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***Regular Article***

# Involvement of $Ca_v3.2$ T-Type $Ca^{2+}$ Channels and the Role of Endogenous Estrogen in Pruritus: Evidence from a Fundamental Study and Cross-Sectional Analysis of Pharmacy Claims Data

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**To clarify the roles of $Ca_v3.2$ T-type $Ca^{2+}$ channels and endogenous estrogen in pruritus, we conducted a fundamental study employing mice and clinical cross-sectional analyses of pharmacy claims data. In mice, intradermal injection of sulfide ($Na_2S$), a $Ca_v3.2$ enhancer, caused itch responses, an effect blocked by KTtp38, a T-type $Ca^{2+}$ channel inhibitor, and deletion of $Ca_v3.2$ gene. KTtp38 also suppressed itch responses following intradermal histamine or chloroquine. The sulfide-induced itch responses in female mice decreased by ovariectomy and/or repeated treatment with letrozole, an aromatase inhibitor. Cross-sectional analyses of pharmacy claims data of 357972 female patients aged 18 years and older, obtained from nationwide branches of a chain pharmacy group, showed significantly lower prescription rates of topical steroids used for treatment of pruritus and/or dermatitis in women 55 years and older than in women under 55 years, and in the users than non-users of estrogen suppressants. Multivariate logistic regression analysis in the users and non-users of estrogen suppressants after propensity score matching indicated significant negative association of topical steroid prescription with the use of estrogen suppressants. Together, the present fundamental and clinical studies suggest the involvement of $Ca_v3.2$ and the promotive role of estrogen in pruritus in mice and/or humans.**

**Key words** $Ca_v3.2$ T-type $Ca^{2+}$ channel, estrogen, itch, pruritus, sulfide

## INTRODUCTION

Itch is an important skin sensation alongside pain as an alerting reaction, but intractable and chronic pruritus could impair sleep, study, and work and might lead to psychological disturbances like depression.[1–4] Currently, topical steroids are best known treatment for itching, but there are only a few alternatives. Itch-sensing primary afferents are unmyelinated, small-diameter C-fiber neurons, and murine itch-sensing neurons have been clustered into three non-peptidergic (NP) afferent populations: MRGPRD-expressing NP1 neurons, MRGPRA3-expressing NP2 neurons, and somatostatin/brain natriuretic peptide (BNP/NPPB)-expressing NP3 neurons.[5] NP2 neurons that express transient receptor potential A1 (TRPA1), TRPV1, and histamine $H_1$ receptors in addition to MRGPRA3 appear to participate in itching accompanying atopic dermatitis and xerosis. There is evidence that genetic deletion or pharmacological inhibition of $Ca_v3.2$ T-type $Ca^{2+}$ channels, known as pronociceptive molecules,[6,7] reduces itch responses following intradermal injection of histamine or chloroquine, an agonist of MRGPRA3.[8] Sulfide is capable of enhancing $Ca_v3.2$ channels through cancellation of zinc inhibition, and participates in the development of intractable somatic and visceral pain.[9–15] Intradermal injection of sulfide produces both pain and itch responses in mice, which are reversible by pharmacological inhibition of $Ca_v3.2$ channels.[16]

There is plenty of clinical and preclinical evidence for sex differences in molecular mechanisms of chronic pain or for sex hormone modulation of pain sensitivity.[17–21] In fact, our clinical and preclinical studies have shown that postmenopausal estrogen decline and ovariectomy aggravate the development of chemotherapy-induced peripheral neuropathy (CIPN) in female cancer patients undergoing paclitaxel-based chemotherapy and female mice receiving repeated administration of paclitaxel, respectively, the latter being reversible by estrogen supplementation.[22,23] The relationship between sex hormones

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and itch is controversial. A fundamental study has shown that supplementation of estrogen, but not progesterone, increases histamine-induced itch response in ovariectomized rats through functional upregulation of gastrin-releasing peptide (GRP)-containing interneurons in the spinal itch pathway,[24] while there is evidence that continuous supplementation of estrogen at high doses suppresses itch responses in ovariectomized mice.[25] Little clinical evidence for the role of estrogen in pruritus is available.

In the present study, we conducted fundamental experiments to test whether ovariectomy and/or chemical inhibition of estrogen production could affect $Ca_v3.2$-dependent itch responses in mice, and then performed a clinical cross-sectional analysis of pharmacy claims data of a nationwide chain pharmacy group to get a clue about the relationship between estrogen and pruritus in humans.

## MATERIALS AND METHODS

**Animals Employed and Ethical Considerations for Animal Experimentation** ICR mice (Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan) were kept under stable temperature (24°C) and 12 h day-night cycle with free access to solid food (MF Oriental Yeast Co., Ltd., Tokyo, Japan) and water. To test the effect of genetic deletion of $Ca_v3.2$, we used $Ca_v3.2^{+/+}$ and $Ca_v3.2^{-/-}$ mice of a C57BL/6J background. $Ca_v3.2^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.) were mated with C57BL/6J ($Ca_v3.2^{+/+}$) mice (Kiwa Laboratory Animals Co., Ltd.), and $Ca_v3.2^{+/-}$ mice were bred to produce $Ca_v3.2^{+/+}$ (wild-type) and $Ca_v3.2^{-/-}$ ($Ca_v3.2$-KO) mice. Animal study protocols were in accordance with the guiding principles of The Japanese Pharmacological Society and the National Institutes of Health (NIH) guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication 86-23), and approved by the Committee for the Care and Use of Laboratory Animals at Kindai University (Approval No.: KAPS-2022-002).

**Major Chemicals for Fundamental Experiments** $Na_2S$ was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), and histamine dihydrochloride and chloroquine diphosphate were from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). These chemicals were dissolved in saline immediately before use. KTtp38 [1-(4-(3-fluorophenyl)butyl)-3-(1-(4-(3-fluorophenyl)butyl)piperidin-4-yl)-1*H*-benzo[d]imidazole-2(3*H*)-one], a T-type $Ca^{2+}$ channel inhibitor, was synthesized in-house, as reported previously,[26] and dissolved in saline containing 1% DMSO and 5% Tween 80. Letrozole and leuprorelin acetate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and were dissolved in 0.3% carboxymethylcellulose. 17$\beta$-Estradiol was purchased from Sigma-Aldrich and dissolved in peanut oil (Nacalai Tesque, Kyoto, Japan).

**Assay of Itch Responses in Mice** Itch responses following intradermal injection of pruritogens in the skin of the cheek or dorsocervical area were assessed, as described previously.[27] It is to be noted that the cheek injection model enabled us to observe itch and pain responses, separately and simultaneously. A few days after the cheek or dorsocervical skin area was shaved under anesthesia with 1.5% isoflurane, the conscious mouse was placed and acclimatized for at least 30 min in an acrylic observation cage (10 × 14 × 30 cm) with mirrors on all four sides and with an overhead video camera for recording of the behavior.

