

## **Rodent Model of Atopic Dermatitis Associated with Itch Sensitization.**

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### **Introduction**

Chronic itch is thought to result from increased sensitivity of itch-signaling pathways, which results in symptoms of spontaneously-occurring itch, itch in response to non-itchy light touch (“alloknesis”), and increased itch to a normally itchy stimulus (“hyperknesis”) such as an insect bite. In conditions of chronic itch such as atopic dermatitis (AD), it is hypothesized that peripheral and/or central itch-signaling neurons become sensitized to provide a stronger itch signal to the central nervous system. However, it is currently not known if chronic itch from diseases such as AD is associated with neuronal sensitization or what the underlying cellular and molecular mechanisms are.

A variety of chemicals can elicit itch via histamine-dependent and histamine-independent pathways (1). Histaminergic itch and non-histaminergic itch require TRPV1 and TRPA1, respectively (2, 3). In the spinal cord, neurokinin-1 receptor (NK1R)- and gastrin releasing peptide receptor (GRPR)-expressing spinal neurons are implicated in acute itch (4, 5). We presently developed behavioral tools to assess itch sensitization in an animal model of AD, and investigated roles for NK1R- and/or GRPR-expressing spinal neurons in chronic itch.

### **Methods**

OVA sensitization and behavioral tests. Experiments were performed using adult male C57BL/6 mice (19-27 g) under a protocol approved by the UC Davis Animal Care and Use Committee. Mice were given an intraperitoneal injection of ovalbumin (OVA; 100 µg), alum (1 mg) and pertussis toxin (300 µg) on the first day. Five days later, they received a subcutaneous injection of 50 µg of ovalbumin or saline alone. Then, local immunization was performed once a day from day 14 to day 39 after the first systemic immunization. The local immunization was conducted as follows. Fur on the rostral back was shaved with electric clippers. Then, gauze (1 × 1 cm) soaked with 0.1% ovalbumin (100 µl) or saline (100 µl) was applied to the shaved skin area. The treated skin area was covered with a patch (Tegaderm, 3M Health Care, St. Paul, MN). The next day the patch was removed, and an identical patch reapplied to the same skin area. This procedure was repeated daily up to day 39. Starting at day 14, mice were videotaped twice a week following the removal of the patch to count scratching behavior. After videotaping, alloknesis testing was conducted. Alloknesis was assessed as follows (6): the mouse received

five separate innocuous mechanical stimuli delivered using a von Frey filament (bending force: 0.7 mN) on the border of gauze treatment area at five randomly selected sites. The presence or absence of a positive response, i.e., a hindlimb scratch bout directed to the site of mechanical stimulation, was noted for each stimulus before the next one was given. The alloknesis score was the total number of positive responses elicited by the five stimuli, i.e., 0, 1, 2, 3, 4, or 5. The patch was left continuously on the rostral part of the back of the mouse, except during the measurement of scratching behavior. To assess hyperknesis, mice received a 10  $\mu$ l intradermal (id) injection of either chloroquine (30  $\mu$ g) or histamine (35  $\mu$ g) within the treatment area 25 days after OVA treatments.

Saporin-injection. To neurotoxically ablate NK1R- or GRPR-expressing spinal neurons, OVA-treated mice received an intrathecal injection of either blank saporin (SAP), substance P-SAP (SP-SAP), or bombesin-SAP (BB-SAP) under isoflurane anesthesia on treatment day 25.

Immunohistochemistry. After completing behavioral testing, the upper cervical spinal cord was dissected under sodium pentobarbital anesthesia following 4% paraformaldehyde perfusion, and was post-fixed in 4% paraformaldehyde followed by 30% sucrose. Thirty-micrometer sections of cervical cord were immunostained with either anti-rabbit GRPR antibody (1:500; LS-A831, MBL) or anti-rabbit NK1R antibody (1:500; AB5060, Millipore, Billerica, MA) using Tyramide Signal Amplification kits (Life Technologies). Briefly, they were incubated with 0.2% Triton X-100 for 10 minutes at room temperature, followed by incubation with peroxidase quenching buffer (PBS + 3% H<sub>2</sub>O<sub>2</sub>) for either 30 minutes for GRPR antibody or 15 minutes for NK1R at room temperature. Then, they were incubated with 1% blocking reagent for 60 minutes at room temperature, followed by incubation with the primary antibody in 1% blocking reagent overnight at 4°C. The next day, sections were incubated with the HRP conjugate secondary antibody solution for 45 minutes at room temperature, followed by incubation with the tyramide working solution for 7 minutes at room temperature. Coverslips were mounted with ProLong® Gold Antifade Mountant (Life Technologies). Images were captured using a fluorescence microscope (Nikon Eclipse Ti; Technical Instruments, San Francisco CA).

