100 μM, Sigma No. M2028) or mustard oil-allyl isothiocyanate (10 μM, Sigma No. W203408) dissolved in PBS, as previously described [17].

Behavioral responses to intradermal pruritogens

The scratch response to intradermal injection of pruritogens was assayed as previously described by Sun *et al* [40]. Briefly, after two hours of cage acclimation mice were injected intradermally with a pruritogen dissolved freshly in sterile PBS (10 microliters) in the nape of the neck and then returned to their cage. Hind limb scratching behavior directed towards the injection site was recorded for 30 minutes at 5-minute intervals. A scratch was defined as the lifting of the hind limb towards the injection sites and then replacing it back on the floor. This is regardless of the number of scratching strokes taking place between the two movements [40]. The pruritogens employed were of the highest purity commercially available and included classical pruritogens (endothelin-1 (25ng/10 μl) (Sigma, Cat. #E7764), serotonin hydrochloride (10 μg/10 μl) (Sigma, Cat. #H9523), compound 48/80 (100μg/10μl) (Sigma, Cat. #C2313), SLIGRL-NH2 peptide (100μg/10 μl) (Tocris, Cat. #1468), chloroquine diphosphate salt (200μg/10 μl) (Sigma, Cat. #C6628), histamine dihydrochloride (500μg/10 μl) (Sigma, Cat. #H7520)), formalin (0.5%/10μl)), Tlr3-related agents (polyinosinic:polycytidylic acid (poly (I:C), Sigma, Cat. #P9582) and polydeoxyinosinic:polydeoxycytidylic acid (poly (dI:dC), Sigma, Cat. #P4929)), bile salt-related pruritogens (deoxycholic acid (25 μg/50 μl) (Sigma, Cat. #D2510), and taurolithocholic acid 3-sulfate disodium salt (25 μg/50 μl) (Sigma, Cat. #T0512)), and the cytokine thymic stromal lymphopoietin (2.5 μg/10 μl) (eBioscience, San Diego, CA, Cat. #148498). Individual mice were used for the testing of no more than three cutaneous or intrathecal pruritogens in the behavioral study, with at least 15 days of rest/washout in their home cage between tests.

Behavioral responses to intrathecal pruritogens

The scratch response to the intrathecal drug injection of pruritogens was performed as described by Hylden and Wilcox [13] using a sterile, disposable 30 gauge, ½ inch needle and a total injection volume of 10 μl [32]. Penetration of the lumbar spinal *dura mater* by the needle was confirmed by the classic tail flick response [14]. Scratching responses were monitored over a period of 45 minutes following injection. Peptides for intrathecal testing were of the highest purity commercially available, and included gastrin-releasing peptide (GRP) (1–5 nmol) (Bachem, Cat. #H3120) and B-type natriuretic peptide (BNP; Nppb) (1–5 nmol) (Sigma, Cat. #B9901) [32]. Individual mice were used for the testing of no more than three cutaneous or intrathecal pruritogens in the behavioral study, with at least 15 days of rest/washout in their home cage between tests.

Atopic dermatitis (eczema) model

A mouse model of atopic dermatitis was employed as described previously with minor modification[23; 40]. Mice were shaved on the nape of the neck and sensitized by painting 0.2 ml of diphenylcyclopropenone (DCP) (1% w/v, dissolved in acetone) once on the shaved skin. Seven days after sensitization mice were again challenged by painting 0.2 ml of DCP (0.5%) on the same location daily, for 10 consecutive days. Thirty minutes following DCP application, mice were injected with saline intraperitoneally and then beginning 30 minutes

post injection scratching behavior directed toward the painted skin area was quantified for the following 30 minutes. Scratching behavior was determined on days 1–5 and on days 8–10 during the 10 consecutive days of daily topical DCP application.

On the 5th, 8th and 10th days, just after quantifying scratching behavior for 30 minutes (after the intraperitoneal saline injection), mice were given an intraperitoneal injection of the specific kappa opioid receptor agonist U50,488 at 0.1, 0.25 and 2.5 mg/kg respectively. Thirty minutes after agonist administration mice scratching behavior directed towards the DCP painted area was counted again for another 30 minutes. The relative scratching response was calculated as the ratio of bouts of scratching following U50,488 injection to that following the preceding saline injection approximately an hour earlier in the same mouse.

Preparation of probes for *in situ* hybridization

Digoxigenin-labeled RNA probes were made by *in vitro* transcription using DIG RNA labeling Kit (SP6/T7) (Roche, Life Science, Cat. # 11175025910). For RNA probe generation from the SP6/T7 dual promoter vector, DNA templates in the range of 500–700 bp derived from cDNA were used. After preparation, probes were analyzed for RNA integrity and stored in aliquots at −70°C. PCR primers used to generate murine cDNA-derived DNA template inserts for the SP6/T7 dual promoter vector were: R7bp, Fwd: 5′-CTG TAC CGA GAG TTG GTC ATT T-3′, Rev: 5′-GAA CCT TCT CTT CCG TCT TCT G-3′; Rgs7, Fwd: 5′-CAT GGC TAC TTC TTT CCC ATC T-3′, Rev: 5′-CCC TCT GTT GAC TTG GTT CTT-3′; Gnb5, Fwd: 5′-ATC TGC CCT CAG GTC ATA GA-3′, Rev: 5′-GCT TGT GGT GGT CTG GSA TAA TA-3′; Rgs9, Fwd: 5′-GAC ACA GAC TAC GCC ATC TAT C-3′, Rev: 5′-CAT CTC TCC ACT CGC ATC TT-3′; Oprk1, Fwd: 5′-AAG TCA GGG AAG ATG TGG ATG T-3′, Rev: 5′-ACT GCA ACT ACT ACC AGC ACC A-3′. Primers used to generate other ISH probes were as previously described [32].

In situ hybridization

Mice were anesthetized with avertin and their spinal cord and dorsal root ganglia were rapidly dissected out. Tissues were frozen with OCT embedding medium (Electron Microscopy Sciences [EMS], Hatfield, PA) and 12 micron-thick sections were cut on a cryostat at −20° C. Pairs of wild type and *R7bp* knockout sections, from either lumbar spinal cord or pooled dorsal root ganglia, were carefully collected onto the same slides and allowed to dry for 10 minutes at room temperature. Sections were fixed in 4%(w/v) paraformaldehyde (EMS) dissolved in RNase-free PBS for 10 minutes. Sections were washed in RNase-free PBS three times and then permeabilized in 10% TritonX-100 (Sigma) (v/v) for 5 minutes. After permeabilization, sections were acetylated in acetylation buffer for 10 min at room temperature. (Acetylation buffer was prepared by combining 675 µl triethanolamine (Sigma Cat. No. 90279) with 125 µl of acetic anhydride (Sigma Cat. No. 320102) in 50 ml of DEPC-treated water; acetylation buffer was prepared fresh and used within 10 min of preparation.) After acetylation, sections were washed in PBS three times. Sections were then incubated with digoxigenin-labeled RNA probes overnight at 68° C. Sections were then washed in 0.2X SSC buffer at 70° C for 30 minutes. Sections were twice washed in a buffer containing 0.1 M maleic acid (Sigma Cat. No. M0375) in 0.15 M NaCl,

brought to pH 7.5 with solid NaOH, and then blocked with 1% (w/v) BSA for 30 minutes. Next, sections were incubated with alkaline phosphatase-labeled anti-digoxigenin antibodies (Roche) for 6–7 hours at room temperature. After incubation the sections were washed extensively and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Roche) developing agent was added. Sections were observed at different time intervals during color development. Pictures were taken using an inverted Leica microscope.

For double fluorescent *in situ* hybridization (FISH), digoxigenin-labeled RNA probes were made for R7bp and fluorescein-labeled RNA probes were made for Oprk1 by *in vitro* transcription as described above. Horseradish peroxidase-conjugated anti-digoxigenin antibody (Roche, Cat. No. 11207733910) and alkaline phosphatase-conjugated anti-fluorescein antibody (Roche, Cat. No. 11426346910) were used for detecting the RNA probes. The HNPP Fluorescent Detection Set (Roche Cat. No. 11758888001) was used for detecting alkaline phosphatase-conjugated antibody and TSA Plus Fluorescence Kit (Perkin Elmer, Cat. No. NEL741001KT) was used for detecting horseradish peroxidase-conjugated antibody. Sections were washed at the end of reaction in PBS 5–6 times and mounted with mounting medium containing DAPI. Sections were immediately analyzed using a Leica inverted fluorescent microscope with capture of digital images.

Quantification of dorsal root ganglion cells expressing pruriceptive markers

Microscopic sections of dorsal root ganglion (DRG) cells expressing MrgprA3 or Nppb by *in situ* hybridization (ISH) were analyzed using the count tool of the Adobe Photoshop CC 2015 software. Images of ISH-stained sections were opened in TIFF format and each stained cell of every DRG section was counted by manually with computer mouse clicks. Six different DRG sections were counted for each genotype (wild type and R7BP knockout mice), with the evaluator blind to the genotype of the mouse from which the section originated.

Induction and quantification of spinal neuron c-Fos expression in response to injected pruritogen

100 μg of compound 48/80 or 200 μg of chloroquine dissolved in PBS in a volume of 50 μl were injected subcutaneously into the nape of the neck by an investigator blind to genotype of the mouse under study. Four to five pairs of wild-type and *R7bp* knockout mouse littermates were analyzed per pruritogen. As a control, 50 μl of PBS vehicle only was injected in wild-type mice to estimate the effect of the skin prick sensation as a background signal. Two hours after injection, mice were anesthetized using Avertin and perfused with 20 ml of cold PBS followed by 25 ml of cold 4% paraformaldehyde dissolved in PBS. Whole spinal cords were dissected and tissues were post-fixed for 1 hr at 4° C, washed extensively with PBS, cryopreserved in 20% sucrose in PBS overnight, embedded in OTC (Electron Microscopy Sciences, PA), and frozen. Serial sections of 20 micron thickness were cut from cervical spinal cord on a cryostat and placed on slides. Tissue sections from wild-type and *R7bp* knockout mice were processed on the same slide to minimize differences in handling. For immunostaining, sections were blocked in 10% BSA and 0.25% Triton X-100 in PBS for 1 hr at RT. Sections were incubated with primary antibodies (c-Fos, Santa Cruz, sc-52; Neu N, Millipore, MAB 377) in blocking solution overnight at 4° C. Slides were washed 4

times 5 min in PBS containing 0.1% Triton X-100. Detection was carried out using Alexa-Fluor-labeled secondary antibodies diluted 1: 500 in blocking solution. Sections were mounted using glycerol containing the nuclear dye DAPI for one minute at room temperature. Sections were visualized using a BZ-9000 fluorescence microscope (Keyence) at 10X magnification and digital images were captured for counting. Four to eleven sections per animal were used for quantification, sampled across ~1200–1500 microns axial distance. Quantification was performed by manual counting of the number of fluorescent c-Fos positive cells in the lateral dorsal horn by a second investigator blind to the genotype. The number of c-Fos cells from each lateral dorsal horn (R and L) in the sections was separately counted. The data presented represents the number of c-Fos positive cells per dorsal horn (spinal sections viewed at 10X magnification) for different mouse genotypes and test substances (compound 48/80, chloroquine, or PBS vehicle).

