

The role of connexin43-gap junction in rat spinal
astrocytes for the regulation of neuropathic pain

(神経因性疼痛に対する脊髄アストロサイト

connexin43-gap junction の役割に関する研究)

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Chapter 1

The down-regulation of connexin 43 expressions in spinal astrocytes contributes to the maintenance of hypersensitivity of neuropathic pain in mice with partial sciatic nerve ligation

1.1. Abstract

Spinal astrocytes are likely to have key roles in maintaining the chronic pain state. One characteristic feature of astrocytes is their high expression levels of connexin 43 (Cx43), a protein that forms gap junction channel, which facilitate direct intercellular communication. Several recent studies have indicated that the change of Cx43 expression level in astrocytes might be associated with the induction of various kinds of neuronal disorder such as multiple sclerosis, epilepsy or chronic pain, however, it is yet unknown which kinds of endogenous molecule could be involved in the change of Cx43 expression, and the mechanism related to the induction of neuropathic pain after the change of Cx43 expression. The current study examined the relationship between the change of Cx43 expression and the regulation of nociceptive responses under neuropathic pain. It was found that the expression of Cx43 in spinal dorsal horn in partial sciatic nerve ligation (PSNL) model was significantly decreased during the maintenance phase, and this response was mediated by tumor necrosis factor- α (TNF- α) dependent pathway. Furthermore, the decrease of Cx43 expression could be related in the induction of mechanical hypersensitivity. Most importantly, it is possible that the enhancement of glutamatergic transmission related in the down-regulation of GLT-1 expression might contribute to the mechanical hypersensitivity evoked by the decrease of Cx43 expression.

1.2. Introduction

Emerging evidence indicates that the enhancement of sensitivity of nociceptive primary afferent neurons and hypersensitivity of nociceptive dorsal horn neurons in spinal cord are associated with pathological chronic pain^{1,2}. Recent studies have demonstrated that spinal glial cells are involved in the initiation of these responses, and crucial players in the development and maintenance of chronic pain related in neuropathic or inflammatory disorders³⁻⁵. Especially, activated spinal astrocytes could contribute to the long-lasting of pain hypersensitivity and the reduction of astroglial activity by intrathecal (i.t.) treatment with fluorocitrate, an inhibitor of astrocytic metabolism, significantly improve abnormal pain responses in rodent neuropathic pain models⁶. There are a number of reports regarding change of astrocytic function under neuropathic pain; activation of MAP kinases, increase of proinflammatory cytokines and chemokines production, or down-regulation of excitatory amino acid transporters (EAATs, Na⁺-dependent glutamate transporters)⁷⁻⁹. Furthermore, it has been demonstrated that these responses could contribute to the maintenance of neuropathic pain state.

It has been recently shown that astrocytes intercellular communication through gap junctions has a crucial role in not only the regulation of astroglial function but also the proper maintenance of neuronal network¹⁰. Gap junctions have important property for molecular exchange and informational communication by passing several signal molecules such as glutamate, ATP, and some second messengers, buffering extracellular Na⁺ or K⁺ spatially, or supplying energy sources between neighboring cells^{11,12}. Gap junctions are formed by two connexons expressed in distinct cell, which are consisted of a hexamer of connexins (Cxs) protein. It is known that 11 of Cxs are detected in mammalian central nervous system, and each cell type possess a distinct set of Cxs. In spinal cord, Cx43 is preferentially

and mainly expressed in astrocytes¹³. Several studies have indicated that astrocytic Cx43 plays an essential role in the modulation of synaptic transduction in central nervous system. In addition, the change of Cx43 expression level in astrocytes might be associated with the induction of various kinds of neuronal disorder. Especially, it has been demonstrated that the decrease of Cx43 protein expression in astrocytes could enhance the neuronal excitability, and this response might contribute to the initiation of neuroinflammation such as multiple sclerosis¹⁴. Emerging evidences have suggested that the up-regulation of Cx43 in spinal cord was induced after inflammatory-related pain models, and the reduction of Cx43 activity by intrathecal delivery of specific siRNA for Cx43 or gap junction inhibitor carbenoxolone could improve the decrease of nociceptive threshold^{15,16}. In contrast, Ohara et al. have also indicated that silencing of Cx43 in the trigeminal ganglion of naïve rat administered by siRNA increased mechanical sensitivity. Thus, relationship between the expression level of Cx43 and the change of nociceptive responses is controversial¹⁷. In addition, it is yet unknown, which kinds of endogenous molecule could be involved in the change of Cx43 expression, and the mechanism related to the induction of neuropathic pain after the change of Cx43 expression.

To understand the relationship between the change of Cx43 expression and the regulation of nociceptive responses under neuropathic pain, the current study utilized partial sciatic nerve ligation (PSNL) model, which mimics some of the major features observed in clinical neuropathic pain. It was found that the expression of Cx43 in spinal dorsal horn following PSNL was significantly decreased during the maintenance phase, but not the induction phase of neuropathic pain, and this response was mediated by tumor necrosis factor- α (TNF- α) dependent pathway. Furthermore, the decrease of Cx43 expression could

be related in the induction of mechanical hypersensitivity. In addition, it is possible that the enhancement of glutamatergic transmission related in the down-regulation of GLT-1 expression might contribute to the mechanical hypersensitivity evoked by the decrease of Cx43 expression.

1.3. Materials and Methods

Animals

Male ddy mice, 5 weeks of age, were used for the experiments. Mice were maintained at a room temperature of 22±2 °C with a 12h light/dark cycle (light on at 8:00 AM), and given access to food and water available *ad libitum* during the experimental period. All experiments by using animals were conducted in accordance with the “Guidelines for the Care and Use of Laboratory Animals” established by Hiroshima University, and the procedures of all animal experiments were approved by the Committee of Research Facilities for Laboratory Animal Science of Hiroshima University.

Construction of partial sciatic nerve ligation model

After anesthetization with sodium pentobarbital (50 mg/kg, i.p.), a tight ligation of approximately one-third or one-half of the diameters of the left sciatic nerve (ipsilateral) was performed with 8-0 silk sutures as described previously¹⁸. In sham-operated mice, the sciatic nerve was exposed without ligation.

Knockdown of Cx43 in the spinal cord

Knockdown of Cx43 was performed by using the hemagglutinating virus of the Japan (HVJ) envelop vector system (HVJ Envelop Vector kit GenomONE-Si, Ishihara Sangyo Kaisya, Ltd., Osaka, Japan). This vector was widely used for in vivo small interfering RNA (siRNA) transfers. The siRNA targeting mouse Cx43 (siGENOME SMARTpool, mouse GJA1, Thermo) or the non-targeting siRNA

(siGENOME Non-targeting siRNA pool #2, Thermo) were incorporated into the HVJ envelop vector according to the manufacture's protocol. In brief, after mixing HVJ envelop vector with enclosing factor, the mixture was centrifuged at 10,000 g, 4 °C, 10 min, and the pellet was suspended by the stock solution of each siRNA (10 µM). Then, this solution was diluted to the concentration used in the current study with sterile saline. Diluted siRNA solution was injected into the subarachnoid space between the L5 and L6 vertebrate (i.t.) of mice.

Measurement of hind paws sensitivity to tactile stimulation and drugs administration

The procedure of measurement of hind paw sensitivity of ipsilateral paw to tactile stimulation was described in previous report. In brief, after surgery for 3, 7, 14, or 21 days, the threshold to tactile stimuli by von Frey monofilaments was measured. About drug treatments, etanercept (TNF-α blocker) were i.t. administrated four times; immediately after sciatic nerve injury, on the day 2, 4, or 6 after the injury. Then, the mechanical sensitivity or the expressions of Cx43 were measured on the day 14 after surgery. TNF-α (mouse recombinant TNF), Carbenoxolone (gap junction blocker) or gap27 (selective Cx43 blocker) was i.t. injected to naïve mice.

The hind paw sensitivity of spinal cord Cx43 knockdown mice were measured after injection of siRNA for 1st, 2nd, 3rd, 5th and 7th day by von Frey monofilaments. About drug treatments, MK-801 (N-methyl-D aspartate receptor antagonist), CP96345 (neurokinin 1 receptor antagonist), CNQX (AMPA receptor antagonist) were i.t. administrated on the day 3 after injected the siRNA.

Western blot

Under anesthesia with ether, mice were decapitated; the lumbar (L4-L6) segments of ipsilateral side of the spinal dorsal horn were removed. These were

immediately frozen in liquid nitrogen and stored at -80 °C until use. Spinal tissues were solubilized in radioimmunoprecipitation assay buffer with inhibitors (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail 2 (Nacalai Tesque, Kyoto, Japan)). The lysates were centrifuged at 14,000 × g for 10 min at 4°C and the supernatant was added to Laemli's buffer and boiled for 5 min. Equal amounts of protein were separated by 7.5 or 10% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Non-specific binding was reduced with blocking buffer, and the membranes were subsequently incubated with a purified polyclonal antibody against rat Cx43, or GLT-1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal antibody against β-actin (1:10,000; Sigma Chemical Co., St. Louis, MO, USA) overnight at 4 °C. After being washed, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. Then, membranes were rinsed and incubated with Luminescence reagent (Thermo Fisher Scientific, Rockford, IL, USA). Finally, the membranes were exposed to X-ray film. For quantification of signals, the densities of specific bands were measured with Science Lab Image Gauge (Fuji Film, Tokyo, Japan).

Reagents

Recombinant mouse TNF-α was obtained from PeproTech Inc. (Rocky Hill, USA) and etanercept was obtained from takeda pharmaceutical co ltd (Osaka, japan). MK801 was purchased from Tocris Bioscience (Minneapolis, MN, USA), CNQX, CP96345 and Carbenoxolone were purchased from Sigma Chemical, Co. (St. Louis, MO, USA). Cabapentin was obtained from Cayman Chemical (A. G.

Scientific, USA)

Statistical analysis

Data are expressed as the mean \pm SE of at least three independent determinations. Differences between means for mechanical hypersensitivity after PSNL or the knockdown of Cx43 were determined using a one-way analysis of variance (ANOVA) with a pairwise comparison by the Tukey-Kramer method. Using student's t-test performed comparison of Cx43, GLT-1, Cx30 or GLAST protein level in the spinal dorsal horn. Differences were considered to be significant when the P value was less than 0.05.

1.3. Results

PSNL evokes mechanical hypersensitivity and decreases the expression of Cx43 protein expression in spinal dorsal horn

It was observed a marked decrease of mechanical threshold of ipsilateral paw to tactile stimuli from days 3 to days 21 after PSNL surgery, compared with withdrawal threshold of corresponding day after sham operation (Fig. 1-1A). About Cx43 expression, three immunopositive bands were detected at approximately 39-44 kDa (Fig. 1-1B) by Western blotting using a Cx43 antibody. "P0" in Fig. 1-1B indicates the non-phosphorylated Cx43, and P1 and P2 correspond to phosphorylated Cx43. In the current study, total Cx43 expression was derived from the sum of the three bands (P0+P1+P2). The amount of Cx43 levels was normalized to that of β -actin levels, which was used as an internal control, and presented as a ratio to vehicle treatment. Then, the expression level of Cx43 protein in spinal dorsal horn was examined on days 3, 7, 14, 21 after surgery by Western blot. As shown in Fig. 1-1B, levels of Cx43 expression in ipsilateral dorsal horn was significantly reduced on days 7, 14, and 21, but not

days 3, after sciatic nerve injury, compared with levels of Cx43 in dorsal horn from sham-operated mice.

Knockdown of Cx43 by siRNA in the spinal cord induces the mechanical hypersensitivity

To confirm the specific down-regulation of Cx43 in spinal dorsal horn exerting mechanical hypersensitivity, the effect of knockdown of Cx43 by siRNA transfer on tactile mechanical threshold was investigated. The significant reduction of Cx43 expression in spinal dorsal horn was observed on days 2 or 3 after injection with Cx43 siRNA with HJV envelop vector compared with that treated with non-targeting siRNA with HJV envelop vector (Fig. 1-2A), and this response was returned on days 5. Furthermore, a significant decrease of mechanical threshold to tactile stimuli was also observed in mice treated with Cx43 siRNA, compared with those in mice treated with non-targeting siRNA. These effects were appeared on days 2 after injection with Cx43 siRNA, sustained on days 5, and disappeared on days 7 (Fig. 1-2B). In addition, the effects of a gap junction blocker (carbenoxolone) on the mechanical threshold were examined. As shown in Fig. 1-2C, i.t. treatment with either carbenoxolone (1 or 3 nmol) markedly decreased the mechanical threshold at 24 h after injection, compared with treatment with vehicle (saline). Thus, these results strongly indicate that the decrease of Cx43-gap junction could induce mechanical hypersensitivity.