Fluorogold injection. To retrogradely label spinal projection neurons, Fluorogold was injected into the ventrobasal thalamus or parabrachial nucleus. Mice were anesthetized with sodium pentobarbital (65 mg/kg ip). The head was fixed in a stereotaxic frame, and the calvarium was exposed by midline scalp incision, and a craniotomy made for introduction of a microinjection cannula into the ventrobasal thalamus (AP:1.8, ML:1.0, DV:-3.6 and AP:1.8, ML:1.6, DV:-3.6) or parabrachial nucleus (AP:5.0, ML:1.27, DV:-3.75 and AP:5.2, ML:1.27, DV:-3.75). The craniotomy consisted of a small burr hole (<2 mm diameter) made through the skull using an electric drill over the intended injection site as determined using stereotaxic coordinates. Eighty nL of Fluorogold was injected at each site. After injection of tracer, the cannula was removed, and the incision was closed with Vetbond (tissue adhesive, 3M). Mice were given an analgesic (buprenorphine, 0.05 mg/kg s.c. at the conclusion of surgery, and again twice daily, for 1 day post- surgery. Animals receiving the tracer injection were then used 1 week later for immunohistochemistry.

Data analysis. Between-group comparisons were made by one-way ANOVA followed by the Bonferroni post-test. In all cases  $p < 0.05$  was considered to be significant.

## Results

Figure 1 shows the macroscopic views of the skin before and 25 days after OVA treatments. OVA treatment induced erythema as well as excoriation. Naive mice exhibited little spontaneous scratching (Fig. 2A). By day 21, counts of spontaneous scratch bouts had increased significantly to a plateau in OVA-treated mice (Fig. 2A). The low level of spontaneous scratching in saline-treated control mice increased only slightly over the same period (Fig. 2A). Naive mice exhibited an alloknosis score of 0 (Fig. 2B). The alloknosis score started to increase by day 18, and was significantly higher by day 25 in OVA-sensitized mice. There was no significant change in the mean alloknosis score in saline-treated mice (Fig. 2B). The number of scratch bouts evoked by acute id injection of chloroquine was significantly greater in OVA-sensitized vs. control mice (Fig. 3). In contrast, there was no significant difference in the number of histamine-evoked scratch bouts between OVA-sensitized and control mice. Selective sensitization of the histamine-independent pathway has been observed in other studies (7, 8). OVA-sensitized mice appear to be a useful model to investigate the neural mechanisms of itch and itch sensitization in AD.

To investigate the role of NK1R- and GRPR-expressing spinal neurons in itch and its sensitization, they were neurotoxically ablated at day 25 when the number of spontaneous scratching and alloknosis score reached to a plateau in OVA-sensitized mice. Figure 4 shows an immunohistochemical image of the dorsal horn of the spinal cord. BB-SAP or SP-SAP treatment resulted in a significant reduction in expression of NK1R- or GRPR-expressing spinal neurons, respectively (Fig. 4). SP-SAP treatment resulted in a significant reduction in spontaneous scratching (Fig. 5). In contrast, BB-SAP treatment did not significantly affect the number of spontaneous scratch bouts. SP-SAP treatment resulted in a significant reduction in the alloknosis score, while BB-SAP did not affect the alloknosis score (Fig. 6). Both BB-SAP and SP-SAP treatments significantly reduced chloroquine-evoked scratching behavior, compared to OVA-sensitized mice receiving blank-SAP (Fig. 7). Blank-SAP did not result in any changes in spontaneous, touch- or chloroquine-evoked scratching compared to OVA-sensitized mice not receiving SAP.

We hypothesized that GRPR-expressing spinal neurons are interneurons and may be upstream of itch signaling mediated through NK1R-expressing spinal neurons. To test whether GRPR-expressing spinal neurons are interneurons, we utilized the retrograde tracer Fluorogold to label spinoparabrachial projection neurons. Ninety one percent of NK-1 expressing spinal neurons were retrogradely labeled with Fluorogold (145/160). Twenty one percent of GRPR-expressing spinal neurons retrogradely labeled with Fluorogold (23/109).

