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A Novel Role of Spinal Astrocytic Connexin 43: Mediating Morphine Antinociceptive Tolerance by Activation of NMDA Receptors and Inhibition of Glutamate Transporter-1 in Rats

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SUMMARY

Aims: Connexin 43 (Cx43) has been reported to be involved in neuropathic pain, but whether it contributes to morphine antinociceptive tolerance remains unknown. The present study investigated the role of spinal Cx43 in the development of morphine tolerance and its mechanisms in rats. **Methods:** Morphine tolerance was induced by intrathecal (i.t.) administration of morphine (15 μg) daily for seven consecutive days. The analgesia effect was assessed by hot-water tail-flick test. Expression of proteins was detected by Western blot and immunohistochemistry assay. **Results:** Chronic morphine markedly increased the expression of spinal Cx43. Gap26, a specific Cx43 mimic peptide, attenuated not only morphine antinociceptive tolerance, but also the up-regulation of spinal Cx43 expression, the activation of astrocytes, and N-methyl-D-aspartic acid (NMDA) receptors (NR1 and NR2B subunits), as well as the decreased GLT-1 expression induced by chronic morphine. MK-801, a noncompetitive NMDA receptors antagonist, suppressed the chronic morphine-induced spinal Cx43 up-regulation, astrocytes activation and decline of GLT-1 expression. **Conclusions:** The spinal astrocytic Cx43 contributes to the development of morphine antinociceptive tolerance by activating astrocytes and NMDA receptors, and inhibiting GLT-1 expression. We also demonstrate that the role of interaction between the spinal astrocytic Cx43 and neuronal NMDA receptors is important in morphine tolerant rats.

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The first two authors contributed equally to this work.

Introduction

Although opioids, including morphine, are commonly used in clinical management of acute and chronic pain, the development of tolerance to its analgesia properties significantly hampers its clinical utility. The molecular and cellular mechanisms responsible for this phenomenon remain elusive. Increasing evidence has shown that astrocytes play important roles in the development of morphine antinociceptive tolerance [1–3]. Chronic morphine induces the activation of astrocytes in the spinal cord, posterior cingulated cortex and hippocampus. Inhibiting astroglial activation by a glial inhibitor (fluorocitrate or propentofylline) significantly attenuated morphine antinociceptive tolerance [1,2].

Astrocytes are characterized by forming gap junction (GJ)-coupled networks [4]. In the central nervous system (CNS), glial GJs play important roles in the molecular exchanges and informational communications between adjacent cells, which are involved in spatial buffering of extracellular potassium ions, glutamate and other signal molecules, energy sources, and mediation of intercellular calcium waves. [5]. GJs are consisted of connexins (Cxs), with the name of respective molecular weights [6]. In the mammalian nervous system, at least 11 Cxs (Cx26, Cx29, Cx30, Cx32, Cx36, Cx43, Cx31, Cx37, Cx45, Cx47, and Cx57) have been identified [7], and different cell populations contain distinctive sets of Cxs. In the spinal cord, Cx43 is the most abundant Cx, mainly expressed in astrocytes [7].

Recently, a number of studies have demonstrated the potential involvement of GJs and Cxs in neuropathic pain [8–16]. The Cx43 expression is increased in several neuropathic pain models, such as spinal cord injury (SCI), chronic constriction injury (CCI), and neuroinflammation [9–11]. Intrathecal delivery of carbenoxolone, a GJ decoupler [12,13], or inhibiting Cx43 expression using RNA interference [14,15], ameliorates neuropathic pain induced by sciatic nerve inflammation or CCI, suggesting that the spinal

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Cx43 and GJs play important roles in neuropathic pain. However, the potential role of the spinal Cx43 in morphine antinociceptive tolerance has not been investigated. As it is hypothesized that opioid tolerance and pathological pain may share common cellular mechanisms [17], we speculate that the spinal Cx43 might mediate morphine antinociceptive tolerance. To confirm this hypothesis, in the present study, we examined the effect of chronic morphine on the spinal Cx43 expression and roles of Cx43 in the development of morphine antinociceptive tolerance in rats.

It is well-known that glutamate [18,19] and N-methyl-D-aspartic acid (NMDA) receptors participate in the development of morphine tolerance [20–25]. Both in patients and in experimental animals with long-term use of morphine, morphine tolerance was developed accompanied by an increase in glutamate level in the cerebrospinal fluid [18,19]. The extracellular glutamate is cleared by glutamate transporters (GTs), which strictly regulate the homeostasis of the extracellular glutamate level [26]. There are at least five indentified GTs. Among them, glutamate transporter-1 (GLT-1) mediates 90% extracellular glutamate uptake in the CNS. Several lines of research have shown that GTs play an important role in the development of morphine antinociceptive tolerance [27–29]. Moreover, it is reported that GJ blockage prevents neuropathic pain by preventing the activation of NMDA receptors [13], and the expression of GLT-1 is up-regulated when Cx43 is knocked out in astrocytes [30]. Therefore, we further investigated the potential roles of NMDA receptors and GLT-1 in the Cx43-mediated morphine antinociceptive tolerance in rats.

