Dynamic effects of TNF-α on synaptic transmission in mice over time following sciatic nerve chronic constriction injury

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Running Head: Reduced effect of TNF-α in CCI mice

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Abstract
Nerve injury–induced central sensitization can manifest as an increase in excitatory synaptic transmission and/or as a decrease in inhibitory synaptic transmission in spinal dorsal horn neurons. Cytokines such as tumor necrosis factor-α (TNF-α) are induced in the spinal cord under various injury conditions and contribute to neuropathic pain. In this study we examined the effect of TNF-α in modulating excitatory and inhibitory synaptic input to spinal substantia gelatinosa (SG) neurons over time in mice following chronic constriction injury (CCI) of the sciatic nerve. Whole-cell patch clamp studies from SG neurons showed that TNF-α enhanced overall excitability of the spinal cord early in time following nerve injury 3 days after CCI compared to that in sham control mice. In contrast the effects of TNF were blunted 14 days after CCI in nerve injured mice compared to sham surgery mice. Immunohistochemical staining showed that the expression of TNF-α receptor 1 (TNFR1) was increased at 3 days but decreased 14 days following CCI in the ipsilateral versus the contralateral spinal cord dorsal horn. These results suggest that TNF-a acting at TNFR1 is important in the development of neuropathic pain by facilitating excitatory synaptic signaling in the acute phases after nerve injury but has a reduced effect on spinal neuron signaling in the later phases of nerve injury induced pain. Failure of the facilitatory effects of TNFα on excitatory synaptic signaling in the dorsal horn to resolve following nerve injury may be an important component in the transition between acute and chronic pain conditions.

Keywords: CCI, hyperalgesia, substantia gelatinosa, spinal synaptic transmission.
Introduction

Nerve injury caused by trauma or surgery can produce persistent neuropathic pain, a chronic condition that is often resistant to conventional analgesics (Sindrup and Jensen 1999; Woolf and Mannion 1999). Allodynia and hyperalgesia are not only the result of peripheral sensitization at the site of injury but also a consequence of hyperactivity or sensitization of neurons in the spinal dorsal horn (Matzner and Devor 1994; Woolf and Mannion 1999). Several mechanisms have been proposed as contributing to central sensitization in neuropathic pain. These include increased excitatory neuronal processes in the spinal cord, parallel decreases in the activity of spinal neuronal inhibitory processes (Costigan et al, 2009; Sandkuhler 2009) and changes in the regulation of the spinal microenvironment by spinal astrocytes and microglia (Gao and Ji 2010; Milligan and Watkins 2009).

Tumor necrosis factor-α (TNF-α) is among the proinflammatory cytokines expressed by a variety of cell types including immune cells and glial cells (Mannel 1986; Ohtori et al, 2004; Xu et al, 2006) that play a critical role in inflammatory and neuropathic pain both in the peripheral as well as in the central nervous system (Cunha et al, 1992; Ferreira et al, 1993; Schafers et al, 2003a; Schafers et al, 2003c; Schäfers et al, 2003; Sommer et al, 1998; Sommer et al, 2001; Watkins et al, 1995; Woolf et al, 1997; Xu et al, 2006). TNF-α is increased at the injury site following chronic constriction injury (CCI) of the sciatic nerve in rats (George et al, 1999; George et al, 2004; Shubayev and Myers 2000) and intra-sciatic injection of TNF-α in rats reproduces CCI-like pain hypersensitivity (Sorkin and Doom 2000; Wagner and Myers 1996). Conversely, CCI-induced hypersensitivity is reversed with peripheral administration of neutralizing antibodies to TNF-α or to TNF receptors (TNFRs), in particular TNFR1 (Sommer et al,
Similarly, intra-peritoneal injection of the TNF-α inhibitor, etanercept, suppresses hypersensitivity after nerve injury (Iwatsuki et al, 2013) and mice lacking TNF-α or TNFR1 show reduced nociceptive sensitivity compared with wild-type littermates after nerve injury (Nadeau et al, 2011).

TNF-α also has an important role within the central nervous system (CNS) in generating neuropathic pain. For example, intrathecal injection of TNF-α induces mechanical allodynia and heat hyperalgesia (Gao et al, 2009; Narita et al, 2008) and TNF-α among other cytokines are released by spinal glial cells (both microglia and astrocytes) that are activated following nerve injury or inflammation (Gao and Ji 2010; Milligan and Watkins 2009). Suppression of this response of spinal glial cells similarly suppresses behavioral hypersensitivity to peripheral stimuli normally observed following nerve injury (Gao and Ji 2010; Milligan and Watkins 2009). Yet, the specific mechanisms underlying the effects of TNF-α within the CNS are not well understood.

