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A p38 Mitogen-Activated Protein Kinase Dependent Mechanism of Disinhibition in Spinal Synaptic Transmission Induced by Tumor Necrosis Factor-α

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Abstract

Tumour necrosis factor-α (TNFα) is a proinflammatory cytokine that contributes to inflammatory and neuropathic pain. The mechanism by which TNFα modulates synaptic transmission in mouse substantia gelatinosa (SG) was studied using whole cell patch clamp and immunohistochemistry. TNFα was confirmed to significantly increase the frequency of spontaneous EPSCs (sEPSCs) in spinal neurons and to also produce a robust decrease in the frequency of spontaneous IPSCs (sIPSCs). The enhancement of excitatory synaptic transmission by TNFα is in fact observed to be dependent upon the suppression of sIPSCs, or disinhibition, in that blockade of inhibitory synaptic transmission prevents the effect of TNFα on sEPSCs but not vice versa. TNFα-induced inhibition of sIPSCs was blocked by neutralizing antibodies to TNF receptor 1 (TNFR1) but not to TNF receptor 2 (TNFR2) and was abolished by the p38 mitogen-activated protein kinase (MAPK) inhibitor SB202190. TNFα rapidly inhibited spontaneous action potentials in GABAergic neurons identified in transgenic mice expressing enhanced green fluorescent protein (EGFP) controlled by the GAD67 promoter. This inhibitory effect was also blocked by intracellular delivery of SB202190 to the targeted cells. The inhibition of spontaneous activity in GABAergic neurons by TNFα is shown as mediated by a reduction in the hyperpolarization-activated cation current (Ih). These results suggest a novel TNFα-TNFR1-p38 pathway in spinal GABAergic neurons that may contribute to the development of neuropathic and inflammatory pain by TNFα.

Keywords

TNFα; MAPK; substantia gelatinosa; disinhibition; GABAergic; Ih

Introduction

Tumour necroscis factor-α (TNFα), one of the major proinflammatory cytokines released in response to injury or inflammation, plays an essential role in the development of inflammatory (Cunha et al., 1992;Ferreira et al., 1993;Watkins et al., 1995;Woolf et al., 1997) and neuropathic pain (Sommer et al., 1998a;Sommer et al., 1998b;Sorkin and Doom, 2000;Lindenlaub et al., 2000;Sommer et al., 2001a;Sommer et al., 2001b;Schäfers et al., 2003c). The effects of TNFα not only include transcriptional regulation of down-stream inflammatory mediators but also include a rapid sensitizing effect on targeted cells. For example, TNFα lowers threshold in Aβ-and C-fibers (Junger and Sorkin, 2000;Liu et al., 2002;Zhang et al., 2002;Schäfers et al., 2003a) and enhances transient receptor potential

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vanilloid receptor 1 (TRPV1) (Nicol et al., 1997) and TTX-resistant sodium currents in primary sensory neurons (Jin and Gereau, 2006). Compared to its peripheral effects, the central effects of TNFα are much less well understood. Intrathecal injection of TNFα induces mechanical allodynia and heat hyperalgesia (Narita et al., 2008;Gao et al., 2009). TNF up-regulates excitatory synaptic spinal transmission and CREB, a pronociceptive gene, in spinal cord dorsal horn (Kawasaki et al., 2008); and recruits Ca^{2+} permeable AMPA receptors in dorsal horn neurons in carrageenan-induced inflammation (Jeong et al., 2010).

P38 mitogen-activated protein kinase (MAPK) is a stress-activated protein kinase with a major role in the development of inflammatory and neuropathic pain (for review, (Ji et al., 2009)). Activation of P38 is common in dorsal root ganglion (DRG) and spinal cord in various pain models (Ji et al., 2002;Jin et al., 2003;Zhuang et al., 2007). The activation of p38 in DRG neurons results in increased expression of TRPV1 (Ji et al., 2002) and enhancement of TTX-resistant sodium currents suggesting a shared effect with TNFα (Jin and Gereau, 2006). In addition, intrathecal application of specific p38 antagonists prevents the development of hyperalgesia and allodynia in inflammatory and neuropathic pain models (Jin et al., 2003;Svensson et al., 2003;Schäfers et al., 2003c;Boyle et al., 2006;Sorkin et al., 2009). The effects of p38 activation in spinal cord remains to be more fully explored, yet there is data to support a role of p38 in spinal synaptic plasticity during central sensitization (Garry et al., 2005).

Since the spinal substantia gelatinosa (SG) is a critical site in nociceptive transmission (Willis and Coggeshall, 2004) and altered functions of SG neurons are implicated in the development and maintenance of inflammatory and neuropathic pain (Castro-Lopes et al., 1993;Ibuki et al., 1997;Eaton et al., 1998;Eaton et al., 1999;Moore et al., 2002), the present study is focused on better defining the mechanisms underlying the modulation of synaptic transmission in SG induced by TNFα and to test the hypothesis that p38 is an important second messenger in mediating its effects in spinal cord. Whole-cell patch clamp and immunohistochemical approaches are used to define the physiological effects of TNFα on subgroups of SG neurons, the receptors mediating these effects, their anatomical localization, and the involvement of p38 in transduction of these signals.

Materials and Methods

Animals

Adult FVB/NJ (4-6 weeks old, Jackson Laboratory, ME) and CB6-Tg (*Gad1*-EGFP) G42Zjh/J mice of either sex were used (Jackson Laboratory, ME). Mice were housed under a 12 h light/dark cycle with free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas M. D. Anderson Cancer Center and were performed in accordance with the National Institutes of Health Guidelines for Use and Care of Laboratory Animals.

Spinal cord slice preparation

Spinal cord slices were prepared as described previously (Zhang et al., 2009). Briefly, mice were deeply anaesthetized with inhaled isoflurane (3%). A laminectomy was performed, the lumber spinal cord was quickly removed and placed into ice-cold oxygenated (95% O_2 + 5% CO_2) ACSF solution consisting of (in mM): 117 sucrose, 3.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 12 glucose. The pia-arachnoid membrane was carefully peeled off and a block of the spinal cord from L3 to S1 was embedded in 4% agar. Transverse slices (300 μm thick) from lumber segments L4 to L5 were cut on a vibratome (series 1000, Technical Products International Inc., St. Louis, MO). The slices were then returned to bubbled Kreb's solution (in mM): 117 NaCl, 3.6 KCl, 1.2 NaH_2PO4, 1.2 $MgCl_2$,

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2.5 $CaCl_2$, 12 glucose, 25 $NaHCO_3$ at room temperature (~ 22°C) and allowed to equilibrate at least for 1 h before recording. The mice were euthanized by anesthetic overdose and exsanguination.

Patch-clamp recording in spinal slices

Whole-cell patch clamp recordings from spinal SG neurons were obtained as previously described (Zhang et al., 2009). An individual slice was transferred to a recording chamber and continually perfused with oxygenated Kreb's solution (3 ml/min) at room temperature. SG Cells were visualized using a 60X water-immersion objective on an upright microscope (Olympus, BX50WI, Japan) equipped with infrared and differential interference contrast (DIC) optics. Patch pipettes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) with resistances of 3-5 MΩ when filled with pipette solutions containing (in mM): 145 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 Mg-ATP and 0.1 Na_3-GTP (PH 7.2 adjusted with KOH) for spontaneous EPSCs recording or 120 Cs_2SO_4, 5 NaCl, 1 $MgCl_2$, 0.5 EGTA, 10 HEPES, 2 Mg-ATP and 0.1 Na_3-GTP (PH 7.2 adjusted with CsOH) for spontaneous IPSCs recording. After establishing the whole-cell configuration, membrane currents were measured with the patch-clamp technique (Hamill et al., 1981). Neurons were held at -70 mV for EPSCs and 0 mV for IPSCs recording. Signals were amplified using a Multiclamp 700B amplifier (Axon Instruments, Sunnyvale, CA), filtered at 2-5 kHz and sampled at 10 kHz in digital forms using a Digidata 1322A digitizing board (Axon Instruments, Sunnyvale, CA) interfaced with a computer system. Access resistance, typically 15-30 MΩ, was measured on establishment of whole-cell configuration and throughout each recording. Cells were abandoned if access resistance changed more than 20%. The input resistance (Rin) of the cell was calculated based on the steady state current change during application of a 10 mV depolarizing and/or 10 mV hyperpolarizing pulse. The membrane capacitance (Cm) was calculated from the transient currents observed during the application of a 10 mV depolarizing or hyperpolarizing pulse, using a single spherical compartment model. The resting membrane potential (Vm) was recorded in current clamp mode. Leak subtraction, capacitance and series resistance compensation were generally not applied except where otherwise noted.