## **Discussion**

OVA-sensitized mice appear to represent a useful model to examine the neuronal mechanisms underlying itch and its sensitization in AD, because of the following reasons. 1) OVA-sensitized mice exhibited an increase in spontaneous scratching over 18 days period,

suggesting that chronic itch occurs in this model. 2) OVA-sensitized mice exhibited alloknesis and hyperknesis, which is a sign of itch sensitization and is observed in human AD. Additionally, alloknesis developed at a delay after the increase in spontaneous scratching. This result supports the idea that alloknesis occurs as a result of itch sensitization. 3) Skin symptoms observed macroscopically were not severer than what we expected. However, there were obvious skin symptoms, such as erythema and excoriation.

Chloroquine-evoked scratching was enhanced in OVA-sensitized mice, while histamine-evoked scratching was not enhanced. Selective sensitization of a histamine-independent pathway has been reported in another mouse study, as well as in a human study (7, 8). Nerve growth factor induced sensitization of cowhage-evoked itch, but not histamine-evoked itch in humans. This sensitization correlated with mechanical hyperalgesia, but not heat hyperalgesia. In a mouse model of dry skin itch, scratching evoked by either serotonin or the PAR2/MRGPRC11 agonist SLIGRL was enhanced, while histamine-evoked scratching was not enhanced. Responses of cultured DRG neurons to either serotonin or the PAR2/MrgprC11 agonist were enhanced, suggesting that peripheral sensitization of the histamine-independent pathway plays a major role in hyperknesis and enhanced itch signals that are presumable conveyed to GRPR-expressing spinal neurons.

Using OVA-sensitized mice, we investigated whether spinal neurons that express molecular receptors of the neuropeptides gastrin releasing peptide and substance P are involved in itch sensitization in the mouse model of AD. SP-SAP abolished NK1R-expressing spinal neurons as well as behavior signs of ongoing itch, alloknesis, and hyperknesis, suggesting that NK1R-expressing spinal neurons have a major role in itch sensitization in AD. It has been reported that NK1R-expressing spinal neurons are involved in conveying acute itch signaling (5). Intrathecal injection of SAP-SP abolished NK1R-expressing spinal neurons as well as scratching evoked by intradermal injection of serotonin in rats. NK1R-expressing spinal neurons are known to be projection neurons in the rat cervical spinal cord (9). The present study confirms this finding in mouse cervical spinal cord. NK1R-expressing spinal neurons plausibly relay itch information to the brain under conditions of both acute and chronic itch.

GRPR-expressing spinal neurons play a critical role in transmission of itch signaling (4). BB-SAP treated mice did not show an increase in spontaneous scratching following repeated treatment with diphenylcyclopropenone, while blank-saporin treated mice exhibited an increase in spontaneous scratching (4), suggesting that GRPR-expressing spinal neurons participate in the development of itch sensitization. In contrast, BB-SAP treatment followed by OVA-treatment only reduced chloroquine-evoked hyperknesis, but not spontaneous scratching or alloknesis, suggesting that 1) GRPR-expressing spinal neurons are not involved in maintenance of itch sensitization and 2) there are different neuronal pathways that underlie ongoing itch, alloknesis and hyperknesis. The latter is supported by our recent study showing that the kappa opioid receptor agonist nalfurafine inhibited spontaneous scratching, but not alloknesis, in chronic dry skin-associated itch in mice (10).

A large population of GRPR-expressing spinal neurons are presumptive interneurons, consistent with a previous study using testicular orphan nuclear receptor 4 (TR4) knockout mice (11). In these mice, a dramatic loss of GRPR-expressing spinal neurons was observed, while projection neurons were preserved. Presumably, NK1R-expressing spinal neurons are downstream of GRPR-expressing spinal neurons are, since most projection neurons express NK1R.

The present study revealed a major role for NK1R-expressing spinal neurons in chronic itch. GRPR-expressing spinal neurons are important for the development, but not the maintenance, of itch sensitization. Desensitization of NK1R-expressing spinal neurons might be a useful approach to treat chronic itch, as an alternative to blocking peripheral molecular targets.

