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A non-opioid pathway for dynorphin-caused spinal cord injury in rats[☆]

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Abstract

Intrathecal injection of dynorphin into rats *via* subarachnoid catheter induces damage to spinal cord tissue and motor function. Injection of the kappa opioid receptor antagonist nor-binaltorphine, or the excitatory amino acid N-methyl-D-aspartate receptor antagonist MK-801 into rats alleviated the pathological changes of dynorphin-caused spinal cord tissue injury and reduced the acid phosphatase activity in the spinal cord. The experimental findings indicate that there are opioid and non-opioid pathways for dynorphin-induced spinal cord injury, and that the non-opioid receptor pathway may be mediated by the excitatory amino acid N-methyl-D-aspartate receptor.

Key Words: spinal cord injury; dynorphin; kappa opioid receptor antagonist; N-methyl-D-aspartate receptor antagonist; motor function; acid phosphatase

Abbreviations: NMDA, N-methyl-D-aspartate; ACP, acid phosphatase; nor-BNI, nor-binaltorphine

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INTRODUCTION

Endogenous opioid peptide is a leading factor of secondary pathological damage following traumatic and irreversible spinal cord injury^[1]. However whether this injury has an underlying mechanism involving an opioid or non-opioid pathway remains unclear^[1-6]. Faden^[6] reported that as an endogenous opioid peptide, dynorphin works mainly depending on its specific receptor, the kappa receptor, and thus this receptor may need to be targeted for treatment of spinal cord injury. Indeed, kappa receptor antagonists can significantly alleviate the degree of spinal cord injury. The non-specific opioid receptor antagonist, naloxone, has been shown to antagonize dynorphin-caused spinal cord injury, although the effect is not significant, indicating that dynorphin-induced spinal cord injury may be achieved *via* opioid-like and non-opioid-like receptor mechanisms^[7-9]. Isaac *et al*^[5] have demonstrated that competitive excitatory amino acid N-methyl-D-aspartate (NMDA) receptor antagonists can block dynorphin-induced loss of tail-flick reflex in the rat. Previous studies performed by our research group have demonstrated that pre-intrathecal injection of excitatory amino acid NMDA receptor antagonist MK-801 could significantly reduce the dynorphin-caused impairment on neurological function and histopathology in rats. Furthermore, this

reduction occurred in a dose-dependent fashion^[10].

This study therefore aimed to verify whether a non-opioid pathway of dynorphin-induced spinal cord injury may be mediated *via* the NMDA receptor.

RESULTS

Quantitative analysis of experimental animals

A total of 81 Sprague-Dawley rats were included in this study, after 11 rats were excluded due to damage in spinal subarachnoid catheterization, the remaining 70 rats were randomly divided into four groups: control group ($n = 16$; intrathecal injection of saline); dynorphin group [$n = 18$; intrathecal injection of saline + dynorphin A (1-13)]; dynorphin + nor-binaltorphine (nor-BNI) group [$n = 18$; intrathecal injection of nor-BNI + dynorphin A (1-13)]; and dynorphin + MK-801 group [$n = 18$; intrathecal injection of MK-801 + dynorphin A (1-13)]. At 3 and 14 days after injection, eight rats from the control group and nine rats from the other three groups were collected and observed. Overall, 70 Sprague-Dawley rats were involved in the result analysis.

Effect of kappa opioid receptor antagonist nor-BNI and NMDA receptor antagonist MK-801 on motor functions with dynorphin-induced spinal cord injury

The critical angle of Rivlin's oblique plate in the dynorphin group was significantly

increased compared with the control group at 3 days post-injection ($P < 0.05$). This critical angle slightly recovered at 14 days, but was still larger than the other three groups ($P < 0.05$). The altered value of critical angle in Rivlin's oblique plate test was significantly reduced in the dynorphin + nor-BNI group and the dynorphin + MK-801 group compared with the dynorphin group at 14 days post-injection ($P < 0.05$; Table 1).

Table 1 Comparison of motor function [critical angle of Rivlin's oblique plate ($^{\circ}$)] in rats

Group	Time after injection (day)	
	3	14
Control	-3.91 ± 2.56	7.65 ± 6.40
Dynorphin	32.26 ± 12.60^a	20.17 ± 7.60^a
Dynorphin+ nor-binaltorphine	33.49 ± 2.17^a	6.38 ± 0.75^{ab}
Dynorphin+MK-801	32.57 ± 5.79^a	5.05 ± 2.70^{ab}

Eight rats were used in the control group and nine rats in the other three groups at each time point. Data were expressed as mean \pm SD and difference between groups was compared with one-way analysis of variance and Student-Newman-Keuls test. ^a $P < 0.05$, vs. control group; ^b $P < 0.05$, vs. dynorphin group.

