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Title:
Footedness for scratching itchy eyes in rodents

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Abstract

The neural bases of itchy-eye transmission remain unclear compared with those involved in body itch. Here, we show in rodents that the gastrin-releasing peptide receptor (GRPR) of the trigeminal sensory system is involved in the transmission of itchy eyes. Interestingly, we further demonstrate a difference in scratching behaviour between the left and right hindfeet in rodents; histamine instillation into the conjunctival sac of both eyes revealed right-foot biased laterality in the scratching movements. Unilateral histamine instillation specifically induced neural activation in the ipsilateral sensory pathway, with no significant difference between the activations following left and right eye instillations. Thus, the behavioural laterality is presumably due to right-foot preference in rodents. Genetically modified rats with specific depletion of *Grpr*-expressing neurons in the trigeminal sensory nucleus caudalis of the medulla oblongata exhibited fewer and shorter histamine-induced scratching movements than controls and eliminated the footedness. These results taken together indicate that the *Grpr*-expressing neurons are required for the transmission of itch sensation from the eyes, but that foot preference is generated centrally. These findings could open up a new field of research on the mechanisms of the laterality in vertebrates, and also offer new potential therapeutic approaches to refractory pruritic eye disorders.

Introduction

Itching, or pruritus, is defined as an unpleasant cutaneous sensation that serves as a self-protective mechanism to prevent the body from being harmed by certain external agents [1-4]. Recently, due to environmental pollution, human diseases associated with itch, redness and edema of the eyes (e.g., allergic conjunctivitis and dry eye) are increasing [5]. Indeed, itch from the eyes, which induces scratching, is a common symptom in patients with conjunctivitis or dry eyes. Decreasing the inflammation pharmacologically reduces the sensations of itch, but some patients report persistent symptoms on current therapies [1]. Treatments that target nerve function may improve symptoms of itch [5, 6].

It has been considered that itch from the eyes is transmitted through mechanisms similar to those transmitting itch from the rest of the body surface, and therefore relatively little research has been done on the mechanisms involved in itchy eyes [7]. In the skin, inflammatory mediators such as substance P, calcitonin gene-related peptide, and neurokinin A activate resident mast cells and macrophages and recruit additional immune-system cells to the site of injury [8-10]. Both the mast cells and macrophages subsequently release itch mediators including histamine [10-16]. Histaminergic and nonhistaminergic itch in the skin are carried by discrete populations of sensory neurons. Although most ocular itch arises from the conjunctiva, the actions of histamine and nonhistaminergic pruritogens converge on a unique subset of sensory neurons, which are clearly distinct from ocular pain arising from the cornea [6]. Thus, neural mechanisms from the sensory neurons should be investigated to develop therapeutic strategies for dealing with itch in the eyes.

Somatosensation from the eyes is conveyed to the brain by trigeminal ganglion (TG) neurons, which terminate in the trigeminal sensory nuclei of the medulla oblongata. We have shown that one of the trigeminal sensory nuclei, the spinal trigeminal nucleus caudalis (Sp5C), contains gastrin-releasing peptide (GRP)-immunoreactive (ir) fibres of TG neurons

and that the GRP receptor (GRPR) is localized to Sp5C in Asian musk shrews (*Suncus murinus*), mice, rats, and macaque monkeys [17-20]. Because the GRP/GRPR system in the spinal somatosensory system contributes to itch sensation from the body region in mice [21-26], we hypothesize that the trigeminal GRP/GRPR system is involved in the transmission of itch from the eyes.

In the present study, we analysed whether the GRPR trigeminal sensory system is involved in the transmission of itch from the eyes, by use of behavioural pharmacology and transgenic rats with toxin receptor-mediated cell knockout (TRECK). Here, we demonstrate a difference in scratching behaviour between the right and left hindfeet in rodents. Unlike the behavioural response there was, however, no laterality in neural activation in the Sp5C, which we show to mediate itching from the eyes. To our knowledge, this is the first demonstration of footedness in rodents. Furthermore, the rodent footedness was right sided, which suggests that the limb laterality is similar to that in humans.

Results

Unilateral histamine instillation induces scratching of itchy eyes by the ipsilateral hindfoot.

When histamine, as a pruritogen, was instilled into the right or left conjunctival sac of the eyes of rats, using saline instillation as a method control (Fig. 1A), we found that the animals used only the ipsilateral hindfoot to scratch. However, there was a difference in the frequency and duration of scratching between the left and right hindfoot (Fig 1B, C). Histamine stimulation of the left eye conjunctiva (Group 1) caused little difference in scratching by the right and left foot; but histamine stimulation of the right eye (Group 2) caused markedly

98 greater scratching by the ipsilateral right foot than by the left foot. There was no significant
99 difference in frequency of grooming behaviour between the groups (Fig. 1D, E).
100
101 **Laterality of scratching behaviours in rats and mice.**
102 To ascertain whether this laterality derives from a preference for using the right rather than
103 the left foot to scratch in rodents, we next examined the laterality of the scratching behaviour
104 in response to histamine instillation into both conjunctival sacs of rats (Fig. 2) and mice (Fig.
105 3) again using saline instillation as a method control. Saline instillation caused little
106 scratching behaviour by either hindfoot (Fig. 2B, C) but histamine instillation into both eyes
107 caused significantly more scratching by the right than by the left hindfoot in rats (Fig. 2B, C).
108 When the experiment was repeated in mice there was, however, only a tendency for
109 scratching more with the right than the left hindfoot after instillation of histamine (Fig 3B,
110 C), and the difference was not as clear-cut as in rats. Thus, ~~When saline was instilled there~~
111 ~~was also an increased amount of scratching with the right hindfoot.~~ a~~A~~ 'right-biased'
112 laterality in hindfoot eye-scratching behaviour was ~~much more~~ obvious only in rats ~~than in~~
113 ~~mice~~. The amount of grooming but not grooming duration increased in the histamine-instilled
114 group of rats (Supplementary Fig. S1). No significant difference in grooming behaviour was
115 observed in mice (Supplementary Fig. S2).
116
117 **Histamine instillation activates the ipsilateral *Grpr*-expressing neurons in the medulla**
118 **oblongata.**
119 Histological analyses of *Grpr*-RFP (red fluorescent protein) transgenic rats using the neural
120 activity marker c-Fos were performed to determine whether *Grpr*-expressing neurons in the
121 medulla oblongata (Sp5C) are involved in the transmission of histamine-induced itchy eyes.
122 We analysed the lateral part of Sp5C, the input area of the ophthalmic nerve and maxillary

