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NO mediated increase of Fos protein and $NMDA_{1A}$ R mRNA expression in rat spinal cord during morphine withdrawal

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KEY WORDS morphine; substance withdrawal syndrome; nitric oxide; proto-oncogene proteins c-fos; spinal cord; *N*-methylaspartate

ABSTRACT

AIM: To investigate the effects of nitric oxide (NO) on activation of the rat spinal cord neurons during naloxone-precipitated morphine withdrawal. **METHODS**: Fos immunocytochemistry, NADPH-d histochemistry, Fos/NADPH-d double-labeling, intrathecal injection, antisense oligonucleotides (AS-ONs) techniques, and RT-PCR were used. **RESULTS**: Acute administration of naloxone and chronic administration of morphine did not change the expression of Fos protein and NADPH-d positive neurons, and there was no expression of Fos/NADPH-d double-labeled neurons in the spinal cord of rats. Morphine withdrawal increased the expression of Fos protein, NADPH-d positive, and Fos/NADPH-d double-labeled neurons, and they were observed in all the laminae of the rat spinal cord. Intrathecal injection of nNOS antisense oligonucleotides (nNOS-AS) inhibited the increase of Fos protein and $NMDA_{1A}$R mRNA expression in the rat spinal cord during morphine withdrawal and decreased the scores of morphine withdrawal symptoms. The effect of nNOS-AS was greater than that of eNOS-AS. There was no effect in nNOS sense oligonucleotides (nNOS-S) group. **CONCLUSION**: NO mediated the increase of Fos protein and $NMDA_{1A}$R mRNA expression in the rat spinal cord during morphine withdrawal.

INTRODUCTION

Opioid dependence is defined as an altered physiological state produced by repeated opiate exposure and cessation of drugs or administration of opioid receptor antagonist, which leads to a withdrawal syndrome characterized by serious physiologic disturbance. According to the traditional view, locus coeruleus (LC) is the main site mediating opioid withdrawal response, and overshoot of intracellular cAMP level is the biochemical base producing opioid withdrawal. However, more and more studies demonstrated that *N*-methyl-*D*-aspartate (NMDA)/NO/cGMP signal transmission pathway in spinal cord played an important role in the development of opioid tolerance, dependence, and withdrawal[1-3]. Rohed *et al*[4,5] reported that the spinal cord neurons of morphine-tolerant and dependent rats were in a state of "latent sensitization" which could be manifested by nociceptive stimulation and naloxone-precipitated withdrawal, and pretreatment with NMDA receptor antagonist and nitric oxide synthase (NOS) inhibitor could prevent neuron sensitization. In the present study, we investigated the effect of NMDA/NO/cGMP pathway on the rat spinal cord neuron sensitization during morphine withdrawal.

MATERIALS AND METHODS

Animals and reagents Male adult Sprague-Dawley rats, weighing 200-250 g, were obtained from Experimental Animal Center of Shanghai (Grade Ⅱ, Certificate № 005). Morphine was purchased from Qinghai Pharmaceutical Factory, China. β-Nicotinamide adenine dinucleotide phosphate (β-NADPH) and nitrotetrazolium blue chloride (NBT) were products of Sigma (USA). Fos antibody, ABC-HRP, and DAB were products of Vector (USA). LipofectinAMINE was purchased from Gibco (USA). Other chemicals were of AR.

Intrathecal cannulation Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). A polythylene catheter (inner diameter of 0.28 mm and outer diameter of 0.61 mm) filled with saline, with a parafilm knot in heat-sealed side, was inserted via an incision of the

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atlanto-occiptal membrane and advanced 5.5 – 6.0 cm caudally to leave its tip in the thoracolumbar level. The catheter was fixed to muscle. Correct catheter placement was confirmed by injection of 15 μL of 2 % lidocaine into the subarachnoid space 20 min after recovery from anesthesia. The catheter was judged to be intrathecal if paralysis and dragging of the hind legs occurred within 30 s of this injection. Animals with negative lidocaine test and neurologic damage after catheter implantation were excluded from the study. The rats were housed individually after surgery and allowed 5 – 6 d recoveries before testing.

