BRAIN
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Inflammation-induced mast cell-derived nerve growth factor: a key player in chronic vulvar pain?

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Provoked vulvodynia (PV) is characterized by localized chronic vulvar pain. It is associated with a history of recurrent inflammation, mast cell (MC) accumulation and neuronal sprouting in the vulva. However, the mechanism of how vulvar-inflammation promotes neuronal sprouting and gene-expression adaptation in the spinal cord, leading to hypersensitivity and painful sensations, is unknown.

Here, we found that vulvar tissue from women with PV ($n = 8$) is characterized by MC accumulation and neuronal sprouting compared to women without PV ($n = 4$). In addition, we observed these changes in an animal study of PV. Thus, we found that repeated vulvar zymosan-inflammation challenges lead to long-lasting mechanical and thermal vulvar hypersensitivity, which is mediated by MC accumulation, neuronal sprouting, overexpression of the pain channels (TRPV1 and TRPA1) in vulvar neurons, as well as a long-term increase of gene expression related to neuroplasticity, neuroinflammation and nerve growth factor (NGF) in the spinal cord/dorsal root ganglia (DRG) (L6–S3). However, regulation of the NGF pathway by stabilization of MC activity with ketotifen fumarate (KF) during vulvar inflammation attenuates the local increase of NGF and histamine, as well as the elevated transcription of pro-inflammatory cytokines and NGF pathway in the spinal cord. Additionally, KF treatment during inflammation modulates MC accumulation, neuronal hyperinnervation and overexpression of the TRPV1 and TRPA1 channels in the vulvar neurons, consequently preventing the development of vulvar pain.

A thorough examination of the NGF pathway during inflammation revealed that blocking NGF activity by using an NGF-non-peptide-inhibitor (Ro08-2750) regulates the upregulation of genes related to neuroplasticity and the NGF pathway in the spinal cord, as well as modulating neuronal sprouting and overexpression of the pain channels, resulting in a reduced level of vulvar hypersensitivity. On the other hand, stimulation of the NGF pathway in the vulvar promotes neuronal sprouting, overexpression of pain channels and increase of gene expression related to neuroplasticity, neuroinflammation and NGF in the spinal cord, resulting in long-lasting vulvar hypersensitivity.

In conclusion, our findings suggest that vulvar allodynia induced by inflammation is mediated by MC accumulation, neuronal sprouting and neuromodulation in the vulvar. Additionally, chronic vulvar pain may involve a long-term adaptation in gene expression in the spinal cord, which probably plays a critical role in central sensitization and pain maintenance. Strikingly, regulating the NGF pathway during the critical period of inflammation prevents vulvar pain development via modulating the neuronal changes in the vestibule and spinal cord, suggesting a fundamental role for the NGF pathway in PV development.

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Introduction

Provoked vulvodynia (PV) is chronic vulvar pain of unknown cause, characterized by localized hypersensitivity and severe pain of the vulvar vestibule upon an attempt at vaginal penetration such as vulvar mechanical allodynia, e.g. intercourse, tampon use.[1] PV affects 7% to 15% of women of all ages, and it is associated with reduced sexual desire and satisfaction, anxiety and depression.[2-5]

The pathogenesis of PV is poorly understood, and the aetiology is uncertain. It may be associated with microbial, genetic components, hormonal and immunological factors.[6-10] Nevertheless, histopathologic studies of vestibular biopsy tissue from women with PV[11-13] and laboratory animals with vulvar pain[14-16] suggest that inflammation plays a role in PV development. The suggested mechanism hypothesizes that recurrent vestibular inflammation leads to long-lasting neuronal alterations in the PNS and CNS, including nerve sprouting, neuromodulation and neuroinflammation.[1,17,18]

Immune cells, particularly mast cells (MCs), are major players in nerve sensitization and sprouting.[19] Several findings have shown that mast cell-nerve interactions contribute to multiple inflammatory pathologies.[19,20] Notably, MC accumulation and neuronal sprouting are frequently reported in vestibular biopsy of women diagnosed with PV.[11,21-23] In response to inflammatory stimuli, MCs discharge various mediators, such as nerve growth factor (NGF) and histamine, that are found to be involved in nerve growth and sensitization.[24,25]

Previous evidence suggests that NGF is critical in MC-neuron interaction and pain development.[26] Thus, the released NGF from MCs during inflammation activates tropomyosin receptor kinase A (TrkA) in peripheral nerve terminals, leading to nociceptor sensitization and upregulation of the pain channels TRPV1-transient receptor potential cation channel vanilloid 1 and TRPA1-transient receptor potential ankyrin 1.[27-31] Furthermore, the overactivation of the NGF signalling pathway from the peripheral sensory neurons to dorsal root ganglion and spinal cord can increase the expression of TRP and sodium ion channels, *N*-methyl-D-aspartate (NMDA) receptor and pro-nociceptive components such as calcitonin gene-related peptide (CGRP), resulting in neuroinflammation, neuroplasticity and central sensitization.[26,32]

The mechanism of how repeated vulvar inflammation promotes sprouting and neuromodulation in vulvar neurons and the spinal cord, which provokes prolonged, painful sensations, is still unknown and remains to be fully elucidated. In the present study, we aimed to investigate the long-term effects of inflammation on vulvar sensitivity, local inflammatory changes and gene-expression adaptation in the spinal cord of an animal model of PV. Specifically, we examined whether and how limiting the NGF pathway via stabilization of MC activity and regulation of NGF signalling during vulvar inflammation prevents chronic vulvar pain development by modulating the neuronal changes. Finally, as previously mentioned, anxiety and depression are frequently reported among women with PV. Therefore, we also examined whether vulvar pain in rodents can promote anxiety-like behaviour.

Materials and methods

Participants

Women with PV were referred to our vulvar clinic because of vulvar pain, including severe superficial dyspareunia, tampon use and gynecological examination. The diagnosis of PV was based on Friedrich's criteria (dyspareunia, burning, itching, swab test), excluding erythema.[33,34] None of the women had received interferon or steroid injections to the vestibule, and none had used local or systemic antihistamine or antidepressant medications during the year before their referral to the clinic. The external genitalia, vagina and cervix were examined colposcopically, and wet mounts were taken from the vagina. Women with an active vaginal infection (e.g. bacterial vaginosis, trichomoniasis, vaginal candidiasis) detected in the wet mount were excluded from the study. Examination of the vestibule for possible PV was carried out using a cotton-tipped applicator, pressing on foci throughout the entire vestibule. Foci found to be sensitive were marked on a schematic drawing of the vestibule. The specimen was excised just inside the hymeneal ring, and the vaginal mucosa was undermined for a distance of 2 cm or more (Fig. 1A). The edge of the undermined vaginal mucosa was exteriorized and reapproximated to the remaining perineal skin and lateral vestibule with the suture line 1 to 2 cm posterior to the fourchette. In the control group, women (without PV) underwent vulvar biopsy procedures for pathological examination following surgery for cystocele/rectocele. The study was approved by the Institutional Review Board (Helsinki Committee) of the Galilee Medical Center (0202-18-NHR, 12 March 2019). All participants signed an informed consent form prior to enrolment.

Animals

Female Sprague-Dawley rats (SD; 250–280 g, 10 weeks of age) were used in this study. All animal procedures were approved by the Animal Care and Use Committee (84-11-2019). The animals were housed in groups of 3–4 rats in a sterilized solid bottom cage with contact bedding under controlled temperature and a 12:12 h light/dark cycle. Animals were maintained on a standard pellet diet, and water was supplied *ad libitum*.

Drug preparation

All reagents were dissolved with saline: NGF-β [13.3 µg/ml in 300 µl subcutaneously (sc) in vulva], anti-NGF-β [10 µg in 500 µl