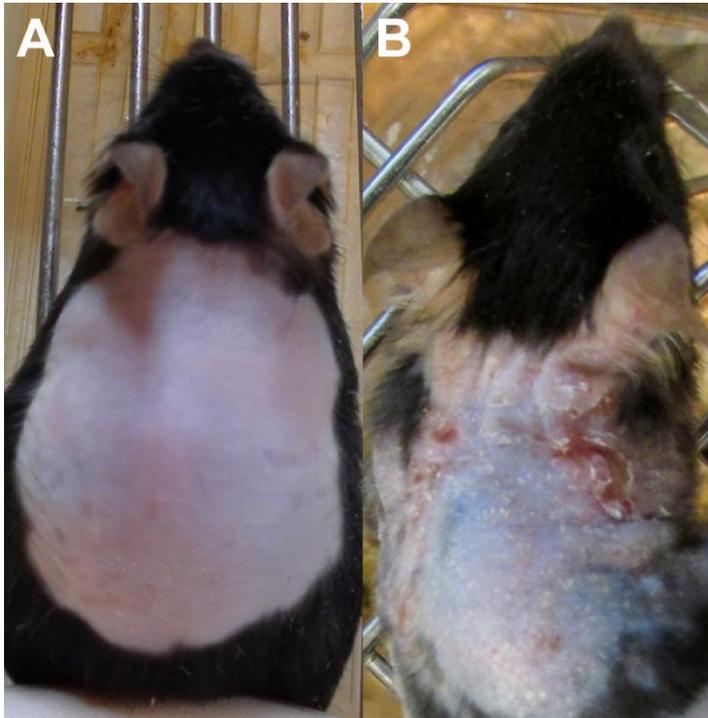


Fig. 1 A typical example of OVA-treated. A: photo taken on day 0 prior to the beginning of OVA treatment. B: OVA treatment day 25.

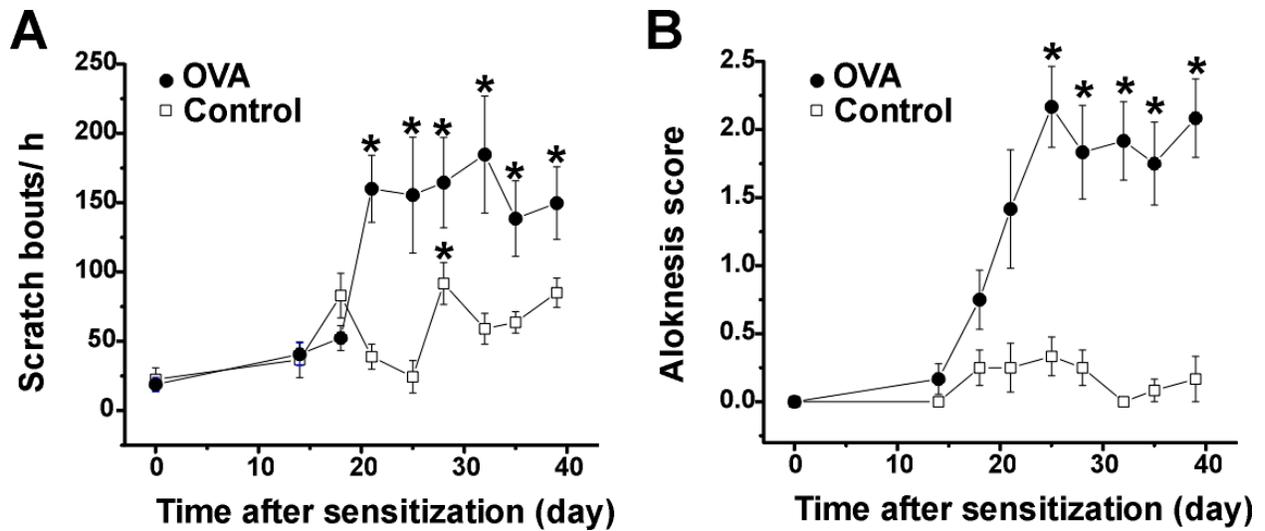


Fig. 2 Time-dependent changes in scratch bouts and alloknesis score in OVA-sensitized mice. A) Spontaneous scratching was measured on pretreatment day 0, and OVA treatment days 14, 18, 21, 25, 32, 35, and 39. Black dots (●) and white squares (□) show, respectively, OVA (OVA-treated) and Control (saline-treated) groups. B) As in A for alloknesis score. Error bars are S.E.M. \* $p < 0.05$ , significantly difference from day 0 (one-way ANOVA followed by Bonferroni test).