The change of Cx30 expression in spinal dorsal horn after PSNL treatment

Previous studies have demonstrated that the enhancement of expression of Cx30, which is another types of Cxs expressed in astrocytes, was induced after the down-regulation of Cx43 in astrocytes¹⁹. Thus, the expression level of Cx30 protein in spinal dorsal horn was examined on days 7, 14 after surgery that Cx43

decreased time point by Western blot. As shown in Fig. 1-3A, the level of Cx30 expressions in ipsilateral dorsal horn was not changed after sciatic nerve injury, compared with levels of Cx30 in dorsal horn from sham-operated mice. In addition, we also examined the effect of knockdown of Cx43 by siRNA transfer on Cx30 expression in spinal dorsal horn. As shown in Fig. 1-3B, the level of Cx30 expressions in spinal dorsal horn also not changed after treated with Cx43 siRNA with HJV envelop vector (Fig. 1-3B).

TNF- α dependent pathway is involved in the PSNL-induced Cx43 down regulation

Several studies have shown that proinflammatory cytokines such as TNF- α released in the spinal cord after nerve injury, and active the astrocyte then induced the neuropathic pain²⁰. Thus, the involvement of endogenous TNF- α in the down-regulation of Cx43 expression after sciatic nerve injury was investigated. As shown in Fig. 1-4A or B, repeat i.t. treatment with etanercept, a TNF- α blocker, was significantly reversed the PSNL-induced mechanical hypersensitivity. Furthermore, it was also found that blockade of TNF- α 's action could significantly prevent the down-regulation of Cx43 expression in spinal dorsal horn of PSNL mice (Fig. 1-4C). Treatment of sham-operated mice with etanercept with same schedule had no effects on the mechanical threshold and Cx43 expression (Fig. 1-4B or C). In addition, single i.t. treatment with recombinant TNF (20 ng) for 24 or 48 hours markedly induced both mechanical hypersensitivity (Fig. 1-4D) and the down-regulation of Cx43 expression (Fig. 1-4E).

The down-regulation of GLT-1 and subsequent enhancement of glutamatergic transmission in spinal dorsal horn is involved in the Cx43-mediated mechanical hypersensitivity

It is speculated that the enhancement of nociceptive transmission at spinal

dorsal horn level might be associated with mechanical hypersensitivity triggered by the down-regulation of Cx43. Therefore, the involvement of glutamate or substance P, which acts as main neurotransmitters of nociceptive transduction in dorsal horn, in Cx43 siRNA-induced mechanical hypersensitivity, was examined by pharmacological approaches. As shown in Fig. 1-5A and B, i.t. treatment with MK-801 (a NMDA receptor antagonist) or CNQX (an AMPA receptor antagonist) led to a significant blockade of the Cx43 siRNA-induced mechanical hypersensitivity. In contrast, i.t. treatment with CP96345 (an NK1 receptor antagonist) did not block the Cx43 siRNA-induced responses (Fig. 1-5C). These receptor antagonists had no effects on the mechanical threshold of mice transfected with non-targeting siRNA (Fig. 1-5A-C). These observations led us to speculate the enhancement of glutamatergic transmission at dorsal level under the situation which spinal Cx43 expression is down regulated. In addition we investigate the effect of gabapentin that enhancer of glutamate release. As shown in Fig. 1-5D, i.t. treatment with gabapentin did not block the Cx43 siRNA-induced responses (Fig.1-5D).

Thus, we next investigated that the expression of glutamate transporters in spinal dorsal horn might be mediated by the change of Cx43 expression level. As shown in Fig. 1-6A, the expression of GLT-1, which is one of glial glutamate transporters and abundantly expressed in astrocytes, was significantly reduced in spinal dorsal horn on days 7, 14 or 21, but not days 3, after peripheral nerve injury, compared with those in sham-operated mice. The current observation was similar with previous reports. Furthermore, it was found by Western blot that the expression of GLT-1 in spinal dorsal horn was dramatically reduced on days 2, 3, 5 after the intraspinal transfection with Cx43 siRNA, compared with those treated with non-targeting siRNA (Fig. 1-6B). This positive relationship between

Cx43 and GLT-1 was also confirmed by pharmacological approach. Previous report has suggested that the prolonged treatment with carbenoxolone reduced not only the function of gap junction but also the expression of connexin43. In the current study, both the expression of Cx43 in spinal dorsal horn and mechanical threshold were markedly reduced after intrathecal administration with carbenoxolone for 24 hours (Fig. 1-6C). In addition, it was found that the expression of GLT-1 was also down regulated by the treatment with carbenoxolone (Fig. 1-6D).

The expression of GLAST, which is another type of glial glutamate transporters, in spinal dorsal horn, was also elucidated. Although the expression of GLAST in spinal dorsal horn was also significantly reduced by the sciatic nerve injury, that was not affected by the knockdown of Cx43 by siRNA (Fig. 1-7A or B).

1.5. Discussion

The current study demonstrated that the TNF- α -modulated down-regulation of Cx43 in spinal dorsal horn could contribute to the PSNL-induced mechanical hypersensitivity during the maintenance phase (7, 14, 21 days), but not the induction phase (3 days), of neuropathic pain. These results are supported by the following observations. The knockdown of Cx43 by transfection with siRNA into mice spinal dorsal horn led to a significant decrease of mechanical threshold. Furthermore, it was found that the down-regulation of glutamate transporter GLT-1 and the subsequent enhancement of glutamatergic transmission in spinal dorsal horn might be pivotal events in the Cx43-mediated mechanical hypersensitivity under sciatic nerve injury. These results indicate that TNF- α -mediated reduction of Cx43 expression in spinal dorsal horn is associated with the maintenance of the neuropathic pain state, and modulation of Cx43

expression could be a unique therapeutic strategy for relieving neuropathic pain.

It has well demonstrated that astrocytic dysfunction could be a crucial response observed under neuropathic pain state. Especially, several previous reports indicated that the excessive activation or gliosis of astrocytes in dorsal horn were observed during the maintenance phase, but not the induction phase, of neuropathic pain^{21,22}, suggesting that astrocytes might play an important role in the formation of persistent pain. The functions of astrocytes are regulated by the activities of various kinds of receptor, ion channel, enzyme, etc. Among them, the emerging evidences have shown that Cx-gap junction is indispensable for the maintenance of astrocytic function²³⁻²⁵. Therefore, it is speculated that the change of Cx43 expression and the subsequent disturbance of gap junction activity might be attributed to the various types of neuronal disorders including neuropathic pain. In fact, previous studies have suggested that the increase of Cx43 expression was observed in spinal cord of nerve-injury-induced neuropathic pain models, and treatment with gap junction blocker carbenoxolone inhibited the nociceptive responses^{15,16}. In the current study, in contrast, the opposite response for the expression of Cx43 in spinal dorsal horn was observed, and i.t. treatment of naïve mice with either carbenoxolone markedly decreased the mechanical sensitivity. In addition, Wu et al. have demonstrated that the reduced astroglial communication by the increase of Cx43 phosphorylation was appeared in the spinal cord of rat after chronic constriction injury of the sciatic nerve²⁶. Moreover, Ohara et al. have also indicated that silencing of Cx43 in the trigeminal ganglion of naïve rat administered by siRNA increased mechanical sensitivity¹⁷. Although there is discrepancy between the up- or down-regulation of Cx43 expression in spinal dorsal horn and nociceptive responses under neuropathic pain state, it may be based on the differences of neuropathic pain models, pain intensity, or

experimental conditions. Previous studies have demonstrated that the enhancement of expression of Cx30 was induced after the down-regulation or knockdown of Cx43 in astrocytes¹⁹. In addition, astrocytic Cx30 plays an important function in neuronal transmission in hippocampus^{11,27}. Therefore, these observations suggest the possibility that the up-regulation of Cx30 expression in spinal dorsal horn might be associated with the Cx43-mediated mechanical hypersensitivity in the PSNL mice. However, this possibility might be excluded, because the current observation revealed that the expression of Cx30 was not changed after the sciatic nerve injury. Thus, the reduction of spinal Cx43 expression from basal level might be one of key responses to induce the mechanical hypersensitivity under neuropathic pain state.

TNF- α is well known to contribute to the induction of neuropathic pain through the activation of astrocytes²⁰. Therefore, we specially focused the role of TNF- α in the regulation of spinal Cx43 expression in PSNL mice. In the current study, the observations obtained by pharmacological approaches using the TNF- α related reagents indicated that the TNF- α might have a crucial role in the reduction of Cx43 expression in spinal dorsal horn under neuropathic pain state. Previous reports demonstrated that the high amount of TNF- α is produced from activated microglia in spinal cord of neuropathic pain models²⁰. It was demonstrated that increased TNF- α could affect synaptic nociceptive transduction via the regulation of various kinds of astrocytic function²⁸. The current observation strongly suggests the relationship between the potential roles of TNF- α as a nociceptive modulator and the regulation of Cx43 expression in spinal astrocytes.

Previous studies have indicated that the down-regulation of GLT-1 in spinal dorsal horn might be involved in the PSNL-induced mechanical hypersensitivity²⁹. Furthermore, it is well known that the enhancement of glutamatergic

transmission in spinal dorsal horn might be pivotal in the nerve-injury-induced hypersensitivity³⁰. However, the molecular mechanism initiating the down-regulation of GLT-1 is unknown until now. The current observations indicate the positive relationship between Cx43 and GLT-1, but not GLAST, and the involvement of the down-regulation of GLT-1 expression in the Cx43-mediated mechanical hypersensitivity by the following reasons. 1) i.t. treatment with antagonists of either NMDA or AMPA receptor, but not gabapentin, which inhibits the release of glutamate from presynaptic nerve, significantly reversed the mechanical hypersensitivity induced by the knockdown of Cx43. 2) The expression of GLT-1 in dorsal horn was observed in not only the PSNL mice but also the Cx43-knockdown mice. Together, these current results revealed that the down-regulation of spinal Cx43 would contribute to the increase of glutamate in synaptic cleft by down-regulation of GLT-1, but not enhancement of presynaptic release, and the following induction of NMDA receptor-dependent glutamatergic transmission in spinal dorsal horn. Then, these responses could affect the nerve injury-induced mechanical sensitivity. Previous study indicated that the expression of GLT-1, but not GLAST, is reduced in cultured cortical astrocytes treated with gap junction blockers or transfected with Cx43 siRNA³¹. Furthermore, the enhancement of glutamatergic current by the decrease of glutamate transporter's activity was observed in hippocampus slice of Cx43/30-double knockout mice²⁷. The molecular mechanisms involving the down-regulation of GLT-1 after the reduction of Cx43 are yet unclear. Although further investigation is necessary, emerging evidences have indicated that Cx43 could also function to provide regulatory crosstalk with other proteins located on cell membranes or cytoskeletons, beside the formation of gap junction to pass several molecules between cells^{32,33}. Furthermore, it was demonstrated that

Cx43-gap junction might be involved in the regulation of other multiple gene expression. In fact, it is reported that the alteration of various kinds of gene is caused in Cx43-null astrocytes³⁴. In addition, Ai et al. have suggested that Cx43 is co-localized with β -catenin in cell membranes; the expression of Cx43 might sequester β -catenin and negatively mediates β -catenin-dependent gene transcription in cardiac myocytes³⁵. The current observation indicated that the blockade of gap junction function by the intrathecal treatment with gap27 did not affect the expression of GLT-1 in cultured spinal dorsal astrocyte. Therefore, it is possible that the down regulation of Cx43 expression might affect the activities of intracellular signal molecules and the following regulation of various gene expressions.

Here, we have provided new information about the mechanisms of neuropathic pain. Under neuropathic pain, Cx43 was decreased via TNF and then induced the GLT-1 down regulation in the spinal dorsal horn. Furthermore, these responses were contribute to the mechanical hypersensitivity via enhanced the glutamatergic transmission.

