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TNFα in the Trigeminal Nociceptive System Is Critical for Temporomandibular Joint Pain

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Abstract

Previous studies have shown that tumor necrosis factor alpha (TNFα) is significantly increased in complete Freund's adjuvant (CFA)-treated temporomandibular joint (TMJ) tissues. However, it is unclear whether TNFα in the trigeminal nociceptive system contributes to the development of TMJ pain. In the present study, we investigated the role of TNFα in trigeminal ganglia (TG) and spinal trigeminal nucleus caudalis (Sp5C) in CFA-induced inflammatory TMJ pain. Intra-TMJ injection of CFA (10 μl, 5 mg/ml) induced inflammatory pain in the trigeminal nerve V2- and V3-innervated skin areas of WT mice, which was present on day 1 after CFA and persisted for at least 10 days. TNFα in both TG and Sp5C of WT mice was upregulated after CFA injection. The CFA-induced TMJ pain was significantly inhibited in TNFα KO mice. The immunofluorescence staining showed that intra-TMJ CFA injection not only enhanced co-localization of TNFα with Iba1 (a marker for microglia) in both TG and Sp5C but also markedly increased the expression of TNFα in the Sp5C neurons. By the methylated DNA immunoprecipitation assay, we also found that DNA methylation at the TNF gene promoter region in the TG was dramatically diminished after CFA injection, indicating that epigenetic regulation may be involved in the CFA-enhanced TNFα expression in our model. Our results suggest that TNFα in the trigeminal nociceptive system plays a critical role in CFA-induced inflammatory TMJ pain.

Keywords Tumor necrosis factor-α · Temporomandibular joint · Inflammatory pain · Trigeminal ganglia · Spinal trigeminal nucleus caudalis

Introduction

Patients with temporomandibular disorders (TMD) often have pain complaints, but TMD-related pain does not always correlate with definitive tissue pathology [1]. This phenomenon may be due to altered nociception processing in the central nervous system (CNS) of TMD patients. Previous studies have shown that tumor necrosis factor alpha (TNFα) levels are increased in the temporomandibular joint (TMJ) synovial fluid of inflammatory TMD patients [2–6], which is accompanied with TMJ pain. TNFα is a proinflammatory cytokine and plays an important role in the development of chronic pain [7–9]. It has been demonstrated that TNFα increases α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

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(AMPAR) GluA1 phosphorylation and trafficking in spinal dorsal horn neurons and contributes to spinal central sensitization and pathological pain [10–12]. However, it is unclear whether TNFα in the trigeminal nociceptive system contributes to the development of TMJ pain.

It has been shown that TNFα mRNA and protein are significantly increased in complete Freund's adjuvant (CFA)-treated TMJ tissues [13, 14] and trigeminal ganglia (TG) [15]. Dysregulated TNFα can promote cytokine proteome profile alteration and orofacial hypersensitivity [16]. TNFα is an inflammatory mediator produced by activated immune cells, glia, and neurons [17–19]. Although these previous studies suggest that TNFα upregulation in the peripheral tissues is involved in the pathogenesis of inflammation and inflammatory pain, and while systemic administration of a monoclonal antibody to TNF partially alleviates TMJ pain as measured by bite force [20], it is still uncertain whether blocking TNFα expression can inhibit inflammatory TMJ pain. In the present study, we used TNFα knockout (KO) mice to investigate the effect of genetic deletion of TNFα on CFA-induced inflammatory TMJ pain. We also analyzed the expression and distribution of TNFα and its receptor in both TG and spinal trigeminal nucleus caudalis (Sp5C) during the CFA-induced inflammatory TMJ pain.

Materials and Methods

Animals

Male C57BL/6 wild-type (WT) and TNFα KO mice from Jackson Laboratory (8–10 weeks) were used in this study. The mice were housed under standard conditions with a 12-h light-dark cycle, with water and food pellets available ad libitum. In all behavioral experiments, mice were acclimated in our animal facility for a minimum of 1 week before use in experiments and acclimated in the laboratory for at least 30–60 min before testing. All experiments were approved by the Institutional Animal Care and Use Committee at Texas A&M University College of Dentistry. We carried out these experiments in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All efforts were made to minimize pain or discomfort and to reduce the number of animals used.

Intra-TMJ Injection

Intra-TMJ injection of CFA (10 μl, 5 mg/ml, Chondrex, Inc.) or saline (10 μl) was conducted under isoflurane anesthesia. We injected bilaterally CFA or saline into the superior joint space of the TMJ as described previously [21].

Orofacial Mechanical Hypersensitivity Test

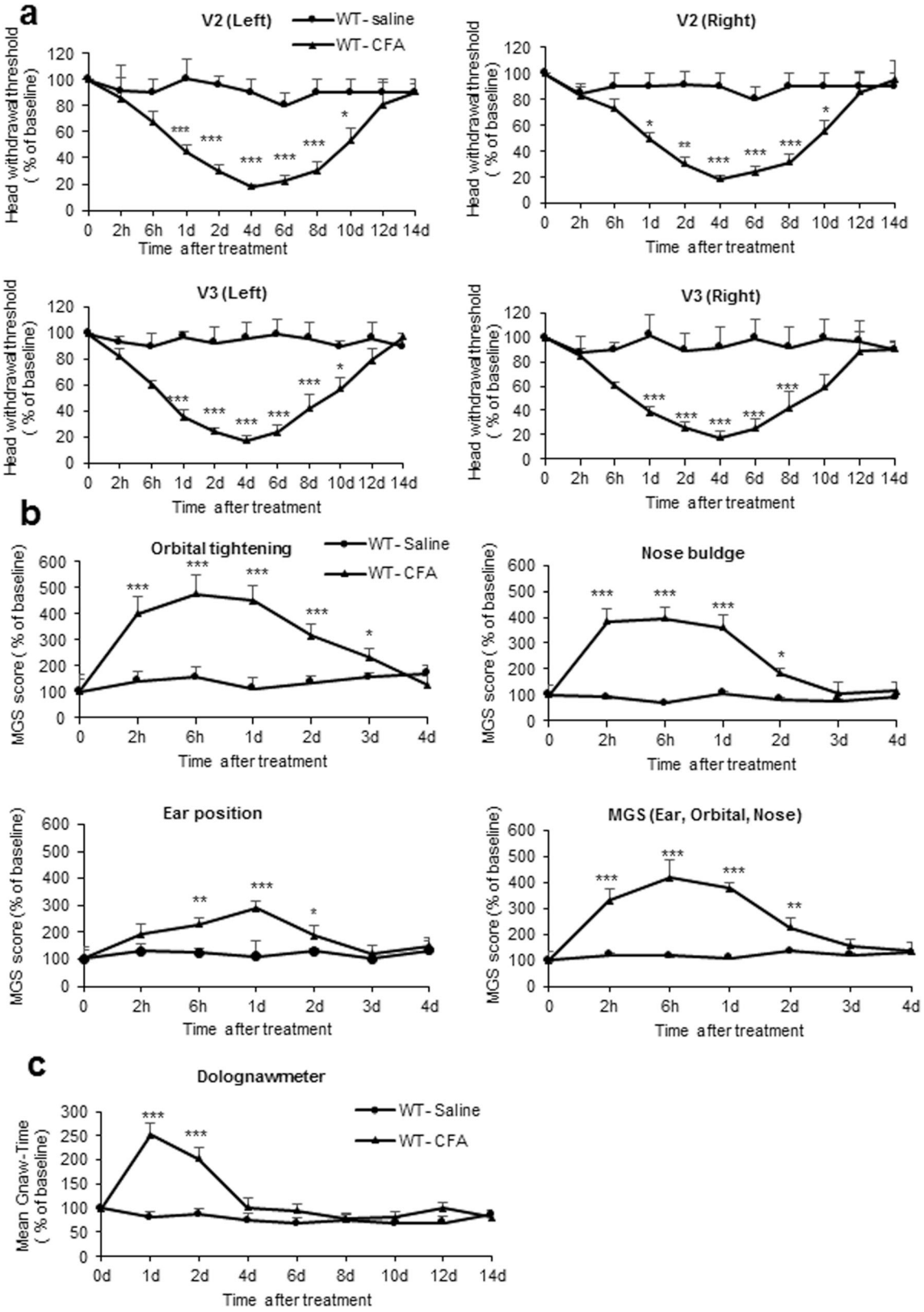
The calibrated von Frey filaments were used to test orofacial mechanical hypersensitivity before treatment and at different time points after intra-TMJ injection of CFA or saline. The mice were placed into a 10-cm-long restraining Plexiglas cylinder and allowed to poke their heads out and forepaws, but the restrainer prevented them from turning around. After acclimation for 5 min, the filament was applied to the skin areas innervated by trigeminal nerve V2 and V3 branches. Each filament was applied five times to the V2- or V3-innervated skin area for 1–2 s with a 10-s interval, starting from the lowest force of filament (0.08 g) and continuing in ascending order. A positive response was defined as a sharp withdrawal of the head upon stimulation. The head withdrawal threshold was calculated as the force at which the positive response occurred in three of five stimuli.

