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Full-length membrane bound TNF alpha acts through TNF receptor 2 to modify the phenotype of sensory neurons

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Abstract

Neuropathic pain resulting from spinal hemisection or selective spinal nerve ligation is characterized by an increase in membrane-bound TNF alpha (mTNF α) in spinal microglia without detectable release of soluble TNF alpha (sTNF α). In tissue culture, we showed that full length transmembrane cleavage-resistant TNF α (crTNF α) construct can act through cell-cell contact to activate neighboring microglia. We undertook the current study to test the hypothesis that mTNF α expressed in microglia might also affect the phenotype of primary sensory afferents, by determining the effect of crTNF α expressed from COS-7 cells on gene expression in primary DRG neurons. Co-culture of DRG neurons with crTNF α -expressing COS-7 cells resulted in a significant increase in the expression of voltage gated sodium channel isoforms Na v 1.7 and Na v 1.8, and voltage gated calcium channel subunit Ca v 3.2 at both mRNA and protein levels, and enhanced CCL2 expression and release from the DRG neurons. Exposure to sTNF α only produced an increase in CCL2 expression and release. Treatment of the cells with an siRNA against TNFR2 significantly reduced crTNF α -induced gene expression changes in DRG neurons while administration of CCR2 inhibitor had no significant effect on crTNF α -induced increase in gene expression and CCL2 release in DRG neurons. Taken together, the results suggest that mTNF α expressed in spinal microglia can facilitate pain signaling by up-regulating expression of cation channels and CCL2 in DRG neurons in a TNFR2 dependent manner.

Introduction

Tumor necrosis factor alpha (TNF α) is a member of the superfamily of type II transmembrane proteins that is expressed in a full-length membrane bound form (mTNF α) that can be cleaved by the inducible TNF α converting enzyme (TACE) to release the diffusible peptide sTNF α [12]. Animal models of neuropathic pain are characterized by neuroimmune activation in the spinal cord associated with increased expression of TNF α in spinal microglia [6; 17; 19]. We previously observed in models both of neuropathic pain resulting from spinal hemisection and after spinal nerve ligation that the increase in TNF α mRNA is accompanied by an increase in mTNF α expression without detectable release of sTNF α in the spinal cord [10; 18]

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In a subsequent study we found that exposure of microglia to substance P (SP) increases the expression of mTNF α without any increase in expression of TACE, and without release of sTNF α . Co-culture of COS-7 cells expressing a mutant TNF α resistant to cleavage by TACE (crTNF α) with microglial cells led to microglial cell activation through direct cell-cell contact [26]. These results suggested a novel pathway through which release of SP by primary afferents activates microglial expression of mTNF α , establishing a feed-forward loop in glia that might contribute to the establishment of chronic pain.

In order to explore whether microglial expression of mTNF α might also affect the phenotype of primary afferents, in the current study we used co-culture of COS-7 cells expressing crTNF α with primary DRG neurons *in vitro* to determine the effect of crTNF α on the expression of genes whose products are implicated in the pathogenesis of chronic neuropathic pain: the cation channel isoforms Na $_V$ 1.7 Na $_V$ 1.8, Ca $_V$ 3.2 and CCL2 [3; 5; 14; 15; 22; 23]. We found that co-culture of DRG neurons with crTNF α -expressing COS-7 cells, but not exposure of the neurons to sTNF α , resulted in an increase in the expression of the voltage gated sodium channel isoforms Na $_V$ 1.7 and Na $_V$ 1.8, and the voltage gated calcium channel isoform Ca $_V$ 3.2. Knockdown of the TNF α receptor TNFR2 in DRG neurons using siRNA but not knockdown of the TNF α receptor TNFR1, abrogated the effect of crTNF α on the neuronal phenotype. Taken together, these results indicate a previously unrecognized mechanism through which microglial activation in the spinal cord may contribute to the development of a pro-nociceptive phenotype in primary afferents.

1. Materials and Methods

2.1. Plasmids

Plasmid pGFP-crTNF α which expresses a crTNF α -GFP fusion protein has been described previously [26]. Plasmid pAcGFP1, which expresses control protein green fluorescent protein (GFP) under the control of cytomegalovirus immediate early promoter, was purchased from Clontech (Mountain View, CA).