Chapter 2

Spinal astrocytes stimulated by tumor necrosis factor- α (TNF- α) and/or interferon- γ (IFN- γ) attenuates connexin 43-gap junction

2.1. Abstract

Spinal astrocytes have important mechanistic contributions to the initiation and maintenance of various neuropathic diseases such as neuropathic pain. Under inflammatory conditions, spinal astrocytes are exposed to cytokines such as tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) and these cytokines could alter astrocytic function by modulating connexin (Cx43), subunits that form channels that modulate intercellular communication in astrocytes. The current study investigated the alteration of Cx43-gap junction in rat primary cultured spinal astrocytes stimulated with cytokines by real-time PCR and Western blotting. The transcriptional and translational levels of Cx43 were significantly but partially reduced 24 and 48 h treatment with either TNF- α or IFN- γ . A mixture of TNF- α and IFN- γ led to a robust decrease of Cx43 expression, moreover, a moderate reduction of gap junction intercellular communication (GJIC), which was evaluated by a scrap loading/dye transfer assay. Both the decrease of Cx43 expression and the reduction in GJIC induced by the mixture of TNF- α and IFN- γ were prevented by blocking c-jun terminal kinase (JNK), but not by blocking extracellular signaling molecules ERK and p38 kinase, indicating a specific role of astrocytic JNK in the response to cytokines. In addition, treatment with cytokines potently induced the phosphorylation of JNK and c-jun in a time-dependent manner. Furthermore, the involvement of ubiquitin-proteasome system on the TNF- α and IFN- γ -induced down-regulation of

Cx43-gap junction in spinal cultured astrocytes also investigated. TNF- α and IFN- γ mixture accelerated Cx43 degradation by used cycloheximide plus assay in rat primary cultured spinal astrocyte. Moreover, proteasome inhibitors MG132 or epoxomicin blocked both the decrease of Cx43 expression and gap junction function. Furthermore, a mixture of TNF- α and IFN- γ led to a significantly increase of proteasome activity in rat primary cultured spinal astrocytes by 20s proteasome activity assay, and the increase of proteasome activity were prevented by blocking JNK signaling. In addition, mixture of JNK inhibitor and proteasome inhibitor were not observed additive inhibitory effect on Cx43 degradation induced by the mixture of TNF- α and IFN- γ . These results indicate that intercellular communication of astrocytes is significantly disrupted in the inflammatory state, and stimulation of spinal astrocytes with inflammatory cytokines led to the significant inhibition of Cx43-GJIC through the activation of JNK signaling pathway and proteasome pathway.

2.2. Introduction

Astrocytes are one of the major non-neuronal cell types in the central nervous system (CNS). One characteristic feature of astrocytes is their high expression levels of connexin 43 (Cx43), a protein that forms two types of channels: gap junction channels, for direct intercellular communication, and hemichannels, for sampling of the extracellular milieu ³⁶⁻³⁹. It is known that the change in cell coupling resulting from tissue injury and pathological conditions is attributed to alterations in connexin expression and the subsequent modulation of gap junction intercellular communication (GJIC) ^{36,40}. In terms of brain astrocyte function, while previous finding highlights the crucial role of Cx43 in the normal physiological state, Cx43 also appears to be crucial in pathological states. For

example, Pu et al. showed that Cx43 expression level was inversely correlated with high-grade astrocytic tumors ⁴¹ and Xu et al. showed that astrocytic gap junction coupling may contribute to epileptogenesis and other neurological deficits in Tuberous Sclerosis Complex ⁴². The modulation of Cx43 and GJIC in astrocytes had been shown to play important roles in the pathology of Parkinson's disease ⁴³. To elucidate mechanisms of neuropathological states and, in turn, to develop effective therapeutics, understanding the mechanism of communication between astrocytes could be as crucial as understanding inter neuronal communication.

Recently, several studies have reported expression of Cx43 in spinal astrocytes in pathological states. Connexin 43 in spinal astrocytes has been shown to be significantly down-regulated in inflamed spinal white matter of mice with an experimental autoimmune encephalomyelitis compared with healthy controls ¹⁴. Lee et al. demonstrated that Cx43 is up-regulated in the spinal cord after spinal cord injury ⁴⁴. Furthermore, prior chapter showed Cx43 of spinal cord astrocyte was involved the neuropathic pain. While changes in Cx43 expression in the pathological state are clearly evident, the mechanism mediating the changes in either expression or function of Cx43 in spinal astrocytes remains unclear.

Studies in brain astrocytes have reported that proinflammatory cytokines released from activated microglia inhibit gap junction activity in brain astrocytes ^{24,45}. Of these cytokines, tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) were identified as most responsible for disrupting astrocytic GJIC ²⁴. Furthermore, prior chapter also showed Cx43 of spinal cord astrocyte was down regulated by TNF in neuropathic pain. Significant release of these particular cytokines in the spinal cord have also been reported following spinal cord injury and peripheral tissue injury (e.g. sciatic nerve injury), which also results in

significant changes in glial function and proliferation ^{46,47}. Whether a similar cascade occurs in spinal astrocytes, involving cytokines, Cx43 and ultimately GJIC, has yet to be clarified.

The current study examined whether proinflammatory cytokines, such as TNF- α , IL-1 β and interferon- γ (IFN- γ), could cause an alteration of Cx43 expression and a subsequent functional modulation of GJIC in cultured rat spinal astrocyte. Mitogen-activated protein kinase (MAPK) is well known to be activated after stimulation with cytokines in astrocytes and could be important in initiating the cascade of intracellular events that leads to various neurological disorders following injury or disease ^{48,49}. Recently, several researches have focused on the role of ubiquitin proteasome system on Cx43 degradation in several cells such as human corneal fibroblasts or rat liver epithelial cell line IAR20 ^{50,51}. The ubiquitin-proteasome system is widely known for its role in intracellular protein degradation, that the proteins after labeled with ubiquities were degradation by proteasome which a large multi-subunit protease ⁵². There are a number of signaling molecules that are activated following cytokine stimulation. Thus, the specific signaling cascade involved in the expression of Cx43 in astrocytes following cytokine stimulation was also elucidated.

2.3. Materials and Methods

Reagents

Recombinant rat TNF- α and IFN- γ were obtained from Wako (Tokyo, Japan) and IL-1 β from PeproTech (Rocky Hill, NJ, USA). Lucifer yellow CH di-potassium salt, H89, N-acetylcysteine (NAC) and Thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Sigma Chemical, Co. (St. Louis, MO, USA). U0126, SB202190, SP600125, PP1, Epoxomicin and genistein were purchased from Tocris

Cookson (Bristol, UK). Gö6983 and AG490 were obtained from Calbiochemical Co. (La Jolla, CA, USA). MG132 and 20s proteasome activity assay kit were obtained from Cayman Chemical (A. G. Scientific, USA). Epoxomicin was purchased from Calbiochemical Co. (La Jolla, CA, USA).

Primary culture of neonatal rat spinal cord astrocytes

Primary spinal astrocytes were prepared from spinal cords of neonatal Wistar rats (1–2 days old) according to a previously reported method ⁵³. In brief, the isolated spinal cords were minced and then incubated with trypsin and DNase I. Dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). Then, cell suspensions were plated in 75 cm² tissue culture flasks (7.5~10×10⁶ cells/flask) precoated with poly-L-lysine (10 µg/ml). Cells were maintained in a 10% CO₂ incubator at 37 °C. After 8~15 days, the mixed glial cells were shaken vigorously and washed with PBS in order to remove other cells such as microglia. Cells were then replanted onto 35 mm diameter dishes (3.2×10⁵ cells) for use in the following experiments. The current method yielded a purity of > 95% astrocytes, as determined by glial fibrillary acidic protein (GFAP) immunoreactivity (Data not shown).

Real-time PCR analysis

According to a previously reported method ⁵⁴, total RNA derived from astrocytes was prepared by a method involving acid guanidinium thiocyanate–phenol–chloroform extraction ⁵⁵ and used to synthesize cDNA with moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and a random hexamer primer. The cDNA synthesized using 1 µg of total RNA in each sample were subjected to real-time PCR assays with specific primers and

EXPRESS SYBER® Green ER™ qPCR SuperMix (Invitrogen, Carlsbad, CA, USA). The sequences of primers were used: Cx43, 5' - CGTGCAGATGCACTGAA -3 (forward); 5' - CCACTGGATGAGCAGGAA -3 (reverse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5' - AGCCAGAACATCATCCCTG -3 (forward); 5' - CACCACCTTCTTGATGTCATC -3 (reverse). Thereafter, real-time PCR assays were conducted using a DNA engine real-time PCR detection system CFX96 (Bio-Rad Laboratories, Tokyo, Japan). A three-step amplification protocol was applied: 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. RNA quantities of target genes were calculated by the Ct method. Ct values of Cx43 amplification were normalized to those of GAPDH amplification.

Western blot analysis and antibodies

Western blot analysis was performed for the detection of Cx43, phospho-JNK and phospho-c-jun in rat spinal astrocytes. After treatment with the various drugs, the cells were solubilized in radioimmunoprecipitation assay buffer with inhibitors (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail 2 (Sigma Chemical, Co.)). The lysates were centrifuged and the supernatant was added to Laemli's buffer and boiled at 95°C for 5 min. Equal amounts of protein were separated by 7.5% (for Cx43 or β-actin) or 10% (for phospho- or total JNK or phospho-c-jun) SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The membranes were incubated with blocking solution, and subsequently incubated with a purified polyclonal antibody against rat Cx43 (1:1000, Cell Signaling Technology, Beverly, MA, USA), phospho-JNK (1:1000, Cell Signaling Technology), total-JNK (1:1000,

Cell Signaling Technology), phospho-c-jun (1:1000, Cell Signaling Technology) or with a monoclonal antibody against β -actin (1:10,000, Sigma Chemical, Co.) overnight at 4 °C. After being washed, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Thereafter, membranes were rinsed and incubated with Luminescence reagent (Thermo Fischer Scientific, Rockford, IL, USA). Finally, the membranes were exposed to X-ray film. For quantification of signals for proteins, the densities of specific bands were measured with Science Lab Image Gauge (Fuji Film, Tokyo, Japan).

Scrape loading/dye transfer assay

Several assays are currently available to determine the GJIC in cultured cells⁵⁶⁻⁵⁸. Among these, the scrap loading/dye transfer (SLDT) assay is based on monitoring the transfer of the fluorescent dye Lucifer yellow from one cell into adjacent cells and has been extensively used to analyze the functional status of gap junction in cell culture systems. The SLDT assay utilizing Lucifer yellow was performed to examine the effect of cytokines on GJIC between spinal astrocytes according to a previously described method⁵⁹. Briefly, cells were incubated in HEPES-buffered salt solution for 10 min containing the following (in mM): 140 NaCl, 5.5 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, pH 7.35. Cells were then washed in Ca²⁺-free HEPES solution for 1 min, and then scrape loading was achieved with a razor blade in the same Ca²⁺-free solution containing Lucifer yellow CH (1 mg/ml; Sigma Chemical, Co). After 1 min, cells were washed several times with the HEPES-buffered salt solution. After scrap loading for 8 min, fluorescent images were captured using an inverted fluorescent microscope equipped for epifluorescence (Olympus, Tokyo, Japan) and images were analyzed using image-analysis software image J (National Institutes of Health, USA). For

each trial, determining the fluorescence area and intensity in time consecutive fields performed quantification of the change in GJIC induced by different treatments.

Proteasome activity

The proteasome activity was analyzed by using a 20s proteasome assay kit according to the manufacturer's instructions. Briefly, seed astrocytes in a 96-well plate at a density of 3×10^5 cell/well. After treatment, astrocytes were lysate by 20s proteasome lysis buffer, then add 20s proteasome substrates SUC-LLVY-AMC to incubate for 1 h at 37 °C. Finally, read the fluorescence intensity of AMC using an excitation and emission wavelengths of 360nm and 480nm filter set in a fluorometer (Perkin Elmer, New York, USA) epifluorescence (Olympus, Tokyo, Japan). The AMC standard curve was generated with a dilution series of purified

Statistical analysis

Data are expressed as the mean \pm SEM of at least three independent experiments. Differences between means were statistically analyzed by one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison analysis using the Tukey–Kramer method. The differences between vehicle and cytokines treatment in the JNK and c-jun phosphorylation studies were analyzed using Student's t-test. A probability value (p) of less than 0.05 was considered to be statistically significant.

2.4. Results

TNF- α and IFN- γ induced a down regulation of Cx43 expression in spinal astrocytes

To elucidate the effects of TNF- α , IFN- γ and IL-1 β on Cx43 expression in spinal astrocytes, changes in Cx43 mRNA and protein expression were quantified. As

shown in Fig. 2-1A, Cx43 expression was significantly decreased with TNF- α treatment at 24 h and 48 h post-treatment and with IFN- γ . Moreover, co-treatment with TNF- α and IFN- γ induced a marked decrease in Cx43 mRNA expression at both 24 and 48 h post-treatment. By contrast, treatment with IL-1 β for either 24 or 48 h did not result any significant change in Cx43 mRNA (Fig. 2-1A).

