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M_2 muscarinic receptor of spinal cord mediated increase of nNOS expression in locus coeruleus during morphine withdrawal

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KEY WORDS morphine; substance withdrawal syndrome; muscarinic receptors; nitric-oxide synthase; spinal cord; locus coeruleus

ABSTRACT

AIM: To investigate the effects of different muscarinic receptor (M) subtypes in the spinal cord on the scores of naloxone-precipitated morphine-withdrawal symptoms and the changes of nNOS expression in locus coeruleus (LC). **METHODS:** nNOS immunohistochemistry, intrathecal injection (it), and antisense oligonucleotides (AS-ONs) techniques were used. **RESULTS:** Intrathecal injection of M_2-antisense oligonucleotides (M_2-AS) decreased the scores of morphine withdrawal symptoms. M_1-AS attenuated morphine-withdrawal symptoms, but the effect was less than that of M_2-AS. The expression of nNOS positive neurons in the LC increased in morphine-dependent rats and increased to a greater extent during morphine withdrawal. Intrathecal injection of M_2-AS inhibited the increase of nNOS expression in LC during morphine-withdrawal, but there was no effect in case of M_1-AS. **CONCLUSION:** M_2 muscarinic receptor of spinal cord mediated the increase of nNOS expression in LC during morphine withdrawal.

INTRODUCTION

Many studies had demonstrated that nitric oxide (NO) contributed to the development of tolerance and physical dependence to morphine. Behaviour reports showed that the inhibitor of nitric oxide synthase (NOS) could effectively delay the development of morphine tolerance and physical dependence, and the donor of NO could improve the procedure of morphine tolerance[1,2]. According to the traditional view, locus coeruleus (LC) is the main site mediating opioid withdrawal response, and the hyperactivity of LC neurons play an important role during opioid antagonist-precipitated morphine withdrawal[3]. Chronic morphine exposure and naloxone-precipitated withdrawal upregulated neuronal nitric oxide synthase (nNOS) in the LC, and NOS inhibitors reduced the LC withdrawal response in

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voltammetric and electrophysiological experiments[4,5]. It is suggested that the expression of nNOS in the neurons of LC showed selective activation during morphine withdrawal.

There are five subtypes of muscarinic receptor M_1–M_5 extensively distributing in the brain and spinal cord. It has been acknowledged that the subtypes of M_1 and M_2 are more important than the other subtypes in the central nervous system, which have many functions such as neural plasticity, acquisition of memory, or brain development. Because of the overlapped expression of muscarinic receptors and lack of sufficient selective muscarinic receptor antagonists, limited information is available concerning the exact function of muscarinic receptor subtypes. It is reported that cholinergic neurons of spinal cord participated in the draw-out of morphine withdrawal symptoms. Intrathecal injection of scopolamine could decrease the scores of morphine withdrawal symptoms, and more interestingly, decrease the expression of NOS mRNA in brainstem[6]. So, it is suggested that the activity of muscarinic receptor of spinal cord was involved in the elicitation and development of physical dependence to morphine by influencing the activity of nNOS of LC. Then, which muscarinic receptor subtype of spinal cord mediates morphine withdrawal symptoms and whether the selective activation of nNOS in LC is modulated by muscarinic receptor of spinal cord are not clear. Antisense oligodeoxynucleotides (ODN) were capable of down-regulating the gene expression, and were used for assessment of gene function and for therapeutic purpose. However, functional efficacy of ODN required not only the selection of an appropriate target sequence, but also a sufficient concentration. Using cationic lipids LipofectinAMINE as carrier could enhance the cellular uptake and nuclear distribution of ODN[7]. In the present study, we investigated the effects of different muscarinic receptor subtypes of spinal cord on the scores of naloxone-precipitated morphine-withdrawal symptoms and the changes of nNOS activity in LC.

