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A Critical Role of Vimentin in Rat Astrocytes After Chronic Constriction Injury

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**Abstract**

Vimentin, among the family of the intermediate filament, it plays as the organiser of some critical proteins involved in migration, attachment and cell-signaling. In this study, the role of vimentin in CCI (chronic constriction injury) was investigated, including the protein expression and so on. Western blot analysis revealed that vimentin shows a significant difference between the injury group and the control group. This difference is particularly prominent in 7days after surgery. Double immunoﬂuorescence staining showed vimentin immunoreactivity was mostly co-localized with astrocytes, not with neurons and microglia. In cell culture, sensory neurons injury stimulated the cultured astrocyte, which leads to the up-regulation of pro-inﬂammation cytokines, p-ERK (Phosphorylated-extracellular signal-regulated protein kinase), and vimentin. However, vimentin gene silencing by siRNA (Small interfering RNA) can reverse this up-regulation and reduce the release of inflammatory cytokines. Overall, stimulated astrocytes released cytokines to promote the development of neuropathic pain via vimentin–ERK signaling. Vimentin might be important for the activation of astrocytes in the neuropathic pain.

**Keywords**

Vimentin; Chronic pain; CCI; Astrocyte; p-ERK

**Introduction**

Neuropathic pain, induced by an injury or inflammation of the somato sensory nervous system, is a kind of peripheral or central nervous system disease and imbalance of chronic condition[[42](#_ENREF_42)]. Hyperalgesia and allodynia, as the most significant features of neuropathic pain, are the two most common symptoms[[40](#_ENREF_40)]. To date, despite considerable progresses have been made, the mechanisms of neuropathic pain have not been fully investigated, and thus, the treatment of it remains a challenge[[23](#_ENREF_23)].

Vimentin, among the family of the intermediate filament, it plays as the organiser of some critical proteins involved in migration, attachment and cell-signaling[[24](#_ENREF_24)]. It can maintain the cell morphology, ensure the integrity of the cytoplasm, and stabilize the interaction between the cytoskeleton[[26](#_ENREF_26), [28](#_ENREF_28), [32](#_ENREF_32)]. It is also involved in the immune response[[3](#_ENREF_3), [5](#_ENREF_5), [7](#_ENREF_7)]. GFAP and Vimentin are two major intermediate filament proteins in astrocytes[[36](#_ENREF_36)].It is previously considered that Vimentin was mainly expressed in immature astrocytes, while GFAP was in the mature astrocytes.[[8](#_ENREF_8)].However, Kim DH et al. propose that vimentin, as well as GFAP, were both upregulated in the adult rat during spinal cord injury[[13](#_ENREF_13), [22](#_ENREF_22)]. These evidence imply that vimentin may be a neglected but critical role in the neuropathic pain.

Mounting evidences have revealed that astrocytes activation can accelerate the development of pain hypersensitivity[[18](#_ENREF_18), [38](#_ENREF_38)]. Upon activation, astrocytes secreted multiple inflammatory mediators such as TNF-a, IL-1β, IL-6[[15](#_ENREF_15), [20](#_ENREF_20), [25](#_ENREF_25)]. Inhibition of such inflammatory cytokines has been shown to effectively relieve neuropathic pain. ERK(extracellular signal-regulated protein kinase), known as an important role of MAPK family, it is acknowledged that activated ERK also involved in neuronal plasticity [[6](#_ENREF_6)] and related learning and memory processes[[14](#_ENREF_14), [35](#_ENREF_35)]. In recent years, our understanding of ERK/MAPK extends to that it can promote neuropathic pain in the dorsal horn and DRG.[[30](#_ENREF_30)].

In this study, using Chronic Constriction Injury (CCI) model, we set out to investigate the expression of vimentin in the spinal cord in neuropathic pain. Our results imply that vimentin plays an essential role in activation of spinal cord astrocytes. Therefore, restricting vimentin in activated astrocytes in spinal cord might be practical for alleviating neuropathic pain after peripheral nerve injury.

**Materials and Methods**

Animals

Adult male Sprague–Dawley rats weighing 200–250g were purchased from the Experimental Animal Center of Nantong University. All animal protocols were carried out according to the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals[[41](#_ENREF_41)]. Animals were housed under a 12 h light–dark cycle at a constant room temperature (21 ± 1 °C). The mice have a free access to water and food.