The mouse received intradermal injection of itch-inducing substances, such as sulfide ($Na_2S$), a $Ca_v3.2$ enhancer, histamine, an agonist of $H_1$ and $H_2$ receptors, and chloroquine, an MRGPRA3 agonist, or saline, in the right cheek (injection volume: 10 $\mu$L) or dorsocervical skin (injection volume: 20 $\mu$L), and the mouse's behavior was recorded for 60 min. In the cheek injection model, itch and pain responses were counted by watching the video later. Scratching of the ipsilateral cheek skin with the hindpaw was counted as an itch response, while wiping of the ipsilateral cheek skin with the forepaw was counted as a pain response. In the dorsocervical injection model, scratching of the dorsocervical area with a hindpaw was counted as an itch response, although pain-like behavior was not detectable. In both models, an itch response was defined as a series of behavior, that is, scratching of the injected site by the hindpaw followed by movement of the hindpaw to the floor or toward the mouth.

**Ovariectomy and Pharmacological Estrogen Deprivation** Female ICR mice were ovariectomized, as described elsewhere.[23] Briefly, the mouse was anesthetized by i.p. administration of medetomidine (0.3 mg/kg) and ketamine (75 mg/kg), and bilateral small incisions were made to expose the ovaries from the retroperitoneum. The areas between the uterus and the oviduct were then ligated with thread and the bilateral ovaries were removed. In sham operation, a mouse was subjected to the corresponding surgery in which the ovaries were exposed but not removed. The ovariectomized mice were examined for evaluation of dorsocervical sulfide-induced itch responses 14 d after the operation.

Given that aromatase inhibitors suppress local estrogen production and the growth of human breast cancer cell line-derived xenograft in ovariectomized mice,[28,29] letrozole, an aromatase inhibitor, at 1 mg/kg was administered i.p. daily for 7 d, starting 7 d after ovariectomy in mice. In a separate experiment, mice received a single s.c. administration of leuprorelin, a gonadotropin-releasing hormone (GnRH) receptor-desensitizer, at 25 $\mu$g/kg, 14 d before repeated treatment with letrozole.

**Statistical Analysis of Animal Experiment Data** Data obtained from animal experiments were expressed as mean ± standard error (S.E.M.). The Mann–Whitney *U* test was used for comparison between two-group data, and the Kruskal–Wallis test was used to evaluate statistical significance between data from three or more groups. Significance was set at a level of $p < 0.05$.

**Pharmacy Claims Data Used for Clinical Research** We used pharmacy claims data (*i.e.*, pharmacy receipt data) of patients who received prescription medication at nationwide 886 community-based pharmacy branches of Sugi Pharmacy Co., Ltd. (Obu, Aichi, Japan), a chain pharmacy group, over a period of 1 month (November 1–30, 2020). Information available from pharmacy claims data in Japan includes patient's age, gender, and prescribed medications, but not the results of diagnosis. We used the encrypted data to maintain

anonymity. Multiple data for one patient were merged to avoid duplications.

**Patient Information Collected for Statistical Analysis and Inclusion/Exclusion Criteria** We collected information about female patients aged 18 years and older ($n=357972$), who got their prescription filled at any of the nationwide community pharmacy branches of the Sugi Pharmacy chain group during 1 month of November 2020 ($n=704953$). In the prescribed medications, "estrogen suppressants" were defined as drugs classified as antiestrogens (*i.e.*, tamoxifen, toremifene), aromatase inhibitors (*i.e.*, letrozole, anastrozole), and GnRH receptor desensitizer nasal sprays (*i.e.*, buserelin, nafarelin) (Supplementary Table S1). It is to be noted that GnRH receptor desensitizers that patients received at the pharmacy branches included nasal sprays or drops for treatment of endometriosis and uterine fibroids (Supplementary Table S1), but not injections of leuprorelin acetate and goserelin acetate for hormone therapy of premenopausal breast cancer.

We also collected information about the medication categories including "topical steroids," "moisturizers (topical protectant)," "oral steroids," "2nd generation $H_1$ inhibitors," "neuropathy medications," "oral non-steroidal anti-inflammatory drugs (NSAIDs)," "opioid analgesics," "anti-depressants, anxiolytics or sleeping drugs," "anti-diabetes medications," "statins" and "anti-hypertensive drugs" (Supplementary Table S1). The data of a patient lacking any information of sex, age, or medication were excluded.

**Statistical Analysis of Pharmacy Claims Data** To investigate the relationship between estrogen and pruritus by univariate analysis, patients were divided into two groups using 55 years as the cutoff age, roughly corresponding to a postmenopausal age, and further classified into users and non-users of estrogen suppressants (Supplementary Fig. S3). Frequency of prescription of topical steroids, which are commonly used to treat pruritus and/or dermatitis, were statistically compared between those four groups, that is, non-user and user of estrogen suppressants under 55 and at 55 and older, using Fisher's exact test and Holm's multiple comparison test. For multivariate logistic regression analysis, the enrolled female patients were classified into two groups, users and non-users of estrogen suppressants, and the patients' age and number of medications other than estrogen suppressors were adjusted between the two groups using 1 : 1 propensity score matching, in order to control the bias due to confounding factors. The association of estrogen suppressant prescription with frequency of prescription of various medication categories including topical steroids was analyzed using Fisher's exact test and multivariate logistic regression models. The caliper coefficient was set to 0.2. The results obtained in the analysis were described as odds ratios (OR) and 95% confidence intervals (CI), and a value of $p<0.05$ was considered statistically significant.

In the subgroup analysis in women aged 55 years and above, the univariate and multivariate analyses were also conducted after propensity score matching, as mentioned above. Statistical analyses of clinical data were performed using EZR (Jichi Medical University Saitama Medical Center, Saitama, Japan, version 1.62),[30] a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 4.3.1). This is an improved version of R commander (version 2.9-1), which is designed to easily perform statistical functions frequently used in biostatistics.

**Ethical Considerations for Real-World Data Analysis in Clinical Research** The present clinical study complied with the ethical standard in the Declaration of Helsinki, and was approved by the Clinical Ethics Committee of the Kindai University Faculty of Pharmacy (Approval No.: 22-209). Considering the retrospective nature, the need for informed consent was waved by the Clinical Ethics Committee of the Kindai University Faculty of Pharmacy, which approved the use of an opt-out method.