A decrease in Cx43 protein expression was observed with treatment with either TNF- α or IFN- γ at both 24 h and 48 h. Similar to the effect observed in Cx43 mRNA expression, a robust reduction of Cx43 protein expression was obtained by a mixture TNF- α and IFN- γ at both 24 and 48 h post-treatment (Fig. 2-1B). By contrast, treatment with IL-1 β did not result in any significant change in Cx43 protein expression at either 24 h or 48 h post-treatment (Fig. 2-1C). As a mixture of TNF- α and IFN- γ appeared to yield a greater effect on both Cx43 transcription and translation than either cytokine alone, the effect of co-incubation with both cytokines on Cx43 expression over time was evaluated. A significant decrease in Cx43 mRNA expression in spinal astrocytes was apparent as early as 3 h post-incubation with the cytokine mixture (Fig. 2-2A). Twelve h following cytokine mixture treatment, Cx43 mRNA expression was reduced to approximately one-third of that of vehicle treated astrocytes. In addition, the subsequently reduction of Cx43 protein expression was obtained by a mixture TNF- α and IFN- γ at 6 h post-treatment (Fig.2-2B).

TNF- α and IFN- γ induced an inhibition of GJIC in spinal astrocytes

Next, gap junction permeability in cultured spinal astrocytes was determined using the SLDT assay. As shown in Fig. 2-3A, Lucifer yellow was transferred through GJIC in vehicle-treated astrocytes, indicating functional gap junctions. After treatment with either TNF- α or IFN- γ for 48 h, a slight reduction of gap

junction permeability was observed but this was not statistically significant compared to vehicle-treated astrocytes ($p>0.05$). By contrast, after co-incubation with the cytokine mixture for 48 h, gap junction permeability between spinal astrocytes was significantly reduced ($61.5 \pm 9.1\%$, $n=7$; $p<0.01$ vs. vehicle-treated; Fig. 2-3B).

JNK plays a critical role in the inhibitory effect of TNF- α and IFN- γ on Cx43-GJIC in spinal astrocytes

The involvement of MAPK in the down-regulation of Cx43-gap junction induced by a mixture of TNF- α and IFN- γ was elucidated by pharmacological approaches. Treatment with SP600125, a potent inhibitor of JNK, significantly blocked the cytokine mixture-induced decrease of both Cx43 mRNA (Fig. 2-4A) and protein levels (Fig. 2-4B and Table 1). In contrast, treatment with either U0126 or SB202190, which are the inhibitors of ERK and p38 MAPK, respectively, had no effect on either Cx43 mRNA (Fig. 2-4A) or protein expression (Fig. 2-4B and Table 1). Moreover, the SLDT assay results demonstrated that treatment with SP600125 reversed the cytokine mixture-induced down-regulation of gap junction function in spinal astrocytes (Fig. 2-5). In contrast, treatment with either U0126 or SB202190 had no effect on GJIC (data not shown).

These findings suggest that the JNK signaling pathway could be involved in the TNF- α and IFN- γ induced down-regulation of both Cx43-gap junction expression and function. Thus, phosphorylation levels of JNK were measured to determine whether JNK was actually activated by both cytokines in cultured spinal astrocytes. Co-treatment with both cytokines markedly induced the phosphorylation of JNK after 30 min, and this response was significantly elevated for at least 12 h (Fig. 2-6A). Thirty minutes pretreatment with SP600125 significantly suppressed cytokine mixture-induced JNK phosphorylation (Fig.

2-6B). In addition, the phosphorylation of c-jun was also analyzed to elucidate a potential effect of JNK activity. Phosphorylation of c-jun was also significantly increased after co-treatment with both cytokines beginning 30 min after incubation, and this response was sustained for at least 12 h (Fig. 2-6C). The cytokine mixture-induced up-regulation of phospho-c-jun (3 h) in spinal astrocytes was reversed by SP600125 (Fig. 2-6D).

TNF- α and IFN- γ promote the degradation of Cx43 expression in spinal astrocytes

Furthermore, to investigate the detail mechanism of Cx43 down-regulation, next elucidate the effect of TNF- α and IFN- γ on degradation of Cx43 in spinal astrocyte used cycloheximide plus assay. As shown in Fig. 2-7, treated with cycloheximide (1 μ m) alone, that a protein synthesis inhibitor caused a time-dependent decrease in Cx43 level, after 6 h the Cx43 protein levels were decreased by 50% compared to the 0 h. However, this effect was accelerated by TNF- α and IFN- γ . In addition, co-treatment with TNF- α and IFN- γ induced a marked acceleration in Cx43 degradation (Fig 2-7). This suggests that TNF- α and IFN- γ induced the Cx43 degradation in spinal astrocytes.

Proteasome plays a critical role in the inhibitory effect of TNF- α and IFN- γ on Cx43-GJIC in spinal astrocytes

Next, the involvement of proteasome in the down-regulation of Cx43-gap junction induced by a mixture of TNF- α and IFN- γ was elucidated by pharmacological approaches. Treatment with MG132, a reversible proteasome inhibitor or epoxomicin, an irreversible proteasome inhibitor, respectively, significantly blocked the cytokine mixture-induced decrease of both Cx43 protein level (Fig. 2-8A) and gap junction function (Fig. 2-8B) in spinal astrocytes. These findings suggest that the proteasome pathway could be involved in the TNF- α and

IFN- γ induced down-regulation of both Cx43-gap junction expression and function. Thus, proteasome activities were measured by used 20s proteasome assay kit to determine whether proteasome was actually activated by both cytokines in cultured spinal astrocytes. Co-treatment with both cytokines significantly increases the activity of proteasome after 4 h, and this response was significantly elevated for at least 12 h. In addition, the cytokine mixture-induced up-regulation of proteasome activity in spinal astrocytes was reversed by SP600125 (Fig. 2-9A). Furthermore, the inhibited role of co-treatment both JNK and proteasome inhibitors on Cx43 down regulation induced by TNF- α and IFN- γ also investigated. As shown in Fig. 2-9B, after co-incubation with the inhibitors mixture did not result any significant change in Cx43 down regulation induced by cytokines-mixture compared the inhibitor single treatment in spinal astrocytes (Fig. 2-9B). These results suggest that TNF- α and IFN- γ induce the proteasome activation via JNK activation and then decrease the Cx43-GJIC in spinal astrocyte.

The involvement of other signaling cascades in the regulation of Cx43 expression which have been reported to be activated after incubation with TNF- α and IFN- γ or regulated the Cx43 expression was investigated by pharmacological approaches. None of the other inhibitors had a significant effect on Cx43 protein expression (Table 2).

2.4. Discussion

The current study demonstrated that TNF- α and IFN- γ , both alone and in combination, caused a significant down-regulation of Cx43 in spinal astrocytes,

leading to decreased gap junction function, as demonstrated by the SLDT assay, which in turn was mediated by JNK-proteasome activation. These results are in accordance with a previous report that demonstrated that lipopolysaccharide (LPS) treatment of rat cortical astrocytes led to a decrease of Cx43, which was mediated by Toll-like receptor 4^{60,61}. Although LPS is known to produce proinflammatory cytokines such as TNF- α and IL-1 β ⁶⁰⁻⁶², there are no reports, which directly demonstrated that TNF- α and IFN- γ trigger the reduction of Cx43 expression. In fact, this study suggests that the cytokine IL-1 β has no role in the reduction of Cx43 expression in spinal astrocytes. Thus, the current study suggests that specific cytokines are involved in the down-regulation of Cx43 following spinal tissue pathology. Furthermore, based on the pharmacological effects, the current study indicates that a specific signaling pathway is involved in the down-regulation of Cx43 expression and function. The findings in total suggest a rational approach to developing treatments for spinal pathologies involving astrocytic dysfunction.

It is known that a network of astrocytes linked by gap junctions could play an important role in maintaining the homeostatic environment of the CNS⁶³. Several cytokines have been reported to contribute to the regulation of Cx43 expression and GJIC in brain astrocytes but these may differ from those that regulate spinal astrocyte Cx43 expression and GJIC. For example, although treatment of mouse cortical astrocytes with TNF- α , IFN- γ and IL-1 β alone did not affect Cx43 and GJIC, co-treatment with TNF- α and IL-1 β markedly reduced the expression of Cx43 and GJIC^{24,59,64}. By contrast, in this study, although IL-1 β was ineffective on Cx43 expression in spinal astrocytes, both TNF- α and IFN- γ alone and in combination in particular exhibited prominent inhibitory effects on Cx43 expression. Furthermore, co-treatment with TNF- α and IL-1 β did not exhibit

additional decrease of Cx43 expression in spinal astrocytes (data not shown). Certainly, it is possible that astrocytes in different region of CNS could have distinct properties and responses to various neurochemical stimuli. A differential effect between brain and spinal astrocytes of cytokines could form the basis of targeted treatment for specific CNS pathologies. Currently, however, there has not been extensive differentiation of phenotypes, between brain and spinal astrocytes, to confirm such a possibility.

The current study is the first to demonstrate that IFN- γ could induce an inhibitory effect on Cx43 function. IFN- γ is produced mainly by T-cells and natural killer cells, and mediates various immune responses to tissue injury. Recently, IFN- γ was shown to be unregulated in the spinal cords of rats with a painful peripheral neuropathy ⁶⁵, in the CNS in a mouse model of amyotrophic lateral sclerosis (ALS) ⁶⁶, and traumatic brain injury ⁶⁷. The expression of IFN- γ in the CNS under various pathological states indicates that it is a ubiquitous cytokine and functional in both brain and spinal cord. Spinal astrocytes in particular can express the IFN- γ receptor, and are therefore sensitive to the effect of IFN- γ ⁶⁸. Thus, it is concluded that IFN- γ alone has an important role in regulating Cx43 expression in spinal cord astrocytes.

Interestingly, this study found that co-treatment of TNF- α with IFN- γ further suppressed the expression of Cx43 in spinal astrocytes as well as further suppressing GJIC. It should be apparent that not one but several cytokines maybe found in the CNS under pathological conditions. To better conceptualize the clinical effect of cytokines on CNS tissues, the use of combinations of cytokines may be more useful rather than individual cytokines. The levels of both TNF- α and IFN- γ have been shown increased in the spinal cord under pathological situations, such as a model of familial ALS ^{66,69,70}. The onset of symptoms

following experimental autoimmune encephalomyelitis correlates with increased TNF- α and IFN- γ in spinal cord ⁷¹. Thus, in clinical spinal neurodegenerative disorders, both TNF- α and IFN- γ are crucial in triggering astrocytic dysfunction.

Although it has been reported that cytokines can reduce Cx43 expression in various cells types, it is yet unclear, which signaling mechanism underlies the cytokine-mediated reduction of Cx43 expression. In the current study, both the dysfunction of Cx43 synthesis and Cx43 degradation were involved the Cx43-gap junction down-regulation induced by TNF- α and IFN- γ . The main focus was on the involvement of MAPK in the TNF- α and IFN- γ induced down-regulation of Cx43, because MAPK is known to be activated after stimulation with cytokines in spinal astrocytes ^{48,49}. The current results indicated that JNK, but not ERK or p38, plays a pivotal role in the TNF- α and IFN- γ induced decrease the mRNA and protein levels of Cx43 and GJIC in spinal astrocytes. Furthermore, treatment with both cytokines markedly induced the phosphorylation of JNK and c-jun, which precedes the down-regulation of Cx43 mRNA expression. The phosphorylation of JNK has been observed under various pathological situations such as neuropathic or inflammatory pain ^{72,73}, suggesting that activation of this kinase in astrocytes is important step in the molecular process that leads, in this case, to a chronic pain state. These observations, combined with the current findings, indicate that JNK is a crucial factor in the cytokine-mediated regulation of Cx43 in spinal astrocytes. Interestingly, in current study suggested that JNK also involved the TNF- α and IFN- γ induced Cx43 degradation via proteasome activity. It is supported by several recent study that TNF- α or IFN- γ mediated astrocyte activity was mediated by proteasome pathway ^{74,75}. Furthermore, C43 degradation in human corneal fibroblasts or rat BWEM cell was also prevented by proteasome inhibitors ^{36,51}. The proteasome system also has been observed under

various pathological situations such as chronic pain ⁷⁶. These observations, combined with the current findings, indicate that proteasome is also a crucial factor in the cytokine-mediated regulation of Cx43 in spinal astrocytes. However, it is also shown that Cx43 ubiquitin was significantly increased in human corneal fibroblasts⁵¹. Thus, the ubiquitin level of Cx43 in spinal astrocyte after treatments with both cytokines are future must be examined.