Materials and methods

Animals

Adult male Sprague-Dawley rats (250–280 g) were housed individually with free access to water and food in a temperature (22 ± 2°C) and light control (12 h light/dark cycle) quiet room. All experimental procedures were approved by the local animal care committee and were carried out strictly in compliance with the NIH guidelines for the care and use of laboratory animals.

Intrathecal Catheter Implantation

For i.t. delivery of drugs, rats were implanted with i.t. catheters according to the methods described previously [31]. Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally), and then, a sterile polyethylene (PE-10) tube filled with 0.9% saline (NS) was inserted through L5/L6 intervertebral space, and the tip of the tube was placed at the spinal lumbar enlargement level.

Induction of Morphine Antinociceptive Tolerance and Behavioral Test

Morphine antinociceptive tolerance was induced by i.t. injection of morphine (15 μg daily, Qing-hai Pharmaceutical Factory, Xining, China) for 7 days. To investigate the effect of Gap26 or MK-801 on morphine tolerance, Gap26 (15 μg, Anaspec, Fremont, CA, USA), MK-801 (3.3 μg, Sigma, St. Louis, MO, USA), or vehicle (NS) was given intrathecally 30 min before each morphine administration. The drug or vehicle solution was i.t. injected in a volume of 10 μL followed by additional 10 μL of NS to flush the cather.

On odd days, morphine antinociception was evaluated by hot-water tail-flick test according to the methods described previously [31]. Briefly, rats were restricted in a plastic container (22 × 6 cm), and distal one-third of the tail was immersed into the water maintained at 50 ± 0.2°C. The latency response was defined by rapid removal of tail from hot water. A cutoff time of 15 second was used to avoid tissue damage. The percentage of maximal possible antinociceptive effect (%MPE) was calculated by comparing the tail-flick latency before (baseline latency, BL) and after drug injection (test latency, TL) using the equation: % MPE = [(TL − BL)/(cut-off time − BL)] × 100.

Immunohistochemistry

Immediately after behavioral test on the indicated days, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused through the ascending aorta with 4% paraformaldehyde. The spinal cord lumbar enlargements were quickly removed and postfixed in the same fixative for 2 h and then transferred into 30% sucrose until sectioning. Transverse spinal sections (20 μm) were cut on a cryostat. The procedure for immunohistochemistry was performed as described previously [31]. In brief, the sections were incubated with the antibody for Cx43 (1:1000; Santa Cruz, Heidelberg, Germany), or GFAP (astrocyte marker, 1:800; Millipore, Billerica, MA, USA) overnight at 4°C. Then, the sections were incubated with corresponding Cy3- or FITC-conjugated secondary antibody (1:800, ZSGB-BIO, Beijing, China) for 2 h at room temperature (RT). For double immunofluorescence, sections were incubated with a mixture antibodies for anti-Cx43 and GFAP, or NeuN (neuronal marker, 1:400, Millipore) or OX42 (microglial marker, 1:100, Millipore) followed by a mixture of Cy3- and FITC-conjugated secondary antibodies. The stained sections were examined with an Olympus fluorescence microscope, and images were captured with a CCD spot camera. Nonspecific staining was determined by omitting the primary antibodies.

Western Blot Assay

On odd days, immediately after behavioral test, rats were killed and the spinal cord lumbar enlargements were rapidly removed. The preparation of proteins and Western blot were performed as described previously [25]. In brief, tissues were homogenized in ice-cold lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease and phosphatase inhibitors (Sigma, USA). Protein samples were separated on SDS–PAGE gradient gel (8–12%) and electrotransfered onto PVDF membranes. The membranes were probed with the antibody for Cx43 (1:5000), GFAP (1:10000), NR1 (1:800, Cell Signaling Technology), NR2B (1:2000, Abcam, Cambridge, UK), p-NR1 (1:1000, Millipore), p-NR2B (1:1000, Millipore), or GLT-1 (1:1000, Cell Signaling Technology, Danvers, MA, USA), and with GAPDH antibody as loading controls. Then, the membranes were incubated with HRP-conjugated secondary antibody, developed in ECL solution and exposed onto X-ray film. For quantification, the films were scanned, and the density of specific bands was measured and normalized with the control band (NS group).

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Statistical Analysis

All data were presented as mean ± SEM. Data were analyzed using repeated measures ANOVA (for the behavioral data) or one-way ANOVA (for Western blot and immunohistochemical data). Whenever applicable, *post hoc* analysis (LSD) was performed for comparisons between specific groups. The significance level was defined as $P < 0.05$.