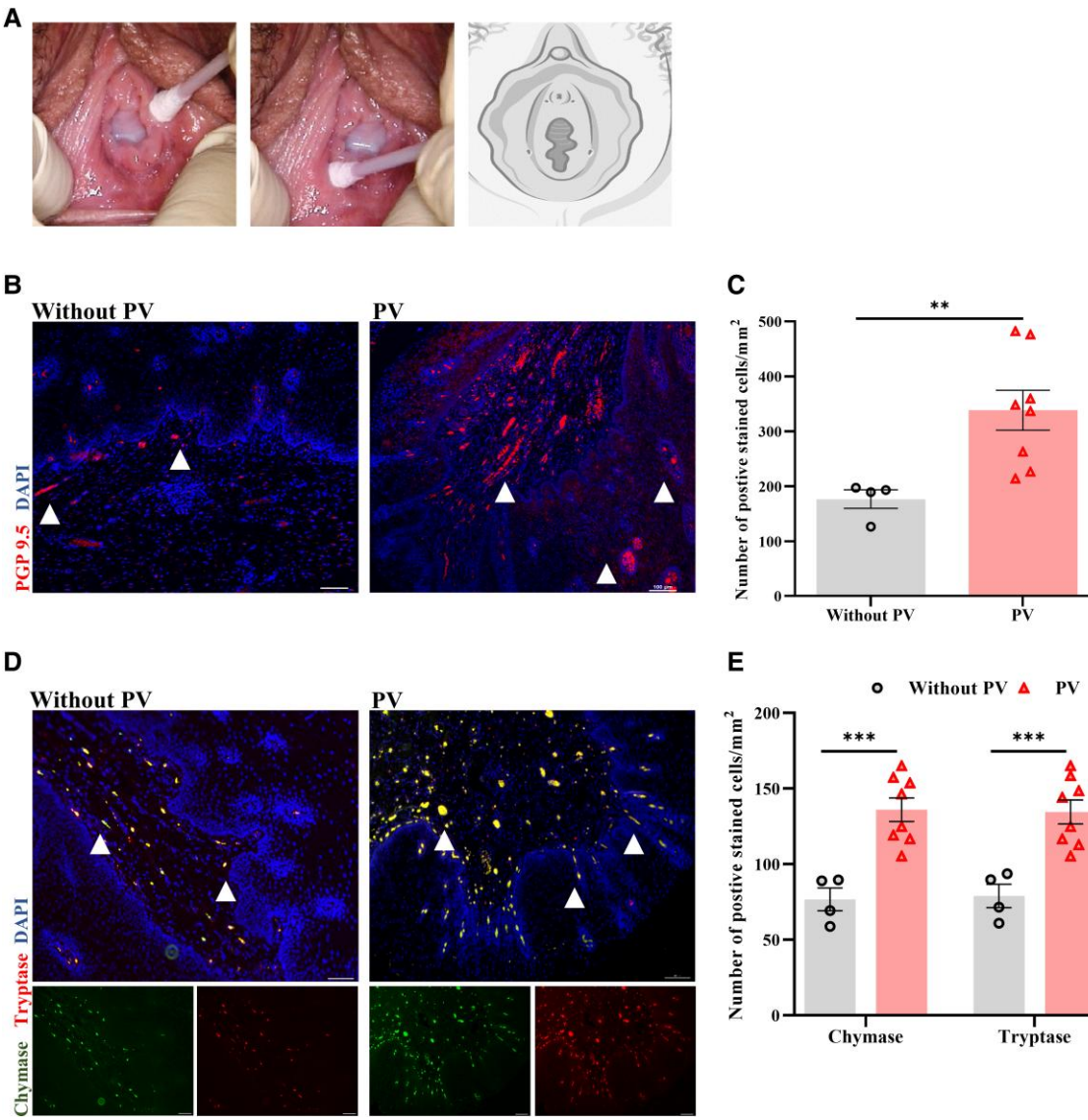

Figure 1 Neuronal sprouting and mast cell abundance in vulvar tissue of women with provoked vulvodynia. (**A**) Illustration of the vulvar pain examination using a cotton-tipped applicator. (**B**) Neuronal sprouting in the vulva tissue of women with provoked vulvodynia (PV) compared to women without PV. White arrows indicate nerve fibres detected by PGP-9.5 (red; white arrows) merged with DAPI stain (blue). Scale bar = 100 µm. (**C**) The number of positively stained nerves by PGP-9.5/mm^2. (**D**) Positively stained cells with mast cell chymase and tryptase in PV and women without PV biopsy vulvar tissue. Mast cell chymase (green; white arrows) and tryptase (red; white arrows) merged with DAPI stain (blue). Scale bar = 100 µm. (**E**) The number of positively chymase or tryptase stained cells of PV and control biopsy vulvar tissue. (n = 4–8 per group). Mean ± standard error of the mean. Student's t-test, $^{*}P < 0.05$, $^{***}P < 0.001$.

intraperitoneally (i.p.) or 1 µg in 100 µl sc in vulva] and zymosan (10 mg/ml in 300 µl sc in vulva). Ketotifen fumarate (KF), Ro08-2750, capsiate and capsaicin were dissolved in DMSO (1% of the final volume), and the stock solution was dissolved with saline [KF, 5 mg/kg in 500 µl i.p.; Ro08-2750, 500 µl of 100 µM i.p., 100 µl of 10 µM sc in vulva or 20 µl of 5 µM intrathecally (i.t.); capsiate or capsaicin, 50 µg in 20 µl i.t.]. Drug delivery and reagent sources are depicted in Supplementary Table 1.

A rat model of vulvodynia

A rat model of vulvodynia was produced using repeated inflammation by zymosan.[18] The zymosan injection (300 µl of 10 mg/ml, 1 ml syringe with a 27G needle) was carried out under isoflurane anaesthesia (Supplementary Fig. 1A).

Behavioural tests and pain assessment

Behavioural tests were performed on animals during the dark phase of the cycle and conducted under red light illumination, following 1 h of habituation in the testing room. All behavioural tests were performed by an observer blind to the experimental condition. The tests were recorded and analysed using a computer-controlled tracking system (EthoVision XT V.15).

Mechanical sensitivity

The mechanical sensitivity was assessed using an electronic Von Frey (VF) device (Cat. No. 38450, Ugo Basile) after 30 min of acclimatization in the testing chambers located 20 cm above the bench. A punctate stimulation was performed on the rat vulva

(Supplementary Fig. 1A). Five values were collected by an observer blind to the experimental condition for each rat.

Thermal sensitivity

The thermal sensitivity test was performed after 30 min of acclimatization in the hot-plate chamber at 35°C (Cat. No. 3515-022, Ugo Basile). After that, the plate temperature increased to 45°C at a rate of 1°C/min. An observer blinded to the experimental condition documented hind paw/vulva lickings, jumping and rearing for each degree interval.

Elevated plus-maze

The elevated plus maze (EPM) consists of two enclosed, opaque Perspex arms [50 (length) × 10 (width) × 30 (height) cm] and two open arms [50 (length) × 10 (width) cm], and both apparatuses are elevated 40 cm above the floor. The animals were placed in the centre of the arms. The session lasted for 5 min.

Open field

The open field (OF) test consists of a large square [70 (length) × 70 (width) × 50 (height) cm]. The animals were placed in the centre of the arena. The session lasted 10 min.

Anxiety index and emotionality Z-score calculation

The Z-normalization methodology was used in the current study as described by Guilloux *et al.*[35] The directionality of the scores was adjusted so that decreased negative Z-score values reflected a high level of emotionality (detailed methods are reported in the Supplementary material):

$$\text{Emotionality Score} = \frac{Z_{OF} + Z_{EPM}}{Number\ of\ parameters} \quad (1)$$

Furthermore, an anxiety index in the EPM was also calculated: the index combines EPM parameters into one unified ratio with values ranging from 0 to 1, with a higher value indicating an increased anxiety level.[36] The following equation was used for the calculation of the anxiety index:

$$\text{Anxiety Index} = 1 - \frac{\left(\frac{\text{Open arms time}}{300\ \text{s}}\right) + \left(\frac{\text{Open arms entry}}{Total\ Entries}\right)}{2} \quad (2)$$

Cell culture and maintenance

Neurons from C1–S2 DRG were collected bilaterally from SD females (1 week age). Sensory neurons were isolated and plated on poly-D-lysine-coated 6- or 96-well tissue culture plates (140675, Thermo Fisher Scientific) and maintained at 37°C in 5% CO_2 (detailed methods are reported in the Supplementary material).

Histology and immunohistochemistry

Tissue collection

The tissue was excised and fixated in 5% paraformaldehyde in phosphate buffer for 1 h and then embedded in paraffin. Sections of 5 μm thickness were cut with a microtome. The sections were then mounted on a Matsunami adhesive glass slide (TM-1190 TOMO, Matsunami).

Immunohistochemistry

Sections were deparaffinized and subjected to heat-induced epitope retrieval. Afterwards, we used a background buster (Cat. No. NB306-50, Innovex), followed by three cycles of wash buffer, and then incubated for 1 h at room temperature with one of the antibodies. After the first immunoreaction, the slides were washed and incubated with the second/or secondary antibody for 1 h at room temperature (antibodies are depicted in Supplementary Table 2). The sections were mounted with 'Flouromount G with DAPI' (eBiosciences) and incubated for 30 s.

Microscopy

Microscopic observation was done using the Eclipse Ci microscope (Nikon Corp.). At least five fields were captured by the Nikon DS-Ri1 camera (Nikon Corp.) with the same microscope settings and exposure time. The images were analysed using NIS Elements analysis software. MC, microglia and neuronal activity were determined by the number of positively stained cells with chymase/tryptase, IBA1 and c-FOS, respectively. The density of TRPV1 and TRPA1 channels that co-localized with PGP-9.5 were determined by fluorescent pixel intensity measurements. Neuronal sprouting was determined by the number of positively stained fibres with S100, and positively stained nerves with PGP-9.5 and CGRP. The morphological score of inflammation in haematoxylin and eosin (H&E)-stained cells was established as described by de Moura Estevão *et al.*[37] The images were analysed by a pathologist blinded to the condition.

Gene expression analysis

The RNA was extracted from the spinal cord/DRG (L6–S3; Supplementary Fig. 1B) by combining TRI reagent (Sigma-Aldrich) and a Purelink TM kit (Thermo Fisher Scientific). cDNA was prepared, and real-time PCR was performed as described in detail in Abu-Ata *et al.*[38] The relative expression of target genes was normalized to β-actin and calculated using the ΔΔCt method, a $\log_{10}$ transformation of the gene expression was performed to ensure symmetric distribution (primers are depicted in Supplementary Table 3).

Biochemistry measurements

NGF and histamine levels were determined in rat vestibule extracts using the rat NGF Sandwich ELISA kit (Cat. No. BE69198, IBL) and the Histamine Competitive-ELISA kit (Cat. No. E-EL-0032, ENCO), according to the manufacturer's instructions.

Mitochondrial function

High-resolution respirometry was performed using the OROBOROS Oxygraph-2k (Oroboros Instruments) on fresh isolated spinal cord (L4–S3; Supplementary Fig. 1B) mitochondria. The mitochondrial oxygen consumption was evaluated as described previously.[39] Detailed methods are reported in the Supplementary material.

Data analysis

No statistical methods were used to predetermine sample sizes, yet the sample sizes were chosen based on our previous studies.[18,40] No explicit randomization method was used to allocate animals to different experimental groups. Statistical analyses were performed

using IBM SPSS statistics and GraphPad Prism. All data are expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed by independent sample *t*-test and one-way ANOVA. Changes in mechanical sensitivity were assessed using a mixed-model repeated-measures ANOVA and a paired sample *t*-test. Significant main effects and interactions were further pursued using Tukey's *post hoc* test and independent sample *t*-test. The accepted significance value for all tests was set at $P < 0.05$.

Results

Nerve sprouting and mast cell accumulation in vestibular tissue of provoked vulvodynia patients

Here, we observed an abnormal remodelling of nerves in the vulvar tissue of women with PV. Thus, there was a robust increase in the density of nerve fibres in women with PV compared to women without PV [$t(10) = 4.03$, $P = 0.003$; Fig. 1B and C and demographic variable depicted in Supplementary Table 4]. Notably, in women with PV, the nerve fibres were found to be intraepithelial. Moreover, we observed a significant increase in MC number in the women with PV compared to women without PV [$t(10) = -4.39$, $P < 0.001$; $t(10) = -4.76$, $P < 0.001$; tryptase, chymase, respectively; Fig. 1D and E].

Mast cell stabilization suppressed vulvar hypersensitivity development but did not relieve vulvar pain

Rats were injected 45 min with KF (i.p; 5 mg/kg) before vulvar inflammation and twice a day to Day 7 after the third inflammation challenge (Fig. 2A). First, there was no significant long-term effect of saline injection in the vulva on the mechanical sensitivity threshold (MST; given that the decrease in the MST of the saline group disappeared after 10 days of the third challenge; Fig. 2B). In the first inflammation challenge, the MST of both zymosan groups recovered to non-allodynic levels (defined as >66% of baseline threshold). However, in the second and third challenges, only the zymosan/KF group recovered to the non-allodynic levels, yet not to the baseline (Fig. 2B). In comparison, the reduction of the MST to the allodynic level in the zymosan group was maintained throughout 60 days after the inflammation challenge compared to the baseline (Fig. 2C). Furthermore, the zymosan group showed a higher thermal sensitivity response (i.e. rearing and vulva licking; Supplementary Fig. 2) than the saline and zymosan/KF group [$F(2,21) = 34.00$, $P < 0.001$; Fig. 2D]. In addition, the mechanical and thermal vulvar hypersensitivity was not due to avoidance or conditioned responses (Supplementary Fig. 3A–C). Moreover, stabilization of MCs in rats with chronic vulvar pain (60 days after the third inflammation challenge) showed no benefit in relieving vulvar pain (Supplementary Fig. 4A and B).

