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Genistein reverses the effect of 17β-estradiol on exacerbating experimental occlusal interference-induced chronic masseter hyperalgesia in ovariectomised rats

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Abstract

Background: Oro-facial pain is more prevalent in women than in men, and oestrogen may underlie this sex difference. Genistein reversed the potentiation of 17β-estradiol (E2) on glutamate-induced acute masseter nociceptive behaviour, but its role in dental experimental occlusal interference (EOI)-induced chronic masseter hyperalgesia remains unclear.

Objective: This study aimed to investigate sex differences, and to explore the role and underlying mechanisms of genistein in E2-potentiated EOI-induced chronic masseter hyperalgesia in rats.

Methods: Female and male rats were prepared to compare the sex differences of masseter hyperalgesia induced by EOI using a 0.4-mm-thick metal crown. Female rats were ovariectomised (OVX) and treated with E2 and genistein, followed by EOI. The head withdrawal threshold (HWT) was examined to assess masseter sensitivity. The protein expression of transient receptor potential vanilloid-1 (TRPV1) in the trigeminal ganglion (TG) was detected using western blotting. Immunofluorescence staining was used to reveal the colocalisation of oestrogen receptors (ERs) with TRPV1 and the percentage of TRPV1-positive neurons in the TG.

Results: To some extent, female rats displayed enhanced sensitivity to EOI-induced chronic masseter hyperalgesia compared with males. Female rats showed the lowest HWT in the pro-oestrus phase. Pre-treatment with genistein antagonised E2 potentiation in EOI-induced masseter hyperalgesia and blocked the effect of E2 by down-regulating TRPV1 protein expression and the percentage of TRPV1-positive neurons in the TG.

Conclusion: Female rats showed greater masseter hyperalgesia than males under EOI. Genistein antagonised the facilitation of EOI-induced chronic masseter hyperalgesia by E2 probably through inhibiting TRPV1 in the TG.

KEYWORDS

17β-estradiol, experimental occlusal interference, genistein, hyperalgesia, sex, TRPV1

1 | BACKGROUND

Temporomandibular disorder (TMD) is a noteworthy health problem that can cause long-term dysfunction and chronic pain, especially in the masticatory muscles and/or temporomandibular joints (TMJs).[1] Although most TMD pain symptoms abate with time, it may take months or even more than years.[2] Patients with chronic painful TMD usually experience psychological distress, such as depression and stress.[3] However, the detailed aetiology of TMD is still controversial and the available therapeutic strategies are far from satisfactory.[4] Current pharmacological treatments for TMD pain include non-steroidal anti-inflammatory drugs, muscle relaxants, corticosteroids, etc.[5] However, the long-term effectiveness of these drugs is not guaranteed after treatment,[6] and their prolonged use due to the chronicity of TMD pain may produce various side effects.[7] Therefore, further research into the development of ideal drugs remains an important direction for clinical intervention of TMD pain.

Women have a greater prevalence of TMD and related pain conditions than men, with a female-to-male ratio of more than 3:1 for seeking treatment.[8] TMD pain primarily affects women aged 20–40 years, often typically beginning at puberty, peaking in the reproductive years, and decreasing after menopause.[9] These observations suggest that high levels of oestrogen may be a risk factor responsible for the pathogenesis of TMD pain. We previously established a dental experimental occlusal interference (EOI) model of rats, which induces bilateral masticatory muscle mechanical hyperalgesia for nearly one month and thus simulates the chronic pain of TMD.[10] We found that both central and peripheral sensitisation mechanisms are involved in EOI-induced sustained hyperalgesia.[11,12] While, as only male rats were used in these previous studies, there are no data on whether sex differences exist in this model.

Genistein, the major biologically active isoflavone derived from soy, is known to be phytoestrogen because of its structural similarity to oestrogen.[13] Genistein exerts anti-oestrogenic activity when the oestrogen level is high and inhibits tyrosine kinase activity at pharmacological doses.[14,15] It has been demonstrated that genistein has a pain relieving effect in inflammatory pain,[16] neuropathic pain,[17] and migraine.[18] In addition, there is also evidence that genistein decreases the number of evoked action potentials, increasing the threshold potential and thus reducing the excitability of nociceptive neurons.[19] Our previous study demonstrated that genistein could reverse the potentiation of 17β-estradiol (E2) on glutamate-induced acute masseter nociceptive behaviour.[20] Therefore, we hypothesise that genistein may also antagonise the effect of E2 on EOI-induced chronic masseter mechanical hyperalgesia.

Oestrogen exerts multiple effects on pain modulation through its action on oestrogen receptors (ERs).[21] ERs are abundantly present in the rat trigeminal ganglion (TG), and ER activation might induce post-translational changes in many pain-related ion channels in peripheral sensory afferents.[22,23] Transient receptor potential vanilloid-1 (TRPV1) is a non-selective cation channel that is activated by thermal, mechanical and chemical stimuli.[24] The expression of TRPV1 receptors in primary sensory neurons significantly decreased in ERα- and ERβ- knockout mice.[25] There was a significant decrease in TRPV1 gene expression in the TGs of ovariectomised (OVX) rats, and oestrogen replacement could significantly increase its expression.[26] However, whether E2 and genistein could affect EOI-induced mechanical hyperalgesia by regulating the expression of TRPV1 in the TG is still unclear.

In this study, we first investigated sex differences in EOI-induced chronic masseter hyperalgesia in rats. Then, we detected whether genistein can antagonise the role of E2 in this form of chronic hyperalgesia. Moreover, we determined the colocalisation of ERs with TRPV1 in the TG and further explored the effect of genistein on TRPV1 in the TG in E2-potentiated EOI-induced chronic masseter hyperalgesia in rats.

2 | METHODS

2.1 | Animals

A total of 80 adult Sprague-Dawley rats initially weighing 200–220 g (14 males and 66 females; Vital River Laboratory Animal Technology Co. Ltd.) were used in this study. The rats were housed in a room at a constant temperature (22 ± 1°C) under a 12-h light/12-h dark cycle with food and water ad libitum. All protocols were conducted with the review and approval of the Institutional Animal Care and Use Committee of Peking University (IACUC number: LA2017129, Beijing, China) and conformed with the Ethical Guidelines of the International Association for the Study of Pain. Concerted efforts were made to minimise the number of experimental animals and their suffering.

2.2 | Model of experimental occlusal interference-induced chronic masseter hyperalgesia

Experimental occlusal interference was used to induce chronic masseter hyperalgesia as previously described.[10] Briefly, the rats were anaesthetised by intraperitoneal (ip) injection of 1% pentobarbital sodium (50 mg/kg), and then metal crowns with a thickness of 0.4 mm were bonded onto the right maxillary first molars with dental adhesive resin cement (Panavia F, Kuraray). The rats in the control groups underwent the same procedure, including anaesthesia and passive mouth opening, except for the application of EOI.