1.1. Cell culture

COS-7 cells, a derivative of African Green Monkey Kidney cells, which do not express endogenous TNF α [26], were maintained and grown in low glucose Dulbecco's modified eagle essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologics, Atlanta, GA) and 100 units/ml penicillin in a 5% CO $_2$ atmosphere [26]. Primary dorsal root ganglion (DRG) neurons were dissociated from DRGs dissected from 17-day rat embryos and cultured in Neurobasal medium (Invitrogen) supplemented with B27, Glutamax I, Albumax, Pstrep, and 7.0S nerve growth factor [1]. Co-culture of primary DRG neurons with COS-7 cells was conducted in the same medium as used for primary DRG neuron culture.

1.2. Transfection

COS-7 cells were transfected with pGFP-crTNF α or pAcGFP1 using lipofectamine 2000 as previously described [26]. To knock down the expression of TNFR1 or TNFR2 in primary DRG neurons, cells were transfected with control siRNA or siRNA specific to rat TNFR1 or TNFR2 (ON-TARGET plusSMARTpool; Dharmacon, Chicago, IL) using lipofectamine 2000 (Invitrogen). One day before transfection, culture medium was changed and cells cultured in antibiotics-free neuronal medium and incubated in a 37°C and 5% CO $_2$ atmosphere overnight. siRNA was diluted by Opti-Mem I (Invitrogen) (250 pmole of siRNA diluted into 0.1 ml by opti-Mem I for transfection of one-well cells) and equal amount of 1:25 diluted lipofectamine 2000 by Opti-Mem I added into diluted siRNA. The mixture was incubated at RT for 20 min and pre-warmed Opti-Mem I (0.2 ml per well-cell transfection)

added into the complex. 0.3 ml of siRNA-lipofectamine 2000 mixture was applied to cells per well after DRG cells was washed by 1 ml of pre-warmed Opti-Mem I. Three days after transfection, cells were harvested for determination of TNFR1 and TNFR2 protein levels. To test the effect of knock-down of TNFR1 or TNFR2 by siRNA on α -TNF α -induced up-regulation of gene expression in DRG neurons, 2 days after siRNA transfection, COS-7 cells transfected with plasmid DNA 4 hrs after transfection were added onto DRG cells and co-cultured cells harvested for determination of gene expression and CCL2 release 1 day after co-culture.

1.3. Western blot

Cells were harvested using a scraper and collected by centrifugation, then washed in $1 \times$ PBS and re-suspended in RIPA buffer supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO) and incubated on ice for 10 min. The cell suspension was sonicated, and the disrupted cells incubated on ice for 10 min. Supernatant was collected by centrifugation at 10,000 RPM at 4° C for 10 min. Protein concentrations in lysates were measured by the BCA method (Thermo Scientific, Rockford, IL), and the proteins separated on 4–20% gradient SDS–PAGE gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Millipore, Medford, MA). Immunoblots were incubated with the primary antibody: anti-Na ν 1.7 or 1.8 (Millipore), anti-Ca ν 3.2 (Sigma), anti-TNFR1, anti-TNFR2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti- β -actin (Sigma) and subsequently incubated with an HRP-conjugated secondary antibody. Protein bands were visualized using an enhanced chemiluminescent substrate (Thermo Scientific). The amount of protein was quantitated using the chemiluminescence values obtained (ChemiDoc, Bio-Rad, Hercules, CA) and protein levels normalized to β -actin and compared to the control group.

1.4. Quantitative PCR (qPCR)

Total RNA was isolated from cell pellets using RNeasy Plus kit from Qiagen (Germantown, MD) and RNA concentrations measured by spectrophotometry. cDNA was synthesized from 2 μ g of total RNA with poly(T) as the primer using the superscript first strand synthesis system (Invitrogen). qPCR was performed using SYBRE green mix (Bio-Rad) under the following conditions: 1 cycle of 95°C/3 min; 40 cycles of 95°C/20s and 60°C/30s. Primers used for qPCR were as follows. CCL2: upper, 5'-ATG CAG TTA ATG CCC CAC TC-3'; lower, 5'-TTC CTT ATT GGG GTC AGC AC-3'. Na ν 1.7: upper, 5'-GCC ATG GAC CCC TAT GAG TA-3'; lower, 5'-CAA TCT GAA TGA CCG CAG AA-3'. Na ν 1.8: upper, 5'-CGA GCT CGA GGA AGA TAT GG-3'; lower, 5'-GCC TGG TGG TTT TCA CAC TT-3'. Ca ν 3.2: upper, 5'-CAG AGC TTC CTG GAC AAA CC-3'; lower, 5'-GGG AGG GCT CAT CTT CTT CT-3'. β -actin upper, 5'-AGC AGA TGT GGA TCA GCA AG-3'; lower, 5'-TTT GCG CAA GTT AGG TTT TG-3'. mRNA levels were normalized to β -actin and the relative mRNA levels compared to the control group.