## RESULTS

**Involvement of $Ca_v3.2$ T-Type $Ca^{2+}$ Channels in Itch Responses in Male and Female Mice** In male ICR mice, cheek intradermal injection of sulfide ($Na_2S$), an enhancer of $Ca_v3.2$ T-type $Ca^{2+}$ channels,[9] in a range of 30–300 $\mu$g, caused both itch and pain responses in a dose-dependent manner (Figs. 1A, 1B), in agreement with the previous report,[16] which were prevented by systemic administration of KTtp38, a T-type $Ca^{2+}$ channel inhibitor,[26] at 10 mg/kg (Fig. 1C). In male and female ICR mice, dorsocervical intradermal injection of $Na_2S$ in the same dose range produced dose-dependent itch responses (Figs. 1D, 1E), which were also significantly reduced by KTtp38 (Fig. 1F). The magnitude of itch responses following dorsocervical $Na_2S$ injection was equivalent between male and female ICR mice (Supplementary Fig. S1). Dorsocervical intradermal injection of $Na_2S$ also produced remarkable itch behavior in male and female wild-type C57BL/6J mice (Fig. 1G), though to a lesser extent than in ICR mice (Fig. 1F), but caused only slight itch responses in male and female $Ca_v3.2$-KO mice of a C57BL/6J background (Fig. 1G). Dorsocervical intradermal injections of histamine and chloroquine significantly increased itch responses at 300 and 300–1000 $\mu$g, respectively, in male and female ICR mice (Figs. 2A, 2B). The itch responses following stimulation with histamine or chloroquine at 300 $\mu$g/site in ICR mice were equivalent between males and females (Supplementary Figs. S1B, S1C), and significantly suppressed by systemic administration of KTtp38 (Figs. 2C, 2D). Collectively, $Ca_v3.2$ T-type $Ca^{2+}$ channels, the activity of which is enhanced by sulfide, are considered to participate in itch behavior following activation of histamine $H_1$ receptors and MRGPRA3 in mice.

**Effects of Estrogen Decline on Itch Responses Caused by Sulfide, a $Ca_v3.2$ Enhancer, in Female ICR Mice** Itch responses between 0 and 60 min or between 10 and 30 min following dorsocervical intradermal injection of $Na_2S$ at 300 $\mu$g tended to decrease in ovariectomized ICR mice, as compared with sham-operated mice (Fig. 3A). The mice subjected to ovariectomy plus repeated i.p. treatment with letrozole, an aromatase inhibitor, at 1 mg/kg exhibited a significant decrease in itch responses between 10 and 30 min after sulfide injection (Fig. 3B). The decreased itch responses between 0 and 60 min or between 10 and 30 min after sulfide injection in the mice subjected to ovariectomy plus letrozole significantly elevated by estrogen supplementation with repeated s.c. administration

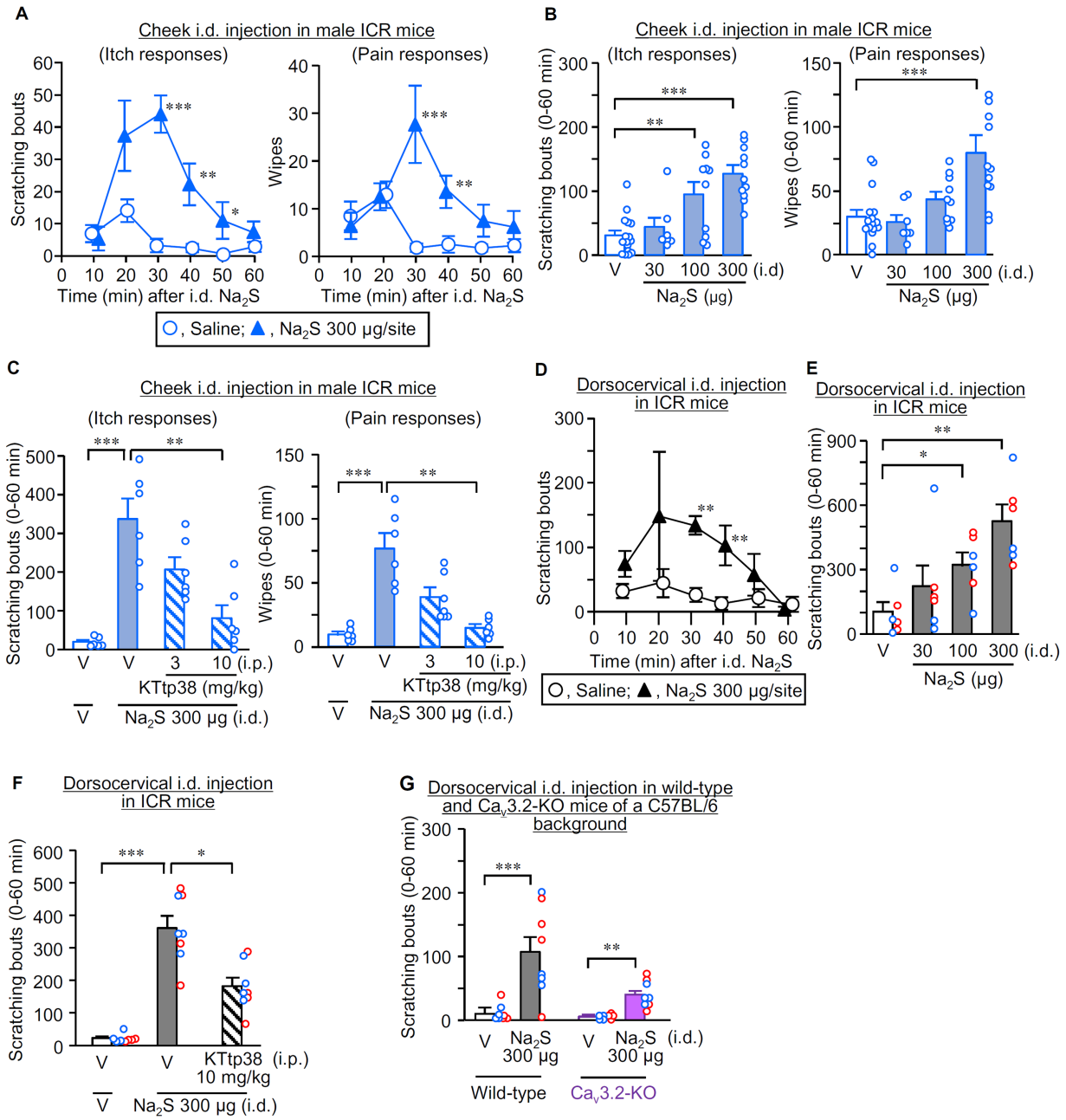


Fig. 1. $Ca_v3.2$-Dependent Itch and/or Pain Responses Induced by Intradermal (i.d.) Injection of $Na_2S$ in the Cheek or Dorsocervical Skin in Mice