As multiple signaling molecules are involved in the regulation of brain astrocytic Cx43, such could also be the case for spinal astrocytes. Previous studies indicated that LPS-induced inhibitory effect on Cx43-GJIC in cortical astrocytes was mediated through a down-regulation of membrane protein caveolin-3 ⁶¹. NF- κ B and the phosphoinositide 3-kinase pathway activated by Toll-like receptor 3 were implicated in the down-regulation of astrocytic Cx43 expression in human fetal brain ⁷⁷. The molecular mechanism of the reduction of Cx43 synthesis combined with JNK activation remains to be clarified, involving either a direct or an indirect mechanism. It is possible that JNK could directly activate a transcriptional repressor to block the expression of Cx43 mRNA, or via an epigenetic mechanism to initiate gene silencing. Indeed, accumulating evidence has shown that epigenetic modulation is associated with Cx43 gene expression ⁷⁸⁻⁸⁰. One possible indirect mechanism is that JNK contributes to the production of inhibitory molecules to block Cx43 transcription, thereby reducing Cx43 protein expression. Several studies have indicated that cytokines utilize a pathway that is highly dependent on treatment with TNF- α and IFN- γ leads to the activation of multiple signaling molecules, including PKA, PKC, JAK, src tyrosine kinase and ROS ⁸¹⁻⁸⁴. However, the current study excluded the possibility of involvement of these molecules by pharmacological approaches. Thus, our data indicate that cytokines utilize a pathway that is highly dependent on JNK phosphorylation,

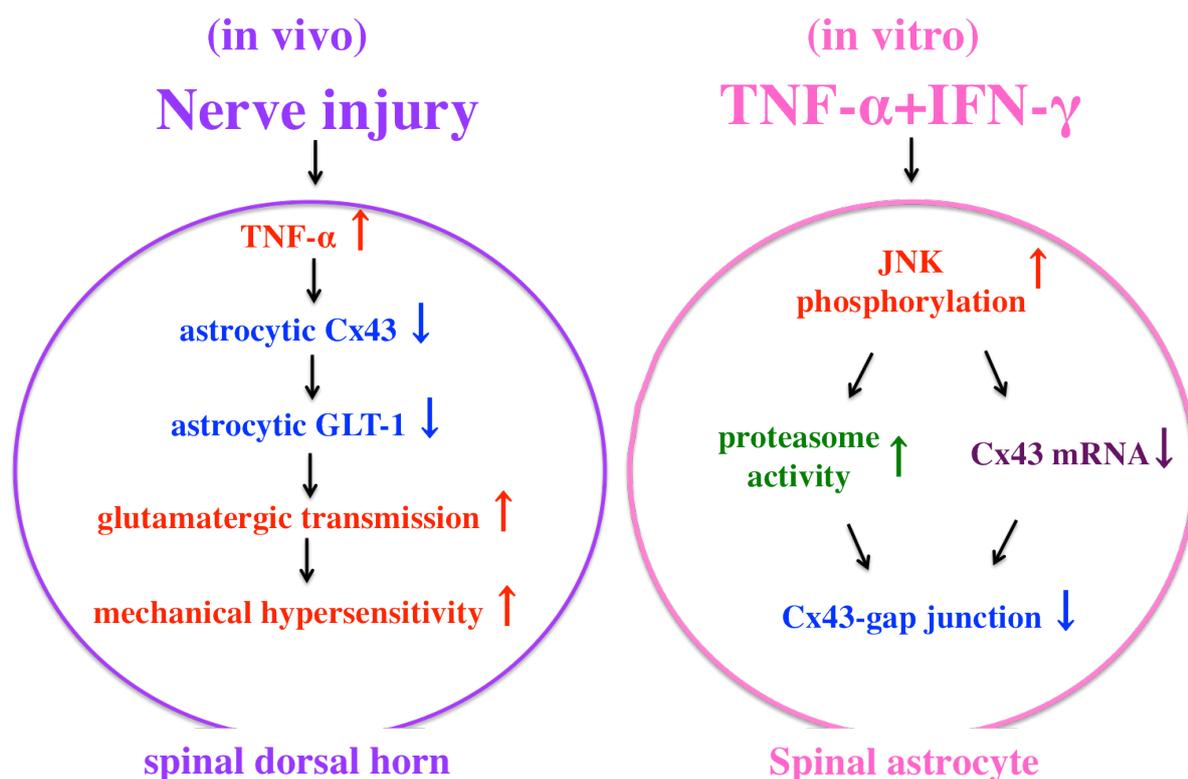
thereby modulating Cx43-GJIC in spinal astrocyte.

In conclusion, the current study demonstrated that TNF- α and IFN- γ , separately and in combination induced a down-regulation of Cx43 expression in spinal astrocytes, which led to decreased gap junction function and that the down-regulation is critically dependent on JNK activation and proteasome activity. As a consequence of diminished functioning of gap junctions, there is a failure of intercellular communication and sensing of the synaptic milieu. Under neurodegenerative or neuroinflammatory conditions, JNK appears to have an essential transcriptional role in dysfunctional spinal astrocytes. Indeed, JNK appears to be involved in a number of processes in both glia and CNS neurons following injury ^{73,85}. In addition, the proteasome activities have an important role on protein degradation. Further elaborating the molecular mechanism that down-regulates the expression of Cx43 might lead to new strategies to rescue CNS tissue in diverse neurological disorders.

Summary

In chapter 1 demonstrated that after nerve injury spinal astrocytic Cx43 was decreased via TNF- α and then induced GLT-1 down regulation. These responses induced neuropathic pain via glutamatergic transmission.

In chapter 2 demonstrated that treatment of spinal astrocyte with mixture of cytokines (TNF- α , IFN- γ) led to a decrease of Cx43 expression via JNK and proteasome pathway.



Schematic illustration of the role of Cx43 on neuropathic pain (in vivo) and the signaling pathway of Cx43 down regulation in spinal astrocyte (in vitro).

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Table.2-1. Effect of MAPK on the cytokines-mediated down-regulation on Cx43 protein expression in spinal astrocytes.

| inhibitor | Cx43 protein expression | | |
|--------------------------------------|-------------------------------|---------------------------------|-------------------|
| | TNF- α + IFN- γ | +Inhibitors | Inhibitors |
| U0125 (10 μ M, P38 inhibitor) | 0.294 \pm 0.048 | 0.414 \pm 0.091 | 0.940 \pm 0.095 |
| SB202125 (10 μ M, ERK inhibitor) | 0.294 \pm 0.048 | 0.214 \pm 0.085 | 0.847 \pm 0.183 |
| SP600125 (10 μ M, JNK inhibitor) | 0.294 \pm 0.048 | 0.667 \pm 0.052 ⁺⁺ | 1.083 \pm 0.105 |

The levels of Cx43 protein in spinal astrocytes incubated with or without inhibitors are expressed as a ratio of protein levels from vehicle-treated astrocytes. Astrocytes were pretreated for 30 min with inhibitor before treatment with the cytokine mixture (TNF- α 10 ng/ml and IFN- γ 5 ng/ml). Data represent the mean \pm SEM for 3 independent experiments. ⁺⁺P<0.01 vs. TNF- α + IFN- γ .

Table.2-2. Effect of several kinds of inhibitors on the cytokines-mediated down-regulation on Cx43 protein expression in spinal astrocytes.

| Inhibitor | Cx43 protein expression | |
|---|-------------------------------|-------------------|
| | TNF- α + IFN- γ | + Inhibitors |
| H89 (10 μ M, PKA inhibitor) | 0.362 \pm 0.012 | 0.357 \pm 0.088 |
| Go6983 (3 μ M, PKC inhibitor) | 0.366 \pm 0.021 | 0.322 \pm 0.092 |
| PP1 (20 μ M, tyrosine kinase inhibitor) | 0.357 \pm 0.017 | 0.411 \pm 0.137 |
| Genistein (50 μ M, tyrosine kinase inhibitor) | 0.380 \pm 0.015 | 0.464 \pm 0.129 |
| NAC (1 mM, Ros inhibitor) | 0.385 \pm 0.111 | 0.459 \pm 0.106 |
| AG490 (10 μ M, JAK inhibitor) | 0.300 \pm 0.027 | 0.338 \pm 0.016 |

The levels of Cx43 protein in spinal astrocytes incubated with or without inhibitors are expressed as a ratio of protein levels from vehicle-treated astrocytes. Astrocytes were pretreated for 30 min with inhibitor before treatment with the cytokine mixture (TNF- α 10 ng/ml and IFN- γ 5 ng/ml). Data represent the mean \pm SEM for at least 3 independent experiments.

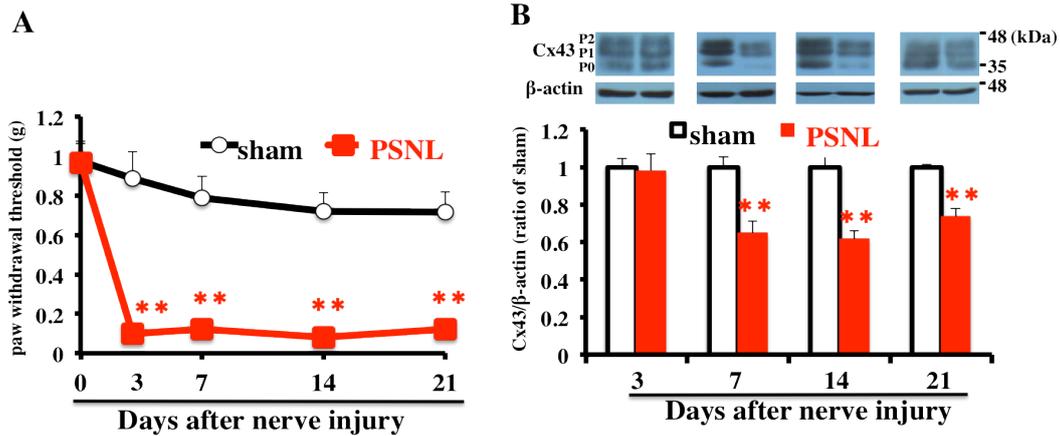


Figure1-1. PSNL induced change of tactile mechanical threshold and Cx43 expression in spinal dorsal horn.

After PSNL treatment, the withdrawal thresholds of the ipsilateral hind (A) and expression of Cx43 in ipsilateral hind spinal dorsal horn were measured by von Frey test and Western blotting, respectively for the periods indicated (3, 7, 14 or 21 day). (A) The values are expressed as the weight of von-Frey filament. (B) In Western blots Cx43 protein levels were normalized to that of β -actin, which served as an internal control, and expressed as ratio of the level of sham group at the corresponding time point. The data represent the mean \pm SEM (bar) of at least three independent experiments. **P < 0.01 vs. sham group.

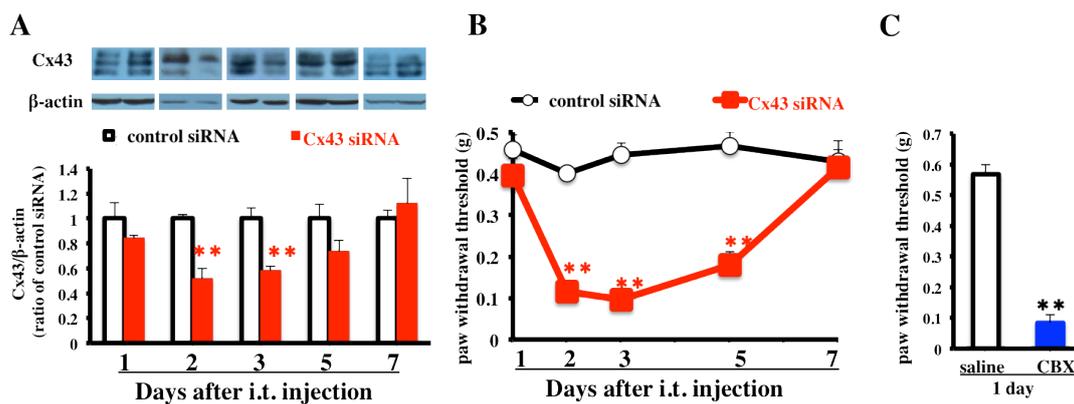


Figure1-2. Cx43 downregulation in spinal cord induced change of tactile mechanical threshold.

After intrathecal injected siRNA (5 μ g), expressions of Cx43 in spinal dorsal horn and mechanical thresholds were measured by western blotting (A) and von-Frey test (B), respectively for the periods indicated (1, 2, 3, 5 or 7 day). After intrathecal injection of carbenoxolone (CBX, 5 μ g), the mechanical thresholds were measured by von-Frey test (C). The data represent the mean \pm SEM (bar) of at least three independent experiments. **P < 0.01 vs. control siRNA group or saline group.

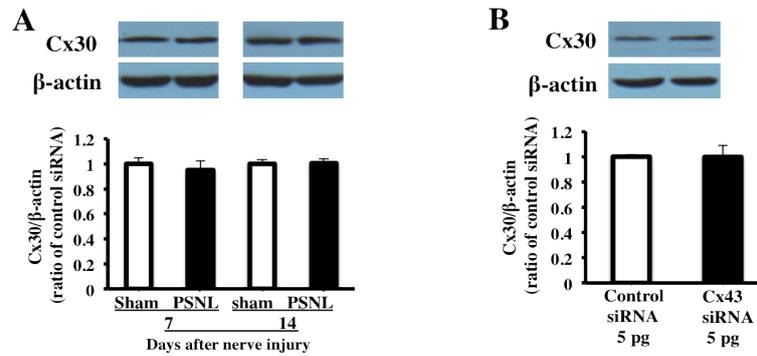


Figure1-3. PSNL induced change of tactile mechanical threshold and Cx43 expression in spinal dorsal horn.