Previous experiments showed that acute application of TNF-α significantly reduced the frequency of spontaneous inhibitory synaptic currents (sIPSCs) and also produced an increase in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in spinal substantia gelatinosa (SG) neurons isolated from naïve mice (Zhang et al, 2010). Acute application of TNF-α directly inhibits the excitability of a subset of spinal GABAergic neurons (Zhang and Dougherty 2011). However, the functional changes of TNF-α on synaptic input to spinal neurons in neuropathic pain conditions have not been tested. This gap in knowledge was addressed in this study.
Materials and Methods

Animals. Sixty-nine C57BL/6 mice of either sex weighing 20-25g were used. All the surgical and experimental protocols were approved by the Animal Care and Use Committee of MD Anderson Cancer Center and conformed to the NIH guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

CCI model and behavioral test. Mice were deeply anesthetized with 2-3% isoflurane. The left sciatic nerve was exposed at the mid-thigh level proximal to the trifurcation and freed from adhering tissue. Three ligatures (chromic gut, 6-0) were tied around the nerve with about 1 mm spacing in between similar to procedures previously used on rats (Carlton et al, 1991; Dougherty et al, 1992; Palecek et al, 1992). The muscle and skin incisions were then closed. In sham animals, the left sciatic nerve was exposed and freed from the connective tissue as in the CCI mice but not surrounded with suture. The response of mice to mechanical stimulation of both hindpaws was evaluated daily beginning 3 days before CCI and then at day 1, 3, 7 and 14 after surgery. Animals were placed under acrylic boxes that were atop wire mesh floors and allowed to habituate for 1 h. Von Frey filaments were applied to the plantar surface of paw and the withdrawal threshold measured using an up-down method beginning with a 0.6g filament (Chaplan, et al., 1994). Mice with confirmed mechanical hypersensitivity in the ipsilateral paw (compared to the contralateral paw) and mice with sham CCI exited the behavioral studies to the terminal electrophysiological or immunohistochemical experiments at either day 3 or 14 after surgery.
**Spinal Cord Slice Preparation.** Mice were anesthetized with 2-3% isoflurane, and lumbar segments 4 to 6 of the spinal cord were rapidly removed through laminectomy. The mice were then killed by inhalation of 5% isoflurane and exsanguination. The spinal cord was immediately placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF) saturated with 95% O$_2$ and 5% CO$_2$. The sucrose ACSF contained 234 mM sucrose, 3.6 mM KCl, 1.2 mM MgCl$_2$, 2.5 mM CaCl$_2$, 1.2 mM NaH$_2$PO$_4$, 12.0 mM glucose, and 25.0 mM NaHCO$_3$. The tissue was then placed in a shallow groove formed in a gelatin block and glued onto the stage of a Vibratome (Products International, Inc., St. Louis, MO). Transverse spinal cord slices (300 μm) through the L5 segment were cut in the ice-cold sucrose ACSF then transferred and incubated in Krebs' solution bubbled with 95% O$_2$ and 5% CO$_2$ at 34°C for at least 1 h before further use. The Krebs' solution contained 117.0 mM NaCl, 3.6 mM KCl, 1.2 mM MgCl$_2$, 1.5 mM CaCl$_2$, 1.2 mM NaH$_2$PO$_4$, 11.0 mM glucose, and 25.0 mM NaHCO$_3$. Slices for study were placed in a glass-bottomed recording chamber (Warner Instruments, Hamden, CT) and fixed with parallel nylon threads supported by a U-shaped stainless steel weight. The slice was continuously perfused with Krebs' solution at 3.0 ml/min at 34°C maintained by an inline solution heater and a temperature controller (TC-344B; Warner Instruments, Hamden, CT).