2.3 | Behavioural assessment of mechanical sensitivity

The head withdrawal threshold was measured using an electronic von-Frey aesthesiometer (BIO-EVF3, Bioseb) to assess the mechanical sensitivity of the masseter muscles as described previously.[10] All behavioural tests were performed by a trained investigator who was blinded to the treatment conditions. The rats were acclimated to the testing environment for 3 consecutive days (30 min/day) prior to baseline evaluation.

Each rat was habituated to standing on its hind paws and leaning against the experimenter's gloved hand. A round cap (diameter of 3 mm) was fixed to a rigid plastic tip that applied pressure to the masseter muscles. The masseter muscle was tested at 10 mm inferior to the central point of the line between the orbit and the tragus. The left and right masseter muscles were tested in a randomised order for each rat. The rats were not restrained and were free to withdraw their heads from the progressively increasing force. The force that elicited a response (in grams) was recorded five times for each muscle. The average of the five values was calculated as the head withdrawal threshold. The pre-treatment baseline mechanical threshold was determined by averaging the head withdrawal thresholds recorded over 3 continuous days.

2.4 | Ovariectomy

After being anaesthetised with 1% pentobarbital sodium (50 mg/kg, ip), the rats in the OVX groups underwent bilateral ovariectomy under aseptic conditions. Briefly, the ovarian fat pads were gently grasped using forceps until the ovaries were exposed through dorsal incisions over both flanks. A ligature was placed below each ovary to prevent bleeding, and then the ovaries were removed. The uterus and remaining part of the oviduct were placed back into the abdominal cavity, and then the muscle, subcutaneous tissue and skin layer were sutured in sequence. The rats were placed on a heated operation bench until they woke from anaesthesia. All animals were intramuscularly injected with penicillin G (10 000 units/kg) after surgery and allowed to recover for 7 days before any other experiments were performed. The rats were fed soy-free food to exclude phytosteroids from their diet.

2.5 | Drug application

The drugs used in this study were 17β-estradiol (E2, E-2758, Sigma) and genistein (Gen, G0272, TCI). E2 and genistein were dissolved in ethanol and diluted to various concentrations in sterile saline. The application of 80 μg/day E2 was selected according to a previous study which found that the plasma E2 levels in OVX rats treated with 80 μg/day E2 were comparable to those in pro-oestrus rats.[27] These drugs were subcutaneously (sc) administered daily in the morning in a volume of 200 μl per rat.

2.6 | Behavioural study protocols

2.6.1 | Sex differences in EOI-induced chronic masseter hyperalgesia

Healthy adult rats of both sexes were randomly divided into four groups (n = 7 per group): the male control group, female control group, male EOI group, and female EOI group. The head withdrawal thresholds of the bilateral masseter muscles were measured on pre-application days 1, 2, and 3 (baseline) and post-application days 1, 3, 5, 7, 14, 21 and 28.

2.6.2 | Masseter nociception in each phase of the oestrous cycle

To evaluate whether masseter muscle nociception fluctuates during the oestrous cycle of intact female rats, the head withdrawal thresholds of intact female rats were tested daily within two consecutive weeks (nearly three oestrous cycles). To minimise the number of experimental animals, the female control group that finished the sex differences experiment (n = 7 rats) was used in this experiment. The oestrous cycle phase was determined by microscopic cytology of vaginal smears from the rats at the end of each mechanical threshold measurement. Then, the head withdrawal threshold data of the bilateral masseter muscles in the different phases of the oestrous cycle (pro-oestrus, oestrous, dioestrus, and metoestrus) were recorded, and data from the first complete oestrous cycle of the female rats were compared.

2.6.3 | Effects of genistein on E2-potentiated EOI-induced masseter hyperalgesia

Forty-eight OVX rats were randomly divided into 6 groups (n = 8 per group), namely, groups A, B, C, D, E, and F. From day 7 after OVX surgery, groups A, B, and C received vehicle (10% ethanol in sterile saline), and groups D, E, and F received various doses (7.5, 15, and 30 mg/kg/day, respectively) of genistein. From day 3 of drug application, genistein or vehicle was injected 2 h earlier for the groups. Then, groups A and B received E2 vehicle, and groups C, D, E, and F received 80 μg/day E2. The rats in all groups except group A underwent EOI after 10 days of E2 or vehicle application. Head withdrawal thresholds of bilateral masseter muscles were measured on day 7 after EOI application.

After the behavioural tests, 30 rats (n = 5 per group) were used for western blotting and 18 rats (n = 3 per group) were used for immunofluorescence staining. Because the protein expression results between bilateral TGs in the western blot experiment are consistent, we then used the data of right TGs for analysis in the subsequent immunofluorescence staining experiment.

2.7 | Vaginal smears

To evaluate the oestrous cycles of intact female rats, vaginal smears were analysed according to the method described by Goldman et al.[28] Briefly, the vaginal openings were flushed with 200 μl of sterile saline, and fresh samples were evaluated under a microscope.

2.8 | Western blot analysis

The bilateral TGs of the rats were homogenised in ice-cold RIPA lysis buffer containing protease inhibitor cocktail (Huaxingbio Science) with a homogeniser (Ultra-Turrax T10, IKA Laboratory Technology). The homogenates were centrifuged at 14 000× *g* for 20 min at 4°C. The supernatants were collected, and the protein concentrations were measured using a BCA protein assay kit (Beyotime Biotechnology). Total protein samples (50 μg/well) were loaded on 8% SDS-PAGE gels, separated electrophoretically and then transferred to polyvinylidine difluoride membranes (Millipore). After they were blocked with 5% bovine serum albumin in TBS/Tween (TBST) for 2 h at RT, the membranes were incubated with a primary antibody against TRPV1 (1:1000, in blocking buffer, Abcam) overnight at 4°C After extensive washing with TBST, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000, Zhong Shan Golden Bridge) for 1 h at RT. An anti-GAPDH antibody (1:2000, Zhong Shan Golden Bridge) was used to measure the level of GAPDH as an internal control. The membranes were visualised using ECL solution (Beyotime Biotechnology) and examined with a luminescent image analyser (Fusion FX, Vilber Lourmat). The densities of the immunoreactive bands were quantified with ImageJ 1.38 software (National Institutes of Health) and normalised to the density of the internal control band.

