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TNF- α contributes to spinal cord synaptic plasticity and inflammatory pain: Distinct role of TNF receptor subtype 1 and 2

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Abstract

Tumor necrosis factor- α (TNF- α) is a key proinflammatory cytokine. It is generally believed that TNF- α exerts its effects primarily via TNF receptor subtype-1 (TNFR1). We investigated distinct role of TNFR1 and TNFR2 in spinal cord synaptic transmission and inflammatory pain. Compared to wild-type (WT) mice, TNFR1 and TNFR2 knockout (KO) mice exhibited normal heat sensitivity and unaltered excitatory synaptic transmission in the spinal cord, as revealed by spontaneous excitatory postsynaptic currents (sEPSCs) in lamina II neurons of spinal cord slices. However, heat hyperalgesia after intrathecal TNF- α and the second-phase spontaneous pain in the formalin test were reduced in both TNFR1- and TNFR2-KO mice. In particular, heat hyperalgesia after intraplantar injection of complete Freund's adjuvant (CFA) was decreased in the early phase in TNFR2-KO mice but reduced in both early and later phase in TNFR1-KO mice. Consistently, CFA elicited a transient increase of TNFR2 mRNA levels in the spinal cord on day 1. Notably, TNF- α evoked a drastic increase in sEPSC frequency in lamina II neurons, which was abolished in TNFR1-KO mice and reduced in TNFR2-KO mice. TNF- α also increased NMDA currents in lamina II neurons, and this increase was abolished in TNFR1-KO mice but retained in TNFR2-KO mice. Finally, intrathecal injection of the NMDA receptor antagonist MK-801 prevented heat hyperalgesia elicited by intrathecal TNF- α . Our findings support a central role of TNF- α in regulating synaptic plasticity (central sensitization) and inflammatory pain via both TNFR1 and TNFR2. Our data also uncover a unique role of TNFR2 in mediating early-phase inflammatory pain.

Keywords

proinflammatory cytokine; central sensitization; TNFR1; TNFR2; formalin; complete Freund's adjuvant

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This study reveals a critical role of TNF- α in regulating spinal cord synaptic plasticity and central sensitization and uncovers distinct role of TNFR1 and TNFR2 in regulating different phases of inflammatory pain.

1. Introduction

Tumor necrosis factor- α (TNF- α) is a major proinflammatory cytokine produced not only in the immune system but also in the peripheral and central nervous system, especially under the pathological conditions [16;48;51;64;67;68;70]. Increasing evidence suggests a critical role of TNF- α in the pathogenesis of pain [56] including neuropathic pain [55;57;62], inflammatory pain [14;68;72], and cancer pain [13;24]. The peripheral effects of TNF- α on nociceptor sensitization (peripheral sensitization) [37] have been well documented. For example, intraplantar, intradermal, endoneurial, or intramuscular injection of TNF- α elicits heat hyperalgesia and mechanical allodynia [14;54;66;68;76]. TNF- α also modulates the activity of multiple ion channels including TRPV1, Na⁺, Ca²⁺, and K⁺ channels [15;36;43] and induces spontaneous activity in primary sensory neurons [52;59].

Several lines of evidence also suggest a central role of TNF- α in producing central sensitization (e.g., enhanced synaptic transmission and hyperexcitability in dorsal horn neurons) [33;39;42;69;75]. First, TNF- α is induced in spinal cord glial cells in several chronic pain conditions [16;27;70]. Second, intrathecal injection of TNF- α produces heat hyperalgesia and mechanical allodynia [23;39;41]. Third, intrathecal injection of TNF- α inhibitor such as etanercept attenuates chronic pain [7;44;55;57]. Fourth, perfusion of spinal cord slices with TNF- α increases the spontaneous excitatory postsynaptic currents (sEPSCs) [39;42;72;75] and enhances NMDA-induced currents in lamina II neurons [13;39;77]. In addition, TNF- α induces the trafficking and surface expression of AMPA receptors (AMPA receptors), leading to enhanced synaptic transmission in hippocampal neurons [3;60]. After spinal cord injury TNF- α induces rapid trafficking of GluR2-lacking AMPARs to the plasma membrane, leading to cell death of spinal cord motor neurons [19]. Recently, Choi et al. have shown that inflammation also induces a TNF- α -dependent surface trafficking of GluR1 AMPARs in the dorsal horn [11].

TNF- α exerts its effects through two structurally related and functionally distinct receptors, the TNF receptor-1 (TNFR1, p55) and the TNF receptor-2 (TNFR2, p75) [6;9]. Compared to TNFR1, little is known about the function of TNFR2. TNFR1 and TNFR2 may play distinct roles in the nervous system. In culture hippocampus neurons TNFR1 and TNFR2 produce cytotoxic and neuroprotective effects, respectively [9;74]. TNFR1 and TNFR2 also play different roles in regulating neuropathic pain [53;65] and cancer pain [13]. However, it remains unclear whether TNFR1 and TNFR2 differentially regulate synaptic transmission and inflammatory pain.

