Cellular/Molecular

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

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Tumor 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 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 on 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 TNFR2 and was abolished by the p38 mitogen-activated protein kinase inhibitor SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole]. TNFα rapidly inhibited spontaneous action potentials in GABAergic neurons identified in transgenic mice expressing enhanced green fluorescent protein 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 (I_h). 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.

Introduction

Tumor necrosis 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,b, 2001a,b; Lindenlaub et al., 2000; Sorkin and Doom, 2000; Schäfers et al., 2003a). The effects of TNFα not only include transcriptional regulation of downstream 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., 2003b) and enhances transient receptor potential 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 upregulates excitatory synaptic spinal transmission and cAMP response element-binding protein (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 (Choi 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, see 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; Schäfers et al., 2003a; Svensson et al., 2003; Boyle et al., 2006; Sorkin et al., 2009). The effects of p38 activation in spinal cord remains to be more fully explored, yet there are data to support a role of p38 in spinal synaptic plasticity during central sensitization (Garry et al., 2005).

Because 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
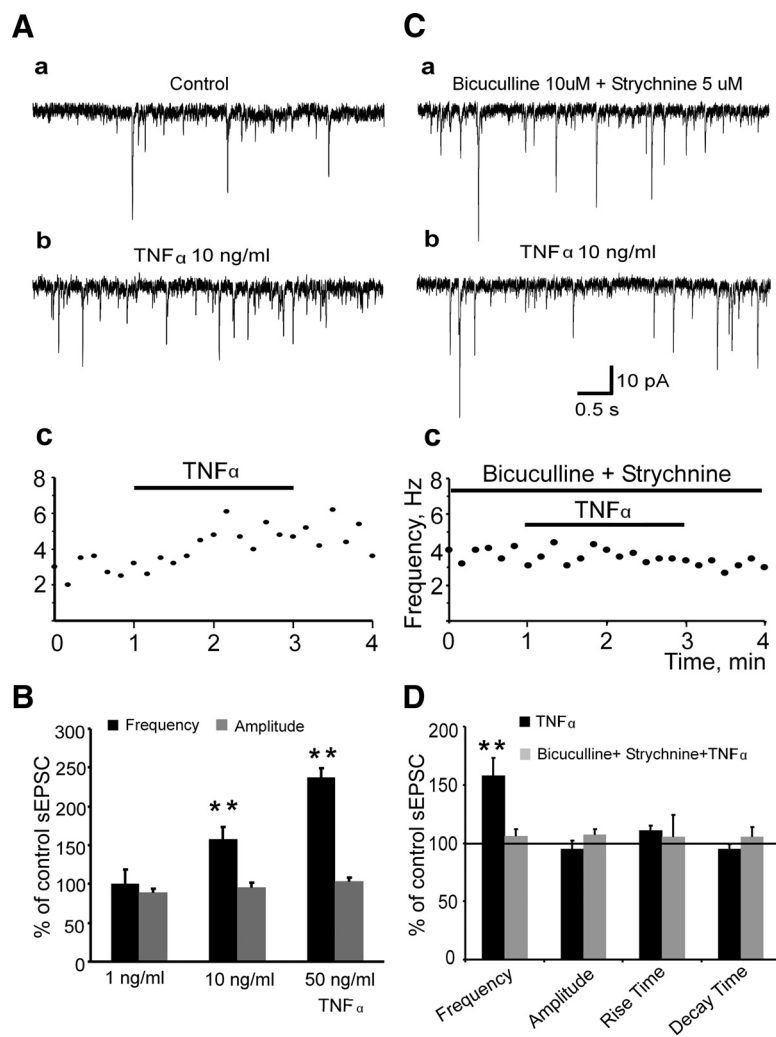
Figure 1. TNFα (10 ng/ml, 2 min) enhances excitatory synaptic transmission in spinal dorsal horn. **A**, sEPSCs recorded from a spinal lamina II neuron before (**a**) and after (**b**) the application of TNFα; **Ac**, frequency of sEPSCs as a function of recording time in the representative cell in **Aa** and **Ab**. Frequency was measured every 10 s. **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 TNFα. ******$p < 0.01$ (one-way ANOVA with *post hoc* Dunn's test). **C**, In the presence of bicuculline (10 μM) plus strychnine (10 μM) 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. ******$p < 0.01$ compared with baseline before application of TNFα (Student's paired *t* test).

et al., 1998, 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; The Jackson Laboratory) and CB6-Tg (*Gad1*–EGFP) G42Zjh/J mice of either sex were used (The Jackson Laboratory). Mice were housed under a 12 h light/dark cycle with access to food and water *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas MD 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 anesthetized with inhaled isoflurane (3%). A laminectomy was performed, and the lumber spinal cord was quickly removed and placed into ice-cold oxygenated (95% O_2/5% CO_2) artificial CSF solution containing the following (in mM): 117 sucrose, 3.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 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–L5 were cut on a vibratome (series 1000; Technical Products International). The slices were then returned to bubbled Krebs' solution (in mM: 117 NaCl, 3.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 12 glucose, and 25 $NaHCO_3$) at room temperature (~22°C) and allowed to equilibrate at least for 1 h before recording. The mice were killed by anesthetic overdose and exsanguination.