Results

Chronic Morphine Treatment Up-regulates the Expression of Spinal Astrocytic Cx43

The results from Western blot assay showed that in morphine-treated rats, i.t. administration of morphine for seven consecutive days induced a significant increase in the expression of spinal Cx43 compared with control rats (Figure 1A). The expression of spinal Cx43 began to increase significantly following 3 days of repeated morphine treatment ($P < 0.001$) and gradually enhanced from 5 to 7 days.

Similarly, immunohistochemistry results showed that the Cx43-immunoreactivity (IR) was up-regulated markedly after chronic morphine treatment compared with the NS group (Figure 1B). Moreover, double immunofluorescent staining showed that the spinal Cx43 was exclusively colocalized with astrocytes (GFAP), but not with neurons (NeuN) or microglial cells (Ox-42) (Figure 1C), which was consistent with the previous reports [32].

Gap26 Attenuates the Development of Morphine Antinociceptive Tolerance and Chronic Morphine-Induced Increase in Spinal Cx43 Expression

To explore whether the inhibition of Cx43 would prevent the development of morphine antinociceptive tolerance, Gap26

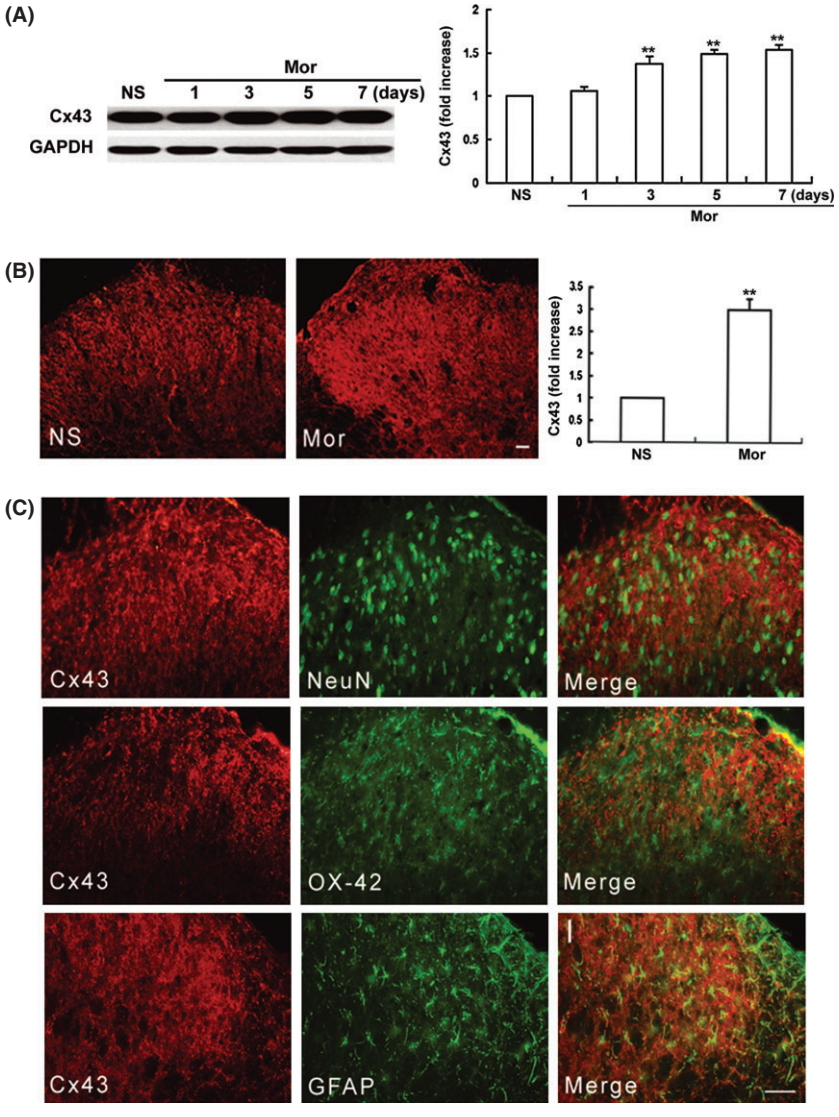

Figure 1 Chronic morphine treatment up-regulates the expression and immunoreactivity of the spinal connexin 43 (Cx43). (**A**) Western blot assay showed that the spinal Cx43 expression was increased progressively after exposure to morphine (15 μg, intrathecal [i.t.]). n = 5. (**B**) Intrathecal injection of morphine for 7 days markedly increased the spinal Cx43 immunoreactivity (IR), n = 6. (**C**) Double immunostaining indicates that the spinal Cx43 was colocalized with GFAP (astrocytes marker), but not with NeuN (neuron marker) or OX-42 (microglia marker). Scale bar: 50 μm.