1.5. Enzyme-linked immunosorbent assay (ELISA)

The amount of CCL2 released from DRG neurons was determined using a commercially available ELISA (Thermo Scientific). This ELISA is specific for the measurement of natural and recombinant rat CCL2 with a detection sensitivity of 5pg/mL.

1.6. Statistical analysis

All experiments were conducted in triplicate. The statistical significance of the difference between groups was determined by Student *t*-test in one parameter experiments and by ANOVA analysis in multiple comparisons. The significance of difference between groups in

multiple comparisons was corrected using Bonferroni's method. Results are expressed as mean \pm SEM.

2. Results

2.1. Co-culture with $\text{crTNF}\alpha$ -expressing COS-7 cells induces expression of voltage gated cation channels and CCL2 in DRG neurons

COS-7 cells in 6-well plates were transfected with the $\text{crTNF}\alpha$ expression plasmid or control GFP-expressing plasmid. 4 hrs later 1.5×10^5 COS-7 cells suspended in DRG neuron culture medium were placed onto primary DRG neurons (3×10^5 cells per well). Cells were harvested after 1-day co-culture. DRG neurons exposed to $\text{crTNF}\alpha$ -expressing COS-7 cells showed an increase in $\text{Na}_V1.7$, $\text{Na}_V1.8$, $\text{Ca}_V3.2$ and CCL2 mRNA expression (Fig. 1A) and $\text{Na}_V1.7$, $\text{Na}_V1.8$, $\text{Ca}_V3.2$ protein levels (Fig. 1B). Co-culture with $\text{crTNF}\alpha$ -expressing COS-7 cells also enhanced the release of CCL2 from those neurons into the medium (109 ± 5.5 ng/ml observed in co-culture of DRG neurons with COS-7 cells expressing $\text{crTNF}\alpha$ versus 42 ± 2.2 ng/ml in co-culture of DRG neurons with COS-7 cells expressing GFP).

2.2. The effect of $\text{crTNF}\alpha$ on neuronal gene expression is distinct from the effect of $\text{sTNF}\alpha$ on the same cells

In order to assess whether the effect of $\text{crTNF}\alpha$ was specific to the transmembrane form of the cytokine, primary DRG neurons were exposed to 15 ng/ml of $\text{sTNF}\alpha$ for 15 hrs. Preliminary studies indicate that the effect of exposure to $\text{sTNF}\alpha$ plateaued after 15 hrs (data not shown). Exposure of DRG neurons to $\text{sTNF}\alpha$ substantially increased CCL2 mRNA level (Fig. 2A) and enhanced the release of CCL2 from DRG neurons into the medium compared with no treatment (49 ± 1.7 versus 19 ± 0.9 ng/ml), but in contrast to the effect of co-culture with $\text{crTNF}\alpha$ -expressing COS-7 cells, there was no change in the mRNA expression of $\text{Na}_V1.7$, $\text{Na}_V1.8$, or $\text{Ca}_V3.2$ in DRG neurons exposed to $\text{sTNF}\alpha$ (Fig. 2A). $\text{sTNF}\alpha$ dose experiments indicated 0.1 ng/ml $\text{sTNF}\alpha$ induced much less CCL2 mRNA expression (Fig. 2B) ($P < .005$) and CCL2 release relative to $\text{sTNF}\alpha$ treatment of higher concentrations (28 ± 1.5 versus $47 \pm 2.8 - 50.5 \pm 3.2$ ng/ml released into the medium). 100 ng/ml $\text{sTNF}\alpha$ resulted in less $\text{Na}_V1.7$ and $\text{Na}_V1.8$ mRNA expression compared with $\text{sTNF}\alpha$ treatment of lower doses ($P < .005$) (Fig. 2B). But identical results in terms of CCL2 and voltage gated cation channel mRNA expression and CCL2 release ($47 \pm 2.8 - 50.5 \pm 3.2$ ng/ml) were found in doses ranging from 1 to 50 ng/ml of $\text{sTNF}\alpha$ (Fig. 2B).