2.9 | Immunofluorescence staining

The rats were anaesthetised and perfused transcardially with 300 ml body temperature physiological saline (0.9%) followed by 200 ml ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The TGs were removed, post-fixed at 4°C overnight, and then transferred to 30% sucrose solution for dehydration. Transverse sections of the TGs (10 μm thickness) were cut on a cryostat at a temperature below −20°C and mounted on adhesive microscope slides (Citoglas). The sections were rinsed 3 times in PBS buffer and blocked with 10% goat serum and 0.3% Triton-X 100 (Sigma) in PBS for 1 h at RT. To evaluate the colocalisation of TRPV1 and ERs in the TGs of female rats, double immunostaining for ERα, ERβ or GPR30 and TRPV1 was performed. The sections were first incubated with a mouse monoclonal anti-TRPV1 antibody (1:500, Abcam) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100, Zhong Shan Golden Bridge). The sections were then incubated with a rabbit polyclonal anti-ERα antibody (1:100, Proteintech), rabbit polyclonal anti-ERβ antibody (1:100, Proteintech) or rabbit polyclonal anti-GPR30 antibody (1:100, Abcam) followed by 90 min with a rhodamine (TRITC)-conjugated goat anti-rabbit secondary antibody (1:100, Zhong Shan Golden Bridge). After extensive washing with PBS, the sections were coverslipped using anti-fade reagent containing DAPI. To determine the percentage of TRPV1-positive neurons in masseter afferent neurons and TG branches (V3 and V1/V2), one of every 5 consecutive sections was collected. Five sections of each TG were used for immunofluorescence staining of TRPV1 using the approach described above. Stained sections were examined under a fluorescence microscope (BX51; Olympus). The primary antibody was omitted from the processing of sections to control for non-specific background staining. Uniform microscope settings were maintained throughout the image capture process.

Image analysis was performed with Image-Pro Plus v6.0 software (Media Cybernetics). Only the labelled neurons showing a discernable nucleus were included in the counts. Neurons with 100 or more contiguous pixels above background threshold levels were considered immunoreactive. Standardised regions of interest were outlined in TG sections to encompass neurons innervating V3 and V1/V2, based on anatomic markers. Neuronal cells were included by counting their distinctive large, round morphology and DAPI-stained nuclei. The percentages of TRPV1-positive neurons in V3 and V1/V2 were calculated by multiplying the number of TRPV1-labelled neurons by 100% and dividing by the total number of neurons. All sections were analysed in a blinded manner.

2.10 | Statistical analysis

Statistical analyses were performed with SPSS 20.0 (SPSS). All data are expressed as the mean ± SEM. Sex differences in EOI-induced masseter mechanical hyperalgesia were evaluated not only for head withdrawal thresholds, but also for changes in head withdrawal thresholds (%, that is, the absolute values of head withdrawal thresholds were normalised to baseline) in rats. Then these data were assessed using multi-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni's post hoc tests. Differences in other data were analysed by one-way ANOVA followed by Bonferroni's post hoc tests for multiple comparisons. $p < .05$ was considered to indicate a significant difference.

3 | RESULTS

3.1 | Sex differences in EOI-induced chronic masseter mechanical hyperalgesia in rats

To assess the impact of EOI on masseter mechanical hyperalgesia between the sexes, we measured head withdrawal thresholds of the bilateral masseter muscles in intact female (presumably in all phases of the oestrous cycle) and male rats following the application of 0.4-mm-thick metal crowns (Figure 1A). The absolute head withdrawal threshold values are shown in Figure 1B. Multi-way repeated-measures ANOVA yielded no significant main effect of muscle ($p > .05$) but revealed significant main effects of sex, treatment, and time ($p < .001$). A significant interaction between treatment and time was observed ($p < .001$). For the control groups, the main effect of time was not significant, indicating that repeated testing did not significantly affect the head withdrawal thresholds in the male control and female control groups. For the EOI groups, the main effect of time was significant, indicating that head withdrawal

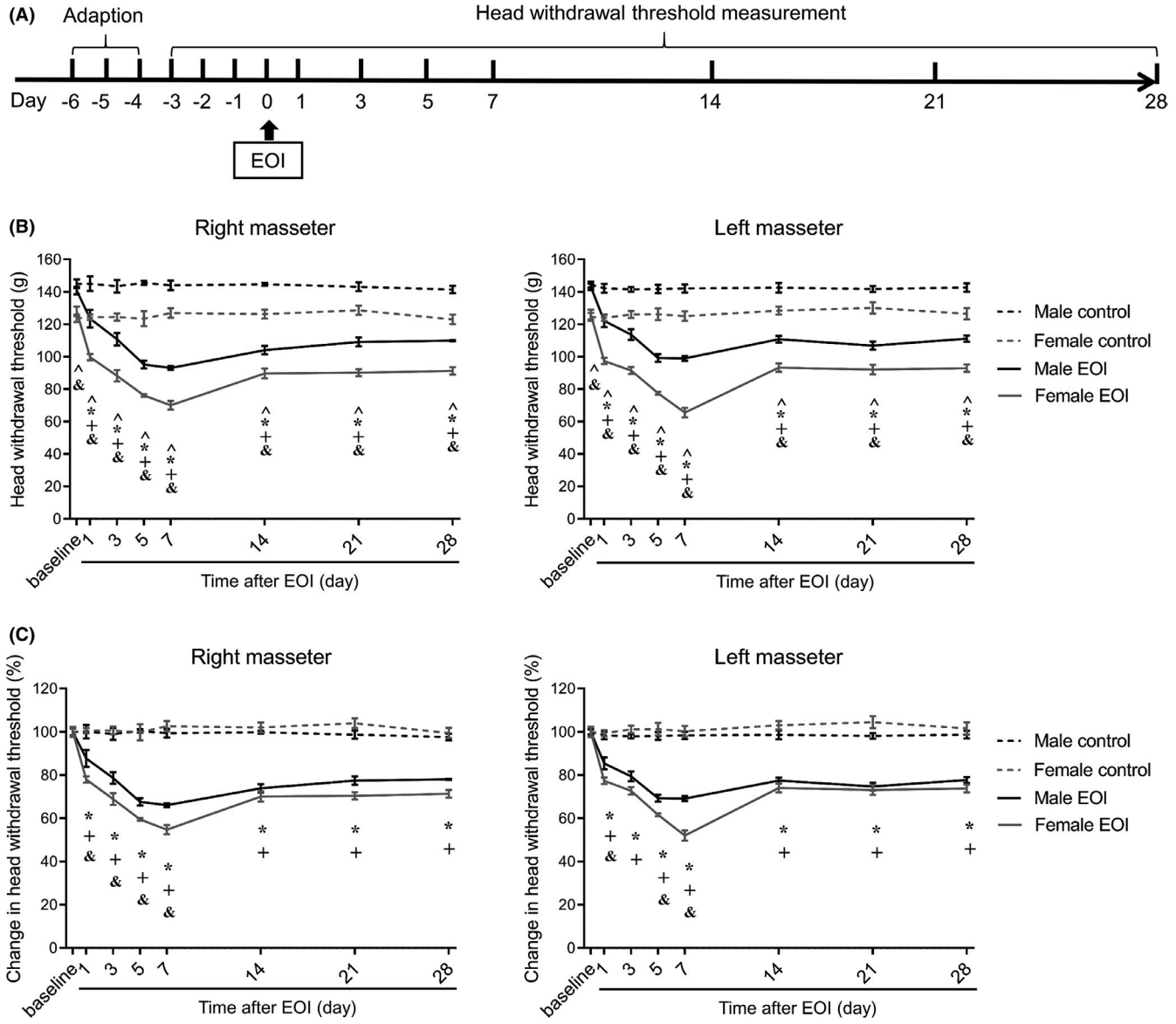

FIGURE 1 Sex differences in EOI-induced chronic masseter mechanical hyperalgesia. (A) Schematic diagram of the experimental time course. (B) Head withdrawal thresholds and (C) changes in head withdrawal thresholds of bilateral masseter muscles following EOI in intact female and male rats ($n = 7$ in each group). ^$p < .05$, between female control group and male control group; *$p < .05$, between the male EOI group and male control group; +$p < .05$, between the female EOI group and female control group; & $p < .05$, between the female EOI group and male EOI group. Multi-way repeated-measures ANOVA followed by Bonferroni's post hoc tests. EOI, experimental occlusal interference