The Mouse Grimace Scale

The Mouse Grimace Scale (MGS), a facial expression-based nociception coding system, has been developed as a reliable measurement approach for spontaneous pain testing in mice [22, 23]. The MGS was performed as described previously [22–24] with minor modification. In brief, after the mice were acclimated in the Plexiglas cylinder for 5 min, we recorded 30-min video with a high-resolution digital video camera before treatment and at different time points after intra-TMJ injection of CFA or saline. The clear face images were selected every 3 min. For each photo, we observed the following three facial expressions [24]: (1) orbital tightening, which is narrowing of the orbital area, with a tightly closed eyelid or an eye squeeze (denoted by wrinkle around eye); (2) nose bulge, which is a rounded extension of skin visible on the bridge of the nose; (3) ear position, which is characterized as the ears pulled apart and back from their baseline position or the formation of vertical ridges owing to the tips of the ears being drawn back. These facial expressions were scored based on the values 0 (not present), 1 (moderately visible), and 2 (obvious). The individual and composite scores were calculated from the mean of the values from 10 images for each mouse per each time point [24].

Functional Allodynia Test

The dolognawmeter, a validated device for oral function measurement [25], was used to test functional allodynia before treatment and at different time points after intra-TMJ injection of CFA or saline. Each mouse was placed into a confinement tube with two obstructing dowels in series. The mouse is required to gnaw through both dowels to escape the device.



Each obstructing dowel is connected to a digital timer, and the timer will automatically stop when the mouse severs the associated dowel. The duration of time required to sever the two dowels and escape the device was recorded. The first six gnawing trials for a mouse are referred to as "training," which allows the animal to learn how to consistently gnaw through

◀ **Fig. 1** CFA induces inflammatory TMJ pain and causes oral dysfunction in WT mice ($n = 6$ for each group). **a** Intra-TMJ injection of CFA significantly decreased the head withdrawal thresholds in both trigeminal nerve V2- and V3-innervated skin areas, starting from day 1 and lasting at least 10 days post-injection (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs the saline control group at the same time point). And the lowest head withdrawal threshold was shown on day 4 after CFA injection. **b** The MGS scores were significantly increased from 2 h to 2 days post-CFA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs the saline control group at the same time point). Among the three facial expressions, orbital tightening was sustained the longest after CFA injection. **c** Gnaw-time in the dolognawmeter test was significantly increased for 2 days post-CFA (***$P < 0.001$ vs the saline control group at the same time point)

the series of two dowels. A baseline gnaw-time was established for each mouse as the mean of the gnaw-times to sever the second dowel during the final three training sessions [25].

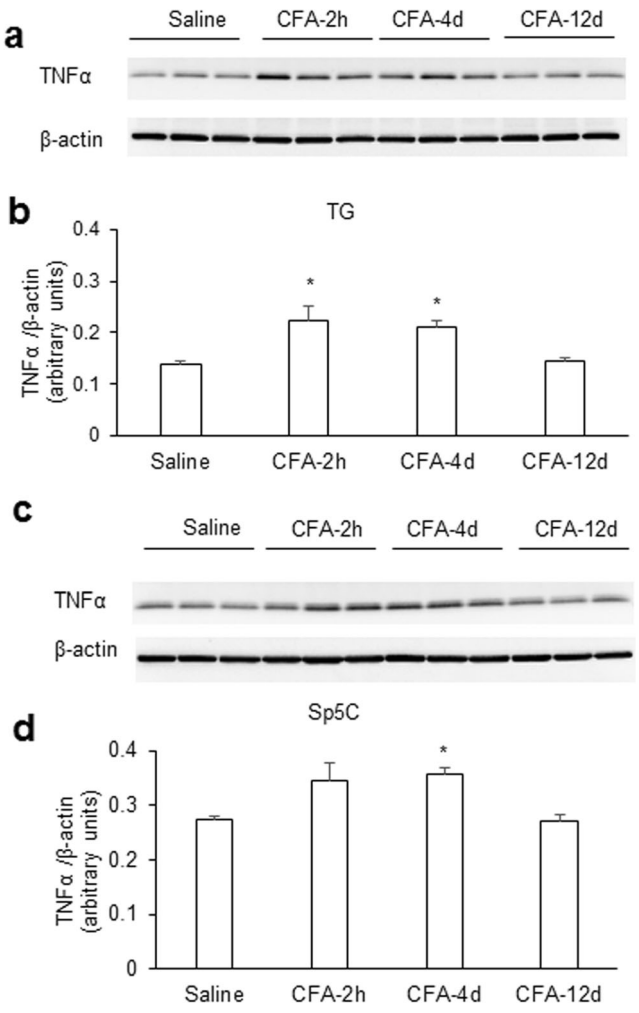


Fig. 2 Intra-TMJ injection of CFA upregulates TNFα expression in both TG and Sp5C ($n = 3$ for each group). **a** In the TG, CFA increased TNFα at 2 h and on day 4 post-injection. **b** Statistical analysis of the data in **a**, *$P < 0.05$ vs the saline control group. **c** In the Sp5C, CFA increased TNFα on day 4 post-injection. **d** Statistical analysis of the data in **c**, *$P < 0.05$ vs the saline control group

Western Blotting

The mice were sacrificed at different time points after intra-TMJ injection of CFA or saline under isoflurane anesthesia. And TG and Sp5C tissues were harvested. The expression of TNFα and TNF receptor 1 (TNFR1) was analyzed with quantitative Western blotting. The affinity-purified antibodies against TNFα (1:2000, ThermoFisher, no. 701135) and TNFR1 (1:2000, Millipore, no. MABC615) were used. β-actin served as a loading control in all Western blot experiments. The intensities of bands in the Western blotting were quantified with densitometry.