Patch-clamp recording in spinal slices. Whole-cell patch-clamp recordings from spinal SG neurons were obtained as described previously (Zhang et al., 2009). An individual slice was transferred to a recording chamber and continually perfused with oxygenated Krebs' solution (3 ml/min) at room temperature. SG cells were visualized using a 60× water-immersion objective on an upright microscope (BX50WI; Olympus) equipped with infrared and differential interference contrast (DIC) optics. Patch pipettes were made from borosilicate glass (World Precision Instruments) with resistances of 3–5 MΩ when filled with pipette solutions containing the following (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 EPSC (sEPSC) 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 IPSC (sIPSC) 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 EPSC recordings and 0 mV for IPSC recording. Signals were amplified using a Multiclamp 700B amplifier (Molecular Devices), filtered at 2–5 kHz, and sampled at 10 kHz in digital forms using a Digidata 1322A digitizing board (Molecular Devices) 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 >20%. The input resistance (R_{in}) 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 (C_m) 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 (V_m) was recorded in current-clamp mode. Leak subtraction, capacitance, and series resistance compensation were generally not applied except when 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 h and then cryoprotected in 30% sucrose solution at 4°C for at least one night. Transverse free-floating spinal cord sections (20 μm) 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 phosphorylated p38 (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 h 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: TNF receptor 1 (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 cyanine 3 (Cy3)- or FITC-conjugated secondary antibodies in 5% NDS and 0.2% Triton X-100 overnight 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 μm) were first prepared as described in patch-clamp recordings in spinal slices. After equilibrating in oxygenated Krebs' 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 h and cryoprotected in 30% sucrose overnight 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 h 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 overnight at 4°C. Slices were then mounted on glass coverslips and were viewed under a confocal microscope (FV5, FluoView V5.0; Olympus). 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 20× objective. Ten to 20 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).

Chemicals. 6,7-Dinitroquinoxaline-2, 3-dione (DNQX) and D-2-amino-5-phosphonopentanoic acid (D-AP-5) were obtained from Tocris Biosciences. Recombinant mouse TNFα was purchased from R & D Systems
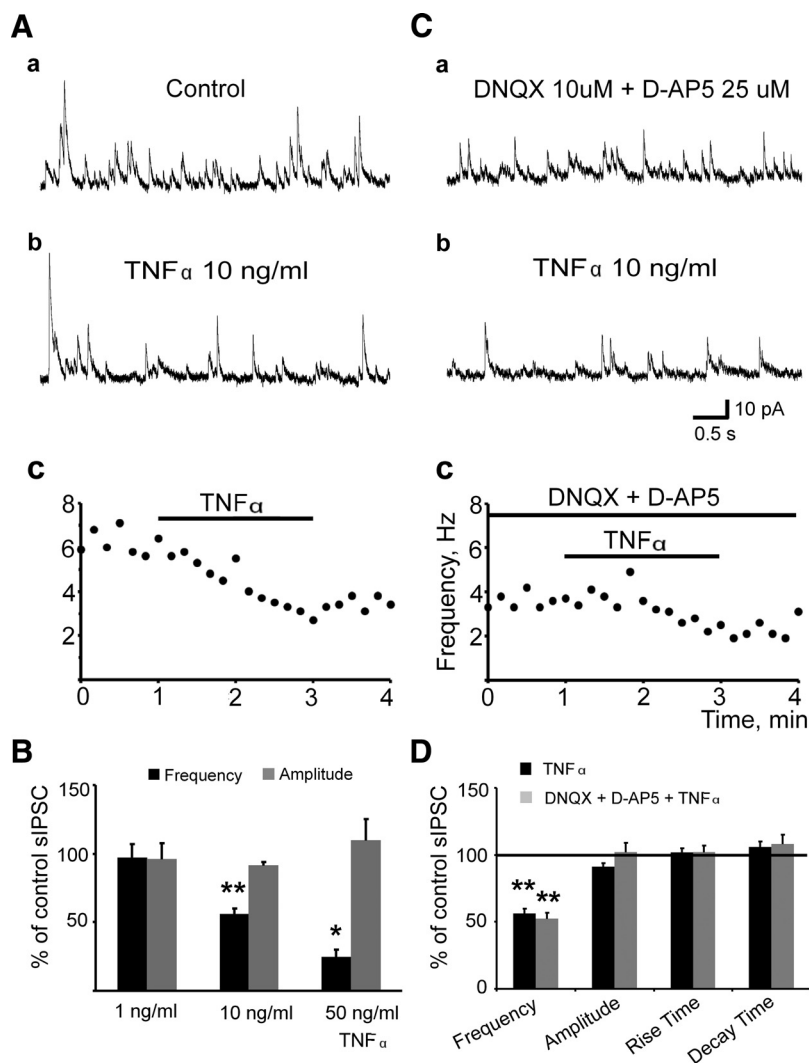

Figure 2. TNFα (10 ng/ml, 2 min) suppresses inhibitory synaptic transmission in spinal dorsal horn. ***A***, sIPSCs recorded from a spinal lamina II neuron before (***a***) and after (***b***) the application of TNFα; ***c***, frequency of sIPSCs as a function of recording time in the representative cell in ***a*** and ***b***. Frequency was measured every 10 s. ***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 TNFα. *$p < 0.05$, **$p < 0.01$ (one-way ANOVA with *post hoc* Dunn's test). ***C***, In the presence of DNQX (10 μM) plus D-AP-5 (25 μM) in the bath, application of TNFα still decreased the frequency of sIPSCs. ***D***, 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-AP-5. **$p < 0.01$ compared with baseline before application of TNFα (Student's paired t test).