**Electrophysiological Recordings.** Recordings of postsynaptic currents were performed using whole-cell voltage-clamp as described previously (Zhang et al. 2010). All recordings were conducted in randomly selected lamina II neurons ipsilateral to the surgery side (CCI or sham). Lamina II was identified by its distinctive translucent appearance and neurons located using a fixed stage microscope (BX51WI; Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. Electrodes
for the whole-cell recordings were triple pulled from borosilicate glass capillary tubes using a horizontal electrode puller (P-97; Sutter Instrument Company, Novato, CA). The impedance of the pipette was 4 to 7 MΩ when filled with internal solution containing 110.0 mM Cs₂SO₄, 5.0 mM KCl, 2.0 mM MgCl₂, 0.5 mM CaCl₂, 5.0 mM HEPES, 5.0 mM EGTA, 5.0 mM ATP-Mg, 0.5 mM Na-GTP, adjusted to pH 7.2 to 7.4 with 1 M CsOH (290–320 mOsm). QX314, a sodium channel blocker, was added to the internal solution to suppress action potentials in targeted neurons. Recordings of postsynaptic currents began approximately 5 min after whole-cell access was established and the current reached a steady state. Input resistance was monitored and recording was abandoned if this changed by more than 15%. Signals were amplified using an MultiClamp700B (Molecular Devices Cellular Neurosciences, Foster City, CA) at a holding potential of 0 mV for sIPSCs and -70 mV for sEPSCs, filtered at 2 kHz, and digitized at 10 kHz (Digidata 1322A, Molecular Devices Cellular Neurosciences, Foster City, CA) and stored on a personal computer for later analysis.

**Immunohistochemistry.** CCI and sham mice (n=4 per group, per time point) were deeply anesthetized with pentobarbital (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 L5 spinal cord segment was 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. All of the sections were first blocked with 10% normal donkey serum (NDS) and 0.2% Triton X-100 in phosphate-buffer saline (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 TNFR1 (rabbit, 1:1000; Abcam) and GFAP (mouse, 1:1000; Cell Signaling Technology). The sections were then incubated with cyanine 3 (Cy3-) and FITC-conjugated secondary antibodies in 5% NDS and 0.2% Triton X-100 overnight at 4°C. To test the specificity of the TNFR1 antibody, a preabsorption experiment was conducted by incubating TNFR1 antibody (1 µg/ml, Abcam) with TNFR1 peptide (5 µg/ml) for 1 hour at room temperature before incubating with spinal cord slices.

**Chemicals.** Recombinant mouse TNF-α was purchased from R&D Systems and prepared as stock solutions at 10 µg/ml in PBS with 0.1% bovine serum albumin. Bicuculline and strychnine were obtained from Sigma (St. Louis, MO).

**Data analysis, Electrophysiology.** Data are presented as the mean ± S.E.M. The sIPSCs and sEPSCs were analyzed off-line. Amplitude and frequency of sIPSCs and sEPSCs were analyzed from 1 min segments collected 30s before, following 2min application and then following 10 to 15min after application of TNF-α. The sIPSCs and sEPSCs were detected as a signal with fast rise time (<200µs) achieving an amplitude 2X threshold above the background noise (MiniAnalysis, Synaptosoft, Decatur, GA). The inter-event intervals of sIPSCs or sEPSCs of each cell (CCI or sham) were then analyzed using the Kolmogorov-Smirnov test to determine and categorize each cell as either responsive or not to TNF-α (MiniAnalysis, Synaptosoft, Decatur, GA). Further statistical analyses of grouped responses were carried out using GraphPad Prism 5.0 software. Fisher's exact test was used to compare the proportion of cells responsive to TNFα in the CCI or Sham groups. Within group (CCI, Sham) differences in responses before and after drug application were evaluated with a paired t-test. Finally, differences in baseline and post-TNF responses between CCI and Sham groups were evaluated with
one-way analysis of variance (ANOVA). $P < 0.05$ was considered in all analyses to indicate statistical significance.

**Data analysis, Immunohistochemistry.** Ten slices were randomly selected from each animal for analysis using NIS Elements imaging software (Nikon) as previously described (Zhang et al, 2011). The area of translucent lamina II both ipsilateral and contralateral to CCI (or sham) was determined and outlined from images captured while viewed under DIC. Fluorescence images from the same orientation first had background subtracted and then the relative brightness of TNFR1- or GFAP- immunoreactivity for the whole of lamina II on both sides was measured for each slice. The exposure times and illumination intensities were identical for all images in a given experiment. Side-to-side differences in fluorescence intensity within the CCI and Sham treatment groups were determined using paired t-tests.

**Results**

**CCI induced prominent mechanical hypersensitivity in mouse**

The withdrawal threshold to mechanical stimulation showed a decrease in the ipsilateral compared to the contralateral paw in CCI mice by 1 day after surgery and this difference lasted at least 14 days. The mechanical threshold significantly decreased in the ipsilateral paw from $1.12 \pm 0.04$ to $0.27 \pm 0.07$ g in CCI mice by 14 days after surgery whereas the withdrawal threshold in the contralateral paw remained at $1.18 \pm 0.04$ (Fig. 1A). Mechanical withdrawal threshold was not altered significantly in sham surgery mice (from $1.24 \pm 0.07$ to $1.11 \pm 0.07$ g) through day 14 after surgery (Fig. 1B).
Effect of TNF-α on inhibitory and excitatory synaptic input to spinal cord SG neurons 3 days after CCI

TNF-α inhibits inhibitory inputs (disinhibits) spinal SG neurons in naïve mice resulting in an increased magnitude of excitatory inputs to these cells (Zhang et al, 2010). To test whether peripheral nerve injury induces plasticity in these effects, the effect of TNF-α (10ng/ml, 3min) on the frequency and amplitude of sIPSCs and sEPSC’s of spinal SG neurons was determined in sham and CCI mice 3 days after surgery.