Immunohistochemistry

Following the perfusion, TG and Sp5C-containing brain tissues were cut at 20 μm with a cryostat (CM1950, Leica, Chicago, IL). Free-floating slices were blocked in a 5% normal goat serum for 1 h followed by incubation with primary antibodies overnight at 4 °C. Next, the slices were washed and placed in a corresponding secondary antibody conjugated to Alexa Fluor 488 or Cy3 for 1 h at room temperature. Immunofluorescent imaging was observed and analyzed under a Leica fluorescence microscope (DMi8, Leica). The following primary antibodies were used in this study: anti-TNFα antibody (1:400, Abcam, no. ab1793), anti-TNFR1 antibody (1:400, Millipore, no. MABC615), anti-Iba1 (1:300; Wako Chemicals, no. 019-19741), anti-glial fibrillary acidic protein (GFAP, 1:400, EMD Millipore, no. MAB3402), and anti-neuron-specific nuclear protein (NeuN, 1:400, Cell Signaling, 12943S). The specificities of antibodies were validated by using positive and negative controls in our immunofluorescent staining. Cell counting was carried out with ImageJ software.

Methylated DNA Immunoprecipitation Assay

The methylated DNA immunoprecipitation (MeDIP) assay was performed using the EpiQuik Tissue MeDIP Kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions. The genomic DNA was sheared to random fragments between 200 and 1000 bp. Immunoprecipitation was performed using a monoclonal antibody against 5-methylcytosine (Epigentek), and normal mouse IgG was used as a negative control. The immunoprecipitated samples were treated with proteinase K for 3 h at 65 °C and the methylated DNA was recovered by phenol-chloroform extraction, followed by ethanol precipitation. And then quantitative polymerase chain reaction (qPCR) amplification was performed. The relative changes in the methylation levels

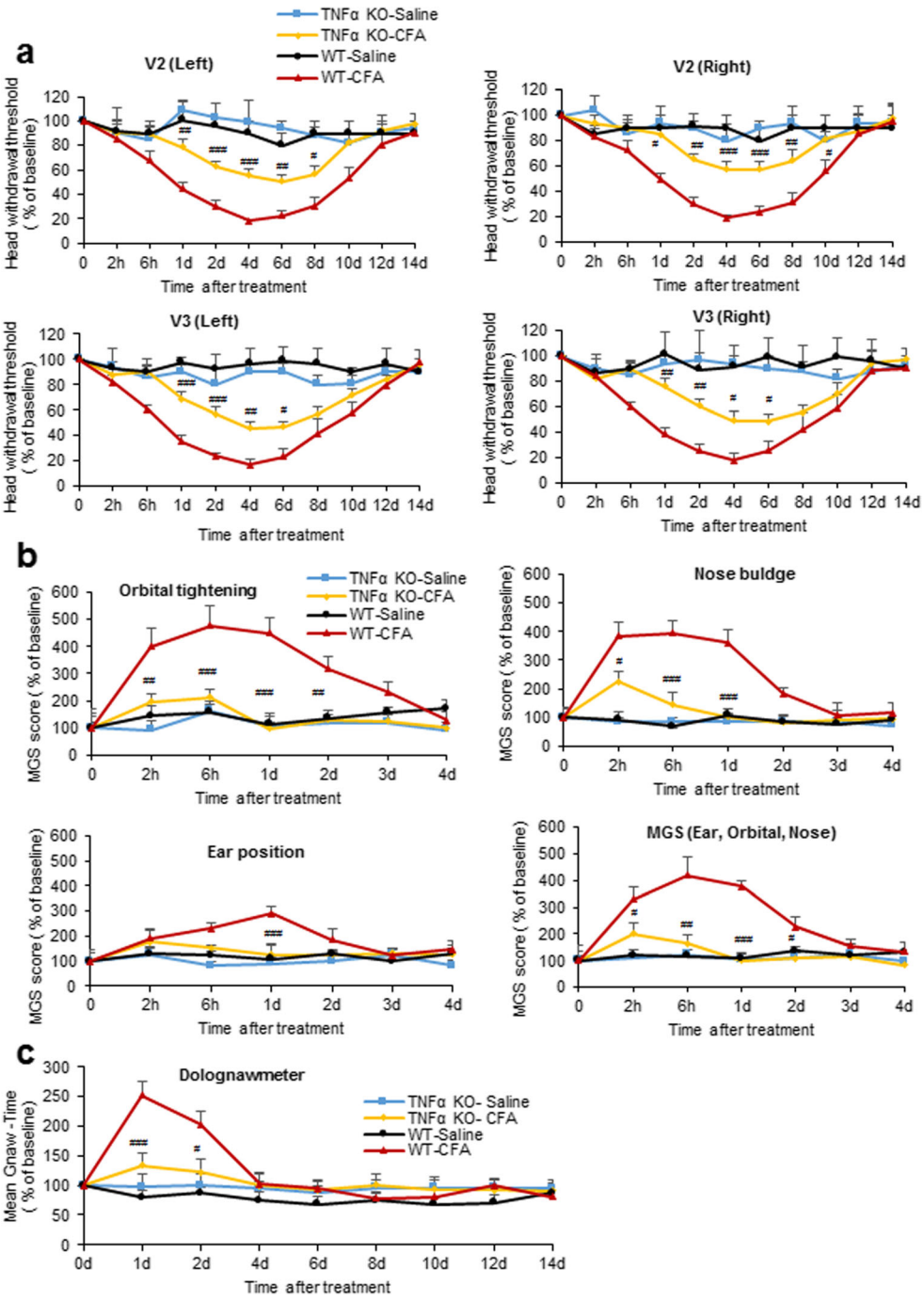


Fig. 3 Genetic deletion of TNFα diminishes CFA-induced TMJ pain ($n = 6$ for each group). **a** Genetic deletion of TNFα in the TNFα KO mice significantly diminished CFA-decreased head withdrawal thresholds in both trigeminal nerve V2- and V3-innervated skin areas ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the CFA group of WT mice at the same time point). **b** In the MGS scoring, genetic deletion of TNFα in the KO mice dramatically reduced CFA-increased MGS scores ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the CFA group of WT mice at the same time point). **c** In the dolognawmeter, genetic deletion of TNFα in the KO mice robustly reduced CFA-increased gnaw-time ($^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs the CFA group of WT mice at the same time point). Note that the data from WT mice are the same as those in Fig. 1

were normalized to the input DNA. The following primers were used in this study: (1) TNFα primer forward CCGAGGGTTGAATGAGAGCTT and reverse TGTTCTCCCTCCTGGCTAGT; (2) mouse β-actin (negative control for MeDIP) forward AGCCAACTTTACGC CTAGCGT and reverse TCTCAAGATGGACCTAATAC GGC; (3) mouse H19 (positive control for MeDIP according to the manufacturer's instructions) forward GCATGGTCCTCAAATTCTGCA and reverse GCATCTGAACGCCCCAATTA; and (4) GAPDH forward TTCACCACCATGGAGAAGGC and reverse GGCATGGACTGTGGTCATGA. The TNFα primers we used detect the TNF gene promoter that is upstream to the transcriptional start site (TSS), which contains five CpG sites. Thus, the altered DNA methylation occurred upstream of the TSS.