nerve (V1 and V2) critical for the transmission of conjunctival sensation, and the dorsal part of Sp5C, and the input area of the mandibular division of the trigeminal nerve (V3) as a control [27] (Fig. 4A). After unilateral instillation of histamine into the conjunctival sac of either the right or the left eye, the numbers of c-Fos-positive ($^+$) neurons in the superficial layers of the lateral (V1 and V2 input) area of the histamine-instilled side of the Sp5C were increased compared to those of the saline-instilled side (Fig. 4B, C and 5A, B), but there was no increase in the dorsal (V3 input) area of Sp5C (Fig. 5C, D and Supplementary Fig. S3). *Grpr*-expressing neurons were, however, widely distributed in both the dorsal and lateral area of Sp5C on both sides. The number of c-Fos$^+$ neurons in *Grpr*-expressing neurons in the histamine-instilled side of the Sp5C was significantly greater than that in the V1- and V2-input area of saline-instilled side of the Sp5C (Fig. 5E, F), but not in the V3 input area (Fig. 5G, H). These results demonstrate that histamine instillation activated *Grpr*-expressing neurons in the ipsilateral lateral part of Sp5C.

Grpr-expressing neurons in the medulla oblongata are essential for itch transmission from the eyes.

Finally, we tested whether *Grpr*-expressing neurons in Sp5C are essential to mediate itch from the eyes. *Grpr*-expressing neurons were specifically lesioned by diphtheria-toxin microinjection locally into the Sp5C, V1-V2 area of the medulla oblongata in *Grpr*-RFP transgenic rats in which the *Grpr* promoter also drives expression of the human-type diphtheria toxin receptor (hDTR) in *Grpr*-expressing neurons (Fig. 6A). Diphtheria-toxin was injected only into the right side of the Sp5C because histamine-induced scratching by the right hindfoot was evaluated as an indicator of itch from the eyes. After the behavioural observations, the extent of the lesion of *Grpr*-expressing neurons in the Sp5C of the right side was assessed by the intensity of the RFP signals. Diphtheria-toxin (DT) treated rats that

showed a more than 80% decrease in RFP signals compared with saline-injected rats (mock control) were used as a DT-lesioned group (Fig. 6B). The DT-lesioned rats exhibited significantly fewer and shorter histamine-induced scratching bouts (Fig. 6C) and duration (Fig. 6D) than before the DT-injection. Again, no significant difference in grooming was observed (Supplementary Fig. S4).

Discussion

Here we demonstrate 'right-footedness' in pruritogen-induced eye-scratching behaviour in both rats and mice. To our knowledge, this is the first demonstration of footedness in scratching behaviour of rodents. The present study also provides histological/pharmacological evidence supporting the hypothesis that the trigeminal GRP/GRPR system is an important mediator of itchy eyes.

Unilateral histamine-instillation specifically induced c-Fos$^+$ neurons in the ipsilateral medulla oblongata (Sp5C, V1–V2 area). Thus, the laterality of scratching behaviour resulting from conjunctival irritation presumably depends on behavioural traits as shown for forefooeet movement of rats [28-30], rather than sensory traits. However, motor laterality including hindfootedness has remained poorly understood in rodents because most behavioural experiments are conducted on their locomotion under freely moving conditions in which it is difficult precisely to separate movements of individual limbs [30]. In our study, movements of one or other hindfoot were clearly observed. We suggest that this lateralized behaviour should be investigated in closely related species in order better to trace trajectories in laterality phylogenesis [31, 32]; furthermore, the "species-fair" test [31] could be used in rodents to elucidate the neuronal basis of locomotor laterality under freely moving conditions. The lateralized footedness in rats was apparently greater and more consistent than

that in mice. In birds, which have been shown to be excellent model organisms in which to study cerebral asymmetries [33], the direction and strength of laterality has been shown to be linked to phylogeny, with strength of laterality closely related to body size [34]. This would be consistent with our demonstration of the more marked lateralization in rats than mice. The reason for this species difference could be that rats are larger and experience more biomechanical difficulty in accessing the eyes with the hindfeet. Güntürkün and Ocklenburg [35] have suggested that the ontogenesis of lateralization, handedness in humans, as well as visual lateralization in birds is mediated by a biased embryonic visual input. Human embryos preferentially turn their heads to the right and suck their right thumbs, giving rise to later handedness [36, 37]. Thus, the "right"-footedness found in eye scratching in rodents might be based on a similar non-genetic mechanism. As a genetic factor, the laterality of paw preference in rodents might depend on the expression of a transcription factor Lim domain only 4 (LMO4), which is expressed asymmetrically in human foetal brains [38]. It is hoped that further exploration of footedness in rodents and other animals will reveal the mechanistic role of genetic/environmental factors in the laterality [39] and answer how lateralization patterns between species or even classes of animals are associated [31].

In histamine-instilled rats, c-Fos induction was most pronounced in the *Grpr*-expressing neurons in the Sp5C but, although trigeminal GRP/GRPR neurons are located in the V3 as well as the V1 and V2 regions [17-19], the number of c-Fos-immunoreactive cells was not increased in the V3 innervated by primary sensory neurons from the cheek [27]. Furthermore, although histamine instillation into the left conjunctival sac of the eye did not induce increased scratching behaviour, it did increase the neural activation of *Grpr*-expressing neurons, suggesting that it is the control of the hindfoot movements in rodents that is lateralized. It has recently been reported that, in mice, Sp5C neuron subpopulations respond to pain-provoking stimuli as well as to pruritic stimulation, but few neurons

responded only to pruritic stimulation [40]. Thus, molecular characterization of the functional differences among *Grpr*-expressing neurons in the Sp5C needs to be investigated.