Effect of nor-BNI and MK-801 on spinal cord morphology in rats with dynorphin-induced spinal cord injury

Hematoxylin-eosin staining showed that edema was visible in the injected spinal cord in the dynorphin group at 3 days after intrathecal injection. Massive hemorrhage

and necrotic tissue were observed in gray matter and white matter. The structure of the central canal was damaged, and neuronal vacuoli were degenerated. Furthermore, some nuclei were concentrated, dissolved, small and triangular in shape. Inflammatory leukocytes infiltrated the white matter, where nerve fibers were disrupted and demyelinated. The distribution of the white matter appeared disordered and the amount was significantly reduced. Spinal cord edema was also apparent in the dynorphin + nor-BNI group and the dynorphin + MK-801 group. The central gray matter developed spotty bleeding, which was more apparent in the dynorphin + MK-801 group. The central canal still appeared to bleed and was damaged, although a small amount of neuronal degeneration, pyknosis, and white matter demyelination were visible (Figure 1).

At 14 days post-intrathecal injection, spinal cord edema disappeared in all groups. In the dynorphin group, a liquefactive necrotic area was wrapped with massive fibers. White matter fibers had proliferated in a disorderly manner. No inflammatory cells had infiltrated the spinal cord, but neuronal degeneration and nuclear pyknosis was still present. By contrast, in the dynorphin + nor-BNI group and the dynorphin + MK-801 group, focal necrosis and gliocytes proliferated. The majority of neurons retained their normal morphology with staining appearing normal. For example, nerve fibers in the white matter were arranged in a tight and orderly fashion. There was no significant difference between the dynorphin + nor-BNI group and the dynorphin + MK-801 group (Figure 2).

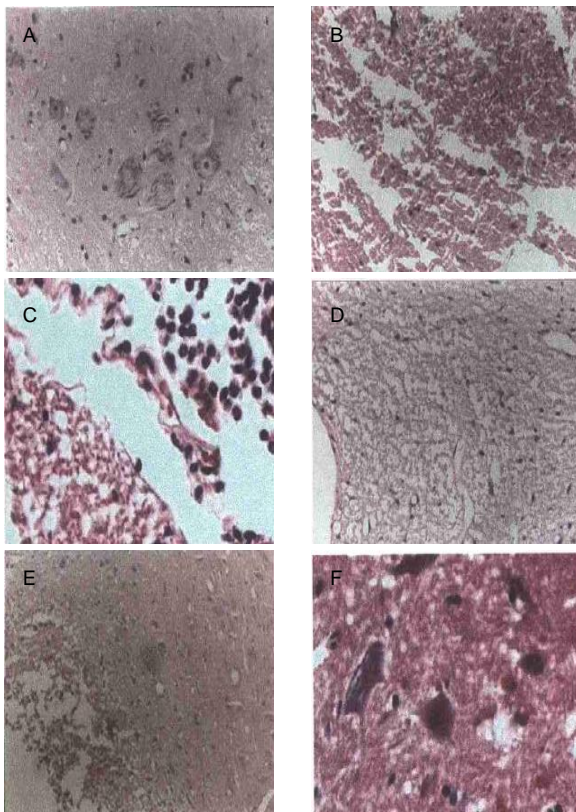


Figure 1 Morphology of rat spinal cord tissue at 3 days after intrathecal injection of MK-801 (N-methyl-D-aspartate receptor antagonist) or nor-binaltorphine (nor-BNI, kappa opioid receptor antagonist) (hematoxylin-eosin staining).

Control group: Gray and white matter were normal (A, $\times 40$).

Dynorphin group: Massive gray matter necrosis and hemorrhage (B, $\times 40$). Severe edema of white matter and inflammatory cell infiltration (C, $\times 40$) were observed.

Dynorphin + nor-BNI group: White matter edema, fiber disorder and demyelination were observed (D, $\times 40$).

Dynorphin + MK-801 group: Gray matter necrosis, hemorrhage, and inflammatory cell infiltration (E, $\times 20$; F, $\times 40$) were observed.