thresholds were significantly different from baseline levels after EOI application ($p < .001$). Comparisons of bilateral masseter muscles were performed at each time point. The female control group consistently exhibited a lower head withdrawal threshold than the male control group ($p < .05$). The head withdrawal thresholds of both the male EOI and female EOI groups were significantly decreased compared with those of the corresponding control groups, with the differences starting on day 1 post-EOI, peaking from days 5 to 7 and persisting for up to 28 days during the observation ($p < .05$).

Moreover, the head withdrawal threshold values were normalised to the baseline to analyse the extent of relative changes in the threshold. As shown in Figure 1C, multi-way repeated-measures ANOVA of the normalised data revealed a significant sex × treatment × time interaction ($p < .001$). The female EOI group exhibited significantly greater reduction than the male EOI group at days 1, 3, 5, and 7 post-EOI ($p < .05$), and the significant difference between sexes was absent at days 14, 21, and 28 after EOI. These results indicated that there were sex-dependent differences in EOI-induced masseter mechanical hyperalgesia in rats.

3.2 | Masseter mechanical thresholds fluctuated with the oestrous cycle in female rats

To provide a necessary foundation for determining the effect of E2 on masseter hyperalgesia in female rats, we compared mechanical

thresholds in different phases of the oestrous cycle (linked to fluctuating physiological E2 levels). As shown in Figure 2A, pro-oestrus smears showed a predominance of nucleated epithelial cells and an absence of leukocytes; oestrous smears were primarily characterised by large numbers of enucleated keratinocytes often stacked together like a leaf; metoestrus smears consisted of a massive number of leukocytes and cornified epithelial cells; and dioestrus smears were primarily characterised by a predominance of leukocytes. Head withdrawal thresholds were lowest in the pro-oestrus phase among the four phases of the oestrous cycle in the bilateral masseter muscles ($p < .05$; Figure 2B).

3.3 | Genistein reversed the facilitatory effect of E2 on EOI-induced masseter hyperalgesia

To examine whether genistein antagonised the effect of E2 on EOI-induced mechanical hyperalgesia of the masseter muscles, rats were pre-treated with 0, 7.5, 15, or 30 mg/kg/day genistein before being treated with 80 μg/day E2 and EOI (Figure 3A). As shown in Figure 3B, head withdrawal thresholds were significantly decreased in all EOI-treated groups compared with OVX rats treated without EOI (group A; $p < .05$). Head withdrawal thresholds were further reduced in the 80 μg/day E2 group (group C) compared with the group that received neither genistein nor E2 (group B; $p < .05$), and this reduction was reversed by 7.5, 15, and 30 mg/kg/day genistein treatment (groups D, E, and F, respectively; $p < .05$). Moreover, pre-treatment with 15 mg/kg/day genistein (group E) displayed a greater alleviation effect than 7.5 mg/kg/day genistein (group D) ($p < .05$). There were no significant differences among the group treated with 15 mg/kg/day genistein (group E), the group treated with 30 mg/kg/day genistein (group F) and the group not treated with genistein and E2 (group B; Figure 3B).

3.4 | Colocalisation of Ers with TRPV1 in the TG of intact female rats

Immunofluorescence labelling confirmed that TRPV1 was coexpressed with ERs (ERα, ERβ, and GPR30) in TG neurons of intact female rats (Figure 4), providing an anatomical framework by which E2 can directly modulate TRPV1 in the TG for oro-facial nociceptive responses.

3.5 | Genistein blocked the effect of E2 through TRPV1 in the TGs

To study how genistein attenuated the effect of E2 on EOI-induced masseter hyperalgesia, we examined its modulation on TRPV1 protein levels in the bilateral TGs. As shown in Figure 5A,B, compared with no genistein treatment (group C), pre-treatment with 7.5, 15, and 30 mg/kg/day genistein (groups D, E, and F, respectively) attenuated E2-induced TRPV1 expression upregulation in the TGs after EOI ($p < .05$). There were no significant differences among the 15 and 30 mg/kg/day genistein groups (groups E, F) and the group not treated with genistein or E2 (group B; $p > .05$).

As EOI may affect TRPV1 expression in both masseter afferent neurons and other peripheral afferents, we further compared the proportion of TRPV1-immunoreactive neurons in the V3 and V1/V2 branches of the TGs. The changes in the percentages of TRPV1-positive neurons in the V3 and V1/V2 branches were similar to the changes in protein levels (Figure 6). Compared with EOI alone, E2 treatment plus EOI (group C) increased the percentage of TRPV1-immunoreactive neurons in the V3 and V1/V2 branches. The percentage of TRPV1-positive neurons in the genistein-treated groups (groups D, E, F) was decreased compared with that in the group not