In this study, we used TNFR1 and TNFR2 knockout (KO) mice, as well as TNFR1/2 double knockout (DKO) mice to determine the distinct role of these two receptors in inflammatory pain conditions following intraplantar injection of formalin or complete Freund's adjuvant (CFA) or intrathecal injection of TNF- α . We also used patch clamp recording in isolated spinal cord slices to investigate whether TNF- α could potentiate excitatory synaptic transmission and NMDA receptor activity via different TNF receptors. Our data demonstrated a critical role of TNF- α in regulating central sensitization and inflammatory pain via both TNFR1 and TNFR2. Our data also revealed a predominant role of TNFR1 in mediating all phases of inflammatory pain and a unique role of TNFR2 in mediating early-phase inflammatory pain.

2. Methods

2.1. Animals and pain models

Knockout mice lacking TNFR1 (*Tnfrsf1a*^{-/-}), TNFR2 (*Tnfrsf1b*^{-/-}), and TNFR1/R2 (*Tnfrsf1a/1b*^{-/-}) were purchased from Jackson Laboratories and bred in the Thorn Research

Building Animal Facility of Harvard Medical School. Because all these KO mice have the same C57BL/6 background, we used C57BL/6 wild-type mice as controls. TNFR1- and TNFR2-KO mice and TNFR1/R2 DKO mice were viable and showed no developmental defects. Adult mice (8-10 weeks) were used for behavioral studies. Young mice (3-5 weeks) were used for electrophysiological studies, because 1) high quality spinal cord slices and patch clamp recordings could be obtained from young animals, 2) spinal cord circuit is fully developed by postnatal age of two weeks [20], and 3) expression of sodium channels such as Na(v)1.8 and Na(v)1.9 reaches adult levels by postnatal day 7 [4]. All animal procedures performed in this study were approved by the Animal Care Committee of Harvard Medical School. To produce acute inflammatory pain, 20 μ l of diluted formalin (5%) was injected into the plantar surface of a hindpaw. To produce persistent inflammatory pain, complete Freund's adjuvant (CFA, 20 μ l, 1 mg/ml, Sigma) was injected into the plantar surface of a hindpaw.

2.2. Drugs and drug administration

TNF- α was purchased from R & D. NMDA, AMPA, and MK-801 were obtained from Sigma. For intrathecal injection, spinal cord puncture was made with a 30G needle between the L5 and L6 level to deliver reagents (10 μ l) to the cerebral spinal fluid [31]. TNF- α was prepared in saline (2 ng/ μ l, pH \approx 7.0) and intrathecally injected at the dose of 20 ng. This dose was based on our previous study showing dose-dependent heat hyperalgesia following intrathecal TNF- α (0.2-20 ng) [23]. For electrophysiological studies, drugs were applied via bath perfusion.

2.3. Quantitative Real-Time PCR

Mice were sacrificed after terminal anesthesia with isoflurane, and the L4-L5 spinal cord segments were rapidly dissected. Total RNA was extracted using RNeasy Plus Mini kit (Qiagen). Quantity and quality of the eluted RNA samples were verified by NanoDrop spectrophotometer (Thermo Fisher Scientific). A total of 0.5 μ g of RNA was reverse transcribed using Omniscript reverse transcriptase according to the protocol of the manufacturer (Qiagen). Specific primers for TNFR1 and TNFR2 as well as GAPDH control were designed using IDT SciTools Real-Time PCR software (Integrated DNA Technologies). The sequences of these primers were described in Table-1. We performed gene-specific mRNA analyses using the MiniOpticon Real-Time PCR system (BioRad). Quantitative PCR amplification reactions contained the same amount of RT product including 7.5 μ l of 2 \times iQSYBR-green mix (BioRad) and 300 nM of forward and reverse primers in a final volume of 15 μ l. The thermal cycling conditions comprised 3 min polymerase activation at 95 $^{\circ}$ C, 45 cycles of 10 s denaturation at 95 $^{\circ}$ C and 30 s annealing and extension at 60 $^{\circ}$ C, followed by a DNA melting curve for the determination of amplicon specificity. All experiments were performed in duplicate. Primer efficiency was obtained from the standard curve and integrated for calculation of the relative gene expression, which was based on real-time PCR threshold values of different transcripts and groups (Berta et al., 2008) [5].

2.4. Spinal cord slice preparation

As we previously reported [1;39], a portion of the lumbar spinal cord (L4-L5) was removed from mice (3-5 week old) under urethane anesthesia (1.5 - 2.0 g/kg, i.p.). The spinal cord segment was kept in pre-oxygenated ice-cold Krebs solution. Spinal segment was placed in a shallow groove formed in an agar block and glued to the bottom of the microslicer stage. Transverse slices (600 μ m) were cut on a vibrating microslicer. The slices were perfused with Kreb's solution (8-10 ml/min) that was saturated with 95% O₂ and 5% CO₂ at 36 \pm 1 $^{\circ}$ C for at least 1-3 h prior to experiment. The Krebs solution contains (in mM): NaCl 117, KCl 3.6, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25, and glucose 11.