Studies using techniques that selectively stimulate or ablate subsets of neurons have provided considerable evidence for itch transmission in the spinal somatosensory system [23, 41, 42]. When we applied the TRECK method to our transgenic rats in order specifically to destroy *Grpr*-expressing projection neurons, histamine-induced eye scratching was markedly attenuated. It was recently shown that neuromedin B (NMB) and its receptor (NMBR) in the trigeminal system are critical molecules for conjunctival itch transmission, but not for corneal pain transmission [6, 43]. Thus, *Grpr*-expressing neurons in the Sp5C may receive itch signals via the NMB-NMBR system to transmit the ocular itch information to the brain (see Figure 6E), as suggested in the spinal somatosensory system [44].

The "cheek model" of rodents has been used as an evaluation method for classification of itch and pain behaviour in the trigeminal sensory system. In this model, wiping of the cheek using the ipsilateral forefoot was induced by nociceptive substances, while hindfoot scratching was induced by some pruritogens [27, 45]. However, behavioural responses to histamine injection are not consistent in the face [27]. In our present study, histamine-evoked itchy eyes were scratched by the ipsilateral hindfoot both in rats and in mice, suggesting high sensitivity of the eye to pruritogens. Together with discrete populations of sensory fibres which transmit conjunctival itch and corneal pain [6], the "eye model" should offer a simpler method for the evaluation of itch behaviour in the trigeminal sensory system.

Conclusions

We propose a behavioural test for itch from the eyes as an evaluation method for footedness in rodents. Handedness and footedness have usually been considered as being unique to

humans, although laterality is seen as a general phenomenon across all bilateralians. Kangaroos apparently show a left-hand preference for many tasks in the wild, although "true" handedness is debated in marsupials [46]. Interestingly, the right preference laterality which we have found in rodents is common to humans; the majority of whom are right-handed. We suggest that our study could open up a new field for research on the mechanisms of laterality in vertebrates including humans. Identification of the medullary system transmitting itch from the eyes also offers new potential therapeutic approaches to refractory pruritic eye disorders, which emerge especially in adolescent females.

Methods

Animals

Young-adult female Wistar rats aged 4 weeks (Charles River Japan, Yokohama, Japan) and female C57BL/6J mice aged 4 weeks (CLEA Japan, Tokyo, Japan) were used since our preliminary experiments showed little sensitivity to histamine in male rats. For experiments probing the expression site of the GRPR, we used monomeric RFP-human heparin-binding epidermal growth factor (hDTR)-expressing BAC transgenic (*Grpr*-RFP) rats [47] in which the *Grpr* promoter driving fusion gene expression was created by pronuclear injection of Wistar rat embryos (Institute of Immunology, Tokyo, Japan). The transgenic rats were identified by standard PCR analysis of extracted ear DNA, using primers detecting the RFP gene. All rats and mice were maintained on a 12-h light/12-h dark cycle and were provided unlimited access to water and rodent chow. All animal experimental procedures were authorized by the Committee for Animal Research at Okayama University and National Institute of Genetics, Japan. All efforts were made to minimize animal suffering and reduce the number of animals used in this study.

Itch behaviour analyses in rats

All rats were habituated singly in an observation cage (a glass chamber, 30 cm x 20 cm x 36 cm) more than 2 times before behavioural observation. In the experiment involving unilateral histamine instillation, 60 µmol histamine (H7125, Sigma, St. Louis, MO) was instilled into the conjunctival sac of either the right or left eye, and 5 µl saline (Hikari, Tokyo, Japan) as a control into the other eye. In the experiment for bilateral histamine instillation, either histamine or saline as a control was instilled into the conjunctival sac of both eyes. We recorded a sequence of movements of the unilateral hindfoot as a bout of scratching behaviour for one hour, using a video camera (HC-W585M, Panasonic, Japan). The left or

right eye was always scratched by the ipsilateral hindfoot, and the laterality of the scratching event was analysed. We also recorded bouts of glooming with both forefeet for one hour. Since both grooming and scratching behaviour occurred as clusters of behaviours with periodic characteristics, with each cycle corresponding to one movement, their bouts and total duration were analysed. All records were made without investigators present. Movies were reviewed by multiple investigators blinded to the treatment, and the number of scratching bouts was counted. A scratch bout was defined as one or more rapid hindfoot motions directed toward and contacting the eye.

Itch behaviour analyses in mice

All mice were habituated singly in an observation cage (an acrylic chamber, 12 cm x 19 cm x 35 cm) more than 2 times before behavioural observation. Saline as a control or 60 μmol histamine in 3 μl saline was instilled into conjunctival sac of both eyes. Scratching behaviour and grooming were recorded by use of SCLABA-Real (Noveltec, Kobe, Japan). All records were made without investigators present and analysed in the same way as for rats.

Tissue preparation of rat brains

Vehicle (5 μl saline as a control) was instilled into the conjunctival sac of one eye and 60 μmol histamine in 5μl saline into the contralateral eye for 90 min during which the animals remained in the observation cage in preparation for the following c-Fos immunofluorescence analysis. The animals were anesthetized with sodium pentobarbital (50–90 mg/kg body weight) and perfused transcardially with either 100 ml (rats) heparinized physiological saline followed by 200 ml (rats) 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for immunofluorescence. Brains were dissected and immersed in the same fixative for 3 h at room temperature, and then immersed in 25% sucrose in 0.1 M PB at 4 ^{o}C until they sank.