Animal model and experimental groups Experiments were carried out on non-cannulated and intrathecal cannulated rats. Non-cannulated rats were used for NADPH-d histochemistry, Fos immunocytochemistry, and Fos/NADPH-d double labeling. Intrathecal cannulation rats were used for Fos immunocytochemistry and RT-PCR.

To set up dependence model, rats were subcutaneously injected with morphine (bid, 5 d). The dose of morphine was 10 mg/kg in the first day and increased by 10 mg/kg each day. On day 6, 4 h after injection of morphine 50 mg/kg, morphine withdrawal syndrome was precipitated by injection of naloxone (4 mg/kg, ip). An equal volume of saline was administered in control group. According to subcutaneous and intraperitoneal injection, all non-cannulated rats were divided into four groups: saline-saline (saline group, $n = 4$), saline-naloxone (naloxone group, $n = 4$), morphine-saline (dependence group, $n = 4$), and morphine-naloxone (withdrawal group, $n = 6$).

In intrathecal cannulated rats, nNOS-AS 0.3 nmol, eNOS-AS 0.3 nmol, nNOS-S 0.3 nmol, (dissolved in 20 μL of 20 % LipofectinAMINE) and 20 μL of 20 % LipofectinAMINE were intrathecally injected at 24 h and 12 h before naloxone-precipitated withdrawal. Intrathecal cannulated rats for Fos immunocytochemistry were divided into four groups: nNOS-AS group ($n = 5$), eNOS-AS group ($n = 5$), nNOS-S group ($n = 4$), and withdrawal group ($n = 4$). In RT-PCR experiment, morphine dependent and non-dependent rats were also included. So we got six groups: control group, dependence group, withdrawal group, nNOS-AS group, eNOS-AS group, and nNOS-S group.

NADPH-d histochemistry One hour after injection of naloxone, all rats for histochemistry and immunocytochemistry were deeply anesthetized with sodium pentobarbital (60 mg/kg, ip) and underwent sternotomy, transcardia aortic needle cannulation, perfusion with saline 100 mL, followed by 400 mL of 4 % ice-cold paraformaldehyde in 0.1 mol/L phosphate buffer (PB). The spinal cord of thoracolumbar level was removed, postfixed in the same fixative for 3 h, then immersed in 30 % sucrose in PB overnight at 4 ℃. Frozen series sections (30 μm) were cut and collected in PB. In normal rats, tissue sections were divided into 3 parts: one part was used for NADPH-d histochemistry, the second for Fos immunocytochemistry, and the third for Fos/NADPH-d double labeling. In intrathecally cannulated rats, sections were only used for Fos immunocytochemistry.

Tissue sections were washed in PB and incubated in PB (pH 8.0) containing 0.3 % Triton X-100, β-NADPH 0.6 mg/kg, and NBT 0.5 mg/kg at 37 ℃ for 40 – 60 min. Then sections were rinsed in phosphate buffer saline (PBS) 0.01 mol/L to stop reaction, mounted on gelatin-coated slides, air dried, dehydrated with 70 % – 100 % alcohol, cleared with xylene, and cover-slipped for microscopy examination.

Fos immunocytochemistry Tissue sections were washed in PBS and incubated in PBS containing 5 % normal goat serum and 0.3 % Triton X-100 at room temperature (25 ℃ ± 2 ℃) for 30 min, followed by rabbit anti-Fos serum (1:1000) at 4 ℃ for 48 h. Then sections were incubated in biotinylated goat anti-rabbit IgG (1:200) at 37 ℃ for 1 h and in avidin-biotin-peroxidase complex (1:100) at 37 ℃ for 2 h. Finally, the sections were reacted with DAB for 5 – 10 min.

Fos/NADPH-d double-labeling Tissue sections were processed with NADPH-d histochemistry, after washing with PBS, followed by Fos immunocytochemistry. Other procedures were the same as mentioned above.

RT-PCR Rats were killed 1 h after injection of naloxone. Total cellular RNA was extracted from the spinal cord of thoracolumber by acid-guanidinium-phenol-chloroform method. The purity of isolated RNA was identified by A_{260}/A_{280} ratio. cDNA was synthesized by the following steps: eppendorf tubes containing 2 μg of total RNA in 10 μL DEPC-treated water were incubated for 5 min at 65 ℃, chilled on ice for 5 min, then each sample was incubated for 60 min at 42 ℃ after adding 4 μL of $MgCl_2$ 25 mmol/L, 10 × buffer 2 μL, dNTP 2 μL, Ribnuclease inhibitor (Promega) 0.5 μL, AMV reverse transcriptase 1 μL, and Oligo (dT) 1 μL in a total volume of 20 μL. Then the samples were incubated for 5 min at 95 ℃. The cDNA was frozen at –20 ℃

until PCR was carried out.