2.3. The effect of $\text{crTNF}\alpha$ on neuronal gene expression is mediated through TNFR2

$\text{TNF}\alpha$ receptors TNFR1 and TNFR2 have different affinities for forms $\text{mTNF}\alpha$ and $\text{sTNF}\alpha$, as well as distinct downstream activation pathways. In order to determine the receptor or receptors involved in mediating the effect of $\text{crTNF}\alpha$ on DRG neurons, we tested the effect of knockdown of TNFR1 or TNFR2 by siRNA on $\text{crTNF}\alpha$ -induced gene expression in DRG neurons. We first confirmed that siRNA specific to TNFR1 or TNFR2 silenced the expression of TNFR1 and TNFR2 effectively as evidenced by much lower protein levels of TNFR1 ($\sim 70 \pm 4$ % knockdown) and TNFR2 ($\sim 75 \pm 4.5$ % knock-down) observed in DRG neurons receiving target specific siRNA compared with those observed in cells treated with control siRNA (Fig. 3A). To determine which receptor is responsible for the effect of $\text{crTNF}\alpha$ on DRG neurons, DRG neurons 2 days after siRNA transfection were co-cultured with COS-7 cells expressing either control GFP or $\text{crTNF}\alpha$ for 24 hrs. Co-culture of DRG neurons receiving control siRNA with $\text{crTNF}\alpha$ -expressing COS-7 cells resulted in increased expression of $\text{Na}_V1.7$ and $\text{Na}_V1.8$ and $\text{Ca}_V3.2$ protein (Fig. 3B) and CCL2 release (105 ± 6 versus 42 ± 2.7 ng/ml) in DRG neurons compared with co-culture with COS-7 cells expressing GFP, but the effect of co-culture on voltage-gated channel protein expression (Fig. 3B) and CCL release (75 ± 3.5 versus 105 ± 6 ng/ml) was significantly reduced in

neurons treated with the TNFR2 siRNA compared with control siRNA. However, up-regulation of gene expression and increase in CCL2 release (99 ± 5.5 versus 105 ± 6 ng/ml) in DRG neurons induced by cr TNF α were not impaired by the treatment of TNFR1-specific siRNA compared with control siRNA (Fig. 3B).

2.4. The effect of cr TNF α on neuronal gene expression is not mediated through induction of CCL2 release

In addition to the observed effect on voltage gated ion channel gene expression, cr TNF α stimulated the expression and release of CCL2 from DRG neurons. In order to determine whether CCL2 acting through CCR2 might be responsible for the changes in expression of voltage-gated channels, DRG neurons were treated with 20 nM CCR2 inhibitor [4; 24] (Santa Cruz Biotechnologies) or vehicle (DMSO) and after 4 hrs of inhibitor treatment co-cultured with COS-7 cells expressing GFP or cr TNF α . One day later the cells were harvested for determination of the $Na_V1.7$, $Na_V1.8$, $Ca_V3.2$ and CCL2 mRNA, $Na_V1.7$, $Na_V1.8$, $Ca_V3.2$ protein levels and CCL2 release. The effect of co-culture with cr TNF α -expressing COS-7 cells on the expression of voltage gated cation channels and CCL2 mRNA (Fig. 4A), the protein levels of $Na_V1.7$, $Na_V1.8$, $Ca_V3.2$ (Fig. 4B) in DRG neurons were not significantly affected by the presence of the CCR2 inhibitor. The CCR2 inhibitor did not influence cr TNF α -induced CCL2 release into the medium compared with vehicle treatment (102 ± 4.8 ng/ml in the presence of CCR2 inhibitor versus 106 ± 6.5 ng/ml in the absence of the inhibitor).

3. Discussion

In this study, we found that: 1) contact with cr TNF α -expressing COS-7 cells, but not exposure to s TNF α , enhances the expression of voltage-gated channel subunits $Na_V1.3$, $Na_V1.8$ and $Ca_V3.2$ at the mRNA and protein levels in DRG neurons; 2) exposure to both cr TNF α and s TNF α upregulates CCL2 mRNA expression in DRG neurons and results in release of CCL2 from those cells; 3) the increase in voltage-gated subunit expression is independent of CCL2/CCR2 signaling; and 4) the effect of cr TNF α on the DRG neuronal phenotype is mediated through TNFR2.

Chronic pain following nerve injury is characterized by spontaneous pain and by peripheral sensitization resulting in allodynia: a phenomenon in which normally innocuous stimuli are perceived as painful, and hyperalgesia, a phenomenon in which normally painful stimuli perceived as more painful than usual. Both spontaneous pain and peripheral sensitization reflect reduced thresholds for activation of peripheral sensory nerves, an effect that is caused in part by alterations in voltage gated channels that are the critical determinants of neuronal excitability [3; 5; 14; 15; 22].