For cryosectioning, tissues were quickly frozen by using powdered dry ice and cut into 30 μm-thick cryostat sections (CM3050 S; Leica, Nussloch, Germany). The sections were then washed several times with phosphate-buffered saline (PBS) for 5 min each.

Immunofluorescence

Immunofluorescence detection of c-Fos was performed according to our established methods [47, 48]. Nonspecific binding components were blocked with 1% normal goat serum and 1% bovine serum albumin in PBS containing 0.3% Triton X-100 for 30 min at room temperature. Sections were incubated with the primary rabbit antiserum raised against human c-Fos (1:5,000; ab190289, Abcam, Cambridge, UK; RRID: AB_2737414) for overnight at 4 ^{o}C. The sections were then incubated for 2 h at room temperature with Alexa Fluor 488-linked anti-rabbit IgG raised in goats (Molecular Probes, Eugene, OR) using a 1:1,000 dilution for detection. Immunoreacted sections were imaged by using a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan). Images were captured and saved as TIFF format. Analyses of c-Fos immunoreactivity of the *Grpr*-expressing neurons in the Sp5C were performed on brainstem cross-sections from female rats. We counted the number of c-Fos^{+}/RFP^{+} neurons (an indicator of *Grpr* expression) in the lateral and dorsal areas of the superficial layers of the Sp5C. Double^{+} neurons were also counted. The cell counts for each signal were obtained by combining data from nine rats with at least five sections each.

***In vivo* depletion of *Grpr*-expressing neurons by TRECK method**

For depletion of *Grpr*-expressing neurons in the Sp5C, *Grpr*-RFP rats which exclusively express the hDTR in *Grpr*-expressing neurons [47] were used for our TRECK experiment. Transgenic rats were anesthetized with sodium pentobarbital and mounted in a stereotaxic frame (ST-7R-HT, Narishige, Tokyo, Japan). To target the Sp5C, the rats were mounted at an

angle such that lambda was 3.5 mm ventral to bregma. The hindbrain of the right side was carefully exposed taking care not to damage the trigeminal nerve. Transgenic rats were treated with 40 ng diphtheria toxin (01-517, CosmoBio, Tokyo, Japan) diluted in 4 μL saline or treated with 4 μL saline as a mock control on day 0. A neurosyringe (701 RN, Hamilton, White Pine, NV) was slowly lowered to the target site to minimize the brain damage. Diphtheria toxin or saline was microinjected into the target site at a low flow rate. After the microinjection, the skin wound was covered with gelfoam, and the incision closed with a surgical suture. One week after surgery, 60 μmol histamine and saline were instilled into the right eye and the left eye of each rat. After the observation of itch behaviour, rats were perfusion-fixed under a deep pentobarbital anesthesia; and depletion of *Grpr*-expressing neurons in the Sp5C was confirmed using 30 μm cross-sections of brains. At least 5 sections per animal were imaged by using a confocal laser scanning microscope (FV1000, Olympus) to assess pixel numbers of RFP signals with ImageJ (Version.45p: National Institutes of Health, Bethesda, MD).

Statistical analyses

All data are expressed as the mean ± standard error of the mean (s.e.m.). In the experiment involving unilateral histamine/saline instillation into the eye of rats, the Wilcoxon signed rank was used to compare data concerning the extent of scratching of the two sides. In the experiment involving histamine/saline instillation into the conjunctival sac of both eyes in rats, the Wilcoxon signed rank test was used to compare data concerning the extent of scratching of the two sides. In the experiment involving histamine-/saline-instillation into both conjunctival sac of the eyes in mice, unpaired Wilcoxon signed rank test was used to compare scratching behaviour on the two sides. The unpaired Wilcoxon signed rank test was used to compare data for c-Fos$^+$ neurons, *Grpr*-expressing neurons, and the percentages of c-

Fos$^+$ and *Grpr*-expressing neurons in rats. The paired *t*-test was used to compare data of parameters of scratching behaviour in *Grpr*-RFP rats. All data analysed by *t*-test were checked for normal distribution and equal variances. All data were analyzed by SPSS Statistics version 27 (IBM, Chicago, IL). Graphs were made using GraphPad Prism 9 (GraphPad Software, San Diego, CA).

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Author contributions:

Y.K., K.T., and H.S. designed the study; Y.K., A.M., and K.T. performed experiments; Y.K., A.M., and K.T. analysed data; T.S., and H.S. performed data curation; Y.K., K.T., and H.S. wrote the paper. K.T., and H.S. conceived and supervised the whole study. All authors discussed the results and had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figures

Figure 1. Scratching behaviour evoked by unilateral instillation of histamine into the conjunctival sac of the eyes of rats.

(A) Schematic showing the instillation of either 60 μmol histamine (His) (n = 6) or saline (Sal) (n = 3) unilaterally into the conjunctival sac of either the left (L) eye (Group 1) or right (R) eye (Group 2). The number (B) and duration (C) of histamine- or saline-evoked hindfoot scratches during 60 min. The number and duration of scratches with the right foot was significantly greater than with that with the left foot (B: P = 0.028, C: P = 0.042). The number (D) and duration (E) of grooming events did not differ significantly in either Group 1 or Group 2 rats. *P < 0.05 with Wilcoxon signed rank test. Data are shown as mean ± s.e.m.

Figure 2. Scratching behaviour evoked by bilateral instillation of histamine into the conjunctival sacs of the eyes in rats.

(A) Schematic showing the bilateral instillation of either saline (n = 5) or 60 μmol histamine (n = 6) into the conjunctival sac of the eyes of rats. The number (B) and duration (C) of saline- or histamine-evoked hindfoot scratches during 60 min. The duration of scratches was significantly greater on the right side (right foot to right eye) compared with the left side after histamine but not saline instillation (B: P = 0.065, C: P = 0.031). *P < 0.05 with Wilcoxon signed rank test. Data are shown as mean ± s.e.m.