There was no significant difference between the baseline sIPSC frequencies between the CCI and sham surgery groups. The baseline frequency of sIPSCs in sham mice was 2.41 ± 0.46 Hz (n=11) and the baseline frequency of sIPSCs in CCI mice was 2.71± 0.46 Hz (n=15). The proportions of neurons showing response to TNF-α was also not changed as 10 of 11 (90.9%) SG neurons responded to TNF-α in sham mice and 14 of 15 (93.3%) SG neurons responded to TNF-α in CCI mice based on analysis of inter-event intervals of sIPSCs before and after drug application (Fig. 2A). TNF-α significantly reduced the frequency of sIPSCs in both sham (from 2.41 ± 0.47 to 1.17 ± 0.20 Hz, n=10, P<0.05) and CCI mice (from 2.71 ± 0.46 to 1.25 ± 0.27 Hz, n=14, P<0.05, Fig. 2B and D). There was no significant difference in the magnitude of inhibition between sham (51.45%) and CCI mice (53.87%). TNF-α had no effect on the amplitude of sIPSCs in either sham or CCI mice (Fig. 2C and D). These results indicate that the effect of TNF-α on inhibitory synaptic transmission to SG neurons was not changed at three days following peripheral nerve injury.

On the other hand the effect of TNF-α on sEPSCs was increased in CCI 3 days after injury in comparison to sham operated mice. A total of 15 neurons in sham and 16 neurons in CCI mice were recorded in SG 3 days after injury. There was no significant
difference between the baseline sEPSC frequency between sham and CCI mice, with the mean baseline frequency of sEPSCs in sham mice at 4.14 ± 0.55 Hz (n=15) and the baseline frequency of sEPSCs in CCI mice at 3.73± 0.48 Hz (n=16). TNF-α significantly increased the frequency of sEPSCs of in 7 of 15 (46.7%) SG neurons from 3.21 ± 0.43 to 5.47 ± 0.72 Hz (n=7, P<0.05, Fig. 2E, F and H) in sham mice but increased the frequency of sEPSCs in 14 of the 16 SG neurons (87.5%) from 3.45 ± 0.51 to 6.03 ± 0.70 Hz (n=14, P<0.05, Fig. 2E and F) in CCI mice. Although the effect on sEPSC rate was not significantly different, with sham rats showing an increase of 170.4% and CCI rats showing an increase to 174.8% of the baseline rate, the overall percentage of neurons responding to TNF was significantly greater in CCI mice than that in sham mice (Fig. 2E). TNF-α again had no effect on the amplitude of sEPSCs (Fig.2G) in either sham or CCI mice. These results indicate that in contrast to the effects on sIPSCs, TNF-α had an augmented effect on excitatory synaptic transmission of spinal SG neurons at 3 days after CCI.

Effect of TNF-α on inhibitory and excitatory synaptic input to spinal cord SG neurons 14 days after CCI

TNF-α reduced the frequency of sIPSCs in 21 of 23 (91.3%) neurons in sham mice (Fig. 3A) from an average of 2.95 ± 0.47 to 1.62 ± 0.28 Hz (n=21, P<0.05, Fig. 3B), a 54.9% reduction from the baseline before TNF-α application. Hence, TNF had the same effect on both the numbers of cells affected and overall decrease in rate at both 3 and 14 days in sham surgery mice. In contrast, only 11 out of 21 cells (52.4%) responded to TNF-α in CCI mice, which was significantly less than the response rate in sham mice (91.3%, Fig. 3A). The effect of TNF-α in those cells that responded was similar with the
mean frequency of sIPSCs dropping from 2.85 ± 0.46 to 1.89 ± 0.36 Hz (n=11, P<0.05, Fig. 3B), or 66.42 ± 0.53% of baseline. Again, there was no significant effect of TNF-α on the amplitude of sIPSCs of SG neurons in sham mice or CCI mice (Fig. 3C). These results suggest that the effect of TNF-α on inhibitory synaptic transmission of spinal SG neurons is reduced 14 days after CCI.