2.5. Patch clamp recordings in spinal slices

The whole cell patch-clamp recordings were made from lamina II neurons in voltage clamp mode [1]. Under a dissecting microscope with transmitted illumination, the substantia gelatinosa (SG, lamina II) is clearly visible as a relatively translucent band across the dorsal horn. Patch pipettes were fabricated from thin-walled, borosilicate, glass-capillary tubing (1.5 mm o.d., World Precision Instruments). After establishing the whole-cell configuration, neurons were held at the potential of -70 mV to record spontaneous excitatory postsynaptic currents (sEPSCs). The stock solution of TNF- α (1000x) was diluted with Krebs' solution (pH \approx 7.2) before the use. After the baseline recordings, neurons were perfused with TNF- α (10 ng/ml, i.e. 0.59 nM) for 2 min. Our pilot study showed that TNF- α at the concentration of 10 ng/ml but not 1 ng/ml induced a marked increase in sEPSCs. We did not intend to use high concentrations, in order to avoid possible neurotoxic effects of TNF- α at higher concentrations (>10 ng/ml) [3;60]. Neuron was held at the potential of -50 mV or -70 mV for recording NMDA- or AMPA-induced current following bath application of NMDA (50 μ M) or AMPA (10 μ M), respectively. The concentration of NMDA and AMPA was chosen according to our previous studies [39;40;72]. The resistance of a typical patch pipette is 5-10 M Ω . The internal solution contains (in mM): potassium gluconate 135, KCl 5, CaCl₂ 0.5, MgCl₂ 2, EGTA 5, HEPES 5, ATP-Mg 5. Membrane currents were amplified with an Axopatch 200B amplifier (Axon Instruments) in voltage-clamp mode. Signals were filtered at 2 kHz and digitized at 5 kHz. Data were stored with a personal computer using pCLAMP 10 software and analyzed with Mini Analysis (Synaptosoft Inc.).

2.6. Behavioral analysis

Animals were habituated to the testing environment daily for at least two days before baseline testing. The room temperature and humidity remained stable for all experiments. Animals were put in plastic boxes and allowed 30 min for habituation before examination. Heat sensitivity was tested by radiant heat using Hargreaves [28] apparatus (IITC Life Science Inc.) and expressed as paw withdrawal latency (PWL). The radiant heat intensity was adjusted so that basal PWL is between 9-12 seconds, with a cut-off of 20 seconds to prevent tissue damage. For formalin test, the time spent in licking and lifting the affected paws was recorded every 5 min for 45 min. The experimenters were blinded to genotypes of mice.

2.7. Statistical analysis

All data were expressed as mean \pm s.e.m. For electrophysiology, those cells showed >5% changes from the baseline levels during drug perfusion were regarded as responding ones and included for statistical analysis [39]. Differences between groups were compared using student t-test or ANOVA followed by Newman-Keuls test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Intrathecal TNF- α induces heat hyperalgesia via both TNFR1 and TNFR2

In order to test the central effects of TNF- α , we delivered TNF- α to the spinal cord intrathecally through spinal cord puncture and measured the paw withdrawal latency (PWL) using radiant heat. TNF- α (20 ng) induced a marked reduction in PWL in WT mice, indicating the development of heat hyperalgesia. Compared to vehicle control, this heat hyperalgesia developed within 1 h, maintained at 3 and 6 h, but recovered at 24 h (Fig. 1). Intrathecal injection of the vehicle (saline) did not induce significant heat hyperalgesia at these time points (Fig. 1). Notably, TNFR1-KO, TNFR2-KO, and TNFR1/R2 DKO mice exhibited normal baseline PWLs ($P > 0.05$, compared to WT mice). However, TNF- α -elicited

heat hyperalgesia was significantly reduced in both TNFR1- and TNFR2-KO mice (Fig. 1). Although the deficit appeared to be more severe in TNFR1-KO mice, only at one time point (6 h) there was significant difference in PWLs between TNFR1 and TNFR2 KO mice ($P < 0.05$, Fig. 1). While TNFR1- and TNFR2-KO mice showed a partial reduction in TNF- α -induced heat hyperalgesia, TNFR1/R2 DKO mice showed a complete loss of heat hyperalgesia (Fig. 1).

3.2. Both TNFR1 and TNFR2 are required for the second-phase spontaneous pain in the formalin test

Intraplantar injection of diluted formalin (5%, 20 μ l) induced characteristic two-phase spontaneous pain behaviors, as indicated by licking and lifting the affected paws in WT mice (Fig. 2). Formalin-induced spontaneous pain in the first-phase (0-5 min) did not alter in TNFR1-KO, TNFR2-KO, and TNFR1/R2 DKO mice (Fig. 2A,B). By contrast, spontaneous pain in the second phase (15-45 min) was reduced in all 3 lines of KO mice (Fig. 2A,B). However, the decrease in the second-phase pain was more robust in TNFR1-KO and TNFR1/R2 DKO-mice than that in TNFR2-KO mice (Fig. 2B). Surprisingly, spontaneous pain in the quiescent inter-phase (5-15 min) was increased in TNFR1- or TNFR2-KO mice but not in TNFR1/R2 DKO mice (Fig. 2).

3.3. Distinct role of TNFR1 and TNFR2 in the development and maintenance CFA-induced heat hyperalgesia

Intraplantar injection of CFA (1 mg/ml, 20 μ l) resulted in a persistent heat hyperalgesia in WT mice, starting on day 1 and maintaining on day 10 (Fig. 3A). CFA-evoked heat hyperalgesia was attenuated in all 3 lines of KO mice lacking TNFR1, TNFR2, and TNFR1/R2 (Fig. 3A). However, the contribution of TNFR1 and TNFR2 to the induction and maintenance phase of heat hyperalgesia was different. TNFR1-KO mice displayed a significant reduction in heat hyperalgesia at all the time points examined from day 1 to day 10, and there was no difference in heat hyperalgesia between TNFR1-KO mice and TNFR1/2-DKO mice at all the time points (Fig. 2A). By contrast, TNFR2 mice exhibited a marked reduction in heat hyperalgesia on day 1 and a mild reduction on day 2, 3, and 7 but no reduction on day 10 (Fig. 2A). Statistically, TNFR1-KO mice showed more reduction in heat hyperalgesia than TNFR2-KO mice on day 3, 7, and 10 ($P < 0.05$, Fig. 2A).