Figure 3. Laterality of scratching behaviour evoked by bilateral instillation of histamine into the conjunctival sacs of the eyes in mice.

(A) Schematic showing the bilateral instillation of saline or histamine into the conjunctival sacs in mice (n = 12). The number (B) and duration (C) of saline- or histamine-evoked hindfoot scratches during 30 min. The number of scratches induced by 60 μmol histamine tended to be increased in the right eye group (P = 0.084) compared with left eye group. There was no significant differences in scratching duration between the right side and the left side in mice (P = 0.140). *P < 0.05 with Wilcoxon signed rank test. Data are shown as mean ± s.e.m.

Figure 4. Histamine instillation induced activation of *Grpr*-expressing neurons in the lateral part of the trigeminal nucleus of the medulla oblongata in rats.

(A) Schematic showing the lateral part (V1 and V2 input area) of the spinal trigeminal nucleus caudalis (Sp5C) in rats. (B, C) Double-fluorescence images showing *Grpr*-expression (RFP, magenta) and c-Fos immunoreactivity (green) in the Sp5C (V1 and V2 area). Histamine instillation into an eye increased c-Fos expression in the ipsilateral side of Sp5C (B), compared to that in the contralateral side of Sp5C (control) (C). White arrows indicate neurons double-positve for c-Fos and *Grpr*. Scale bar, 10 μm.

Figure 5. Quantitation of c-Fos-immunoreactive neurons in the trigeminal nucleus of the medulla oblongata in rats.

Histamine instillation increased the number of c-Fos-immunoreactive neurons in the V1 and V2 area of the ipsilateral side in Sp5C [(A) left side instillation of histamine (L), n = 4; P = 0.028, (B) right side instillation of histamine (R), n = 5; P = 0.008]. The number of c-Fos-immunoreactive neurons in the V3 area was not changed (C and D). The number of cells

529 double positive for c-Fos and *Grpr* expressed as a proportion of the total number of *Grpr*-
530 expressing neurons was significantly higher in the histamine-instilled side than in the control
531 side in the V1 and V2 area of ipsilateral side [(E) left side instillation of histamine (L); P =
532 0.028] [(F) right side instillation of histamine (R); P = 0.015]. The number of c-Fos-
533 immunoreactive neurons was not changed in the V3 area (G and H). *P < 0.05 with
534 Wilcoxon signed rank test. Data are shown as mean ± s.e.m.
535
536
537 **Figure 6. Scratching behaviour was reduced by the depletion of *Grpr*-expressing**
538 **neurons in the medulla oblongata in rats.**
539 (A) Schematic showing the initial instillation of 60 μmol histamine into the conjunctival sac
540 of the right eye and saline into the left eye of *Grpr*-RFP transgenic rats (pre). One day later,
541 saline (n = 3) or diphtheria toxin (DT) (n = 5) was injected locally into the right trigeminal
542 nucleus caudalis (Sp5C). Seven days after the toxin injection, 60 μmol histamine was again
543 instilled in the conjunctival sac of the right eye and saline was instilled in the conjunctival sac
544 of the left eye to examine the scratching behaviour (post). (B) RFP (*Grpr* expression)
545 fluorescent signals in the right Sp5C of the saline-injected rats (left, vehicle) and right Sp5C
546 of the DT-injected rats (right, DT-lesioned). Scale bar, 100 μm. The number (C) and duration
547 (D) of scratches with the right hindfoot were significantly decreased in the post-DT injection
548 group in compared with the pre-DT injection group at 60 min (n = 5) (C: P = 0.027, D: P =
549 0.038). *P < 0.05 with t-test. Data are shown as mean ± s.e.m. (E) Hypothetical model of
550 NMB-NMBR and GRP-GRPR pathways for histaminergic itch transmission in the eye.
551 Histamine in the eye activates GRP-GRPR and NMB-NMBR systems via pruriceptors. As
552 suggested in the spinal sensory pathway of the body itch [6, 49], *Grpr*-expressing neurons in
553 the Sp5C might integrate multiple lines of information, and transmit histaminergic itch

554 information to the brain. Possible interaction between NMB-NMBR and GRP-GRPR systems
555 should be investigated in future studies of ocular itch. Broken lines indicate possible actions
556 in the itch transmission.
557