Culture of dorsal root ganglion cells and intracellular calcium mobilization assay

Preparation and dissociation of mouse dorsal root ganglion cells was performed following the procedure of Malin *et al* [29], with minor modification as noted. Wild-type and *R7bp* knockout cage-matched littermate mice were anesthetized using avertin and the dorsal root ganglia were rapidly dissected (without prior animal perfusion). The sensory ganglia were washed once in cold Hanks' Balanced Salt solution without calcium or magnesium (GIBCO) and incubated with papain (NeuroPapain, Cat. No. NM100200, Genlantis, San Diego) for 10 min at 37 degrees followed by collagenase, Type 2 (Cat. No. LS004174, Worthington Biochemical Corp., Lakewood, NJ) and dispase II (Cat. No. 17-105-041, GIBCO) at 37 degrees. The ganglia were suspended in culture Ham's F12 medium containing 10% fetal calf serum, penicillin and streptomycin and triturated with fire polished Pasteur pipettes to isolate single neurons. The neurons with medium were plated onto Poly-D-lysine/laminin coated cover slips (Cat. No. 354087, Corning, NY) and grown in culture at 37 degrees with 5% CO_2.

A fluorescence-based assay for detecting intracellular calcium mobilization in the cultured dorsal root ganglia cells employed the Rhod-4 No Wash Calcium Assay Kit (Abcam, Cat No. ab112157), following the manufacturer's instructions for intracellular calcium assay. In short, culture medium was removed and Rhod-4 dye containing buffer was gently added to cells, followed by incubation at 37 degrees for 30 min followed by 20 min at room temperature. For imaging and quantification of ligand-induced intracellular calcium mobilization, confocal images were acquired using a Leica DMI 6000 confocal microscope (Leica Microsystems, Exton, PA) enabled with 20X multi-immersion objective NA 1.25. Images were acquired using high sensitive hybrid detectors to achieve a maximum signal to noise ratio and the time lapse images of the confocal scan were acquired every second over a three minute period. Sensory neuronal cultures were treated with either chloroquine or histamine (50 μ M, final concentration), added after the first 10 seconds to establish a baseline and observe the steady- state changes in calcium mobilization in response to the ligand treatment. Acquired confocal images were post-processed using Imaris image processing software (Bitplane USA, Concord, MA). Fluorescent intensity changes were quantified using fixed parameters across the entire set of captured images.

Statistical methods

No sample size calculation was used to predetermine sample sizes. Sample size was chosen as a balance between establishing confidence in reproducibility (on one hand) and practical considerations, such as the time required to breed animals of a particular age, gender, and genotype (on the other). Sample size is reported in the legend to each figure. Data distribution was assumed to be normal, but this was not formally tested. For comparison of two data sets, the two-tailed unpaired Student's t-test or two-way ANOVA test was employed, as appropriate. No data points were excluded. Significance level was set at $P < 0.05$ and all data are reported as mean ± S.E.M. Prism computer software, Version 5.0f (GraphPad Software, Inc.) was employed for the statistical analysis.

Results

Nociceptive somatosensation is largely preserved in mice lacking R7bp

R7bp is thought to function as a component of a heterotrimeric complex with Gβ5 and R7-RGS proteins (Fig. 1A), and R7bp and Rgs7 proteins are expressed in mouse dorsal root ganglia (DRG)[24]. The expression of R7bp and its potential functional partners in the heterotrimeric Gβ5/R7-RGS/R7BP complex was verified by *in situ* hybridization (ISH) in wild-type and *R7bp* KO mice. R7bp, Gβ5, Rgs7 and Rgs9 transcripts were readily identified in wild-type DRG (Fig. 1B upper panel), and knockout of *R7bp* did not appear to significantly affect the expression of the latter transcripts (Fig. 1B lower panel). We therefore wondered if the R7bp present in sensory neurons might regulate one or more modality of somatosensation.

Vertebrates utilize the capsaicin–sensitive Trpv1 channel expressed in peripheral sensory neurons for thermal nociception. DRG harvested from wild-type and *R7bp* KO mice showed no apparent difference in the expression of Trpv1 (Fig. 1C). Acute thermal nociception, as determined by the eye wipe test with the Trpv1 agonist capsaicin and the acute tail immersion assay, respectively, were unchanged in *R7bp* KO mice (Fig. 1D,E). Thermal nociception using the hot plate test was diminished in the *R7bp* KO mice as evidenced by enhanced basal latency of paw withdrawal (Fig. 1F), as previously reported [48].

In adult mice, sensory neurons expressing the G protein-coupled receptor Mrgprd are required for the behavioral response to noxious mechanical stimuli [5]. There was no obvious difference in the expression of Mrgprd in DRG from *R7bp* KO mice versus control (Fig. 1G). Furthermore mechanical nociception was preserved in *R7bp* KO mice (Fig. 1H,I).

Acute chemical nociception in response to mustard oil (acting through Trpa1 receptors) also appeared unchanged in *R7bp* KO mice (Fig. 1J). Since nociception was largely preserved in *R7bp* KO mice we investigated if the sense of itch was altered.

R7bp-knockout mice exhibit impaired pruriception

The scratching response to the intradermal application of a variety of classical histamine-dependent and histamine-independent pruritogens acting directly and indirectly through GPCRs was markedly deficient in *R7bp* KO mice compared to that of their wild-type

littermates (Fig. 2A–E). To exclude motor difficulties that might impact their ability to scratch, we confirmed that mice lacking R7bp exhibited normal motor coordination and locomotor activity, consistent with previous reports (data not shown)[1]. We proceeded to study a more diverse set of cutaneous pruritogens in order to better demarcate the pruriceptive defect in these mutant mice.

In wild-type mice we confirmed that the toll-like receptor TLR3 agonist polyinosinic:polycytidylic acid (poly (I:C)), a structural analog of double-stranded (ds) RNA such as that found in dsRNA viruses, but not the TLR3-inactive synthetic double-stranded DNA analog polydeoxyinosinic:polydeoxycytidylic acid(dI:dC), was a weak pruritogen (Fig. 2F) as was previously reported [27]. Mice lacking R7bp failed to respond to the pruritogenic effects of poly (I:C) (Fig. 2G).

The *R7bp* KO mice also failed to respond to the cytokine thymic stromal lymphopoietin [TSLP], implicated in the pathogenesis of atopic dermatitis in humans [15] and shown to evoke scratching behavior in mice [44] (Fig. 2H), endothelin-1, a paracrine factor released from endothelial cells (Fig. 2I), and the bile salts deoxycholate (DCA) and taurolithocholate (TLCA) implicated in the pruritus characteristic of cholestatic liver disease[20] (Fig. 2J,K).

One explanation for the above findings could be a loss of pruriceptive sensory neurons in mice lacking R7bp. Using DRG sections labeled by ISH, we found that specific pruriceptive sensory neuronal markers, such as natriuretic peptide B (Nppb)[32] and mas-related GPCR MrgprA3[25], appeared to be expressed normally in DRG from *R7bp* KO mice (Fig. 2L). Cell counting experiments showed that knockout of R7bp caused no reduction in the number of DRG cells expressing either pruriceptive marker (Fig. 2M, N). If anything in fact, the *R7bp* KO mice tended to have more DRG cells expressing the pruriceptive markers than controls, a difference that did not however achieve statistical significance.

Another explanation for the loss of scratching behavior observed in the *R7bp* KO mice could be a loss of sensitivity to pruritogens in primary pruriceptive neurons. To test this hypothesis, DRG were harvested from WT and *R7bp* KO mice, and primary sensory neurons were isolated by gentle protease treatment and cultured *in vitro* for testing, using ligand-induced mobilization of intracellular calcium as a functional assay. Treatment of the isolated sensory neurons with the prototypical pruritogens histamine and chloroquine triggered intracellular calcium release in a subset of sensory cells from both WT and *R7bp* KO mice over the three minute study period (Fig. 2O, P), with no significant difference between genotypes detected ($P = 0.73$, histamine; $P = 0.24$, chloroquine, by two-way ANOVA).

Given that loss of R7bp diminished the scratching behavior in response to multiple pruritogens acting through disparate classes of receptors, including non-GPCRs (Table 1), we questioned our underlying hypothesis linking loss of R7bp's canonical function, namely facilitation of Gαi/o-directed GAP activity, to the pruriceptive defects. We therefore sought independent confirmation of a linkage between loss of Gαi/o-directed GAP activity and loss of pruriception.

Mice heterozygous for an RGS protein-insensitive mutant of Gαo exhibit a pruriceptive defect similar to that of *R7bp* knockout mice

Within R7bp- and Gβ5/R7-RGS complex-expressing neurons, the amplitude of the intracellular signal transduced from agonist-activated Gi/o-coupled GPCRs represents a balance between receptor-driven Gi/o activation and Gi/o de-activation (Fig. 3A). Signaling in neurons from mice lacking R7bp (Fig. 3B) or harboring an RGS protein-insensitive mutant form of Gαi Gαo (Fig. 3C) might be similarly enhanced.

Given the similarity of the predicted signaling phenotypes, we hypothesized that the pruriceptive phenotype of mice expressing a Gαi/o subunit insensitive to the GAP activity of RGS proteins might be similar to that of *R7bp* KO mice if, in fact, the *R7bp* KO phenotype was due to loss of its canonical role in facilitating the Gαi/o-directed GAP activity of Gβ5/R7-RGS complexes.