A robust increase in anxiety-like behaviour was observed in the zymosan group compared to the saline and zymosan/KF group. Thus, in the EPM test, the zymosan group spent significantly more time in the closed arms and less time in the open arms compared to the saline group [$F(2,21) = 4.36$, $P = 0.026$; $F(2,21) = 8.62$, $P = 0.002$, respectively; Fig. 2E]. However, there was no significant difference in the time spent in arms between the zymosan/KF and the zymosan group (Fig. 2E). In addition, a significantly higher anxiety score was observed in the zymosan group compared to the saline and zymosan/KF group [$F(2,21) = 11.06$, $P < 0.001$; Fig. 2F].

In the OF test, there was a significant difference in the distance moved and time spent in the centre zone of the arena between the three groups [$F(2,21) = 5.40$, $P = 0.01$ 3; $F(2,21) = 4.23$, $P = 0.028$, respectively; Fig. 2G and H]. Thus, the zymosan and zymosan/KF groups showed a significant decrease in distance moved in the centre of the arena compared to the saline group (Fig. 2H). Regarding the time spent in the centre, there was only a significant difference between the saline and the zymosan/KF group (Fig. 2G). No significant difference was found in the total distance moved in the arena between the groups (Supplementary Fig. 5). Z-score analyses revealed a significant reduction in the integrated total emotionality of the zymosan group but not the zymosan/KF group compared to the saline group [$F(8,66) = 6.07$, $P < 0.001$; Supplementary Fig. 6B].

KF treatment modulates mast cell accumulation, nerve sprouting and neuromodulation of vulvar nerves

MC accumulation and neuronal sprouting were observed following repeated vulvar inflammation. We found a significant increase in the MC number in the zymosan group compared to the saline and zymosan/KF group [$F(2,11) = 44.23$, $P < 0.001$; $F(2,11) = 29.87$, $P < 0.001$; tryptase, chymase, respectively; Fig. 2I and J]. In addition, there was no significant difference in MC number between the zymosan/KF and saline group (Fig. 2I and J). Furthermore, in the zymosan group, we observed a significant increase in the density of the nerve fibres that were stained with S100 and PGP9.5, but not CGRP, compared to the saline and zymosan/KF group [$F(2,8) = 37.84$, $P < 0.001$; $F(2,11) = 16.419$, $P < 0.001$; $F(2,9) = 2.10$, $P = 0.17$; S100, PGP9.5, CGRP, respectively; Fig. 2K and L]. Additionally, an increase in the expression of TRPA1 and TRPV1 were noted in the zymosan group compared to the saline and zymosan/KF group [$F(2,11) = 8.05$, $P = 0.007$; $F(2,10) = 13.76$, $P < 0.001$, respectively; Fig. 2M and N].

Mast cell stabilization regulates protein and gene expression related to inflammation and neuromodulation

Since MCs are a primary source of NGF and histamine, we examined the effects of MC stabilization with KF during vulvar inflammation on the local concentration of NGF and histamine. Rats were injected with KF (i.p; 5 mg/kg) 45 min before vulvar inflammation and 12, 24 and 36 h after the zymosan challenge (Fig. 3A). We found that KF treatment attenuated the upregulated level of histamine. Thus, after 6 and 48 h of each vulvar inflammation challenge, the histamine concentration was significantly higher in the zymosan group compared to the zymosan/KF group (excluding 48 h after the third inflammation challenge; Fig. 3B). In addition, we found KF treatment attenuated the upregulated level of NGF. Thus, after 6, 24 and 48 h of the first and the second inflammation challenge, the NGF concentration was significantly higher in the zymosan group compared to the zymosan/KF (excluding 48 h in the second inflammation challenge; Fig. 3C). However, in the third inflammation challenge, there was no significant difference in NGF concentration between groups (Fig. 3C).

Moreover, to determine the effects of MC stabilization on vulvar inflammation severity, neuroinflammation and hyperexcitability in the spinal cord/DRG (L6-S3), rats were injected with KF (i.p., 5 mg/kg) 45 min before vulvar inflammation and twice a day until Day 7 after the third vulvar inflammation challenge (Fig. 3D). The inflammation assessment analysis revealed moderate chronic vulvar-inflammation in the zymosan/KF group compared to a

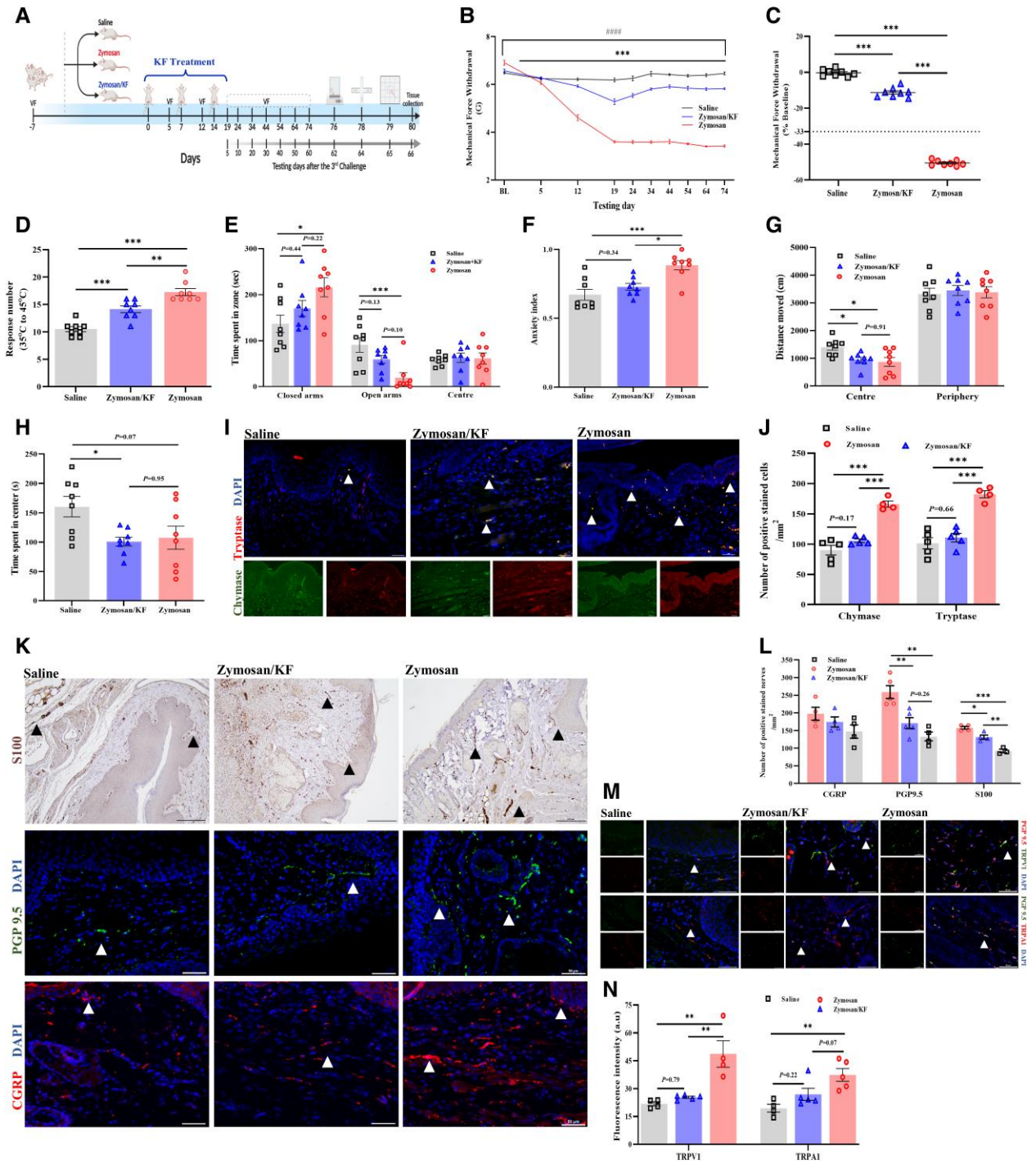

Figure 2 Treatment with ketotifen during inflammation suppressed the development of vulvar hypersensitivity. (**A**) The experimental timeline. Vulvar rats were injected with zymosan or saline on Days 0, 7 and 14; overall, three injections. The zymosan/ketotifen fumarate (KF) group was injected with KF 45 min before vulvar inflammation and twice daily to Day 7 after the third vulvar inflammation challenge. Vulvar mechanical sensitivity was measured by Von Frey (VF). A hot plate test was performed on Day 76, and elevated plus maze (EPM) and open field (OF) tests were performed on Days 78 and 79. (**B**) Mechanical force withdrawal (G) of the zymosan, zymosan/ketotifen, and saline group. (**C**) The mechanical force withdrawal test of Day 74 was normalized to the baseline. (**D**) The sum of nociceptive response in the hot plate test. (**E**) Time spent in open/closed arms and the centre of the EPM (seconds). (**F**) EPM-Anxiety index of the zymosan, the zymosan/KF, and the saline group (0 = low anxiety level; 1 = high anxiety levels). (**G**) Time spent in the centre of the OF (seconds). (**H**) Distance moved in the OF centre and periphery (cm). (**I**) Positively stained cells with MC chymase (green; white arrows) and tryptase (red; white arrows) merged with DAPI stain (blue). Scale bar = 100 µm. (**J**) The number of positively stained cells with chymase or tryptase after 60 days of inflammation challenge. (**K**) Neuronal sprouting, as detected by S100, PGP 9.5 (green) and CGRP (red), merged with DAPI stain (blue). Scale bar = 100 µm, 50 µm. (**L**) Neuronal sprouting assessment by nerve stained/mm^2. (**M**) The expression of TRPV1 and TRPA channels in the vulvar neurons. Co-expression of TRPV1 channel (green) and PGP-9.5 (red; white arrows) merged with DAPI stain (blue), and TRPA1 channel (red) and PGP-9.5 (green; white arrows) merged with DAPI stain (blue). Scale bar = 50 µm. (**N**) Fluorescence intensity (arbitrary units) of TRPV1/PGP-9.5 and TRPA1/PGP-9.5. Behavioural assessment ($n = 8$ per group), IHF ($n = 4$–5 per group). Mixed-model ANOVA: one-way ANOVA followed by Tukey's test. Mean ± standard error of the mean. $^{###}P < 0.001$ compared to baseline; $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$ difference between groups.

