Behavioral and Morphological Evidence for the Involvement of Glial Cells in the Antinociceptive Effect of Najanalgesin in a Rat Neuropathic Pain Model

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Neuropathic pain is a devastating neurological disease that seriously affects patients’ quality of life. Despite a high level of incidence, the underlying mechanisms of neuropathic pain are still poorly understood. However, recent evidence supports the prominent role of spinal glial cells in neuropathic pain states. In our laboratory, we observed that najanalgesin, a novel peptide isolated from the venom of Naja naja atra, exerts significant analgesic effects on acute pain in mice and neuropathic pain in rats. The objective of the present study was to determine whether spinal glia are associated with the antinociceptive effect of najanalgesin in an L5 spinal nerve ligation (SNL) rodent model of neuropathic pain. Mechanical allodynia developed after surgery, and hypersensitivity was significantly attenuated by the intrathecal administration of najanalgesin. The inhibitory effect of najanalgesin was significantly (p<0.05) enhanced after pretreatment with fluorocitrate (a glial cell antagonist). In addition, the astrocyte activation was attenuated following najanalgesin treatment in the dorsal horn of neuropathic rats, as assessed by immunohistology and Western blotting. The tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) content of cerebral spinal fluid and cell culture supernatants changed significantly after najanalgesin administration. The results suggest that najanalgesin may exert its anti-allodynic effect by altering astrocyte cell function.

Key words snake venom; neuropathic pain; astrocyte cell; cytokine

Neuropathic pain is one of the most significant health problems in the world. Although numerous treatments such as tricyclic antidepressants, antiepileptics, and opioids have been applied in the clinic, these therapeutic approaches are not completely effective for the relief of neuropathic pain, which impairs the patients’ quality of life. Therefore, the discovery of novel therapeutic alternatives or adjuncts would be beneficial.

Traditionally, neuropathic pain is viewed as being mediated solely by neurons. However, accumulating evidence suggests that glial cells in the spinal cord contribute to the development and maintenance of neuropathic pain. Immunohistochemical studies have shown that spinal glia are activated in various animal models of pathological pain, including subcutaneous formalin injection, spinal nerve injury, and peripheral nerve injury. The activation of spinal glia is causally related to pathological pain states because the pharmacological inhibition of glial activation prevents the development of pain. Activated astrocytes are a source of numerous biological mediators, including proinflammatory cytokines (e.g., tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β)), prostaglandin (PGE2) and nerve growth factor (NGF), which may contribute to the initiation and maintenance of pain hypersensitivity after nerve injury.

Pharmacological studies have demonstrated that protein and peptides isolated from snake venom have strong analgesic effects. In our laboratory, we investigated that najanalgesin, a single polypeptide isolated from cobra venom, has an analgesic effect on many different types of pain, including those produced by heat and chemical stimulants, and by L5 spinal nerve ligation (SNL). The purpose of the present study was to determine whether najanalgesin decreases allodynia in a rat model of neuropathic pain and whether the putative effects of najanalgesin are associated with the glial activation state and the expression of proinflammatory cytokines within the spinal cord.

MATERIALS AND METHODS

Drug Administration Fluorocitrate (FC) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). All drugs except fluorocitrate were dissolved in physiological saline and given in a total volume of 10 µL for intrathecal injection (i.t.). FC (4 mg) was dissolved in 0.5 mL 2 M hydrochloric acid by adding 1–2 drops of sodium sulfate followed by 1 mL phosphate buffer (PB). The resulting solution was centrifuged at 12000 rpm for 5s. The supernatant was removed, and saline was added to obtain a volume of 4.8 mL (1 mmol/µL). The doses for intrathecal najanalgesin were selected according to the results of a previous study and our pilot study.

Animals All of the experiments were performed using male Sprague-Dawley rats (200–220 g) purchased from the laboratory animal center of Sun Yat-sen University. All of the procedures were performed in accordance with the National Institute of Health guidelines on animal care. The rats were housed in separate cages with free access to food and water and were maintained on a 12/12 h light/dark cycle. All of the behavioral experiments were performed between 11:00 and 17:00, and the animals were acclimated to the facilities for one week to reduce stress-related analgesia. All efforts were made to minimize the number of animals used in the experiments and their suffering.

Surgery All of the experimental procedures were performed on rats that were deeply anesthetized with sodium
pentobarbital (50 mg/kg, intraperitoneally (i.p.)). For intrathecal injection, rats were implanted with catheters according to the method described by Watanabe et al. In brief, a sterile polyethylene catheter (PE-10, Portex) was inserted through the gap between the L4/L5 vertebrae and was extended to the rat’s subarachnoid space of lumbar enlargement. Nerve injury was induced in accordance with the procedure of Kim and Chung. The L5 spinal nerve was tightly ligated with a silk suture. In sham-operated rats, the nerve was exposed without ligation.