We therefore tested pruriception in mice heterozygous for the G184S mutant of Gαo (*Gnao1* G184S/+) [8]. This approach afforded the opportunity to test the hypothesis without any knowledge regarding the upstream Gi/o-coupled GPCR(s) that might be involved in R7bp-dependent pruriception. Because of the single amino acid mutation, the G184S mutant form of Gαo fails to bind RGS proteins with high affinity and is insensitive to RGS protein GAP activity [22]. Mice homozygous for this mutation are not viable [8].

We found the scratching response to the intradermal application of both compound 48/80 and chloroquine was markedly deficient in *Gnao1* G184S heterozygous mice (Fig. 3D and E respectively). The similarity of the pruriceptive phenotype of *Gnao1* G184S/+ mice to that of *R7bp* KO mice strongly suggested that it was loss of R7bp's canonical role as facilitator of Gαi/o-directed GAP activity that was critical to the phenotype of the latter mice.

To help explain why the scratching response to such a diverse array of cutaneously applied pruritogens was affected in *R7bp* KO mice (Table 1), and to account for the apparent lack of effect of *R7bp* KO on the number or sensitivity of primary pruriceptors (Fig. 2L–P), we decided to consider possible effects of R7bp loss in the central nervous system. To evaluate this possibility, we quantified behavioral responses to intrathecally-administered pruritogens.

Mice lacking R7bp show reduced scratching behavior in response to intrathecally-administered pruritogens

The itch sensation is conveyed by primary sensory neurons that release Nppb onto secondary pruriceptors in the spinal dorsal horn (Fig. 4A)[32]. Accordingly the intrathecal administration of Nppb induces scratching behavior in mice [32]. Secondary pruriceptors release gastrin-releasing peptide (GRP) onto tertiary pruriceptors in the superficial dorsal horn that express gastrin-releasing peptide receptors (Grpr) (Fig. 4A) [32; 39; 40]. Scratching behavior resulting from the intrathecal administration of Nppb is blocked by pretreatment with a Grpr antagonist [19]. Wild-type and *Nppb* −/− mice, but not *Grpr* −/− mice, exhibit scratching behavior in response to the intrathecal administration of GRP [32; 39].

We studied the scratching behavior in response to intrathecal Nppb and GRP in *R7bp* KO mice. The intrathecal administration of either Nppb (Fig. 4B) or GRP (Fig. 4C) stimulated scratching behavior that was greatly diminished in mice lacking R7bp (Fig. 4B,C) (Table 1). We observed that, as in *R7bp* KO mice, *Gnao1* G184S/+ mice also exhibited reduced scratching behavior in response to intrathecal GRP (Fig. 4D).

The diminished responsiveness to intrathecal pruritogens observed suggested that the pruriceptive defect in *R7bp* KO mice involves R7bp actions at the spinal level. If this model were true, *R7bp* KO mice should also exhibit reduced activation of spinal neurons in response to cutaneously applied pruritogens. We tested this using an immunohistochemical assay for the activation of spinal neurons.

Mice lacking R7bp show impaired activation of spinal cord dorsal horn neurons in response to cutaneous pruritogens

We first confirmed the expression of R7bp and other components of the heterotrimeric Gβ5/R7-RGS/R7BP complex in the spinal cord by ISH (Fig. 5A). Transcript for *R7bp* was expressed widely in cells throughout the dorsal horn and intermediate zone of grey matter in wild-type mice (Fig. 5A upper panel, left). Knockout of *R7bp* did not affect the level or pattern of expression of transcripts for Gβ5, Rgs7 and Rgs9 (Fig. 5A, lower panel).

Cutaneous pruritogens can activate neurons in the superficial dorsal horn as evidenced by induction of the immediate early gene *c-Fos* in neuronal nuclei [10; 45]. We therefore reasoned that if the pruriceptive defect in *R7bp* KO mice resulted from diminished inter-neuronal signaling at the spinal level, there might be a corresponding reduction in the pruritogen-induced activation of *c-Fos* expression in neurons present in the superficial dorsal spinal cord. Intradermal administration of compound 48/80 induced *c-Fos* immunoreactivity in lateral spinal dorsal horn neurons of wild-type mice (Fig. 5B upper panel; 5C). Significantly fewer neurons demonstrated *c-Fos* induction in 48/80-treated *R7bp* KO mice or in PBS-treated wild-type mice (Fig. 5B lower panel; 5C). Spinal lateral dorsal horn neuronal *c-Fos* induction in response to intradermal chloroquine administration was also reduced in *R7bp* KO mice (Fig. 5D).

Taken together, the results above were most consistent with a model in which the impaired scratching behavior in response to the cutaneous application of pruritogens in *R7bp* KO mice resulted (a) primarily from disruption of the itch sensory circuitry at the spinal level, and (b) from the loss of the canonical function of R7bp to facilitate GAP activity directed against Gai/o. We therefore considered candidate Gi/o-coupled GPCR-regulated pathways in the spinal cord that were normally *inhibitory* to pruriception. Loss of R7bp-facilitated GAP activity in such a pathway could lead to an exaggeration of the normal anti-pruriceptive inhibitory signal, resulting in a loss-of-pruriception phenotype.

Knockout of the kappa-opioid receptor rescues the pruriceptive defects of *R7bp* knockout mice

Recently, a population of inhibitory interneurons in the dorsal horn was identified that express the transcription factor *Bhlhb5* and are abolished by knockout of *Bhlhb5* during development [36]. These spinal interneurons, termed B5-I neurons, are involved in the tonic

inhibition of itch[17; 36]. B5-I neurons, thought to be GABAergic or glycinergic, also utilize dynorphin as an inhibitory neuromodulator [17; 36]. Dynorphin is the endogenous ligand for the kappa-opioid receptor, a Gi/o-coupled GPCR encoded by *Oprk1*. Loss of R7bp from neurons expressing kappa-opioid receptors and positioned downstream from B5-I neurons in the pruriceptive signaling pathway could lead to an exaggeration of normal inhibitory neuromodulation and a loss-of-pruriception phenotype. We therefore hypothesized that, if this were the case, knockout of the kappa-opioid receptor might reverse the loss-of-pruriception phenotype in *R7bp* KO mice.

To explore this possibility, pruriception was tested in mice singly deficient for Oprk1 or for R7bp, in *Oprk1/R7bp* double KO mice, and in their wild-type littermates. As previously shown, the scratching response to the intradermal application of both compound 48/80 and chloroquine was deficient in *R7bp* KO mice compared to their wild-type littermates (Fig. 6A,B). While the knockout of *Oprk1* alone had no effect, the double knockout of *Oprk1* and *R7bp* completely reversed the deficiency of scratching behavior in response to intradermal 48/80 and chloroquine observed in mice deficient for R7bp alone (Fig. 6A, B).

In response to intrathecal GRP, *R7bp* KO mice exhibited diminished scratching behavior, as previously shown (Fig. 6C). Mice deficient for Oprk1 also showed a diminished response to intrathecal GRP, however mice doubly knocked out for *Oprk1* and *R7bp* showed a normal scratching response to intrathecal GRP, indicating mutual rescue of the diminished scratching responses seen with either single gene deficiency (Fig. 6C). That single knockout of *Oprk1* resulted in diminished scratching in response to intrathecal GRP was an incidental and unexpected finding, not readily explained by current models.

Mice lacking R7bp exhibit diminished scratching behavior and enhanced sensitivity to kappa opioid agonists in a model of atopic dermatitis

We showed above that *R7bp* KO mice exhibit diminished scratching in response to cutaneous application of the cytokine TSLP (Fig. 2I). Since TSLP signaling has been implicated in the pathogenesis of atopic dermatitis [15], we studied the scratching behavior *R7bp* KO mice and their wild-type littermates in a mouse model of atopic dermatitis induced by the chronic topical application of diphenylcyclopropenone (DCP)[23]. Following the sensitization period, mice lacking R7bp exhibited significantly diminished scratching behavior over the 10 days of the trial compared to control ($P < 0.0001$, two-way ANOVA) (Fig. 6D).

Since we previously showed the effects of *R7bp* KO on acute pruritogen-induced scratching behavior depended on the presence of kappa opioid receptors, we tested the effect of U50,488, a specific kappa receptor agonist, on chronic itch during the latter portion of DCP trial. Since during this time the control and R7bp KO mice had different levels of scratching behavior, we compared the relative scratching behavior following U50,488 injection to that following a preceding saline injection an hour earlier in the same mouse. Control mice exhibited no difference in relative scratching behavior in response to the 0.1 mg/kg and 0.25 mg/kg doses of U50,488, whereas *R7bp* KO mice showed a significant reduction (Fig. 6E). A heightened sensitivity to U50,488 treatment in the R7bp KO mice was particularly evident

with the 0.25 mg/kg dose following which the mutant mice showed a nearly 90% reduction in relative scratching behavior compared to control (Fig. 6E).

Discussion

We demonstrate here that R7bp critically regulates the acute and pathologic sensation of itch *in vivo*. Our results indicate that the loss-of-pruriception phenotype seen in *R7bp* KO mice results primarily from regulatory actions by R7bp on itch signaling at the level of the spinal cord, or more centrally. This is because the loss of R7bp fails to reduce the number or sensitivity of primary pruriceptive sensory neurons yet results in inhibition of the scratching response to intrathecally-applied Nppb and GRP. Regulation by R7bp occurring at a central node in the pruriceptive pathway can explain why R7bp loss affects responses to a diverse set of peripherally administered pruritogens (Table 1). Our findings do not exclude however the possibility that R7bp expressed in primary sensory neurons, at their cutaneous or spinal nerve terminals, may play a secondary role in pruriception.

A loss-of-pruriception phenotype very similar to that seen in R7bp KO mice was observed in *Gnao1* G184S heterozygotes, expressing an RGS protein-insensitive mutant of Gαo. This indicates that the central effect of R7bp loss likely results from loss of R7bp's canonical function as facilitator of Gαi/o-directed GAP activity.

If this is true, and since our results show that R7bp effects on pruriception depend on Oprk1 expression, the simplest model would be that R7bp normally enhances pruriception in the CNS by inhibiting Gi/o protein signaling directly coupled to kappa-opioid receptors (Fig. 7B). Our observation that *R7bp* KO potentiates the antipruritic effects of exogenous kappa opioid agonists would be consistent with this model. Our preliminary expression analysis by double fluorescent ISH in the spinal cord identified a subset of R7bp+ cells in the dorsal horn that were also Oprk1+, a finding also consistent with our proposed model (Fig. 7A).

