Estrogen alleviates acute and chronic itch in mice

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Abstract. Itching is associated with various skin diseases, including atopic dermatitis and allergic dermatitis, and leads to repeated scratching behavior and unpleasant sensation. Although clinical and laboratory research data have shown that estrogen is involved in regulating itch, the molecular and cellular basis of estrogen in itch sensation remains elusive. In the present study, it was found that estrogen-treated mice exhibited reduced scratching bouts when challenged with histamine, chloroquine, the proteinase-activated receptor-2 activating peptide SLIGRL-NH2 (SLIGRL), compound 48/80, and 5-hydroxytryptamine when compared with mice in the placebo group. Moreover, estrogen also suppressed scratching bouts in the mouse model of chronic itch induced by acetone-ether-water treatment. Notably, consistent with the behavioral tests, the present RNA-seq analysis showed that estrogen treatment caused significantly reduced expression levels of itch-related molecules such as Mas-related G-protein coupled receptor member A3, neuromedin B and natriuretic polypeptide b. In addition, estradiol attenuated histamine-induced and chloroquine-induced calcium influx in dorsal root ganglion neurons. Collectively, the data of the present study suggested that estrogen modulates the expression of itch-related molecules and suppresses both acute and chronic itch in mice.

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Introduction

Itching is 'an unpleasant sensation that evokes a desire to scratch' (1). ~10-20% of individuals will cope with itching in their lifetime, which seriously affects the patients' quality of life, mood, and sleep (2-4). According to a survey of global disease, the economic burden caused by chronic pruritus ranks it among the top 50 most serious diseases (5). However, the itch has been severely underestimated in the past several decades, resulting in vague mechanisms of itch development. Without effective drugs in clinical application, pruritus has become an urgent clinical problem to be solved.

Women have unique hormonal changes over time, which may be correlated with the periodic changes in the elemental composition of the skin throughout the age range. Notably, it has been also reported that the incidence of pruritus increases in postmenopausal women (6), and up to 55% suffer from pruritus and dryness associated with chronic skin diseases including psoriasis, dermatitis and infection, causing severe discomfort (7,8). Changes in estrogen levels may affect the hydration, collagen content and glycosaminoglycan concentration in the skin (9,10), and the number and types of estrogen receptors expressed by epithelial keratinocytes (11). Notably, it was shown that estrogen receptors are also widely expressed in the dorsal root ganglion (DRG) and superficial dorsal horn (12,13). The application of estrogen causes changes in intracellular Ca^{2+} concentrations in DRG cells of sensory neurons by binding to receptors and is involved in the transduction of nociception (14). However, whether and how estrogen is involved in developing chronic itch in females still needs to be understood in an improved way.

Unmyelinated small-diameter C fibres are critically involved in the generation of itch sensation (15-17). Among them, three pruriceptive small-diameter DRG neuron subtypes have been identified by both functional studies and RNA-sequencing (RNA-seq) analysis. NP1 neurons are classified by the expression of Mas-related G-protein coupled receptor (Mrgpr)D, NP2 neurons are classified by the expression of MrgprA3 and MrgprC11, and NP3 neurons are rich in other unique markers, including the neuropeptides natriuretic polypeptide b (Nppb), neurotensin and somatostatin. To further characterize

the relationship between estrogen and the expression of those itch-related genes, RNA-seq analysis was employed and the key components in hormonal change-related itch sensation development were determined.

In summary, it was aimed to decipher the function of estrogen in acute and chronic itch. In a behavioural study, estrogen application rescued the increased scratching bouts in ovariectomized mice in both acute and chronic itch models. RNA-seq analysis indicated that loss of estrogen expression in the ovariectomized mice resulted in significantly increased levels of itch signalling molecules, and this effect could be reversed by the implantation of estrogen sustained-release tablets. The present study uncovered the critical role of estrogen in itch modulation and provided a reliable theoretical basis for developing safe and effective drugs for treating pruritus.

Materials and methods

Animals. A total of 50 healthy C57BL/6J female mice (9~10 weeks-old; weight, 20~25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were housed in an SPF environment, maintained at room temperature at 23~25˚C with a diurnal 12 h rhythm, and had free access to water and food. Experiments were performed one week after adaptive feeding.

All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all experiments complied with the Animal Research: Reporting *in vivo* Experiments (ARRIVE) guidelines. The present study was approved (approval no. ZX201898) by the Animal Experimental Ethics Committee of the Plastic Surgery Hospital, Chinese Academy of Medical Sciences (Beijing, China).

Experimental design. The aim of the present experiment was to investigate the effect of estrogen on pruritus at a slightly higher than physiological level. Embedding estrogen sustained-release tablets can result in a relatively stable estrogen level in mice. By observing the state of the mice, measuring the mouse body weight (Fig. S1), detecting the activity of the mice with the rotarod test (Fig. S2), and synthesizing the previous research, it was considered that this dose of estrogen will not affect the health of the mice. The mice exhibited favorable recovery, and their health status was also favorable. To achieve the purpose of the experiment, an appropriate dose of estrogen sustained-release tablets should be selected. According to previous studies (11,18), three doses of estrogen sustained-release tablets were used in the preliminary experiment: 0.36, 0.1 and 0.01 mg per tablet. The levels of estrogen in the body were 402±86.39, 109±2.89, and 24.64±12.10 pg/l, respectively. Finally, 0.1 mg was selected as the experimental dose.