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(15 μg, i.t.), a mimetic peptide of Cx43, served as a relatively specific blocker of Cx43 [33–35], was administered 30 min before each morphine treatment for consecutive 7 days. As shown in Figure 2A, morphine injection produced robust analgesia to noxious stimuli on day 1. Repeated morphine treatment produced a progressive decline of antinociception, indicating the development of tolerance to morphine analgesia. Gap26 alone did not affect the basal pain threshold. However, coadministration of Gap26 with morphine considerably delayed the decline of morphine antinociception. On day 7, the %MPE in Gap-Mor group (45.83 ± 4.17) was markedly higher than the one in NS-Mor group (23.88 ± 2.81) ($P < 0.001$). Moreover, the Western blot data showed that coadministration of Gap26 with morphine inhibited the chronic morphine-induced increase in the spinal Cx43 expression (Figure 2B). These data suggested that Cx43 is involved in the development of morphine antinociceptive tolerance.

Gap26 Ameliorates the Established Morphine Antinociceptive Tolerance

To investigate whether Gap26 would reverse the established morphine antinociceptive tolerance, Gap26 (15 μg, i.t.) was administrated 30 min before morphine injection on day 8 and continued for the next 8 consecutive days. As shown in Figure 2C, on day 9, 11, 13, and 15, the analgesic effect of i.t. morphine in NS-Mor group were further declined, having no statistical difference as compared with NS-NS group ($P > 0.05$). However, morphine produced significant analgesic efficacy in Gap26-Mor group ($P < 0.001$ vs. NS-Mor group) on day 9, 11, 13, and 15. These results indicated that Gap26 could partly ameliorate the established morphine antinociceptive tolerance.

Gap26 Suppresses Chronic Morphine-Induced Activation of Astrocytes

As the spinal astrocytes have been found to be activated by chronic morphine treatment and Cx43 is mainly expressed in astrocytes, we examined the effect of Gap26 on morphine-induced astrocyte activation. Immunoblotting assay (Figure 3A) indicated that the spinal GFAP expression was obviously increased after 7 days of morphine exposure ($P < 0.05$), indicating that astrocytes are activated by chronic morphine treatment. The increased expression of GFAP was blocked by coadministration of Gap26 with morphine ($P < 0.001$). Similarly, the immunofluorescence staining showed that Gap26 also considerably attenuated chronic morphine-induced increase in the spinal GFAP-IR level (Figure 3B).

Gap26 Inhibits Chronic Morphine-Induced Down-Regulation of Spinal GLT-1

Figure 4 shows an obvious decrease in the spinal GLT-1 expression after 7 days of continuous morphine infusion ($P < 0.001$), indicating that GLT-1 is down-regulated by chronic morphine treatment. However, this inhibitory effect was distinctly attenuated by coadministration of Gap26 with morphine ($P < 0.001$). Gap26 alone did not alter the basal expression of spinal GLT-1.

Gap26 Prevents Chronic Morphine-Induced Phosphorylation of NR1 and NR2B Subunits of NMDA Receptors

As it has reported that the GJ blockage prevents neuropathic pain by preventing the activation of NMDA receptors [13,36], the effects of Gap26 on chronic morphine-induced alterations in

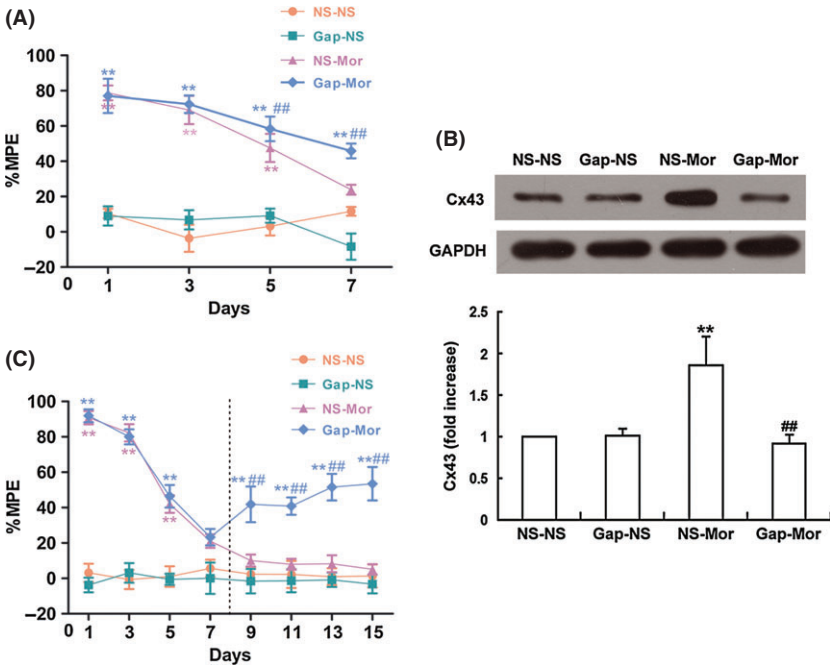

Figure 2 Effects of Gap26 on the development of morphine antinociceptive tolerance (**A**), chronic morphine-induced increase in the spinal connexin 43 (Cx43) expression (**B**), and the established morphine antinociceptive tolerance (**C**). Gap26 (15 μg), a specific Cx43 mimic peptide, or vehicle (10 μL) was intrathecally injected 30 min before daily morphine (15 μg) treatment. The vertical dashed line in (**C**) indicated that Gap26 administration was initiated on day 8 following the development of morphine tolerance and continued for the next 8 days. MPE%, percentage of maximal possible antinociceptive effect. $^{**}P < 0.001$ versus NS-NS group; $^{\#\#}P < 0.001$ versus NS-Mor group. n = 6.