Dynorphin is the endogenous ligand of the kappa-opioid receptor whose knockout reverses the dominant loss-of-pruriception phenotype observed in *R7bp* KO mice. Ross and co-workers showed that dynorphin is synthesized and utilized as a neuromodulator by B5-I spinal interneurons that are inhibitory to itch sensation[17]. Our model would further be consistent with the possibility that the functionally relevant kappa-opioid receptors, whose knockout reverses the dominant loss-of-pruriception phenotype in *R7bp* KO mice as demonstrated here, are normally responsive to the dynorphin released from these B5-I spinal interneurons (Fig. 7B). If that is true, it implies that dynorphin is released tonically from B5-I cells under basal conditions. Basal activation of kappa-opioid receptors by dynorphin released from B5-I spinal interneurons could explain how KO of *R7bp* produces a loss-of-pruriception phenotype under un-stimulated conditions. Confirmation and rigorous testing of this model will require at least two parallel sets of experiments utilizing R7bp conditional KO mice. One set of experiments would target and limit the KO of R7bp to anatomically and spatially defined subpopulations of neurons within the peripheral and central nervous system. The other line of experiments would restrict the KO of R7bp to neurons functionally defined by their expression of specific signaling molecules, such as G o-coupled GPCRs

(particularly including Oprk1), specific R7-RGS subfamily RGS proteins, or other signaling molecules and their regulators.

Current therapeutic options for pathologic itch are suboptimal [46]. The pruritus associated with diseases such as cholestatic liver disease, uremia, neuropathies, and eczematous, psoriasiform, and other types of dermatitis frequently does not respond to antihistamines or general anti-inflammatory agents. Centrally acting antipruritic therapies are being explored and clinical studies suggest that the kappa-opioid receptor agonists hold promise for the treatment of uremia-associated pruritus [21; 42]. Pharmacologic agents acting at the kappa-opioid receptor likely mimic the antipruritic action of the endogenous itch-inhibitory dynorphinergic B5-I neurons identified by Ross and co-workers [17].

Our findings suggest therefore that an endogenous kappa opioid pathway signals tonically to inhibit itch, and that manipulation of that tone can have striking effects on pruriception and the response to exogenous kappa opioids. Since our studies indicate that loss of R7bp diminishes pruriception to a wide range of pruritogens and has pronounced effects on both acute and chronic itch, inhibitors of R7bp-dependent GAP activity could represent a novel and more specific therapeutic strategy for pathological itch.

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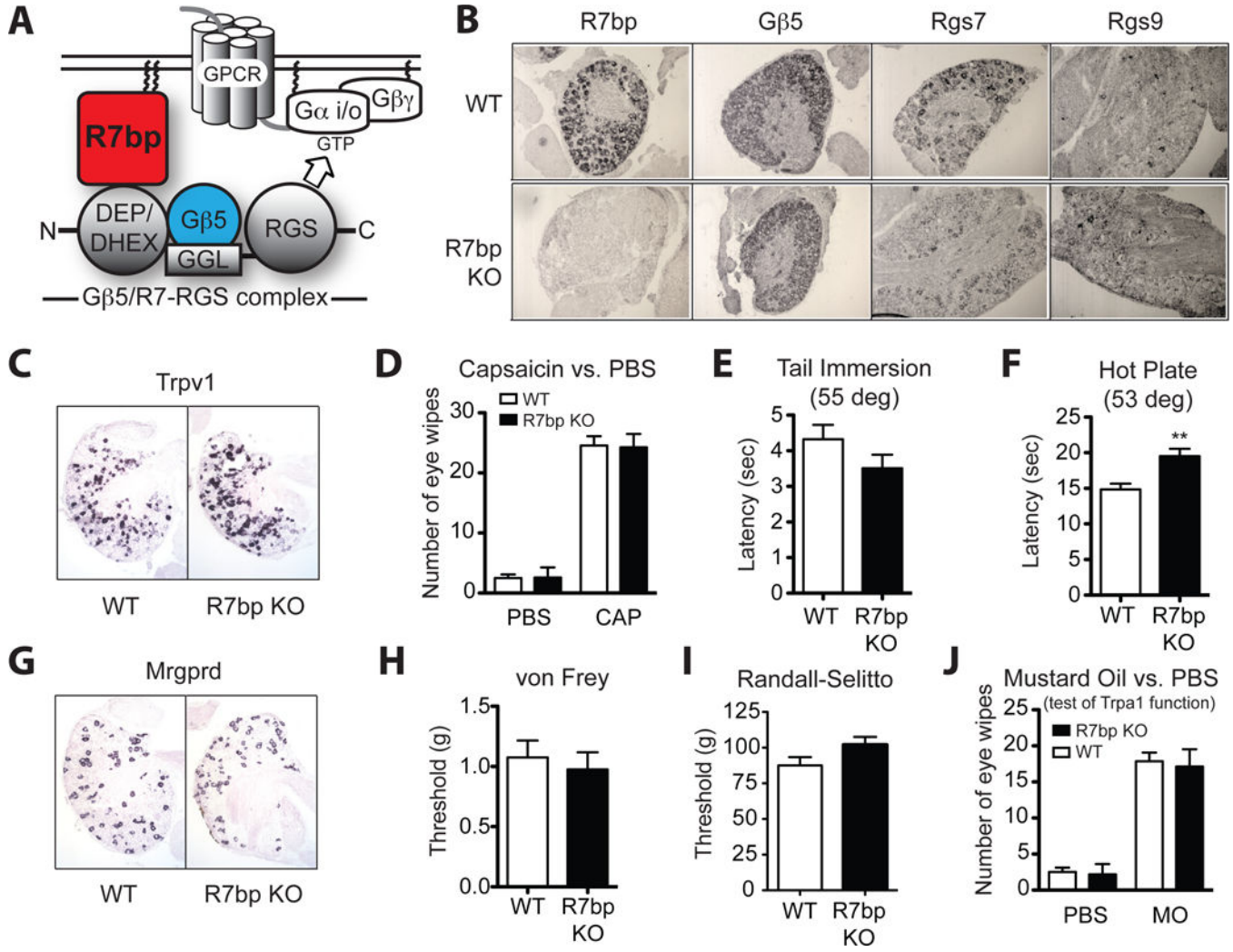

Fig. 1. Nociceptive somatosensation is largely preserved in mice lacking R7bp

(**A**) Schematic illustration of R7bp, in complex with R7-RGS and Gβ5 proteins, and heterotrimeric G protein Gαi/o subunit, the target of Gβ5/R7-RGS mediated GTPase activating protein (GAP) activity, positioned near the inner face of the plasma membrane. R7bp facilitates the GAP activity of the Gβ5/R7-RGS complex through its membrane-anchoring function. Following Gi/o activation by agonist-activated Gi/o-coupled G protein coupled receptor (GPCR), the GAP activity of the Gβ5/R7-RGS complex (arrow) helps to terminate signaling through Gαi/o and Gβγ. R7bp is shown anchored to the plasma membrane by dual palmitoylation (two squiggles), Gαi/o by myristoylation (single squiggle), and Gβγ by γ-isoprenylation (single squiggle). The R7-RGS subfamily of RGS proteins (RGS6, RGS7, RGS9 and RGS11) shares a similar domain architecture: an N-terminal DEP/DHEX domain (the putative binding site of R7bp), followed by a G-gamma-like domain (GGL) that imparts the ability to bind Gβ5, and a C-terminal RGS core domain that possesses GAP activity directed against Gαi/o (arrow). (**B**) Sections through lumbar dorsal root ganglia (DRG) harvested from wild type (WT) (upper panel) and *R7bp* knockout (KO) (lower panel) mice and hybridized *in situ* with antisense probes against Gβ5, R7bp,

and the R7-RGS family members Rgs7 and Rgs9 showed no obvious differences in transcript expression. (**C**) Sections through DRG from WT and *R7bp* KO mice hybridized *in situ* with an antisense probe against the capsaicin-binding transient receptor potential cation channel subfamily V member 1 (Trpv1) showed no obvious differences in transcript expression. (**D**) No significant differences between WT and *R7bp* KO littermates were observed in the eye wipe assay using the Trpv1 agonist capsaicin (CAP). Results using phosphate-buffered saline (PBS) are shown for comparison. (**E**) No significant differences between WT and *R7bp* KO littermates were noted in the tail immersion test of thermal nociception. (**F**) *R7bp* KO mice exhibited an increased latency in 53° C hot plate testing compared to WT littermates. (**G**) No obvious differences in mas-related receptor Mrgprd transcript expression in DRG from WT versus *R7bp* KO mice were observed by *in situ* hybridization. (**H**) No significant differences between WT and *R7bp* KO littermates were seen with Von Frey filament behavioral testing of mechanical nociception. (**I**) Results of the tail withdrawal assay using the Randall-Selitto test showed no significant difference between WT and *R7bp* KO littermates. (**J**) No significant differences between WT and *R7bp* KO littermates were observed in the eye wipe test of chemical nociception using the Trpa1 agonist mustard oil (MO). Results using phosphate-buffered saline (PBS) are shown for comparison. Statistics and representative figure reporting: For panels C and G, the hybridization experiment was repeated twice with similar results, using previously published probes[32]. For B, the hybridization experiment was repeated three times for each probe with similar results. In D–F, and H–J, the evaluator was blind to the genotype of the mouse being tested. In D–F, and H–J, the two-tailed unpaired Student's t-test was employed, with bars indicating mean ± S.E.M. For panels D, H, and J, n= number of mice from each genotype tested (for panel D, PBS WT n= 6; CAP WT n= 9; PBS KO n= 5; CAP KO n= 8; for panel H, n=8; for panel J, PBS WT n= 6; MO WT n= 7; PBS KO n= 6; MO KO n= 7). For panels E, F, and I, n= number of mice tested (for panel E, WT n= 7, KO n= 8, with mice tested 2 or 3 times, with results from individual mice averaged; for panel F, WT n= 14, KO n= 10, with mice tested 3 times, with results from individual mice averaged; for panel I, WT n= 8, KO n= 7, with mice tested 3 times, with results from individual mice averaged.) P values: D, for PBS, P= 0.95, for CAP, P= 0.91; E, P =0.1; F, *** P=0.0002; H, P =0.63; I, P= 0.27; J, for PBS, P= 0.84, for MO, P= 0.80.