Estrogen replacement

Ovariectomy via the dorsal route bilaterally. Anesthesia was induced with 80 mg/kg ketamine plus 8 mg/kg xylazine (19). The hair was removed, and a smooth incision ~1 cm long was made in the skin. The skin and subcutaneous tissue were separated. The color of the ovary is slightly lighter than that of the surrounding muscle. An incision of ~0.5 cm was made in that location, which exposed the adipose tissue surrounding the ovary and the uterine horn closely connected to the ovary. Curved forceps were used to gently clamp the adipose tissue to pull it away from the wound. Then, the fallopian tube was ligated at the upper and lower uterine horns, the uterine horn was cut off, and the ovary was removed and dissected (20). Then, estrogen sustained-release tablets or placebo tablets were implanted into the ovary position simultaneously. The adipose tissue was put back into its original position, and 7.5 mg/kg Rimadyl was diluted in saline injection subcutaneously before the mouse woke up (21,22). The subcutaneous tissue and skin were sutured, and the sutured wound surface was disinfected with iodophor. After surgery, the mice were placed in a cage at 30˚C separately. A total of 80% of the mice could wake up within 1-2 h, the remaining other mice could wake up within 3 h at the latest, and the wounds could heal in 3 days. Absorbable sutures were used for intradermal sutures, and the sutures of the skin fell off within 4-6 days as the mice moved, and new hair gradually grew at the sutures. No abnormalities were observed after one week. The cage was kept dry and the wounds were checked every day.

Slow-release pellet of 17β-estradiol. Based on our preliminary test results and previous studies (11,18), a slow-release pellet of 17β-estradiol (0.1 mg/pellet, 60-day release, Innovative Research of America) or the placebo was placed back in the ovary position, respectively. These are tablets with a diameter of 3 mm and a release cycle of 60 days, or placebo tablets of the same texture and size. The pellets produce a slow release of the active drug over 60 days (1.7 μg per day) and have been shown to maintain estrogen plasma levels of ~125 pg/ml for the duration of the pellet. The results of the plasma estradiol concentrations are shown in Fig. S3.

Itch behavioral tests

Acute itch behavioral tests. The acute itch behavior tests were performed similarly to a previous study (23). Intradermal injection with different pruritogens was used to evaluate the acute itch behavior. Pruritogens included 200 μg histamine, 100 μg chloroquine (CQ), 50 μg compound 48/80, 50 nmol 5-hydroxytryptamine (5-HT) and 100 nmol SLIGRL-NH2 dissolved in phosphate buffered saline, respectively. The pruritogens doses used in the acute itch experiments were examined in the preliminary experiments, and the data are demonstrated in Fig. S4.

Protocols of estrogen effect on acute itch. A total of 50 mice were used in the acute itch tests, which included five experiments. In every experiment, 10 littermate mice were randomly assigned to 2 groups, namely the estrogen group (E group, n=5) and placebo group (P group, n=5); the E group was implanted with estrogen sustained-release tablets, and the P group was implanted with the placebo tablets. The acute behavioural tests were completed within one week.

Chronic itch behavioral tests. The acetone/ether/water (AEW) model was established as the chronic itch model (24,25). The mice were shaved (2x2 cm) to expose the skin, and the exposed skin was covered with acetone-ether mixed liquid (AE)-soaked cotton for 15 sec, followed by water-soaked cotton for 30 sec twice a day at 9 am and 5 pm for seven days. The skin was observed and images were captured to assess the dry-skin-evoked changes, including excoriation and dry

scales. Under sterile conditions, the skin of each group was collected, H&E staining was performed at room temperature (stain with haematoxylin 5 min and stain with eosin 1 min), the samples were observed under a light microscope (DM3000; Leica Microsystems), and images were recorded. The relative thickness of the epidermis was analyzed by Image-Pro Plus 6.0 (Media Cybernetics).

Protocols of estrogen effect on chronic itch. A total of 10 days after the ovariectomy and implanted surgery, 16 littermate mice were randomized into two groups, the E group (n=8) and the P group (n=8); the grouping method was the same as the acute itch behavioural test. The AEW chronic itch model was completed within ten days when the chronic itch model was successfully established. A total of six mice in each group were used for the RNA-seq analysis, and two mice in each group were subjected to H&E staining.

How animal count was decided. Behavioral experiments were performed by recording the number of bouts of scratching. The hair on the nape of the neck was shaved three days prior to the itch behavior test. The mice were placed in behavior chambers for 30 min for acclimation before the acute or chronic itch behavior test. A scratching bout is defined as follows (26): The mice raise the hind leg towards the treated sites (chemical-injected site in acute itch and AEW-treated site in chronic itch), scratching once or continuously scratching for several times and then put down the leg. This series of movements was counted as one scratching bout. Scratching towards other parts is omitted.

Microarray analyses of the DRG. A total of 15 days after ovariectomy and estrogen replacement surgery, DRG neurons on both sides of the lower cervical and upper thoracic segments (C4~T2) of the mice were chosen. According to the manufacturer's instructions, total RNA was extracted from the mice using the RNeasy Mini Kit (cat. no. 74104; Qiagen) and RNA-sequencing was analyzed by Novogene Co., Ltd. FeatureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene and then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis of two conditions was performed using the DESeq2 package (1.16.1). The P-values were adjusted using the Benjamini & Hochberg method (27). All differentially expressed genes were determined by Padj <0.05.