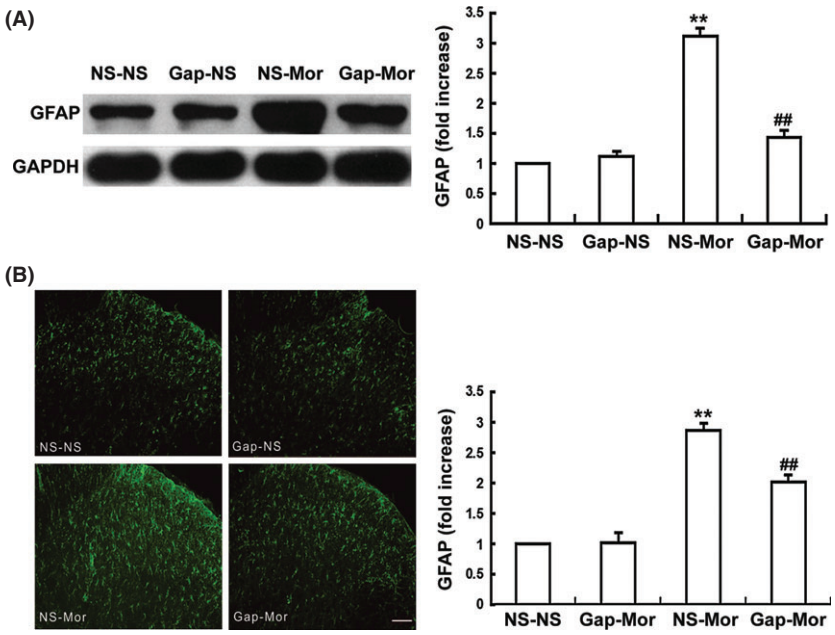

Figure 3 Gap26 suppresses chronic morphine-induced activation of the spinal astrocytes. (**A**) Western blot showed that coadministration of Gap26 (15 μg, intrathecal [i.t.]) with morphine significantly prevented the chronic morphine-induced spinal GFAP up-regulation. (**B**) Immunofluroescence assay showed that chronic morphine induced an increase in spinal GFAP-IR. Gap26 attenuated the chronic morphine-induced increase in spinal GFAP-IR. Scale bar: 50 μm. Data are the mean ± SEM (n = 6) $^{**}P < 0.001$ versus NS-NS group. $^{\#\#}P < 0.001$ versus NS-Mor group.

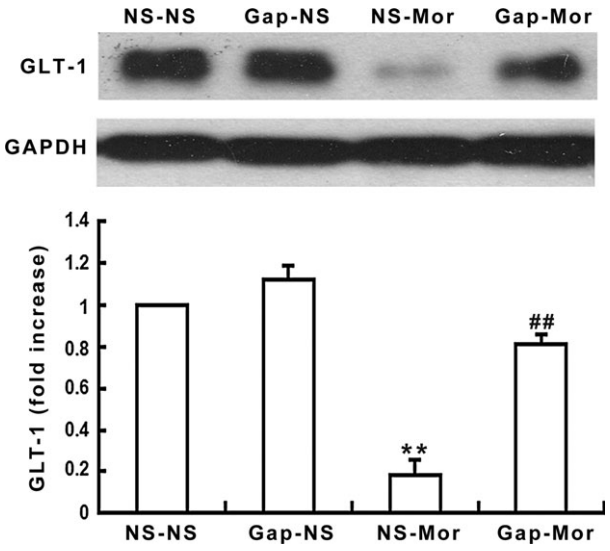

Figure 4 Gap26 inhibits chronic morphine-induced down-regulation of spinal GLT-1 expression. The spinal GLT-1 expression was decreased after chronic morphine administration. Gap26 significantly suppressed the decreased GLT-1 expression. Data are presented as mean ± SEM (n = 4). $^{**}P < 0.001$ versus NS-NS group. $^{\#\#}P < 0.001$ versus NS-Mor group.

NR1 and NR2B expressions were therefore examined. As shown in Figure 5, chronic morphine not only up-regulated the expressions of NR1 and NR2B (Figure 5A and B), but also increased the phosphorylations of NR1 and NR2B (Figure 5C and D) ($P < 0.001$) in the spinal cord compared with the NS group ($P < 0.001$). These increases in the expression levels and the phosphorylations of the two subunits were inhibited by Gap26 coadministration ($P < 0.001$). Gap26 or NS alone did not induce changes in the basal expression levels and phosphorylations of NR1 and NR2B.