The effect of TNF-α on the sEPSCs was also tested 14 days after nerve injury. TNF-α significantly increased the frequency of sEPSCs in 11 of 16 (68.7 %) neurons in sham mice (Fig. 3E) with the mean frequency increasing from 3.39 ± 0.62 to 5.37 ± 0.89 Hz (n=11, P<0.05, Fig. 3F) or 164.57 ± 12.66% of baseline, an effect similar to that observed at 3 days following the sham surgery. In CCI mice, sEPSCs were recorded from a total of 15 spinal SG neurons and 7 cells (46.7%, Fig. 3E) responded to TNF-α where the mean frequency of sEPSCs increased from 3.46 ± 0.37 to 4.88 ± 0.39 Hz (n=7, P<0.05 Fig. 3F) or 143.96 ± 6.31% of baseline. Although TNF-α affected a smaller proportion of the neurons from CCI than sham mice, this difference did not achieve statistical significance. Finally, once again, TNF-α had no significant effect on the amplitude of sEPSCs in either sham mice or CCI mice (Fig. 3G).

**Effect of TNF-α on GABAergic and glycinergic inhibitory input to SG neurons in the spinal cord dorsal horn**

Inhibitory neurotransmission in the spinal cord is primarily mediated by GABA and glycine. The possible differential effect of TNF-α on these inhibitory synaptic processes was examined pharmacologically by adding bicuculline (10 µM) or strychnine (1 µM) in the recording chamber. As shown in Fig. 4A, GABAergic sIPSCs were defined by their characteristic long duration, with a mean rise time of 4.74 ± 0.38 ms and mean
decay time of 19.29 ± 1.88 ms. TNF-α significantly decreased the frequency of GABAergic IPSCs by 65.6% (from 1.89 ± 0.61 to 1.19 ± 0.39 Hz, n=7, P<0.05, Fig. 4C) in 7 of 9 neurons without changing the amplitude (19.56 ± 3.51 to 17.60 ± 2.69 pA, 92.87% of baseline). As shown in Fig. 4B, glycinergic postsynaptic currents were defined by their characteristic short duration, with a mean rise time of 3.73 ± 0.42 ms and mean decay time of 4.87 ± 0.33 ms. TNF-α also decreased the frequency of glycinergic IPSCs by 59.34% in 8 of 10 neurons (from 1.56 ± 0.67 to 0.86 ± 0.34 Hz, n=8, P<0.05, Fig. 4C) without significant effect on the amplitude (from 29.74 ± 4.18 to 27.03 ± 3.88 pA, 92.13% of control). These results suggest that TNF-α indiscriminately inhibits both GABAergic and glycinergic inhibitory inputs to SG neurons.
Expression of TNFR1 in the spinal cord dorsal horn over time after CCI

TNF-α receptors are normally expressed in the spinal cord of naive animals (Holmes et al, 2004; Ohtori et al, 2004). Previous work confirmed that TNFR1 is expressed throughout the spinal cord gray matter in both neurons and astrocytes, whereas TNFR2 is expressed only in large ventral horn neurons of naive mice (Zhang et al., 2010). As shown in Fig. 5, the expression of TNFR1 and GFAP was upregulated in the spinal dorsal horn ipsilateral to injury side by 3 days after CCI (Fig. 5A and D). TNFR1 and GFAP expression in specifically in the ipsilateral SG increased to $125 \pm 6.3\%$ ($n=24$, $P<0.05$) and $172.3 \pm 12.3\%$ ($n=24$, $P<0.05$) compared to the contralateral SG at 3 days after injury (Fig. 5F). In contrast, at 14 days after CCI, the expression of TNFR1 was downregulated in the ipsilateral dorsal horn (Fig. 5B and E). The levels in SG specifically were reduced on the ipsilateral side to $85.3 \pm 2.3\%$ ($n=32$, $P<0.05$) of the level on the contralateral side (Fig. 5F). Pre-incubation of TNFR1 peptide and TNFR1 antibody prevented the specific staining of TNFR1 in spinal cord (Fig. 5C). These data indicate that the expression of TNFR1 ipsilateral to the CCI injury is up-regulated at day 3 consistent with previous reports, but surprisingly down-regulated at day 14.