Immunohistochemistry

Animals were deeply anesthetized with pentobarbital sodium (Nembutal, 100 mg/kg i.p.) and perfused through the ascending aorta with warm saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. The L4 – L5 spinal cord segments were removed and postfixed in 4% paraformaldehyde for 4 hr and then cryoprotected in 30% sucrose solution at 4°C for at least one night. Transverse free-floating spinal cord sections (20 um) were cut in a cryostat and processed for immunofluorescent staining. Thirty sections per mouse were used unless otherwise stated. Double-immunofluorescence labeling was used to study the distribution of TNFα receptors and p-p38 MAPK in spinal dorsal horn cells. All of the sections were first blocked with 10% normal donkey serum (NDS) and 0.2% Triton X-100 in PBS for 1 hr at room temperature. The sections were then incubated over two nights at 4°C in 5% NDS and 0.2% Triton X-100 in PBS containing the primary antibodies for the following targets: TNFR1 (rabbit, 1: 1000, Abcam), TNFR2 (rabbit, 1:500, Abcam), GFAP (mouse, 1:1000, Cell Signaling Technology), neuronal-specific nuclear protein (NeuN) (mouse, 1:1000, Millipore), microtubule-associated protein 2 (MAP2) (mouse, 1:1000, Millipore), and CD11b (rat, 1:500, Serotec). The sections were then incubated with Cy3-or FITC-conjugated secondary antibodies in 5% NDS and 0.2% Triton X-100 over night at 4°C. For double immunofluorescent staining, sections were incubated with two different primary antibodies followed by a mixture of appropriate Cy3- and FITC-conjugated secondary antibodies. For p-p 38 staining, spinal cord slices (300 um) were first prepared as described in *path-clamp recordings in spinal slices*. After

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equilibrating in oxygenated Kreb's solution at least for 1 h at room temperature, slices were stimulated with TNFα (10 ng/ml) for 5 or 30 min. Slices were then postfixed in 4% paraformaldehyde for 4 hr and cryoprotected in 30% sucrose over night at 4°C. For immunohistochemical staining, slices were first incubated with 10% NDS and 1% Triton X-100 for 30 min followed by 10% NDS and 0.2% Triton X-100 for 1 hr at room temperature. Slices were then incubated over two nights at 4°C with anti-p-p38 antibody (rabbit, 1:100, Cell Signaling Technology) and Alexa Fluor 488 conjugated anti-GFAP antibody (mouse, 1:500, Cell Signaling Technology) or Alexa Fluor 488 conjugated anti-NeuN antibody (mouse, 1:500, Millipore) or Alexa Fluor 488 conjugated anti-CD11b antibody (mouse, 1:100, Serotec). After washing with PBS, slices were incubated with Cy3-conjugated secondary antibody over night at 4°C. Slices were then mounted on glass coverslips and were viewed under a confocal microscope (Olympus, FV5, FluoView V5.0, Japan). To quantify the level of p-p38 in the spinal cord, the brightness of fluorescent staining in spinal cord dorsal horn (laminas I-II) was measured from images captured under 20X objective. Ten to twenty slices from L4-L5 spinal cord segments were randomly selected for each treatment. For each slice, background was first subtracted to differentiate positively stained cells and the brightness of all positive cells in laminas I-II was measured. The area of spinal lamina I-II was determined when the slice was viewed under DIC. For a given experiment, the exposure times and illumination intensities were identical when images were captured. All images were analyzed using NIC Elements imaging software (Nikon, Japan).

Chemcials

6,7-Dinitroquinoxaline-2, 3-dione (DNQX) and D-2-Amino-5-phosphonopentanoic acid (D-AP5) were obtained from Tocris (St. Louis, MO, USA). Recombinant mouse TNFα was purchased from R&D System (Minneapolis, MN, USA) and prepared as stock solutions at 10 μg/ml in PBS with 0.1% BSA. TNFα receptor antagonizing antibodies were kind gifts from Dr. Bharat Aggarwal (University of Texas MD Anderson Cancer Center, Houston, TX, USA). SB202190 and its inactive analog SB202474 were obtained from Calbiochem (La Jolla, CA, USA) and dissolved in DMSO (final concentration less than 0.1%). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Statistical analysis

All results are presented as means ± S.E.M. Differences between means were tested for significance using Student's paired *t*-test or ANOVA with an alpha value of $P < 0.05$.

Results

TNFα increases excitatory synaptic transmission by suppressing inhibitory synaptic transmission in spinal dorsal horn

Spontaneous EPSCs (sEPSCs) and IPSCs (sIPSCs) were recorded from randomly selected SG neurons. The effect of TNFα on excitatory synaptic transmission was tested first. Rapid bath application of TNFα (10 ng/ml, 2 min) significantly increased the frequency of sEPSCs in 11 of 14 recorded neurons, to 158.3 ± 15.0% of control (from 2.4 ± 0.6 to 3.3 ± 0.7 Hz, n= 11; $p < 0.005$) (Fig. 1A, D). TNFα had no effect on other characteristics of sEPSCs including amplitude, rise time or decay time constant (Fig. 1D). Since capacitance and series resistance were not compensated, subtle changes in rise time kinetics induced by TNF may not have been observed. TNFα evoked greater enhancement in sEPSCs when tested at 50 ng/ml but no effect was observed at a concentration of 1 ng/ml (Fig. 1B). The frequency of sEPSCs was increased to 237.3 ± 12.4% of control (n=4/5; $p < 0.01$) at 50 ng/ml TNFα but did not change at 1 ng/ml TNFα (99.8 ± 18.7% of control, n=5). These findings are

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consistent with the previously reported enhancement of excitatory synaptic transmission in SG neurons induced by TNFα (Kawasaki et al., 2008).

Spinal SG is composed of both excitatory and inhibitory neurons interconnected in a complicated but not fully characterized circuit, yet the overall valence in the circuit is considered inhibitory. Hence the increased frequency of sEPSCs by TNFα was initially hypothesized as due to alterations in inhibitory processes. To test this hypothesis, the $GABA_A$ receptor blocker (bicuculline, 10 μM) and the glycine receptor blocker (strychnine, 5 μM) were added to the bath before the application of TNFα. The addition of bicuculline and strychnine significantly increased the frequency of sEPSCs in 8/10 cells from 2.8 ± 0.1 to 3.7 ± 0.2 Hz (n=8; $p < 0.01$). TNFα added following bicuculline and strychnine resulted in no further change in the frequency of sEPSCs when inhibitory synaptic transmission was blocked (n=8, Fig. 1C, D).

These results suggested that TNFα increases sEPSCs in SG by inhibiting sIPSCs. Indeed TNFα was found to produce a robust inhibitory effect of sIPSCs in SG neurons. In 22 of 23 recorded neurons, bath application of TNFα (10ng/ml, 2 min) significantly decreased the frequency of sIPSCs to 55.4 ± 4.4% of control (from 7.2 ± 1.0 to 3.0 ± 0.5 Hz, n=22; $p < 0.01$) (Fig. 2A, D). This inhibitory effect of TNFα on sIPSCs was also dose dependent, with significant inhibitory effect seen at 10 and 50 but not 1 ng/ml of TNFα (Fig. 2B). The frequency of sIPSCs was decreased to 25.1 ± 5.5% of control (n=5; $p < 0.05$) at 50 ng/ml TNFα, reaching the maximal inhibition. To test if the inhibitory effect of TNFα on sIPSCs was dependent on the excitatory synaptic transmission, the AMPA-kainate receptor blocker DNQX (10 uM) and the NMDA receptor blocker D-AP5 (25 uM) were added into the bath before the application of TNFα. As shown in Fig. 2C and D, the inhibitory effect of TNFα on sIPSCs persisted during blockade of excitatory synaptic transmission as the frequency was reduced to 52.4 ± 5.4% of control for the 12 of 14 neurons tested (from 2.1 ± 0.4 to 1.2 ± 0.3 Hz, n=12; $p < 0.001$). In either condition, TNFα had no effect on the amplitude, rise time or decay time constant of sIPSCs (Fig. 2D).