Calcium imaging test

Isolation and preparation of DRG cells. Nerve fibers were cut off, and the DRG was minced and placed in calcium-free and magnesium-free (Hanks' balanced salt solution; HBSS) buffer followed by 5 ml of digestive enzymes, which contained 0.5 mg/ml trypsin (Type III; Thermo Fisher Scientific, Inc.), 1 mg/ml collagenase (Type IA, Sigma-Aldrich, USA.) and 0.1 mg/ml DNase (Type III, Sigma-Aldrich, USA.), and placed in a 37˚C incubator for rough stirring at a speed of 10~11 rpm for no more than 30 min. Then, the samples were centrifuged at 4˚C (60 x g; 2 min), and the digestive enzymes were aspirated. Next, 1 ml NBM culture medium was added, the samples were gently stirred and aspirated and allowed to stand for 1~2 min, and the supernatant was aspirated and placed into a 15 ml centrifuge tube. The samples were repeatedly stirred and aspirated three times, after which the precipitate was discarded. The samples were centrifuged at 800 rpm for 4 min, and the supernatant was discarded. NBM culture medium was added, the samples were stirred evenly and aspirated, the dispersed cells were added to the cell climbing sheet coated with poly-lysine, and the samples were precipitated in incubators at 37˚C and 5% CO_2 for 1 h. Another 2 ml of NBM culture medium was added to the culture dish, and the samples were cultured overnight at 37˚C in a 5% CO_2 incubator. The cells were divided into a control group (C group) and an estrogen group (E group), and 100 nM β-estradiol was added to the estrogen group.

Calcium imaging. Before calcium imaging experiments, cultured DRG neurons were loaded with 4 μM Fura-2 AM (Life Technologies) for 60 min at 37˚C. Cells were washed three times and placed in HBSS for 30 min at room temperature. During the experiment, fluorescence at 340 and 380 nm excitation was imaged using an inverted Nikon Ti-E microscope controlled by NIS-elements imaging software (Naikon Instruments, Inc.). The Fura-2 ratio (F340/F380) was used to reflect the change in $[Ca^{2+}]_i$ after stimulation, and the activation threshold was defined as 3 SD above the mean (~20% above baseline).

Plasma estradiol concentrations. Plasma estradiol concentrations were tested by Radioimmunoassay by the Beijing North Institute of Biotechnology company. Mice in the E and P group were enucleated bilaterally under sevoflurane anesthesia, blood was instilled into an EP tube, allowed to stand at room temperature for more than 1 h, centrifuged speed at x1,900 x g for 10 min, and serum was removed and rapidly frozen at -70˚C. This kit uses competitive radioimmunoassay to perform competitive binding reactions between radiolabeled antigen and non-labeled antigen to be tested and a limited amount of specific antibody simultaneously. By separating unbound labeled antigens, the radioactivity count of the labeled antigen-antibody complex is determined. The content of the substance to be exmined in the sample is calculated using the standard curve and the mathematical model of RIA (28). Plasma estradiol concentrations were tested twice on 10th day after implanted surgery and when all the experiments were finished.

Euthanasia. After mice were anaesthetized with 7-8% sevoflurane by a delivery machine at an airflow rate of 1 l/min for 5-7 min when the experiments were completed, reflexes disappeared, respiratory arrest and pupil dilation occurred, and the mice were euthanized by cervical dislocation.

Statistical methods. Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software, Inc.). All data are expressed as the mean ± SEM. Unpaired two-tailed Student's t-test or one-way analysis of variance followed by Dunnett's was used to evaluate differences. P<0.05 was considered to indicate a statistically significant difference for all tests.

Results

Estrogen mitigates chemical-induced acute itch sensation in mice. The ovariectomy was first performed and tablets were implanted, either the estrogen sustained-release or placebo