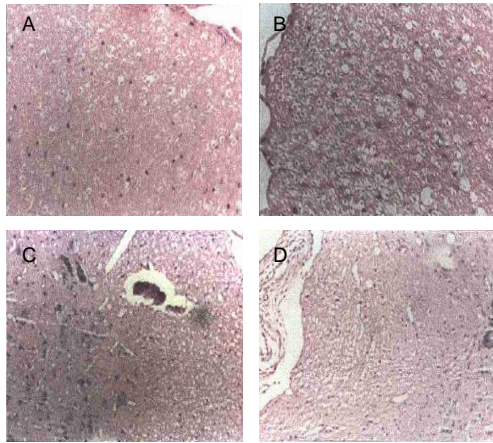


Figure 2 Morphology of rat spinal cord tissue at 14 days after intrathecal injection of MK-801 (N-methyl-D-aspartate receptor antagonist) or nor-binaltorphine (nor-BNI, kappa opioid receptor antagonist) (hematoxylin-eosin staining, $\times 20$).

- (A) Gray matter was normal in the control group.
- (B) In the dynorphin + nor-BNI group, white matter edema disappeared and fibers were slightly demyelinated.
- (C) A small area of liquefaction necrosis was visible in the dynorphin + MK-801 group.
- (D) In the dynorphin group, liquefaction necrosis was wrapped with fibers.

Effect of nor-BNI and MK-801 on acid phosphatase (ACP) expression in rats with dynorphin-induced spinal cord injury

Immunohistochemical detection analysis showed that ACP activity in spinal cord anterior horn cells was normal in control rats at 3 days post-injection. Enzyme reaction particles were small, yellow, and uniformly distributed in the cytoplasm. The nuclei and nucleoli were clearly visible. In the dynorphin group, ACP activity in the spinal cord anterior horn cells was significantly enhanced and the number of reaction particles was increased ($P < 0.05$). Particles were thick and brownish and the nuclei were covered with dense enzyme reaction particles. ACP activity was slightly increased in the dynorphin + nor-BNI and dynorphin + MK-801 groups. Enzyme reaction particles were tiny, but the number of particles was higher in these groups than in the control group and lower than the dynorphin group ($P < 0.05$). There was no significant difference between the groups treated with nor-BNI and MK-801 (Table 2, Figure 3). At 14 days after injection, the ACP activity decreased in all groups. The number of enzyme reaction particles reduced and particles were evenly distributed. The ACP activity was still at high levels in some sections from the dynorphin group, but no significant difference was observed compared with other groups (Table 2, Figure 3).

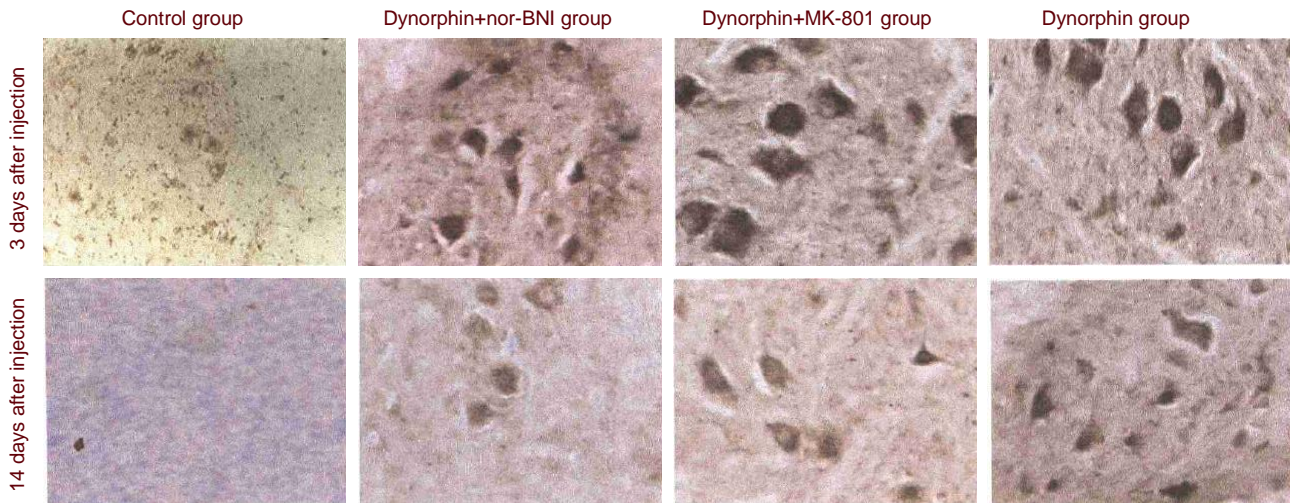


Figure 3 Acid phosphatase (ACP) activity in the spinal anterior horn after intrathecal injection of MK-801 (N-methyl-D-aspartate receptor antagonist) or nor-binaltorphine (nor-BNI, kappa opioid receptor antagonist) (histochemical staining, $\times 40$).