**Behavioral Testing** Mechanical sensitivity was assessed using the up-down method described in a previous study, and a set of von Frey hairs (Ugo Basile, Italy) was used to apply logarithmically increasing stiffnesses ranging from 3.61 (0.41 g) to 5.18 (15.14 g). Briefly, rats were placed inside acrylic cages on a wire mesh grid floor. The probe was applied to the middle of the left hind paw to determine the stimulus intensity threshold stiffness. Quick withdrawal in response to the stimulus was considered to be a positive response.

**Immunohistochemistry** Immunohistochemistry was performed on 25-µm, free-floating L5 spinal cord sections. An antibody to glial fibrillary acidic protein (GFAP) (1:1000, Cell Signaling Technology, CA, U.S.A.) was used to label the astrocytes. Spinal cord sections were incubated overnight at 4°C with primary antibody and were subsequently incubated with a fluorescein isothiocyanate-conjugated secondary antibody (1:6000, Boshide, China). To assess non-specific staining, control sections were incubated in the absence of primary antibodies. The quantification of immunofluorescent GFAP staining (GFAP-IR) in spinal cord sections was measured in the dorsal horn using the computerized image analysis system Image-Pro Plus. The mean value of GFAP-IR was obtained within spinal dorsal horn laminae I–III, and six randomly selected sections from each animal were assessed.

**Western Blotting** Animals were rapidly killed, and the lumbar sacral spinal cord was mid-sagittally subdivided into two halves to perform two separate protein analyses, and the samples were flash-frozen in liquid nitrogen. Equal amounts of the samples were loaded in each lane, separated using 12% dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with GFAP antibodies (1:1000; Cell Signaling Technology) and the monoclonal primary antibody for β-actin (1:500; Boshide) in 5% bovine serum albumin in TBST.

**Enzyme-Linked Immunosorbent Assay (ELISA)** The cerebral spinal fluid (CSF) was collected by aspiration and was flash-frozen in liquid nitrogen. The supernatants from purified astrocyte cell cultures were collected. ELISA kits for TNF-α and IL-1β were used according to the manufacturer’s instructions to detect the levels of these proteins in the supernatant and CSF, as described previously.

**Primary Astrocyte Culture** To eliminate in vitro neurons, oligodendrocytes and fibroblasts, the culture flasks were shaken on a rotating shaker (200 rpm, 24 h) on day 9. Further subculture enrichment was achieved by eliminating contaminating microglial cells using a replanting procedure and by seeding the cells at a low cell density (5000 cells/cm²), which further reduced the number of contaminating microglial cells. Anti-GFAP immunofluorescence staining was used to identify the purity of rat spinal astrocytes. Astrocytes (1×10⁶ cells/well) were incubated in a 96-well plate on the third passage to study the effect of different concentrations of najanalgesin on the proliferation of spinal astrocytes at 24 h and 48 h and determine the concentrations required for drug induction experiments.

**Statistical Analysis** All data are expressed as the mean ± S.E.M. The behavioral test data were analyzed using a Friedman analysis of variance (ANOVA) for repeated measures, and Wilcoxon tests of matched pairs were conducted when appropriate. One-way ANOVAs followed by Tukey’s tests were used for immunohistochemistry, Western blotting, and ELISA data. A probability of 0.05 was accepted as significant.

**RESULTS AND DISCUSSION**

**Effects of Fluorocitrate (FC) or Concomitant FC and Najanalgesin Treatment on SNL-Induced Behavioral Hypersensitivity** Thirty minutes to 12 h after the administration of 1 nmol FC, the paw withdrawal threshold (PWT) significantly increased (Fig. 1). A low dose of FC (0.1 nmol) or saline did not alter the allodynia of either hind paw. In Fig. 2, when a combination of FC and najanalgesin was given, rats received FC (i.t.) 2.5 h prior to najanalgesin treatment. Experimental groups were compared 30 min to 24 h after administration of najanalgesin. However, by using receiver operating characteristic (ROC) curve analysis, the area under the curve (AUC) 0.844, 95% confidence interval 0.640–1.048 with a combination of 0.1 nmol FC + 40 ng/kg najanalgesin.
was significantly greater than the effect of 40 ng/kg najanalgesin alone ($p<0.05$). And the area under the curve (AUC) 0.875, 95% confidence interval 0.646–1.104 with a combination of 0.1 nmol FC + 40 ng/kg najanalgesin, was significantly greater than the effect of 0.1 nmol FC alone ($p<0.05$).