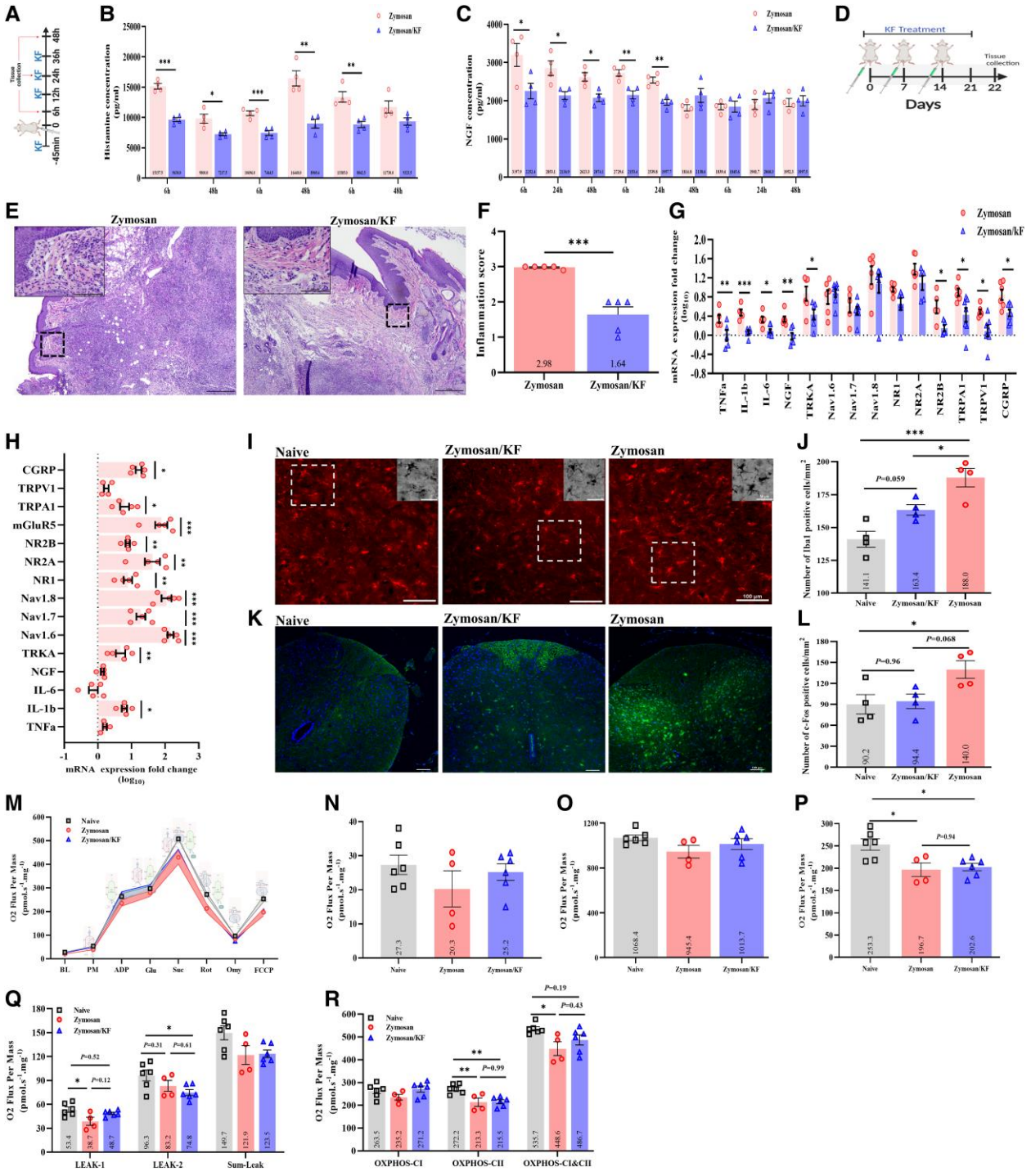

Figure 3 Treatment with ketotifen modulates protein and gene expression of inflammation and neuroplasticity in the vulva and spinal cord/dorsal root ganglia (L6–S3) after vulvar inflammation challenge. (**A**) The experimental timeline. Vulvar rats were injected with zymosan on Days 0, 7 and 14; overall, three injections. Ketotifen fumarate (KF) was injected 45 min before each injection, 12, 24 and 36 h following the zymosan injection. Vulvar tissue was collected after 6, 24 and 48 h of the zymosan injection. (**B**) Histamine concentration in the vulva after 6 and 48 h of each inflammation challenge in the zymosan and zymosan/KF group ($n = 8$ per time point; $n = 4$ per group). (**C**) NGF concentration in the vulva after 6, 24 and 48 h of each inflammation challenge in the zymosan and zymosan/KF group ($n = 8$ per time point; $n = 4$ per group). (**D**) Rats were injected with ketotifen (i.p; 5 mg/kg) 45 min before each vulvar inflammation and twice daily to Day 21 after the third inflammation challenge; rats were sacrificed on Day 22. (**E**) Visual inspection of haematoxylin and eosin (H&E)–stained vulvar tissue sections of zymosan and zymosan/KF group after 7 days of the third inflammation challenge. Scale bar = 50 µm, 200 µm. (**F**) Inflammation score of zymosan and zymosan/KF group. (**G**) The expression of genes related to neuroinflammation, neuroplasticity, and NGF pathway in the spinal cord/DRG (L6–S3) after 7 days of the third inflammation challenge (fold-change $\log_{10}$, $n = 6$–7 per group). (**H**) The expression of genes related to neuroinflammation, neuroplasticity and NGF pathway in the spinal cord/DRG (L6-S3) after 60 days of the third inflammation challenge in the zymosan group (fold-change $\log_{10}$, $n = 5$). (**I**) Iba1 (red) staining of microglia in the spinal cord 7 days after the third vulvar inflammation challenge. Scale bar = 100 µm, 50 µm. (**J**) The number of positively stained cells with Iba1 in the spinal cord. (**K**) Staining of neuronal marker activity c-Fos (green) merged with DAPI stain (blue) in the spinal cord 7 days after the third vulvar inflammation challenge. Scale

(Continued)

severe chronic vulvar-inflammation in the zymosan group [$t(8) = -6.10$, $P < 0.001$; Fig. 3E and F]. Nevertheless, the difference in vulvar inflammation severity was less detectable after 66 days of the third vulvar challenge (Supplementary Fig. 7A and B).

Furthermore, we found that vulvar inflammation promotes a significant upregulation in the transcription of pro-inflammatory cytokines and neuropeptide (IL-6, IL-1β, TNF-a, CGRP; Fig. 3G), sodium-ion channels ($Na_v1.6$-1.8; Fig. 3G), NMDA receptor (Fig. 3G), NGF and TrkA receptor (Fig. 3G) and TRP channels (TRPA1, TRPV; Fig. 3G) in the spinal cord/DRG (L6–S3). Notably, we confirmed the expression of the TRP channels and $Na_v1.8$ in the isolated neuronal tissue from the spinal cord/DRG (Supplementary Fig. 8A and B). In addition, we found that activation of the TRP channels in the spinal cord (L5–L6) induces vulvar and paw mechanical hypersensitivity without affecting motor function (Supplementary Fig. 9A–F).

Regarding MC regulation during vulvar inflammation (Fig. 3D), we found that KF treatment significantly regulates the overexpression of the TRP channels (Fig. 3G), NMDA receptor (Fig. 3G), NGF and TrkA receptor (Fig. 3G) pro-inflammatory cytokines and neuropeptide (Fig. 3G). Moreover, long-term increase in the expression of the TRP, sodium-ion channels, TrkA receptor, NMDA receptor, pro-inflammatory cytokine and neuropeptide were noted in the spinal cord/DRG in vulvar allodynic rats after 66 days of the vulvar inflammation challenges (Fig. 3H).

The neuroinflammation process, hyperexcitability and cellular stress in the spinal cord following vulvar inflammation were validated using Iba1, c-Fos staining and mitochondrial function analysis. Thus, a significant increase in the number of microglia (Fig. 3I and J), neuronal activation marker staining (Fig. 3K and L) and an impairment in mitochondrial oxygen consumption (Fig. 3M–R) were noted in the zymosan group compared to the naïve and zymosan/KF group.

NGF promotes vulvar hypersensitivity development

Upregulation of the NGF pathway after repeated vulvar-inflammation challenges suggests that the NGF pathway is involved in developing vulvar pain.[18] We thus investigated the effect of exogenous NGF challenge on vulvar hypersensitivity. First, we injected NGF (13.3 µg/ml in 300 µl) or saline into the vulva (three injections overall, 7 days between each injection) (Fig. 4A). After the first challenge, the NGF group tended to show recovery to baseline (Fig. 4B). However, in the second and third challenges, there was a significant decrease in the mechanical threshold, with no evidence of healing to the non-allodynic levels in the NGF group (Fig. 4C). During the three vulvar challenges with NGF, and also afterwards, there was a significant difference in mechanical sensitivity between the saline group and the NGF group (excluding 5 days after the first challenge). In comparison to the saline group, the NGF group showed thermal hypersensitivity [$t(14) = -8.07$, $P < 0.001$; Fig. 4D].

Anxiety-like behaviour was observed in the NGF group. Thus, in the EPM, the NGF group spent more time in the closed arms and less time in the open arms compared to the saline group [$t(14) = -4.54$, $P < 0.001$; $t(14) = 4.53$, $P < 0.001$, respectively; Fig. 4E]. In addition, significantly higher anxiety score was observed in the NGF group compared to the saline group [$t(14) = -4.76$, $P < 0.001$; Fig. 4F]. In the OF test, there was a significant reduction in distance moved and time spent in the centre of the arena among the NGF group compared to the saline group [$t(14) = 3.14$, $P = 0.007$; $t(14) = 3.24$, $P = 0.006$, respectively; Fig. 4G and H]. There was no difference in the total distance moved in the arena (Supplementary Fig. 3). The emotionality *Z*-score analyses revealed a significant reduction in the emotionality score of the NGF group compared to the saline group [$F(8,66) = 6.07$, $P < 0.001$; Supplementary Fig. 6B].