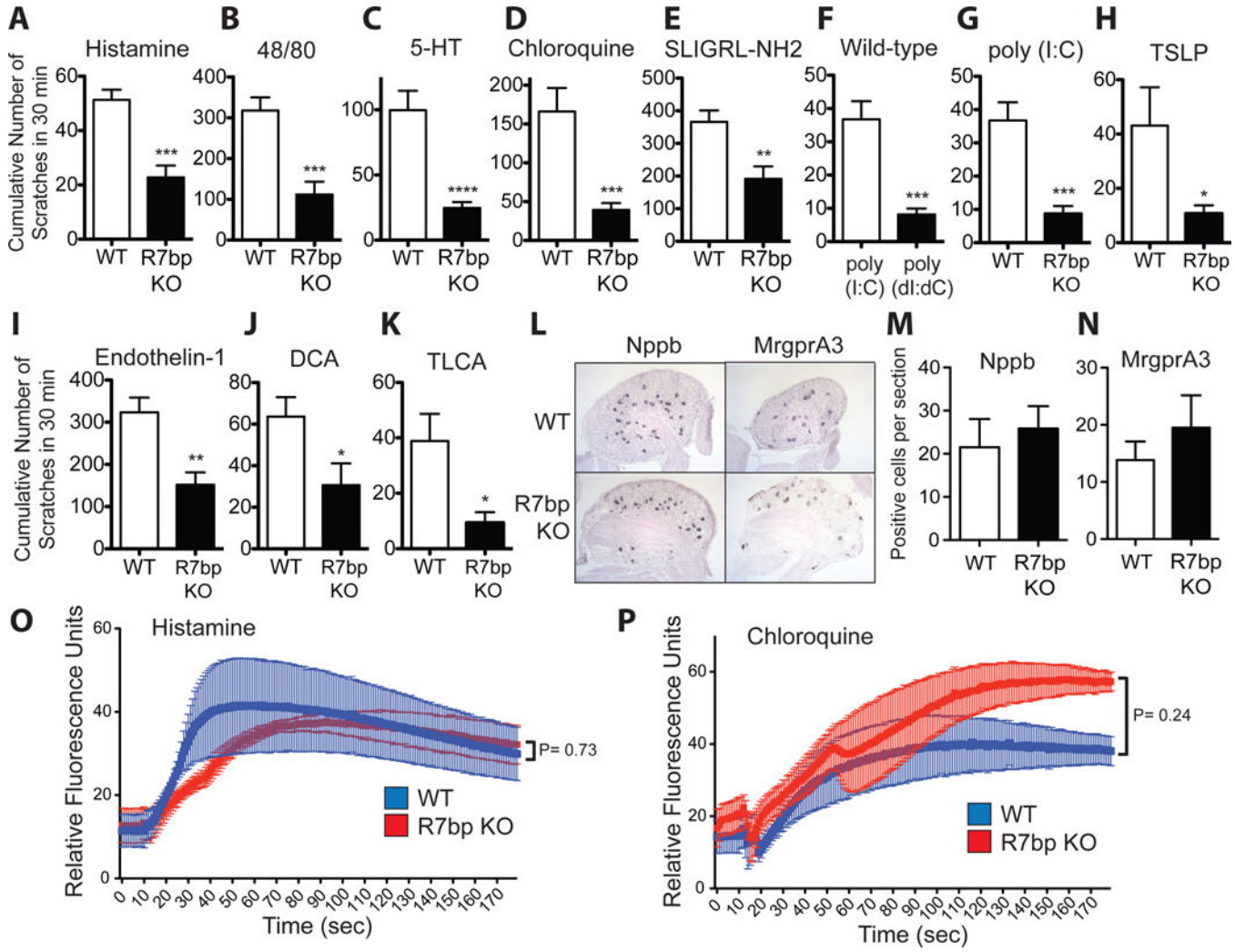

Fig. 2. R7bp-knockout mice exhibit impaired pruriception but retain peripheral sensory neuronal responsiveness to pruritogens

(**A,B**) Cumulative scratching behavior induced by intradermal histamine, a prototypical pruritogen that signals through the H1 and H4 subclasses of histamine GPCR (**A**) and compound 48/80, which acts indirectly via histamine release from mast cells (**B**) over 30 minutes was significantly decreased in *R7bp* knockout (KO) mice (filled bars) compared to their wild-type (WT) littermates (open bars). (**C**, **D**, and **E**) Cumulative scratching behavior to the pruritogens serotonin (5-HT) (**C**), chloroquine, a 4-aminoquinoline antimalarial drug that signals itch through MrpgrA3 and Trpa1 [25; 43] (**D**), and SLIGRL-NH2, a synthetic peptide that signals itch through MrpgrC11 and Trpa1 [26; 43] (**E**) over 30 minutes was significantly decreased in *R7bp* KO mice compared to their WT littermates. (**F**) In wild-type mice, intradermal application of the TLR3 receptor agonist and double stranded RNA viral mimetic polyinosinic: polycytidylic acid (poly (I:C)) induced more cumulative scratching behavior (open bars) relative to intradermal injection of the TLR3-inactive double stranded DNA analog deoxypolyinosinic: deoxypolycytidylic acid (poly (dI:dC)) (filled bars) when observed for 30 minutes. (**G**) *R7bp* KO mice had significantly reduced cumulative scratching behavior in response to intradermal poly (I:C) (filled bars) compared to their WT

littermates (open bars) when observed for 30 minutes. (**H**) The cumulative scratching response over 30 minutes following intradermal injection of the pruritogenic cytokine thymic stromal lymphopoietin (TSLP) was significantly lower in *R7bp* KO mice (filled bar) compared to their WT littermates (open bar). (**I**) *R7bp* KO mice (filled bar) showed significantly reduced cumulative scratching behavior in response to the intradermal injection of endothelin-1, a paracrine peptide released from endothelial cells, compared to their WT littermates (open bar) when observed for 30 minutes. (**J, K**) The cumulative scratching response over 30 minutes following intradermal injection of the pruritogenic bile salts deoxycholic acid (DCA) (**J**) or taurolithocholic acid (TLCA) (**K**) was significantly lower in *R7bp* KO mice (filled bar) compared with their WT littermates (open bar). (**L**) Dorsal root ganglion cells from WT (upper panel) and *R7bp* KO (lower panel) mice hybridized *in situ* with antisense probes against Nppb (left) and the mas-related G protein-coupled receptor MrgprA3 (right), specific markers of primary pruriceptive neurons, showed no obvious differences in transcript expression. (**M, N**) Quantification of Nppb-positive (**M**) and MrgprA3-positive (**N**) neurons in sections from DRGs harvested from WT and *R7bp* KO mice, following *in situ* hybridization, showed no significant differences. (**O,P**) Time course of intracellular calcium mobilization in isolated DRG cells harvested from WT (blue symbols) or *R7bp* KO (red symbols) mice in response to the *in vitro* application of histamine (**O**) or chloroquine (**P**). Statistics and representative figure reporting: For A-K the evaluator was blind to the genotype of the mouse being tested. For the cell counting experiments shown in M and N, the evaluator was blind to the genotype of the mouse from which the DRG section originated. In A–K, and M, N the two-tailed unpaired Student's t-test was employed, with bars indicating mean ± S.E.M. The hybridization experiment in panel L was repeated twice with similar results, using previously published probes[32]. For O and P, two-way ANOVA was employed with data points indicating mean ± S.E.M. For panels A–K, n= number of mice from each genotype tested (for panel A, WT n= 10, KO n= 9; for panel B, WT n= 8, KO n= 9; for panel C, WT n= 10, KO n= 11; for panel D, WT n= 8, KO n= 10; for panel E, n= 8; for panel F, n= 8 for each compound; for panel G, n= 8; for panel H, n=7; for panel I, n= 9; for panel J, WT n= 8, KO n =6; for panel K, n= 8). For M and N, n = the number of sections per genotype counted (n = 6). For O and P, n = 4 calcium mobilization experiments per genotype per drug. P values: A, *** P= 0.0001; B, *** P =0.0004; C, **** P < 0.0001; D, *** P = 0.0004; E, ** P = 0.004; F, *** P = 0.0005; G, *** P= 0.0002; H, * P =0.04; I, ** P=0.002; J, * P = 0.04; K, * P =0.014; M, P = 0.23; N, P = 0.06; O, P = 0.73; P, P = 0.24.