**Najanalgesin Attenuates the Expression of GFAP in the Spinal Dorsal Horn** As previously observed, low basal constitutive expression of GFAP was observed in naïve spinal cords. A robust increase in GFAP-IR cells was observed in L5 spinal nerve ligation rats. However, the intrathecal administration of najanalgesin effectively attenuated nerve injury-induced hypertrophy and inhibited an increase in GFAP expression (Fig. 3).

Changes in GFAP expression were confirmed by Western blot analysis (Fig. 4). Consistent with the results obtained by immunohistochemistry, nerve injury induced a significant increase in GFAP levels, but najanalgesin significantly inhibited this effect in the ipsilateral spinal cord at 3 h, 6 h, and 9 h, and these results correlated to the observed changes in immune responses.

**Astroglial Cell Cultures** The survival rate of rat spinal primary astrocytes was 75–85%. A density of 0.3–0.7×10^6 cells per rat spinal tissue was obtained, and the viability and attachment efficiency was greater than 75%. The purity of rat primary astrocytes was greater than 98%. Najanalgesin

![Image](https://via.placeholder.com/150)

**Fig. 3. Changes in the Expression of GFAP in the Ipsilateral Spinal Dorsal Horn Detected by Immunohistochemistry after SNL-Induced Neuropathic Pain and Najanalgesin Treatment**

Images of GFAP immunostaining in the ipsilateral spinal dorsal horn of the control group, SNL group, najanalgesin group and FC group are presented (a–l). An image with a lower magnification showing the area of the dorsal horn was also analyzed (total). A small number of GFAP cells were observed in the spinal cord of control rats (a, d, and g). Prominent astrocytic activation was observed in rats treated with intrathecal saline after nerve injury (b, e, and h). These responses were remarkably inhibited in rats treated with intrathecal najanalgesin at 3 h, 6 h and 9 h (c, f, and i) or intrathecal FC (j, k, and l) (a–l: 20× magnification). The results obtained at all of the time points were quantified and are shown in the figure. Data are represented as the mean±S.E.M. ($n=6$), **$p<0.01$ vs. the control group; $^*p<0.05$, $^{**}p<0.01$ vs. the SNL group).

![Image](https://via.placeholder.com/150)

**Fig. 4. Changes in GFAP Protein Levels in the Ipsilateral Dorsal Horn of the Spinal Cord, as Detected by Western Blot**

(A) Protein bands corresponding to GFAP in the spinal dorsal horn of the control group, SNL group and najanalgesin group at the indicated time points are shown. (B) Quantification of the immunoblot results at different time points. The protein level was expressed as the ratio to β-actin. (C) The image shows protein bands corresponding to GFAP after intrathecal FC treatment. Data are represented as the mean±S.E.M. ($n=4$), **$p<0.01$ vs. control group; $^*p<0.05$, $^{**}p<0.01$ vs. SNL group).
inhibited the proliferation of astrocytes, and the optimal najanalgesin concentration was 2 μg/mL (data not shown).

**Najanalgesin Inhibits Proinflammatory Cytokine Protein Expression**  Eight days after SNL, an induction in the release of IL-1β and TNF-α into the CSF was detected. However, intrathecal najanalgesin significantly inhibited the release of IL-1β and the level of TNF-α at 6h, 12h, and 24h (Table 1). After lipopolysaccharide (LPS) treatment, the protein level of IL-1β and TNF-α was upregulated in control astroglial monocultures. This increase was significantly inhibited by treatment with najanalgesin in astrocytic cultures (Table 2).

**DISCUSSION**

In the present study, we found that coapplication of najanalgesin with intrathecal injection of a glial metabolic inhibitor fluorocitrate synergistically antagonized rat neuropathic pain. FC, a non-specific glial metabolic inhibitor, inhibits glial cells without directly affecting neurons.23) The preceding results showed that PWT was not influenced by i.t. injection of 0.1 nmol FC in neuropathic pain rats. When a combination of 0.1 nmol FC with najanalgesin was given, PWT had a robust increase, compared with the effects obtained in either the najanalgesin or 0.1 nmol FC alone group, suggesting a significant inter-reinforcing of najanalgesin and disrupting spinal glial function.