MK-801 Attenuates Chronic Morphine-induced Up-regulation of the Spinal Cx43 and GFAP Expression, and Down-regulation of the GLT-1 Expression

Interestingly, we also observed that MK-801, a noncompetitive antagonist of NMDA receptors, not only prevented the development of morphine antinociceptive tolerance (Figure 6A), but also significantly attenuated chronic morphine-induced increases in the spinal Cx43 (Figure 6B) ($P < 0.001$) and GFAP (Figure 6C) ($P < 0.05$) expressions and the decrease in the spinal GLT-1 expression (Figure 6D) ($P < 0.001$) when coadministrated with morphine. MK-801 alone did not affect the basic levels of their expressions.

Discussions

Glial cells are now recognized as important contributors in the development of morphine antinociceptive tolerance. In the present study, we provide a novel mechanism that the spinal astrocytic Cx43 mediates morphine antinociceptive tolerance by activating astrocytes and NMDA receptors (including NR1 and NR2B subunits), and inhibiting GLT-1 expression.

In the CNS, astrocytes are the most abundant cells which communicate with each other mainly through GJs [37]. Cx43 is the major component of astrocytic GJs [7]. Accumulating evidence indicates that Cx43 and GJs are involved in neuropathic pain. Wu et al. [38] reported that the expression levels of GFAP (a marker of astroglia) and Cx43 in the spinal cord are significantly increased after CCI of the sciatic nerve in rats. Similarly, Cx43 expression is up-regulated in the trigeminal ganglion in rats with a CCI of the

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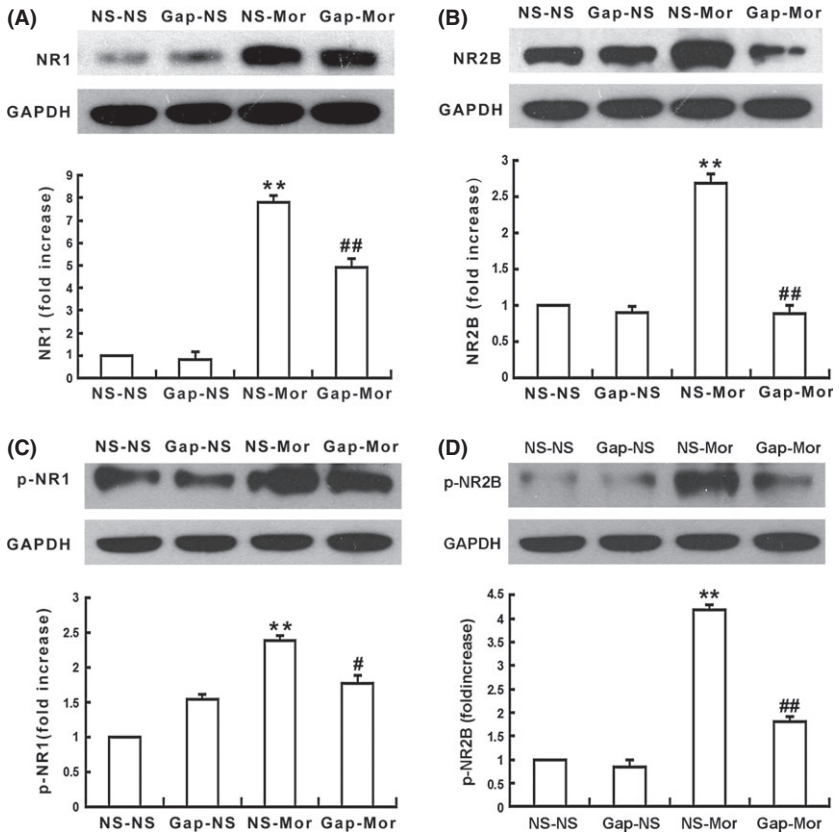

Figure 5 Gap26 prevents chronic morphine-induced increases in the expressions and phosphorylations of spinal N-methyl-D-aspartic acid (NMDA) receptor NR1 and NR2B subunits. Chronic morphine treatment up-regulated the expressions of spinal NR1 (**A**) and NR2B (**B**), and increased phosphorylate (p) NR1 (p-NR1) (**C**) and NR2B (p-NR2B) (**D**); Gap26 significantly prevented the chronic morphine-induced up-regulation of NR1, p-NR1, NR2B, and p-NR2B expressions, but Gap26 alone did not affect the basal levels of them. Data are presented as mean ± SEM (n = 4). $^{**}P < 0.001$ versus NS-NS group. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ versus NS-Mor group.