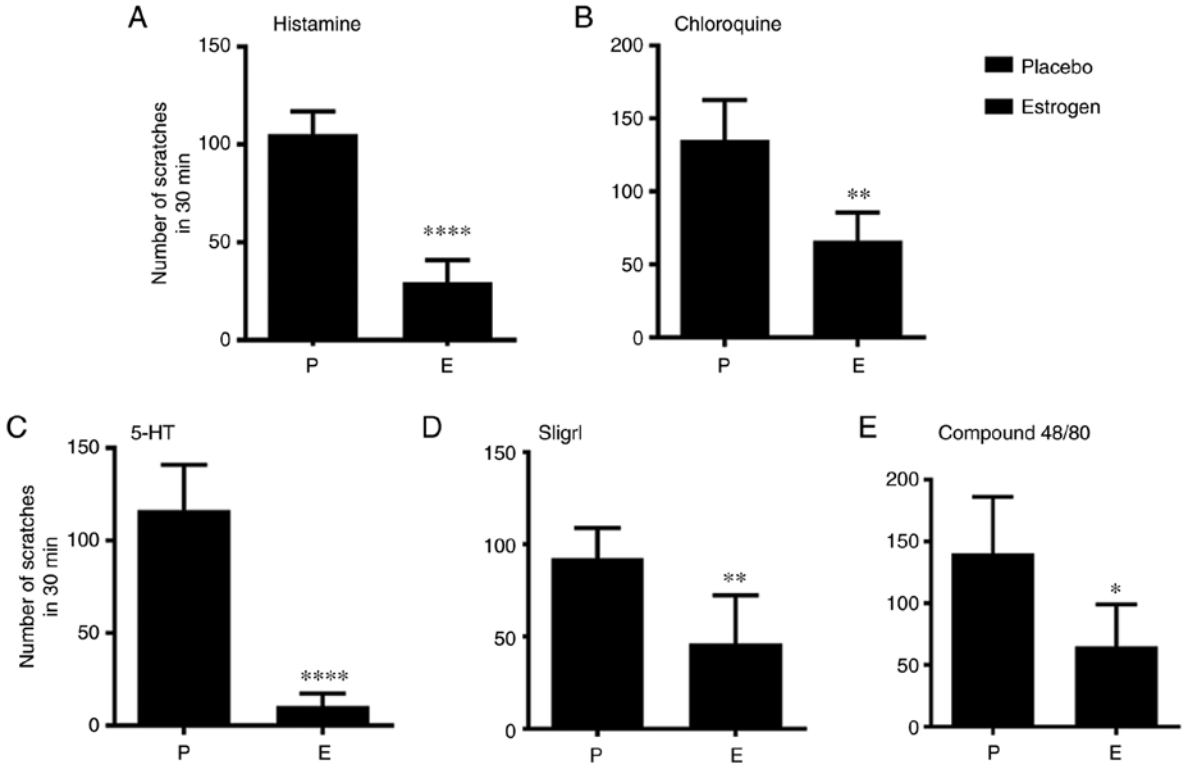

Figure 1. Estrogen mitigates chemical-induced acute itch sensation in mice. (A) Histamine-induced scratching behavior was inhibited in E group (200 μg/50 μl). (B) Chloroquine-evoked scratching behavior was reduced in E group (100 μg/50 μl). (C) Scratching behavior evoked by 5-HT was significantly reduced in E group (50 nmol/50 μl). (D) The Par-2 agonist sligrl-evoked scratching behavior was reduced in E group (100 nmol/50 μl). (E) Compound 48/80-evoked scratching behavior was inhibited in E group (50 μg/50 μl). n=5 mice for each group. *P<0.05, **P<0.01 and ****P<0.001. Two-tailed unpaired Student's t-test. Error bars represent the SEM. 5-HT, 5-hydroxytryptamine; E, estrogen; P, placebo.

pallets. Then, the acute itch model was established by intradermal injection of histamine, CQ, SLIGRL, compound 48/80 and 5-HT in the P group and E group. The number of scratches was counted by individuals unaware of the grouping.

Estrogen treatment significantly reduced the number of scratches in mice injected with histamine (P<0.05) (Fig. 1A), CQ (P<0.01) (Fig. 1B), 5-HT (P<0.01) (Fig. 1C), SLIGRL (P<0.05) (Fig. 1D) and compound 48/80 (P<0.01) (Fig. 1E), suggesting that estrogen treatment mitigates chemical-induced acute itch sensation.

Estrogen attenuates chronic itch in a mouse model of AEW. Next, the effects of estrogen on scratching behavior in the AEW mouse model were evaluated; a schematic experimental protocol is presented in Fig. 2A. Notably, the number of scratches was reduced in the estrogen treatment group compared with the placebo group over the 7-day period. (P<0.001); (Fig. 2B). Moreover, AEW-induced skin hyperproliferation was also reduced, as the thickness of the epidermal layers was significantly thinner in the E group than that in the P group (Fig. 2C and E) and photo displaying the area of treatment in the cervical back of mice, hyperplasia and dryness is milder in the estrogen group than in the placebo group (Fig. 2D).

Estrogen suppresses itch-related gene expression in the DRG. To exclude the endogenous expression of estrogen, the ovariectomy was first performed and sustained-release tablets filled with either vehicle control or estrogen were implanted in mice. Then, the overall effect of estrogen on gene expression was assessed by RNA-seq analysis in the placebo group (P) and the estrogen group (E). The gene expression pattern of known genes involved in itch sensation was examined. The first group analyzed was GRCR family members. Strikingly, MrgprA3, a well-known pruritogen receptor in both acute and chronic itch, was highly enriched in the P group compared with the E group. The expression of some other Mrgprs with unknown functions, such as MrgprB13, was also reduced in the E group (Fig. 3A).

A total of 25 known genes that are essential to itch sensation were then analyzed. Although the expression of two histamine receptors (Hrh1 and Hrh2) was not affected by estrogen, neuromedin B and Nppb, the neuropeptides required for scratching behaviors induced by histamine, compound 48/80, and 5-HT were significantly reduced in the E group compared with the P group, suggesting that estrogen treatment may be involved in itch signal transduction (Fig. 3C).

Transient Receptor Potential (TRP) channels are a class of cationic channels that act as signal transducers by altering membrane potential or intracellular calcium concentration and function as important initiators in itch sensation. The current data showed that neither TRPA1 nor TRPV1 was affected by estrogen treatment. Notably, TRPC3 expression was significantly upregulated in the E group. However, its role in itch remains to be determined (Fig. 3B).