For amplification of the desired cDNA, the rat $NMDA_{1A}$ receptor ($NMDA_{1A}R$) primers (Sangon CO, Shanghai) (forward: 5'CAT GCA CCT GCT GAC ATT 3', backward: 5'CGA GTC TAC AAC TGG AAC 3', predicted size: 475 bp) and β-actin primers, as an internal standard, (forward: 5'TCA TGA AGT GTG ACG TTG ACA TCC GTA AAG 3', backward: 5'CCT AGA AGC ATT TGC GGT GCA CGA TGG ACG 3', predicted size: 409 bp) were used. All PCR were done in a volume of 25 μL. The PCR mixture contained 0.2 μg of the reverse transcription products in a volume of 10 μL, 10 × buffer 2.5 μL, 2.5 mmol/L dNTP 2 μL, H_2O 7 μL, each primers 50 pmol (0.5 μL) and Taq DNA polymerase 2.5 U (2.5 μL). Subsequent RT-PCR was performed in DNA thermal cycler (AMPLITRON, USA). The cycle program was set to denature at 94 ℃ for 30 s, to anneal at 51 ℃ for 30 s, and to extend at 72 ℃ for 1 min. At the end of 35 cycles, further extension for 10 min at 72 ℃ was performed. The PCR products were seperated by electrophoresis on 1.5 % agarose gel and stained by ethidium bromide. The specific amplified fragments were detected under UV light (FOTODYNE, USA) and analyzed by an autogel analysis system (STORM 860, USA).

Synthesis of ODN and selection of sequence targets The sequences of antisense oligonucleotides (ODN) against nNOS and eNOS were the initiation of translation sites of rat nNOS mRNA and eNOS mRNA. nNOS antisense: 5'CTT CCA TGG TAT CTG TGT 3', sense: 5'ACA CAG ATA CCA TGG AAG 3', eNOS antisense: 5'TCC CCA TGA GTG AGG CAG 3'. Sense ODN was used as control. ODN was synthesized by Sangon Co Ltd, Shanghai, and modified with phosphorothioate.

Scores of morphine withdrawal response Within 1 h after naloxone precipitated withdrawal, the scores of morphine withdrawal response were obtained according to morphine withdrawal syndrome as following: wet dog shaking, teeth chatting, abnormal position, irritability, weight lose, and autonomic nerves symptoms, *etc*.

Counting of positive neurons To study the distribution of positive neurons, four regions of the spinal cord were defined as following: superficial laminae (laminae Ⅰ – Ⅱ), nucleus proprious (laminae Ⅲ – Ⅳ), neck of dorsal horn (laminae Ⅴ – Ⅵ), and ventral laminae (laminae Ⅶ – Ⅹ). We selected 5 spinal cord sections per animal showing the greatest number of positive neurons. One parameter was made for every rat: total numbers of positive neurons in bilateral spinal cord. All positive neurons were counted without considering the intensity of the staining.

Statistical analysis The data were presented by $\bar{x} \pm s$ and compared with one-way ANOVA.

RESULTS

Fos immunocytochemistry Fos protein expression was very low in the rat spinal cord of saline group, naloxone group, and dependence group, and mainly distributed in superficial laminae (laminae Ⅰ – Ⅱ) of the spinal cord. Compared to other three groups, the number of Fos protein was significantly increased ($P < 0.01$) and distributed in total laminae of bilateral spinal cord in morphine withdrawal group (Tab 1).

Tab 1. Expression of Fos, NADPH-d positive, and Fos/NADPH-d-double-labeled neurons in the rat spinal cord during morphine withdrawal. $n = 5$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* mor-sal group.