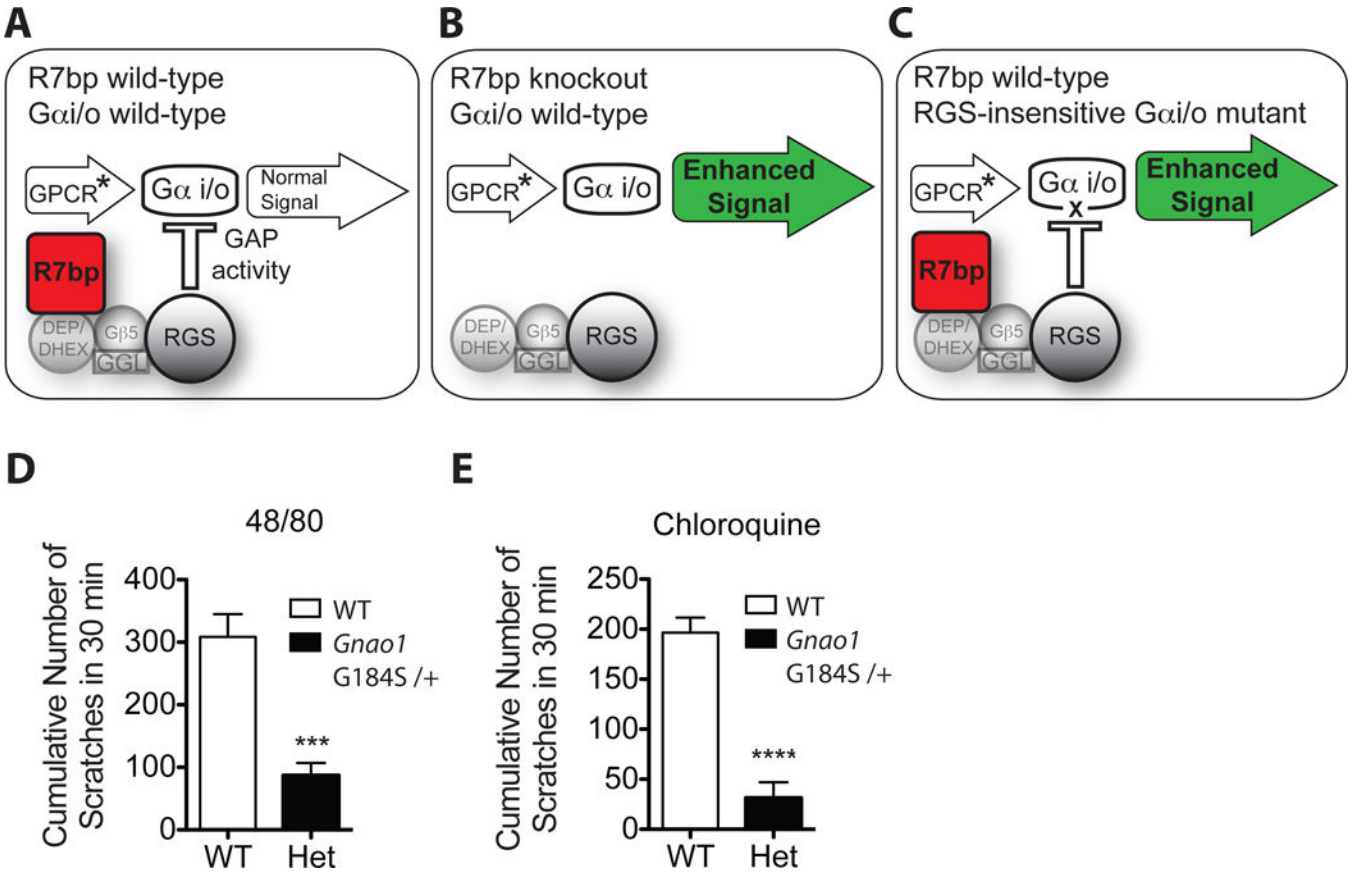

Fig. 3. Mice heterozygous for an RGS protein-insensitive mutant of Gαo (*Gnao1* G184S/+) show impaired responsiveness to cutaneous pruritogens

(**A**) In neurons expressing the Gβ5/R7-RGS/R7bp complex and wild-type G protein signaling components, a normal signal results from the balance between agonist-stimulated, G protein-coupled receptor (GPCR*)-mediated Gi/o activation, and R7-RGS protein RGS domain-mediated Gαi/o-directed GTPase-activated protein (GAP) activity that de-activates Gi/o (cf. Fig. 1A). For simplicity, only the Gαi/o component, and not the Gβγ complex, of the Gi/o heterotrimer is shown. (**B**) An enhanced signaling phenotype is predicted from knockout of R7bp, resulting in loss of R7bp-facilitated, Gβ5/R7-RGS complex-mediated GAP activity directed against Gαi/o. (**C**) A similar enhanced signaling phenotype is predicted from introduction of a mutation in Gαi/o that blocks RGS protein binding, renders the subunit RGS protein-insensitive, and prevents GAP activity. (**D, E**) Intradermal administration of the histaminergic pruritogen compound 48/80 (**D**) and the non-histaminergic pruritogen chloroquine (**E**) evoked significantly fewer scratching responses in *Gnao1* G184S/+ mice (Het) (filled bars), expressing an RGS protein-insensitive mutant of Gαo, compared to their wild type (WT) littermates (open bars). Statistics reporting: In D and E, the evaluator was blind to the genotype of the mouse being tested. In D and E, the two-tailed unpaired Student's t-test was employed, with bars indicating mean ± S.E.M. For panels D and E, n= number of mice from each genotype tested (n = 8). P values: D, *** P=0.0001; E, **** P < 0.0001.

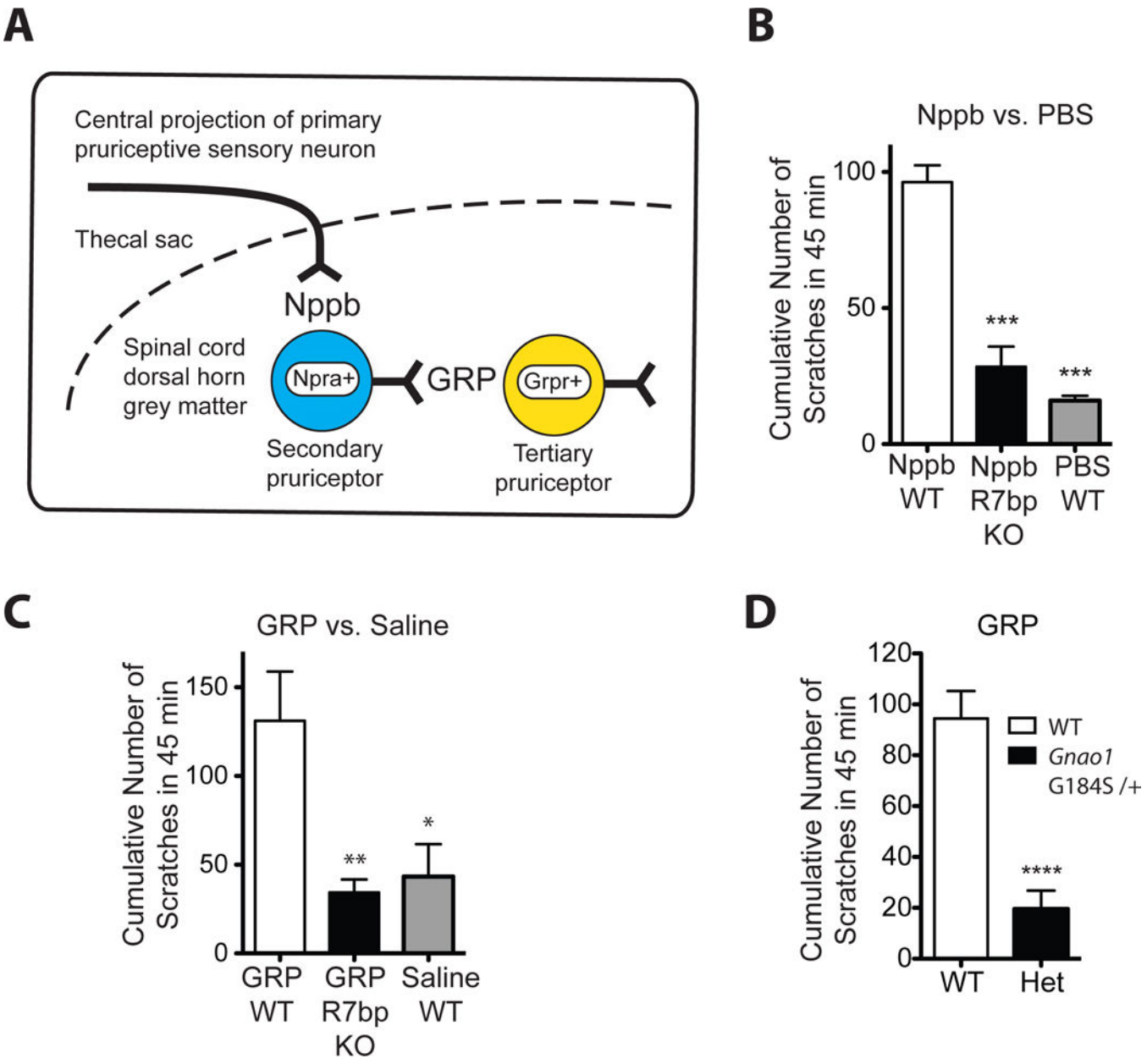

Fig. 4. Mice lacking R7bp, or heterozygous for the RGS protein-insensitive *Gnao1* G184S mutation, show impaired scratching responses to intrathecal pruritogens

(**A**) Illustration of the relationship between primary peripheral pruriceptors, and the secondary (Npra+) and tertiary (Grpr+) pruriceptors located in the grey matter of the spinal cord dorsal horn (boundary of the spinal cord indicated by dashed line) [32]. The itch-specific peptides natriuretic peptide type B (Nppb) (released from the primary pruriceptors) and gastrin-releasing peptide (GRP) (released from the secondary pruriceptors) are indicated. Drugs and peptides injected into the cerebrospinal fluid within the thecal sac can act directly on neurons in the dorsal horn grey matter, including secondary and tertiary pruriceptors. (**B**) Intrathecal injection of Nppb induced significantly more scratching behavior (open bar) than did the PBS vehicle control (grey bar) in wild type (WT) mice.

Intrathecal Nppb induced significantly less scratching behavior in *R7bp* knockout (KO) mice (filled bar) as compared to their WT littermates (open bar). (**C**) Intrathecal injection of GRP induced significantly more scratching behavior (open bar) than did the saline vehicle control (grey bar) in WT mice. Intrathecal GRP induced significantly less scratching behavior in *R7bp* KO mice (filled bar) as compared to their WT littermates (open bar). (**D**) Intrathecal GRP produced significant fewer scratching responses in mice heterozygous for the *Gnao1* G184S mutation (Het) (filled bar) compared to their WT littermates (open bar). Statistics reporting: In B–D, the evaluator was blind to the genotype of the mouse being tested. In B–D, the two-tailed unpaired Student's t-test was employed, with bars indicating mean ± S.E.M. For panels B–D, n= number of mice from each genotype tested (for panel B, Nppb WT n= 8, Nppb KO n= 8; PBS WT n = 6; for panel C, GRP WT n= 8, GRP KO n= 8; Saline WT n = 8; for panel D, n= 8). P values: B, Nppb WT vs. Nppb KO *** $P < 0.0001$, for Nppb WT vs. PBS WT *** $P < 0.0001$; C, GRP WT vs. GRP KO ** $P = 0.0046$ GRP WT vs. Saline WT * $P = 0.02$; D, **** $P < 0.0001$.