In addition to suppressing sIPSCs, TNFα was also found to suppress mini IPSCs (mIPSCs). TNFα at 10 ng/ml significantly decreased the frequency of mIPSCs to 61.6 ± 6.9% (from 3.8 ± 0.8 to 2.4 ± 0.7 Hz, n=7; $p < 0.05$) (Fig. 3A, C) and there was a strong rightward shift in the cumulative inter-event interval distribution (Fig. 3B). The amplitude of mIPSCs was decreased but did not reach statistical significance (Fig. 3B, C).

TNFα induces disinhibition in synaptic transmission through activation of TNFR1

TNFα exerts its action through two receptors, the TNF receptor 1 (TNFR1, p55) and the TNF receptor 2 (TNFR2, p75) (Tartaglia et al., 1991;Tartaglia and Goeddel, 1992), with the majority of the its effects being transmitted through TNFR1 (Vandenabeele et al., 1995). TNFR1 and TNFR2 are expressed in both the peripheral and central nervous systems (Shubayev and Myers, 2001;Pollock et al., 2002;Holmes et al., 2004). The roles of these receptors in TNFα induced suppression of sIPSCs in SG neurons were defined in experiments using spinal slices preincubated with antagonizing antibodies to either TNFR1 or TNFR2 (Higuchi and Aggarwal, 1992). As shown in Figure 4, TNFα had no effect on sIPSCs when spinal slices were preincubated with antagonizing antibody to TNFR1 (1:500, n=13) (Fig. 4A, C). In contrast, TNFα caused a significant decrease in the frequency of sIPSCs when spinal slices were preincubated with antagonizing antibody to TNFR2 (1:500, 55.6 ± 7.2% of control, n=11; $p < 0.001$) (Fig. 4B, C). This suggests that TNFα inhibits spinal inhibitory synaptic transmission via the activation of TNFR1 not TNFR2.

Although it has been reported that TNFα receptors are generally expressed in spinal cord of naïve animals (Ohtori et al., 2004;Holmes et al., 2004), the baseline expression and the exact

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location of TNFα receptors in naïve animals is not clear. To address this question, the location of TNFα receptors in spinal cord was determined by double immunohistochemistry staining of TNFR1 or TNFR2 along with cellular markers for neurons (NeuN and microtubule associated protein-2, MAP2), astrocytes (GFAP) and microglia (CD11b). The specificity of the antibodies used in the present study were previously defined (Khan et al., 2008;Vince et al., 2009). TNFR1 was confirmed to be expressed throughout the spinal cord gray matter while TNFR2 was expressed only in ventral horn of naïve mouse (Fig. 5B and Fig. 6B, respectively). It was further observed that TNFR1 was expressed on astrocytes and spinal dorsal horn neurons but not microglia (Fig. 5C-F). In contrast, TNFR2 was only expressed in large ventral horn neurons that were consistent in location and morphology to motor neurons (Fig. 6C-F).

TNFα induced disinhibition of spinal synaptic transmission is dependent on activation of p38 mitogen-activated protein kinase in spinal dorsal horn neurons

P38 MAPK is activated in spinal cord following tissue injury and inflammation, and blockade of p38 MAPK activation prevents the development of neuropathic and inflammatory pain (Schäfers et al., 2003c;Svensson et al., 2005;Boyle et al., 2006;Sorkin et al., 2009). The possible involvement of p38 MAPK activation in mediating the effects of TNFα on spinal synaptic transmission was explored by examining the levels of active (phosphorylated) p38 (p-p38) in mouse spinal cord following treatment with TNFα. Spinal cord slices were prepared and bathed with TNFα as in the electrophysiological recordings (10 ng/ml in Kreb's solution for 5 or 30 min) and then processed using double immunohistochemistry. As shown in Figure 7A, a modest baseline expression of p-p38 was confirmed present in superficial dorsal horn in control slices (Svensson et al., 2008). Application of TNFα was found here to induce a marked increase of pp38 expression in spinal dorsal horn of slices incubated with TNFα for as little as 5 minutes and thirty minutes' treatment with TNFα further increased the expression of p-p38 (Fig. 7B-D). The cellular distribution of p-p38 was primarily colocalized with the neuronal marker NeuN (Fig. 7E) and only to a much less extent with the astrocytic marker GFAP (Fig. 7F). No co-expression of pp38 was observed with the microglial marker CD11b (Fig. 7G).

The involvement of p38 in mediating the effects of TNFα was further explored by testing the effect of TNFα on sIPSCs in SG neurons using spinal cord slices preincubated with the p38 inhibitor SB202190 (20 μM). The spinal slices were incubated with antagonist beginning at least 30 min before recording and perfused with antagonist throughout each experiment. As shown in Figure 8, TNFα had no effect on sIPSCs when p38 was blocked by SB202190 (n=15). In contrast, TNFα significantly decreased the frequency of sIPSCs in slices pretreated with SB202474 (20 uM), an inactive structural analog of SB202190 (Lee et al., 1994) (n=5; $p < 0.005$).

TNFα inhibits spontaneous action potentials in GABAergic neurons in spinal dorsal horn by reducing hyperpolarization-activated (Ih) currents

The inhibitory effect of TNFα on sIPSCs suggested that the outputs from inhibitory neurons with spontaneous activity are decreased by TNFα treatment. The effect of TNFα on the activity of identified spinal GABAergic neurons was tested to examine this possibility. Transgenic mice expressing the "enhanced" derivative of green fluorescent protein (EGFP) at the upstream regulatory sequence from the murine *Gad1* gene (encoding the GABA synthesizing enzyme GAD67) were used in these experiments. The EGFP-labeled neurons were observed throughout the central nervous system including spinal cord dorsal horn (data not shown). Synaptic transmission was blocked by adding DNXQ (10 μM), D-AP5 (25 μM), bicuculline (10 μM) and strychnine (5 μM) in the bath. GABAergic neurons were first studied in cell-attached mode. As shown in Figure 9A, a subset of GABAergic neurons

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(4/10) displayed intrinsic spontaneous activity (SA) that was independent of synaptic transmission. Rapid application of TNFα inhibited this spontaneous activity, reducing the frequency of SA from 6.8 ± 0.9 to 3.2 ± 0.4 Hz (n=3; $p < 0.01$). In whole-cell patch-clamp recordings, 8 of 25 recorded GABAergic neurons displayed intrinsic SAs and TNFα significantly decreased the frequency of SAs in 6 neurons from 3.8 ± 0.3 to 1.5 ± 0.2 Hz (n=6; $p < 0.001$) (Fig. 9B). To examine whether the inhibitory effect of TNFα on GABAergic neurons is mediated by p38 MAPK, SB202190 (20 uM) was added to patch pipette solution in another series of experiments. As shown in Figure 9C, TNFα no longer had any inhibitory effect on GABAergic neurons when p38 MAPK was blocked inside the recorded cells (n=4), strongly suggesting that intra-neuronal activation of p38 MAPK-dependent pathway is involved in the modulation of neuronal activities induced by TNFα.