Groups	Fos	NADPH-d	Fos/NADPH-d
Sal-sal	4.3 ± 1.6	18 ± 6	0
Sal-nal	5.8 ± 2.0	21 ± 8	0
Mor-sal	10 ± 4	26 ± 7	0
Mor-nal	532 ± 154^c	58 ± 17^b	8 ± 4^c

NADPH-d histochemistry NADPH-d-stained somata, fibers, and terminals were located in superficial laminae of spinal dorsal horn and the gray matter surrounding central canal. The quantity of NADPH-d positive neurons showed no difference in saline group, naloxone group, and dependence group ($P > 0.05$), but was significantly increased in morphine withdrawal group ($P < 0.01$, Tab 1).

Fos/NADPH-d double-labeling No double-labeled neurons were expressed in saline group, naloxone group, and dependence group. However, a lot of double-labeled neurons were expressed in total laminae of spinal cord in morphine withdrawal group and mainly distributed in the superficial laminae spinal dorsal horn (Tab 1 and Fig 1).

Effect of intrathecal injection of NOS-AS on the scores of morphine withdrawal symptoms Both intrathecal injection of nNOS and eNOS antisense

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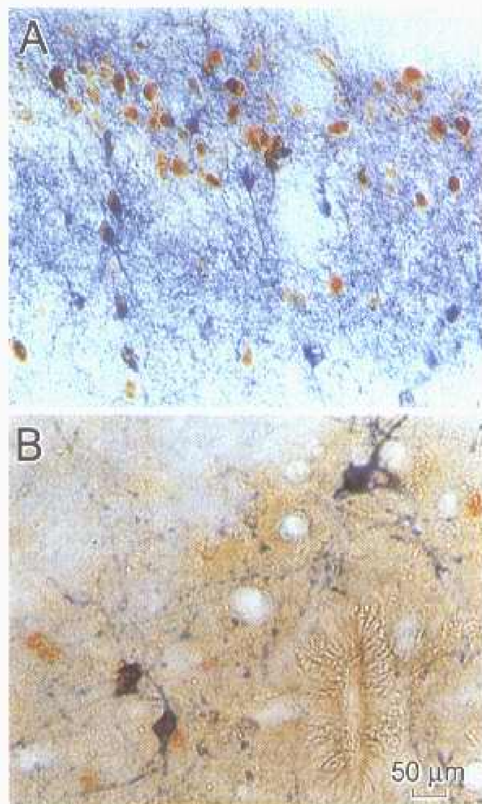

Fig 1. Fos/NADPH-d double-labeled neurons were expressed in the total laminae of bilateral spinal cord in rats during morphine withdrawal. A: laminae Ⅰ – Ⅳ, B: the gray matter surrounding central canal. ×120.

oligonucleotides could decrease the scores of morphine withdrawal symptoms, but the effects of injection of eNOS-AS were less than that of nNOS-AS ($P < 0.05$). Injection had no effect in nNOS-S group (Fig 2).

Effect of intrathecal injection of NOS-AS on the expression of Fos protein Intrathecal injection of nNOS antisense oligonucleotides didn't change Fos protein expression in the spinal cord of morphine dependence and non-dependence rats (data not provided), but could significantly inhibit the expression of Fos protein in the spinal cord of morphine-withdrawal rats ($P < 0.01$). There was a trend inhibiting the expression of Fos protein in eNOS-AS group, but there was no statistical significance ($P > 0.05$). Injection had no effect in nNOS-S group (Tab 2 and Fig 3).

Effect of intrathecal injection of NOS-AS on the expression of $NMDA_{1A}$ R mRNA Compared with control group, $NMDA_{1A}$R mRNA expression in the spinal cord of morphine-dependent rats was increased and progressively increased in morphine-withdrawal group. Both intrathecal injection of nNOS and eNOS antisense oligonucleotides inhibited the increase of $NMDA_{1A}$ R mRNA expression in the rat spinal cord during morphine-withdrawal ($P < 0.05$), but the inhibitory effect was more significant in the former ($P < 0.05$). There was no significant effect in nNOS-S group (Fig 4 and 5).

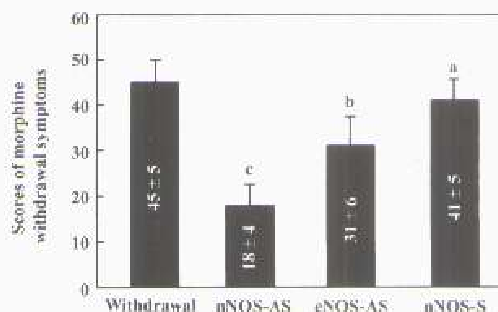

Fig 2. Effect of intrathecal injection of NOS antisense oligonucleotide on scores of morphine withdrawal symptoms. $n = 8$ rats. $\bar{x} \pm s$. [a]$P > 0.05$, [b]$P < 0.05$, [c]$P < 0.01$ *vs* morphine withdrawal group.