Numerous other sensory neuro markers were also analyzed but did not provide any informative results (Fig. 3D). In addition, it was found that several neuropeptides,

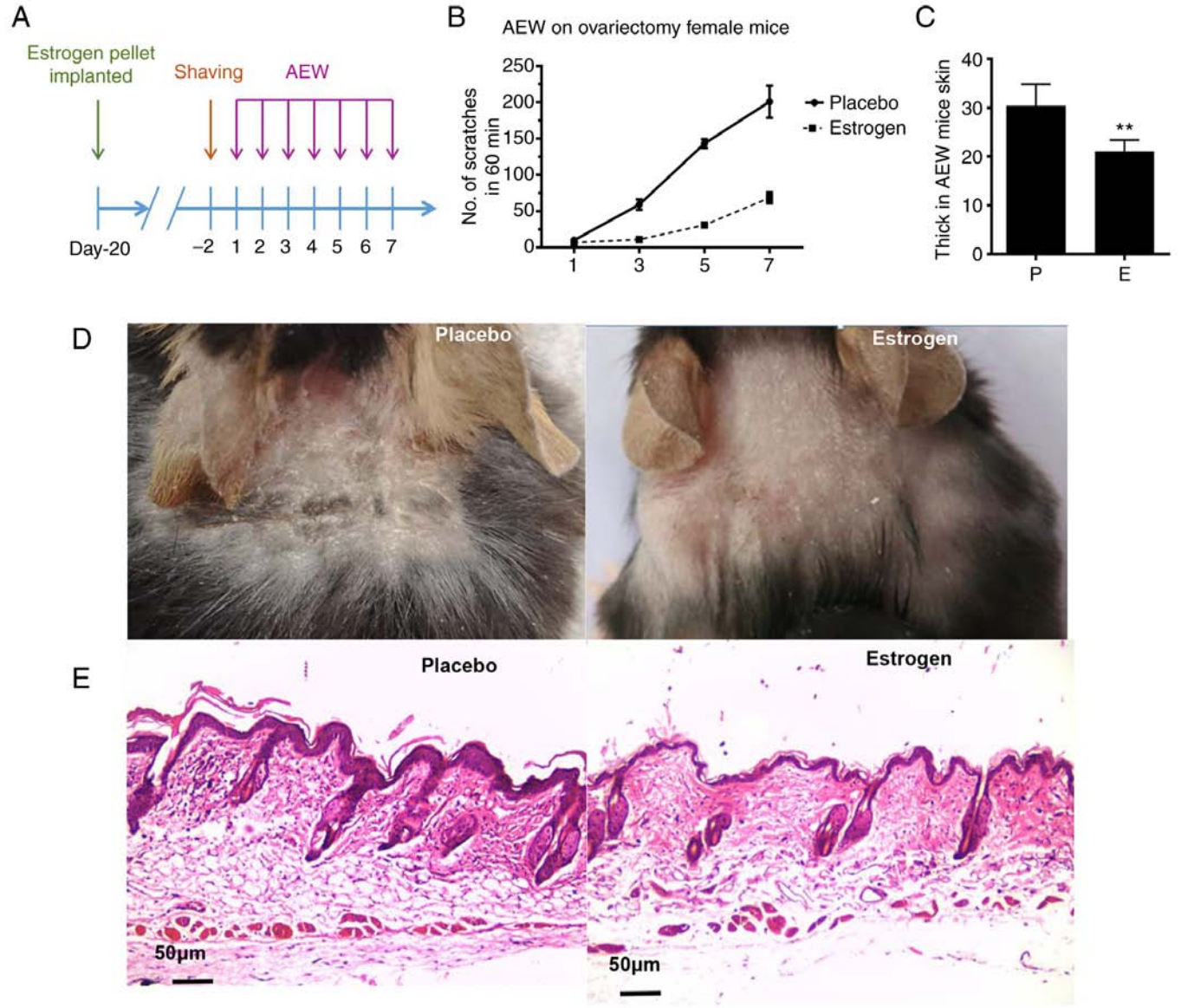

Figure 2. Estrogen attenuates chronic itch in a mouse model of AEW. (A) Schematic diagram for generating a chronic itch mouse model of AEW in E or P group. (B) Number of AEW-induced scratches in the E and P groups. n=6 mice per group, one-way ANOVA. (C) Quantitative analysis of the thickness of the nucleated epidermal layers in the E and P groups. Mean ± SD. **P<0.01. Two-tailed unpaired Student's t-test. (D) Representative images of AEW-treated mice in the E and P groups. (E) H&E staining showing the nucleated epidermal layers of AEW-treated mice from the P group and E group. A total of 16-24 sections from two mice per group. AEW, acetone/ether/water; E, estrogen; P, placebo; H&E, hematoxylin and eosin.

including Calca, TAC1 and Calcb (Fig. 3E), were reduced in the E group, suggesting a potential role of estrogen in the modulation of somatic sensation and neurogenic inflammation (Fig. 3F).