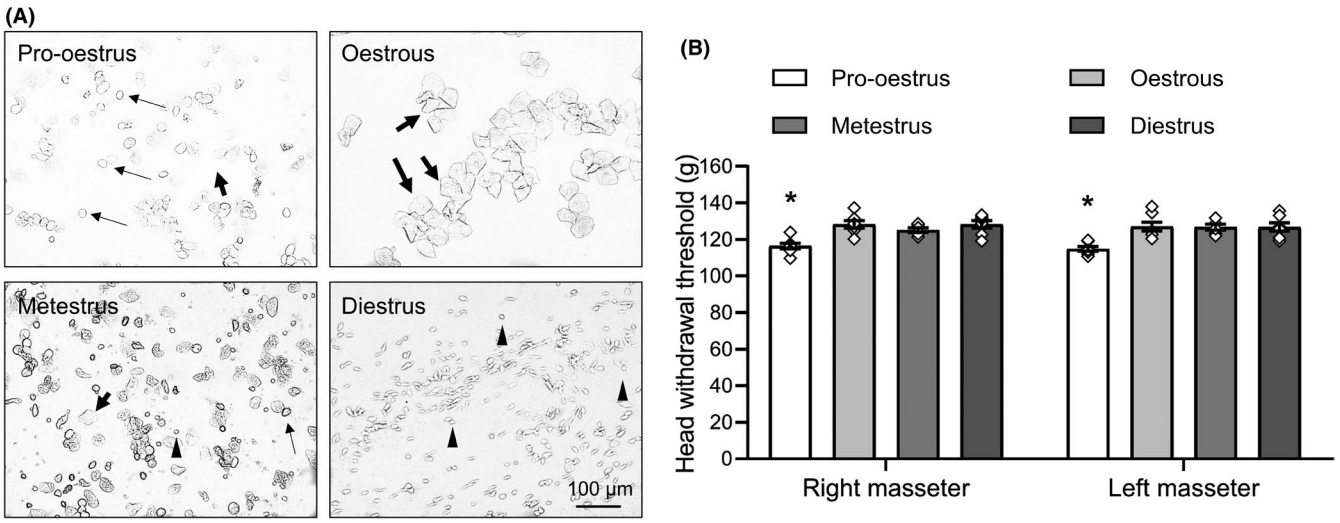

FIGURE 2 Masseter mechanical thresholds fluctuated with the oestrous cycle in intact female rats. (A) Photomicrograph of representative vaginal smears from intact oestrous cycling rats. The long thin arrows indicate nucleated epithelial cells, the long thick arrows indicate enucleated keratinocytes, and the triangular arrows indicate leucocytes. (B) Head withdrawal thresholds of the bilateral masseter muscles during different oestrous phases ($n = 7$ in each group). *$p < .05$, compared with other phases. One-way ANOVA followed by Bonferroni's post hoc tests

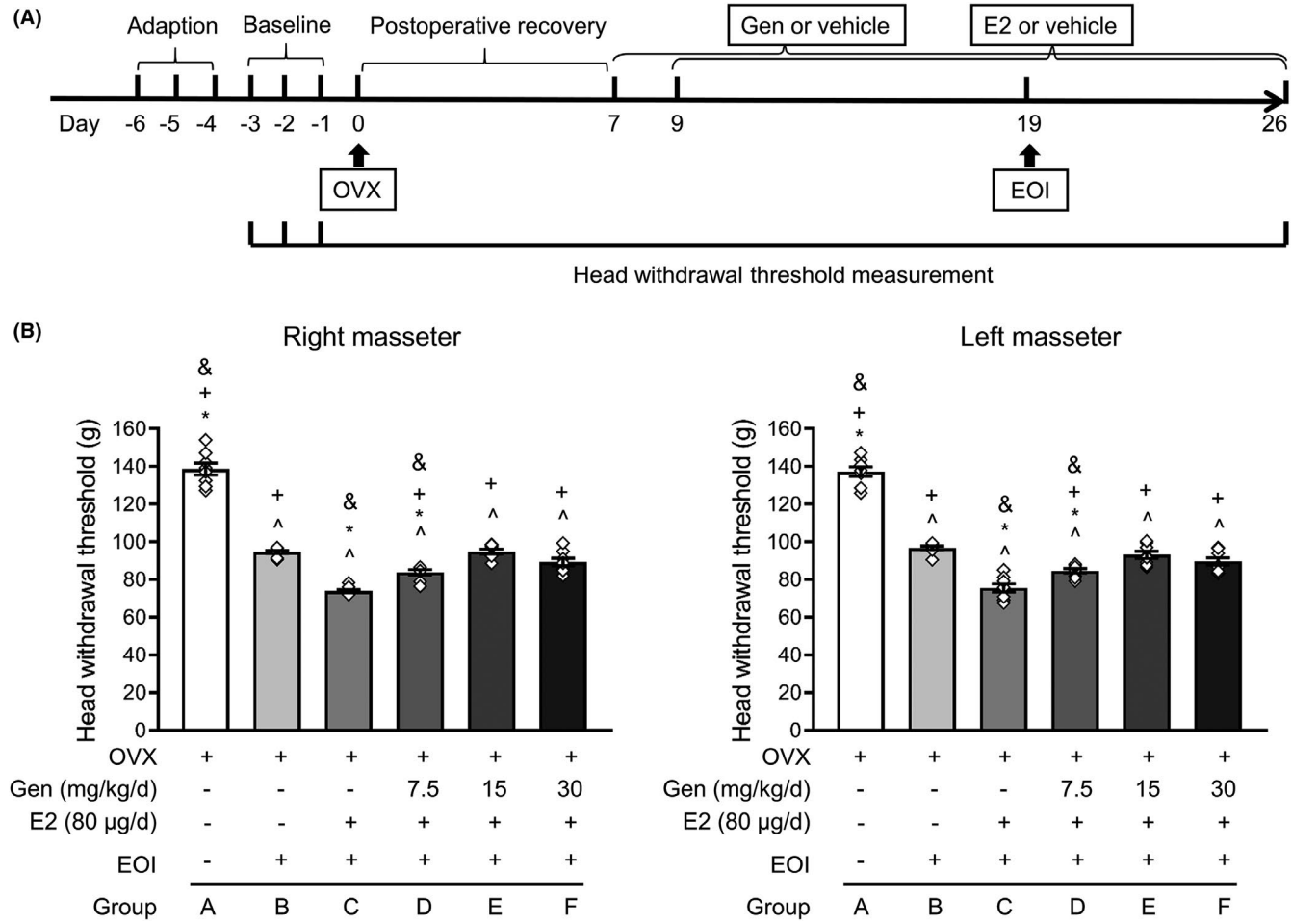

FIGURE 3 Genistein alleviated E2-potentiated EOI-induced masseter mechanical hyperalgesia in OVX rats. (A) Schematic diagram of the effects of genistein on E2 and EOI-induced masseter hypernociception. (B) The effects of genistein on E2-potentiated EOI-induced masseter mechanical hyperalgesia on the 7th day after application of EOI ($n = 8$ in each group). ^$p < .05$ vs. group A; *$p < .05$ vs. group B; +$p < .05$ vs. group C; & $p < .05$ vs. group E. One-way ANOVA followed by Bonferroni's post hoc tests. E2, 17β-estradiol; EOI, experimental occlusal interference; Gen, genistein; OVX, ovariectomy

treated with genistein (group C; $p < .05$) and was the same as that in the group not treated with genistein or E2 (group B; $p > .05$).

4 | DISCUSSION

Injection of endogenous or exogenous chemical irritants is the most common approach used to establish experimental muscle pain models. Several previous studies have demonstrated that there are sex differences in pain tolerance and sensitivity of the masseter muscle following injection of glutamate or nerve growth factor,[29,30] which mirror the sex differences observed in TMD-related pain to a certain extent. Intramuscular injection of irritants may be more likely to mimic some pain symptoms than to simulate the pathogenesis of pain conditions. In rats, EOI can result in masseter hyperalgesia for at least 28 days by imitating clinical dental conditions. This model is more close to the chronicity of occlusion-related pain.