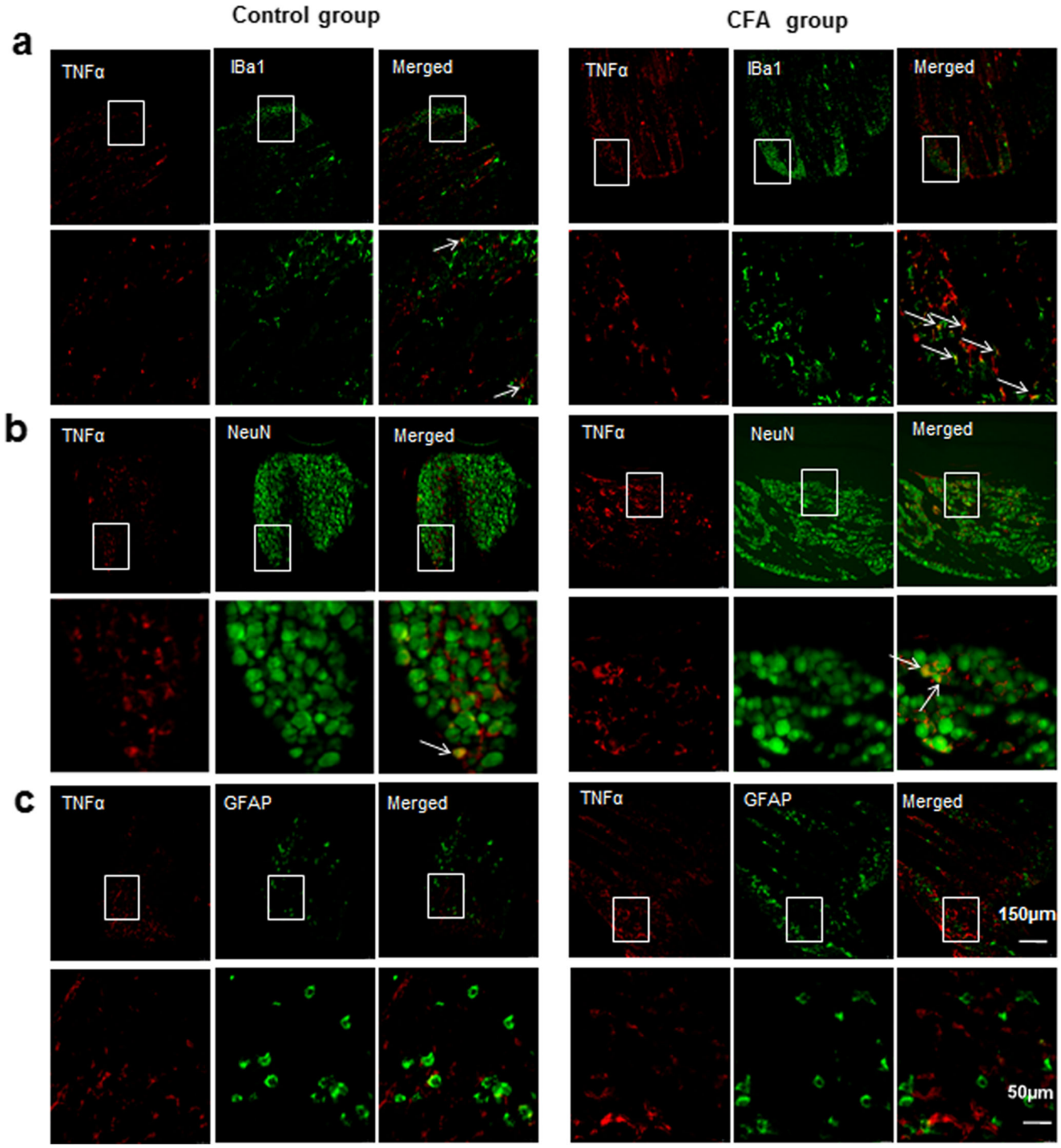


Fig. 4 Intra-TMJ injection of CFA alters the distribution of TNFα in both TG and Sp5C ($n = 3$ for each group). **a** The co-expression of TNFα with Iba1 in the TG was enhanced on day 4 after CFA injection. Statistical analysis of the data was described in the "Results" section. **b** The co-expression of TNFα with NeuN in the TG was similar between the saline and CFA groups. **c** TNFα was not co-labeled with GFAP in the TG of the saline- or CFA-treated mice. **d** The co-expressions of TNFα with Iba1 in the Sp5C were enhanced on day 4 after CFA injection. **e** The co-expressions of TNFα with NeuN in the Sp5C were also enhanced on day 4 after CFA injection. Statistical analysis of the data in **d** and **e** was described in the "Results" section. **f** TNFα was not co-labeled with GFAP in the Sp5C of the saline- or CFA-treated mice

Statistical Analysis

Data are expressed as the mean ± SEM. One-way analyses of variance (ANOVA) was performed for Western blotting data, and two-way ANOVA with repeated measures was performed for behavioral testing data. The Student-Newman-Keuls method was used for post hoc test of ANOVA. The unpaired Student *t* test was used to analyze the data from the immunostaining results