During the last decade, growing evidence has supported the prominent role of glial cells in the mechanism of neuropathic pain. Peripheral nerve injury leads to the activation of glial cell in the spinal cord.22) Intrathecal injection of general inhibitors of glial function attenuate neuropathy- or inflammatory-induced hyperalgesia and allodynia.23) Activated astrocytes release proinflammatory cytokines such as TNF-α, IL-1β and IL-6 and other substances that enhance neuronal central sensitization and nerve injury-induced persistent pain.24)

Along this line, we considered whether najanalgesin might modulate spinal glial function to produce a higher efficiency under pathological pain conditions. Therefore, we performed immunohistochemical and westernblotting studies to demonstrate that the anti-allodynic effect of najanalgesin may be linked to a reduction in spinal astrocyte activation. In agreement with previous studies, the present results showed significant astrocyte activation in the spinal cord horn due to nerve injury. However, najanalgesin suppresses SNL-induced activation of spinal astrocytes at 3h, 6h and 9h.

As mentioned above, the release of proinflammatory cytokines and other algesic substances from activated astrocytes in the spinal cord with peripheral nerve injury. In our study, najanalgesin inhibited the release of the proinflammatory cytokines TNF-α and IL-1β at 6h and 12h, indicating that the antinociceptive effect of najanalgesin can be attributed to its ability to suppress the central proinflammatory immune response. In addition, the level of cytokines was significantly inhibited by najanalgesin treatment in astrocytic cultures. Several lines of evidence indicate that crototoxin (CTX) displays immunomodulatory and anti-inflammatory activities that affect the cellular and vascular component of inflammatory and immune responses,25,26) by interfering with the activity of leukocytes.27) This toxin also inhibits cytokine release (e.g., IL-2, IL-4 and IL-10) in human serum albumin-immunized mice.

According to these results, we include that najanalgesin suppressed the increases in the immunoreactivities of activated astrocytes. In addition, najanalgesin attenuated the production of proinflammatory cytokines (including TNF-α, IL-1β). However, there exist the inconsistencies in time. The peak time of najanalgesin in analgesic action did not correlate with those of GFAP expression and cytokines. These data suggested that main mechanism of analgesic action of najanalgesin is different from that of FC. Therefore, the antinociceptive effect of najanalgesin is related to the state of spinal astrocyte

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**Table 1. Effects of Intrathecal Injection of Najanalgesin on Protein of Proinflammatory Cytokines IL-1β and TNF-α in the CSF of Neuropathic Rats (pg/mL)**

<table>
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<th>Con</th>
<th>SNL</th>
<th>Najanalgesin</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>6h</td>
<td>1764.61±222.36**</td>
<td>718.17±72.99*</td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td>1678.87±209.76**</td>
<td>543.25±105.08*</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>1867.87±1889.76**</td>
<td>1162.46±138.23*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>6h</td>
<td>2764.72±246.07**</td>
<td>1168.14±148.11**</td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td>2871.54±271.63**</td>
<td>669.11±244.38**</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>2875.67±244.38**</td>
<td>1728.67±260.64</td>
</tr>
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</table>

The mean values obtained after L5 spinal nerve ligation were greater than that of the control group. Compared to the SNL group, najanalgesin had an inhibitory effect on IL-1β and TNF-α in CSF at 6h and 12h. Data are represented as the mean±S.E.M. (**p<0.01 vs. control group; *p<0.05 vs. SNL group).**

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**Table 2. Effects of Najanalgesin on the LPS-Induced Protein Expression of IL-1β and TNF-α in Astrocytes (pg/mL)**

<table>
<thead>
<tr>
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<th>Con LPS</th>
<th>Najanalgesin</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>6h</td>
<td>18.68±1.04</td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td>23.40±1.10</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>28.30±1.60</td>
</tr>
<tr>
<td>TNF-α</td>
<td>6h</td>
<td>17.47±1.27</td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td>21.51±1.01</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>25.74±1.95</td>
</tr>
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</table>

LPS was added to astrocytic cells 30 min after the addition of najanalgesin. The effect of LPS on protein expression after 6h, 12h and 24h of treatment was measured. Compared to the LPS group, najanalgesin had an inhibitory effect on IL-1β at 6h, 12h and 24h and TNF-α at 24h in astrocytes. Data are represented as the mean±S.E.M. (**p<0.01 vs. control group; *p<0.05 vs. LPS group).
activation, but not the main mechanism. There may be other mechanisms to najanalgesin’s antinociception, such as impact-
ing on neurons. The early references on snake venom were
described that the venom affects neuronal function to produce
antinociceptive effect, such as cholinergic receptor or opioid
receptor on neuron. The present results that co-application
of najanalgesin with low dose glial metabolic inhibitor syner-
gistically antagonized neuropathic pain may provide a poten-
tial strategy for the treatment of neuropathic pain.

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