Estradiol attenuates chemical-induced calcium influx in mouse DRG neurons. To elucidate the cellular basis of the inhibitory effect of estrogen on itch sensation, cultured mouse DRG neurons were first incubated in 100 nM β-estradiol overnight. As revealed in Fig. 4, histamine or CQ elicited a significant neuronal Ca^{2+} influx in the vehicle-treated DRG neurons. By marked contrast, 100 nM β-estradiol treatment abolished histamine or CQ-induced calcium influx DRG neurons. Taken together, these data suggested that estrogen treatment could reduce pruritogen-induced DRG neuron activation.

Discussion

The acute chemical itch can be relieved by scratching or antihistamine therapy, but the chronic itch is challenging to cure because the mechanisms are unclear, and the chronic itch is resistant to conventional treatments (e.g., antihistamines) (29,30). Numerous patients suffer from chronic itch, and the development of effective antipruritic drugs is imminent (31). Numerous studies have shown that estrogen is involved in the regulation of pruritus, including clinical research, animal experiments and cellular experiments (8,32), but its specific mechanism remains unknown. In the present study, it was revealed that estrogen could suppress the expression of itch-related receptors and neuropeptides and inhibit pruritogen-induced calcium influx in DRG neurons, contributing to both acute and chronic itch relief in estrogen-treated mice.

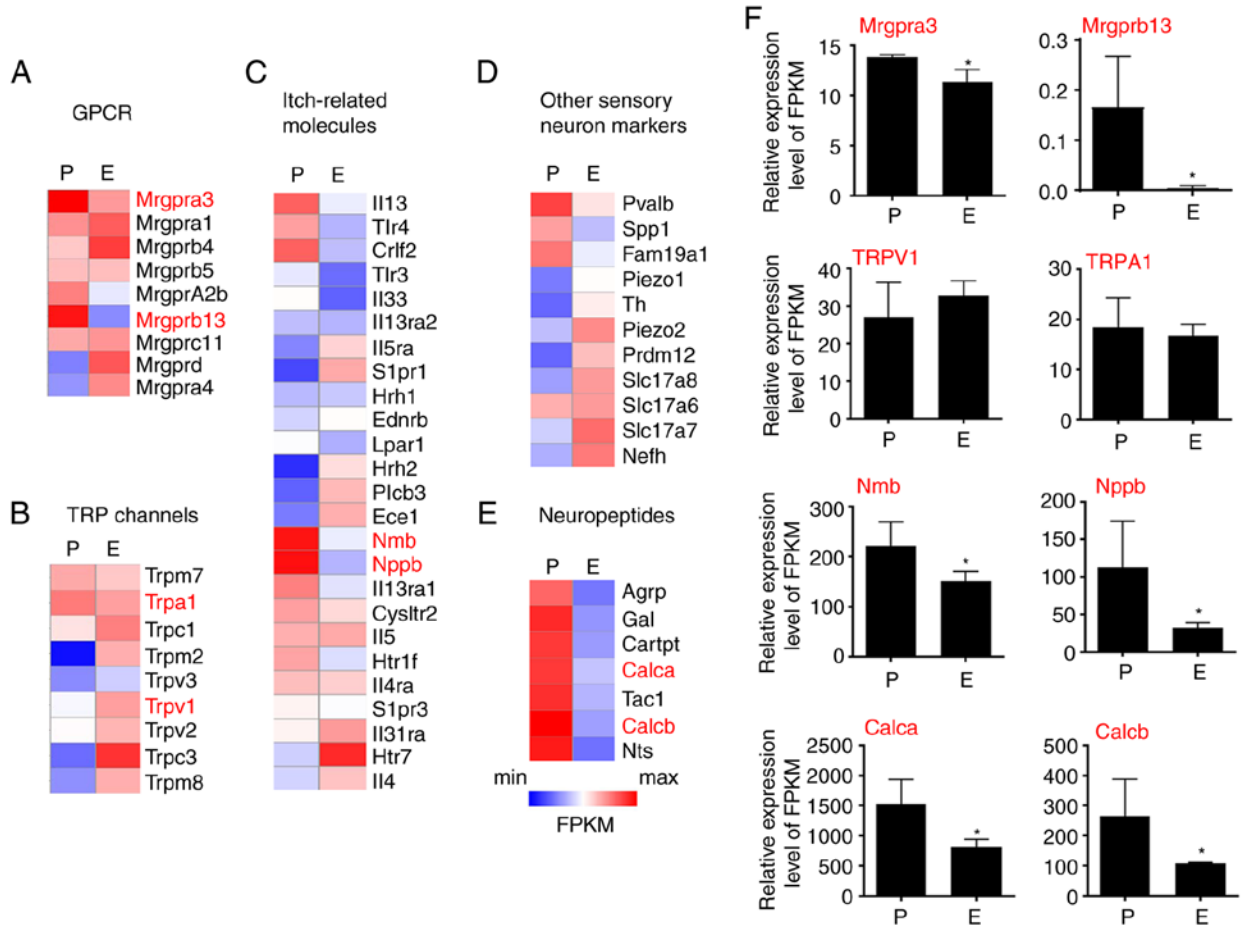

Figure 3. Heatmaps showing the RNA-sequencing analysis of itch-related genes in the dorsal root ganglion between the P and E groups. (A) GRCR family member expression. (B) TRP channels. (C) Itch-related molecules. (D) Other sensory neuron markers. (E) Neuropeptides. (F) The expression changes of eight representative genes relative to nociceptive responses. FPKM: Fragments Per Kilobase Million, n=3 mice for each group. *P<0.05. Two-tailed unpaired Student's t-test. Error bars represent the SEM. TRP, Transient Receptor Potential.