There is substantial evidence to indicate that peripheral nerve injury results in activation of microglia in the spinal cord, and increased expression of inflammatory cytokines and chemokines by those cells including TNF α [16; 17; 25]. But in our previous studies in models of neuropathic pain we found that the substantial increase in TNF α mRNA expression in the spinal cord after nerve injury is not accompanied by measurable release of s TNF α [10; 18]. This result correlates with the observation in microglial cells *in vitro* that exposure to substance P increases the expression of TNF α mRNA and full-length mTNF α protein, but does not cause increased expression of the TNF α cleaving enzyme (TACE) or release of s TNF α from those cells [26]. In our previous study we observed that full-length non-cleavable TNF α (cr TNF α) localized in the cell membrane, acting through cell-cell contact, was fully capable of activating neighboring microglia, indicating one mechanism through which spread of sensitization might occur at the spinal level [10; 18]. The current study extends those results by indicating mTNF α expressed in the membrane of microglial

cells, through cell-cell interactions with afferent nerve terminals, may modulate the expression of voltage-gated channels in the DRG neurons projecting to the dorsal horn.

What mechanism might be responsible for the differential effects of sTNF α and mTNF α that we observed? In other model systems it has been shown that sTNF α rapidly binds to TNFR1 with high affinity (K_d 19 pm) and a slow dissociation from the receptor once bound ($t_{1/2}$ =33 min), a process which efficiently activates TNFR1. The dissociation kinetics of sTNF from native TNFR2 is approximately 20 – 30 fold faster than from TNFR1 and the affinity significantly less than sTNF's affinity for TNFR1 [7; 9]. It is not clear how the binding characteristics of membrane-bound TNF at TNFR1 and TNFR2 compare to the binding characteristics of sTNF α , but it is well-known that slight structural changes in the TNF α sequence can lead to dramatic changes in its binding characteristics to TNF receptors. In DRG neurons specific effects of sTNF α acting through TNFR1 have been reported [13], and distinct effects of mTNF α acting through TNFR2 have been identified in the immune system [2].

We demonstrated in this study that full-length uncleaved TNF α produces an increase not only in mRNA but also in protein levels of Na ν 1.3, Na ν 1.8 and Ca ν 3.2 voltage-gated channel proteins in DRG neurons. In this study we have not directly assessed the function of these channels in cultured neurons, but all of these alterations by increasing the number of available channels would be expected to increase neuronal excitability and thus could serve to produce both spontaneous pain and the hypersensitive state characteristic of neuropathic pain.

Peripheral nerve hyperexcitability is characteristic of the hypersensitivity state that is observed in models of inflammatory pain, a process in which peripheral release of sTNF α and other cytokines have been shown to play an important role [17]. In the current study, we found that the effect of crTNF α on gene expression in DRG neurons is distinct from the effect of exposure of the same cells to sTNF α . By knockdown experiments we found evidence that the effect of crTNF α on neuronal gene expression is achieved through selective activation of the TNF α receptor TNFR2. This result is consistent with studies in immune and neuron cells that indicate that sTNF α preferentially activates TNFR1 [2; 11; 20; 21] while mTNF α typically acts through TNFR2 [8].

The observations in the current study indicating that mTNF α can activate DRG neurons to upregulate the expression of voltage-gated channel subunit proteins and the chemokine CCL2 through TNFR2 have potentially important implications for understanding mechanisms that would facilitate the persistence of neuropathic pain. Further studies will be required to explore this effect in vivo, and to determine whether selective block of this interaction may provide a novel therapy for the treatment of neuropathic pain.