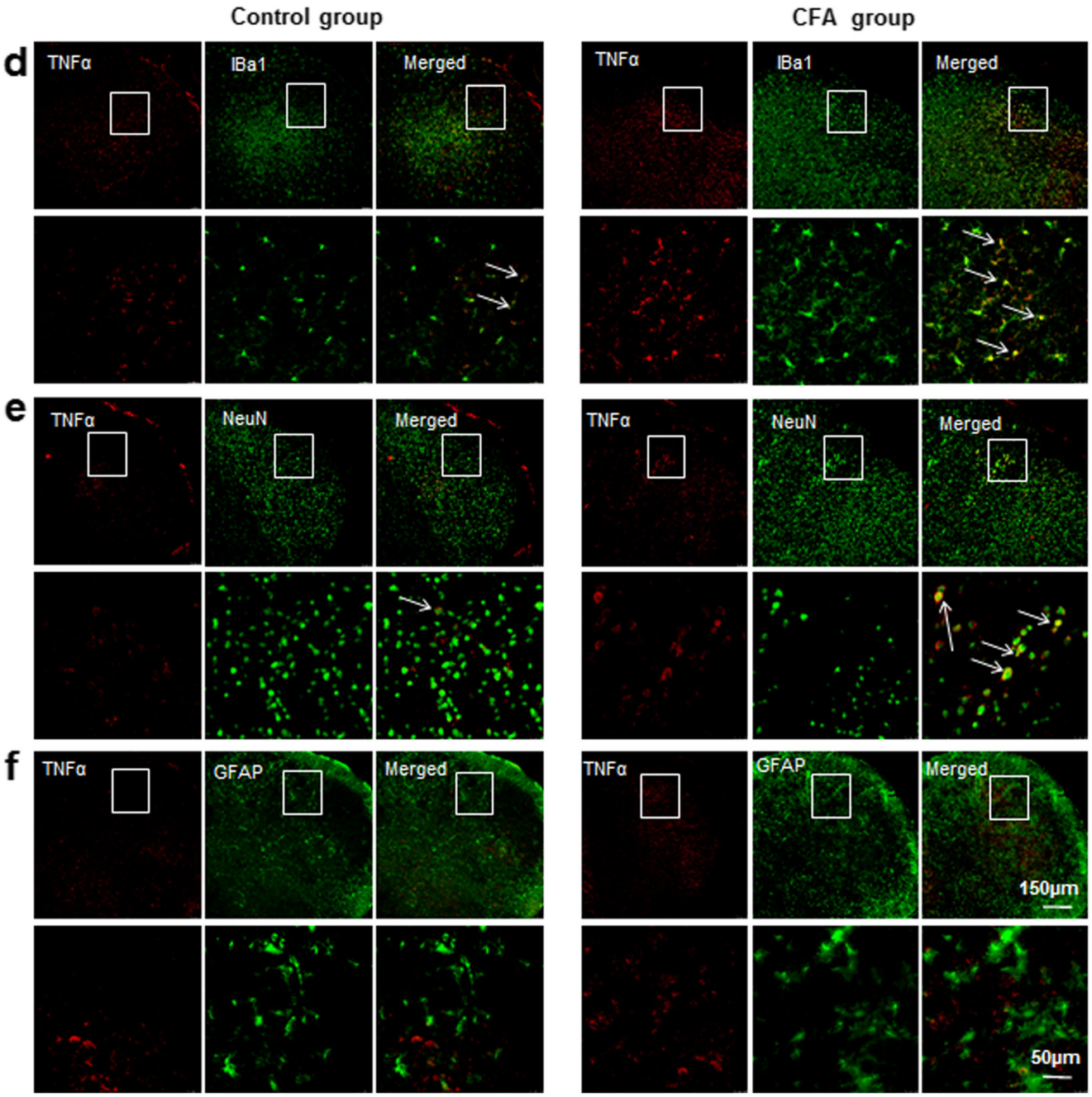


Fig. 4 continued.

and MeDIP qPCR. $P < 0.05$ was considered statistically significant.

Results

CFA Induces Inflammatory TMJ Pain and Causes Oral Dysfunction in WT Mice

To determine whether bilateral intra-TMJ injection of CFA produces orofacial mechanical hypersensitivity and affects oral function, we used different approaches to test nociception behaviors and gnawing activity of mice before and after CFA or saline injection. Using von Frey testing, we observed that the CFA injection significantly decreased the head withdrawal threshold in both trigeminal nerve V2- and V3-innervated skin areas compared with the saline control group, which started from day 1 and lasted at least 10 days post-injection (Fig. 1a). And the lowest head withdrawal threshold was shown on day 4 after CFA injection (Fig. 1a). By the MGS, we analyzed facial expression-indicating spontaneous pain following the CFA injection. The MGS scores were significantly increased

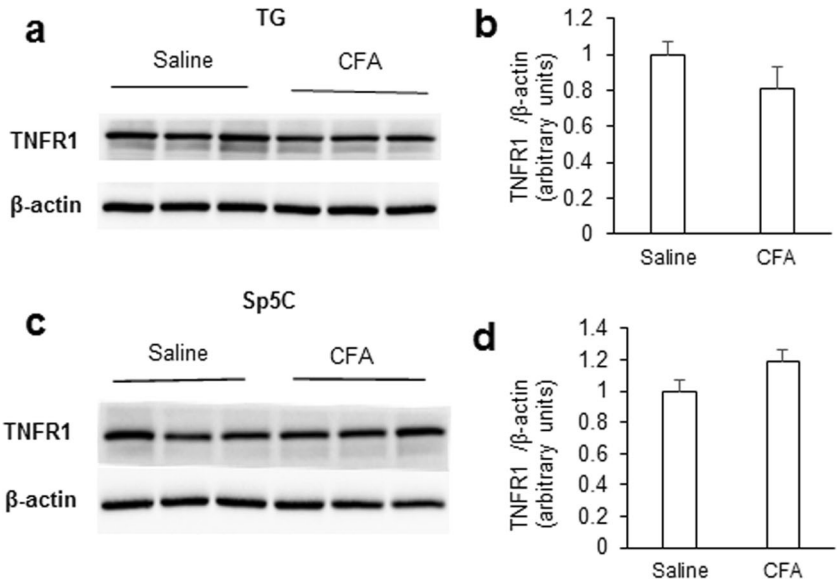


Fig. 5 Intra-TMJ injection of CFA has no effect on TNFR1 expression in both TG and Sp5C ($n = 3$ for each group). Using Western blotting, we showed that the CFA injection did not affect the amount of TNFR1 protein in both TG and Sp5C. **a** In the TG, CFA did not alter the expression of TNFα. **b** Statistical analysis of the data in **a**. **c** In the Sp5C, CFA did not alter the expression of TNFα. **d** Statistical analysis of the data in **c**

from 2 h to 2 days post-CFA compared with the saline control group (Fig. 1b). Among the three facial expressions, orbital tightening was sustained the longest after CFA injection (Fig. 1b). Moreover, we used the dolognawmeter to test functional allodynia following CFA injection. We found that gnawing time was significantly increased for 2 days post-CFA compared with the saline control group (Fig. 1c), indicating that the intra-TMJ injection of CFA induces functional allodynia and causes oral dysfunction.

Intra-TMJ Injection of CFA Upregulates TNFα Expression in Both TG and Sp5C

To verify whether TNFα in the trigeminal nociceptive system is involved in CFA-induced inflammatory TMJ pain, we analyzed the expression of TNFα in TG and Sp5C at different time points after CFA injection. Using quantitative Western blotting, we found that intra-TMJ injection of CFA upregulated TNFα protein levels in both TG and Sp5C (Fig. 2). In the TG, CFA significantly increased TNFα at 2 h and on day 4 post-injection compared with the saline control group (Fig. 2a, b), and in the Sp5C, CFA significantly increased TNFα on day 4 post-injection compared with the saline control group (Fig. 2c, d).

Genetic Deletion of TNFα Diminishes CFA-Induced TMJ Pain

To determine whether TNFα contributes to the pathogenesis of CFA-induced inflammatory TMJ pain, we used TNFα KO mice to investigate the effect of blocking TNFα expression on the inflammatory TMJ pain. We found that genetic deletion of TNFα in the KO mice significantly inhibited the CFA-induced TMJ pain compared with that in the WT mice. In the von Frey testing, genetic deletion of TNFα in the KO mice markedly diminished CFA-decreased head withdrawal thresholds in both trigeminal nerve V2- and V3-innervated skin areas compared with those in the WT mice (Fig. 3a). In the MGS scoring, genetic deletion of TNFα in the KO mice dramatically reduced CFA-increased MGS scores compared with those in the WT mice (Fig. 3b). In the dolognawmeter test, genetic deletion of TNFα in the KO mice robustly reduced CFA-increased gnawing time compared with that in the WT mice (Fig. 3c). Our supplemental experiment with intra-Sp5C injection of a specific TNFα antagonist (R-7050, Cayman Chemical) showed that Sp5C TNFα antagonism significantly increased CFA-decreased head withdrawal threshold (Supplemental Fig. 1), indicating that TNFα in the trigeminal nociceptive system contributes to CFA-induced inflammatory TMJ pain.