(A) Time-course of itch (scratching) and pain (wiping) responses following cheek i.d. $Na_2S$ at 300 $\mu$g in male ICR mice. (B) Total itch and pain responses for 60 min after cheek i.d. $Na_2S$ in male ICR mice. (C) Effect of KTtp38, a T-type $Ca^{2+}$ channel inhibitor, on itch and pain responses for 60 min after cheek i.d. $Na_2S$ in male ICR mice. KTtp38 at 10 mg/kg was administered i.p. 30 min before i.d. $Na_2S$. (D, E) Time-related (D) and total (E) itch (scratching) responses for 60 min following dorsocervical i.d. $Na_2S$ in male and female ICR mice. (F) Effects of KTtp38 on itch responses following dorsocervical i.d. $Na_2S$ in male and female ICR mice. KTtp38 was administered as described above. (G) Itch responses for 60 min following dorsocervical i.d. $Na_2S$ in wild-type and $Ca_v3.2$-knockout (KO) male/female mice of a C57BL/6J background. Blue and red open circles indicate the individual data of male and female mice, respectively. Data show the mean with S.E.M. for 11–16 (A), 7–16 (B), 6 (C), 6 (male=3, female=3) (D, E), 8 (male=4, female=4) (F, G) mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

of 17$\beta$-estradiol at 0.15 mg/kg (Fig. 3C). It was also confirmed that a single s.c. administration of leuprorelin, a GnRH receptor desensitizer, at 25 $\mu$g/kg, followed by repeated treatment with letrozole, showed a remarkable tendency for reducing sulfide-induced itch responses in female ICR mice (Supplementary Fig. S2).

**Difference in Prescribed Estrogen Suppressants by Age in Pharmacy Claims Data Employed for a Cross-Sectional Clinical Study** Pharmacy claims data in Japan include information about medications and patients' gender and age, but not diagnosis. Estrogen suppressants include GnRH receptor desensitizers, antiestrogens (estrogen receptor antagonists), and aromatase inhibitors (Supplementary Table S1). Of the 704953 patients who visited 886 nationwide chain pharmacy branches during 1 month of November 2020, 357972 female patients aged 18 years and older were enrolled in this study, and divided into 3894 users and 354078 non-users of estrogen suppressants (Supplementary Fig. S3).

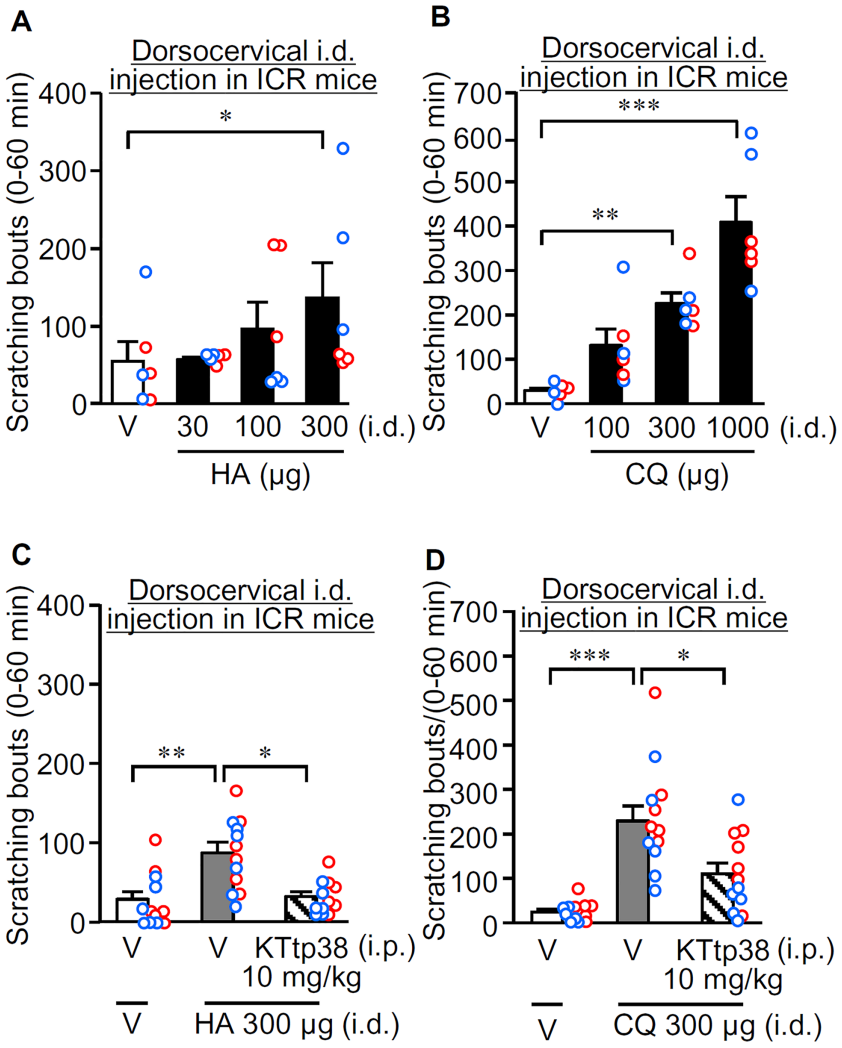


Fig. 2. Effect of KTtp38, a T-Type $Ca^{2+}$ Channel Inhibitor, on Itch Responses Following Dorsocervical Intradermal (i.d) Injection of Histamine (HA) or Chloroquine (CQ) in Male and Female ICR Mice

KTtp38 at 10 mg/kg was administered i.p. 30 min before i.d. HA or CQ. Blue and red open circles indicate the individual data of male and female mice, respectively. Data show the mean with S.E.M. for 6 (male=3, female=3) (A, B) or 12 (male=6, female=6) (C, D) mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Antiestrogens were often prescribed to women in their 20s, 30s, 40s, and 50s, while aromatase inhibitors were often prescribed to women in their 50s, 60s, and 70s (Supplementary Fig. S4). A very small number of women only in their 50s or younger were prescribed with GnRH receptor desensitizers (Supplementary Fig. S4), which were nasal sprays for treatment of endometriosis and uterine fibroids. It is to be noted that prescriptions of injections of GnRH receptor desensitizers including leuprorelin acetate and goserelin acetate for hormone therapy of premenopausal breast cancer are generally filled at the hospital pharmacy, but not at external pharmacies, in Japan.

**Association of Prescription Rates of Topical Steroids and Antihistamines with Use of Estrogen Suppressants or Age** Given that topical steroids are used to treat pruritus and/or dermatitis, we examined the relationship of prescription rates of topical steroids with the use of estrogen suppressants. The rate of topical steroid monotherapy was significantly and greatly lower in the users than in non-users of estrogen suppressants (Fig. 4A, left). There were not many patients who received topical antihistamine preparations (Fig. 4A, center), which might be often used for treatment of pruritus rather than dermatitis. Nonetheless, the rate of prescription of topical antihistamines was also significantly lower in the estrogen suppressant users than in non-users (Fig. 4A, center). Similarly, the prescription rate of a combination of topical steroids and topical antihistamines tended to decrease in the users of estrogen suppressants (Fig. 4A, right). We also analyzed the relationship of topical steroid prescription with age (<55 or ≥55), and found that the prescription rates of topical steroids in non-users of estrogen suppressants were significantly lower in women 55 years and over than in women under 55 years (Fig. 4B). The topical steroid prescription rate in each of the two different age groups, that is, aged 55+ and under 55 years, was significantly lower in users than in non-users of estrogen suppressants, respectively (Fig. 4B).