At 3 days after intrathecal injection, ACP reaction particles were light yellow and evenly distributed in the control group. In the dynorphin + nor-BNI group, ACP reaction particles were brownish yellow and cellular morphology was normal. Similar observations were seen in the dynorphin + MK-801 group. ACP reaction particles were brownish black and thick, with an irregular cell morphology being seen in the dynorphin group.

At 14 days after intrathecal injection, ACP reaction particles were uniformly distributed and stained as light yellow in the control group. In the dynorphin + nor-BNI group, ACP reaction particles at 14 days after intrathecal injection were stained lighter than at 3 days, showing a light yellow color. The ACP reaction particles at 14 days after intrathecal injection in the dynorphin + MK-801 group were stained lighter than at 3 days. ACP reaction particles at 14 days after intrathecal injection were stained brown yellow in the dynorphin group, which was lighter than at 3 days, and cellular morphology was normal.

Table 2 Comparison of acid phosphatase activity in spinal anterior horn lateral neurons of rats

Group	Mean gray value (%)		Equivalent circular diameter of positive particles (nm)	
	3 days	14 days	3 days	14 days
Control	36.8±1.2	37.6±1.2	20.3±5.1	20.5±5.7
Dynorphin	48.3±1.4 ^a	43.8±1.6	36.0±5.0 ^a	28.1±5.3
Dynorphin+ nor-binaltorphine	40.2±1.8 ^{ab}	37.4±1.6	28.6±5.1 ^{ab}	24.8±6.1
Dynorphin+ MK-801	41.0±1.5 ^{ab}	38.9±1.5	30.2±5.6 ^{ab}	25.1±6.0

Lower mean gray value indicates a lower acid phosphatase activity. Eight rats were used in the control group and nine rats were used in the other three groups at each time point.

Data were expressed as mean ± SD and difference between groups was compared with one-way analysis of variance and Student- Newman-Keuls test. ^aP < 0.05, vs. control group; ^bP < 0.05, vs. dynorphin group.

DISCUSSION

ACP is a lysosomal marker and can be released when cells are injured^[11]. Enhanced activity of ACP reflects the increased number and active function of lysosomes. The increased release of lysosomal enzymes leads to cell damage and even death^[11-12]. In this study, ACP activity was reduced or returned to normal after treatment with the NMDA receptor antagonist, MK 801, and the kappa receptor antagonist, nor-BNI. In addition, the impairment of motor function and spinal cord tissue was also significantly improved after intrathecal injection. This is evidence that the kappa receptor antagonist and NMDA receptor antagonist can alleviate dynorphin-induced spinal cord injury. This is consistent with previous findings^[13]. Nor-BNI and MK-801 can be used to treat injury, but they respectively depend on blocking kappa and NMDA receptors to do so^[14-21], indicating that the effects of dynorphin are interrelated with kappa and NMDA receptors. This is possibly why nor-BNI and MK-801 cannot completely block the pathological effects of dynorphin. The underlying mechanism could be as follows^[22-24]: (1) subarachnoid space injection of dynorphin results in decreased local spinal cord blood flow, causing ischemia. The secretion of excitatory amino acids, such as glutamate and aspartic acid, increases after spinal cord ischemia, thus causing over-excitation of the NMDA receptor. This excitation in turn causes massive Ca²⁺ entry into postsynaptic neurons and induces membrane depolarization. The free fatty acid content in neurons is then increased, ultimately leading to neuronal damage. (2) Dynorphin is dependent on a presynaptic mechanism that increases excitatory amino acid concentrations or enhances excitatory amino acid activity, rather than directly causing spinal cord ischemia. (3) Dynorphin can directly act on certain sites of the

NMDA receptor and trigger NMDA receptor depolarization.

In conclusion, this study suggested that there are two pathological mechanisms underlying the action of dynorphin, an opioid and a non-opioid pathway. The potential receptor involved in the non-opioid pathway is the NMDA receptor. Further studies are required to conclusively determine the exact role of NMDA receptors in non-opioid mechanisms underlying the action of dynorphin. The interaction between this non-opioid pathway and the opioid pathway also needs further elucidation. Additionally, studies regarding whether dynorphin enhances NMDA receptor activity through excitatory amino acid modulation or directly through the NMDA receptor, are also required.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed in the Animal Center of the Second Military Medical University of Chinese PLA, China.