Intra-TMJ Injection of CFA Alters the Distribution of TNFα in Both TG and Sp5C

Using double immunofluorescence staining, we showed that TNFα was co-expressed with Iba1 (a marker for infiltrating macrophages in the TG and microglia in the Sp5C) and NeuN (a maker for neurons) in both TG and Sp5C of the saline- or CFA-treated mice (Fig. 4). The co-expression of TNFα with Iba1 in the TG was enhanced on day 4 after CFA injection compared with that in the saline control group (Fig. 4a), and the percentage of double-labeled cells in total Iba1-positive cells of the TG was significantly increased ($18.07 \pm 0.89\%$ for the CFA-treated group vs $5.69 \pm 0.71\%$ for the saline control group, $P < 0.001$). The extent of co-expression of TNFα with NeuN in the TG was similar between the saline and CFA groups (Fig. 4b), and the percentage of double-labeled cells in total NeuN-positive cells of the TG was not significantly changed ($6.8 \pm 1.89\%$ for the CFA-treated group vs $4.66 \pm 0.9\%$ for the saline control group,

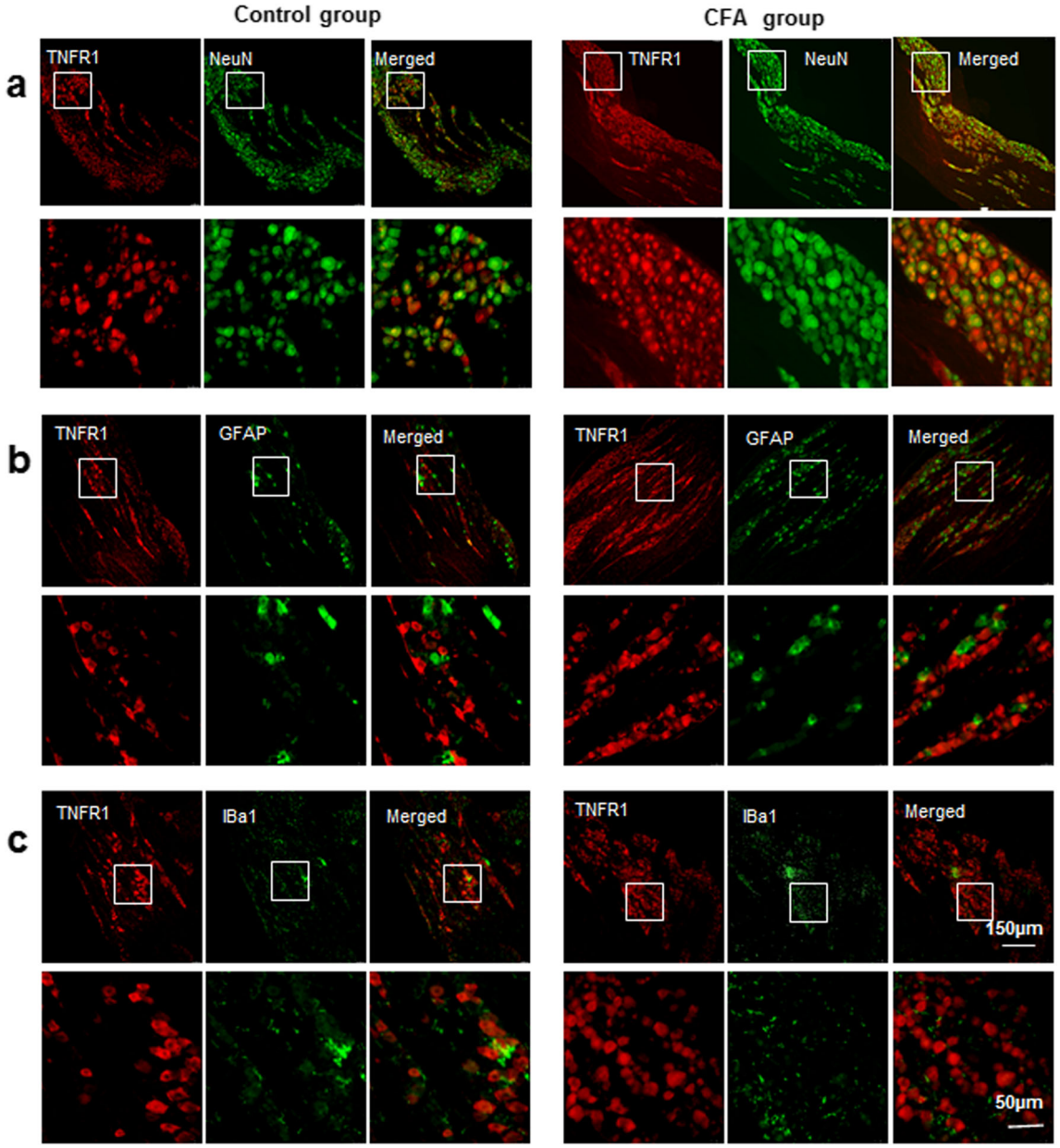


Fig. 6 Intra-TMJ injection of CFA has no effect on TNFR1 distribution in both TG and Sp5C ($n = 3$ for each group). Using double immunofluorescence staining, we showed that TNFR1 was co-expressed with NeuN in both TG and Sp5C of the saline- or CFA-treated mice. **a** In the TG, CFA did not affect the co-expression of TNFα with NeuN. Statistical analysis of the data was described in the "Results" section. **b** TNFR1 was not co-expressed with GFAP in the TG of the saline- or CFA-treated mice. **c** TNFR1 was not co-expressed with Iba1 in the TG of the saline- or CFA-treated mice. **d** In the Sp5C, CFA also did not affect the co-expression of TNFα with NeuN. Statistical analysis of the data was described in the "Results" section. **e** TNFR1 was not co-expressed with GFAP in the Sp5C of the saline- or CFA-treated mice. **f** TNFR1 was not co-expressed with Iba1 in the Sp5C of the saline- or CFA-treated mice

$P > 0.05$). However, the co-expression of TNFα with both Iba1 and NeuN in the Sp5C was enhanced on day 4 after CFA injection compared with those in the saline control group (Fig. 4d, e), and the percentage of double-labeled cells in total Iba1-positive cells of the Sp5C was significantly increased ($50.58 \pm 7.85\%$ for the CFA-treated group vs $15.03 \pm 6.53\%$ for the saline control group, $P < 0.05$) and the percentage of double-labeled cells in total NeuN-positive cells of the Sp5C was also significantly increased ($10.21 \pm 2.2\%$ for the CFA-treated group vs $4.1 \pm 0.13\%$ for the saline control group, $P < 0.05$). The co-localization of TNFα with Iba1 and NeuN was further confirmed by confocal Z-stacking (Supplemental Fig. 2). We also observed that TNFα was not co-labeled with GFAP (a marker for satellite cells in the TG and astrocytes in the Sp5C) in both TG and Sp5C of the saline- or CFA-treated mice (Fig. 4c, f).