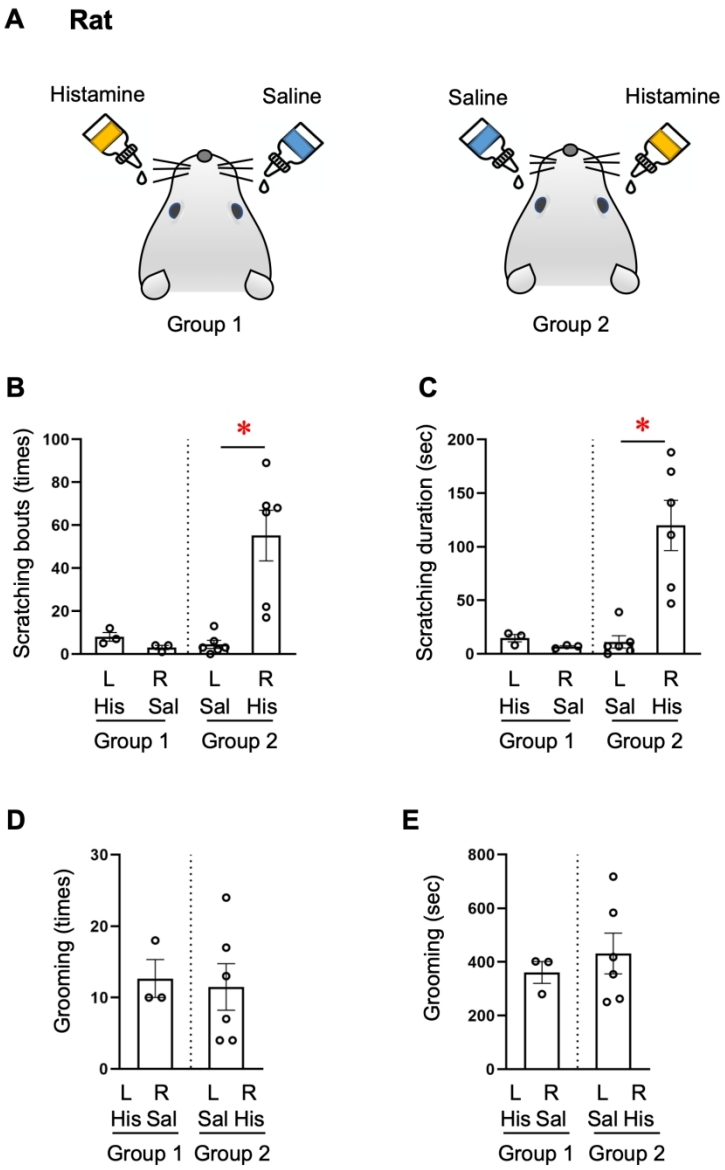

Figure1

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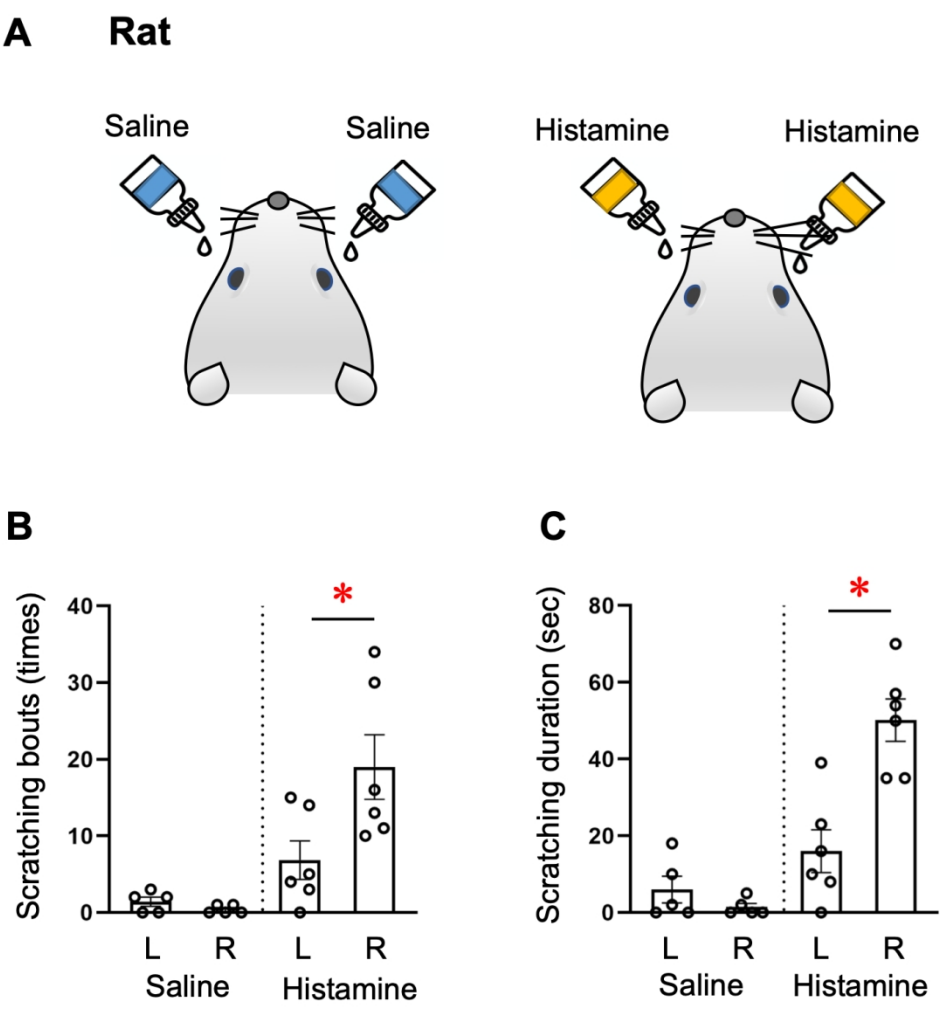