Tab 2. Effect of intrathecal injection of NOS-AS on the expression of Fos protein in the rat spinal cord during morphine withdrawal. $n = 5$. $\bar{x} \pm s$. [a]$P > 0.05$, [c]$P < 0.01$ *vs* withdrawal group.

	Withdrawal	nNOS-AS	eNOS-AS	nNOS-S
Fos protein	543 ± 156	122 ± 32[c]	385 ± 105[a]	512 ± 178[a]

DISCUSSION

The proto-oncogene c-*fos* is one of the immediate-early genes, which can be activated in neurons by increasing intracellular cAMP level, Ca^{2+} concentration, and other intracellular messengers. Fos protein, the product of c-*fos* gene, has been used as a maker for neuronal activation in the CNS. The present results showed that Fos protein expression was significantly increased and distributed in total laminae of bilateral spinal cord of rats during morphine withdrawal. It suggested that a state of hypersensitization had developed in the spinal cord neurons of morphine withdrawal rats, which may be the reason of resulting in morphine withdrawal response.

Opioid receptors are located on the terminals of small-diameter primary afferent, projection neurons and

Fig 3. Effect of intrathecal injection of NOS-AS on the expression of Fos protein in the rat spinal cord during morphine withdrawal. A: Withdrawal group, B: nNOS-S group, C: eNOS-AS group, D: nNOS-AS group. ×60.

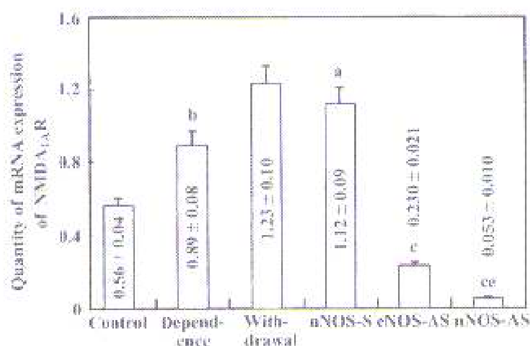

Fig 4. Intrathecal injection of NOS-AS inhibited the expression of $NMDA_{1A}R$ mRNA of the rat spinal cord during morphine withdrawal. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* morphine withdrawal group. $^eP < 0.05$ *vs* eNOS-AS group.

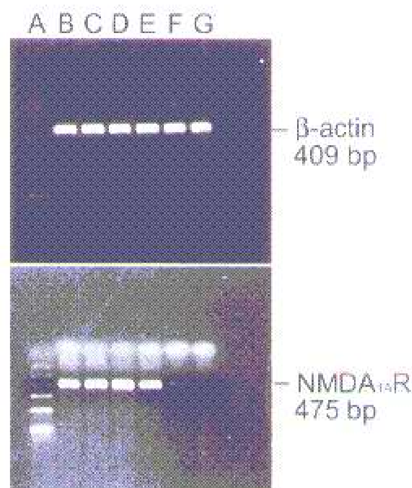

Fig 5. Effects of intrathecal injection of NOS-AS on the expression of $NMDA_{1A}R$ mRNA in the rat spinal cord during morphine withdrawal. Lane A: maker; Lane B: control; Lane C: dependence; Lane D: withdrawal; Lane E: nNOS-S; Lane F: eNOS-AS; Lane G: nNOS-AS.

the interneurons of the superficial laminae of spinal dorsal horn[6]. Chronic opiate exposure can lead to a compensatory up-regulation of cAMP pathway in these neurons.

After administration of opioid receptor antagonist, an overshoot of cAMP level would happen and induce Fos protein expression by activating CREB (cAMP-responsible element binding protein). With this view, we could interpret the change of Fos protein expression in the superficial laminae of spinal dorsal horn of morphine withdrawal rats. However, how to interpret Fos protein expression in other spinal cord laminae without opioid receptor?