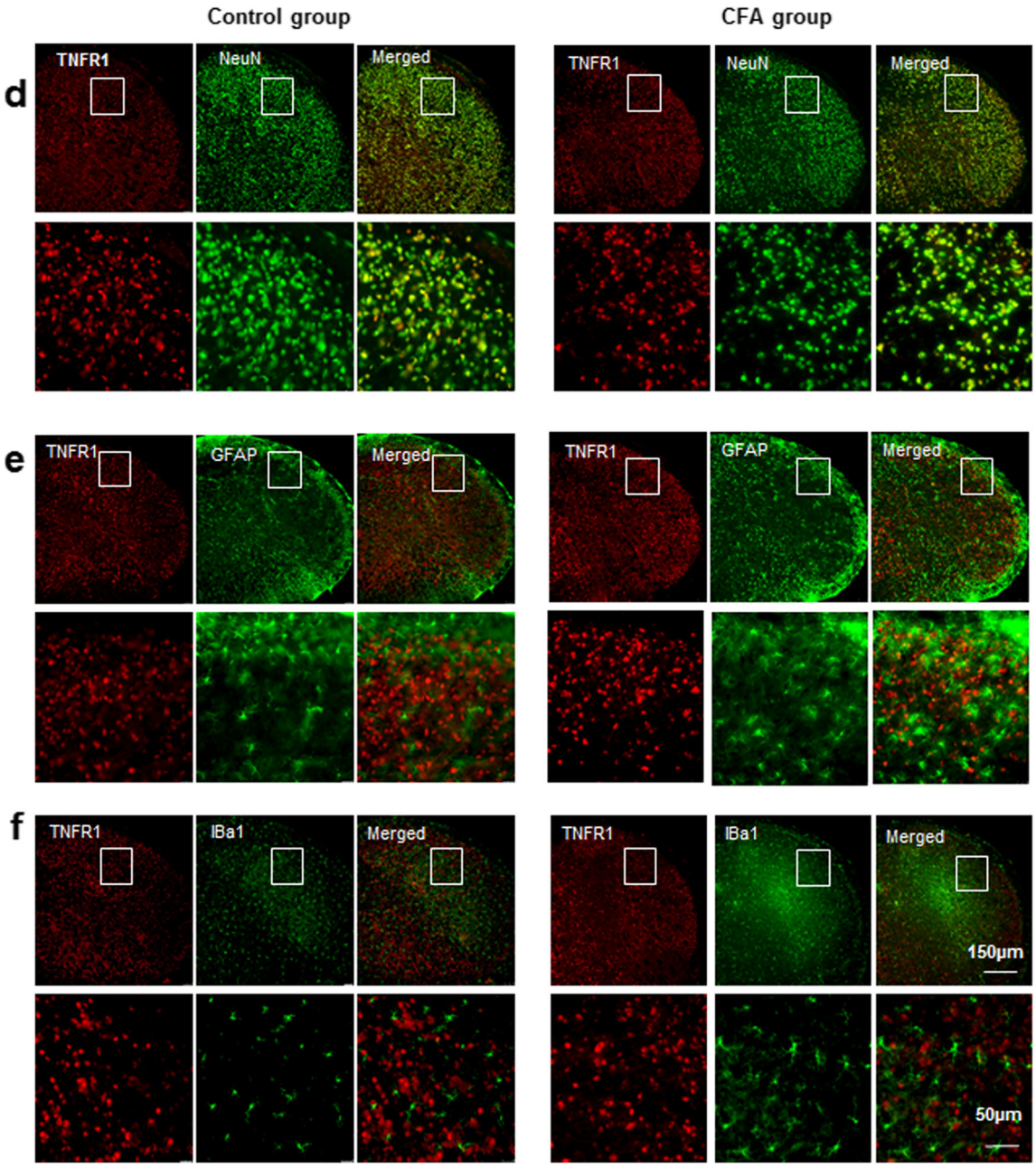


Fig. 6 continued.

Intra-TMJ Injection of CFA Has No Effect on TNFR1 Expression and Distribution in Both TG and Sp5C

To investigate whether intra-TMJ injection of CFA alters the expression and localization of TNFα receptor TNFR1 in the trigeminal nociceptive system, we examined TNFR1 protein level and its distribution in both TG and Sp5C. Using Western blotting, we showed that the CFA injection did not significantly affect the amount of TNFR1 protein in either TG (Fig. 5) or Sp5C (Fig. 5c, d). We further assessed the distribution of this receptor using double immunofluorescence staining. And we showed that TNFR1 was co-expressed with NeuN in both TG and Sp5C of the saline- or CFA-treated mice, and the CFA injection had no effect on the distribution of the receptor on TG or Sp5C neurons (Fig. 6a, d). The percentage of double-labeled cells in total NeuN-positive cells of the TG was not significantly changed ($87.23 \pm 3.22\%$ for the CFA-treated group vs $85.09 \pm 2.7\%$ for the saline control group, $P > 0.05$), and the percentage of double-labeled cells in total NeuN-positive cells of the Sp5C was also not significantly changed ($45.15 \pm 2.77\%$ for the CFA-treated group vs $44.74 \pm 2.57\%$ for the saline control group, $P > 0.05$). We also observed that TNFR1 was not co-expressed with Iba1 and GFAP in both TG and Sp5C of the saline- or CFA-treated mice (Fig. 6b–f).

Intra-TMJ Injection of CFA Inhibits DNA Methylation Specifically at the TNF Gene Promoter Region

To reveal whether epigenetic regulation contributes to the CFA-enhanced TNFα expression in the trigeminal nociceptive system, we conducted MeDIP to analyze DNA methylation at the TNF gene promoter region. We found that intra-TMJ injection of CFA significantly diminished DNA methylation on day 4 post-injection in the TG specifically at the TNF gene promoter region (Fig. 7a, b), indicating that the CFA injection increases TNFα expression through inhibiting TNF DNA methylation.

Discussions

In the present study, we showed that intra-TMJ injection of CFA markedly upregulates the expression of TNFα in both TG and Sp5C and that genetic deletion of TNFα significantly inhibits CFA-induced mechanical hypersensitivity in the trigeminal nerve V2- and V3-innervated skin areas, spontaneous pain measured by the MGS, and oral dysfunction measured by dolognawmeter. Because von Frey filaments were used to measure mechanical hypersensitivity, the MGS was used to measure spontaneous pain with facial expression alteration, and the dolognawmeter was used to measure functional allodynia, we observed different patterns of response to CFA injection in these tests. Our results demonstrate that TNFα upregulation in the trigeminal nociceptive system plays a critical role in CFA-induced inflammatory TMJ pain. Thus, TNFα could be used as a potential target for developing a new approach to treat inflammatory TMJ pain.

TNFα is a proinflammatory cytokine and plays an important role in the development of chronic pain [7–9]. TNFα is first synthesized as a transmembrane protein and cleavage of the extracellular domain of transmembrane precursor TNFα by the matrix metalloprotease TNFα-converting enzyme releases bioactive soluble TNFα [26]. The biologic activities of TNFα are mediated by two TNFRs, including the widely expressed TNFR1 and the limited constitutively expressed TNFR2 [16]. Glia-synthesized TNFα may directly act on primary afferent neurons via TNFR1 but not TNFR2 [27]. Previous studies have shown that the upregulation of TNFα and its receptor TNFR1 in dorsal root ganglia and spinal cord dorsal horn contributes to the development of chronic neuropathic pain [19, 28]. A recent study identified TNFα as a prominent mediator in oral cancer-induced nociception and inflammation [29]. Therefore, TNFα could be a common