Figure2

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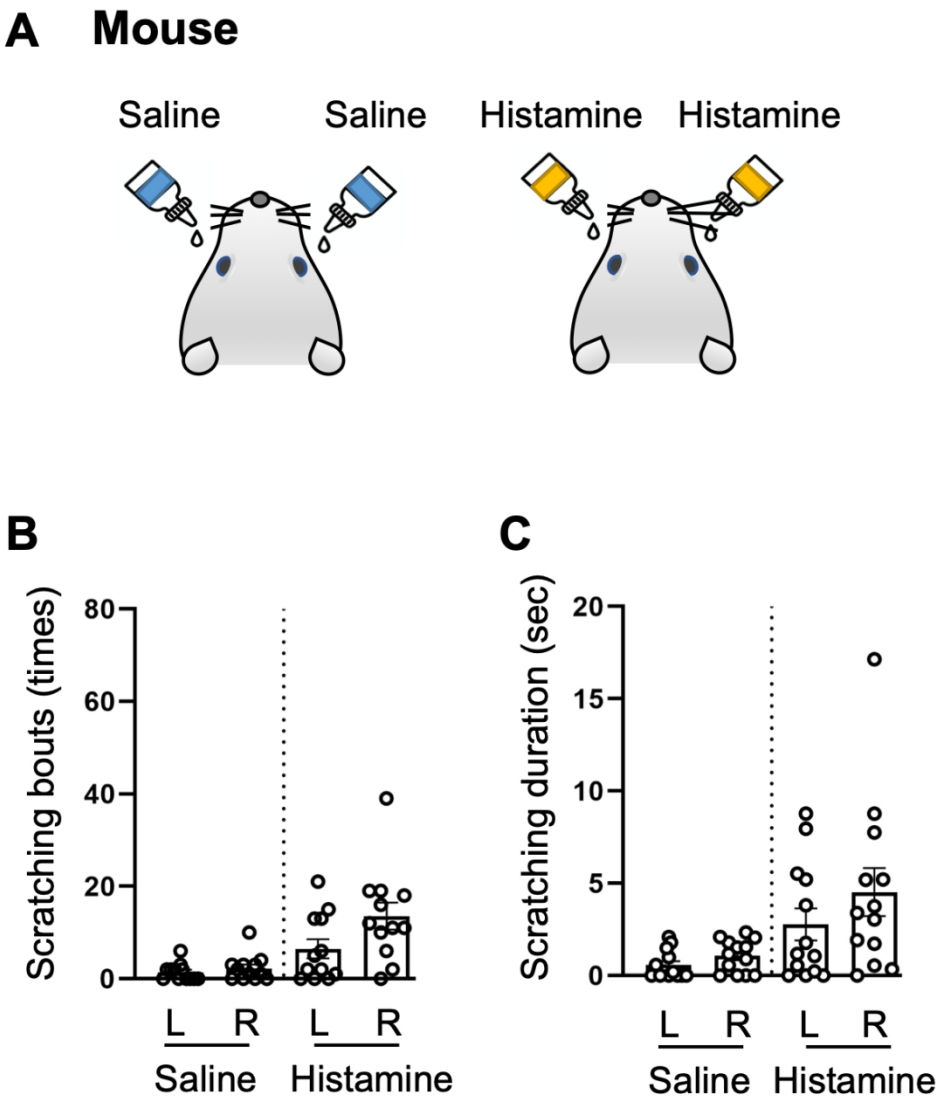

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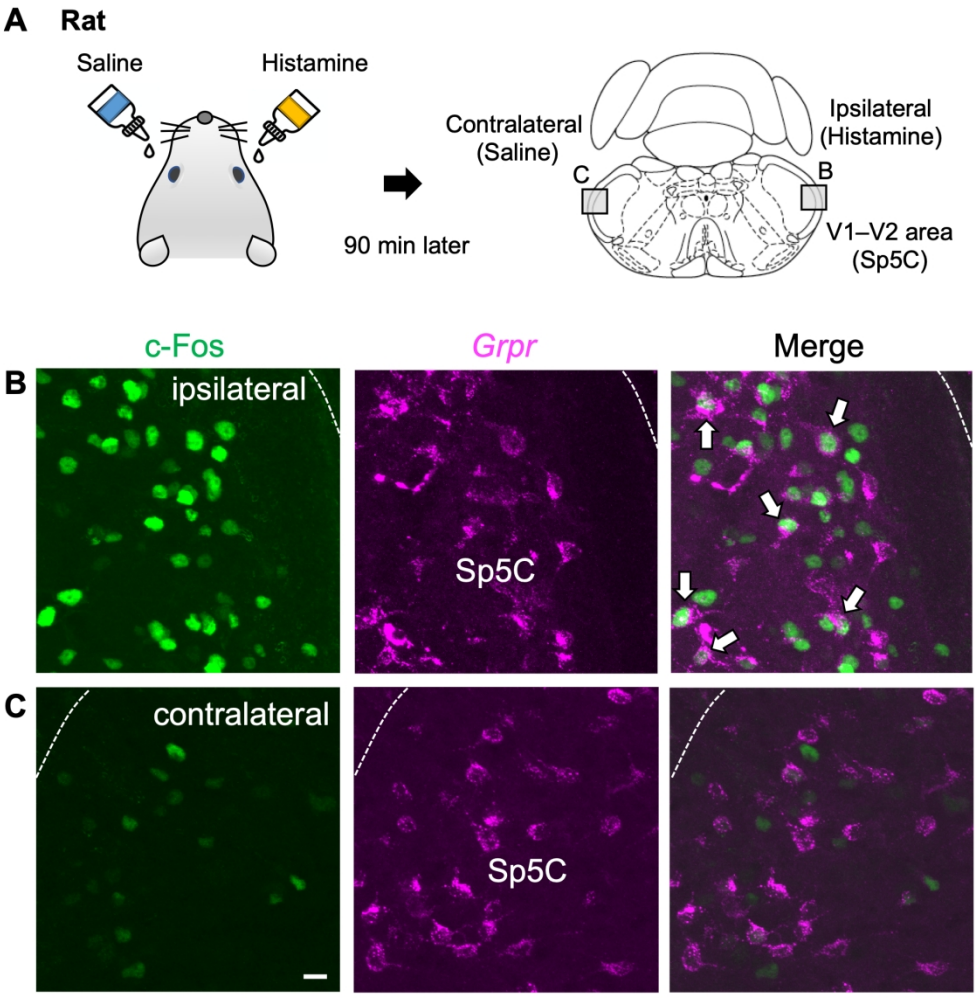