Our previous works have shown that NO was involved in the increase of formalin-induced Fos protein expression in the spinal cord of morphine-tolerant rats[7]. In the present study, NADPH-d positive neurons, fibers and terminals were significantly increased in the superficial laminae of spinal dorsal horn in rats during morphine withdrawal. Importantly, Fos/NADPH-d double-labeled neurons were expressed in total laminae of spinal cord. Intrathecal injection of nNOS antisense oligonucleotides could significantly inhibit the expression of Fos protein and decrease the scores of morphine withdrawal symptoms. It suggested that NO played an important role in inducing Fos protein expression in spinal cord, especially in other laminae except the superficial laminae during morphine withdrawal. In other words, NO participated in the rat spinal cord neuron sensitization during morphine withdrawal.

Machelska *et al*[8,9] reported that chronic morphine exposure and naloxone-precipitated withdrawal increased the expression of nNOS mRNA and upregulated nNOS immunoreactivity in several brain regions and spinal cord of rats. The present study was consistent with their results. We considered that NOS was in a state of latent hyperactivation resulting from the increase of NOS gene expression in the spinal cord neurons in morphine dependent rats, which could be revealed by naloxone precipitated-withdrawal. NOS hyperactivation could increase the release of NO. NO could diffuse to the adjacent cells and other laminae of spinal cord, then sensitize the spinal cord neurons, induce Fos protein expression, and mediate morphine withdrawal response through NO/cGMP pathway. Moore *et al*[10,11] reported that 7-NI, a high selected inhibitor of nNOS, was more effective than other NOS inhibitor in preventing the development of morphine tolerance and dependence. We also found that nNOS activation played a more important role than eNOS did in inducing Fos protein expression and morphine withdrawal response.

Many studies had demonstrated that NMDA receptor activation and associated intracellular cascades were critically important in the development and maintenance of morphine withdrawal-induced neuron sensitization[12,13]. In agreement with this view, in the present experiment, morphine dependence and withdrawal increased the expression of $NMDA_{1A}$R mRNA in spinal cord of rats. The increase of $NMDA_{1A}$R mRNA expression could further up-regulate NMDA receptor function and mediate the spinal cord neuron hypersensitization in morphine withdrawal rats. Pretreatment with NOS antisense oligonucleotides could significantly inhibit the increase of $NMDA_{1A}$R mRNA expression during morphine withdrawal. It suggested that NO was involved in modulating the expression of NMDA receptor during morphine withdrawal. The change of $NMDA_{1A}$R mRNA expression was paralleled to that of Fos protein expression. However, whether Fos protein, as a transcription factor, mediated the effect of NO on modulating the expression of $NMDA_{1A}$R mRNA were not clear. It would be proved in our following experiment.

In conclusion, our results suggested that NO mediated the increase of Fos protein and $NMDA_{1A}$R mRNA expression in the rat spinal cord during morphine-withdrawal.

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NO介导吗啡戒断大鼠脊髓 Fos 蛋白和 $NMDA_{1A}$ 受体mRNA 表达的增加

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关键词 吗啡；物质禁断综合症；一氧化氮；原癌基因蛋白质 c-fos 类；脊髓；*N*-甲基天冬氨酸

目的：观察 NO 在纳洛酮催促吗啡戒断大鼠脊髓神经元活动变化中的作用. **方法**：采用 Fos 免疫组织化学、NADPH-d 组织化学、Fos/NADPH-d 双标、鞘内注射、反义寡核苷酸和 RT-PCR 技术. **结果**：急性应用纳洛酮和慢性应用吗啡对大鼠脊髓 Fos 蛋白及 NADPH-d 阳性神经元表达无明显影响，二者也无 Fos/NADPH-d 双标神经元表达；纳洛酮催促吗啡戒断大鼠脊髓 Fos 蛋白、NADPH-d 阳性神经元、纤维和终末表达明显增加，且出现 Fos/NADPH-d 双标神经元表达. 预先鞘内注射 nNOS 反义寡核苷酸明显降低吗啡戒断症状评分，减少吗啡戒断大鼠脊髓 Fos 蛋白及 $NMDA_{1A}R$ mRNA 表达. **结论**：NO 介导吗啡戒断大鼠脊髓 Fos 和 $NMDA_{1A}R$ mRNA 表达的增加.

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