To determine the role of estrogen in modulating the expression of itch-related genes, spinal DRG neurons were isolated for high-throughput sequencing. It was found that high concentrations of estrogen caused a large number of gene expression changes in mice. Among the numerous genes with changes, MrgprA3, but not TRPA1 or TRPV1, displayed significantly different expression levels between the P group and E group, which was consistent with behavioral results. MrgprA3 is a receptor for the antimalarial drug CQ that directly activates DRG neurons, causing site-directed scratching in response to CQ (33). Since multiple itch receptors are highly enriched in MrgprA3-positive neurons, MrgprA3-positive neurons play critical roles in the itch responses induced by at least four different pruritogens, including CQ, histamine, BAM8-22 and SLIGRL-NH2, as well as in AEW-induced chronic itch (34). Although it was shown that estrogen induced upregulation of TRPA1 and TRPV1 in the rat endometrium (35), no change was detected in mouse DRG neurons, suggesting that estrogen may modulate TRP channel expression with regional specificity.

To explore the role of estrogen in itch sensation, ovariectomies were performed in mice and the *in vivo* estrogen concentration was manipulated by embedding β-estradiol sustained-release tablets or a placebo. With this approach, it was surprisingly found that high estrogen levels can reduce pruritic sensation in not only the acute itch model but also the chronic itch model. This result is consistent with numerous clinical observations that ~50% of elderly women develop vulvar pruritus (36), which is closely correlated with the reduction of estrogen levels in serum, and the local application of low-dose estrogen can safely and effectively treat vulvar pruritus (37). A multicenter, randomized, double-blind study of 550 female patients for 12 weeks showed that topical estrogen could also effectively relieve vulvar dryness and reduce pruritic sensation in patients (7).

The DRG is an important site of visceral afferent convergence and cross-sensitization (38,39) and mediates somatic sensation, including pain, touch and itching (40). The present experimental results revealed that estrogen inhibits histamine-induced calcium influx in neuronal cells supplemented with estrogen in calcium imaging buffer. These results indicated that estrogen reduces the response of neuronal cells. Estrogen binding to different receptors in different parts of the body can produce a variety of biological effects (41,42). A large number of studies support that estrogen attenuates nociceptive responses by regulating the intracellular calcium concentration $[Ca^{2+}]_i$ in DRG neurons (43). For example, ATP/capsaicin-induced nociceptive responses can cause