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). SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole] and its inactive analog SB202474 [4-ethyl-2(*p*-methoxyphenyl)-5-(4′-pyridyl)-1*H*-imidazole] were obtained from Calbiochem and dissolved in DMSO (final concentration <0.1%). All other chemicals were obtained from Sigma-Aldrich unless otherwise noted.

Statistical analysis. All results are presented as means ± SEM. Differences between means were tested for significance using Student's paired t test or ANOVA with an α value of $p < 0.05$.

Results

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

sEPSCs and sIPSCs were recorded from randomly selected SG neurons. The effect of TNFα on excitatory synaptic transmission
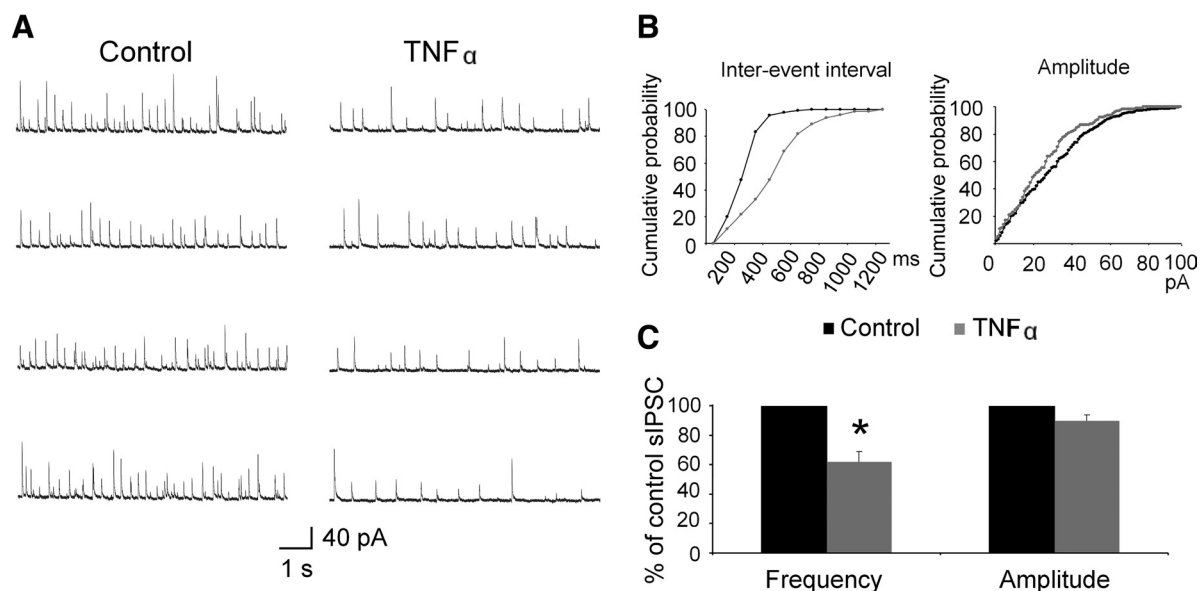
Figure 3. TNFα (10 ng/ml, 2 min) decreases the frequency of mIPSC in spinal dorsal horn. ***A***, Examples of mIPSCs before and after the application of TNFα. ***B***, Cumulative probability of mIPSC interevent interval and amplitude from the same cell shows a significant rightward shift in the distribution of interevent 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).