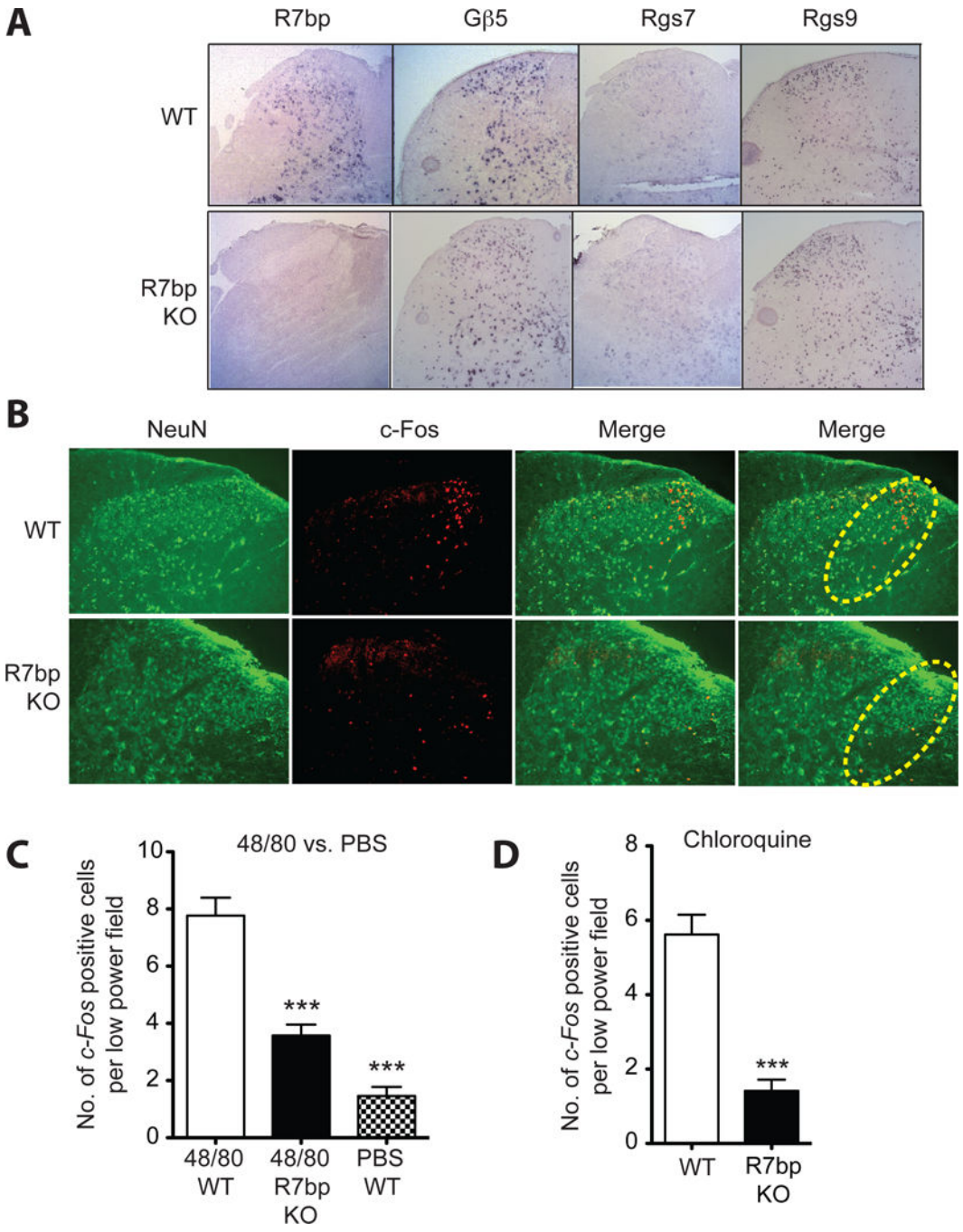

Fig. 5. Mice lacking R7bp show impaired activation of spinal cord dorsal horn neurons in response to cutaneous pruritogens

(**A**) Sections through lumbar spinal cord of *R7bp* knockout (KO) and wild-type (WT) mice showed no obvious differences in the expression of Gβ5, Rgs7 and Rgs9 by *in situ* hybridization (ISH) with the corresponding antisense probes. R7bp expression was lacking in *R7bp* KO mice. (Shown are lateral, dorsal quadrants of coronal lumbar spinal cord sections following ISH with the indicated probes.) (**B**) Intradermal nuchal injection of compound 48/80 induced a lower expression of *c-Fos*, a marker of neuronal activation, in the

cervical spinal lateral dorsal horn of in *R7bp* KO mice when compared to WT littermates. Staining for the neuronal marker NeuN was used to identify neurons in the dorsal horn of cervical spinal cord and is indicated by green fluorescence. Red fluorescence indicates *c-Fos* expression in 48/80-responsive neurons. The dashed yellow ellipses in the rightmost panels demarcate the region of the lateral dorsal horn within which c-Fos positive cells were counted for quantification. (**C, D**) Quantification of *c-Fos* expression when analyzed after intradermal application of compound 48/80 (**C**) or chloroquine (**D**) revealed a significant decrease in *c-Fos* expression in *R7bp* KO mice (filled bars) compared to WT controls (open bars). Intradermal injection of the PBS vehicle control in WT mice was also analyzed for comparison purposes (stippled bar in C). Statistics and representative figure reporting: For panel A, the hybridization experiment was repeated three times with similar results, and no limitation on reproducibility. For panel B, the experiment was repeated in each of 5 mice per genotype (5 to 9 sections per mouse) with no limitation on reproducibility. In panels C and D, the evaluator was blind to the genotype of the mouse from which the spinal sections being analyzed were derived. In panels C and D, the two-tailed unpaired Student's t-test was employed, with bars indicating mean ± S.E.M. For panels C and D, n= number of cell counts (for panel C, WT 48/80 n= 73 cell counts from sections from 5 mice (5 to 9 sections per mouse), WT PBS n= 25 cell counts from sections from 4 mice (4 to 10 sections per mouse), KO 48/80 n= 78 cell counts from sections from 5 mice (5 to 9 sections per mouse); for panel D, WT CQ n= 79 cell counts from sections from 4 mice (8 to 11 sections per mouse), KO CQ n= 70 cell counts from sections from 4 mice (8 to 11 sections per mouse)). P values: C, WT 48/80 vs. KO 48/80 *** $P < 0.0001$, WT 48/80 vs. WT PBS *** $P < 0.0001$; D, *** $P < 0.0001$.

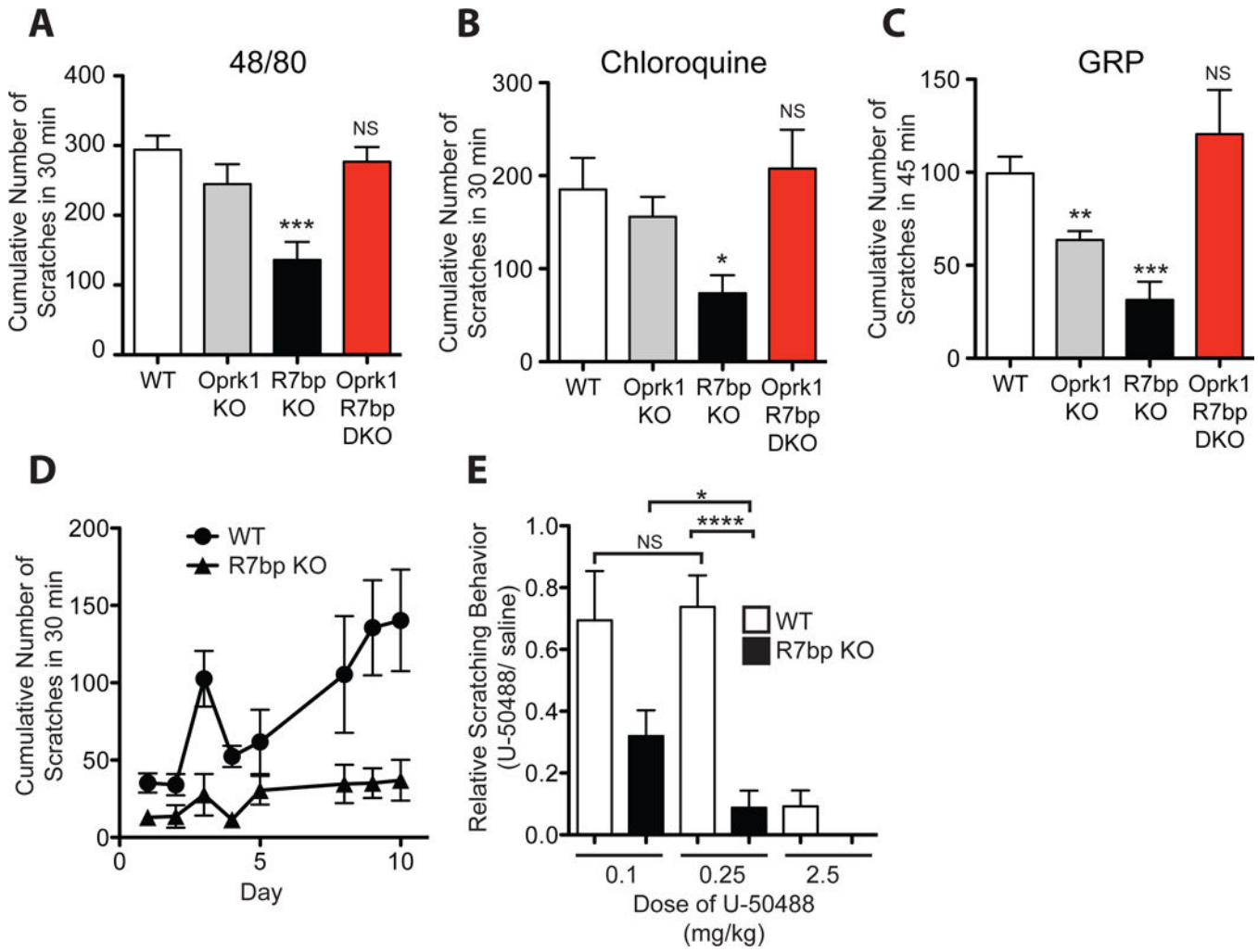

Fig. 6. Knockout of *Oprk1* reverses the loss-of-pruriception phenotype in *R7bp* knockout mice and mice lacking R7bp show diminished scratching behavior and enhanced sensitivity to kappa opioid agonists in a model of atopic dermatitis