**Univariate and Multivariate Analyses of Prescription Rates of Various Medications Including Topical Steroids in Users and Non-users of Estrogen Suppressants** In 3894 users and 354078 non-users of estrogen suppressants,

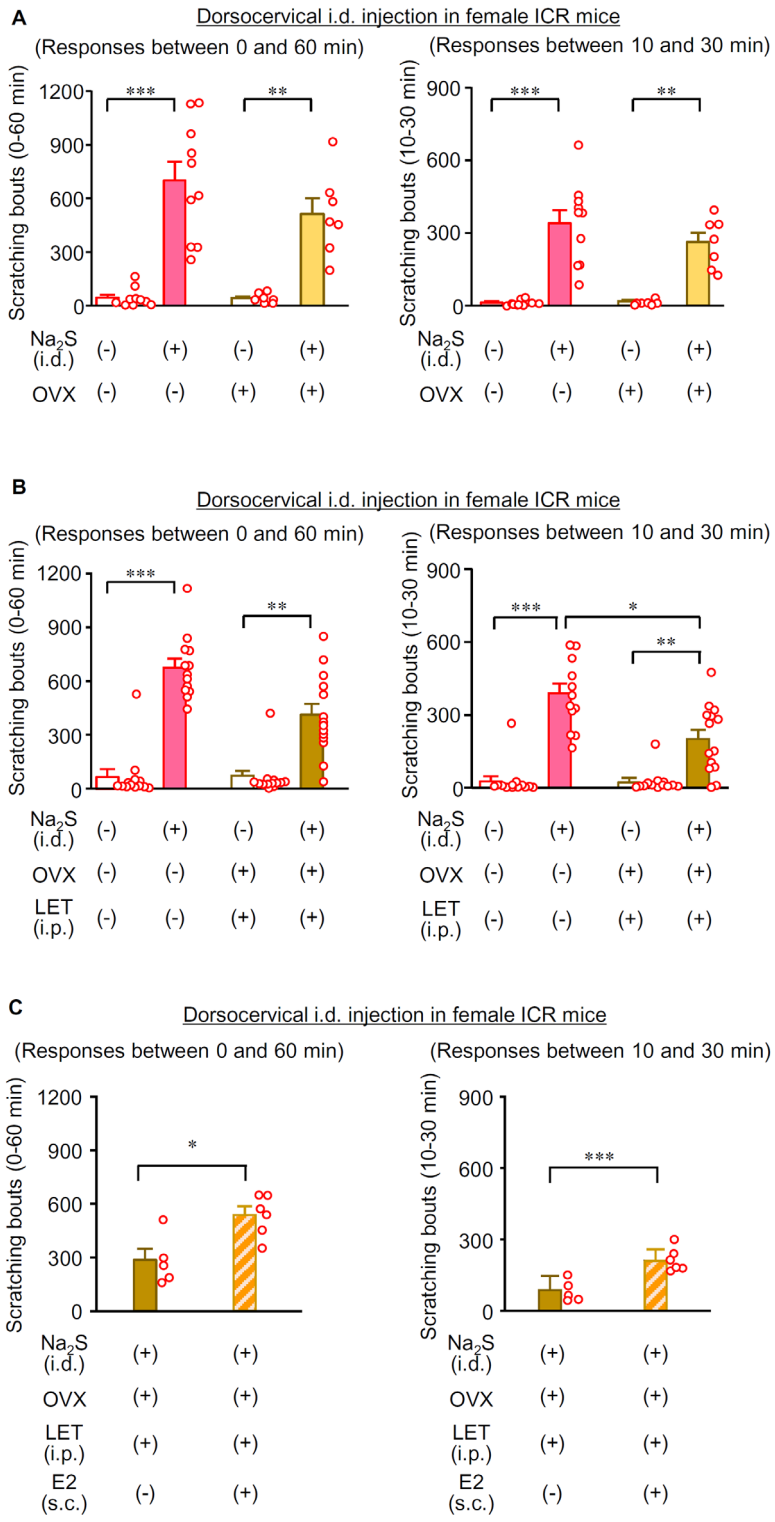


Fig. 3. Effect of Ovariectomy and/or Letrozole, an Aromatase Inhibitor, on Dorsocervical i.d. Sulfide-Induced Itch Responses in Female ICR Mice

Itch responses during 0–60 min and 10–30 min after i.d. $Na_2S$ are shown (A–C). (A) Female ICR mice were subjected to ovariectomy (OVX) or sham operation 2 weeks before observation of sulfide-induced itch responses. (B) Seven days after OVX or sham operation, female ICR mice received daily i.p. administration of letrozole (LET) at 1 mg/kg, 7 times in total, and thereafter, were tested for sulfide-induced itch responses. (C) Seven days after OVX or sham operation, female ICR mice received daily s.c. administration of 17$\beta$-estradiol (E2) at 0.15 mg/kg in addition to daily i.p. administration of LET, each 7 times in total, and thereafter, were tested for sulfide-induced itch responses. Red open circles indicate the individual data of female mice. Data show the mean with S.E.M. for 7–10 (A), 13–14 (B) and 5–6 (C) mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

the median age was 60 and 54, and the median number of medications other than estrogen suppressants was 3 and 0, respectively (Table 1). These variables were balanced by propensity score matching between the two groups. After the matching, the number of patients in each group was 1672, and the median of age and the number of medications other than estrogen suppressants were 59 and 2, respectively (Table 1). Then, prescription rates of various medications in users and