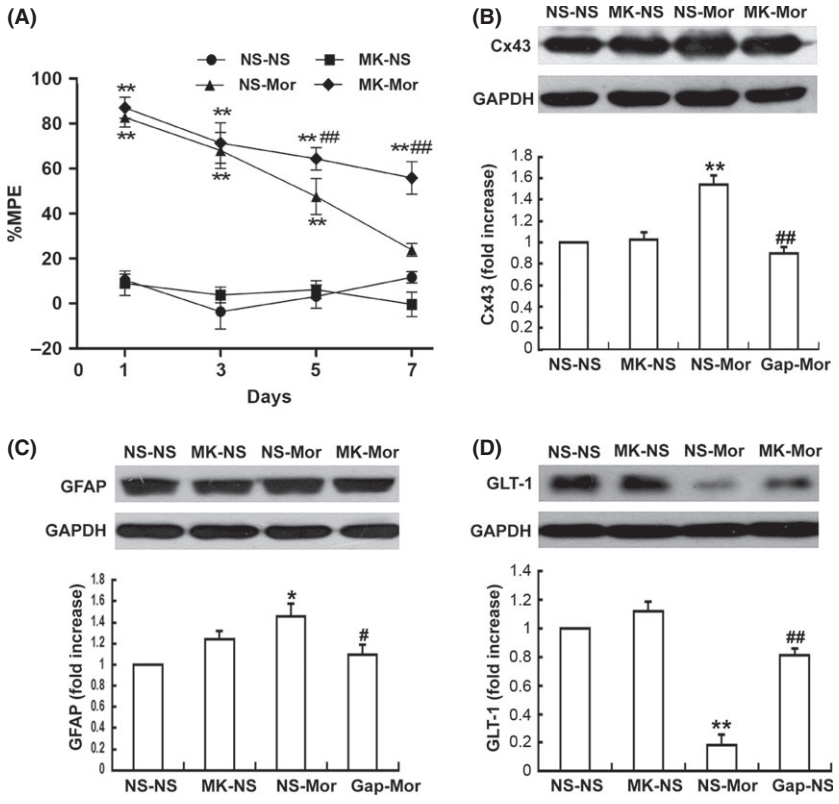

Figure 6 Effects of MK-801 on morphine antinociceptive tolerance and chronic morphine-induced changes of the spinal Cx43, GFAP and GLT-1. (**A**) MK-801 (3.3 μg, intrathecal [i.t.]) was injected 30 min before daily morphine (15 μg, i.t.) treatment. MK-801 distinctly reduced the chronic morphine-induced increase in levels of the spinal Cx43 (**B**) and GFAP expression (**C**), and the decrease in GLT-1 expression (**D**). Data are presented as mean ± SEM (n = 4). $^{*}P < 0.05$, $^{**}P < 0.001$ versus NS-NS group. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ versus NS-Mor group.

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infraorbital nerve and genetic inactivation of Cx43 by RNAi attenuates the pain-like behavior in this model [14,15]. Intrathecal or intracisternal treatment with carbenoxolone, a GJ decoupler, markedly suppresses subcutaneous formalin-induced thermal hyperalgesia [39] and thrombotic ischemia-induced mechanical allodynia in rats [40]. Collectively, the above results suggest that Cx43 and GJs contribute to the development of neuropathic pain. However, whether the spinal Cx43 participates in the development of morphine antinociceptive tolerance is unknown.

In the present study, we provided the first evidence that the spinal astrocytic Cx43 contributes to morphine antinociceptive tolerance in rats. This is supported by the following results: (1) the spinal Cx43 expression and Cx43-IR are significantly enhanced by chronic morphine treatment; (2) the spinal Cx43 is exclusively colocalized with astrocytes (GFAP), which is consistent with the previous studies [7]; and (3) i.t. treatment with Gap26, a specific Cx43 mimic peptide, markedly attenuates the development of morphine antinociceptive tolerance, and partly attenuates the established morphine antinociceptive tolerance. As it was shown that the spinal astrocytic Cx43 is involved in the development of neuropathic pain [12–15,38–40], this study provides another novel evidence to confirm the well-known hypothesis that opioid tolerance and phathological pain may share common cellular mechanisms [17].

As the spinal astrocytes have been shown to interconnect by Cx43 and to be colocalized with Cx43, we explore the effect of chronic morphine-induced increase in Cx43 on the spinal astrocytic activation. Consistent with the previous studies [1–3], we found that chronic morphine induced astrocyte activation, as evidenced by increased GFAP expression and astrocyte hypertrophy in the spinal dorsal horn. Noteworthily, we found that Gap26 also markedly reduced chronic morphine-induced increases in GFAP expression and GFAP-IR level in the spinal cord, suggesting the stimulatory effect of Cx43 on the spinal astrocyte activation. Astrocytes, the principal glial cell type, have been shown to participate in the development of morphine tolerance [1–3,41–43]. However, the biological molecules by which chronic morphine induces the activation of astrocytes are still incompletely clear. Based on our above results, it was suggested that the spinal astrocytic Cx43 may be an inducer for the activation of astrocytes in the chronic morphine-treated rats. Notably, increasing studies confirmed that the activated glia can release some proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF)-α, which participate in the development of morphine tolerance [2,3,44–46]. Therefore, we speculated that chronic morphine-induced up-regulation of the spinal Cx43 expression activates astrocytes, which in turn trigger release of these mediators, leading to the tolerance to morphine analgesia. To confirm this hypothesis, further studies are needed.