Involvement of the NGF pathway in neuronal sprouting and neuromodulation

Neuronal sprouting and neuromodulation were observed in the vulvar tissue following NGF administration (Fig. 4A). We found a significant increase in the expression of TRPA1 and TRPV1 channels in vulvar neurons in the NGF group compared to the saline group [$t(6) = -3.23$, $P = 0.01$; $t(6) = -3.30$, $P = 0.01$, respectively; Fig. 4I and J]. Regarding the neuronal sprouting, we found a significant increase in the density of nerve fibres only in the NGF group compared to the saline group [$t(6) = -4.05$, $P = 0.007$; Fig. 4K and L]. To further pursue the exploration of the NGF signalling overactivation effect in the vulvar on gene expression in the spinal cord/DRG (L6-S3), we injected NGF (13.3 µg/ml in 300 µl) into the vulva, overall three injections, 7 days between each injection (Fig. 4A). After 7 days of the third NGF injection in the vulvar (Fig. 4A), we found a robust increase in the transcription of TRP and sodium-ion channels (Fig. 4M), NMDA receptors (subunits-NR1-NR2B), NGF and TrkA receptor, and pro-inflammatory cytokines and neuropeptide (Fig. 4M) in the spinal cord/DRG (L6–S3). In addition, intraepithelial neuronal sprouting was observed in the vulvar tissue 7 days following the third NGF injection (Fig. 4N).

Furthermore, *in vitro* examination of the NGF pathway revealed that activation of the pain channel with capsaicin (1 µm) or capsiate (1 µm) for 30 min in a sensory neurons culture that was incubated for 10 min with NGF (100 ng/ml) significantly upregulate the mRNA-expression of TRPV1, NGF and c-Fos in the capsaicin group (Fig. 4O), and the transcription of c-Fos and TRKA in the capsiate group compared to the control group (Fig. 4O). In addition, the sensitization of the pain channels by NGF was validated using c-Fos staining. Thus, a significant increase in the number of neuronal activation marker stainings (Fig. 4P and Q) was noted in the capsaicin group that was pre-incubated with NGF (Fig. 4P and Q).

Modulating vulvar hypersensitivity development by interrupting the NGF pathway

Following the previous results, we speculated that blocking the NGF pathway would prevent chronic vulvar pain development. To examine this hypothesis, Ro08-2750 (500 µl of 100 µM i.p. or 100 µl of 10 µM sc in vulvar) and a non-peptide NGF inhibitor, anti-NGF (10 µg in 500 µl i.p. or 1 µg in 100 µl sc in vulvar) were administered at the early inflammation phase (4, 24 and 48 h after zymosan injection) (Fig. 5A).

Figure 3 Continued

bar = 100 µm. (**L**) The number of positively stained cells with c-Fos in the spinal cord. (**M–R**) Mitochondrial oxygen consumption in the spinal cord 7 days after the third vulvar inflammation challenge. Respiration states [LEAK, oxidative phosphorylation (OXPHOS) and electron transfer (ET)] by stimulation/inhibition complex-I (NADH), complex-II (succinate) and ATP synthase. Pyruvate and malate (PM), ADP, glutamate (Glu), succinate (Suc), rotenone (Rot), oligomycin (Omy), uncoupler (FCCP) (**M**). Basal mitochondrial respiration (**N**). Maximal mitochondrial respiration following CI + CII and ATP synthase stimulation (**O**). Mitochondrial respiration in the ET capacity state (**P**). Mitochondrial respiration in the non-phosphorylation resting states-LEAK (**Q**). Mitochondrial respiration in the OXPHOS states is coupled to the phosphorylation of ADP to ATP in the presence of saturating ADP with CI, CII and CI + CII stimulation (**R**). Protein concentration ($n = 4$ per group); H&E analysis ($n = 5$ per group); real-time PCR ($n = 4$–7 per group); IHF ($n = 4$ per group); mitochondrial function analysis ($n = 4$–6 per group). One-way ANOVA followed by Tukey's test; Student's *t*-test. Mean ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

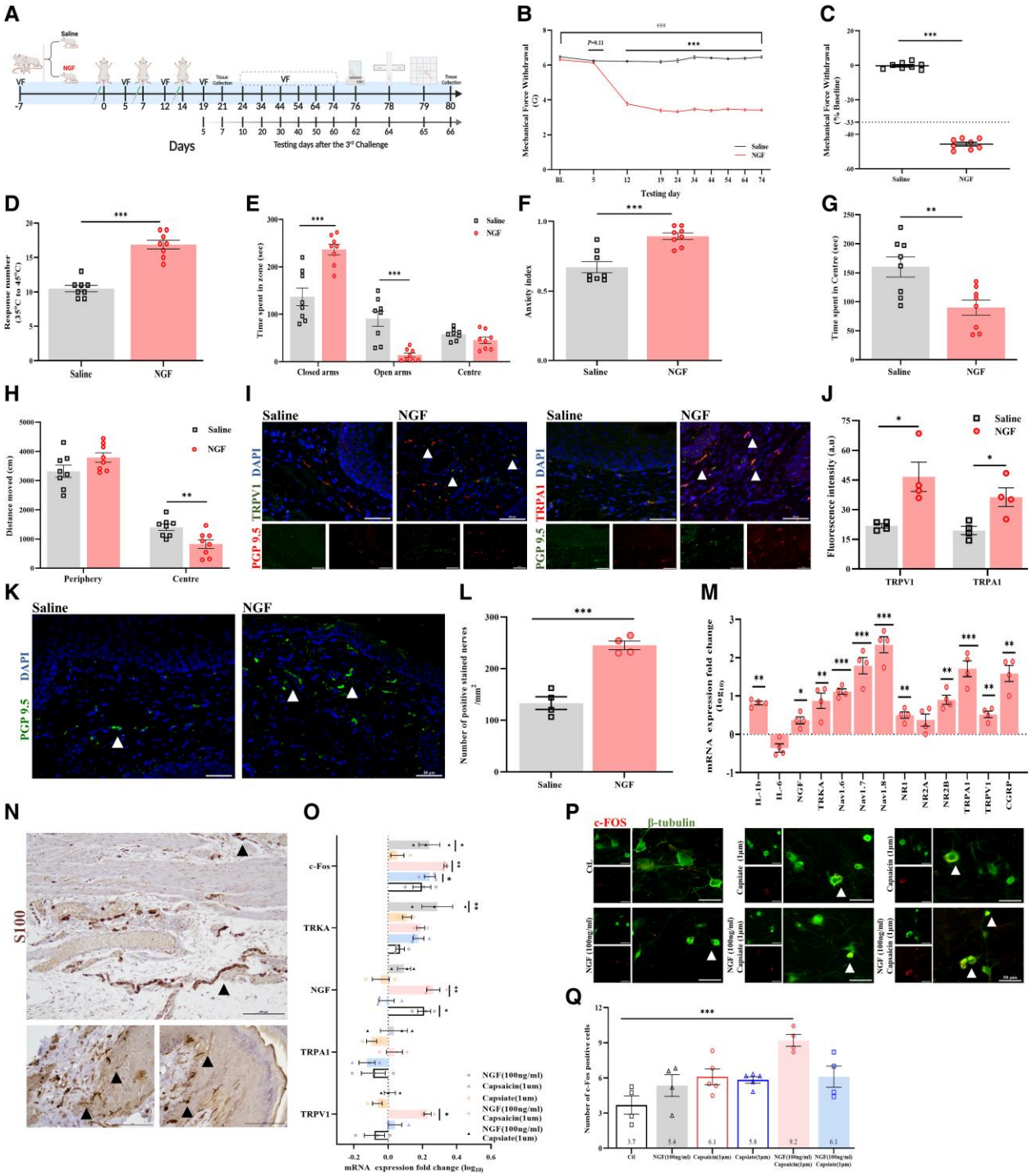

Figure 4 Vulvar hypersensitivity induced by local injection of NGF. (**A**) The experimental timeline illustrates the experimental procedures across three rounds of the NGF vulvar challenge. Vulvar mechanical sensitivity was measured by Von Frey (VF). A hot plate test was performed on Day 76, and elevated plus maze (EPM) and open field (OF) tests were performed on Days 78 and 79. (**B**) Mechanical force withdrawal (G) of the saline and NGF groups. (**C**) The mechanical force withdrawal test of Day 74 was normalized to the baseline. (**D**) The sum of nociceptive response in the hot plate test. (**E**) Time (s) spent in open/closed arms and the centre of the EPM. (**F**) EPM-Anxiety index of the saline and NGF group (0 = low anxiety level; 1 = high anxiety levels). (**G**) Time (s) spent in the centre of the OF. (**H**) Distance moved in the centre and the periphery in the OF (cm). (**I**) The expression of TRPV1 and TRPA1 channel in vulva neurons. Co-expression of TRPV1 channel (green) and PGP 9.5 (red; white arrows) merged with DAPI stain (blue); co-expression of TRPA1 channel (red) and PGP-9.5 (green; white arrows) merged with DAPI stain (blue). Scale bar = 50 μm. (**J**) Fluorescence intensity (arbitrary units) of TRPV1/PGP-9.5 and TRPA1/PGP-9.5 expression. (**K**) Neuronal sprouting in the vulvar tissue, the white arrows indicate nerve fibres detected by PGP-9.5 (green; white arrows) merged with DAPI stain (blue). Scale bar = 50 μm. (**L**) Neuronal sprouting assessment by nerve stained/mm^2. (**M**) The expression of genes related to neuroinflammation neuroplasticity and NGF pathway in the spinal cord/DRG (L6-S3) after 7 days of the third NGF challenge (fold-change $\log_{10}$, $n = 4$). (**N**) Intraepithelial neuronal sprouting 7 days following the third NGF challenge. (**O**) The expression of genes related to NGF, TRP and activation marker in the sensory neuronal culture that was incubated with capsaicin (1 μm) or capsiate (1 μm) for 30 min with/without previous incubation with NGF (100 ng/ml) for 10 min (fold-change $\log_{10}$, $n = 3$). (**P**) Staining of neuronal marker activity c-Fos (red, white arrows) merged with neural marker-β-tubulin (green). Scale bar = 50 μm. (**Q**) The number of positively stained cells with c-Fos. Behavioural assessment ($n = 8$ per group), IHF ($n = 3$–4 per group). Mixed-model ANOVA, followed by Student's t-test; one-way ANOVA followed by Tukey's test. Mean ± standard error of the mean. $^{###}P < 0.001$ compared to baseline; $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$ difference between groups.