The change of Cx30 expression in ipsilateral hind spinal dorsal horn at 7 and 14 days after PSNL treated mice (A) and in spinal dorsal horn at 3 days after Cx43 siRNA treated mice (B) were measured by Western blotting. The Cx30 protein levels were normalized to that of β-actin, which served as an internal control, and expressed as ratio of the level of sham group or control siRNA group. The data represent the mean ± SEM (bar) of at least three independent experiments.

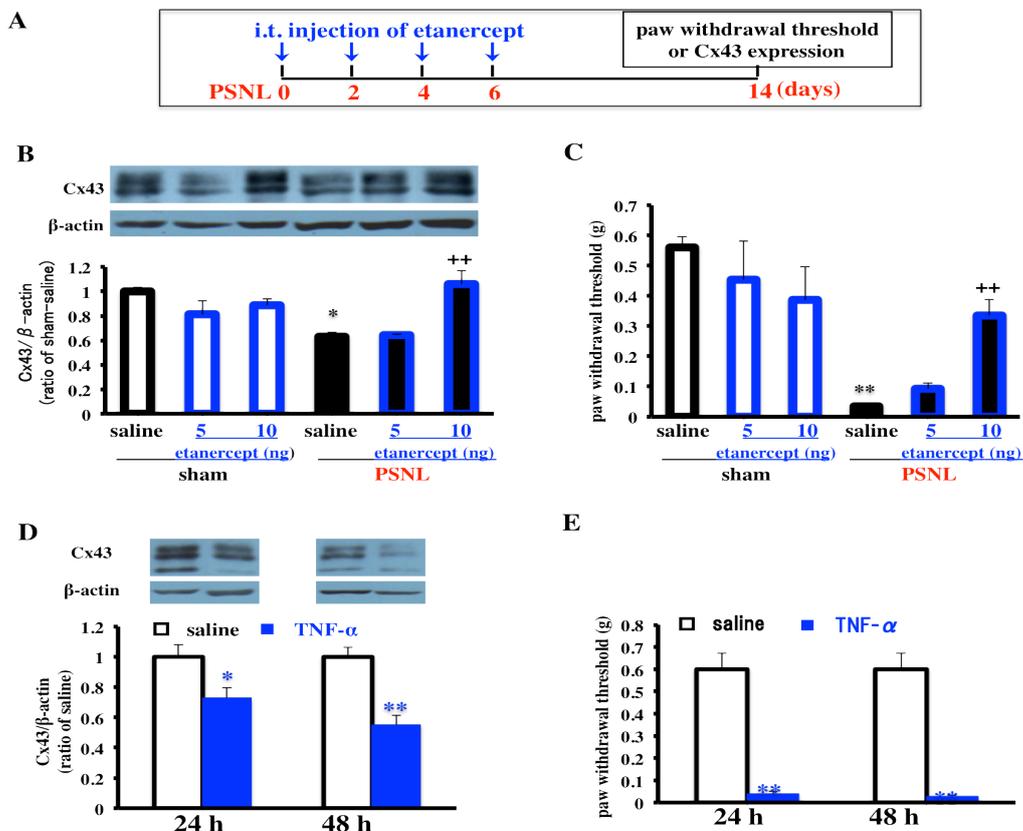


Figure1-4. TNF-α involves the PSNL induced down regulation of Cx43 in spinal dorsal horn.

The protocol of repeated-intrathecal injected with etanercept for PSNL mice (A). The

expression of Cx43 in the spinal dorsal horn in PSNL mice (B) or TNF- α treated mice (D) were measured by western blotting. The change of withdrawal thresholds after etanercept treatment mice (C) or TNF- α treated mice (E) was measured by von Frey test. The data represent the mean \pm SEM (bar) of at least three independent experiments. **p<0.01 vs. sham-saline group or saline group.

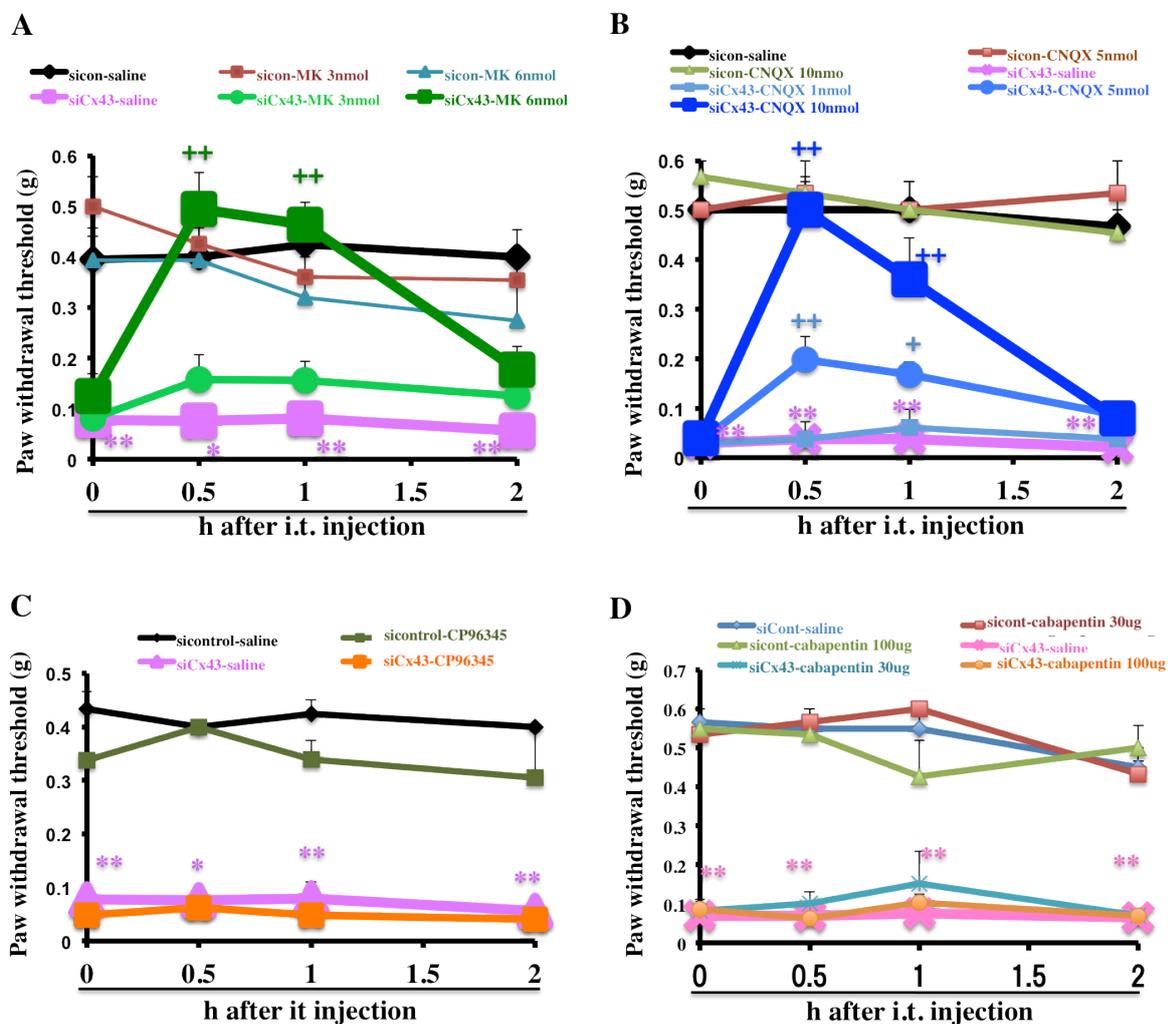


Figure1-5. Glutamate receptors antagonists attenuate the Cx43 siRNA-induced mechanical hypersensitivity

After treated several antagonists including MK801 (A), CNQX (B), CP96345 (C) or cabapentin (D), the change of mechanical hypersensitivity induced by Cx43 siRNA were measured by von Frey test. Values are expressed as the weight of von-Frey filament. The data represent the mean \pm SEM (bar) of at least three independent experiments. *p<0.05, **p<0.01 VS. sicon-saline group. +p<0.05, ++p<0.01 VS. siCx43-saline group.

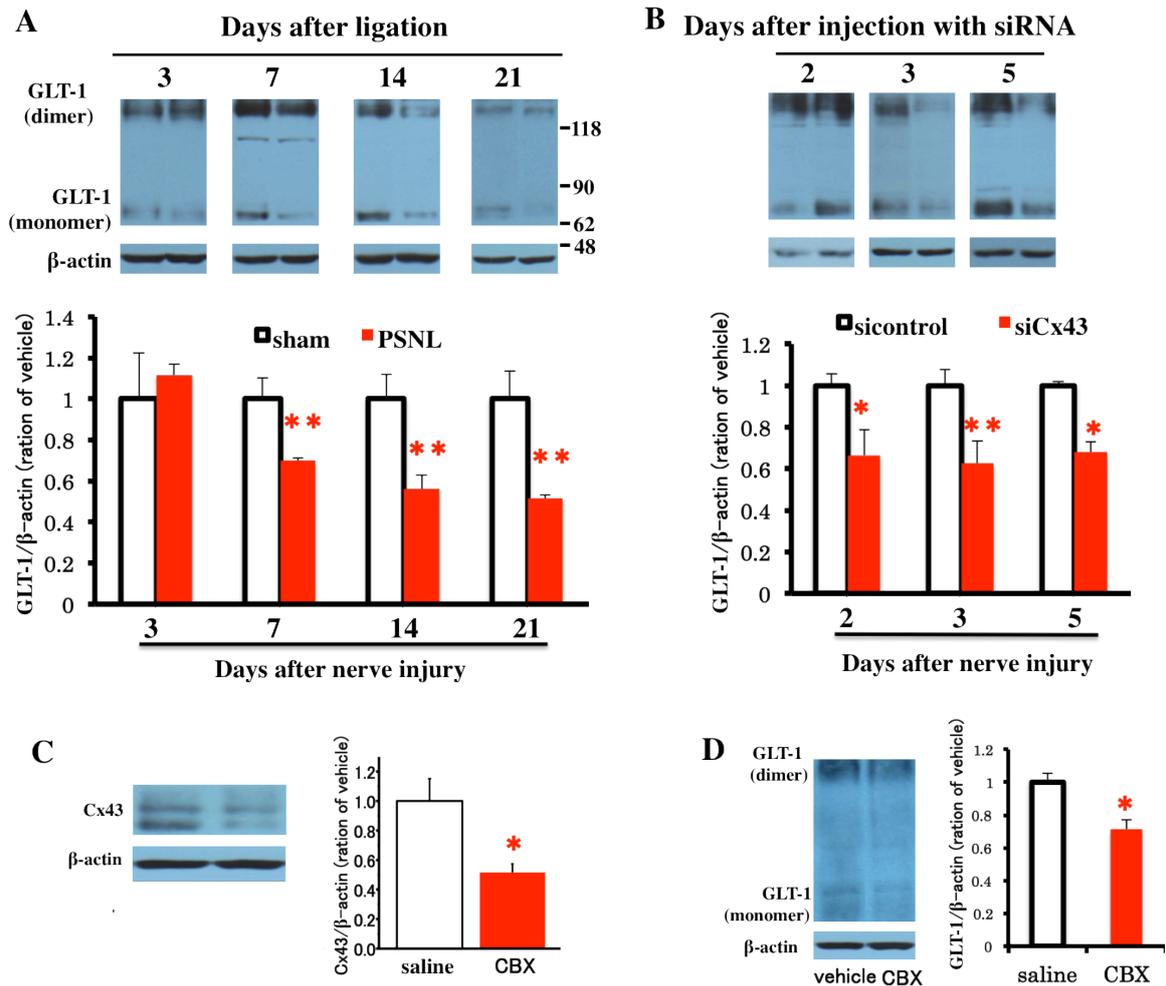


Fig.1-6. The down-regulation of Cx43 expressions were related GLT-1 decreasing in spinal dorsal horn.

After treatment with PSNL (a) or Cx43 siRNA (b), the expression levels of GL-1 in spinal dorsal horn were measured by Western blotting. After treatment with carbenoxolone (CBX) the Cx43 expression (c) and GLT-1 expression (d) were also measured by Western blotting. The data represent the mean \pm SEM (bar) for at least three independent experiments, **P<0.01 vs. sham group, control-siRNA group or saline group.