The rapid inhibitory effect of TNFα on GABAergic neurons suggests a modulation of basic ionic mechanisms of spike generation. Superimposition of action potential spikes recorded before and after application of TNFα revealed minimal effects on the properties of action potential waveforms (Fig. 10Aa) but revealed significantly prolonged interspike intervals resulting in the attenuation of firing frequency of GABAergic neurons (Fig. 10Ab). TNFα reduced the slope of the depolarization ramp leading to action potential threshold, suggesting a possible decrease of the hyperpolarization-activated cation current (Ih). To study the effect of TNFα on Ih currents of GABAergic neurons, cells were held at resting membrane potential and then hyperpolarized by a long current test pulse (200pA, 2s). A slow depolarizing “sag” was elicited during a time-dependent inward rectification following the hyperpolarization and represented the activation of Ih current (Harris et al., 1989;Harris, 1992). A sag current was elicited by hyperpolarizing currents in 14 of 16 GABAergic neurons (Fig. 10Ba, black arrow) but only in 4 of 22 non-EGFP (GAD67 negative) neurons, suggesting Ih current is predominantly expressed in GABAergic neurons. The application of TNFα (10 ng/ml) reduced the sag current in 9 of 11 GABAergic neurons (Fig. 10Ba, red arrow, Fig. 10Bb) but only in 1 of 4 non-EGFP neurons showing a sag current. Hyperpolarization-induced sag was often followed by a slow afterhyperpolarizing tail that was accompanied by rebound action potentials, reflecting activation of a transient outward current (Fig. 10Ba) (Harris et al., 1989;Harris, 1992). TNFα also reduced the number of rebound action potentials from 9.2 ± 0.8 to 3.1 ± 0.5 (n= 9; $p < 0.01$) (Fig. 10Ba, Bc). The effect of TNFα on sag and rebound action potentials mimics the effect of ZD7288 (50 μM, n=4) (Fig. 10C), a specific Ih blocker, suggesting that TNFα has a selectivity for Ih in spinal GABAergic neurons.

Discussion

Several studies have revealed that both central and peripheral administration of TNFα induces mechanical allodynia and thermal hyperalgesia (Cunha et al., 1992;Perkins and Kelly, 1994;Woolf et al., 1997;Schäfers et al., 2003a;Schäfers et al., 2003b;Wacnik et al., 2005;Jin and Gereau, 2006;Gao et al., 2009). The peripheral mechanisms of TNFα-induced hypersensitivity have been well documented. TNFα promotes heat-induced CGRP release from nociceptor terminals in skin (Opree and Kress, 2000), sensitizes peripheral Aβ- and C-fibers (Junger and Sorkin, 2000;Liu et al., 2002;Zhang et al., 2002;Schäfers et al., 2003a), sensitizes TRPV1 of primary sensory neurons (Nicol et al., 1997), and enhances TTX-resistant sodium currents in isolated primary sensory neurons (Jin and Gereau, 2006). Although TNFα has been shown to modulate excitatory and inhibitory synaptic transmission in hippocampus in different ways in early studies (Stellwagen et al., 2005), potential spinal effects of TNFα had not been explored until recently when direct application of TNFα was shown to enhance excitatory synaptic transmission and increase AMPA and NMDA-evoked EPSCs in spinal dorsal horn (Kawasaki et al., 2008) . The increase in spontaneous excitatory synaptic transmission induced by TNFα shown in the present study is consistent with these

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previous observations. Importantly, it is further shown here that the enhancement of spinal excitatory synaptic transmission evoked by TNFα is due to inhibition of on-going inhibitory synaptic transmission. The similar inhibitory effect of TNFα on miniature IPSCs of spinal dorsal horn with that in hippocampus suggested the decreased release of neurotransmitters from presynaptic terminals (Stellwagen et al., 2005) and was consistent with the inhibitory effect of TNFα on presynaptic voltage-dependent calcium channels (Czeschik et al., 2008;Motagally et al., 2009). Disinhibitory processes in the dorsal horn have been previously claimed as important to the development and maintenance of neuropathic pain (Yaksh, 1989;Castro-Lopes et al., 1993;Sivilotti and Woolf, 1994;Ibuki et al., 1997;Eaton et al., 1998;Eaton et al., 1999;Moore et al., 2002). Thus, the data shown here implicates TNFα as a potential mediator key to this process.

TNFα has two receptors: TNFR1 and TNFR2, with TNFR1 being the dominant pathway for the major effects of TNFα (Vandenabeele et al., 1995). Our data indicate that TNFα-induced disinhibition of spinal synaptic transmission is mediated through TNFR1 but not TNFR2. TNFR1 but not TNFR2 was observed expressed in spinal dorsal horn neurons and astrocytes and only the neutralizing antibodies to TNFR1 were found to block the effects of TNFα on SG neurons. These findings are in concert with previous suggestions that TNFR1 is the major receptor for TNFα in promoting pain signal processing after nerve injury or inflammation (Sommer et al., 1998b;Parada et al., 2003;Cunha et al., 2005;Jin and Gereau, 2006). The expression of TNFR1 on spinal dorsal horn neurons and astrocytes suggested that both types of cells could be activated by TNFα and may contribute to the downstream effects following TNFα.

P38 MAPK has been shown to play an important role in the development of inflammatory and neuropathic pain (Ji et al., 2002;Jin et al., 2003;Svensson et al., 2003;Schäfers et al., 2003c;Tsuda et al., 2004;Svensson et al., 2005;Boyle et al., 2006;Zhuang et al., 2007;Svensson et al., 2008;Sorkin et al., 2009;Fitzsimmons et al., 2010). Although the activation of p38 in spinal cord has been previously suggested as mediating many effects of TNFα (Schäfers et al., 2003c;Svensson et al., 2005;Boyle et al., 2006), direct evidence at the spinal level has been lacking. The data shown here where TNFα-induced disinhibition of spinal synaptic transmission was prevented by blocking the activation of p38, and where bath application of TNFα activated p38 predominantly in spinal dorsal horn neurons strongly implicate p38 as a key downstream mediator following TNFα-TNFR1 activation. The TNFα-TNFR1-p38 pathway may involve additional molecules including ASK1, MEKK4 and possibly MKK3/6. Activated p38 is also translocated to the nucleus and can activate transcriptional factors such as ATF2, c-Jun and CREB (Ji et al., 2009). In agreement with our data, it has been shown that application of TNFα increased the expression of activated CREB in spinal dorsal horn neurons (Kawasaki et al., 2008). Although activated p38 was exclusively observed in spinal microglia following nerve injury (Ji et al., 2002;Jin et al., 2003;Schäfers et al., 2003c;Svensson et al., 2005;Zhuang et al., 2007), the findings here that the activation of p38 was predominantly in spinal dorsal horn neurons and to a much less extent, in spinal astrocytes is in line with other studies using inflammatory pain models (Boyle et al., 2006;Svensson et al., 2008;Sorkin et al., 2009). The variances in the pattern of p-p38 expression might reflect the different contributions of spinal neurons, astrocytes and microglia to the development of pain under different initiating conditions of the various models or may simply reflect different expression patterns among the various cellular elements in the spinal cord over time.

The inhibitory tone in spinal cord dorsal horn is determined by the output from the inhibitory interneurons including GABAergic and glycinergic neurons. Transgenic mice expressing the “enhanced” derivative of green fluorescent protein (EGFP) in the upstream regulatory sequence from murine *Gad1* gene (encoding the GABA synthesizing enzyme

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GAD67) (Chattopadhyaya et al., 2004) were used here to focus on a subgroup of identified GABAergic SG neurons. Under this condition, the labeled neurons account for about one third of GABAergic population in spinal SG (Heinke et al., 2004). A population of these GABAergic neurons in spinal SG was found to exhibit spontaneous repetitive action potential firing. These discharges were observed to persist while synaptic inputs were blocked, indicating they were intrinsic to these neurons and suggesting these subgroups provide tonic inhibitory tone within the dorsal horn. The inhibitory effect of TNFα on spontaneous firings of GABAergic neurons is consistent with the reduced spinal inhibitory synaptic transmission induced by TNFα. The involvement of p38 in the TNFα-induced inhibition of spontaneous firings of GABAergic neurons further supported previous data showing the role of p38 in mediating the TNFα-induced inhibition on sIPSCs and the increased expression of p-p38 in spinal dorsal horn neurons after TNFα. Together, these data clearly indicate that TNFα acting on TNFR1 on neurons is sufficient to suppress spontaneous action potentials in a subgroup of GABAergic neurons through a p38-mediated mechanism.