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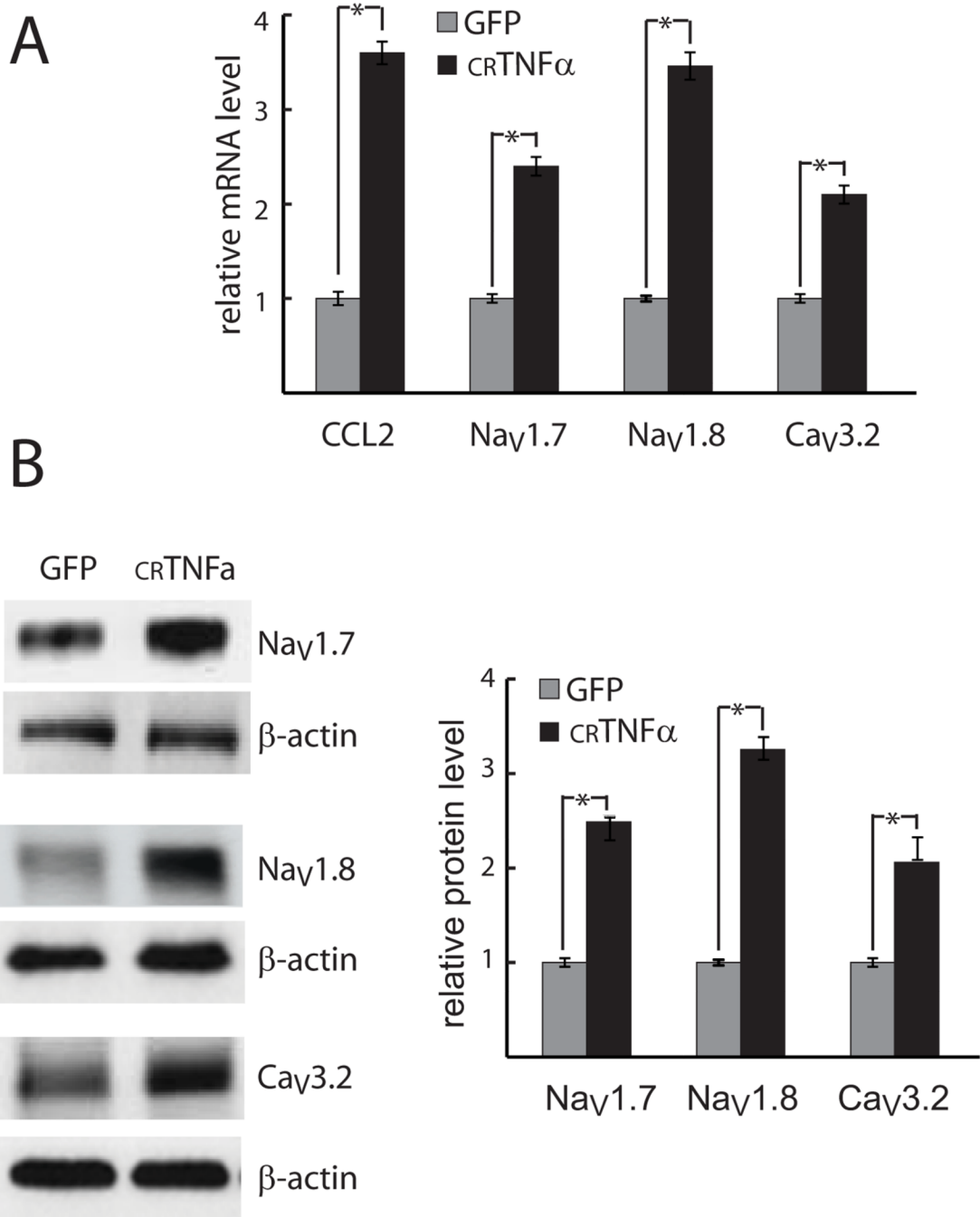


Fig. 1. crTNF α increases expression of genes in DRG neurons

Primary DRG neurons were co-cultured with crTNF α or control (GFP-expressing) COS-7 cells. The expression of voltage-gated channel subunits and CCL2 in DRG neurons was determined at the mRNA (A) and protein (B) levels. * $p < .05$.