Surgical Procedures

Animals were anesthetized with isoflurane (4% for induction; ~1.5% during surgery). The whole procedure was aseptically. All rats were performed on the right side. Extra care was taken to eliminate the risk of inflammation and infection. Peripheral nerve injury frequently leads to neuropathic pain, and chronic constriction injury was done. For CCI (chronic constriction injury) model, the right common sciatic nerve of each rat was exposed, and then ligated with 4–0 silk suture, with identical surgery in the sham group as described previously[[2](#_ENREF_2)].

Behavioral Testing

Before the test, we make the animals accustomed to the testing environment for at least 3 days. The behavioral test (n = 5 animals/group) was done from 3 days before surgery to 3 weeks after the CCI surgery. For measuring mechanical sensitivity, animals were put on a metal mesh ﬂoor 15 minutes before test . We then use the Von Frey hairs (Stoelting, Wood Dale, IL) to stimulate the plantar surface of each hind paw for 2-3s (5 min interval). The 50% withdrawal threshold was conformed as described in Dixon’s up–down method [[4](#_ENREF_4)]. As for heat sensitivity, rats were also put on the plate to adapt to the environment before test. Then the plantar surface of rats was exposed to the radiant heat (Life Science Model 390G; IITC Life Science Inc, Woodland Hills,CA),we then record the value as paw withdrawal latency (PWL).The temperature was maintained at 30 °C during the whole process. The base line latencies were set to 10–14s with a maximum of 20s as cut-off to prevent tissue injury.

Western Blotting

Western blotting was prepared from the naïve group, sham group, and the injury group ,the L5 level of spinal cord were separated at different time points from 1 day to 3 weeks (n = 5 for each time point). We then weighed the frozen tissue samples and minced it to prepare lysates. The samples were then homogenized in lysis buffer. Then homogenates were centrifuged at 12,000 rpm for 20 min. The supernatant ws collected at last. Proteins were quantified, separated on a 10% sodium dodecyl sulfate-agarose gel, and transferred to a nitrocellulose membrane. The membranes were blocked with 5 % non-fat dried skim milk for 2h at room temperature, and then were incubated with primary antibody against Vimentin (1:1000; Abcam)**,** p-ERK(1:500; Cell signaling), ERK（1:500; Cell signaling）; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Santa Cruz ), TNF-a (1:500; Santa Cruz),and β-actin(anti-mouse, 1:2000; Sigma) at 4℃, respectively. membranes were washed and incubated with horseradish peroxide-conjugated secondary antibody accordingly. After 2h incubation, blots were visualized and quantified using an enhanced chemiluminescence-western blotting detection system (ECL kit, Amersham Biosciences).

Sections

Rats were deeply anesthetized with pentobarbital (50mg/kg,i.p.)and perfused pericardially with 500 ml 0.9 % saline and 4 % paraformaldehyde. After that, the L4-6 spinal cords were dissected and put in the 20% sucrose for 2–3 days, 30% sucrose for 2–3 days follows. When needed, the specimens were cut at 7 μm as mentioned in our previous study and the sections were stored at −20 °C until use.

Double Immunofluorescent Labeling

Sections were applied for the double immunofluorescence. The sections were placed in the oven for 1h at 37 °C, and then were blocked with solution containing 10 % normal serum, 3%(w/v) bovine serum albumin (BSA), 0.1% Triton X-100, and 0.05% Tween-20. After 2h incubation, the sections were incubated with primary antibodies against Vimentin(rabbit,1:50;Abcam),mouse antibody anti-NeuN(a marker of neuron, 1:200; Millipore), anti-glial fibrillary acid protein (GFAP) (a marker of astrocytes,1:200; Sigma), anti-Iba1 (a marker of microglial,1:200; Millipore). After washing with phosphate buffer solution (PBS) three times for 5 min each, corresponding secondary antibodies were added in dark room and incubated for 2 h at 4 °C. Finally, the sections were washed as previous and were assayed using Leica microscope (Leica, DM 5000B; Leica CTR 5000; Germany).

siRNA Knockdown of vimentin Expression

Control siRNA, vimentin-siRNA were designed and synthesized by Oligobio (Beijing, China). The lipofectamine and plus reagent in Opti-MEM(Invitrogen) was used to perform siRNA transfection according to the manufacture protocol. The siRNA sequence were as follows: vimentin, sense 5′-TCAGACAGGATGTTGACAAT-3′ and antisense 5′-GACATGCTGTTCCTGAATCT-3′. Control siRNA is an irrelevant siRNA with random nucleotides and no known specificity.