Hyperpolarization-activated cation currents (Ih), encoded by a family of genes called hyperpolarization-activated and cyclic nucleotide-sensitive cation nonselective (HCN), has been identified in various central and peripheral neurons (Pape, 1996). Most of the spinal GABAergic neurons recorded here displayed Ih currents, in line with previous studies (Yoshimura and Jessell, 1989;Grudt and Perl, 2002;Hantman et al., 2004). HCN channel subunit 2 (HCN2) is widely expressed in spinal dorsal horn revealed by in situ hybridization and immunohistochemical approaches (Santoro et al., 2000;Papp et al., 2006). Since Ih current contributes to pacemaker activity (spontaneous repetitive action potential firing or rhythmic-oscillatory activity) in CNS neurons (McCormick and Pape, 1990;Maccaferri and McBain, 1996;Pape, 1996;Gasparini and DiFrancesco, 1997;Luthi and McCormick, 1998), it may also modulate activities of GABAergic neurons in spinal dorsal horn. After the application of TNFα, Ih currents of GABAergic neurons were decreased; suggesting TNFα may reduce the intrinsic spontaneous discharges of these GABAergic neurons through inhibition of Ih currents. The fact that ZD7288, a specific Ih current blocker, produced the same effect as of TNFα on inhibiting Ih current is in line with this conclusion. The data that only a small proportion of non-EGFP neurons have Ih currents suggests that this current may be useful in prospectively indentifying GABAergic neurons and hence focus the effects of TNF on these cells.

The expression of TNFR1 and p-p38 in spinal astrocytes suggest that astrocytes could be involved in the TNFα-induced spinal disinhibition. Spinal astrocytes have been recently found to actively contribute to the development of neuropathic pain following peripheral nerve injury (Zhuang et al., 2006;Gao et al., 2009). Upon activation, spinal astrocytes can release various molecules which can activate spinal dorsal horn neurons (for review, (Watkins and Maier, 2003)). The data shown here revealed that the activation of TNFα-TNFR1-p38 pathway in spinal neurons is sufficient to induce the inhibition of spinal inhibitory transmission, but the possible role of spinal astrocytes can not be excluded and may need further study.

In conclusion, the present study suggests a unique cellular mechanism that mediates the modulation of spinal synaptic transmission induced by TNFα. First, TNFα directly suppressed inhibitory synaptic transmission in spinal SG region which consequently resulted in the enhancement of the excitatory synaptic transmission. Second, disinhibition of spinal synaptic transmission induced by TNFα was mediated by the activation of TNFR1 in spinal cord dorsal horn followed by the activation of neuronal p38 MAPK. Third, $GAD67^{+}$ GABAergic neurons in transgenic mice were found to display spontaneous intrinsic action potentials that were directly inhibited by TNFα via a p38 dependent pathway. This inhibition

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of spontaneous activities of GABAergic neurons was accompanied with a reduction of Ih currents, suggesting TNFα may inhibit neuronal activities through reducing Ih currents of GABAergic neurons. The cellular and molecular pathway underlying the TNFα-induced spinal inhibition provides a presynaptic mechanism for central sensitization in the development and maintenance of neuropathic and inflammatory pain.

Acknowledgments

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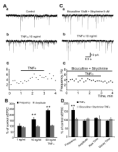

Figure 1.

TNFα (10 ng/ml, 2 min) enhances excitatory synaptic transmission in spinal dorsal horn. **A**, SEPSCs recorded from a spinal lamina II neuron before (**a**) and after (**b**) the application of TNFα; **c**, Frequency of sEPSCs as a function of recording time in the representative cell in **a** and **b**. Frequency was measured every 10s. **B,** TNFα enhances sEPSCs in a concentration-dependent manner. The significant effect on sEPSCs was observed at 10 (n=11) and 50 (n=4) but not 1 (n=5) ng/ml of TNFα. * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA with *post hoc* Dunn's test). **C**, In the presence of bicuculline (10 uM) + strychnine (10 uM) in the bath, application of TNFα had no effect on sEPSCs. **D**, Grouped data showed the percentage change (mean ± SEM) in frequency, amplitude, rise time and decay time of sEPSCs after application of TNFα with (n=8) and without (n=11) bath application of bicuculline and strychnine. * $p < 0.05$, ** $p < 0.01$ compared with baseline before application of TNFα (student's paired *t*-test).

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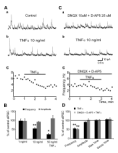

Figure 2.

TNFα (10 ng/ml, 2 min) suppresses inhibitory synaptic transmission in spinal dorsal horn. **A**, SIPSCs recorded from a spinal lamina II neuron before (**a**) and after (**b**) the application of TNFα; **c**, Frequency of sIPSCs as a function of recording time in the representative cell in **a** and **b**. Frequency was measured every 10s. **B,** TNFα inhibits sIPSCs in a concentration-dependent manner. The significant effect on sIPSC was observed at 10 (n=22) and 50 (n=5) but not 1 (n=7) ng/ml of TNFα. * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA with *post hoc* Dunn's test). **C**, In the presence of DNQX (10 uM) + D-AP5 (25 uM) in the bath, application of TNFα still decreased the frequency of sIPSCs. **C**, Grouped data showed the percentage change (mean ± SEM) in frequency, amplitude, rise time and decay time of sIPSCs after application of TNFα with (n=12) and without (n=22) bath application of DNQX and D-AP5. * $p < 0.05$, ** $p < 0.01$ compared with baseline before application of TNFα (student's paired *t*-test).

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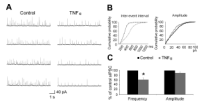

Figure 3.

TNFα (10 ng/ml, 2 min) decreases the frequency of mIPSC in spinal dorsal horn. **A**, Examples of mIPSCs before and after the application of TNFα. **B**, Cumulative probability of mIPSC inter-event interval and amplitude from the same cell shows a significant rightward shift in the distribution of inter-event interval but only a slight leftward shift in the distribution of amplitude after TNFα treatment (black: before TNFα; gray: after TNFα). **C**, TNFα significantly decreases the frequency but not the amplitude of mIPSC (n=7). * $p < 0.05$ compared with baseline before application of TNFα (student's paired *t*-test).

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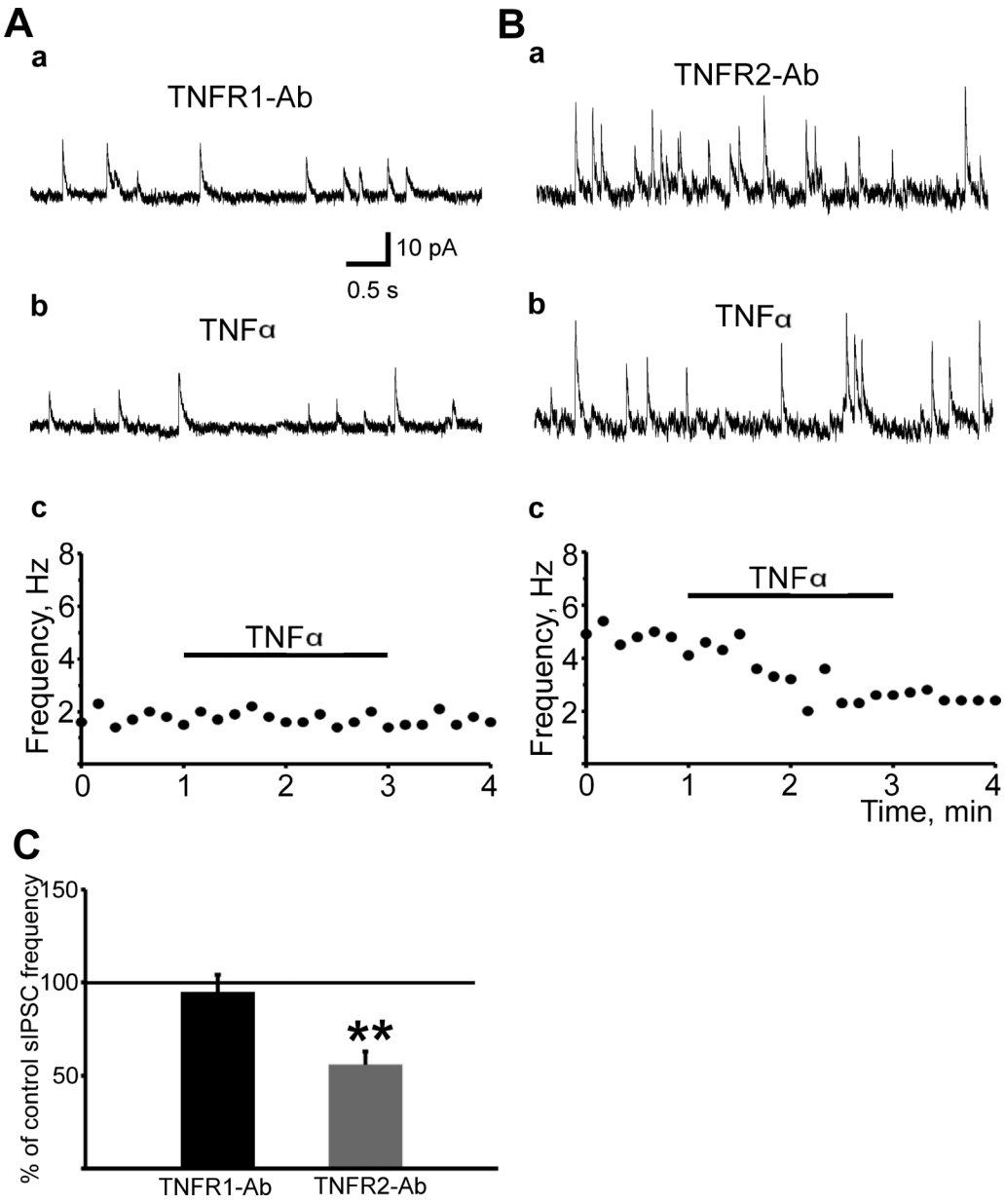