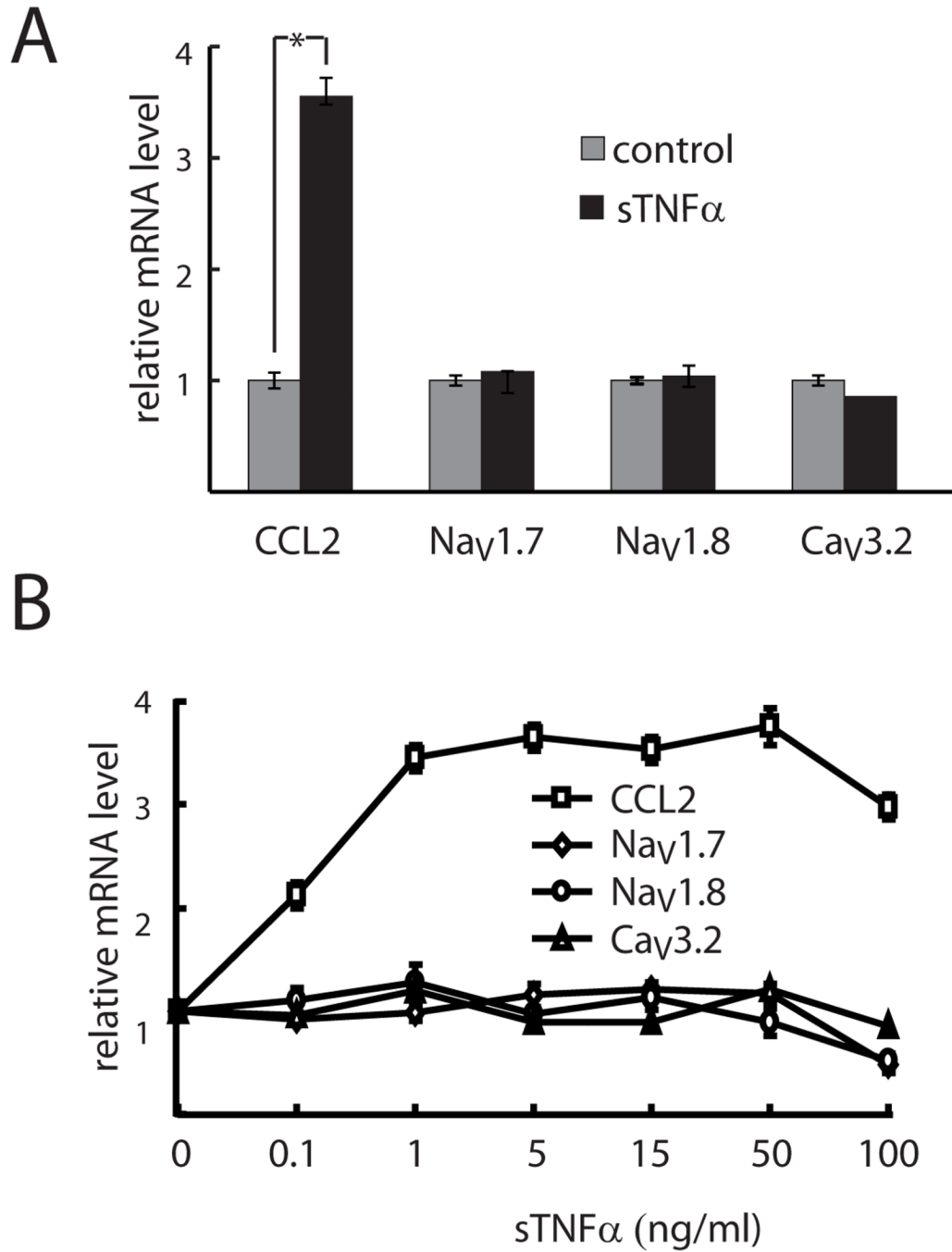


Fig. 2. Exposure of DRG neurons to sTNF α does not produce the same changes in gene expression

Effect of sTNF α on the expression of voltage-gated channel subunits and CCL2 mRNA in DRG neurons. A: Effect of 15 ng/ml sTNF α . B: Effect of sTNF α doses. * $p < .05$.