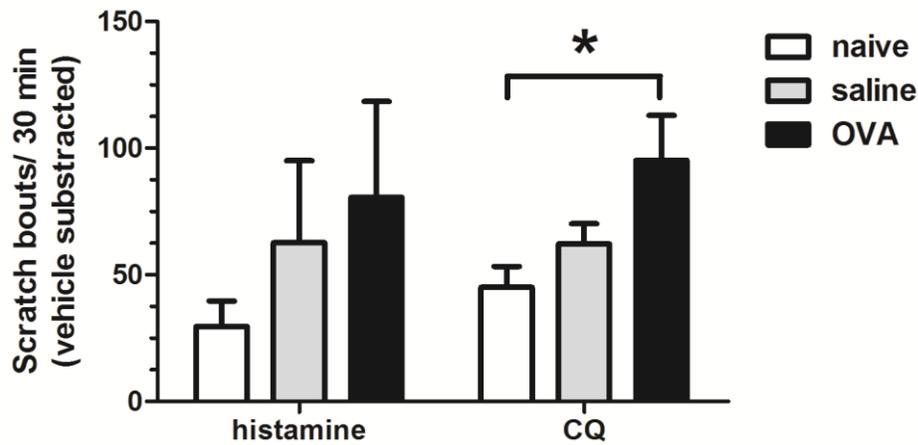


Fig. 3 Stimulus-selective hyperknesis in ovalbumin (OVA)-sensitized mice. Either histamine or chloroquine was intradermally injected into the nape of the neck. Following the injection scratch bouts were counted over a 30 min period. White, gray, and black columns show, respectively, naive, saline-treated, and OVA-treated groups. Error bars are S.E.M. \* $p < 0.05$ , significant difference from naïve (one-way ANOVA followed by Bonferroni test).

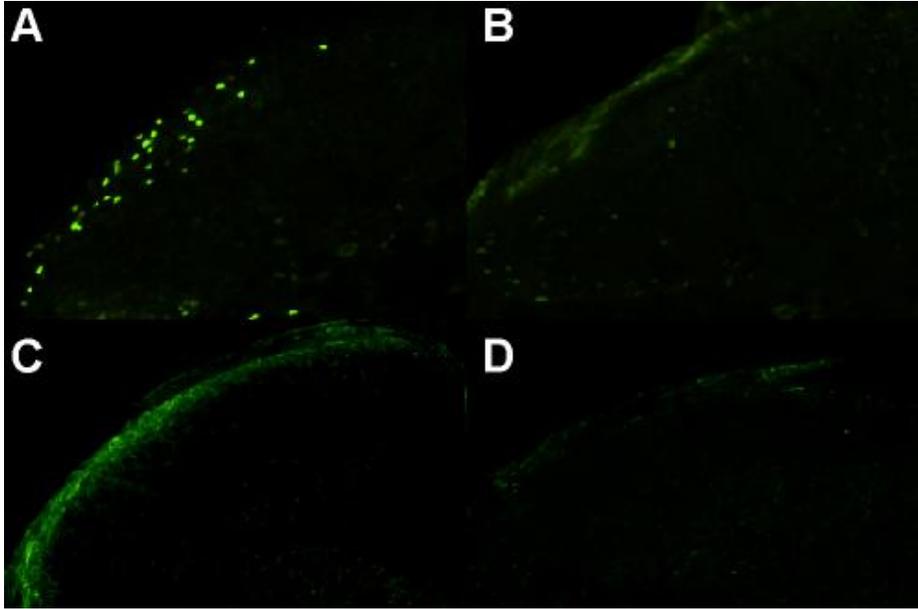


Fig. 4 A typical example of the gastrin-releasing peptide receptor (GRPR) and neurokinin 1 receptor (NK1R) expression in the spinal cord of saporin-treated mice. A) GRPR expression in the spinal cord of mice treated with blank saporin. B) GRPR expression in the spinal cord of mice treated with bombesin saporin. C) NK1R expression in the spinal cord of mice treated with blank saporin. D) NK1R expression in the spinal cord of mice treated with substance P saporin.