Real-Time PCR revealed distinct regulation of TNFR1 and TNFR2 mRNA expression in the spinal cord following inflammation (Fig. 2B). CFA induced a transient increase in TNFR2 mRNA levels on day 1 but not on day 3 and 7. However, CFA did not change TNFR1 mRNA levels at all these times (Fig. 2B). Notably, the copy number of TNFR1 mRNA was more than 80 times higher than that of TNFR2 mRNA in naïve animals (Fig. 2C, $P < 0.05$, $n = 4$).

3.4. TNF- α enhances excitatory synaptic transmission (sEPSC) in spinal lamina II neurons via both TNFR1 and TNFR2

To address how TNF- α mediates central sensitization and inflammatory pain, we prepared spinal cord slice and performed whole-cell patch clamp recordings in lamina II neurons where many nociceptive neurons are localized [1;38]. We examined excitatory synaptic transmission by recording spontaneous EPSCs (sEPSCs) in WT and TNFR1- and TNFR2-KO mice. We did not find any difference in basal synaptic transmission between WT and TNFR1- or TNFR2-KO mice: both the frequency and amplitude of sEPSCs were comparable (Table II).

We further assessed the role of TNFR1 and TNFR2 in TNF- α -induced enhancement of excitatory synaptic transmission. Superfusion of spinal cord slices with TNF- α (10 ng/ml,

i.e. 0.59 nM) caused a robust (>100%) increase in sEPSC frequency (Fig. 4A), as a result of enhanced glutamate release from presynaptic sites [39;45;73]. This frequency increase by TNF- α was almost abolished in TNFR1-KO and TNFR1/2-DKO mice but only partially reduced in TNFR2-KO mice (Fig. 4A,B). In contrast to frequency changes, TNF- α did not induce amplitude changes in sEPSCs either in WT mice or in TNFR1- and TNFR2-KO mice (Fig. 4A, C).

3.5. TNF- α enhances NMDA-induced currents in spinal lamina II neurons via TNFR1 but not TNFR2

Excitatory synaptic transmission is mediated by AMPA and NMDA receptors of glutamate [72]. Thus, we also examined whether the activity of AMPA and NMDA receptors could be modulated by TNF- α via TNFR1 or TNFR2. We bath applied AMPA and NMDA to spinal cord slices to stimulate AMPA and NMDA receptors. AMPA (10 μ M), at the holding potential of -70 mV, induced a robust inward current in WT mice (Fig. 5A). However, TNF- α (10 ng/ml, 2 min) failed to potentiate this current either in WT mice or in TNFR1- or TNFR2-KO mice (Fig. 5B).

Perfusion of NMDA (50 μ M), at the holding potential of -50 mV, also induced a robust inward current in WT mice. Unlike AMPA-induced current, NMDA-induced current was greatly potentiated by TNF- α (10 ng/ml, 2 min) in WT mice (Fig. 6A,B). This potentiation of NMDA current by TNF- α was abrogated in TNFR1-KO mice but not in TNFR2-KO mice (Fig. 6B), indicating a role of TNFR1 but not TNFR2 in NMDA receptor sensitization.

Finally, to determine a behavioral consequence of NMDA receptor sensitization by TNF- α , we examined whether administration of the NMDA receptor antagonist MK-801 could attenuate TNF- α -induced pain. MK-801 (10 nmol) intrathecally administered prior to TNF- α injection (20 ng, intrathecal), prevented the development of TNF- α -induced heat hyperalgesia (Fig. 6C).

4. Discussion

We have demonstrated an essential role of TNF- α in regulating acute and persistent inflammatory pain, as well as central sensitization, indicated by TNF- α -evoked increases in both sEPSC frequencies and NMDA-induced currents in lamina II neurons in spinal cord slices. We have also revealed similar and distinct roles of TNFR1 and TNFR2 in mediating inflammatory pain and TNF- α -evoked synaptic and neuronal changes in lamina II neurons. First, neither TNFR1 nor TNFR2 was required for producing baseline heat pain in naive animals and mediating excitatory synaptic transmission (sEPSC) in the normal (non-injured) conditions. Second, both TNFR1 and TNFR2 were necessary for inducing the second-phase spontaneous pain in the formalin test and heat hyperalgesia following intrathecal TNF- α injection and CFA inflammation. Third, both TNFR1 and TNFR2 were required for TNF- α -induced sEPSC frequency increase, whereas only TNFR1 was required for TNF- α -induced NMDA current increase. Fourth, CFA induced a transient increase (day 1) in TNFR2 mRNA expression in the spinal cord without changing TNFR1 mRNA expression. Finally, in the CFA model TNFR1 was required for inducing heat hyperalgesia at all the time points (1-10 d) examined, but TNFR2 was mainly required for the early-phase development of heat hyperalgesia.