Figure 4.

The inhibitory effect of TNFα on sIPSCs in spinal dorsal horn was mediated by the activation of TNF receptor 1 (TNFR1) but not TNF receptor 2 (TNFR2). **A**, SIPSCs recorded from a spinal lamina II neuron before (**a**) and after (**b**) the application of TNFα when the slice was preincubated with TNFR1 antibody to neutralizing TNFR1; **c**, Frequency of sIPSCs as a function of the recording time in the representative neuron in **a** and **b**. Frequency was measured every 10s. **B**, SIPSCs recorded from a spinal lamina II neuron before (**a**) and after (**b**) the application of TNFα when the slice was preincubated with TNFR2 antibody to neutralizing TNFR2; **c**, Frequency of sIPSCs as a function of the recording time in the representative neuron in **a** and **b**. Frequency was measured every 10s. **C**, Averaged data showed that neutralizing TNFR1 (n=13) but not TNFR2 (n=11) prevented the inhibitory effect of TNFα on sIPSCs. * $p < 0.05$, ** $p < 0.01$ compared with baseline before application of TNFα (student's paired *t*-test).

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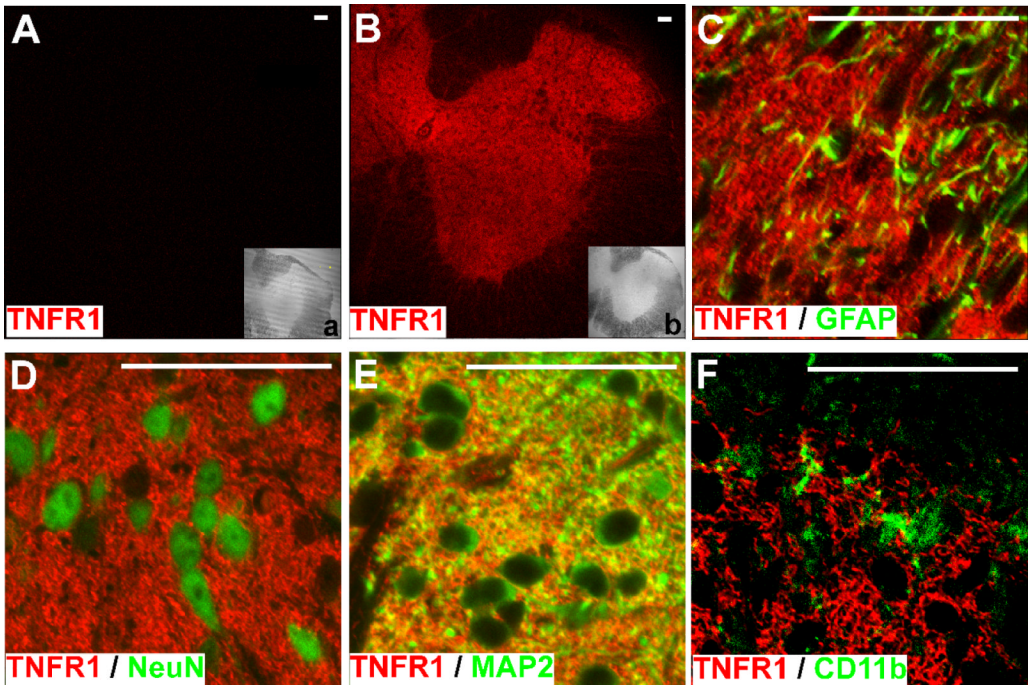

Figure 5.

Localization of TNFR1 in spinal cord of naïve mice. **A**, A negative control for TNFR1 staining when the primary antibody to TNFR1 is not added; **B**, TNFR1 is expressed throughout the spinal cord gray matter. **a** and **b**, view of the slices under the light microscope. **C**, Double immunofluorescence in spinal dorsal horn shows colocalization of TNFR1 (red) and GFAP, an astrocyte marker (green). **D**, Double immunofluoscence in spinal dorsal horn shows no colocalization of TNFR1 (red) and NeuN, a neuronal soma marker (green). **E**, Double immunofluorescence shows colocalization of TNFR1 (red) and MAP2, a neuronal dendrite marker (green). **F**, Double immunofluorescence shows no colocalization of TNFR1 (red) and CD11b, a microglia maker (green). Scale bar = 5μm.

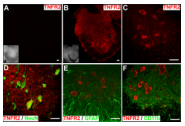

Figure 6.

Localization of TNFR2 in spinal cord of naïve mice. **A**, A negative control for TNFR2 staining when the primary antibody to TNFR1 is not added; **a**, view of the same slice under the light microscope. **B**, TNFR2 is expressed predominantly in spinal cord ventral horn; **b**, view of the same slice under the light microscope. **C**, A magnifying view shows TNFR2 is expressed in spinal ventral horn neurons. **D-F**, Double immunofluorescence in spinal dorsal horn shows the colocalization of TNFR2 with NeuN (**D**) but not with GFAP (**E**) or CD11b (**F**). Scale bar = 5μm.

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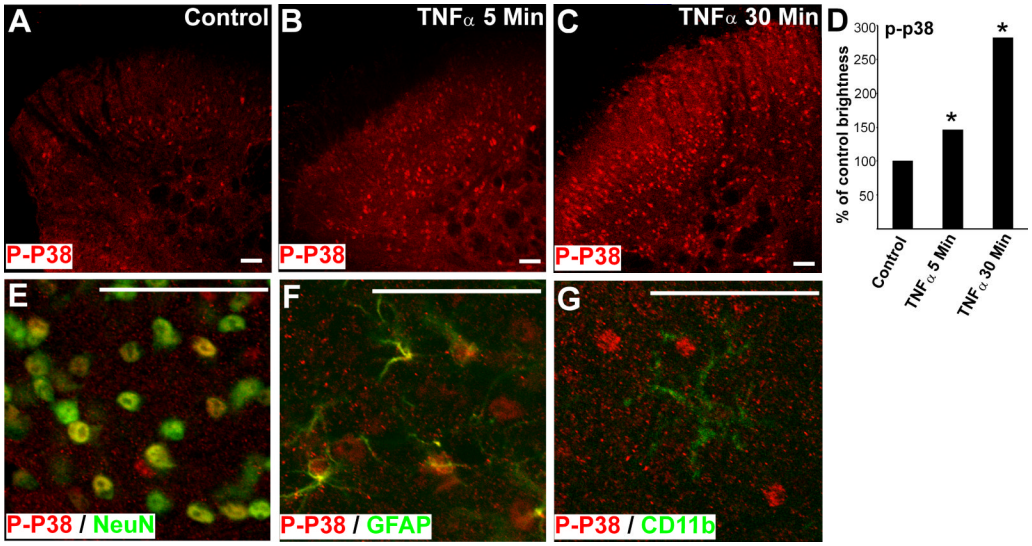

Figure 7.