In this study, we showed that EOI-treated rats, of both sexes, present chronic mechanical hyperalgesia in the right and left masseter muscles. This finding denotes that EOI produces generalised masseter nociception in a similar manner, thus further confirming the validity of this model. Although the trend of the mechanical responses of female rats was similar to that of male rats in the presence and absence of EOI, female rats tended to show lower baseline mechanical sensitivity and greater hypersensitivity than male rats. These findings are consistent with the clinical results showing that healthy female subjects have a lower pressure pain threshold of the bilateral masseter muscles than healthy male subjects[31] and that the incidence of pain and tenderness on masticatory palpation is higher in female TMD patients than in male TMD patients.[32] The extent of mechanical hyperalgesia was greater in female rats in the early phase (before day 7 post-EOI), and no difference was found between females and males in the later maintenance phase (from day 7 to day 28 post-EOI), suggesting that different sex-dependent mechanisms in the phases of pain development and maintenance need to be investigated further.

Given the fluctuations in E2 levels during the different phases of the oestrous cycle, we further compared the effect of the oestrous

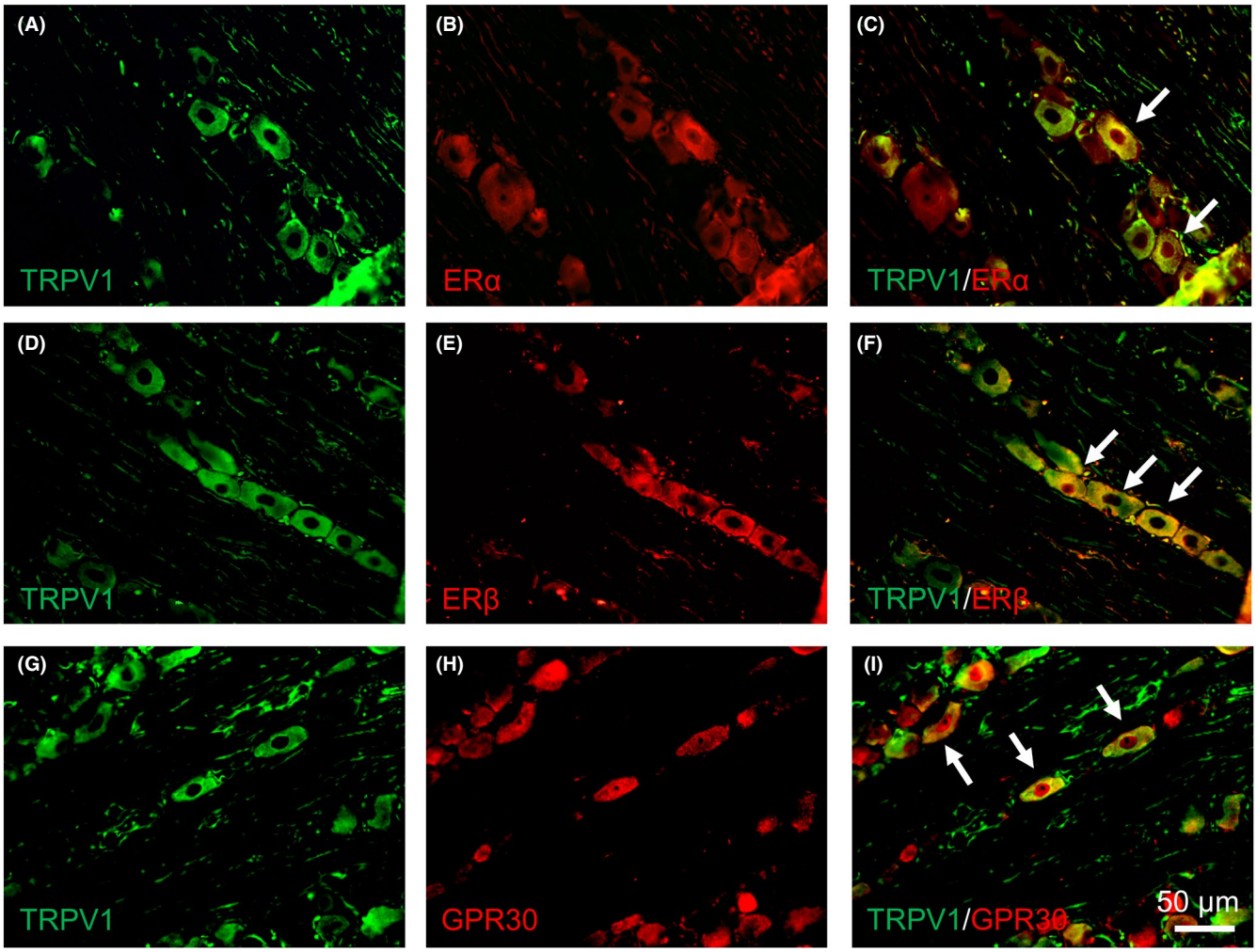

FIGURE 4 Immunofluorescence staining revealed that TRPV1 (green) was colocalised with oestrogen receptors (ERα, ERβ, and GPR30, red) in the TG neurons of intact female rats. TRPV1 immunoreactivity in neurons, which was mainly observed in the cytoplasm of small- and medium-sized neurons, is shown in A, D, and G. ERα, ERβ and GPR30 immunoreactivity in neurons is shown in B, E and H and in the merged double-labelled images in C, F, and I. The arrows indicate double-labelled neurons

cycle phase on masseter muscle pain responses in female rats. Our study showed that head withdrawal thresholds were significantly lower in the pro-oestrus (when E2 was higher) than in other phases, which is similar to a previous study in rat TMJ nociception.[33] This result may be explained by the increased neuronal excitability of the trigeminal system in the pro-oestrus.[34] Together, sex and oestrous cycle differences data support the notion that females tend to be more sensitive to nociceptive stimuli than males, and pain level for females increases when physiological E2 peaks. The results provide a foundation for determining the underlying roles of E2 in masseter muscle nociception.

We used OVX rats treated with injections of 80 μg/day E2 to resemble the physiological E2 level of rats in pro-oestrus. Plasm E2 levels of OVX rats treated with different doses of E2 were determined in a previous study,[27] and 80 μg/day E2 was appropriate for examining the effect of physiological E2 on EOI-induced chronic masseter muscle mechanical hyperalgesia. Genistein, a natural phytoestrogen, has been found to potentially relieve inflammatory and neuropathic pain.[17,18,35,36] It has previously been shown that subcutaneous injection of genistein (7.5, 15, and 30 mg/kg) could relieve the thermal hyperalgesia and mechanical allodynia induced by chronic constriction injury in C57BL/6J male mice.[17] Genistein binds to ERs that can act as an oestrogenic property when E2 levels are low and as an anti-oestrogenic property when E2 levels are high in both in vivo and in vitro models.[14,37] Our previous study demonstrated that pre-treatment with a high dose of genistein (60 mg/kg) slightly decreased the head withdrawal thresholds in OVX rats without E2 replacement, which may play a E2-like effect; a low dose of genistein (2 mg/kg) did not reverse the decreases in the head withdrawal thresholds in OVX rats with 80 μg/day E2 replacement; and medium doses of genistein (7.5, 15, and 30 mg/kg) effectively prevented the E2-potentiated glutamate-evoked acute masseter hyperalgesia.[20] Therefore, we selectively used these three medium doses of genistein in this study.