4.1. TNF- α induces central sensitization

Central sensitization plays an essential role in the development and maintenance of persistent pain [33;69]. Central sensitization could be enhanced and maintained by activation of spinal cord glial cells such as microglia and astrocytes and release of

proinflammatory cytokines (e.g., TNF- α and IL-1 β), chemokines (e.g., CCL2), or growth factor (e.g., BDNF) from these glial cells [17;22;29;34;46;63]. In the spinal cord, TNF- α is mainly produced by microglia, although other cell types such as astrocytes also produce TNF- α [27;34;71]. Activation of p38 MAP kinase in spinal microglia was implicated in the synthesis and release of TNF- α [30;35;61;78]. We provided both behavioral and electrophysiological evidence to support a critical role of TNF- α in regulating central sensitization. In support of our previous studies [39;72], intrathecal TNF- α induced marked heat hyperalgesia (Fig. 1). In an antigen-induced arthritis model, spinally administered etanercept acutely reduces pain-related behavior and attenuates both the development and the maintenance of inflammation. Of interest intrathecal etanercept is more effective in reducing pain and inflammation than systemic etanercept [7].

Formalin-induced spontaneous pain has two phases, separated by an inter-phase. While the first-phase pain is a result of direct activation of nociceptors and peripheral sensitization, the second-phase pain could be a result of central sensitization [12;18;32;49]. A relatively quiescent inter-phase is thought to be mediated by a descending antinociceptive mechanism from the brainstem [21]. Our data showed that both TNFR1 and TNFR2 were required for the second-phase spontaneous pain (Fig. 2), supporting a role of TNF- α in central sensitization. By contrast, the first-phase pain was completely intact in TNFR1-KO, TNFR2-KO, and TNFR1/2-KO mice. A possible explanation is that after formalin injection TNF- α may not be immediately released in the first phase. Surprisingly, the inter-phase response was enhanced in both TNFR1 and TNFR2-KO mice (Fig. 2B), suggesting a possible involvement of TNF- α in brainstem descending inhibition [21].

How can TNF- α modulate central sensitization? TNF- α might promote central sensitization by enhancing glutamate release from presynaptic terminals and increasing the activity of NMDA receptor, a key player in central sensitization [69]. The frequency of sEPSCs in lamina II neurons drastically increased following TNF- α treatment (Fig. 4), as a result of enhanced glutamate release from presynaptic terminals. Several lines of evidence suggest that activation of TRPV1 contributes to the sEPSC frequency increase by TNF- α . First, TNF- α activates TRPV1 [36;47], and TRPV1 activation by capsaicin substantially increases the sEPSC frequency [73]. Second, the TRP antagonist capsazepine suppresses the TNF- α -induced sEPSC frequency increase [72]. Third, heat hyperalgesia elicited by intrathecal TNF- α is abolished in TRPV1 KO mice [72]. We also found a marked increase in NMDA-induced currents in lamina II neurons following TNF- α perfusion, which was supported by our behavioral result that MK-801 prevented heat hyperalgesia induced by intrathecal TNF- α . Thus TNF- α can also induce heat hyperalgesia via the activation of spinal NMDA receptors. Consistently, formalin-induced second-phase pain was reduced by both TNFR deletion and NMDA receptor blockade [26]. However, we should not exclude the involvement of other ion channels, since our recording conditions for sEPSC (-70 mV) and NMDA current (-50 mV) may not be optimal for the activation of some Na⁺ channels (e. g., Nav1.9).

Inflammation has also been shown to induce TNF- α -dependent surface trafficking of AMPA receptor (GluR1) in the dorsal horn [11]. However, TNF- α failed to increase the amplitude of sEPSC (Fig. 4C), which is mediated by postsynaptic AMPA receptors [73]. Neither did TNF- α enhance AMPA-induced currents in dorsal horn neurons (Fig. 5), in agreement with previous recordings in dorsal horn neurons [39;75]. This discrepancy in potentiation of AMPA receptors by TNF- α may result from different types of spinal neurons examined (lamina II neurons vs. other lamina neurons). Also, we should not rule out that TNF- α may enhance AMPA currents under injury conditions.

One limitation of blinded patch clamp recordings is we cannot be certain about the chemical properties of the neurons we recorded, since there are at least 4 types of neurons in the lamina II (substantia gelatinosa): islet (inhibitory), radial, central, and vertical neurons [25]. However, excitatory interneurons dominate the lamina II [50]. A recent study by Zhang et al. (2010) reported that TNF- α could also inhibit spontaneous inhibitory postsynaptic currents (sIPSCs) in GAD67-positive inhibitory neurons in the lamina II [77]. In contrast, we did not observe any effect of TNF- α on IPSCs lamina II neurons in rats [39] and mice (data not shown). One possible explanation for this discrepancy is different types of neurons (excitatory vs inhibitory) were recorded in the present study and the recent study by Zhang et al. (2010) [77].

4.2. Distinct role of TNFR1 and TNFR2 in central sensitization and inflammatory pain

Although TNFR1 and TNFR2 have different signaling mechanisms, they have cross-talk through their intracellular parts [8]. TNFR1 has been regarded as a predominant receptor, whereas the role of TNFR2 was underestimated. TNFR1 is constitutively expressed in all tissues, whereas TNFR2 expression is tightly regulated [8;9]. TNFR1 has high affinity for soluble TNF, which is commonly used as TNF- α receptor agonist [2;8;9]. TNFR1 contains a death domain and mediates TNF- α -induced cell death in hippocampal neurons. Conversely, TNFR2 does not contain death domain and instead mediates TNF- α -induced neuronal survival [9;74]. Interestingly, in the Alzheimer's disease brain TNFR1 levels and binding affinity are increased, but TNFR2 levels and binding affinity are decreased [10].