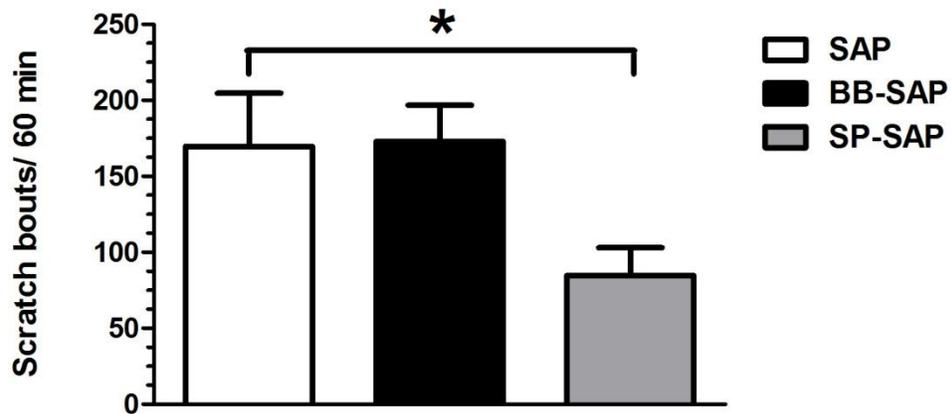


Fig. 5 Substance P saporin reduced spontaneous scratching in ovalbumin (OVA)-sensitized mice. Saporin was intrathecally injected on the day 25 in OVA-sensitized mice. White, black, and gray columns show, respectively, SAP (blank saporin-treated), BB-SAP (bombesin saporin-treated), and SP-SAP (substance P saporin-treated) groups. Error bars are S.E.M. \* $p < 0.05$ , significant difference from SAP (one-way ANOVA followed by Bonferroni test).

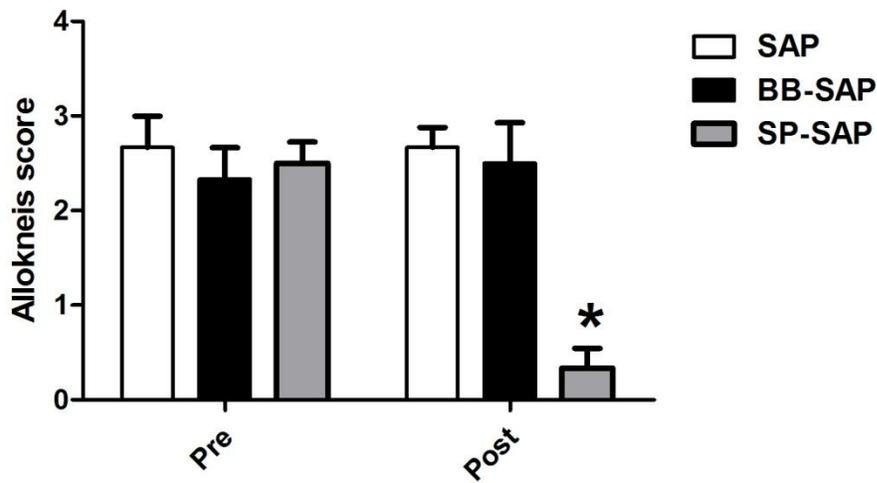


Fig. 6 Substance P saporin reduced alloknesis score in ovalbumin (OVA)-sensitized mice. Saporin was intrathecally injected on day 25 in OVA-sensitized mice. White, black, and gray columns show, respectively, SAP (blank saporin-treated), BB-SAP (bombesin saporin-treated), and SP-SAP (substance P saporin-treated) groups. Error bars are S.E.M. \* $p < 0.05$ , significantly difference from SAP. One-way ANOVA followed by Bonferroni test.