Cell Culture

Primary astrocyte cells of rat spinal cord were prepared as the previous study[[21](#_ENREF_21)]. Briefly, the spinal cord was firstly isolated from the rat, and then fully digested by the trypsin, at last plated in the medium supplemented with 1 mm HEPES, 10% fetal bovine serum, 2 mM glutamine,and 1% antibiotic/antimycotic. Cells were used for experiments after 2 weeks. Untreated cells served as controls. Cells pretreated with vimentin siRNA or U0126, inhibitor for ERK were used.

The dorsal root ganglia were extracted and cultured as described previously[[29](#_ENREF_29)]. 5 dorsal root ganglia in vitro were cultured in a 35-mm tissue culture dish containing 200 μl of culture medium (Neurobasal medium [Invitrogen, Carls-bad, CA] with 100 ng/ml nerve growth factor). At the second day, adding 10 μM 5-ﬂuoro-20-deoxyuridine (Sigma) reaction medium for 24 hours, the common medium was replaced. 7 days later, when the neurite reached the maximum growth, DRG cells were added to the astrocyte cell cultures.

ELISA

For the measurement of TNF-α、IL-1β、IL-6 levels, the astrocytes were stimulated by damaged sensory neurons for 3 hours. Then cultured cells were collected, and cell lysates were prepared as the same with western blot. The supernatants of cells were isolated by centrifugation at 4000g for 10 min. The levels of TNF-α、IL-1β、IL-6 were detected using an enzyme linked immunosorbentassay (ELISA) kit (Jiancheng Biotech, Nanjing, China) according to the manufacturer’s instructions. TNF-α、IL-1β、IL-6 concentrations were expressed as pg/ml in the serum.

Statistical Analysis

Datas are presented as means ± S.E. Statistical significance of differences was determined by Student t- test or analysis of variance with post hoc test. A p value of ＜0.05 was considered statistically significant.

**Results**

Decrease of allodynia and hyperalgesia in rats after CCI

Firstly, we choose the CCI model as previously described[[2](#_ENREF_2)] to explore the possible role of vimentin during neuropathic pain. As shown in Fig.1a, surgery to the right sciatic nerve induced a rapid and lasting mechanical allodynia. In contralateral and the sham group, the value of paw withdrawal threshold(PWT) stay stable, however, in the ipsilateral-operated rats, PWT decreased sharply and remained at the low level until 3 weeks(p<0.05,fig.1a). Moreover, CCI also caused the decrease of heat hyperalgesia in rats. The value of the paw withdrawal latency (PWL) has fallen quickly after the surgery and maintained until 3 weeks in the operation-group(P<0.05,fig.1b).These data demonstrated that the neuropathic pain model was successfully established by CCI on rats.

Changes in protein expression for Vimentin after CCI by western blot assay

We then checked Vimentin protein expression from 1 to 21 days after CCI by Western blotting. Compared to naïve group, the expression of vimentin was significantly up-regulated from 5 to 10 days, and declined at 21days after CCI(p<0.05,fig.2a,b). Sham group show no differnence on Vimentin expression in the spinal cord with the naïve group(fig.2b). As shown in fig2, CCI increase Vimentin protein expression in spinal cord, suggesting that Vimentin may play an important role during the neuropathic pain.

Vimentin was colocalized with astrocytes in the spinal cord after CCI

Double-labeling immunofluorescence was performed to figure out which cell type was vimentin colocalized with. According to the previous results, the spinal cord tissue at 7 days after CCI was chosen for this detection. As shown in fig3, CCI induced a marked increase of Vimentin-immunoreactive (IR) in the ipsilateral side of the surgery group compared to the sham group (Fig.3a,b). Vimentin-IR cells were mainly found in the superficial layers (laminae I–III) of the dorsal horn. To conform the cellular distribution of Vimentin, we performed double staining of Vimentin with different cell markers: NeuN, Iba1, and GFAP. Vimentin-IR was mostly colocalized with the astrocyte marker GFAP (Fig.3c–e) but not with neuronal marker NeuN (Fig. 3f–h) or microglial marker Iba1 (Fig.3i–k), suggesting the localization of Vimentin in spinal cord.