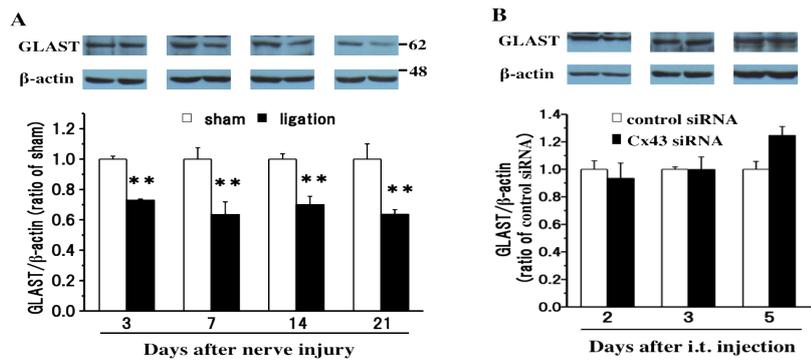


Figure1-7. The expression of GLAST in spinal dorsal horn not changed following Cx43 down regulation.

The expression of GLAST in the ipsilateral hind spinal dorsal horn at PSNL mice (A) and in spinal dorsal horn in Cx43 siRNA treated mice (B) were measured by Western blotting. The GLAST protein levels were normalized to that of β -actin, which served as an internal control, and expressed as ratio of the level of sham group or control siRNA group. The data represent the mean \pm SEM (bar) of at least three independent experiments. **P<0.01 vs. sham group.

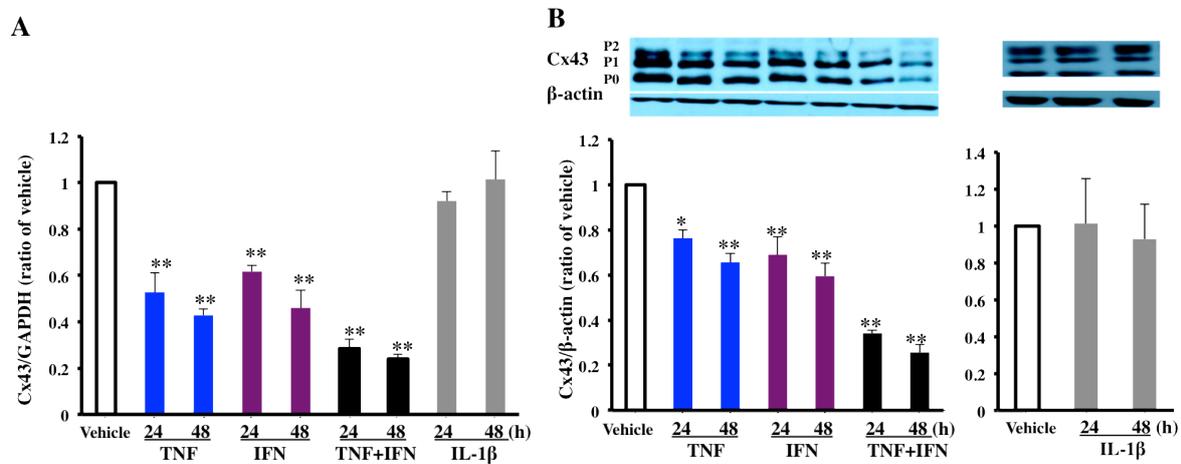


Fig.2-1. Effect of cytokines on the expression of Cx43 in cultured rat spinal astrocytes.

After treatment of spinal astrocytes with either TNF- α (TNF, 10 ng/ml), IFN- γ (IFN, 5 ng/ml), IL-1 β (10 ng/ml) or a mixture of TNF- α and IFN- γ (TNF+ IFN) for 24 or 48 h, the expression levels of Cx43 mRNA (A) and protein (B) were analyzed by real-time PCR and Western blotting, respectively. (A) The expression of Cx43 mRNA was normalized to that of GAPDH mRNA, used as an internal control. Data are expressed as a ratio of the level of vehicle-treated astrocytes. (B) In Western blots Cx43 protein levels were normalized to that of β -actin, which served as an internal control, and expressed as ratio of the level of vehicle-treated astrocytes. The data represent the mean \pm SEM (bar) of at least 3 independent experiments, *P < 0.05, **P < 0.01 vs. vehicle-treated.

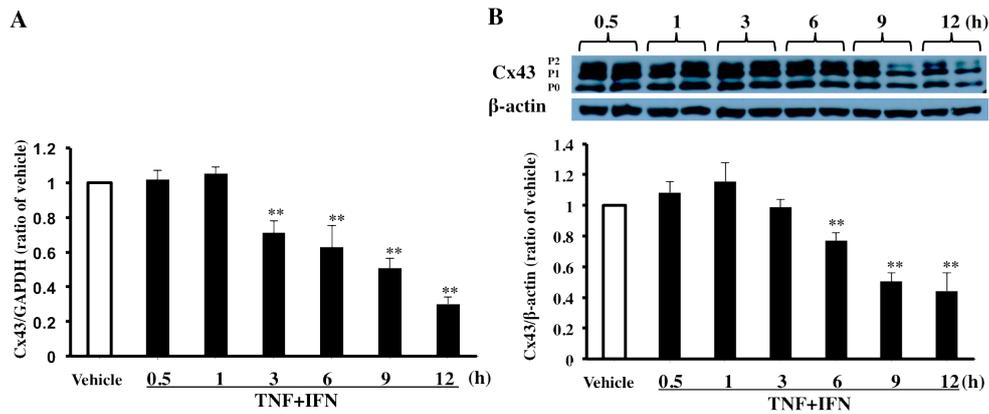


Fig.2-2. Time dependent effects of co-treatment with TNF- α and IFN- γ on Cx43 mRNA and protein levels.

Spinal astrocytes were treated with a mixture of TNF- α (TNF, 10 ng/ml) and IFN- γ (IFN, 5 ng/ml) for the periods indicated (0.5, 1, 3, 6, 9 or 12 h). The expressions level of Cx43 mRNA (A) and protein (B) were measured by real-time PCR and Western blotting, respectively. Data represent the mean \pm SEM (bar) at least 3 independent experiments, **P < 0.01 vs. vehicle-treated.

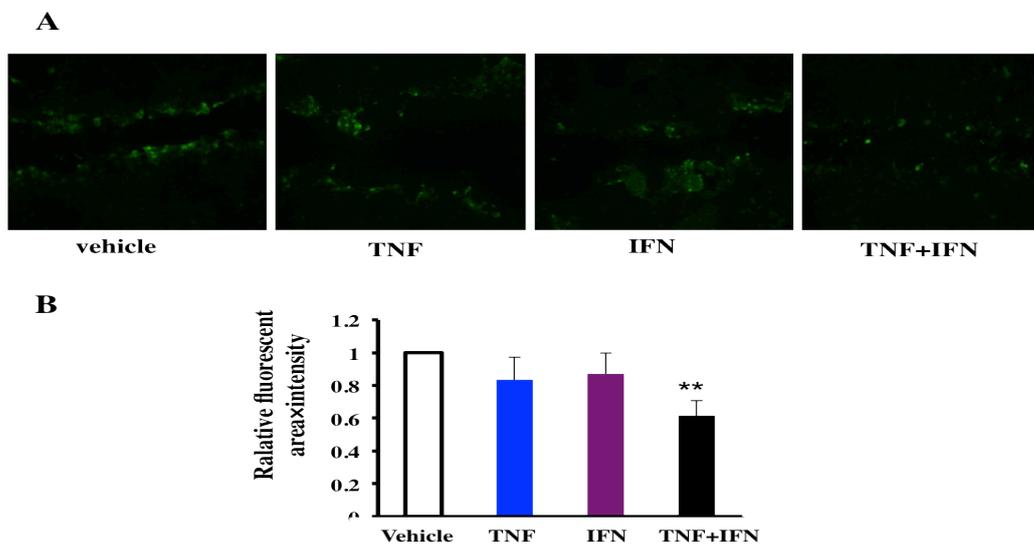


Fig.2-3. Effect of co-treatment with TNF- α and IFN- γ on the function of GJIC in cultured spinal astrocytes.

Spinal astrocytes were treated with either TNF- α (TNF, 10 ng/ml), IFN- γ (IFN, 5 ng/ml) or both for 48 h. After incubation, cells were cut with a razor blade in the presence of Lucifer yellow. (A) Spreading of the dye was taken as an indicator for intercellular junction coupling. Scale bar, 100 μ m. (B) The graph represents the quantitation of the spreading fluorescent area \times intensity. The values are expressed as a ratio of vehicle-treated astrocytes. The data represent the mean \pm SEM (bar) for at least 3 independent experiments, **P < 0.01 vs. vehicle-treated.

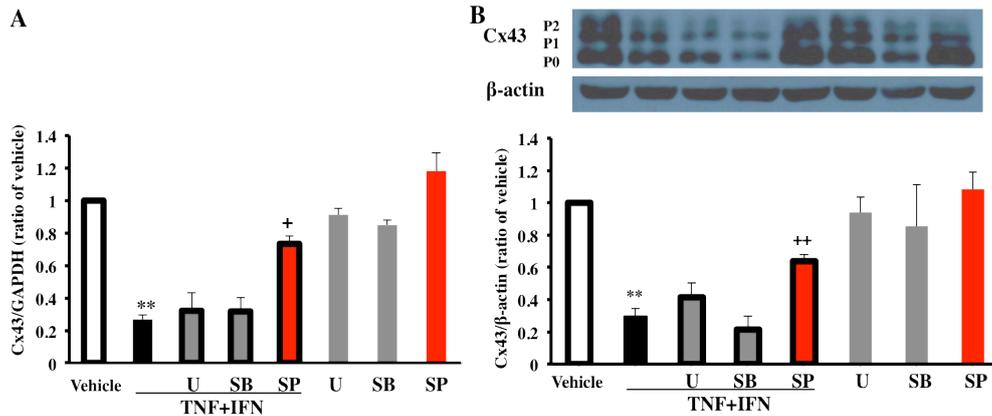


Fig.2-4. Effect of MAP kinase inhibitors on cytokine-mediated down-regulation of Cx43 in cultured spinal astrocytes.

After treatment with U0126 (U, 10 μ M), SB202190 (SB, 10 μ M) or SP600125 (SP, 10 μ M) for 30 min, spinal astrocytes were stimulated with a mixture of cytokines (TNF- α 10 ng/ml, IFN- γ 5 ng/ml) for 48 h. The expression levels of Cx43 mRNA and protein were measured by real-time PCR (A) and Western blotting (B), respectively. The data represent the mean \pm SEM (bar) for at least 3 independent experiments, **P<0.01 vs. vehicle-treated. +P<0.05 vs. TNF- α + IFN- γ . ++P<0.01 vs. TNF- α + IFN- γ .

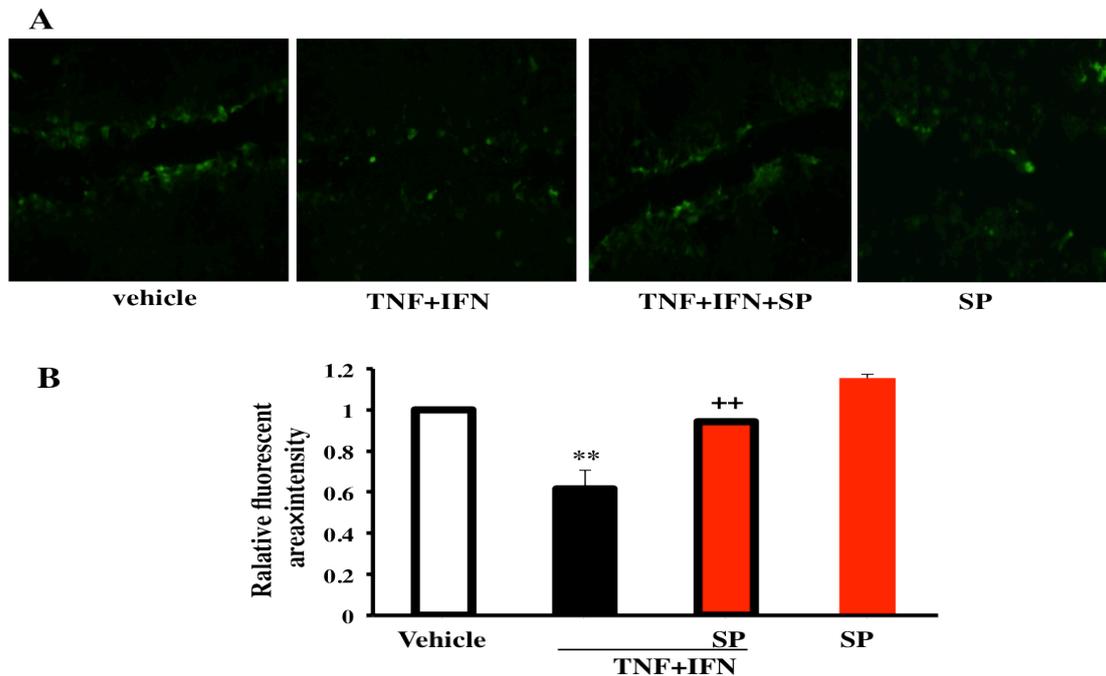


Fig.2-5. Effect of JNK inhibitors on cytokine-mediated down-regulation of GJIC in cultured spinal astrocytes

After incubation with SP600125 (SP, 10 μ M) for 30 min, cells were stimulated with a mixture of TNF- α (TNF, 10 ng/ml) and IFN- γ (IFN, 5 ng/ml) for 48 h. Then, GJIC between spinal astrocytes was measured by the SLDT assay. Upper panels indicate representative

results. Scale bar, 100 μ m. The graph in the lower part of figure 6 is quantitation of the spreading fluorescent area \times intensity. The values are expressed as a ratio of vehicle-treated. The data represent the mean \pm SEM (bar) for at least 3 independent experiments, ** $P < 0.01$ vs. vehicle-treated. ++ $P < 0.01$ vs. TNF- α + IFN- γ .