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 $158.3 \pm 15.0\%$ 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). Because 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 $237.3 \pm 12.4\%$ of control ($n = 4$ of 5; $p < 0.01$) at 50 ng/ml TNFα but did not change at 1 ng/ml TNFα ($99.8 \pm 18.7\%$ of control; $n = 5$). These findings are 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 attributable 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 of 10 cells from 2.8 ± 0.1 to 3.7 ± 0.2 Hz ($n = 8$; $p < 0.01$). TNFα added after bicuculline and strychnine resulted in no additional 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 (10 ng/ml, 2 min) significantly decreased the frequency of sIPSCs to $55.4 \pm 4.4\%$ 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 TNFα (Fig. 2B). The frequency of sIPSCs was decreased to $25.1 \pm 5.5\%$ of control ($n = 5$; $p < 0.05$) at 50 ng/ml TNFα, reaching the maximal inhibition. To test whether the inhibitory effect of TNFα on sIPSCs was dependent on the excitatory synaptic transmission, the AMPA–kainate receptor blocker DNQX (10 μM) and the NMDA receptor blocker D-AP-5 (25 μM) were added into the bath before the application of TNFα. As shown in Figure 2, C and D, the inhibitory effect of TNFα on sIPSCs persisted during blockade of excitatory synaptic transmission as the frequency was reduced to $52.4 \pm 5.4\%$ 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 miniature IPSCs (mIPSCs). TNFα at 10 ng/ml significantly decreased the frequency of mIPSCs to $61.6 \pm 6.9\%$ (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 interevent 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, TNFR1 (p55) and 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 PNS and CNS (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. 4 B,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 naive animals (Holmes et al., 2004; Ohtori et al., 2004), the baseline expression and the exact location of TNFα receptors in naive 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 MAP2), astrocytes (GFAP), and microglia (CD11b). The specificity of the antibodies used in the present study were defined previously (Khan et al., 2008; Vince et al., 2009). TNFR1 was confirmed to be expressed throughout the spinal cord gray matter, whereas TNFR2 was expressed only in ventral horn of naive mouse (Figs. 5 B, 6 B, respectively). It was further observed that TNFR1 was expressed on astrocytes and spinal dorsal horn neurons but not microglia (Fig. 5 C–F). In contrast, TNFR2 was only expressed in large ventral horn neurons that were consistent in location and morphology to motor neurons (Fig. 6 C–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 after tissue injury and inflammation, and blockade of p38 MAPK activation prevents the development of neuropathic and inflammatory pain (Schäfers et al., 2003a; 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 after treatment with TNFα. Spinal cord slices were prepared and bathed with TNFα as in the electrophysiological recordings (10 ng/ml in Krebs' 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 p-p38 expression in spinal dorsal horn of slices incubated with TNFα for as little as 5 min, and 30 min 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 coexpression of p-p38 was observed with the microglial marker CD11b (Fig. 7G).
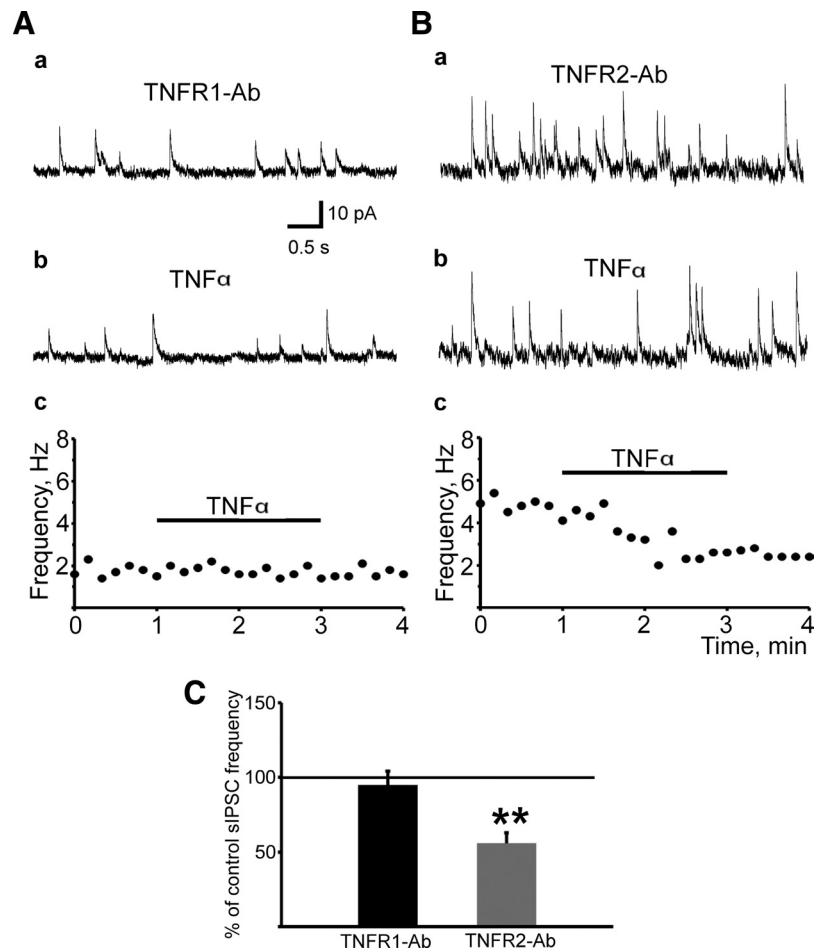

Figure 4. The inhibitory effect of TNFα on sIPSCs in spinal dorsal horn was mediated by the activation of TNFR1 but not TNFR2. A, sIPSCs recorded from a spinal lamina II neuron before (a) and after (b) the application of TNFα when the slice was preincubated with TNFR1 antibody (Ab) to neutralizing TNFR1; c, frequency of sIPSCs as a function of the recording time in the representative neuron in a and b. Frequency was measured every 10 s. 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 10 s. C, Averaged data showed that neutralizing TNFR1 ($n = 13$) but not TNFR2 ($n = 11$) prevented the inhibitory effect of TNFα on sIPSCs. **$p < 0.01$ compared with baseline before application of TNFα (Student's paired t test).