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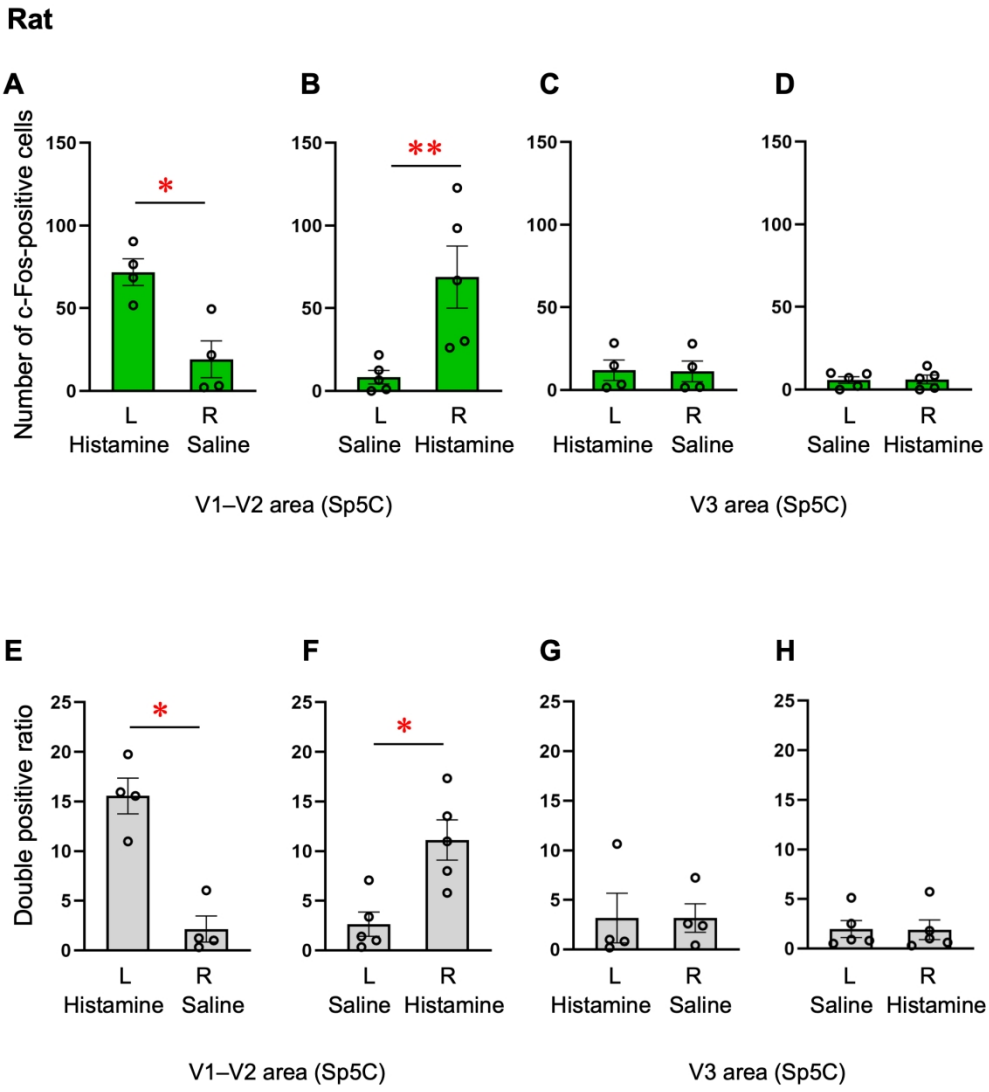

Figure5

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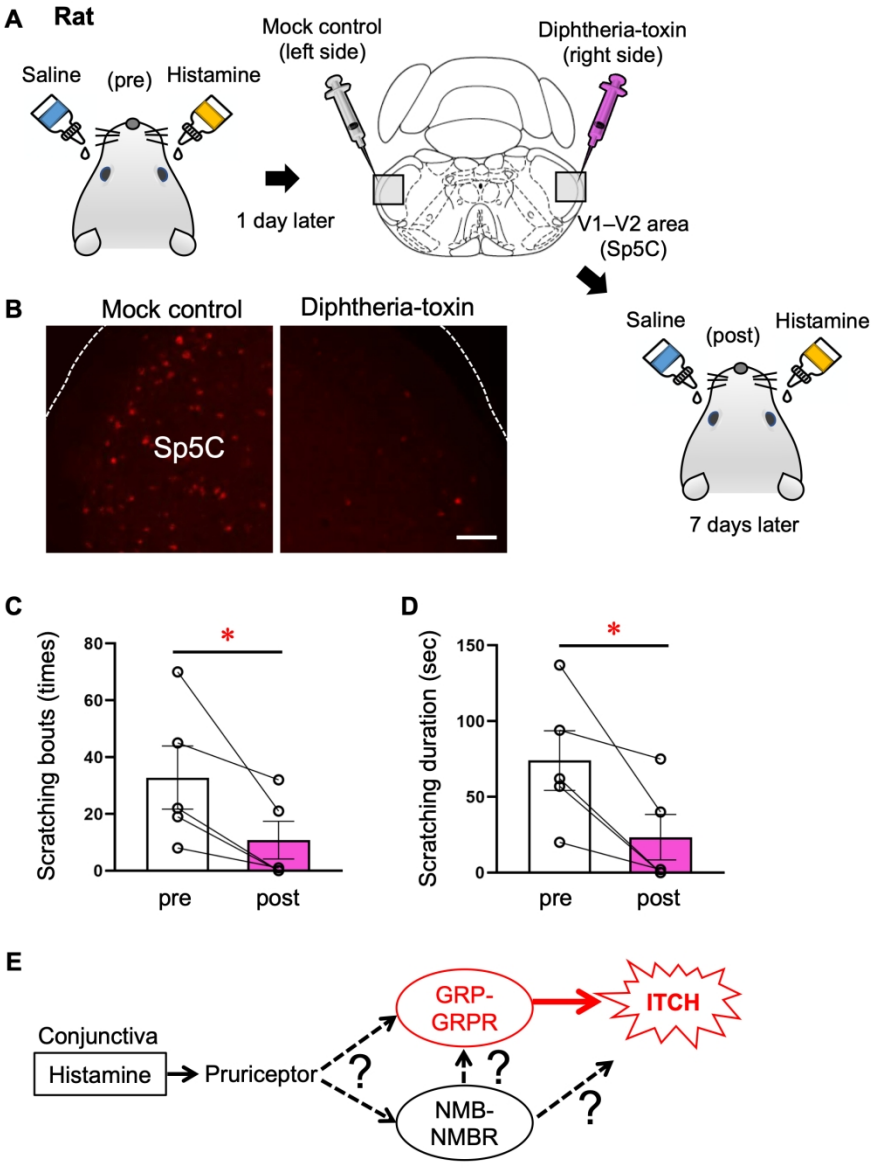

Figure6

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