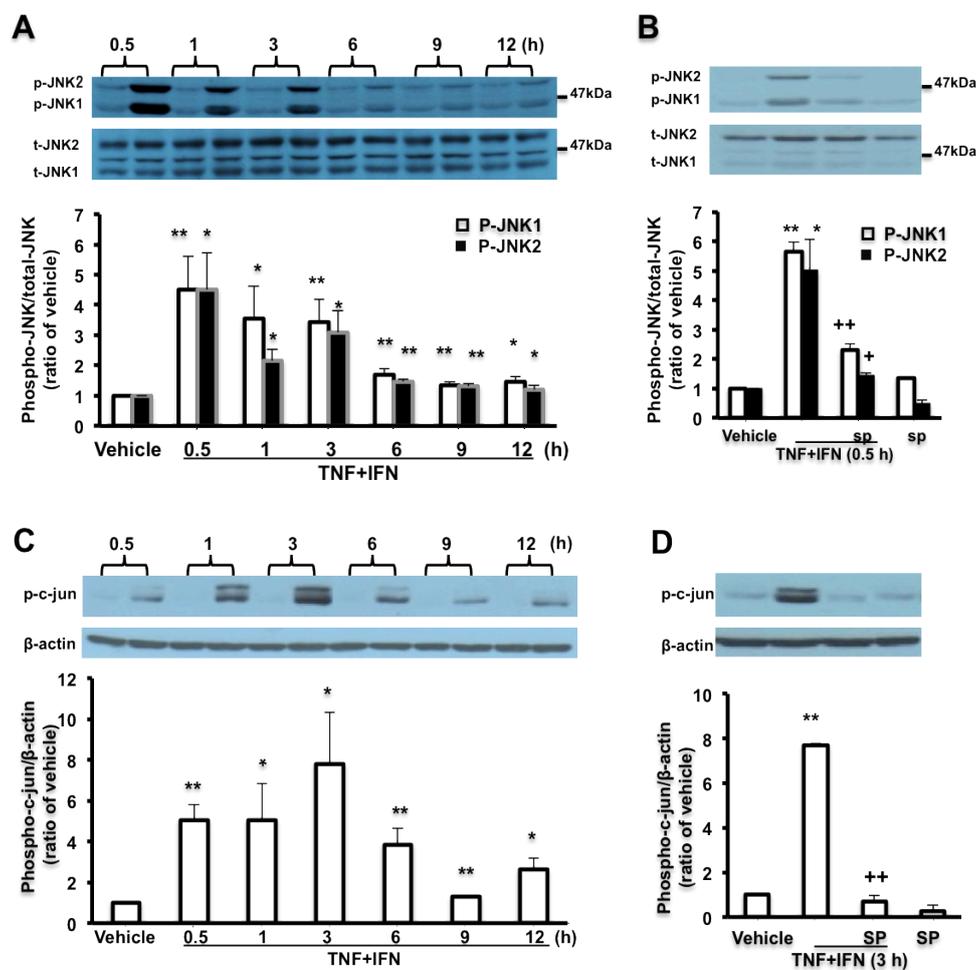


Fig.2-6. Effect of co-treatment with TNF- α and IFN- γ on JNK and c-jun phosphorylation levels in cultured spinal astrocytes.

The time-courses (hours) of JNK phosphorylation (A) and c-jun phosphorylation (C) after co-incubation with TNF- α (TNF, 10 ng/ml) and IFN- γ (IFN, 5 ng/ml) are shown. Upper panel are representative blots. The graph in the lower part of the figure represents quantitative data for each blot. Each level of JNK phosphorylation was normalized for total JNK and c-jun phosphorylation was normalized for β -actin and expressed as a ratio of vehicle-treated levels. The effect of SP600125 on the phosphorylation levels of JNK (B) after stimulation with both cytokines for 30 min and phosphorylation levels of c-jun (D) after stimulation with both cytokines for 3 h are shown. Data represent the mean \pm SEM (bar) for at least 3 independent experiments, * $P < 0.05$, ** $P < 0.01$ vs. vehicle-treated, + $P < 0.05$ vs. TNF- α + IFN- γ , ++ $P < 0.01$ vs. TNF- α + IFN- γ .

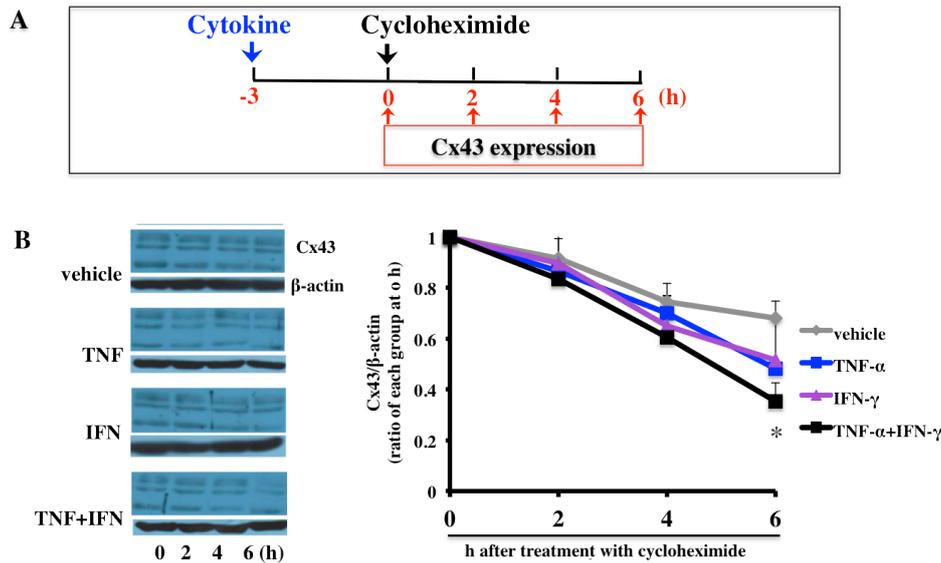


Figure2-7. Effect of TNF- α and IFN- γ on the Cx43 degradation in spinal astrocytes.

Spinal astrocytes treated with cycloheximide (1 μ M) for the periods indicated (0, 2, 4 or 6 h) after 3 h treated TNF- α (TNF, 10 ng/ml), IFN- γ (IFN, 5 ng/ml) or TNF- α +IFN- γ (TNF+IFN) (A). The expression levels of Cx43 protein were measured by Western blotting (B). Data represent the mean \pm SEM (bar) at least 3 independent experiments, *P < 0.05 vs. vehicle-treated at respectively h.

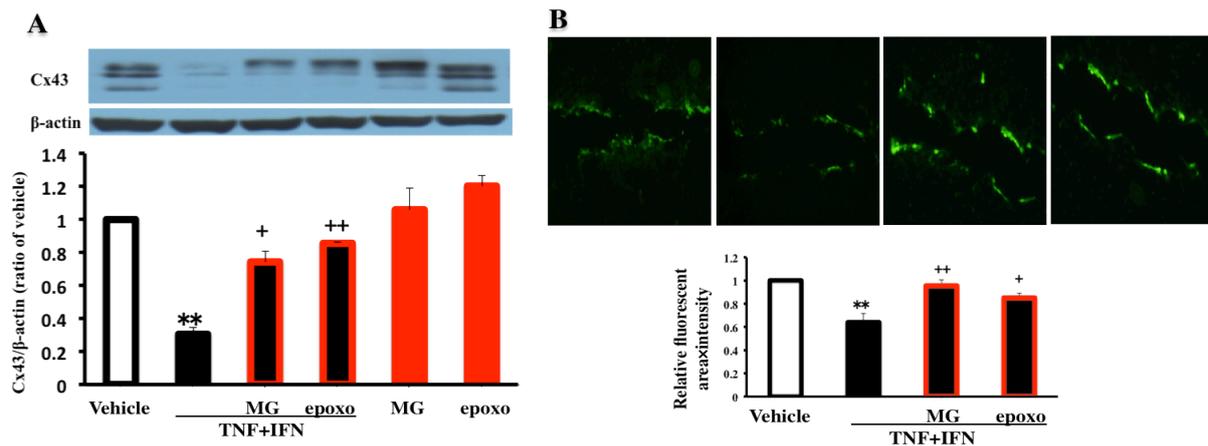


Fig.2-8. Effect of proteasome inhibitors on cytokine-mediated down-regulation of Cx43-gap junction in cultured spinal astrocytes.

After treatment with either MG132 (MG, 5 μ M) or epoxomicin (epoxo, 25 nM) for 1 h, spinal astrocytes were stimulated with a mixture of cytokines (TNF- α , TNF 10 ng/ml, IFN- γ , IFN 5 ng/ml) for 24 h. The expression levels of Cx43 protein and gap junction function were measured by Western blotting (A) and SLDT assay (B), respectively. The data represent the mean \pm SEM (bar) for at least 3 independent experiments, **P<0.01 vs. vehicle-treated. +P<0.05 vs. TNF- α + IFN- γ . ++P<0.01 vs. TNF- α + IFN- γ

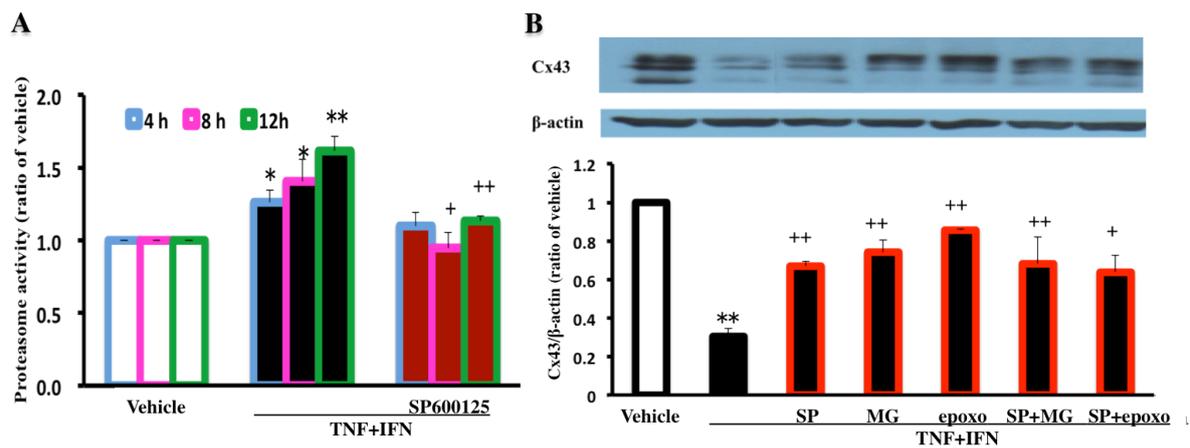


Fig.2-9. The JNK activity plays an important role in the cytokines-induced proteasome activity.

After treatment with SP600125 for 30 min, astrocytes were treated a mixture of cytokines (TNF- α , TNF 10 ng/ml, IFN- γ , IFN 5 ng/ml) for 4, 8 or 12 h. the proteasome activity was measured by using 20s proteasome assay kit (A). After treatment with either SP600125 (SP, 10 μ M), MG132 (MG, 5 μ M), epoxomicin (epoxo, 25 nM), a mixture of SP600125+MG132 or a mixture of SP600125+epoxomicin for 1 h, cells were stimulated with a mixture of cytokines for 24 h. Data represent the mean \pm SEM (bar) for at least 3 independent experiments, *P < 0.05, **P < 0.01 vs. vehicle-treated, +P<0.05 vs. TNF- α + IFN- γ , ++P<0.01 vs. TNF- α + IFN- γ .