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 μM), 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 (I_h) currents

The inhibitory effect of TNFα on sIPSCs suggested that the outputs from inhibitory neurons with spontaneous activity (SA) 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 reg-
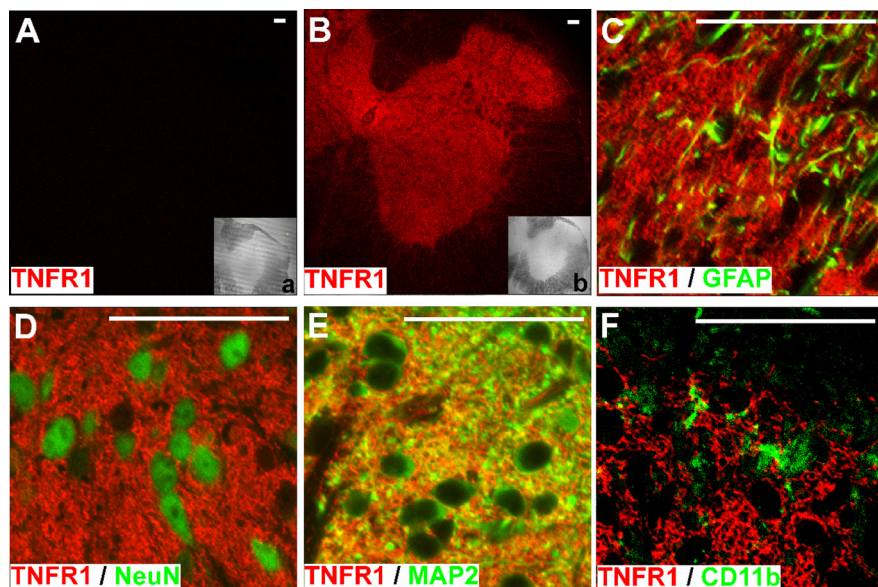
Figure 5. Localization of TNFR1 in spinal cord of naive mice. **A**, A negative control for TNFR1 staining when the primary antibody to TNFR1 is not added; **B**, TNFR1 is expressed throughout the spinal cord gray matter. **a, b**, View of the slices under the light microscope. **C**, Double immunofluorescence in spinal dorsal horn shows colocalization of TNFR1 (red) and GFAP, an astrocyte marker (green). **D**, Double immunofluorescence 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 bars, 5 μm.
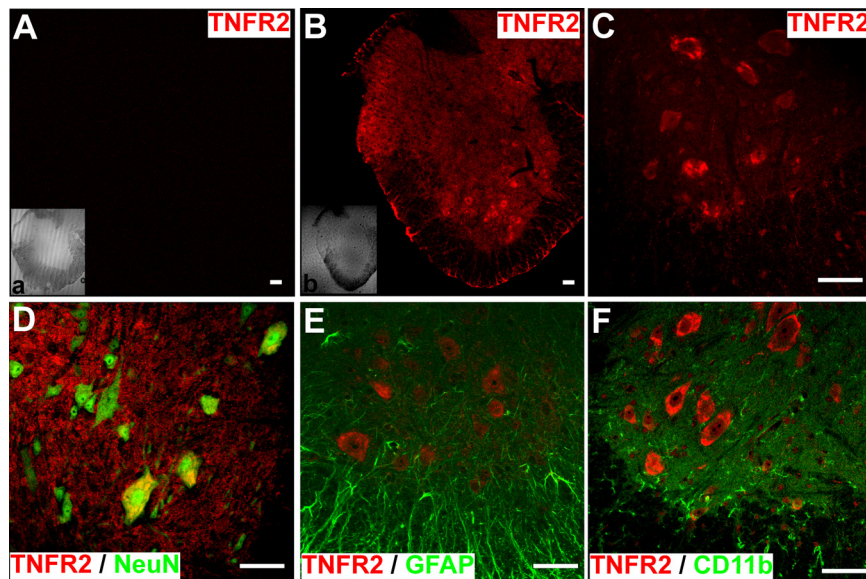

Figure 6. Localization of TNFR2 in spinal cord of naive mice. **A**, A negative control for TNFR2 staining when the primary antibody to TNFR1 is not added; **a**, view of the same slice under the light microscope. **B**, TNFR2 is expressed predominantly in spinal cord ventral horn; **b**, view of the 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 bars, 5 μm.

ulatory 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 CNS, including spinal cord dorsal horn (data not shown). Synaptic transmission was blocked by adding DNXQ (10 μM), D-AP-5 (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 (4