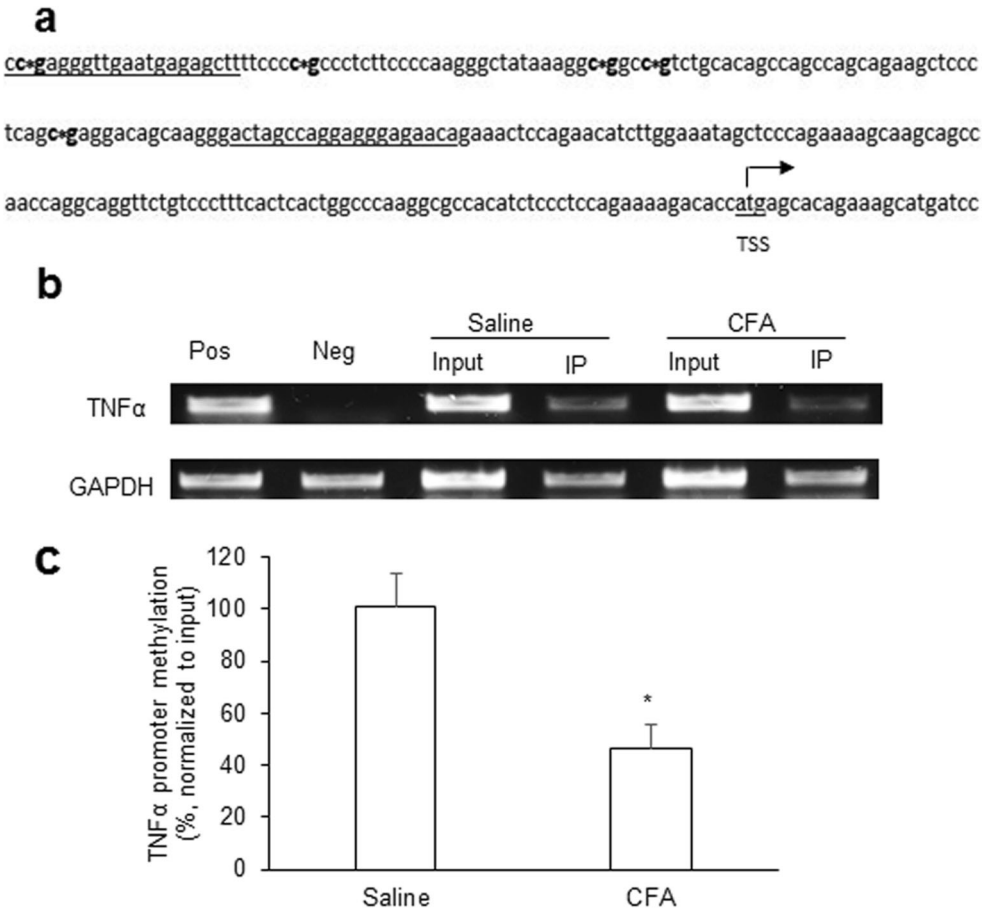


Fig. 7 Intra-TMJ injection of CFA inhibits DNA methylation specifically at the TNF gene promoter region ($n = 3$ for each group). **a** The TNF gene promoter fragment upstream to the transcriptional start site (TSS) contains five CpG sites that are marked with "asterisk" and in bold. The TNF primers we used are underlined. **b** CFA diminished DNA methylation on day 4 post-injection in the TG specifically at the TNF gene promoter region. **c** Statistical analysis of the data in **a**. *$P < 0.05$ vs the saline control group

molecule that is involved in the pathogenesis of different types of pain.

Our data showed that intra-TMJ injection of CFA not only upregulates TNFα expression in the trigeminal nociceptive system but also enhances cell type-specific distribution of increased TNFα. In the TG, CFA injection dramatically elevated the distribution of TNFα in Iba1-labeled macrophages; in the Sp5C, CFA injection significantly increased the distribution of TNFα in both Iba1-labeled microglia and NeuN-labeled neurons. Although the expression of TNFR1 was not altered by intra-TMJ injection of CFA in our model, this receptor potentially linked glia-neuron crosstalk during the CFA-induced TMJ pain because it was exclusively expressed on the neurons in both TG and Sp5C. Our recent experiment showed that TNFR1 co-localized with AMPAR GluA1 in neurons (unpublished data). It has been reported that TNFα increases AMPAR GluA1 phosphorylation and trafficking in the spinal dorsal horn neurons and contributes to inflammatory pain [10, 12]. Moreover, AMPAR phosphorylation and trafficking can alter synaptic AMPAR subunit composition and lead to AMPAR switch from Ca^{2+}-impermeable to Ca^{2+}-permeable receptors [30, 31]. TNFα has been shown to induce a rapid membrane insertion of Ca^{2+}-permeable AMPARs via phosphatidylinositol 3-kinase and protein kinase A-dependent mechanisms [32]. A reduction in Ca^{2+}-permeable AMPARs in the superficial dorsal horn is accompanied by a loss of nociceptive plasticity, whereas an increase in spinal Ca^{2+}-permeable AMPARs facilitates nociceptive plasticity and enhances long-lasting inflammatory hyperalgesia [33, 34]. Therefore, the TNFα-TNFR1-AMPAR pathway-mediated synaptic AMPAR switch from Ca^{2+}-impermeable to Ca^{2+}-permeable receptors is likely one of the underlying mechanisms for the CFA-induced TMJ pain in our current study.

TNFα not only enhances AMPAR synaptic activities but also causes gamma-aminobutyric acid type A ($GABA_A$) receptor endocytosis and results in fewer surface $GABA_A$ receptors and a decrease in inhibitory synaptic strength in the CNS [35]. Thus, TNFα may alter the balance of synaptic excitation and inhibition, which could further promote central sensitization in the trigeminal nociceptive system and contribute to the CFA-induced TMJ pain. Additional experiments will be necessary to determine whether intra-TMJ injection of CFA can strengthen excitatory synapses and weaken inhibitory synapses by enhancing TNFα production and regulating both AMPAR and $GABA_A$ receptor activities accordingly.

DNA methylation has been indicated as an important epigenetic regulatory mechanism in controlling human TNFα transcriptional expression in periodontal disease [36] and in persistent breast pain following breast cancer surgery [37]. The two human studies showed that increased DNA methylation in the TNF gene promoter correlates with periodontal inflammation and breast pain [36, 37]. Compared to other transient epigenetic regulatory mechanisms, such as histone modifications and non-coding RNA modulation, DNA methylation is the least reversible epigenetic regulation. It has been demonstrated that the degree of DNA methylation present in gene promoter regions is usually inversely related to the transcriptional levels of those genes [38, 39]. Our data showed that intra-TMJ injection of CFA robustly diminished DNA methylation specifically at the TNF gene promoter region, which indicates that the CFA injection increases TNFα expression in the trigeminal nociceptive system through inhibiting TNF DNA methylation. The discrepancy about the regulation of DNA methylation in the TNF gene promoter between previous human studies [36, 37] and the present study using a mouse pain model could be due to the following reason: The TNF gene promoters in humans and mice may contain transcriptional binding elements for different transcriptional factors, therefore the increased DNA methylation in the human TNF gene promoter in previous human studies [36, 37] and diminished DNA methylation in the mouse TNF gene promoter in this study may modulate the binding of those different transcriptional factors and then correlate with pain and inflammation accordingly. Further studies will be needed to illustrate which DNA methyltransferases are involved in the epigenetic regulation and whether DNA demethylation also contributes to the CFA-enhanced TNFα expression.

In conclusion, our results demonstrate that intra-TMJ injection of CFA induces inflammatory TMJ pain by increasing TNFα in the trigeminal nociceptive system, which is regulated by TNF DNA methylation. Targeting the epigenetic regulation of TNFα expression may be employed to develop an efficient therapy for inflammatory TMJ pain.

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Compliance with Ethical Standards

All animal procedures were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the Texas A&M University College of Dentistry Institutional Animal Care and Use Committee.

Conflict of Interest The authors have no conflicts of interest to declare.

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