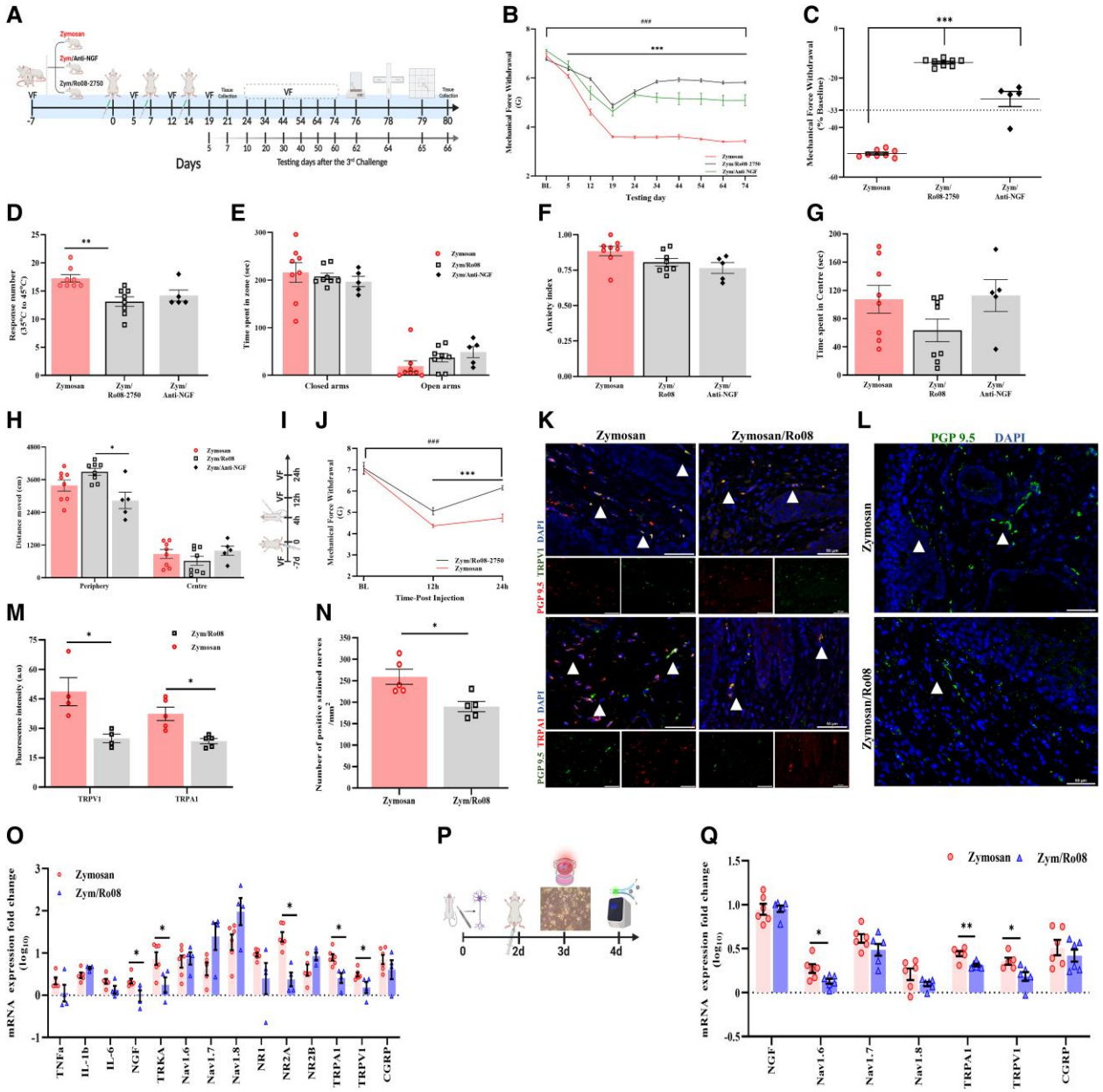

Figure 5 Blocking the NGF signalling pathway during inflammation modulates vulvar hypersensitivity development. (**A**) The experimental timeline illustrates the experimental procedures across three rounds of inflammation vulvar challenge. Ro08-2750 or Anti-NGF was injected 4, 24 and 48 h after each zymosan injection in the vulvar. (**B**) Mechanical force withdrawal (**G**) of the zymosan, zymosan/Ro08-2750 and zymosan/anti-NGF groups. (**C**) The mechanical force withdrawal test on Day 74 was normalized to baseline. (**D**) The sum of the nociceptive response in the hot plate test (35°C to 45°C); only the zymosan/Ro08-2750 group showed a lower thermal sensitivity response compared to the zymosan group. (**E**) Time (s) spent in open/closed arms and the centre of the elevated plus maze (EPM); no significant difference was observed in time spent in closed and open arms [$F(3,25) = 0.42$, $P = 0.73$; $F(3,25) = 2.08$, $P = 0.12$, respectively]. (**F**) Anxiety index of zymosan, zymosan/Ro08-2750 and zymosan/anti-NGF groups [$F(2,18) = 3.38$, $P = 0.057$; 0 = low anxiety level; 1 = high anxiety levels]. (**G**) Time (s) spent in the centre of the open field (OF); no significant difference was observed between groups [$F(2,18) = 2.06$, $P = 0.15$]. (**H**) Distance moved in the centre and the periphery in the OF test (cm). A slight decrease in the distance moved in the periphery was noted in the zymosan/anti-NGF group compared to the zymosan/Ro08-2750 group [$F(2,18) = 6.24$, $P = 0.009$; $F(2,18) = 1.23$, $P = 0.31$; centre, periphery, respectively]. (**I**) The experimental timeline. Vulvar rats were injected with zymosan. After 4 h, Ro08-2750 (5 μM in 20 μl) was injected intrathecally (i.t.) into the spinal cord L5–L6. Mechanical force withdrawal was elevated after 12 and 48 h of the vulvar inflammation challenge. (**K**) The TRPV1 and TRPA1 channel expression in the vulvar neurons. Co-expression of TRPV1 channel (green) and PGP-9.5 (red; white arrows) merged with DAPI stain (blue); Co-expression of TRPA1 channel (red) and PGP-9.5 (green; white arrows) merged with DAPI stain (blue). Scale bar = 50 μm. (**L**) Neuronal sprouting of the vulvar nerves, white arrows indicate nerve fibres as detected by PGP-9.5 (green; white arrows) merged with DAPI stain (blue). Scale bar = 50 μm. (**M**) Fluorescence intensity (arbitrary units) of TRPV1/PGP-9.5 and TRPA1/PGP-9.5 expression. (**N**) Neuronal sprouting assessment by nerve stained/mm^2. (**O** and **Q**) The expression of genes related to neuromodulation and neuroinflammation in the spinal cord (L6–S3) after 7 days of the third inflammation challenge in the zymosan and the zymosan/Ro08-2750 group normalized to the control group (fold-change $\log_{10}$, $n = 4$) (**M**) and in the sensory neuronal culture that was incubated with inflamed-tissue combined with/without Ro08-2750 treatment (fold-change $\log_{10}$, $n = 3$–5) (**P**–**Q**). Behavioural assessment ($n = 5$–8 per group), IHF ($n = 4$–5 per group). Mixed-model ANOVA; one-way ANOVA followed by Tukey's test; Student's t-test. Mean ± standard error of the mean. $^{###}P < 0.001$ compared to baseline; $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$ difference between groups.

First, it should be noted that the local treatment was more effective in modulating the development of chronic vulvar pain than the systemic treatment (Supplementary Fig. 10A–D). Hence, in the local treatment of the NGF blocking groups (Ro08-2750, anti-NGF), more rats recovered to non-allodynic levels (defined as >66% of baseline threshold) compared to the systemic treatment (Supplementary Fig. 10C). In addition, the systemic treatment of blocking the NGF pathway reduced locomotor activity (Supplementary Fig. 5) but not cognitive performance (Supplementary Fig. 10K).

The local treatment with Ro08-2750 (100 µl of 10 µM) and anti-NGF (1 µg in 100 µl), contributed to modulating the development of chronic vulvar pain. In the first inflammation challenge, all groups (the zymosan, zymosan/Ro08-2750 and zymosan/anti-NGF groups) recovered to non-allodynic levels (Fig. 5B). In the second vulvar-challenge, 80% of the rats in the zymosan/anti-NGF and all rats in the zymosan/Ro08-2750 group recovered to non-allodynic levels, compared to 25% in the zymosan group. In the third challenge, 40% of the zymosan/anti-NGF and 87% of the zymosan/Ro08-2750 group recovered to non-allodynic levels. Furthermore, there was a recovery trend to non-allodynic levels; thus, at the end of the experiment (60 days after the third inflammation challenge), all rats in the zymosan/Ro08-2750 and 40% of the zymosan/anti-NGF group were recovered to non-allodynic levels, while no evidence for healing or recovery was observed in the zymosan group. Additionally, the zymosan/Ro08-2750 group showed a lower thermal sensitivity response (i.e. rearing and vulva licking) compared to the zymosan group [$F(2,18) = 7.95$, $P = 0.003$; Fig. 5D].

Regarding the anxiety level and locomotion, there was no significant difference in time spent in the closed/open arms of the EPM or the distance moved and time spent in the centre zone of the arena in the OF between the zymosan, zymosan/Ro08-2750 and zymosan/anti-NGF groups (Fig. 5D–H). However, the zymosan/anti-NGF group tended to have a lower anxiety index score compared to the zymosan group (Fig. 5F).

To explore the beneficial effects of Ro08-2750 treatment in modulating pain development, the Ro08-2750 (20 µl of 5 µM) group was injected intrathecally in the spinal cord (L5–L6) 4 h following the zymosan injection in the vulvar (Fig. 5I). We found a significant improvement in mechanical vulvar hypersensitivity after 12 and 24 h of the zymosan injection (Fig. 5J).

The NGF role in gene-expression upregulation, neuronal sprouting and neuromodulation

Since interrupting the NGF pathway during inflammation modulates vulvar pain development, we investigated whether blocking this pathway modulates the neuronal change in the vulvar and spinal cord. We found that 66 days after the inflammation challenge (Fig. 5A), the zymosan/Ro08-2750 group expressed lower levels of TRPA1 and TRPV1 channels in the vulvar neurons, as well as reduced levels of nerve fibre density compared to the zymosan group [$t(8) = 3.76$, $P = 0.005$; $t(6) = 3.18$, $P = 0.01$; $t(7) = 2.94$, $P = 0.02$, TRPA1, TRPV1, PGP9.5, respectively; Fig. 5K–N].

To explore of the beneficial effects of Ro08-2750 treatment in modulating pain development, we assessed the transcription level in the sacral nerves and spinal cord (L6–S3) 7 days after the third vulvar-inflammation challenge in rats that were treated with Ro08-2750 (100 µl of 10 µM, sc in vulvar). We found that Ro08-2750 treatment modulated the mRNA upregulation of TRP channels, NGF and TrkA receptor and NMDA receptor (subunits NR2A), with no significant effects on the expression of sodium ion channels, pro-inflammatory cytokines and neuropeptide (Fig. 5O). Furthermore, *in vitro* examination of the NGF pathway revealed that Ro08-2750 (1 µM) treatment in cultured sensory neurons that were incubated for 24 h with vulvar-inflamed tissue significantly regulated the mRNA-upregulation of TRP channels and neuropeptide (CGRP), with no significant effects on the expression of sodium-ion channels (Fig. 5P).