TNFR2 also plays a unique role in neuropathic pain [58;65] and cancer pain [13;24]. Epineural injection of TNFR1 but not TNFR2 neutralizing antibody reduces heat hyperalgesia and mechanical allodynia in the chronic constriction injury (CCI) model [58]. CCI-induced thermal hyperalgesia is absent in TNFR1-KO mice, whereas CCI-induced mechanical and cold allodynia are reduced in both TNFR1- and TNFR2-KO mice [65]. Of interest intrathecally injected TNFR1 agonist but not TNFR2 agonist slightly reduces mechanical and thermal withdrawal thresholds in rats, but co-injection results in an additive pain behavior [53]. Further, TNFR1 agonist induces discharge of sensory neurons in normal conditions, whereas TNFR2 agonist increases the discharge after nerve injury, suggesting a unique role of TNFR2 in injury-induced excitation of sensory neurons [53]. In a mouse skin cancer model, TNFR2 gene deletion results in attenuated heat hyperalgesia and reduced TRPV1 expression in tumor-bearing mice, whereas TNFR1 gene deletion has only a minor effect [13]. However, evoked pain and spinal gliosis in a bone cancer pain model require both TNFR1 and TNFR2 [24].

We examined the involvement of TNFR1 and TNFR2 in three different inflammatory pain conditions. Our data supported an important role of TNFR2 in mediating central sensitization and inflammatory pain. TNFR2-KO mice exhibited the following phenotypes: (1) reduced heat hyperalgesia after intrathecal TNF- α , (2) decreased second-phase pain in the formalin test, (3) reduced early-phase heat hyperalgesia in the CFA model, and (4) abrogated sEPSC frequency increase by TNF- α . Of interest TNFR2 but not TNFR1 was transiently up-regulated on day 1 after CFA injection, suggesting a distinct role of TNFR2 in early phase development of inflammatory pain.

However, our data also showed a predominant role of TNFR1 in mediating TNF- α 's effects. First, TNFR1 was required for all the effects involving TNFR2. Second, TNFR1 but not TNFR2 mediated TNF- α -induced potentiation of NMDA currents and CFA-induced heat hyperalgesia in the late phase (day 10). Third, TNFR1-KO mice and TNFR1/2-DKO mice displayed almost identical phenotypes with the exception of intrathecal TNF- α -induced heat hyperalgesia.

In summary, we have demonstrated a critical role of TNF- α in inflammatory pain by regulating central sensitization, specifically illustrated as synaptic plasticity (sEPSC frequency increase as a result of increased glutamate release) and neuronal plasticity (NMDA receptor hyperactivity) in spinal lamina II neurons. TNF- α may initiate inflammatory pain via TNFR1/TNFR2-mediated glutamate release and maintain inflammatory pain via TNFR1-mediated activation of NMDA receptors in the dorsal horn. Although TNFR1 plays a predominant role in mediating all the effects of TNF- α , TNFR2 has a particular role in mediating early development of inflammatory pain. Given an important central role of TNF- α in regulating synaptic and neuronal plasticity, targeting TNF- α signaling in the central nervous system, in addition to the peripheral nervous system and immune system, would increase the analgesic efficacy of the anti-TNF- α treatments.

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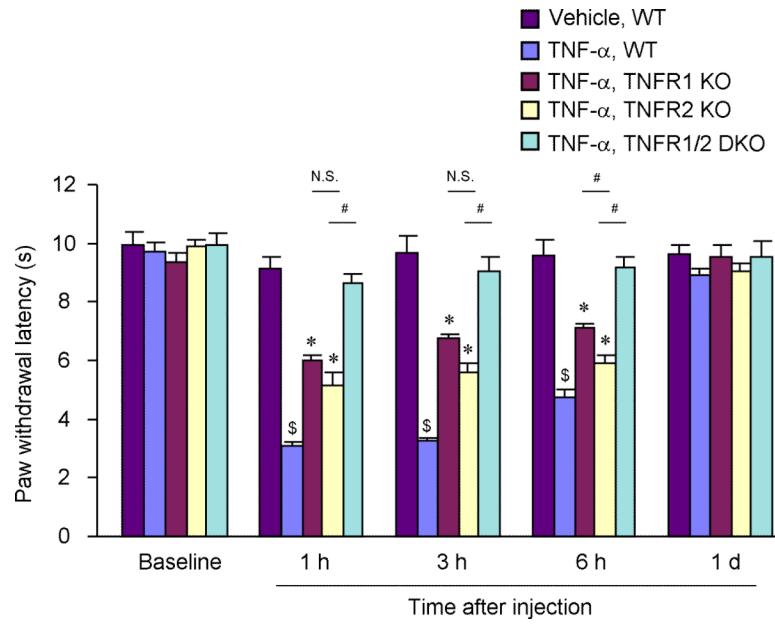
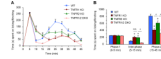


Figure 1.