Discussion

TNF-α has many well documented roles in promoting inflammatory and nerve-injury related pain, especially in the peripheral nervous system, but less is known concerning the effects TNF-α might have in altering spinal synaptic transmission in promoting nociceptive processing. In the present study, TNF-α was shown to induce a number of changes in spinal inhibitory and excitatory synaptic processes that would be consistent with a net excitatory effect on synaptic transmission and some of these effects
showed plasticity over time in a mouse model of CCI. TNF-α produces a prominent
disinhibitory effect through suppression of sIPSC’s both in sham mice and in mice 3
days after CCI. Not surprisingly, TNF-α also enhanced excitatory synaptic transmission
in sham mice and this effect was exaggerated in CCI mice 3 days after nerve injury now
affecting a larger population of neurons that normally observed in naïve mice. In
contrast to the net excitatory effect of TNF-α on SG synaptic physiology at day 3 after
CCI, these effects were blunted on both inhibitory and excitatory synaptic transmission
at 14 days after nerve injury. Parallel to the physiological effects, immunohistochemical
staining shows that TNFR1 is upregulated at day 3 but downregulated at day 14 in the
ipsilateral versus contralateral side of the dorsal horn in CCI mice.

A number of behavioral studies have suggested that TNF-α plays a significant
role in the development of inflammatory and neuropathic pain. Mice with genetic
deletion of TNF-α or TNFR1 show reduced nociceptive sensitivity compared with wild-
type littermates after nerve injury (Nadeau et al, 2011); and perineural or intrathecal
injection resulted in hyper-responsiveness to peripheral heat and mechanical stimuli
hypersensitivity is reversed with peripheral administration of neutralizing antibodies to
TNF-α or TNF receptors (TNFRs), in particular TNFR1 (Sommer et al, 1998; Sorkin and
Doom 2000). Similarly, intra-peritoneal injection of the TNF-α inhibitor, etanercept,
suppresses hypersensitivity after nerve injury (Iwatsuki et al, 2013) and mice lacking
TNF-α or TNFR1 show reduced nociceptive sensitivity compared with wild-type
littermates after nerve injury (Nadeau et al, 2011). Parallel to these behavioral studies,
TNF-α was found to be increased in both DRG and spinal cord following nerve injury
(Xu et al, 2006; (Cha et al, 2012).
The physiological bases underlying the behavioral effects of TNFα are still to be fully defined. TNFα directly activates primary afferent fibers when applied along peripheral axons (Schafers et al, 2003a) and modifies synaptic activity of spinal cord neurons in naïve mice (Kawasaki et al, 2008; Zhang et al, 2010). More specifically at the spinal level, acute application of TNF-α suppresses sIPSC’s and increases sEPSC’s in SG neurons and this effect appears largely driven by a direct inhibition of the excitability of a subset of spinal GABA neurons (Zhang and Dougherty 2011). The net effect of TNFα in both the peripheral and central nervous system would be consistent with promoting nociceptive processing and hence contributing to primary and secondary forms of hyperalgesia.

In the present study, TNF-α very similarly suppressed inhibitory synaptic transmission in SG neurons in mice with sham or CCI nerve injury as in naïve mice, but was found to have an exaggerated effect on the excitatory synaptic transmission in CCI mice as indicated by the larger percentage of neurons showing increases in sEPSC’s. In that the increase of sEPSC’s in naïve mice were entirely accounted for by suppression of sIPSC’s (Zhang et al, 2010) the more pronounced increase on sEPSC’s in the context of similar levels of suppression of sIPSC’s in CCI mice would suggest an acquired direct effect of TNFα on subsets of excitatory spinal neurons. The idea that under conditions of nerve injury TNFα might have a preferential effect on a subset of spinal neurons is not without precedent as BDNF and CCI injury differentially affect the activities of SG neurons identified by spike-burst properties (Lu et al, 2009; Lu et al, 2012).

On the other hand, at day 14 after CCI, the effect of TNF-α became blunted with reduced percentages of neurons showing either disinhibitory or excitatory effects. Combined, the plasticity in effects of TNFα on spinal synaptic processes would suggest a
role in promoting spinal nociceptive processing (sensitization) early in the time course following CCI of the sciatic, but a diminishing role in the longer-term stages following injury. Indeed, failure of the effects of TNFα to resolve over time following nerve injury may be a key event in the transition between acute and chronic pain conditions. A caveat in these results is that equal samples of neurons were collected at the different time points. The distribution of sexes of the mice in the two samples was equal so a potential of a differential effect of TNFα on spinal synaptic processing between male and female mice seems unlikely. The cells that were selected for study were also equal among the regions of the SG and so it would also seem unlikely that unbalanced populations of excitatory versus inhibitory neurons were sampled in the 3 day and 14 day CCI mice. Moreover, given that sIPSC's and sEPSC's are reflective of the summation of inputs from large groups of up-stream neurons, it seems unlikely that a sampling bias affected the final results.