of 10) displayed intrinsic 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 six 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 μM) 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 intraneuronal 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 (I_h). To study the effect of TNFα on I_h currents of GABAergic neurons, cells were held at resting membrane potential and then hyperpolarized by a long current test pulse (200 pA, 2 s). A slow depolarizing "sag" was elicited during a time-dependent inward rectification after the hyperpolarization and represented the activation of I_h 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 that I_h 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, Bb) 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 ($n =$
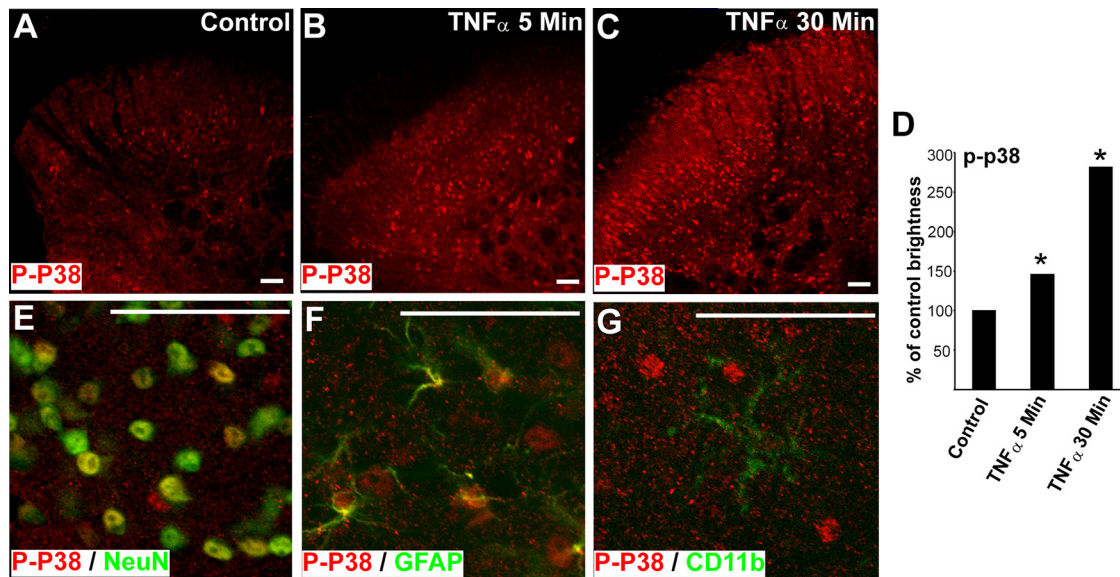
Figure 7. Expression of p-p38 in spinal dorsal horn induced by the application of TNFα. **A**, A baseline expression of p-p38 is detected in spinal dorsal horn in naive mice. **B**, **C**, A robust increase in p-p38 expression in spinal dorsal horn is detected after spinal slices are incubated with TNFα (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 5 min ($n = 15$) and 30 min ($n = 18$) treatment with TNFα significantly increased the expression of p-p38 in spinal cord laminas I–II compared with control ($n = 11$). The expression of p-p38 was significantly increased after 30 min treatment of TNFα compared with 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 bars, 5 μm.

9; $p < 0.01$) (Fig. 10 *Ba,Bc*). The effect of TNFα on sag and rebound action potentials mimics the effect of ZD7288 (4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride) (50 μM; $n = 4$) (Fig. 10*C*), a specific I_h blocker, suggesting that TNFα has a selectivity for I_h 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., 2003b,c; 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 calcitonin gene-related peptide release from nociceptor terminals in skin (Oprée and Kress, 2000), sensitizes peripheral Aβ- and C-fibers (Junger and Sorkin, 2000; Liu et al., 2002; Zhang et al., 2002; Schäfers et al., 2003b), 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 previous observations. Importantly, it is further shown here that the enhancement of spinal excitatory synaptic transmission evoked by TNFα is attributable to inhibition of ongoing 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 claimed previously 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, 1999; Moore et al., 2002). Thus, the data shown here implicate 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 after 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, 2005, 2008; Schäfers et al., 2003a; Tsuda et al., 2004; Boyle et al., 2006; Zhuang et al., 2007; Sorkin et al., 2009; Fitzsimmons et al., 2010). Although the activation of p38 in spinal cord has been suggested previously as mediating many effects of TNFα (Schäfers et al., 2003a; Svensson et al., 2005; Boyle et al., 2006), direct evidence at the spinal level has been lacking. The data shown here in which TNFα-induced disinhibition of spinal synaptic transmission was prevented by blocking the activation of p38 and in which bath application of
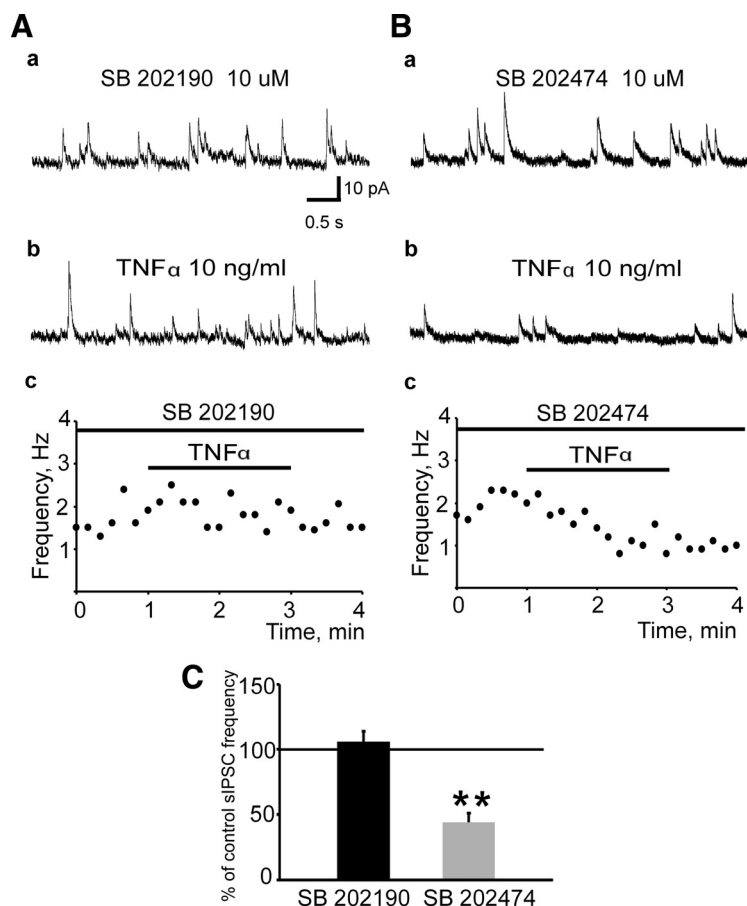
Figure 8. TNFα-induced inhibition of spinal inhibitory synaptic transmission is mediated by the activation of p38 MAPK. ***A***, The inhibitory effect of TNFα on sIPSCs was prevented by blocking the activation p38. sIPSCs recorded from a spinal lamina II neuron before (***a***) and after (***b***) the 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 10 s in both ***A*** and ***B***. ***C***, Averaged data showed the percentage change of the frequency of sIPSCs after the application of TNFα when the slices were preincubated with SB202190 ($n = 15$) or SB202474 ($n = 5$). **$p < 0.01$ compared with baseline before application of TNFα (Student's paired t test).