Expression of p-p38 in spinal dorsal horn induced by the application of TNFα. **A**, A baseline expression of p-p38 is detected in spinal dorsal horn in naïve mice. **B** and **C**, A robust increase in p-p38 expression in spinal dorsal horn is detected after spinal slices are incubated with TNFα (10 ng/ml) for 5 and 30 min, respectively. **D**, The expression of p-p38 in spinal dorsal horn with different treatment was quantified and represented as percentage of control. Both 5min (n=15) and 30min (n=18) treatment with TNFα significantly increased the expression of p-p38 in spinal cord laminas I-II, compared to control (n=11). The expression of p-p38 was significantly increased after 30 min treatment of TNFα compared to 5 min treatment. * $p < 0.01$, ANOVA. **E-G**, Double immunofluorescence shows that the expression of p-p38 is predominantly in neurons (**E**) and in a small number of astrocytes (**F**) but not in microglia (**G**). Scale bar = 5μm.

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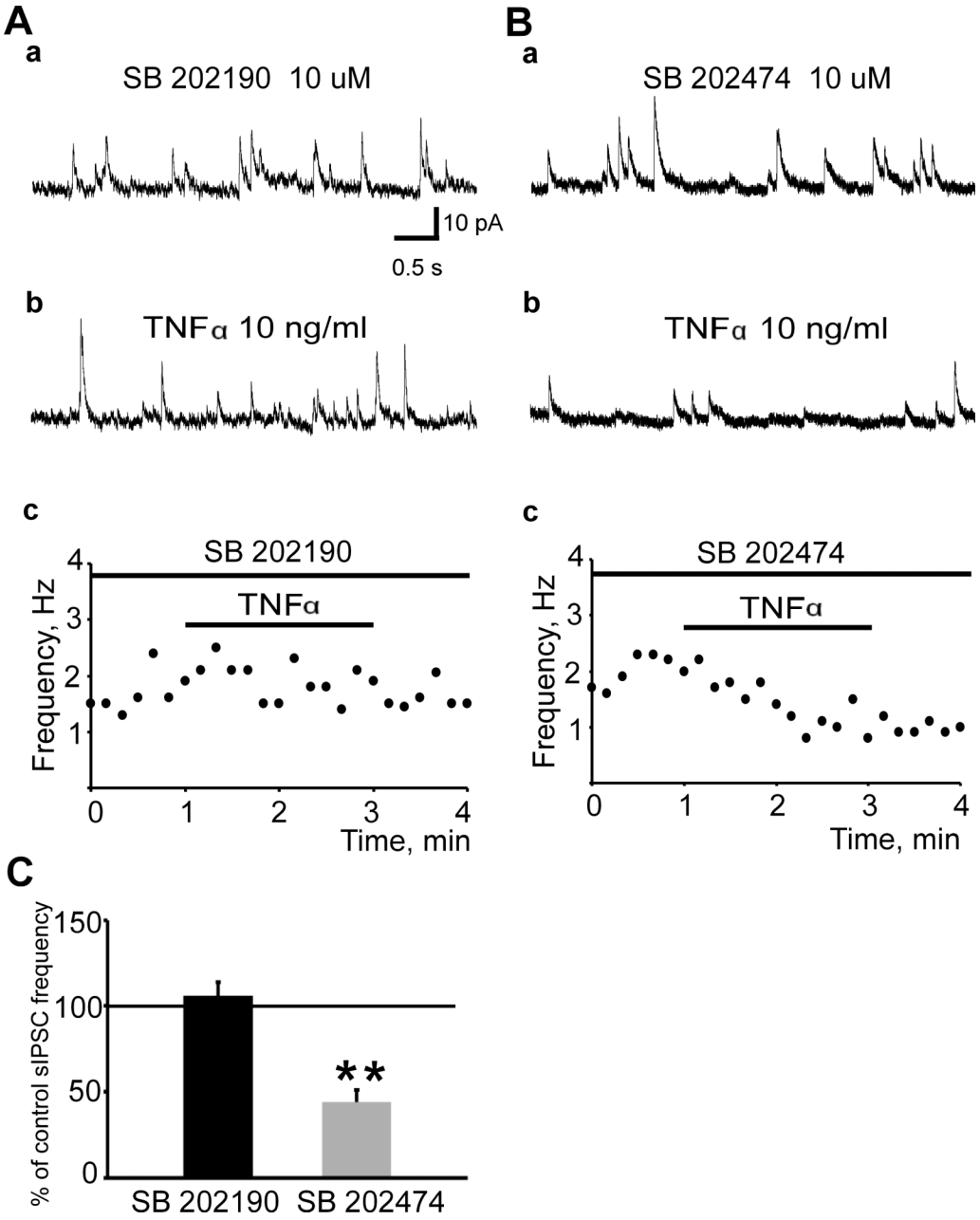

Figure 8.

TNFα-induced inhibition of spinal inhibitory synaptic transmission is mediated by the activation of p38 MAPK. **A**, The inhibitory effect of TNFα on sIPSCs was prevented by blocking the activation of p38. SIPSCs recorded from a spinal lamina II neuron before (**a**) and after (**b**) the application of TNFα when the slice was preincubated with SB202190, a p38 antagonist; **c**, Frequency of sIPSCs as a function of the recording time in the representative neuron in **a** and **b**. **B**, When the spinal slice was preincubated with SB202474, a p38 blocker inactive analog, application of TNFα significantly decreased the frequency of sIPSCs. Recordings of sIPSCs from a spinal lamina II neuron before (**a**) and after (**b**) the application of TNFα; **c**, Frequency of sIPSCs as a function of the recording time in the representative neuron in **a** and **b**. Frequency was measured every 10s in both **A** and B. **C**, Averaged data showed the percentage change of the frequency of sIPSCs after the

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application of TNFα when the slices were preincubated with SB202190 (n=15) or SB202474 (n=5). * $p < 0.05$, ** $p < 0.01$ compared with baseline before application of TNFα (student's paired *t*-test).

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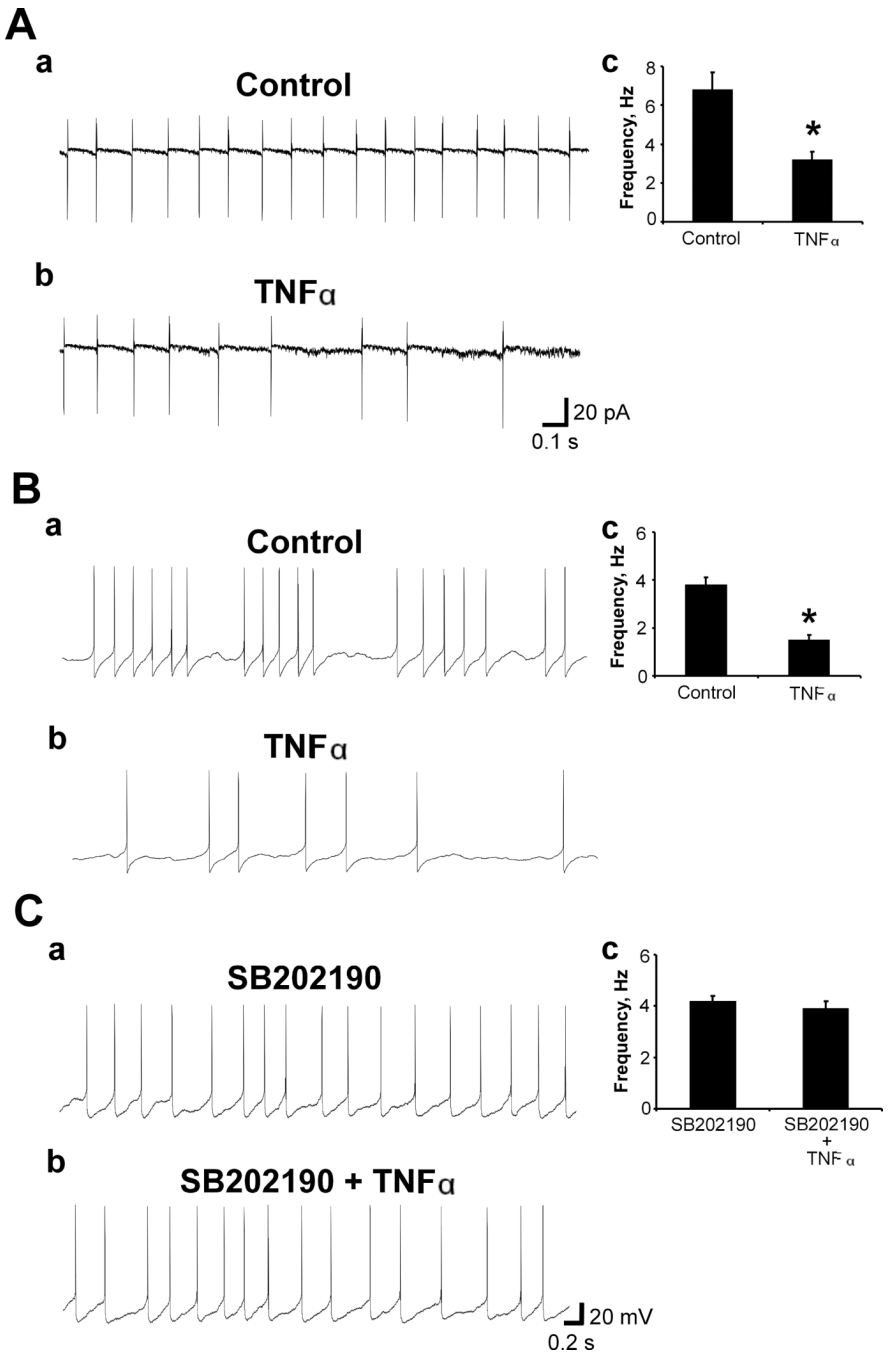