Spinal cord astrocyte activation after CCI surgery

Spinal cord glial cells can be activated by injured axons even the death of neuronal cell after CCI surgery[[21](#_ENREF_21)] .We then extracted the primary astrocyte from rat spinal cord and stimulated them with neuritis of DRG. As we predicted, the injury of DRG neuronal cell body can induce the upregulation of vimentin, TNF-a in the spinal cord astrocyte(fig.4a). The levels of TNF-α、IL-1β、IL-6 were measured by ELISA in the cultured cell (Fig.4b,c,d) of mice at 3 hours after stimulation. TNF-α(fig4b)、IL-1β(fig4c)、IL-6(fig4d) levels were increased in all stimulated groups (p < 0.05 or p < 0.01) compared with the control group of mice. Thus it can be seen that sensory neurons injury synthetized and secreted astrocytes activating factor(s), which might promote the activation of astrocytes and the occurrence of inflammation.

Features of vimentin knockdown in rat spinal astrocyte

To explore the role of vimentin in astrocytes, we then designed specific siRNA for rat vimentin. In addition, we designed a control siRNA to overcome the potential effects of the transfection. Western blot was used to asses the expression of vimentin protein. After the transfection of vimentin siRNA(20nM),a significant down-regulation of vimentin protein was observed(fig.5a). Rat astrocytes transfected with vimentin–siRNA were then stimulated by damaged sensory neuron for 3h,we observed that the protein level of TNF-α was down-regulated(fig.5c). Similarly, the up-regulation of TNF-α、IL-1β、IL-6 by damaged sensory neurons were reversed(fig.4b,c,d)，suggesting that vimentin is critical in pro-inflanmation cytokines secreted in astrocyte activation.

Role of vimentin in Damaged Sensory neurons stimulated ERK1/2 Activation

We then observe that the expression of ERK1/2 increased after the CCI surgery(fig.6a,b). As shown in fig6c, a sharp decrease of ERK phosphorylation indicated that vimentin is critical in ERK activation. Surprisingly, U0126 could not inhibit the up-regulated of vimentin caused by damaged sensory neurons(fig.6e,f). These results imply that sensory neurons injury regulate the development of pain by the Vimentin–ERK signal path of spinal cord astrocyte cells.

**Discussion**

Neuropathic pain that resulted from nerve injury, inﬂammation, or cancer is a great challenge to human health. Neuroinﬂammation, mediated by inﬂammatory mediators, including chemokines and cytokines, has been recently recognized to play an important role in the pathogenesis of neuropathic pain[[27](#_ENREF_27), [33](#_ENREF_33), [39](#_ENREF_39)].

Our research group have investigated the role of vimentin in the activated astrocytes in the model of neuropathic pain. The protein expression of vimentin in spinal cord were increased significantly. Then, we use the sections to conduct immunofluorescence,we found that vimentin colocalized with astrocytes but not with neuron and microglia. In order to know how vimentin affects the spinal astrocytes, we stimulated the astrocytes with the damaged primary sensory neurons, which can be viewed as the CCI model in vitro. Then, we found that sensory neurons injury synthetized and secreted proinﬂammation cytokines, Vimentin, p-ERK were also increased. vimentin gene silencing by siRNA reverse this up-regulation and inhibits the activation of astrocytes.

Researches in recent year suggest that astrocytes in central nervous system (CNS) play an important role in the development and maintenance of neuropathic pain[[10](#_ENREF_10), [18](#_ENREF_18)]. The activation of astrocytes may promote the occurrence and development of neuropathic pain. At present, it is believed that the regulation of astrocyte on pain is mainly through the regulation of the following 3 kinds of activation. Firstly, changes in the intracellular signaling pathways, such as mitogen- activated protein kinase ( MAPK) phosphorylation levels, changes in the expression of transcription factors[[17](#_ENREF_17)]. Secondly, the expression of receptors and channel proteins, such as up-regulation of inflammatory factor receptor and gap junction protein, down regulation of glutamate transporter. Lastly, synthesis and release of glial derived mediators such as chemokines, cytokines, proteases and growth factors [[10](#_ENREF_10), [17](#_ENREF_17), [18](#_ENREF_18), [19](#_ENREF_19)]. Our study shows that vimentin is coexpressed with GFAP, a marker of astrocytes. Astrocytes are important in the maintenance of neuropathic pain. That vimentin–IR is co-localized with astrocytes at 7days indicates that vimentin may contribute to neuropathic pain in some way.