Abundant evidence suggests that glutamate and NMDA receptors participate in the development of morphine tolerance. The level of extracellular glutamate is actively and tightly regulated by GTs [26]. GLT-1 mediates 90% extracellular glutamate uptake in the CNS. The spinal GTs are down-regulated in chronic morphine-treated rats [27–29]. Pharmacological inhibition of GT by trans-pyrrolidine-2-4-dicarboxylate potentials, whereas activation of GT by riluzole reduces the development of morphine antinociceptive tolerance [27]. In addition, ceftriaxone inhibits morphine antinociceptive tolerance through activating GLT-1, increasing glutamate uptake, and attenuating extracellular glutamate levels [47]. GLT-1 is mainly expressed in astrocytes, which is in accordance with Cx43 [7,48]. Noteworthily, a recent study demonstrated that GLT-1 expression in cerebral cortex is increased in the conditional Cx43 knockout mice, suggesting that Cx43 may have a regulatory effect on GLT-1 [30]. However, it was unclear the relationship between spinal Cx43 and GLT-1 in the chronic morphine-treated rats. Therefore, the present study explored the effect of spinal Cx43 activation on GLT-1 in morphine tolerant rats. In agreement with the previous studies [27–29,47], our data showed that chronic morphine treatment induced down-regulation of the spinal GLT-1 expression. Importantly, blocking Cx43 by Gap26 markedly depressed the chronic morphine-induced decrease in the spinal GLT-1 expression, suggesting that the spinal Cx43 may regulate GLT-1 expression, and the decreased GLT-1 may be implicated in the spinal Cx43-mediated morphine antinociceptive tolerance. Conceivably, the chronic morphine-induced down-regulation of GLT-1 expression would enhance the availability of extracellular glutamate and then would increase the probability of excitatory amino acid receptors, such as NMDA receptors. The activation of NMDA receptors and downstream signal transduction pathway plays a critical role in tolerance to morphine analgesia [20,49–51].

Due to the above-mentioned findings that the spinal astrocytic Cx43 regulates GLT-1 expression which regulates the functional activity of NMDA receptors [36], we further investigated the effect of spinal Cx43 on the activation of NMDA receptor subunits (NR1 and NR2 subunits) and the possible interaction between them in the chronic morphine-treated rats. Consistent with the previous studies [20–25], this study showed that both NMDA receptor NR1 and NR2B subunits participated in morphine antinociceptive tolerance. Importantly, we found that blocking the spinal Cx43 with Gap26 not only attenuated morphine antinociceptive tolerance, but also inhibited chronic morphine-induced increases in expressions and phosphorylations of NR1 and NR2B subunits, indicating that the spinal Cx43 may contribute to the increased activation of NMDA receptors by chronic morphine. Moreover, MK-801 coadministration with morphine attenuated the chronic morphine-induced increase in spinal Cx43 expression, suggesting the involvement of NMDA receptor activation in the increased expression of the spinal Cx43 by chronic morphine.

As the spinal Cx43 is mainly colocalized with astrocytes and NMDA receptors are mainly expressed on the membrane of postsynaptic neurons, we proposed that the spinal Cx43-mediated astrocyte–neuron signaling may represent a novel mechanism underlying the development of morphine antinociceptive tolerance in rats. We speculated that there might be a feedback mechanism to modulate the astrocyte–neuron signaling communication. That is, chronic morphine-induced increase in Cx43 activates astrocytes and reduces extracellular glutamate uptake by down-regulating the GLT-1 expression, and accelerates extracellular glutamate accumulation, resulting in activation of NMDA receptors (including NR1 and NR2B subunits); and the activated NMDA receptors enhance the Cx43 expression, reduce the GLT-1 expression, and activate astrocytes. This interaction between the spinal astrocytic Cx43 and neuronal NMDA receptors might form a posi-

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tive feedback and aggravate the tolerance to morphine analgesia in chronic morphine-treated rats. However, the mechanisms of the neuron–astrocyte interaction in the development of morphine antinociceptive tolerance are complicated and require further studies.

In conclusion, the present study demonstrated for the first time that the spinal astrocytic Cx43 contributes to the development of morphine antinociceptive tolerance by activating astrocytes and NMDA receptors (NR1 and NR2B subunits) and inhibiting GLT-1 expression in the spinal cord of rats. Although the role of the spinal Cx43 in the tolerance to morphine analgesia may involve multiple pathways, the spinal Cx43-NMDA receptors pathway may be an important one. The interaction between the spinal astrocytic Cx43 and neuronal NMDA receptors may reflect the functional significance of astrocyte–neuron signaling in the development of morphine antinociceptive tolerance in rats.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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