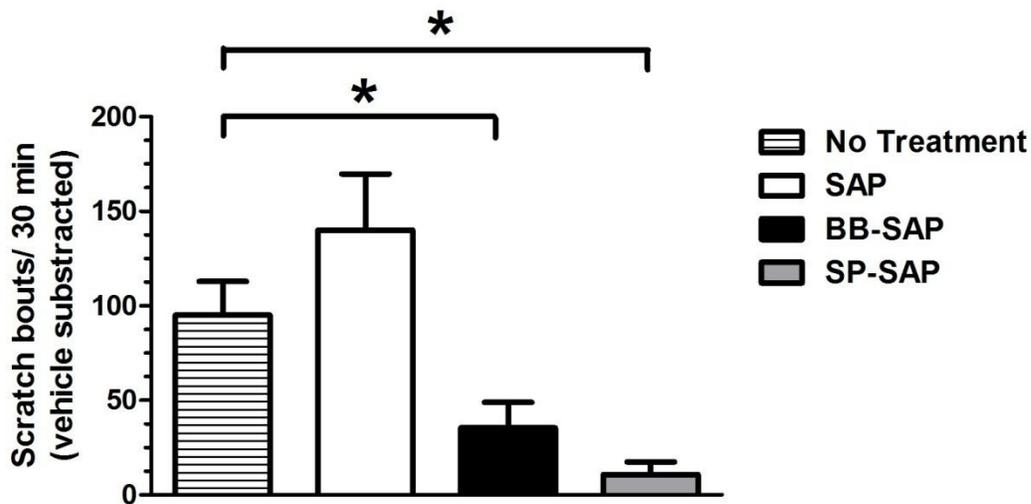


Fig. 7 Bombesin saporin and substance P saporin reduced chloroquine-evoked scratching in ovalbumin (OVA)-sensitized mice. Saporin was intrathecally injected on day 25 in OVA-sensitized mice. Chloroquine was intradermally injected in OVA-treated skin. Striped, white, black, and gray columns show, respectively, no treatment, SAP (blank saporin-treated), BB-SAP (bombesin saporin-treated), and SP-SAP (substance P saporin-treated) groups. Bar graph plots mean number of scratch bouts recorded over a 30-min period following id injection of chloroquine with vehicle-associated scratching subtracted. Error bars are S.E.M. \* $p < 0.05$ , significantly difference from no treatment group. One-way ANOVA followed by Bonferroni test.

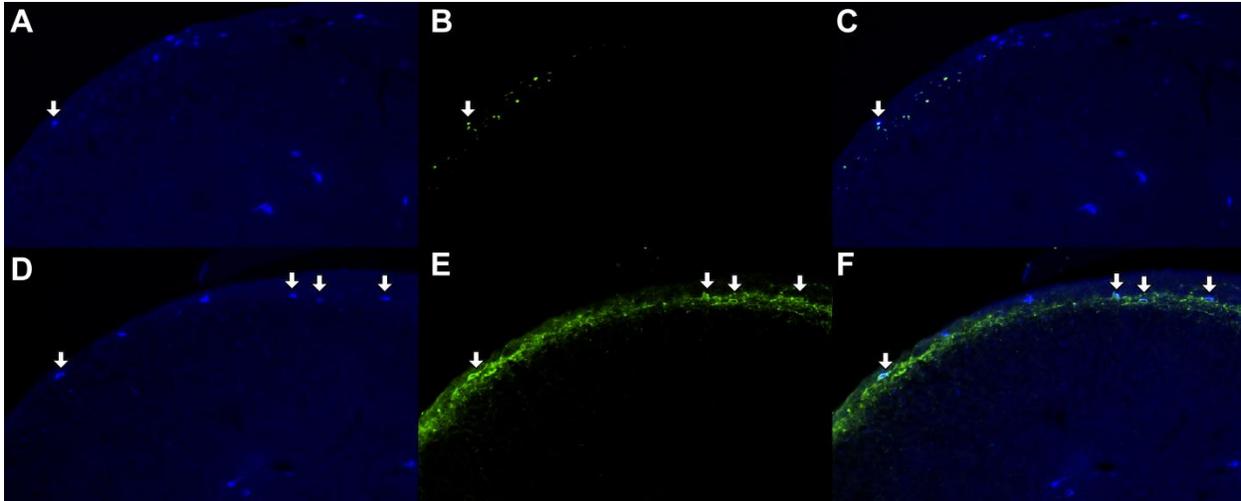


Fig. 8 A typical example of gastrin-releasing peptide receptor (GRPR) and neurokinin 1 receptor (NK1R) expressed by retrogradely-labelled spinoparabrachial projection neurons. Fluorogold was injected into lateral parabrachial nucleus. One week later, the upper cervical spinal cord was processed with either GRPR antibody or NK1R antibody. A) Fluorogold image of the spinal cord. B) GRPR image. C) Merged image of A and B. D) Fluorogold image of the spinal cord. E) NK1R image. F) Merged image of E and F. Arrow shows double-labeled spinal neurons.

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