As expected, we found that 7.5, 15, and 30 mg/kg genistein alleviated the facilitatory effect of E2 on EOI-induced chronic mechanical hyperalgesia, which is in line with our previous evidence on acute masseter hyperalgesia.[20] While, our behavioural evidence suggested

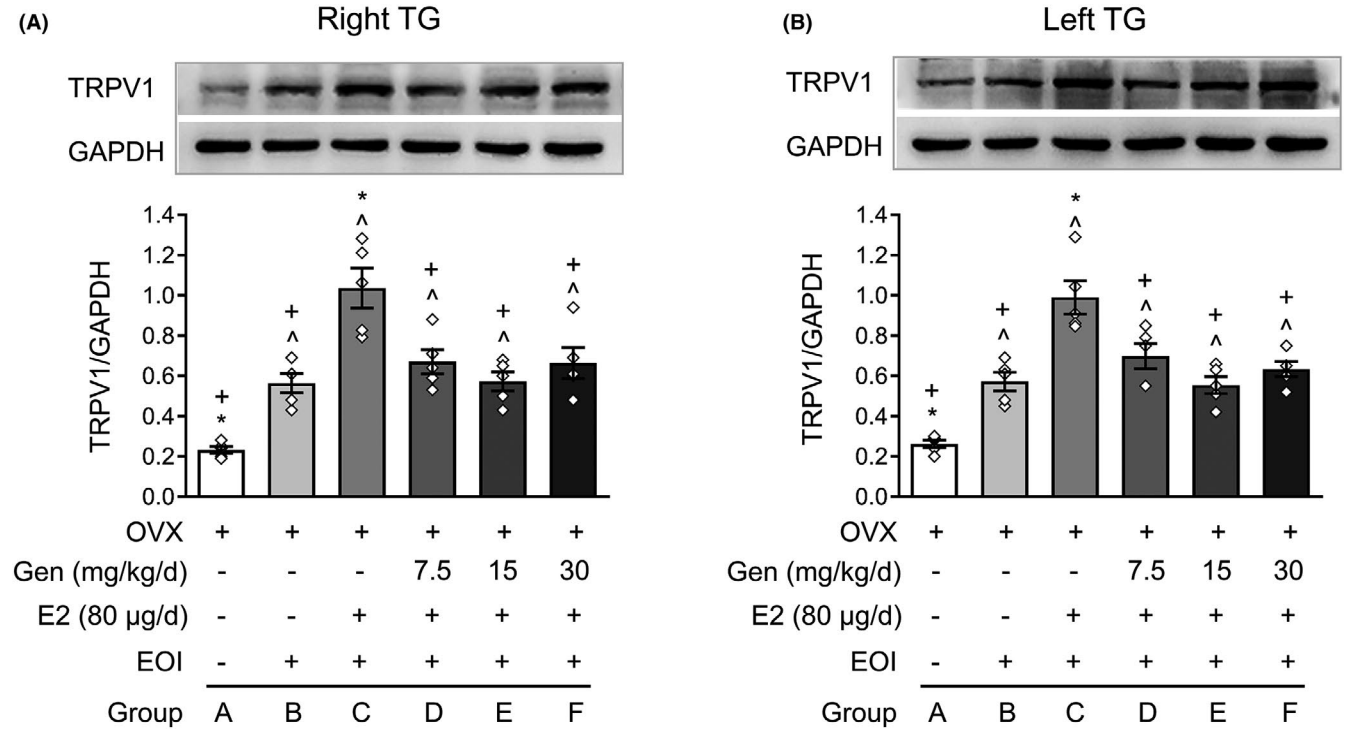

FIGURE 5 Protein levels of TRPV1 in the bilateral TGs were downregulated by different doses of genistein in OVX rats that had undergone consecutive E2 replacement and EOI for 7 days. (A, B) Example western blots. Quantification of protein levels confirmed that genistein decreased TRPV1 protein levels in the bilateral TGs of OVX rats treated with E2 and EOI. The data were normalised to GAPDH as an internal control, and protein levels were calculated relative to those in group C ($n = 5$ in each group). ^$p < .05$ vs. group A; *$p < .05$ vs. group B; +$p < .05$ vs. group C. One-way ANOVA followed by Bonferroni's post hoc tests. E2, 17β-estradiol; EOI, experimental occlusal interference; Gen, genistein; OVX, ovariectomy

that the effect of genistein at both 15 and 30 mg/kg is stronger than that of 7.5 mg/kg genistein, which is not completely consistent with the findings from several previous studies.[17,20] The different dose responses to genistein suggest that the complex effects of genistein treatment may be related to different animals, different pain models, and different drug applications. As previous studies of genistein mainly involved males, which are different from females in the types and levels of sex hormones and the distribution and number of ERs in tissues, it is of importance to explore the roles of genistein under E2 conditions. This study gave priority to investigate the effect of genistein on E2-potentiated EOI-induced masseter hyperalgesia, while further studies are also needed to explore its role on pure EOI-induced masseter hyperalgesia in OVX rats and male rats, so that we would get more information about whether the functions of genistein involved other mechanisms besides the E2 signal.

It has previously been reported that intrathecal pre-administration of genistein decreases visceral pain hypersensitivity in a dose-dependent manner.[38] A phytoestrogen-containing soy diet has been shown to have analgesic effects in rats when administered before but not after partial sciatic nerve ligation injury.[39] Some investigators have shown that genistein can reverse allodynia in mice with neuropathic pain even after the onset of nociception.[17,36] In this study, we used genistein pre-treatment to study its role. It would be interesting to determine in the future whether genistein treatment after EOI also has a pain relieving effect because this situation is more consistent with the clinical treatment of patients with TMD-related pain.