Intrathecal injection of TNF- α induces heat hyperalgesia via both TNFR1 and TNFR2. Spinal infusion of TNF- α (20 ng, i.t.) elicits robust heat hyperalgesia in wide-type (WT) mice, which is reduced in both TNFR1 and TNFR2 knockout (KO) and abolished in TNFR1/2 double knockout (DKO) mice. ^{\$} $P < 0.05$, compared with WT control with vehicle injection; ^{*} $P < 0.05$, compared to WT control with TNF- α injection; [#] $P < 0.05$, $n = 5$ mice.

**Figure 2.**

Formalin-induced spontaneous pain in the second-phase requires both TNFR1 and TNFR2. **(A)** Time course of formalin-induced spontaneous measured every 5 min for 45 min. **(B)** Spontaneous pain plotted in the first phase (0-5 min), inter-phase (5-15 min), and the second phase (15-45 min). Intraplantar formalin (5%) induces biphasic spontaneous behaviors (licking/lifting the affect paws) in WT mice. The second-phase but not the first-phase pain behaviors are attenuated in both TNFR1-KO, TNFR2-KO and TNFR1/2 DKO mice. Notably, the inter-phase response is increased in TNFR1-KO and TNFR2-KO mice. * $P < 0.05$, compared to WT control; # $P < 0.05$, $n = 6$ mice. N.S., no significance.

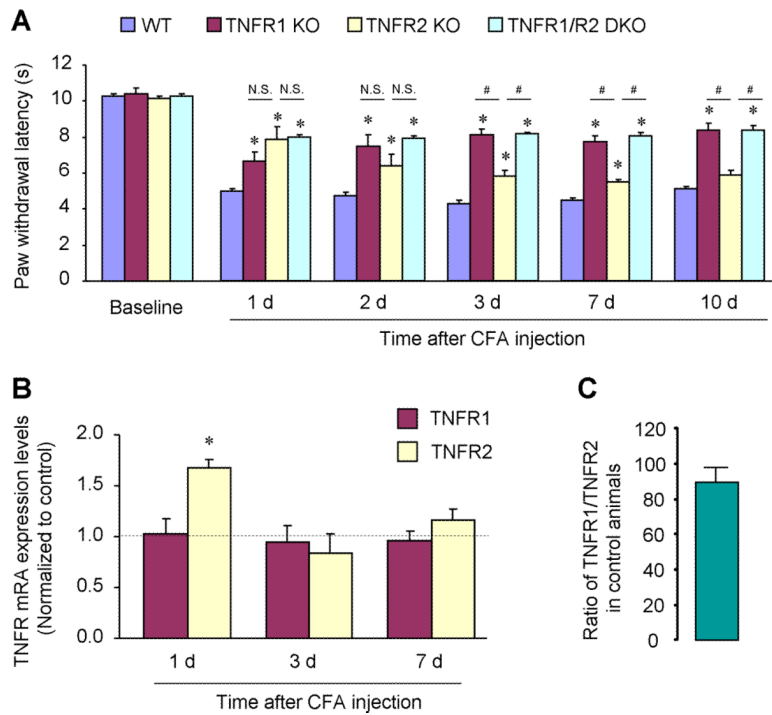


Figure 3.

CFA-induced heat hyperalgesia differentially requires TNFR1 and TNFR2. **(A)** Intraplantar CFA induces persistent heat hyperalgesia for > 10 days in WT mice, which is reduced in TNFR1-KO and TNFR1/2-DKO mice at all the times examined. Of note TNFR2-KO mice only show deficit in the first several days, especially on day 1. * $P < 0.05$, compared to WT control; # $P < 0.05$; N.S., no significance. $n = 5$ mice. **(B)** Real-time RT-PCR reveals transient increases in TNFR2 mRNA levels in the spinal cord on CFA day 1. There is no change in TNFR1 mRNA levels after CFA inflammation. * $P < 0.05$, compared to naive control, $n = 4$ mice. **(C)** Ratio of spinal mRNA copies of TNFR1/TNFR2 in the naïve control. $n = 4$ mice.

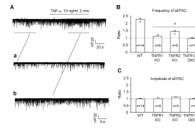


Figure 4.

TNF- α enhances excitatory synaptic transmission, i.e. spontaneous EPSC frequency (sEPSC) in spinal lamina II neurons via both TNFR1 and TNFR2. **(A)** Patch-clamp recording in spinal cord slices shows an increase in the frequency of sEPSC after bath application of TNF- α (10 ng/ml, 2 min). Aa and Ab are enlarged recordings before and after TNF- α treatment, respectively. **(B)** TNF- α increases the frequency of sEPSC in WT mice. This frequency increase is abolished in TNFR1-KO and TNFR1/2 -DKO mice and partially reduced in TNFR2-KO mice. * P <0.05, compared to pretreatment baseline; # P <0.05, compared to WT. The number of recorded neurons is indicated inside each column. **(C)** TNF- α fails to increase the amplitude of sEPSC in WT mice, in TNFR1-KO and TNFR2-KO mice, as well as in TNFR1/2-DKO mice. All the data are expressed as the ratio of corresponding baseline. The number of recorded neurons is indicated inside each column.