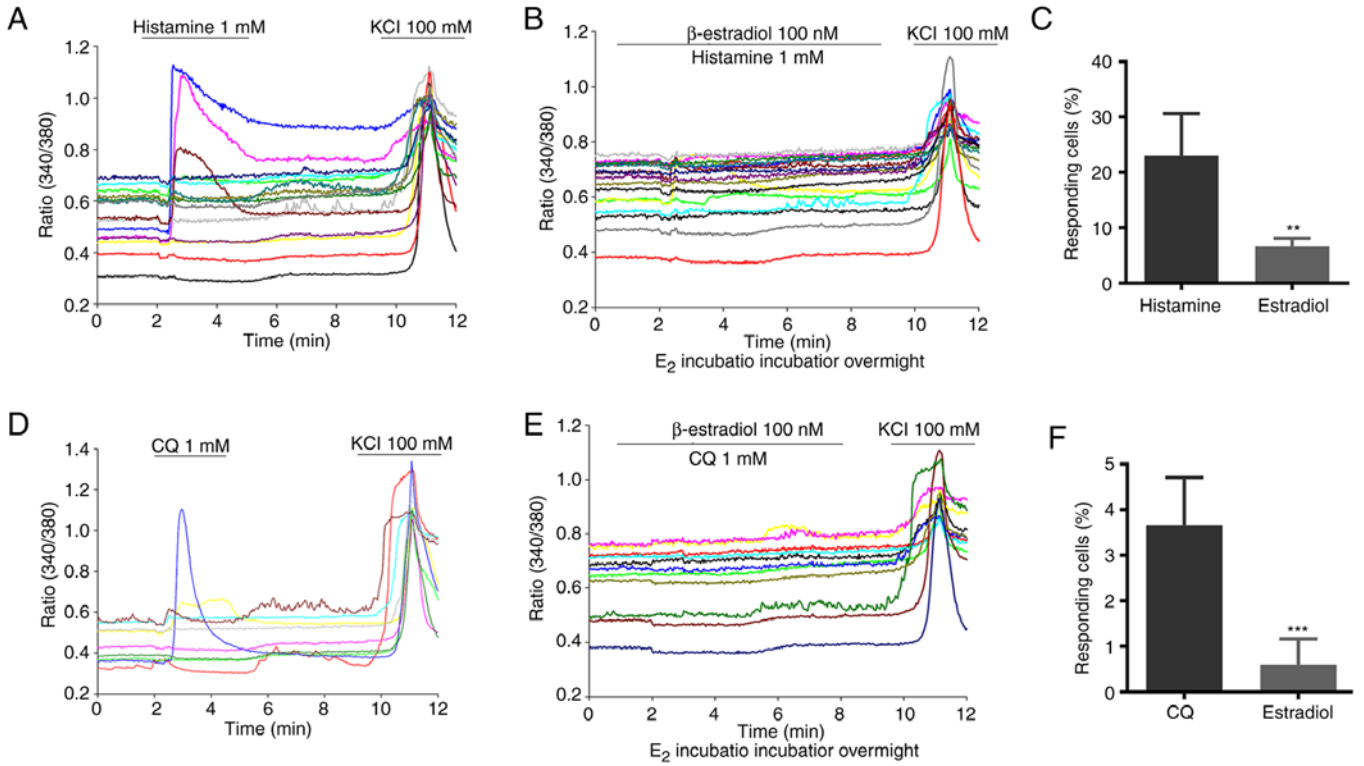

Figure 4. Estradiol attenuates calcium influx in mouse DRG neurons. (A) Histamine-induced $[Ca^{2+}]_i$ in vehicle-treated DRG cells; (B) Histamine-induced $[Ca^{2+}]_i$ in 100 nM β-estradiol-treated DRG cells; (C) Percentage of DRG neurons in response to histamine in the absence and presence of β-estradiol; (D) CQ-induced $[Ca^{2+}]_i$ in vehicle-treated DRG cells; (E) CQ-induced $[Ca^{2+}]_i$ in 100 nM β-estradiol-treated DRG cells; (F) Percentage of DRG neurons in response to CQ in the absence and presence of β-estradiol. Mean ± SD. **P<0.01 and ***P<0.001. Two-tailed unpaired Student's t-test. DRG, dorsal root ganglion; CQ, chloroquine.

increased intracellular calcium concentrations in DRG cells and cause significant $[Ca^{2+}]_i$ responses, and β-estradiol (E2) inhibits nociceptive responses caused by TRPV1-induced calcium influx in adult rat nociceptor neurons by regulating estrogen receptor β-signaling (44); it has also been shown that E2 can gate primary afferent responses to increase or decrease nociception, depending on the input of nociceptive signals (45). These results indicate various functions of estrogen in different sensory neuron-innervated areas, which are related to the cell type, location and proportion of estrogen receptors expressed by the innervating neurons (46), further illustrating the complexity of estrogen regulation in the peripheral nervous system.

The present study revealed that estrogen could modulate the expression of itch-related signaling and inhibit both acute and chronic itch in mice. Notably, Takanami *et al* (32) showed that ovariectomy females treated with 17β-estradiol had a serum concentration estradiol of 60.18±13.54 pg/ml. These researchers further demonstrated that estradiol modulated the GRP-GRPR signalling at the spinal cord level. By marked contrast, in the present study the dose of 17β-estradiol was further increased up to 100 pg/ml in the serum. Notably, with the RNA-seq technique, it was identified that a higher concentration of 17β-estradiol suppresses the expression of itch-related genes in the peripheral DRG level. Combined with the aforementioned results, these data together revealed that the inhibitory or excitatory roles of 17β-estradiol are tightly correlated with their serum concentration, implicating the complexity of 17β-estradiol in itch modulation.

Moreover, estrogen acts *in vivo* mainly through three receptors, ERα, ERβ and GPER, and estrogen receptor expression has been found in various systems throughout the body (46,47), which may be caused by estrogen acting on different receptors. More in-depth subsequent studies are needed to clarify this issue. Furthermore, different animal models\interventions\observation methods affect the effects of estrogen (48,49), and there are some differences in the present and previous studies (30): i) The experimental animals in previous studies were rats, but mice were used in the current study; ii) The previous researchers used estradiol from 15 mm Silastic capsules containing crystalline 17β-estradiol with the blood concentrations of estradiol within the normal range of the estrus cycle in adult female rats from the previous studies. One to two months after surgery, rats were used for behavioural analysis. However, slow-release matrix pellets were used in the present experiment, 0.1 mg/pellet, 60-day release, and plasma estradiol concentrations were slightly higher than the normal range of the estrus cycle. Behavioural analysis was performed 10 days after surgery. Moreover, there are differences between the two kinds of pellets according to a previous study (18). In the aforementioned study, histamine was used at a dose of 3 g/100 μl, but the current dose was 200 μg/50 μl, which is different. In summary, the effect of estrogen on pruritus needs to be further explored, and understanding the role of estrogen in pruritus can provide theoretical support for understanding the generation and regulation of pruritus and can also provide a theoretical basis for developing personalized drugs for the treatment of pruritus.

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Availability of data and materials

The related RNA-seq data have been deposited to GEO (accession number: GSE222059) and datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

FX, GY, JJ and LL designed the project. FX, JJ, LL and GY wrote the manuscript. JJ and LL performed the experiments and analyzed the data. JJ, KL, CW and LL performed behavioural tests. YW, QuL and QiL assisted with Ca^{2+} imaging. WL and AQ assisted with data analysis. YW, LL and KL assisted with cell isolation experiments. All authors discussed the manuscript, commented on the project and contributed to manuscript preparation. JJ and LL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. ZX201898) by The Animal Experimental Ethics Committee of the Plastic Surgery Hospital, Chinese Academy of Medical Science (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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