TNFα activated p38 predominantly in spinal dorsal horn neurons strongly implicate p38 as a key downstream mediator after 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 after nerve injury (Ji et al., 2002; Jin et al., 2003; Schäfers et al., 2003a; 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 EGFP in the upstream regulatory sequence from murine *Gad1* gene (encoding the GABA synthesizing enzyme 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 approximately 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 that 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 (I_h), 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 I_h 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). Because I_h 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; Lüthi and McCormick, 1998), it may also modulate activities of GABAergic neurons in spinal dorsal horn. After the application of TNFα, I_h currents of GABAergic neurons were decreased, suggesting that TNFα may reduce the intrinsic spontaneous discharges of these GABAergic neurons through inhibition of I_h currents. The fact that ZD7288, a specific I_h

current blocker, produced the same effect as of TNFα on inhibiting I_h current is in line with this conclusion. The data that only a small proportion of non-EGFP neurons have I_h 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 found recently to actively contribute to the development of neuropathic pain after peripheral nerve injury (Zhuang et al., 2006; Gao et al., 2009). After activation, spinal astrocytes can release various molecules that can activate spinal dorsal horn neurons (for review, see Watkins and Maier, 2003). The data shown here revealed that the activation of the TNFα–TNFR1–p38 pathway in spinal neurons is sufficient to induce the inhibition of spinal inhibitory transmission, but the possible role of spinal