(**A, B**) Intradermal application of pruritogens 48/80 (**A**) and chloroquine (**B**) in *R7bp* knockout (KO) mice (black bars) led to a significant loss of scratching behavior compared to wild type (WT) littermates (open bars). However, *Oprk1* single knockout mice (grey bars) and double knockout (*Oprk1 R7bp* DKO) mice (red bars), missing both R7bp and the kappa-opioid receptor, responded like WT mice to the cutaneous pruritogens. (**C**) Mice lacking R7bp alone (R7bp KO) (black bar) and the kappa-opioid receptor alone (Oprk1 KO) (grey bar) showed significantly less scratching behavior compared to wild WT littermates (open bar) following intrathecal gastrin-releasing peptide (GRP) administration. However, double knockout (Oprk1 R7bp DKO) mice missing both R7bp and the kappa-opioid receptor responded normally to intrathecal GRP (red bar). (**D**) Mice lacking R7bp (R7bp KO)(filled triangles) or their wild-type littermates (WT)(filled circles) were evaluated for scratching behavior during a ten-day period of daily topical application of diphenylcyclopropenone (DCP) in a model of atopic dermatitis, over a period of 30 minutes on days 1–5 and 8–10, as described in Methods. (**E**) The relative scratching behavior of wild-type (open bars) and *R7bp* KO mouse littermates (filled bars) was calculated as the ratio of scratching behavior

following injection of the specific kappa opioid receptor agonist U50,488 injection to that following a preceding saline injection in the same mouse on day 5, day 8, and day 10 of topical DCP treatment in a model of atopic dermatitis (see panel D) as described in Methods. Mice were given an intraperitoneal injection of U50,488 at a dose of 0.1, 0.25 and 2.5 mg/kg on day 5, day 8, and day 10 respectively. Statistics reporting: For panels A–E, the evaluator was blind to the genotype of the mouse being tested. In A–C and E, the two-tailed unpaired Student's t-test was employed, with bars indicating mean ± S.E.M. In D, two-way ANOVA was employed with bars indicating mean ± S.E.M. For panels A–E, n= number of mice from each genotype tested (A–C, WT n= 6, R7BP KO n= 7, DKO n= 6; D and E, WT n= 10, R7BP KO n= 8). P values: panel A, WT vs. OPRK1 KO P = 0.2, WT vs. R7bp KO *** P= 0.0007, WT vs. DKO P = 0.57; panel B, WT vs. OPRK1 KO P = 0.46, WT vs. R7bp KO * P= 0.01, WT vs. DKO P = 0.69; panel C, WT vs. OPRK1 KO ** P= 0.004, WT vs. R7bp KO *** P= 0.0004, WT vs. DKO P = 0.42; for A–C, NS = not significant vs. the wild-type; panel D, WT vs. R7BP KO, P < 0.0001; panel E, WT 0.1 mg/kg vs. 0.25 mg/kg P = 0.82 (NS = not significant between doses), R7BP KO 0.1 mg/kg vs. 0.25 mg/kg * P = 0.04, WT vs. R7BP KO for 0.25 mg/kg dose **** P < 0.0001.

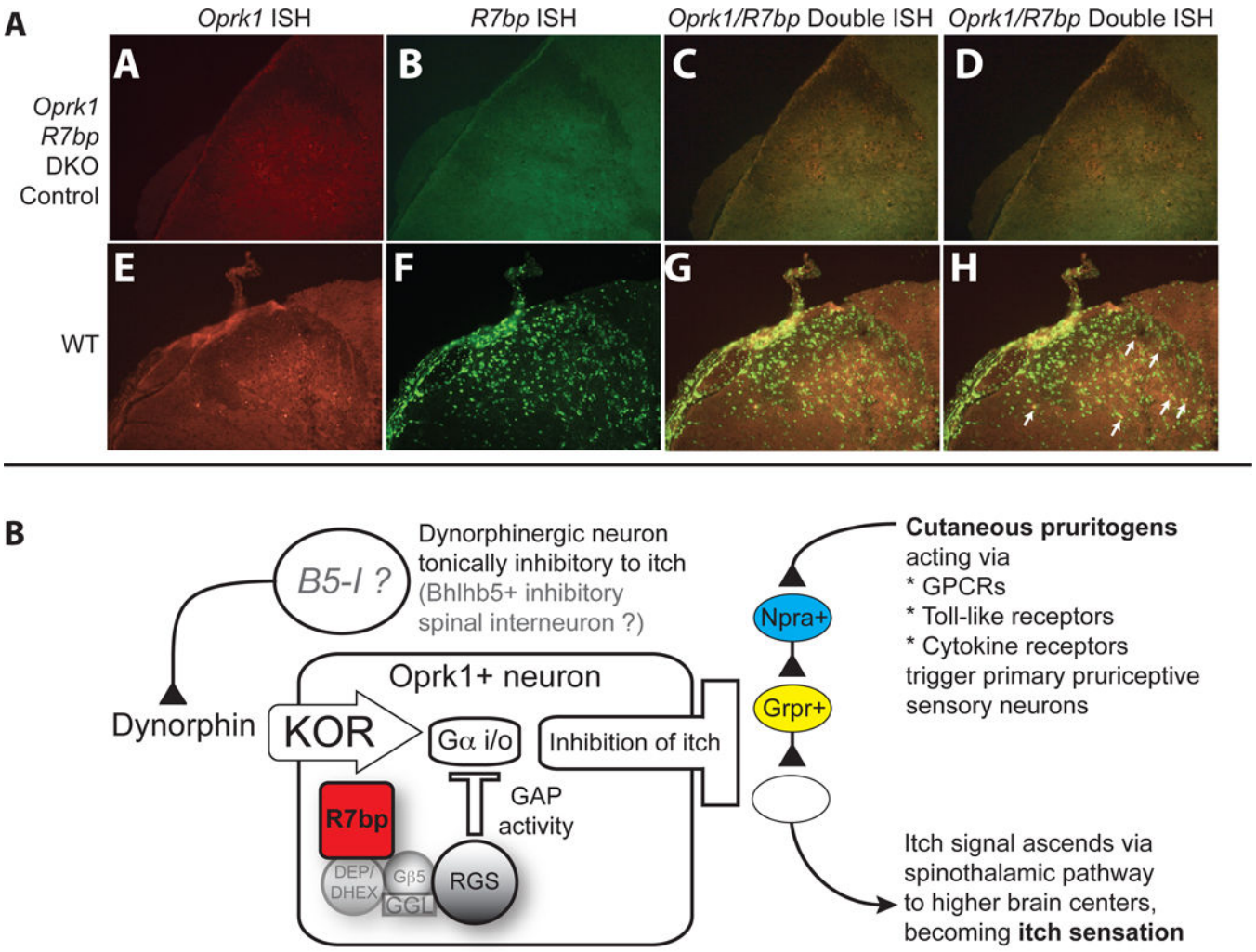

Fig. 7. R7bp and Oprk1 are coexpressed in a subset of spinal dorsal horn neurons and proposed model of R7bp regulation of the pruriceptive signaling pathway

(**A**) Microscopic sections through lumbar spinal cord were collected from *Oprk1/R7bp* double knockout control (DKO) (A–D) and wild type (WT) (E–H) mice and hybridized *in situ* with antisense probes against Oprk1 (red signal) (A, E), R7bp (green signal) (B, F) (cf. B,F to Fig. 5A), or both (C, D, G, H) as described in Methods (ISH = *in situ* hybridization). Shown is the portion of the section containing the spinal dorsal horn. White arrows in H indicate a subset of cells in WT sections positive for both R7bp and Oprk1 probes (yellow signal). (**B**) Schematic diagram showing a proposed model in which R7bp regulates pruriception. Primary pruriceptors activated by cutaneous pruritogens activate Npra+ secondary pruriceptors (blue) that in turn activate Grpr+ tertiary pruriceptors (yellow). The itch signal then traverses a yet-to-be-defined spinal circuit before it ascends via the contralateral spino-thalamic tract to higher brain centers resulting in conscious pruriception. Our model hypothesizes the presence of Oprk1+ neurons containing R7bp (red) that facilitates Gβ5/R7-RGS complex-mediated GTPase activating protein (GAP) activity targeting Gi/o coupled to the kappa opioid receptor (KOR). The KOR in this model is activated by its endogenous ligand, dynorphin, released from a yet-to-be-identified spinal

neuron located upstream that is tonically inhibitory to the pruriceptive pathway. A candidate for this dynorphinergic neuron in this model is the Bhlhb5+ inhibitory spinal interneuron (B5-I) previously identified by Ross and co-workers [17; 36]. Activation of the KOR inhibits itch, and knockout of R7bp in these cells promotes forward signalling through the KOR that exaggerates (a) the basal tonic dynorphinergic inhibition of itch resulting in a loss-of-pruriception phenotype, and (b) the antipruritic effects of exogenous kappa opioid agonists. Since R7bp knockout results in loss-of-pruriception to intrathecally-administered gastrin-releasing peptide (GRP), R7bp-regulated inhibition of itch sensation must act at the level of, or central to, the Grpr+ tertiary pruriceptors (yellow).

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Table 1

Pruritogens with Scratching Responsiveness Sensitive to R7bp Knockout in Mice

	Pruritogen	Receptor	Receptor Class	G protein mediators	Comments
Cutaneous pruritogens	Histamine	H1 H4	GPCR GPCR	Gq/11 Gi/o	Released from mast cell granules upon activation by allergens
	Compound 48/80	H1 H4	GPCR GPCR	Gq/11 Gi/o	Actions indirect via degranulation of mast cells
	Serotonin (5-hydroxy-tryptamine; 5-HT)	5HT2 5HT7	GPCR GPCR	Gq/11 Gs	Released from mast cell granules upon activation by allergens
	Chloroquine	MrpgrA3	GPCR	Gq/11	Synthetic 4-aminoquinoline antimalarial drug
	SLIGRL-NH2	MrgprCll	GPCR	Gq/11	SLIGRL-NH2 also activates PAR2 GPCR implicated in nociception
	Polyinosinic:polycytidylic acid (poly (I:C))	Tlr3	Toll like receptor	N/A	Structural analog of double-stranded RNA such as that found in dsRNA viruses
	Thymic stromal lymphopoietin (TSLP)	Crlf2/IL7Ra heterodimer	Type 1 cytokine receptor	N/A	TSLP is implicated in pathogenesis of atopic dermatitis (eczema)
	Endothelin-1 (ET-1)	ETAR	GPCR	Gq/Gs	Paracrine peptide released from endothelial cells
	Deoxycholic acid (DCA)	Gpbarl	GPCR	Gs	DCA and TLCA are secondary bile acids. Gpbarl is also known as Tgr5
	Taurolithocholic acid (TLCA)				
Intrathecal pruritogens	B-type natriuretic peptide (BNP; Nppb)	Npra	Transmembrane guanylyl cyclase	N/A	Nppb/BNP is released from primary pruriceptive sensory neurons
	Gastrin-releasing peptide (GRP)	Grpr	GPCR	Gq/11	GRP is released from secondary pruriceptive sensory neurons. Grpr is also known as bombesin type 2 receptor (BB2R)