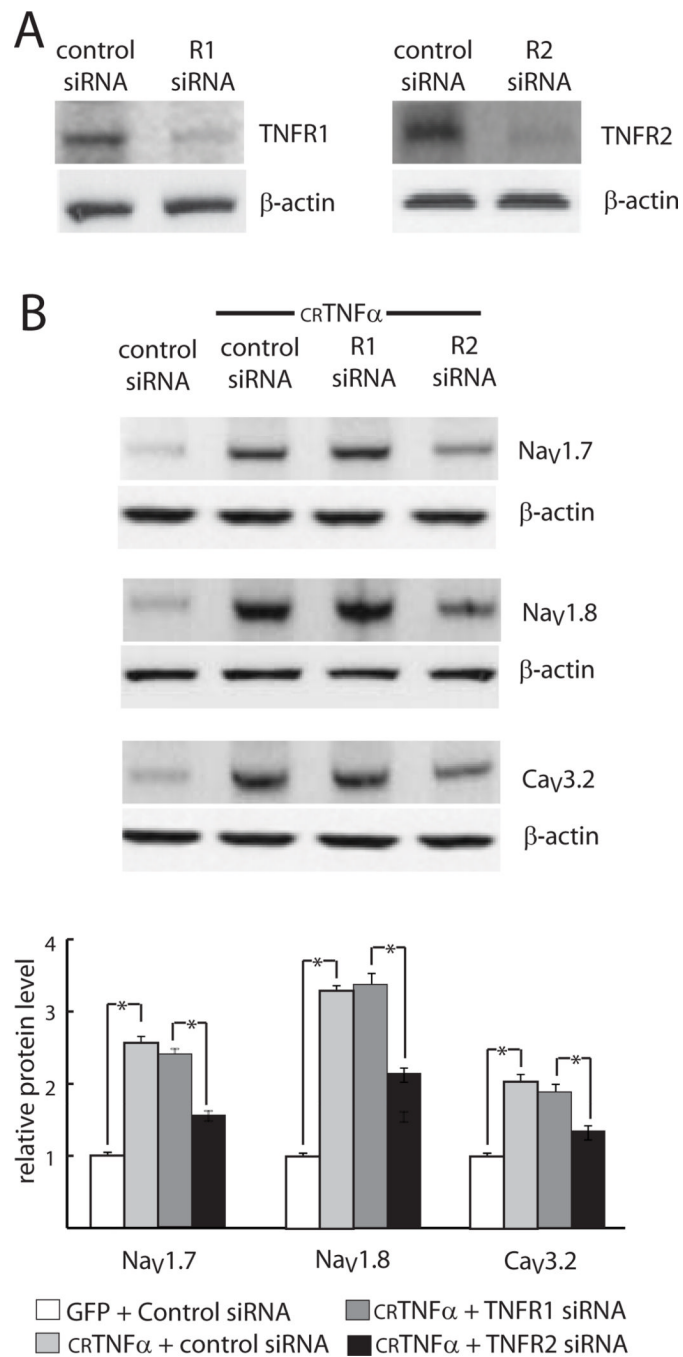


Fig. 3. crTNF α acts through TNFR2

Effect of knock-down of TNFR1 and TNFR2 by siRNA on crTNF α -induced up-regulation of gene expression in DRG neurons. A: Knockdown of TNFR1 and R2 by siRNA (Western blot). B: Protein levels of voltage-gated channel subunits. * $p < .05$.

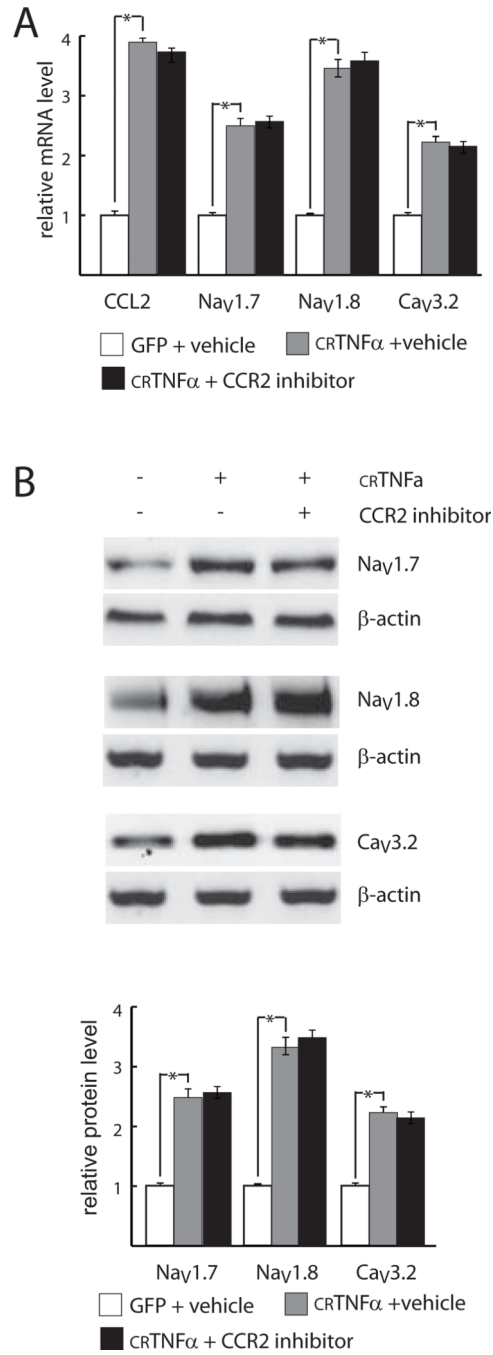


Fig. 4. The effects of crTNFα are not mediated through CCL2/CCR2
 Effect of CCR2 inhibition on crTNFα-induced up-regulation of gene expression in DRG neurons. A. mRNA. B. Protein. * p < .05.