Materials

A total of 81 healthy, male Sprague-Dawley rats, aged 2 months, weighing 300–350 g, of clean grade, were provided by the Animal Center of the Second Military Medical University of Chinese PLA, China (license No. SCXK (Hu) 2002-0006). All rats were housed under natural light/dark cycle, allowing free access to food and water. All experimental disposal of animals was in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[25].

Methods

Subarachnoid cannulation

Subarachnoid cannulation was performed using the modified Lopachin method^[26]. In brief, when clear cerebrospinal fluid outflow was visible, a PE-10 polyethylene tube filled with 7 µL normal saline was inserted into the subarachnoid space (7.0 cm) until it reached the level of T₁₀₋₁₁. Soft tissue was sutured together, layer by layer, and the polyethylene tube was fixed in the soft tissue around the wound. Rats recovered for 3 days, and were allowed free movement.

Drug injection

Control group: Rats were intrathecally injected with 10 µL saline. Dynorphin group: Rats were intrathecally injected with saline + 30 nM dynorphin A (1–13) (Sigma, St. Louis, MO, USA). Dynorphin + nor-BNI group: Rats were intrathecally injected with 100 nM nor-BNI^[27] (Sigma) + 30 nM dynorphin A (1–13). Dynorphin + MK-801 group: Rats were intrathecally injected with 100 nM MK-801^[27] (Sigma) + 30 nM dynorphin A (1–13). All injected drugs were prepared with saline into 10 µL solution prior to use, dynorphin A (1–13) was given at 15 minutes after intrathecal injection of nor-BNI or MK-801. All drugs were

injected within 2 minutes.

Assessment of motor function in rats by Rivlin's oblique plate test

We used a Rivlin oblique plate^[28]. This plate was a wooden half-arc structure, 40 cm wide and 50 cm long. Angles were marked on both sides, and the moveable plate was designed with an adjustable angle of 0–90°. The plate surface was covered with a rubber pad, 40 cm long and 30 cm wide. This pad was attached with several parallel shallow grooves, at a depth of 0.3 cm and at 1.6 cm intervals (supplementary Figure 1 online). At 3 and 14 days after injection, rat motor function was investigated using a modified Rivlin method^[28]. The rat head faced forward, the body's longitudinal axis was perpendicular to the oblique plate longitudinal axis. The angle between the plate and the horizontal plane was then gradually increased, until the rats could maintain just 5 seconds in a constant position, deemed the critical angle. The critical angle was recorded and the change in angle before and after injection was calculated. The gripping ability of the rat limb determines the angle, thus reflecting spinal cord nerve function in rats. Each rat was measured three times and the mean value was calculated.

Morphology of spinal cord T₁₀₋₁₂ segments

After the oblique plate test was completed, rats were killed under deep anesthesia via intraperitoneal injection of 70 mg/kg phenobarbital sodium. T₁₀₋₁₂ segments of spinal cord were fixed, paraffin-embedded and cut into consecutive slices at 10 μm thickness. Three serial slices were randomly selected from each tissue for hematoxylin-eosin staining and observation under a light microscope (Olympus, Tokyo, Japan).

Histochemical staining of ACP

T₁₀₋₁₂ segments of the rat spinal cord were cut into consecutive frozen slices (20 μm), and three consecutive slices were randomly selected from each tissue for ACP histochemical staining. In brief, frozen slices were dried, incubated with culture medium at 37°C for 3 hours, and rinsed with double distilled water. Slices were then incubated with 2% acetic acid for 1 minute and rinsed again with double distilled water, whereupon they were incubated with 1% ammonium sulfide for 1 minute and repeatedly rinsed with tap water. After rinsing, slices were incubated with 2% methyl green counter-stain (for nuclei) for several minutes and then dried and dehydrated with 96% and 100% ethanol, respectively. Following this, slices were fixed using xylene transparency and gum cementing. The mean gray and equivalent circle diameter of ACP positive particles in spinal cord anterior horn neurons were automatically measured with a computer image analysis system (Institutes of Biomedical Sciences at Fudan University, Shanghai, China; consisting of Smartscap image acquisition card and Petium III master station)^[29].

Statistical analysis

Data are expressed as mean ± SD and differences between groups were compared with one-way analysis

of variance and Student-Newman-Keuls test.

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Author contributions: All authors were responsible for study design, implementation and assessment.

Conflicts of interest: None declared.

Ethical approval: The experiments were approved by Animal Ethics Committee at the Second Military Medical University of Chinese PLA in China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 11, 2012 item after selecting the "NRR Current Issue" button on the page.

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