The biological effects of E2 are mediated through direct genomic and indirect non-genomic pathways, which occur in a receptor-specific manner by the classical ERs (ERα and ERβ) and membrane-associated G protein-coupled receptor (GPR30/GPER1), respectively.[40] TRPV1 is principally expressed in the peripheral and central nervous systems and plays a critical role in the development of pain hypersensitivity, and accumulating evidence has demonstrated that E2 could enhance TRPV1 expression and induce mechanical sensitisation.[27,41,42] In our previous study, the increased protein level of TRPV1 in TGs was verified in EOI-induced masseter mechanical hyperalgesia in male rats.[11] Here, our data demonstrated that E2 (80 μg/day) significantly increased the expression of TRPV1 in the TGs of EOI-treated OVX rats, and that genistein reversed the effects of E2 by downregulating the TRPV1 protein level and decreasing the percentages of TRPV1-positive neurons in the V3 and V1/V2 branches of the TGs. To our knowledge, this is the first direct evidence showing that genistein downregulates the expression of TRPV1 in E2-potentiated chronic masseter hyperalgesia. Genistein may exert its effect by competing with E2 to bind ERs. We observed the colocalisation of TRPV1 and all three ERs (ERα, ERβ, and GPR30) in the TG, and provided

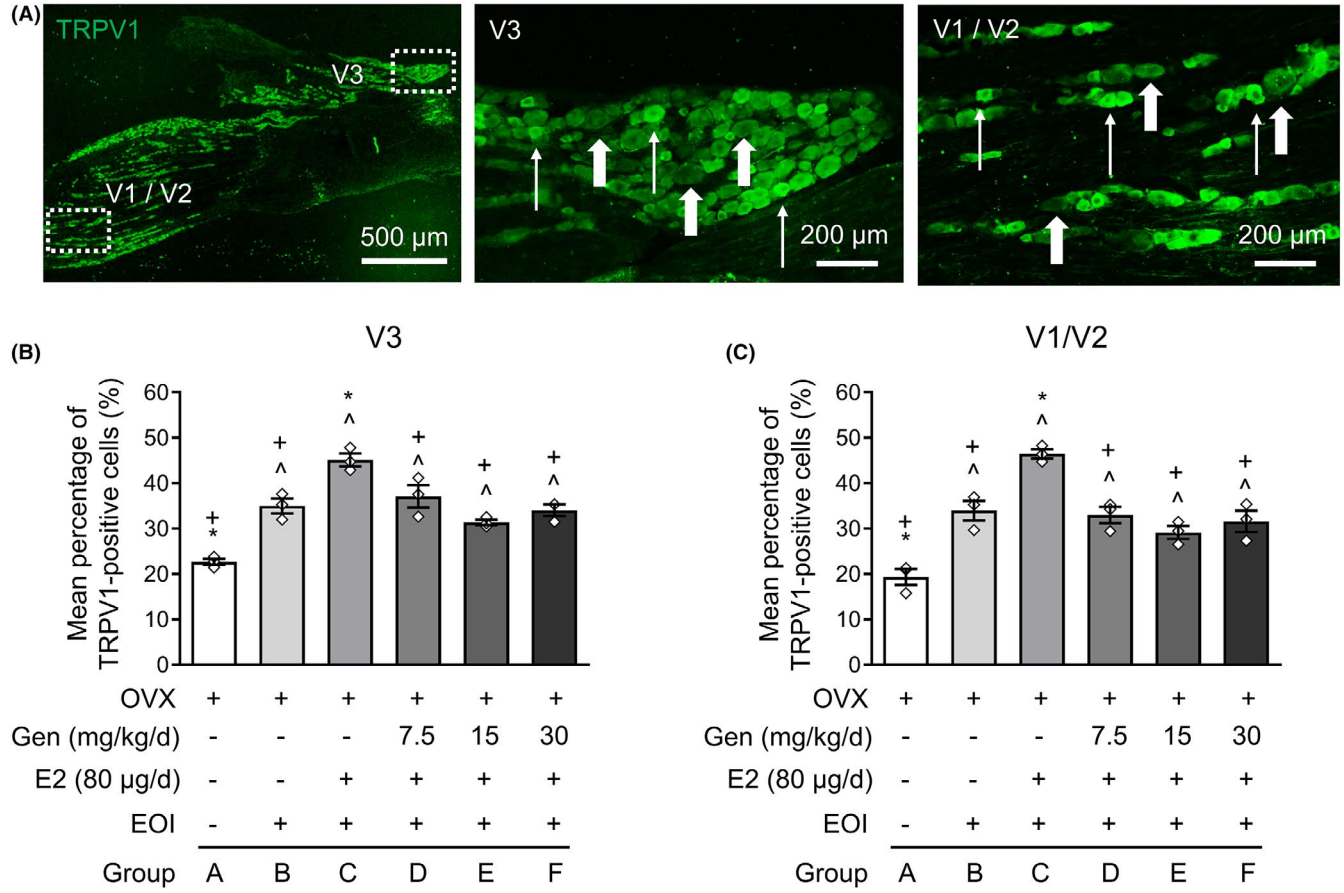

FIGURE 6 Percentages of TRPV1-positive neurons in the TGs were downregulated by different doses of genistein in rats that had undergone consecutive E2 replacement and EOI for 7 days. (A) Representative fluorescence photomicrographs of the expression of TRPV1-positive (green) neurons in the V3 and V1/V2 branches of the TG. The long thin arrows indicate TRPV1-immunoreactive neurons, and the long thick arrows indicate neurons not expressing TRPV1. (B, C) Quantitative analysis of the mean percentages of TRPV1-positive neurons in the V3 (B) and V1/V2 (C) branches of the right TG ($n = 3$ in each group). ^$p < .05$ vs. group A; *$p < .05$ vs. group B; +$p < .05$ vs. group C. One-way ANOVA followed by Bonferroni's post hoc tests. E2, 17β-estradiol; EOI, experimental occlusal interference; Gen, genistein; OVX, ovariectomy

evidence that genistein and E2 likely modulate nociception through TRPV1 expression levels, but the subtypes of ERs involved in this mechanism were not explicated. Further studies are needed to go into more details.

5 | CONCLUSION

In summary, the present study demonstrated sex differences in EOI-induced masseter mechanical hyperalgesia in rats. Female rats displayed an enhanced sensitivity to the EOI-induced nociceptive response compared with male rats. E2 replacement exacerbated EOI-induced masseter hyperalgesia in OVX rats and genistein could antagonise the potentiation on EOI-induced masseter hyperalgesia by E2 via regulation of TRPV1 in the TG. The findings from this study offer important clues to develop sex-specific pharmacological treatment, and the good safety and effectiveness of genistein makes it a promising analgesic candidate for women with chronic pain in masseter muscles.

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CONFLICT OF INTERESTS

All authors declare no conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS

Y. Liu, contributed to the initial design of the study, performed most of the animal and laboratory experiments, and drafted the manuscript; X.Y. Zhang, contributed to the design of experiments, and helped to revise the manuscript; Y.Y. Fan, helped to perform the animal behaviour experiments and the statistical analyses; X.X. Xu, helped with the data analysis and interpretation, and critically revised the manuscript; Q.F. Xie, contributed to the concept of the study and the coordination of all experiments, and revised the manuscript. All authors read and approved the final manuscript.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The detailed data of this study are available from the corresponding author upon reasonable request.

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