astrocytes cannot be excluded and may need additional 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 of spontaneous activities of GABAergic neurons was accompanied with a reduction of I_h currents, suggesting that TNFα may inhibit neuronal activities through reducing I_h 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.
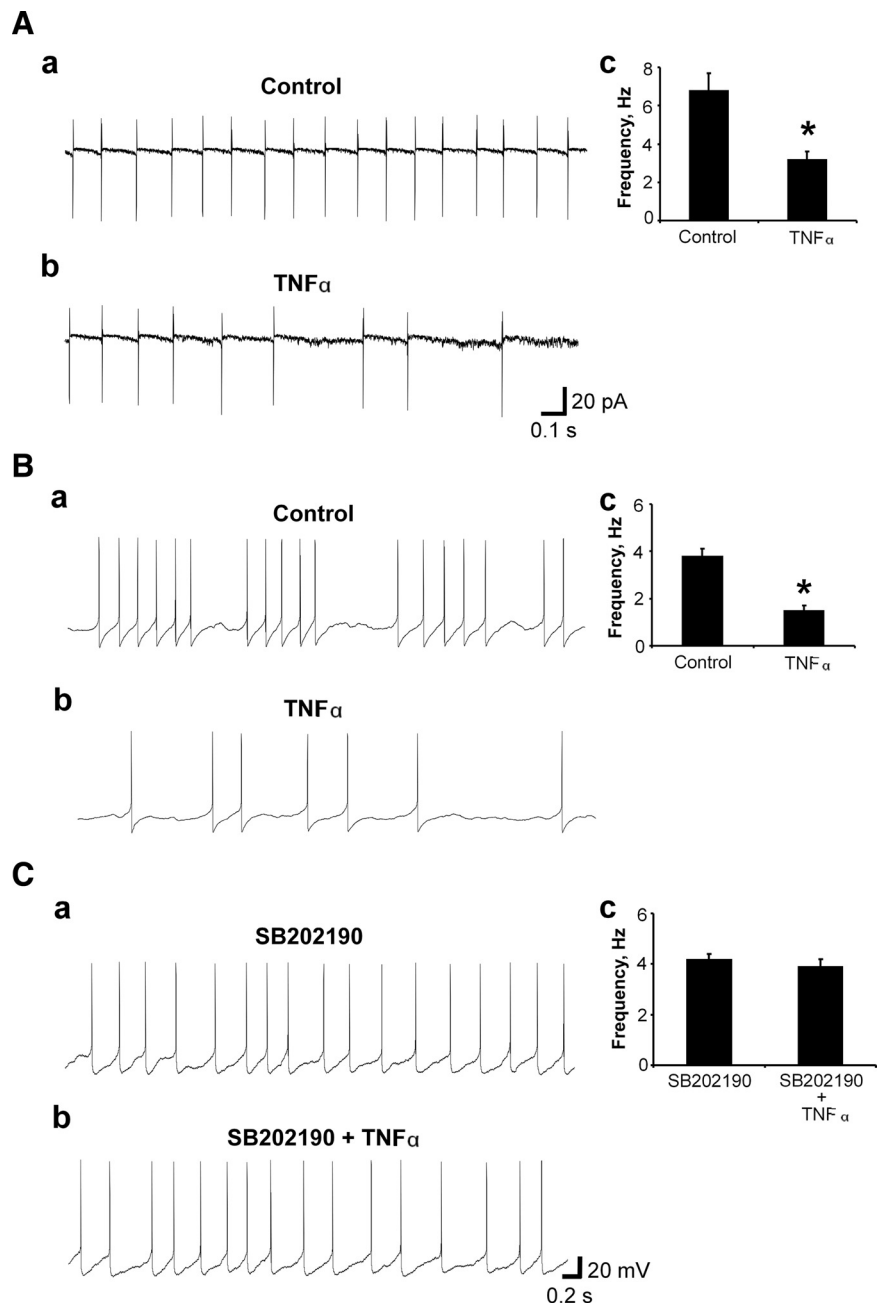
Figure 9. TNFα decreases the frequency of spontaneously active GABAergic neurons in spinal cord dorsal horn. **A**, Recorded in cell-attach mode, a GAD67^{+} spinal dorsal horn neuron showed spontaneous discharges when bicuculline, strychnine, DNQX, and D-AP-5 were added in the bath (**a**). Application 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 the frequency of spontaneous discharges of GABAergic neurons (n = 6). Bicuculline, strychnine, DNQX, and D-AP-5 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**, Additional 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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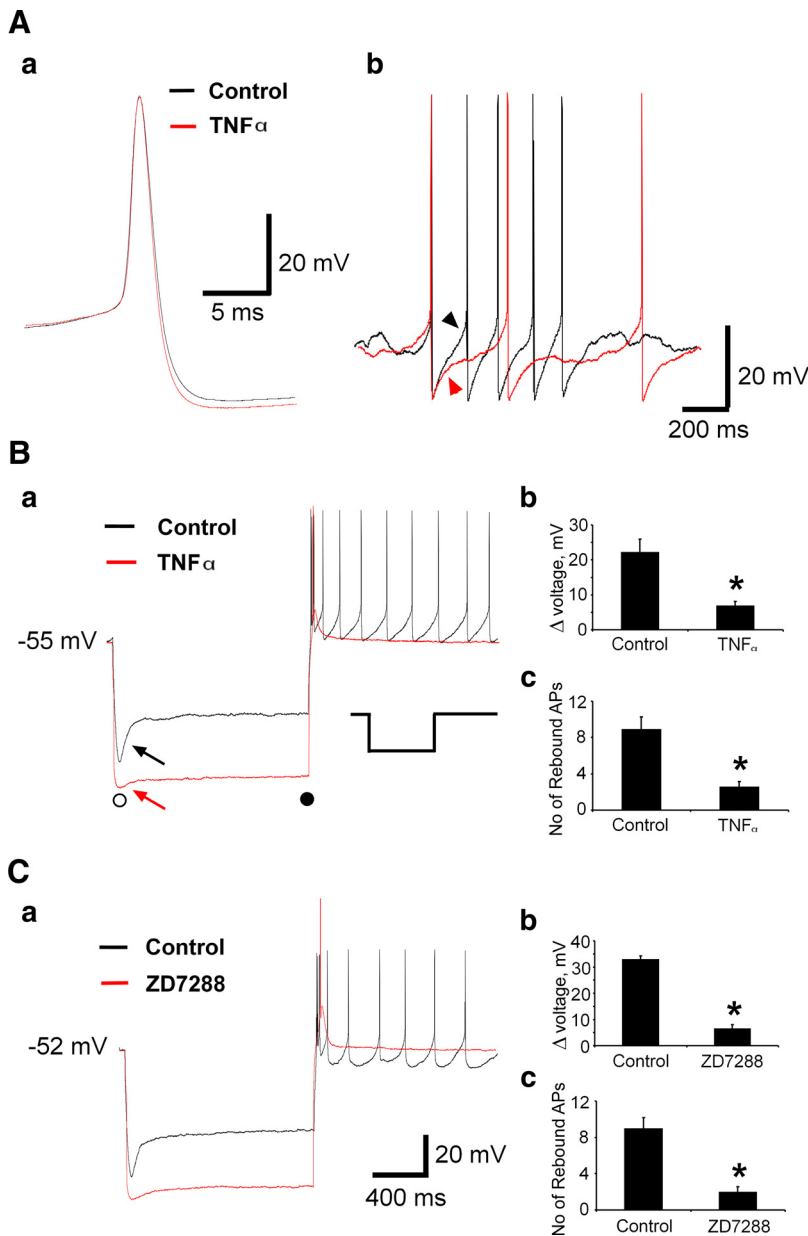
Figure 10. TNFα inhibits the hyperpolarization-activated cation currents (I_h) of GABAergic neurons of spinal dorsal horn. *Aa*, Action potentials of a GAD67 $^+$ neuron recorded in control solution (black) and after the application of TNFα (red) were superimposed to show no modification of action potential waveforms. *Ab*, The depolarization ramp leading to action potential threshold was prolonged after the application of TNFα (red arrowhead) compared with control solution (black arrowhead), which led 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 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*, *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 (○). *C*, ZD7288, a specific I_h 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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