Accumulating evidence indicates that activated spinal cord astrocytes release proinflammatory cytokines and chemokines such as TNF-α、IL-1β、IL-6, which contributes to the maintenance of pain states[[9](#_ENREF_9), [11](#_ENREF_11)]. Researches found that intrathecal injection of exogenous TNF can promote pain, leading to thermal hyperalgesia and mechanical allodynia[[12](#_ENREF_12)], and intrathecal injection of TNFR antibody, especially TNFR1 antibody, can reverse the formation of hyperalgesia[[16](#_ENREF_16)]. The same results can also occur in IL-1βand IL-6[[34](#_ENREF_34), [37](#_ENREF_37)]. In our study, the damaged sensory neurons stimulated the primary cultured spinal astrocytes, which induce the up-regulation of cytokines forementioned ,including the vimentin. There shows that vimentin may be involved in the pathogenesis of neuropathic pain. We speculate boldly that vimentin can promote the synthesis and release of cytokines, which can accelerate the development of pain. Behavioral tests also validate this result, with up-regulation of vimentin, the paw withdrawal threshold(PWT) was decreased, and the paw withdrawal latency(PWL) was shortened.

Emerging evidence indicates that activated ERK/MAPK contribute to nociceptive responses following inflammation and/or nerve injury[[30](#_ENREF_30)]. The expression of the phosphorylated ERK (p-ERK) in the dorsal horn was induced following chronic constriction injury(CCI) of the sciatic nerve[[1](#_ENREF_1)]. Intrathecal injection of the MEK1 inhibitor U0126 reversed CCI induced mechanical allodynia[[31](#_ENREF_31)]. In our experiment, we found that blocking the expression of vimentin by siRNA decreased the up-regulation of p-ERK and the release of cytokines. Then, U0126, the inhibitor of ERK, did not have impact on the increased expression of vimentin. Therefore, the vimentin–ERK pathway may be essential for the astrocytes activation under neuropathic pain.

Overall, activated astrocytes released cytokines such as TNF-a 、IL-1β、IL-6 after neuropathic pain via vimentin–ERK signaling. This provides us with a new treatment for neuropathic pain. As previously mentioned, vimentin plays a role in cytoskeletal arrangement.But here we did not do further exploration, however, we cannot exclude the possibility that vimentin may play another role in morphological changes. It will be noted in our future study.

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**Figure caption**

**Fig.1** Decrease of mechanical allodynia and heat hyperalgesia in rats after CCI. a CCI induces a significant decrease in PWT compared to the preoperative value and the sham groups. b The value of PWL is also decreased in the postoperative group as the same above. \*P＜0.05, \*\*P＜0.01 versus baseline. Two-way ANOVA followed by Bonferroni test. n = 5/group

**Fig.2** CCI induced Vimentin protein up-regulation in rats spinal cord. a, b Western blot results showed that Vimentin protein expression was up-regulated in CCI animals than that in sham-operated animals at 5, 7 and 10 days. \*P＜0.05 versus naive, #P ＜0.05 versus sham. Student’s t test. n = 5/group.

**Fig.3** Double immunofluorescence staining for Vimentin with different phenotype-specific markers in the spinal cord after CCI. a,b Immunofluorescence showed that Vimentin-IR was increased at 7 days after CCI compared to the naïve animals. Double staining immunofluorescent showed that Vimentin-IR was largely colocalized with astrocytic marker GFAP(fig.3c,d,e), not with not with neuron marker NeuN(fig.3f,g,h) and microglial marker Iba1(fig3i,j,k) in the spinal cord at 7 days after CCI.

**Fig.4**  The damaged sensory induced pro-inﬂammatory gene expression in astrocytes. supernatant was collected at 3h after transfection. a Western blot results showed that Vimentin、TNF-α protein expression was significantly increased in treatment group than that in control group. TNF-α(fig4b)、IL-1β(fig4c)、IL-6(fig4d) were measured by ELISA in all of the treatment groups than the control group. \*p < 0.05, \*\*p < 0.01 compared with the control group.

**Fig.5** Vimentin gene silencing inhibited sensory neurons injury mediated up-regulation of pro-inﬂammation cytokines. a Western blot showed siRNA knockeddown Vimentin expression in the transfected cell(fig5a). \*P<.05 versus control group, Student’s t test. c the effect of Vimentin gene silencing on TNF-α、Vimentin protein expression in damaged sensory neurons-stimulated astrocytes.

**Fig.6** Astrocytes were transfected with a nonspeciﬁc siRNA or Vimentin siRNA. After exposed to damaged sensory neuron, cell lysates were prepared and subjected to immunoblot analysis. Western blots were used the antibodies recognizing the total ERK proteins and GAPDH. Values represent as the means ± SE mean. e,f Cells were pretreated with U0126 (20μM) for 1 hour, then exposed to damaged sensory neuron. Western blots were assayed using the antibodies recognizing the Vimentin proteins and GAPDH.