TNF-α has two receptors, TNFR1 and TNFR2. Activation of TNFR1 triggers the dominant pathway for the major effects of TNF-α (Vandenabeele et al, 1995) and several lines of evidence suggest that TNFR1 is the major receptor promoting pain signal processing by TNFα after nerve injury or inflammation (Jin and Gereau 2006; Sachs et al, 2002; Sommer et al, 1998). Consistent with these findings the TNF-α-induced disinhibition of spinal synaptic transmission was also shown as mediated through TNFR1 but not TNFR2 (Zhang et al, 2010). The expression of TNFR1 was found to show plasticity over time following CCI that paralleled the changes in physiological responses discussed above. Hence, the expression of TNFR1 was increased at day 3 after CCI when the physiological effects of TNFα were also exaggerated, but then receptor expression was reduced by day 14 where the physiological effects of TNFα
were also becoming blunted. The dynamic change of the expression of TNFR1 in spinal
cord dorsal horn following CCI is intriguing. Several lines of evidence have shown that
TNFR1 increases early in time following nerve injury. For example, Schafers et al. found
that TNFR1 mRNA levels in both DRG and the spinal dorsal horn increased 1 day after
spinal nerve ligation and returned to control levels by 7 and 14 days, respectively
(Schafers et al, 2003b). Lee et al. reported the same temporal pattern for the TNF
receptors as well as TNFα in DRG and spinal cord with CCI of the sciatic nerve (Lee et
al, 2004). Finally, TNF-α and TNFR1 immunoreactivity increased in both DRG and
spinal cord by 1 day after transection of the L5 ventral root and persisted for about 2
weeks (Xu et al, 2006). The observation here that TNFR1 expression in dorsal horn was
increased and the effect of TNF-α on the frequency of sEPSCs was enhanced 3 days after
CCI is consistent with these previous studies and clearly show that the time course of
increased TNFR1 expression is shorter than that of mechanical hyper-responsiveness
produced by nerve injury. Thus, increased TNFR1 expression and enhanced
physiological effects of TNF-α appear to have important roles in increasing the overall
excitability of the spinal dorsal horn contributing to pain after in the early stages
nerve injury, but have a reducing influence in maintaining nerve injury pain.

Inhibitory tone in the spinal cord dorsal horn is determined by the output of
inhibitory GABA and glycine containing interneurons. A recent study showed that GABA
neurons are tonically inhibited by glycine or GABA in a regionally distinct fashion
(Takazawa and MacDermott 2010). Inhibitory neurons at the lamina II/III border were
shown as under tonic glycine-mediated inhibition, whereas the more dorsal lamina I
and IIo inhibitory neurons were primarily under GABAergic inhibition. The findings
shown here did not seem consistent with this differential pattern of inhibitory
connections, but rather were more consistent with results by others (Labrakakis et al, 2009), in that cells throughout SG showed both GABA and glycine mediated IPSCs and these were equally sensitive to TNFα. Glycine and GABA have a common vesicular transporter (vesicular inhibitory amino acid transporter; VIAAT) and thus can be co-packaged into the same synaptic vesicles and co-released from the same terminals (Wojcik et al, 2006). Thus, the pure GABAergic or glycinergic IPSCs are actually dependent on the receptors (GABA_A receptor or glycine receptor, respectively), which are expressed in postsynaptic neurons (Jonas et al, 1998; Mitchell et al, 2007). The current study showed TNF-α equally inhibited both GABAergic and glycinergic inputs indicating that these neurons have both receptors throughout the lamina II regardless of position in the SG. An important caveat however, is that TNF-α was bath applied and may have activated affected inhibitory inputs to these cells from multiple other dorsal horn areas.

In conclusion, electrophysiology and immunohistochemistry results indicate that the effect of TNF-α on the regulation of overall excitability of spinal dorsal horn is enhanced in the acute phase and attenuated in the late phase of CCI neuropathy. The plasticity in the physiological effects of TNF-α on the excitability of spinal cord neurons following nerve injury appears mediated by plasticity in the expression of its key receptor TNFR1 expression at different phase. These results suggest that TNF-α and TNFR1 are important in the development but may not be as central in the maintenance of nerve injury related pain.

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Matzner O, Devor M. Hyperexcitability at sites of nerve injury depends on voltage-sensitive 


Figure Captions

Figure 1. The scatter and line plots show the mechanical withdrawal threshold (y-axis, in g) of the ipsilateral (filled circles) and contralateral paws (open circles) in mice with either a CCI sciatic nerve injury (A) or sham sciatic nerve injury (B) over time (x-axis, in days). The mechanical withdrawal threshold of the paw ipsilateral to the CCI injury was significantly reduced at post-operative day (POD) 1 and this remained across the time interval observed (A). The threshold of hindpaw withdrawal showed no change in sham mice (B).