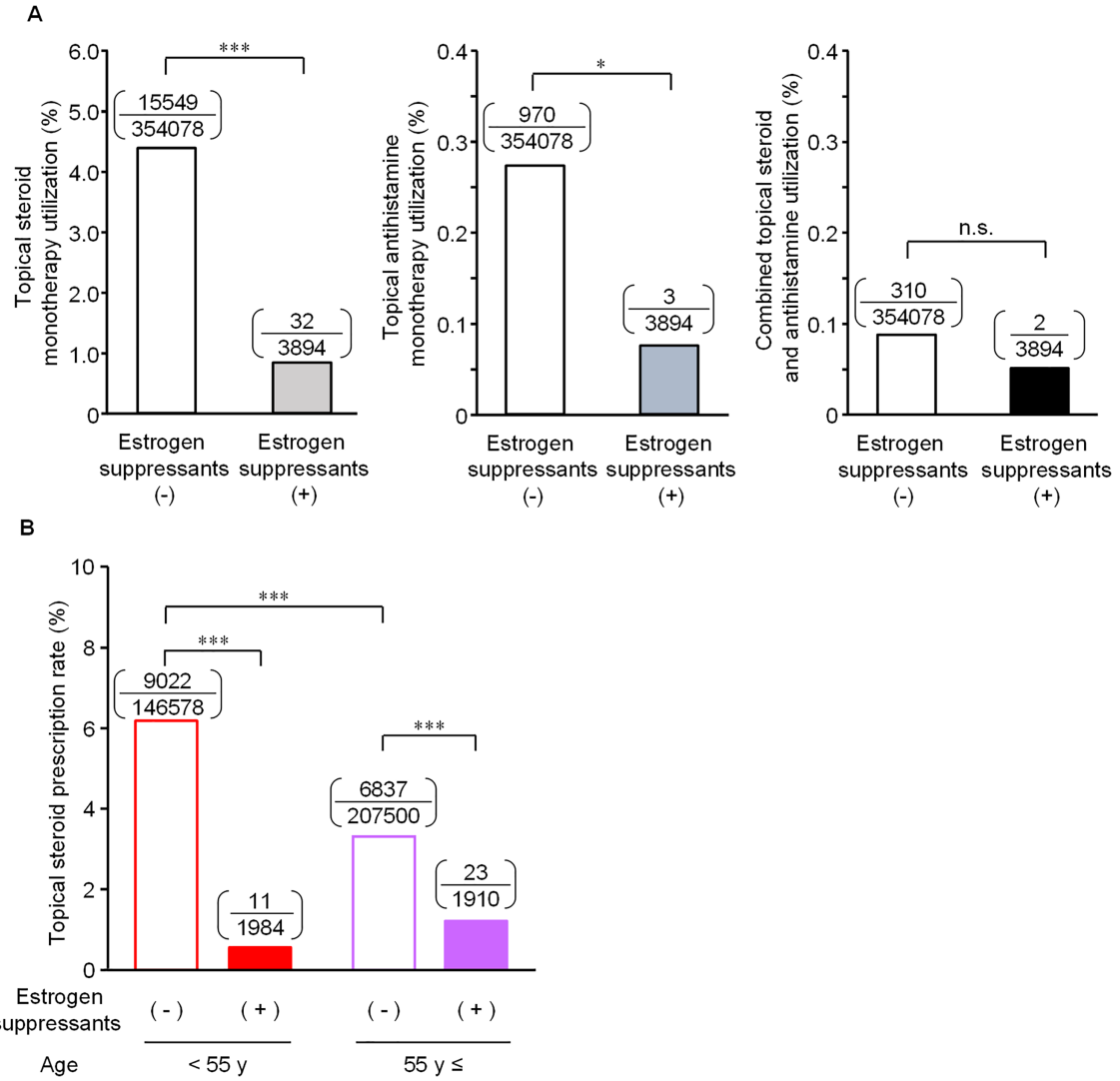


Fig. 4. Prescription Rates of Topical Steroids and Topical Antihistamines in the Users and Non-users of Estrogen Suppressants at Different Ages

(A) Prescription rates of topical steroids (left), topical antihistamines (center) or their combination (right) in the users and non-users of estrogen suppressants among female patients aged 18 years or older. (B) Topical steroid prescription rates in women 55+ years and under 55 years with or without the use of estrogen suppressants. The parenthesis in the top of each column shows [sample size (N)]/[population size (N)]. Statistical comparisons between 2 groups were performed using Fisher's exact test, while comparisons between 4 groups were conducted by Fisher's exact test, followed by Holm's multiple comparison test. *$p<0.05$, ***$p<0.001$.

Table 1. Patient Characteristics in Users and Non-users of Estrogen Suppressants before and after Propensity Score Matching

| Factor | Before propensity score matching | | | After propensity score matching | | |
|---|---|---|---|---|---|---|
| | Estrogen suppressants | | | Estrogen suppressants | | |
| | (–) | (+) | *p* | (–) | (+) | *p* |
| *n* | 354078 | 3894 | | 1672 | 1672 | |
| Age, median [range] | 60 [18–108] | 54 [18–102] | <0.001 | 59 [18–102] | 59 [18–102] | 1.00 |
| Number of medications other than estrogen suppressants, median [range] | 3 [1–20] | 0 [0–17] | <0.001 | 2 [1–20] | 2 [1–17] | 0.93 |

Statistical comparisons of age and number of prescribed medications other than estrogen suppressants between users and non-users of estrogen suppressants were performed by Mann–Whitney *U* test.

non-users of estrogen suppressants were assessed by univariate and multivariate analyses (Table 2). Univariate analysis using Fisher's exact test indicated significant decreases in prescription rates of topical steroids and 2nd generation $H_1$ inhibitors, and increase in a prescription rate of moisturizers (topical protectant) in the users of estrogen suppressants. Multivariate logistic analysis indicated significant positive or negative association of prescription of topical steroids, moisturizers (topical protectant), 2nd generation $H_1$ inhibitors, and opioid analgesics with the use of estrogen suppressants, showing adjusted odds ratios of 0.40 [95% confidence interval (CI): 0.25–0.63, $p<0.001$], 3.77 [2.79–5.11, $p<0.001$], 0.68 [0.50–0.92, $p=0.01$],

Table 2. Univariate and Multivariate Analyses of Prescription Rates of Various Medications Including Topical Steroids in users and Non-users of Estrogen Suppressants in Population after Propensity Score Matching

| Factor | Estrogen suppressants | | Fisher | Multivariate logistic analysis | |
|---|---|---|---|---|---|
| | (−) | (+) | *p* | Adjusted odds ratio [95% CI] | *p* |
| | Among 1672 | Among 1672 | | | |
| Topical steroids, *n* (%) | 59 (3.5) | 34 (2.0) | **0.01** | **0.40** [0.25–0.63] | **<0.001** |
| Moisturizers (topical protectant), *n* (%) | 65 (3.9) | 194 (11.6) | **<0.001** | **3.77** [2.79–5.11] | **<0.001** |
| Oral steroids, *n* (%) | 33 (2.0) | 21 (1.3) | 0.13 | 0.67 [0.38–1.17] | 0.16 |
| 2nd generation $H_1$ inhibitors, *n* (%) | 116 (6.9) | 81 (4.8) | **0.01** | **0.68** [0.50–0.92] | **0.01** |
| Neuropathy medications, *n* (%) | 64 (3.8) | 53 (3.2) | 0.35 | 0.76 [0.52–1.12] | 0.16 |
| Oral NASIDs, *n* (%) | 145 (8.7) | 155 (9.3) | 0.59 | 1.06 [0.83–1.35] | 0.66 |
| Acetaminophen, *n* (%) | 49 (2.9) | 51 (3.1) | 0.92 | 0.98 [0.65–1.48] | 0.92 |
| Opioid analgesic, *n* (%) | 29 (1.7) | 46 (2.8) | 0.06 | **1.83** [1.11–2.99] | **0.02** |
| Anti-depressants, anti-anxiety drugs, sleeping drugs, *n* (%) | 176 (10.5) | 167 (10.0) | 0.65 | 0.94 [0.75–1.18] | 0.59 |
| Anti-diabetes medications, *n* (%) | 69 (4.1) | 84 (5.0) | 0.25 | 1.31 [0.93–1.85] | 0.13 |
| Statins, *n* (%) | 188 (11.2) | 176 (10.5) | 0.54 | 0.92 [0.72–1.17] | 0.48 |
| Anti-hypertensive drugs, *n* (%) | 292 (17.5) | 281 (16.8) | 0.65 | 0.97 [0.80–1.19] | 0.80 |