Repeated acute inflammation challenges induced vulvar hypersensitivity development

To investigate the clinical scenario in which women experience recurrent vulvar inflammation with a healing period between inflammation outbreaks, vulvar rats were challenged with zymosan. Following each challenge, we assessed the mechanical vulvar hypersensitivity, and rats were re-challenged with zymosan only if vulvar sensitivity had recovered to baseline (i.e. less than 10% change compared to baseline; Fig. 6A). There was no significant long-term effect of saline injections in the vulva on MST given that the decrease in the mechanical threshold of the saline group disappeared after 10–15 days of each injection (Fig. 6B). Overall, four vulvar-inflammation challenges were required to induce chronic vulvar pain. In the first inflammation challenge, mechanical vulvar sensitivity recovered to baseline after 15 days of the vulvar challenge. In the second challenge, mechanical vulvar sensitivity recovered to baseline after 45 days, and after 55 days of the third vulvar challenge. However, in the fourth inflammation challenge, persistent allodynia was present in all rats, and the decrease in vulvar mechanical threshold was maintained throughout 135 days after the fourth inflammation challenge. In comparison to the saline group, the zymosan group showed thermal hypersensitivity after 136 days of the fourth vulvar inflammation challenge (Fig. 6D).

In addition, there was a significant increase in anxiety-like behaviour in EPM and OF tests in the zymosan group. Thus, in the EPM, the zymosan group spent less time in the open arms compared to the saline group (Fig. 6E). Moreover, the EPM anxiety-index analysis revealed a significant increase in the zymosan group compared to the saline group (Fig. 6F). In the OF test, there was a significant decrease in the time spent, and the distance moved, in the centre zone in the zymosan group compared to the saline group (Fig. 6G and H).

To reveal the molecular aspects of the current PV model, the transcription level in the spinal cord/DRG (L6–S3) was evaluated. A significant increase in the transcription of TRP and sodium ion channels, NMDA receptor and IL-1β (Fig. 6I) was noted 140 days after the fourth vulvar-inflammation challenge.

Chronic vulvar pain promotes anxiety-like behaviours

Pearson correlation analysis indicates significant correlations between the reduction in the vulvar mechanical threshold and some parameters of the EPM and OF test (Fig. 6J). Critically, however, a negative correlation regarding the anxiety index ($r = -0.636$, $P < 0.001$; Fig. 6J) such that a more substantial decrease in vulvar mechanical threshold was associated with a robust increase in the anxiety index (Fig. 6J). Moreover, a positive correlation emerged concerning the integrated total emotionality *Z*-scores ($r = 0.452$, $P < 0.001$; Fig. 6K). Thus, the decrease in vulvar mechanical threshold was associated with a substantial reduction in the emotionality score (Fig. 6K).

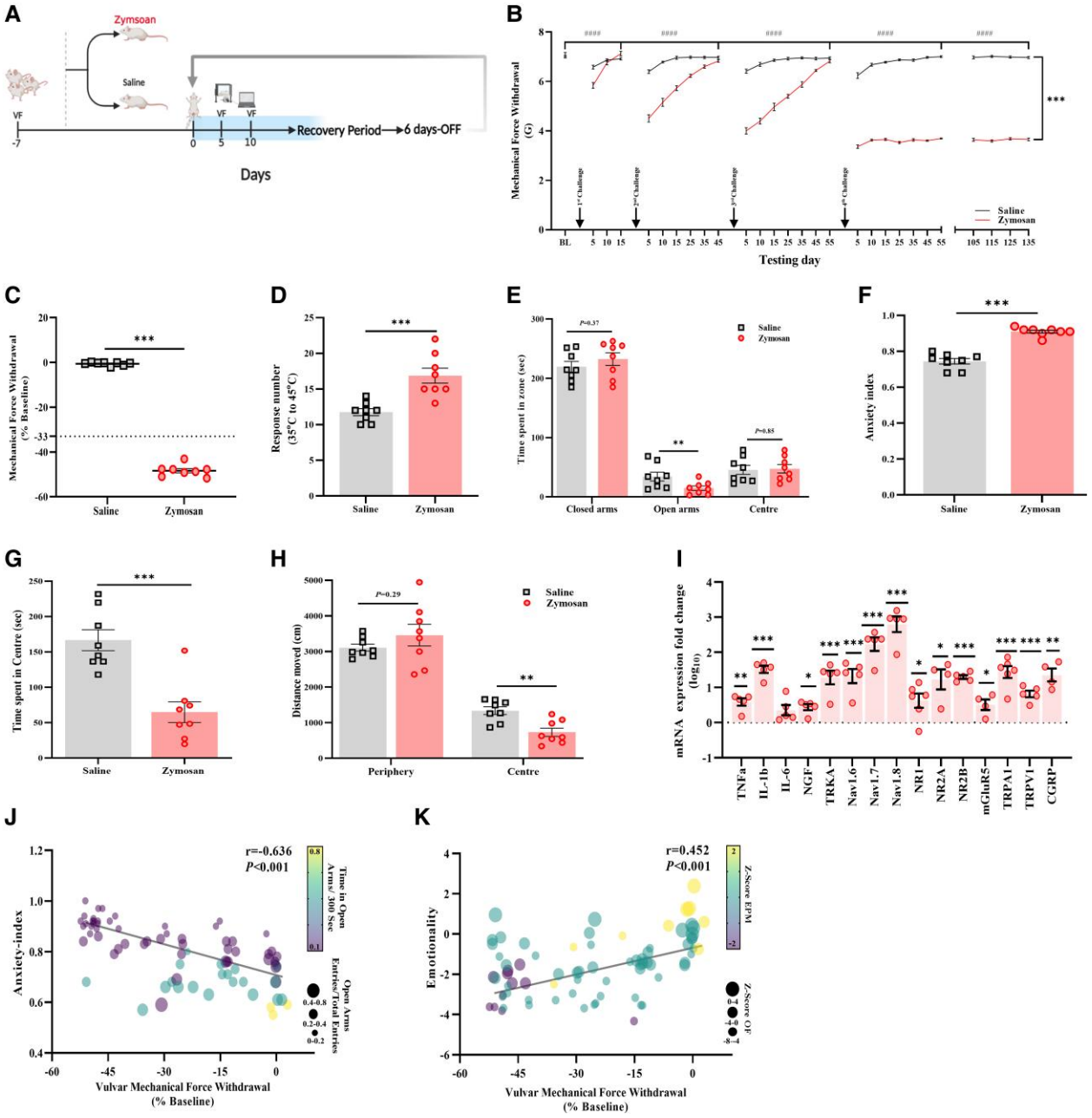

Figure 6 Development of vulvar hypersensitivity after repeated inflammation challenge and healing period. (**A**) The experimental timeline illustrates the experimental procedures across four rounds of vulvar inflammation challenge. (**B**) Mechanical force withdrawal (G) of zymosan and saline group. (**C**) Mechanical force withdrawal test on Day 135 after the fourth vulvar inflammation challenge normalized to the baseline. (**D**) The sum of nociceptive response in the hot plate test (35°C to 45°C) after 136 days after the fourth inflammation challenge [$t(14) = 4.38$, $P < 0.001$]. (**E**) Time (s) spent in open arms [$t(14) = 2.34$, $P = 0.03$], closed arms [$t(14) = -0.92$, $P = 0.37$] and the centre of the elevated plus maze (EPM) [$t(14) = 0.85$, $P = 0.85$]. (**F**) EPM-Anxiety index of the zymosan and saline group [$t(14) = -9.34$, $P < 0.001$; 0 = low anxiety level; 1 = high anxiety levels]. (**G**) Time (s) spent (seconds) in the centre of the open field test (OF) [$t(14) = 4.90$, $P < 0.001$]. (**H**) Distance moved in the centre [$t(14) = 3.84$, $P = 0.002$] and the periphery in the OF (cm). (**I**) The expression of genes related to neuromodulation and neuroinflammation in the spinal cord (L6–S3) after 7 days of the third inflammation challenge in the spinal cord of the zymosan-challenged rats after 135 days of the fourth vulvar inflammation challenge (fold-change $\log_{10}$, $n = 5$–6). (**J**) Negative correlation between the EPM anxiety index and changes in vulvar mechanical threshold in all experiments. (Higher values reflected a high level of anxiety; circle size represents the ratio of entries to open arms; colour represents the ratio of time spent in open arms, $n = 75$). (**K**) Positive correlation between the integrated total emotionality Z-scores (Z-score of EPM and OF tests) and changes in vulvar mechanical threshold in all experiments (negative Z-score values reflected a high level of emotionality; the circle size represents the calculated Z-score of the OF test; colour represents the calculated Z-score of the EPM test, $n = 75$). Behavioural assessment (**B**–**H**, $n = 8$ per group). Mixed-model ANOVA; Student's t-test. Mean ± standard error of the mean. $^{###}P < 0.001$ compared to baseline; $^{**}P < 0.05$, $^{***}P < 0.001$ difference between groups.

Discussion

The current study further supports the hypothesis that inflammation plays a role in the development of PV.[1] Here, we found that repeated vulvar inflammation challenges with/without a healing period led to long-lasting mechanical and thermal hypersensitivity long after the resolution of the inflammation in the challenged rats. Vulvar allodynia was found to be mediated by neuronal alteration, including neuronal sprouting, neuromodulation and