Figure 9.

TNFα decreases the frequency of spontaneously active GABAergic neurons in spinal cord dorsal horn. **A**, Recorded in cell-attach mode, a GAD67^{+} spinal dorsal horn neuron showed spontaneous discharges when bicuculline, strychnine, DNQX and D-AP5 were added in the bath (**a**). Application of TNFα reduced the frequency of spontaneous discharges (**b**); **c**, Averaged data showed decreased frequency of spontaneous discharges of GABAergic neurons after the application of TNFα (n=3). **B**, Whole-cell patch clamp recordings from GAD67+ spinal dorsal horn neurons. **a**, A representative recording from GAD67^{+} neurons showed spontaneous discharges. **b**, After the application of TNFα, the frequency of spontaneous discharges was reduced. **c**, Averaged data showed TNFα significantly reduced

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the frequency of spontaneous discharges of GABAergic neurons (n=6). Bicuculline, strychnine, DNQX and D-AP5 were added in the bath. **C**, Intracellular blockade of p38 prevented the TNFα-induced inhibition of spontaneous discharges of GABAergic neurons. **a**, A representative recording from a $GAD67^{+}$ spinal dorsal horn neuron showing spontaneous discharges with SB202190 (20 μM) added in the pipette solution. **b**, Further application of TNFα had no effect on the spontaneous discharges of the recorded neuron. **c**, Averaged data showed the frequency of spontaneous discharges before and after the application of TNFα (n=4). * $p < 0.01$ compared with baseline before application of TNFα (student's paired *t*-test).

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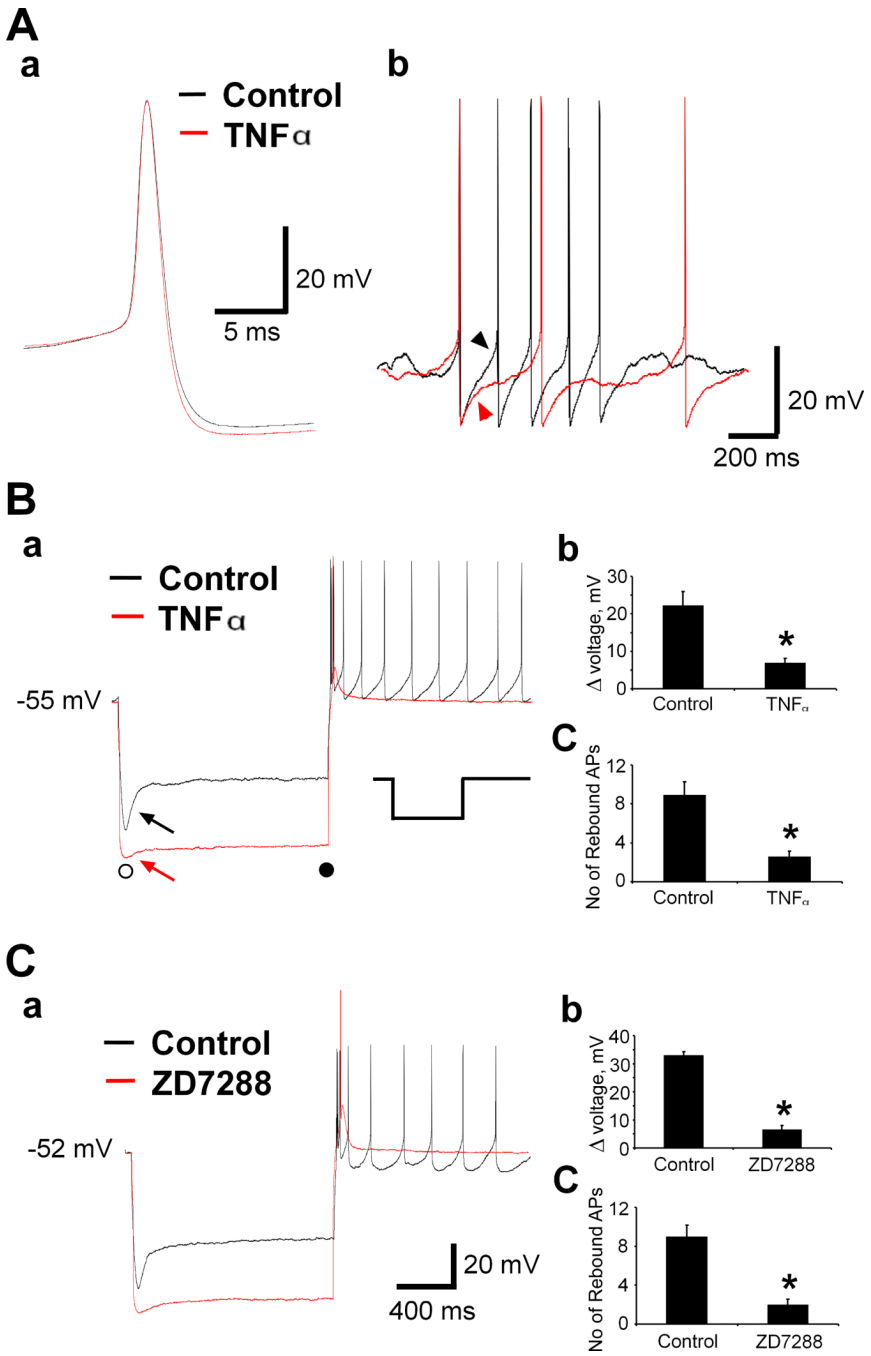

Figure 10.

TNFα inhibits the hyperpolarization-activated cation currents (Ih) of GABAergic neurons of spinal dorsal horn. **Aa**, Action potentials of a GAD67^{+} neuron recorded in control solution (black) and after the application of TNFα (red) were superimposed to show no modification of action potential waveforms. **Ab**, The depolarization ramp leading to action potential threshold was prolonged after the application of TNFα (red arrow head) compared to control solution (black arrow head), which leaded to a lower frequency of spontaneous discharges. **Ba**, In current-clamp recordings, a hyperpolarizing current step (inset) induced a “sag” (black arrow) and rebound action potentials from a GAD67^{+} neurons (black line). Application of TNFα reduced both “sag” (red arrow) and the number of rebound action

potentials in the same cell (red line). **Bb** and **Bc**, Averaged data of the changes in “sag” and the number of rebound action potentials after the application of TNFα (n=9), respectively. The amplitude of “sag” was measured by subtracting the voltage induced at the end of the hyperpolarizing step (●) from the voltage induced at the initial of the hyperpolarizing step (O). **C**, ZD7288, a specific Ih blocker, had similar effect with TNFα (n=4). * $p < 0.01$ compared with baseline before application of TNFα (student's paired *t*-test).

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