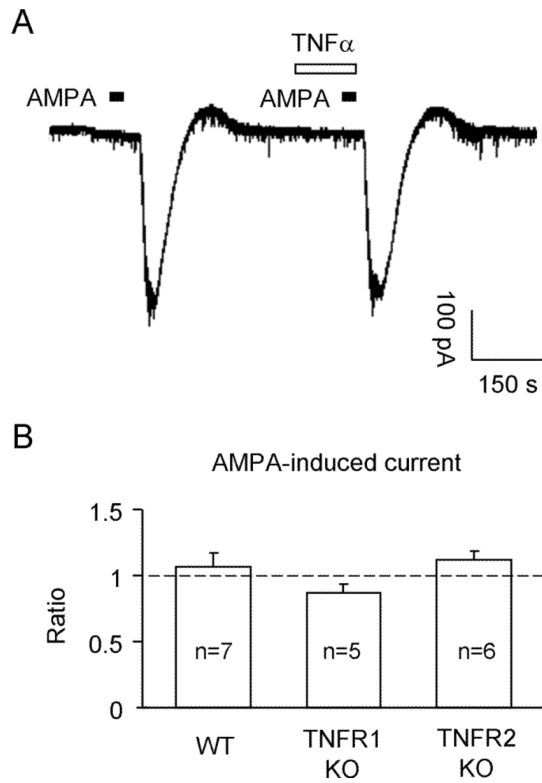


Figure 5.

(A) Patch clamp recording in lamina II neurons of spinal cord slices shows no potentiation of AMPA (10 μ M)-induced current by TNF- α (10 ng/ml, 2 min). (B) Quantification of AMPA-induced currents in WT mice, TNFR1-KO, and TNFR2-KO mice following TNF- α treatment. The data are expressed as the ratio of the amplitude of AMPA current (after vs. before TNF- α stimulation). The number of recorded neurons is indicated inside each column.

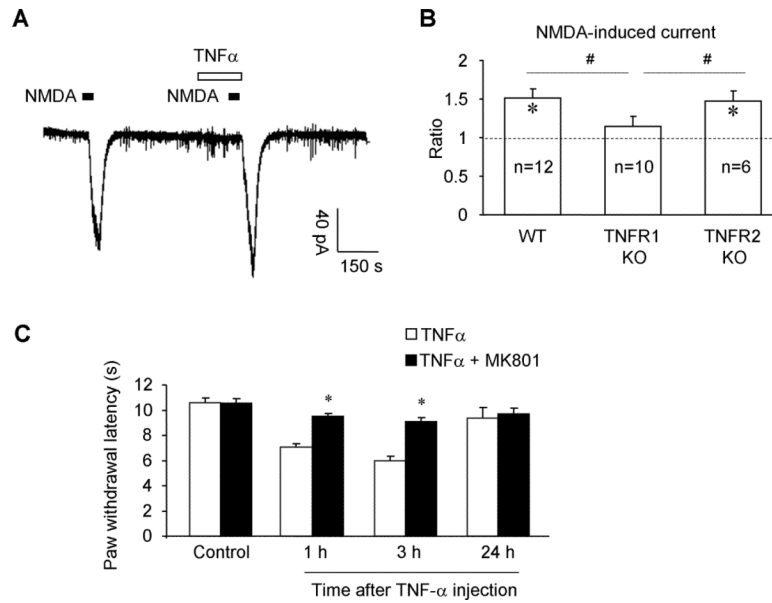


Figure 6. TNF- α enhances the NMDA-induced current in lamina II neurons of spinal cord slices via TNFR1. (A) Patch clamp recording shows potentiation of NMDA-induced current after perfusion of TNF- α (10 ng/ml, 2min) in spinal cord slices of WT mice. (B) Quantification of NMDA-induced currents in lamina II neurons of WT mice and TNFR1-KO and TNFR2-KO mice, following TNF- α treatment. The data are expressed as the ratio of the amplitude of NMDA current. * $P < 0.05$, compared to the first NMDA-induced current; # $P < 0.05$. Note that TNF- α enhances NMDA currents in WT and TNFR2-KO mice but not in TNFR1-KO mice. The number of recorded neurons is indicated inside each column. (C) Intrathecal injection of TNF- α (10 ng) induces heat hyperalgesia in naïve mice, which is prevented by intrathecal pretreatment of the NMDA receptor antagonist MK-801 (10 nmol), given 30 min before TNF- α injection. * $P < 0.05$, compared to TNF- α , $n = 6$ mice.

Table I

Sequences of DNA primers for Real-time PCR.

Target Gene	Forward Primers	Reverse Primers	Genbank No.
TNFR1	TGAGTGCCTCCCTTGCAGCC	AACCAGGGGCAACAGCACCG	NM011609
TNFR2	GTCATGGCGGAGGCCCAAGG	GCGCTGGCTTGGGAAGAGCA	NM011610
GAPDH	TCCATGACAACCTTGGCATTG	CAGTCTTCTGGGTGGCAGTGA	XM001473623

Table II

Frequency and amplitude of spontaneous EPSC (sEPSC) recorded in lamina II neurons in spinal cord slices of wild-type (WT), TNFR1 and TNFR2 knockout (KO) mice. $P > 0.05$, compared to TNFR1-KO and TNFR2-KO mice. $n = 14, 16, 13$ neurons in WT, TNFR1-KO, and TNFR2-KO group, respectively.

	Frequency (Hz)	Amplitude (pA)
WT	6.07 ± 1.56	12.37 ± 1.08
TNFR1 KO	6.32 ± 1.63	12.50 ± 1.52
TNFR2 KO	6.13 ± 1.58	12.10 ± 0.99