Figure 2. The pie charts, bar graphs and representative whole cell recordings summarize the effect of TNF-α on synaptic transmission in spinal SG neurons in mice at 3 days following CCI compared to sham sciatic nerve injury in mice. A and E show the percentages of cells that responded to TNF-α with a change in frequency of sIPSCs (A) and sEPSCs (E) in sham (left hand side of each pie chart) and CCI mice (right hand side of each pie chart). A significantly larger proportion of SG neurons in CCI mice showed a change in sEPSC frequency than in sham mice (E). The bar graphs in B and F summarize the mean (and standard error) change in frequency of sIPSCs (B) and sEPSCs (F) before (black bars), following 10 minutes application of TNF-α (open bars) and following washout (gray bars) for those cells showing response in sham (left hand set of bars) and CCI mice (right hand set of bars). The bar graphs in C and G show the mean (and standard error) amplitude of sIPSCs (C) and sEPSCs (G) before (black bars), after 10 minutes application of TNF-α (open bars) and following washout (gray bars) in
in sham (left hand set of bars) and CCI mice (right hand set of bars). The stars in the
bar graphs indicate significant differences to the baseline control recording. Finally,
representative examples of analog whole cell recordings for SG neurons before (1,
expanded time base inline two) and then following application of TNFα (2, expanded
time base in line 3) for cells showing suppression of sIPSC’s (D) and increase of
sEPSC’s (H) at the bottom of the figure. The washout segment is not shown. *= p<0.05.

Figure 3. The pie charts, bar graphs and representative whole cell recordings summarize
the effect of TNF-α on synaptic transmission in spinal SG neurons in mice at 14 days
following CCI compared to sham sciatic nerve injury in mice. A and E show the
percentages of cells that responded to TNF-α with a change in frequency of sIPSCs (A)
and sEPSCs (B) in sham (left hand side of each pie chart) and CCI mice (right hand side
of each pie chart). A significantly smaller proportion of SG neurons in CCI mice showed
a change in sIPSC frequency than in sham mice (A). The bar graphs in B and F
summarize the mean (and standard error) change in frequency of sIPSCs (B) and
sEPSCs (F) before (black bars), following 10 minutes application of TNF-α (open bars)
and following washout (gray bars) for those cells showing response in sham (left hand
set of bars) and CCI mice (right hand set of bars). The bar graphs in C and G show the
mean (and standard error) amplitude of sIPSCs (C) and sEPSCs (G) before (black bars),
after 10 minutes application of TNF-α (open bars) and following washout (gray bars) in
in sham (left hand set of bars) and CCI mice (right hand set of bars). The stars in the
bar graphs indicate significant differences to the baseline control recording. Finally,
representative examples of analog whole cell recordings for SG neurons before (1,
expanded time base inline two) and then following application of TNFα (2, expanded
time base in line 3) for cells showing suppression of sIPSC’s (D) and increase of sEPSC’s (H) at the bottom of the figure. The washout segment is not shown. *= p<0.05.

Figure 4. The representative analog recordings and bar graphs summarize the effect of TNF-α on GABAergic (A and solid bars in C) and glycinergic (B and open bars in C) inputs to SG neurons. The representative analog recordings in the top line of A and B show a long time base sample before, during (soild line above) and following application of TNFα whereas the expanded time base in 1 and 2 show samples before and then late during TNFα application. *= p<0.05.

Figure 5. The representative immunohistochemical sections show the expression of TNFR1 (A and B) and GFAP (D and E) in the ipsilateral (Ipsi, left side) and contralateral (Contra, right side) spinal dorsal horn 3 (A and D) and 14 days (B and E) after CCI. No signal was detected when an excess of TNFR1 was pre-incubated with the primary antibody (C, Inset is DIC view of the slice for orientation). The bar graphs summarize the grouped quantification data where the ratio of fluorescence in the ipsilateral to contralateral SG are compared to indicate down regulation of TNFR1 and activation of GFAP in the ipsilateral compared with contralateral spinal SG at day 3 (D3) and day 14 (D14) following CCI. *= p<0.05.
Fig. 1

Graph A: CCI Ipsilateral vs. CCI Contralateral
- Baseline
- POD1
- POD3
- POD7
- POD14
- n=10

Graph B: Sham Ipsilateral vs. Sham Contralateral
- Baseline
- POD1
- POD3
- POD7
- POD14
- n=8

Hindpaw Withdrawal Threshold (g)
Fig. 5