Statistical comparisons of prescription rates of various drugs between users and non-users of estrogen suppressants were performed by univariate analysis using Fisher's exact test and by multivariate logistic regression analysis. CI: confidence interval.

and 1.83 [1.11–2.99, $p=0.02$], respectively (Table 2). Thus, topical steroids were less frequently prescribed in users than in non-users of estrogen suppressants, while moisturizers were more frequently prescribed in the users.

**Comparison of Prescription Rates of Topical Steroids or Moisturizers between Female Patients Receiving Anticancer Drugs and Those Receiving No Such Treatment** To test the relationship of the use of topical steroids or moisturizers with cancer or cancer-related treatment strategies, we compared their prescription rates in female patients receiving anticancer drugs and those receiving no such drugs. Interestingly, the prescription rate of moisturizers was significantly higher in females receiving anticancer drugs than in those receiving no such treatment, while the prescription rate of topical steroids was not different between the two groups (Supplementary Table S2).

**Subgroup Analysis of the Association of Prescription Rates of Various Medications Including Topical Steroids with the Use of Estrogen Suppressants in Female Patients Aged 55+** Information about prescriptions of injections of GnRH receptor desensitizers for hormone therapy of premenopausal breast cancer, which are generally filled at the hospital pharmacy, cannot be obtained from pharmacy claims data of external pharmacies. Considering this undeniable bias, we conducted subgroup analysis in female patients aged 55+ years, because injections of GnRH receptor desensitizers are used for treatment of premenopausal, but not postmenopausal, breast cancer. Multivariate logistic regression analysis after propensity score matching in women aged 55+ years indicated significant negative association of topical steroid prescription and significant positive association of moisturizers and opioid analgesics with the use of estrogen suppressants, showing the adjusted odds ratio of 0.48 [0.27–0.85, $p=0.01$], 2.76 [1.89–4.03, $p<0.001$], and 3.05 [1.65–5.61, $p<0.001$] (Supplementary Table S3). Thus, decreased frequency of topical steroid prescription was associated with the use of estrogen suppressants in postmenopausal women who never undergo hormone therapy using GnRH receptor desensitizers for treatment of breast cancer.

## DISCUSSION

Our previous study has demonstrated that sulfide-induced functional upregulation of $Ca_v3.2$ T-type $Ca^{2+}$ channels expressed in nociceptor neurons promote somatic and visceral pain, which are blocked by KTtp38, the novel T-type $Ca^{2+}$ channel inhibitor.[26] In the present fundamental study using mice, we demonstrated that intradermal administration of sulfide, an enhancer of $Ca_v3.2$ T-type $Ca^{2+}$ channels, caused prompt $Ca_v3.2$-dependent itch responses in addition to pain responses, and that pharmacological inhibition of T-type $Ca^{2+}$ channels by KTtp38 reduced itch responses following stimulation of $H_1$ receptors with histamine and of MRGPRA3 with chloroquine, known to be expressed in itch-sensing neurons, but not nociceptors. Thus, it is considered that KTtp38 reduces pain and itch by inhibiting T-type $Ca^{2+}$ channels expressed in nociceptors and itch-sensing neurons, respectively. It is to be noted that KTtp38 had no effect on the general behavior or motor functions, as assessed by the rota-rod test in our previous study.[26] Considering the evidence that $Ca_v3.2$-positive primary neurons project to mouse and human skin,[31] our data suggest a key role of $Ca_v3.2$ in excitation of itch-sensing neurons (Fig. 5). Furthermore, our animal study also showed that ovariectomy and/or inhibitors of estrogen generation reduced the $Ca_v3.2$-dependent itch responses by sulfide, an effect restored by estrogen supplementation. These findings suggest the pro-pruritic role of estrogen in mice, which is in agreement with previous reports using a histamine-induced acute itch model in female rats[24] and a toluene-2,4-diisocyanate-induced allergic dermatitis-related pruritus model in male mice,[32] but inconsistent with the report using acute and chronic itch models in mice.[25] Although the mechanisms by which estrogen controls itch sensitivity are still largely open to question, there is evidence that estrogen causes functional

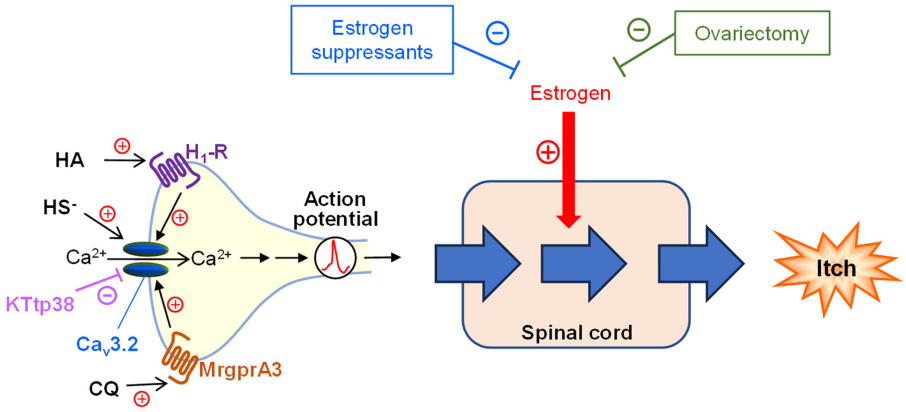


Fig. 5. Hypothetical Scheme for the Involvement of $Ca_V3.2$ T-Type $Ca^{2+}$ Channels and the Role of Estrogen in the Itch Transmission Pathway

In the peripheral terminals of itch-sensing primary afferent nerves, $Ca_V3.2$ mediates neuronal excitation in response to sulfide ($HS^-$), a $Ca_V3.2$ enhancer, and following stimulation of $H_1$ receptors ($H_1$-R) with histamine (HA) and of MRGPRA3 with chloroquine (CQ). KTtp38, a T-type $Ca^{2+}$ channel inhibitor, is considered to suppress $Ca_V3.2$-dependent excitation of itch-sensing neurons. Estrogen is considered to promote itch transmission at the spinal level, according to the previous report.[24] The estrogen-dependent acceleration of the spinal itch transmission pathway might be reduced by estrogen suppressants.