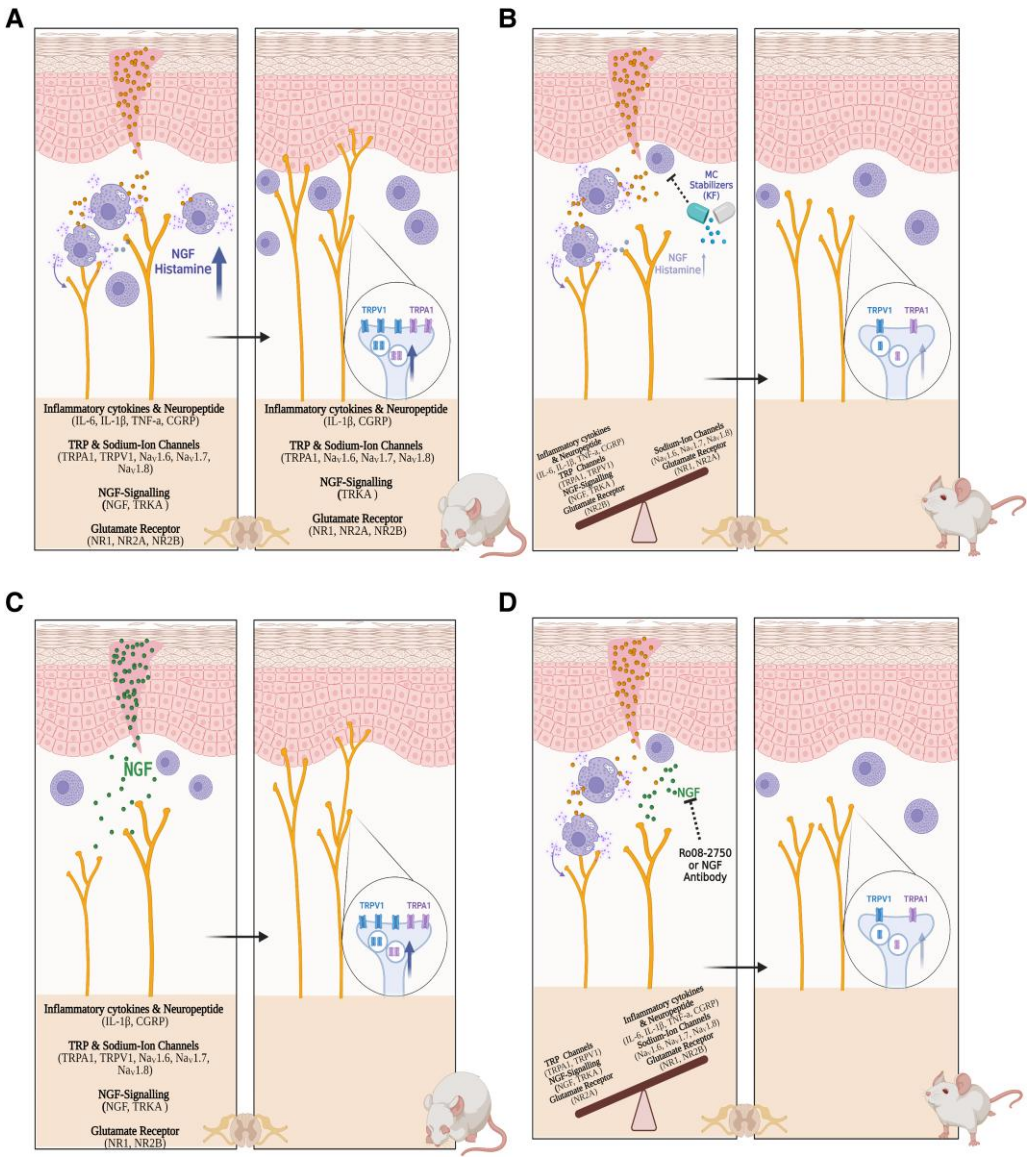

Figure 7 Diagram illustrating the development of chronic vulvar pain induced by repeated inflammation challenge. (**A**) Vulvar inflammation induced by zymosan leads to NGF and histamine release from the inflammation area and increases the transcription of neuropeptide, pro-inflammatory cytokines, glutamate receptors, TRP and sodium ion channels in the spinal cord during inflammation. Strikingly, the inflammation challenge promotes long-term mast cell (MC) accumulation, hyperinnervation, upregulation in the pain channels (TRPA1 and TRPV1) in the vulvar neurons and gene expression adaptations in the spinal cord, resulting in vulvar hypersensitivity that promotes anxiety-like behaviour. (**B**) Stabilization of MC activity by ketotifen fumarate (KF) during vulvar-inflammation challenge attenuates the upregulated level of NGF and histamine, regulates the over-transcription of genes in the spinal cord and modulates MC accumulation and neuronal modification in the vulvar neurons. (**C**) Vulvar-NGF challenge increases the transcription of genes involved in hyperexcitability, neuroinflammation and long-term potentiation and promotes hyperinnervation and overexpression of the pain channels in the vulvar neurons, resulting in vulvar hypersensitivity that promotes anxiety-like behaviour. (**D**) Blocking the NGF signalling pathway during a vulvar-inflammation challenge regulates the over-transcription of genes in the spinal cord and modulates hyperinnervation and overexpression of pain channels in the vulvar neurons.

neuroinflammation in the PNS and CNS (Fig. 7A). Notably, neuronal sprouting was also observed in the vulvar tissue of women with PV.

Unsurprisingly, we found that during vulvar inflammation, there is a significant increase in the expression of pro-inflammatory cytokines, NGF signalling pathway, sodium ion channels, TRP channels and NMDA receptor in the spinal cord. Nevertheless, unexpectedly, the upregulation of these genes continued over two months after the inflammation challenges in the inflammation challenges without a healing period model and strikingly after five months in the inflammation challenges with a healing period model. Repeated vulvar inflammation possibly causes a robust nociceptive signal, as we confirmed by the c-FOS staining, resulting in a long-lasting increase in synaptic efficacy (long-term potentiation) via NMDA receptor and TRP channels.[41-43] In addition, it also might lead to a dramatic neuroinflammatory response and neuronal stress due to the increase of releasing glutamate and CGRP, which can recruit and stimulate the release of pro-inflammatory cytokines from microglia,[44-46] as we confirmed by the Iba1 staining. Together, these changes are likely involved in the mechanisms driving the transition from acute to chronic pain, central sensitization[47] and pain maintenance among allodynic rats.

Nevertheless, we found that limiting MC activity during inflammation by KF, an antiallergic and antihistaminic agent that

inhibits the calcium-dependent degranulation of MCs and non-competitively blocks histamine at the H1-receptor,[48] contributes to the prevention of chronic pain development by modulating inflammation severity, local upregulation of the NGF and histamine concentration, neuronal sprouting, overexpression of pain channels in the vulvar neurons and more importantly regulates the neuroinflammation and neuromodulation process in the CNS. The current results suggest that limiting MC activation is crucial to resolving the ongoing peripheral inflammation and, consequently, modulation of chronic vulvar pain through regulating the neuronal changes in the vulvar and spinal cord (Fig. 7B).

Interestingly, MC activation could establish a positive feedback loop that might also contribute to pain maintenance.[25] This notion is currently being challenged and may deserve reconsideration given the current results that show no significant effect of MC stabilization for 2 weeks in relieving vulvar pain in rats. Nevertheless, the analgesic effects of MC stabilization might be dose-dependent.[49] Therefore, future studies are needed to examine the dose-dependent analgesic effects.

The regulation of NGF concentration in the vulvar and the mRNA expression of NGF and its receptor (TrkA) in the spinal cord by KF during inflammation may be one of the mechanisms to control vulvar pain development.[50] To gain further insight into the role of the NGF pathway in pain development, we explored the effects of vulvar challenge on behavioural and molecular aspects with exogenous NGF. Our results demonstrate that repeated NGF administration in the vestibule upregulate the transcription level of genes that coded several proteins and ion channels that are involved in hyperexcitability, neuroinflammation and neuroplasticity. Notably, the overexpression of these genes is associated with the development of peripheral and central sensitization.[51] As expected, we found that the NGF challenge causes long-lasting vulvar hypersensitivity. Additionally, among NGF-challenged rats, we observed a robust increase in nerve sprouting, pain channel expression and activity (Fig. 7C). These results are in line with previous evidence that has shown that NGF signalling pathway is a major player in promoting neuronal modification (e.g. increasing TRPV1/TRPA1 expression, neuronal growth and sprouting) that probably contributes to the development of chronic pain.[28,52-54]

More importantly, our results show that all the compounds (Ro08-2750; anti-NGF) and strategies (local treatment, systemic treatment) that targeted the NGF pathway during the inflammation were effective in mitigating the development of chronic vulvar pain. Nevertheless, the local treatment was more effective in regulating vulvar pain and safer than the systemic treatment, as was suggested previously.[55] Hence, we found that systemic blocking of the NGF pathway reduces locomotor activity (with no effect on cognitive performance) compared to the control group, although previous evidence has shown no such an effect.[56] Considering the modest group size ($n = 7–8$), which may influence the interpretation of the findings, replication among a larger sample is critical.

Regarding vulvar hypersensitivity, we found that the local treatment with Ro08-2750 or anti-NGF during the early phase of the inflammation modulated the development of mechanical allodynia. Remarkably, we found a significantly lower expression of pain channels and nerve sprouting in vulvar tissue in the zymosan/Ro08-2750 treatment group compared to the zymosan group. These results suggest that blocking NGF signalling during inflammation may decrease hypersensitivity through the downregulation of TRPV1 and TRPA1 channels and modulation nerve growth, as was proposed before.[57] Yet, injection of Ro08-2750 in the spinal cord reverses mechanical sensitivity following the vulvar inflammation challenge, suggesting a downstream role for the NGF pathway in spinal cord pain signalling.

The regulation of gene-expression in the spinal cord via blocking the NGF pathway during peripheral inflammation may be one of the mechanisms to control chronic pain development.[57-59] We found that local treatment of Ro08-2750 during vulvar inflammation significantly diminished the mRNA upregulation of gene expression related to neuroplasticity, neuroinflammation, and nerve growth factor in the spinal cord/DRG (Fig. 7D). Unsurprisingly, the beneficial effects of Ro08-2750 treatment were observed in regulating the transcription level in the sensory neurons co-cultured with inflamed-tissue. Together, these results suggest that the NGF pathway in the sensory neurons during inflammation plays a critical role in the development of chronic pain by promoting long-lasting neuronal alterations in the peripheral and central nervous systems.

Interestingly, current results suggest that the underlying mechanisms of PV may also involve psychological distress contributing to pain maintenance.[40,60-63] Strikingly, we found that chronic vulvar pain in rats causes anxiety-like behaviour and emotional disorder with no effect on locomotor activity. More critically, our results show that the vulvar pain severity was associated with an increase in the anxiety level, as well as a reduction in emotionality score, suggesting that chronic vulvar pain can trigger anxiety and mood disruption, which might contribute to exacerbating the pain symptoms.

In conclusion, current findings suggest that vulvar allodynia induced by inflammation is mediated by MC accumulation, peripheral neuronal alterations including nerve sprouting, and an increase in pain channel expression. In addition, chronic vulvar pain was found to be accompanied by long-term adaptations in gene expression related to neuroinflammation and neuroplasticity in the spinal cord, which could play a role in central sensitization and pain maintenance (Fig. 7A–C). Crucially, reducing NGF signalling by limiting MC activity and blocking NGF activity during vulvar inflammation modulates the neuronal alteration in peripheral (vulvar nerves) and spinal cord, consequently preventing chronic vulvar pain development. On the other hand, stimulation of NGF signalling in the vulvar promotes transcription changes in the spinal cord, vulvar neuronal sprouting and neuromodulation. Given the fundamental role of NGF in chronic pain development, the analgesic effects of drugs targeting the NGF pathway need to be explored in other pre-clinical pain models.

Data availability

The datasets included in the study and the code for statistical analysis are available from the corresponding author upon request.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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