upregulation of GRP-expressing interneurons in the spinal itch transmission pathway.[24] Therefore, we speculate that estrogen regulation of sulfide-induced itch responses observed in this study may be due to acceleration of itch transmission at the spinal levels (Fig. 5). In the clinical cross-sectional analysis of pharmacy claims data that we conducted to get a clue to clarify the role of estrogen in human pruritus, decreased prescription rates of topical steroids used for treatment of pruritus and/or dermatitis were associated with age of 55 years and over, that is, the postmenopausal generation, and with the use of estrogen suppressants (Fig. 4). Furthermore, multivariate logistic regression analysis of population after propensity score matching indicated negative and positive association of prescription of topical steroids and moisturizers (topical protectants), respectively, with the use of estrogen suppressants (Table 2), which reminded us that endogenous estrogen might promote itch sensitivity and/or dermatitis, but help with skin hydration in humans. The increased prescription of moisturizers in the user of estrogen suppressants is understandable, considering previous reports showing that estrogen decline leads to a decrease in the skin's water retention capacity and collagen content, thereby suggesting the role of estrogen in maintaining the structure of the skin.[33–36] It thus appears that estrogen might play a dual role in the skin, that is, maintaining healthy skin and promoting itch sensation and/or dermatitis, in humans. Together, our fundamental and clinical cross-sectional studies provide apparently consistent evidence for a pro-pruritus role of estrogen in mice and humans.

It is to be noted that the cross-sectional analysis of the pharmacy claims data in this study does not directly demonstrate a causal relationship between estrogen suppression and reduced pruritus susceptibility. Since many estrogen suppressants are used for treatment of breast cancer, the cancer itself or concomitant therapies including cytotoxic chemotherapy might worsen the skin condition, leading to the increased use of moisturizers. Furthermore, we cannot rule out the possibility that the observed negative association between prescription of topical steroids and the use of estrogen suppressants might be an artifact of different treatment strategies for cancer patients rather than the effect of estrogen suppressants. Nevertheless, our data (Supplementary Table S2) showed the increased use of moisturizers, but no change in the prescription rate of topical steroids, in female patients receiving anticancer drugs, compared with those receiving no such treatment. These findings suggest possibilities that moisturizers might be used to prevent or treat skin damage due to cancer or cancer-related therapies, as mentioned above, and that the use of topical steroids might not be, in general, affected by cancers or related therapies.

In this study, we could not know the information about the use of GnRH receptor desensitizer injection formulations, such as leuprorelin and goserelin, prescribed in hospital pharmacies in Japan. In general, GnRH receptor desensitizers are used for premenopausal women, but not for postmenopausal women who would receive oral aromatase inhibitors. Therefore, we assume that the large control group (354078 patients who did not receive estrogen suppressants) would include some patients who were prescribed with GnRH receptor desensitizer injection formulations in hospital pharmacies (Table 1), leading to a bias. Nonetheless, the multivariate analysis of the subgroup of females aged 55 years and over, that is, postmenopausal women, who never received GnRH receptor desensitizers, still showed significant negative and positive associations of estrogen suppressant prescription with the use of topical steroids and moisturizers, respectively (Supplementary Table S3).

It is not possible to differentiate itch (symptom) from dermatitis (inflammation) as the therapeutic purposes of topical steroid preparations. Therefore, the notion that estrogen promotes "itch" in humans based solely on topical steroid prescription might be an overinterpretation, but supported, to a certain extent, by our findings that the prescription rate of topical antihistamines, which might be often used for treatment of pruritus rather than dermatitis, was also lower in the users than in non-users of estrogen suppressants (Fig. 4A). The history of diseases including diabetes and atopic dermatitis is not available in pharmacy claims data. Diabetes could be associated with skin infection or damage, whereas the

multivariate logistic regression analysis indicated significant association of decreased and increased prescription rates of topical steroids and moisturizers, respectively, with the use of estrogen suppressants, which would be independent of the prescription of anti-diabetes medications (Table 2). There is evidence that estrogen enhances Th2 immunity and regulatory T cell activity, and reduces skin barrier impairment.[37] Therefore, it is also likely that estrogen suppressants modulated dermatitis itself and/or promoted skin barrier damage, followed by changes in prescription rates of topical steroids and/or moisturizers, which could be confounding bias.

The relationship between estrogen and pain in the present clinical cross-sectional analysis is controversial, because the users of estrogen suppressants had significantly increased prescription of opioid analgesics, but not neuropathy medications (Table 2). The same tendency was also detected in the subgroup analyses in female patients aged 55 years and older (Supplementary Table S3). Aromatase inhibitors that occupied the majority of estrogen suppressants prescribed to women 55+ years (Supplementary Fig. S4) are used for treatment of postmenopausal breast cancer. Therefore, the increased prescription of opioid analgesics is considered to reflect the increased proportion of cancer patients in the users of estrogen suppressants, compared with the non-users. Our cross-sectional analysis of pharmacy claims data also detected significantly decreased prescription of 2nd generation $H_1$ inhibitors in the users of estrogen suppressants (Table 2).

There are several limitations of the clinical cross-sectional analyses of pharmacy claims data in the present study. Pharmacy claims data obtained from nationwide chain pharmacy branches in Japan include information about each patient's prescription, gender, and age, but not diagnosis. Therefore, we had to analyze prescription patterns, for example, prescription of estrogen suppressants, topical steroids, moisturizers (skin protectants), and neuropathy medications or opioid analgesics, as surrogate indicators of decreased estrogen, pruritus/dermatitis, dehydrated skin, neuropathic, or cancer pain, respectively. In the analyses of pharmacy claims data, it is not possible to demonstrate the causal relationship between the use of estrogen suppressants and the prescription of topical steroids, and to clarify the therapeutic purposes of topical steroids, that is, for treatment of itch, dermatitis, or both. There was no information available about prescriptions of GnRH receptor desensitizer injections for treatment of premenopausal breast cancer, which are generally filled at the hospital pharmacy, but not at external pharmacies. Patient's information available for confounder adjustment was age and the number of prescribed medications other than estrogen suppressants. Considering these limitations and their retrospective nature, the present findings obtained from pharmacy receipt data analysis need to be ascertained by prospective studies, in order to clarify causal relationships.

In summary, the present fundamental and clinical cross-sectional studies suggest the possible involvement of $Ca_v3.2$ T-type $Ca^{2+}$ channels in the itch transmission pathway and a promotive role of endogenous estrogen in pruritis or dermatitis in mice and/or humans. The ultimate goal of our study is to clarify whether estrogen plays a role in itch sensitivity in humans, as does in laboratory animals. A prospective clinical study is necessary to ascertain the present evidence from a retrospective study using pharmacy claims data.

## DECLARATIONS

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** This article contains supplementary materials.

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