MATERIALS AND METHODS

Animals and reagents Male adult Sprague-Dawley rats, weighing 200–250 g, were obtained from Shanghai Experimental Animal Center (Grade II, Certificate No 005). Morphine was purchased from Shenyang Pharmaceutical Factory, China. nNOS antibody was product of Santa Cruz (USA). Biotinylated goat anti-rabbit IgG, avidin-biotin peroxidase complex (ABC), and 3,3'-diaminobenzidine (DAB) were purchased from Vector (USA). LipofectinAMINE was purchased from Gibco (USA). Other chemicals were of analytical grade.

Intrathecal cannulation Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). A polyethylene 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 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 in intrathecal cannulated rats. To set up dependence model (bid, 5 d), the dose of morphine was 10 mg/kg at the first day and increased by 10 mg/kg each day. An equal volume of saline was administered in the control group. On d 6, 4 h after injection of morphine 50 mg/kg, morphine withdrawal syndrome was precipitated by injection of naloxone (4 mg/kg, ip). M_1-AS 2 nmol, M_2-AS 2 nmol (dissolved in 20 μL of 20 % LipofectinAMINE), and 20 μL of 20 % LipofectinAMINE were intrathecally injected at 24 h before naloxone-precipitated withdrawal. So we got five groups: control group, dependence group, withdrawal group, M_1-AS group, and M_2-AS group.

nNOS immunohistochemistry One hour after injection of naloxone, all rats for immunohistochemis-

try were deeply anesthetized with sodium pentobarbital (50 mg/kg, ip) and underwent sternotomy and transcardial aortic needle cannulation, then perfused with saline 100 mL, followed by 400 mL of 4 % ice-cold paraformaldehyde in phosphate buffer (PB) 0.1 mol/L. The brainstem was removed quickly, postfixed for 3 h in the same solution of paraformaldehyde at room temperature, then immersed in 30 % sucrose in PB overnight at 4 ℃. Frozen series sections (25 μm) were cut and collected in PB. Tissue sections were washed in phosphate-buffered saline (PBS) and incubated in PBS containing 5 % normal goat serum and 0.3 % Triton X-100 at room temperature (25±2 ℃) for 30 min, and then in rabbit anti-nNOS serum diluted at 1:800 in PBS/Triton X-100 at 4 ℃ for 48 h. After several washes in PBS, the sections were incubated with 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.

Synthesis of ODN and selection of sequence targets The sequences of ODN against M_1 and M_2 were the initiation of translation sites of rat M_1 mRNA and M_2 mRNA. M_1 antisense: 5'-TGT GCA TCC CTG TGT GCT-3'; M_2 antisense: 5'-TAT TCA TTT TGT GTT CAG-3'. ODN was synthesized by Sangon Co Ltd, Shanghai, and modified with phosphorothioate[8,9].

Scores of morphine withdrawal response After the last injection of morphine, the rats were placed in a plexiglass observation chamber to allow for acclimatization to the environment. Following naloxone precipitated withdrawal, response was obtained according to morphine withdrawal syndrome. Two classes of withdrawal signs were measured: counted signs and observed signs. The counted signs were made every 15 min for 1 h. During each period of 15 min, the number of bouts of wet dog shakes, teeth chatting, and irritability (jumping and attack) were counted and a withdrawal score for each sign was assigned as follows: 0=no occurrence; 1=1 occurrence; 2≥2 occurrences. Abnormal position was observed over periods of 2 min, with two points being given for the presence during each period. The scores of four periods showing the signs were added. Diarrhea was evaluated and given a score based on the severity of the response observed (0=absent, 4=mild and moderate, 8=severe). The score of salivation was based on the severity of the response observed (0=absent, 1=mild and moderate, 2=severe). The withdrawal score of body weight loss was determined by the weight difference (ΔW) before and 60 min after administration of the naloxone, which was assigned as follows: 0=no change; 1=ΔW<2 %; 5=ΔW <4 %; 10=ΔW<6 %; 15=ΔW<8 %; 20=$\Delta W \geq 8$ %.

Quantification For each animal, five LC sections were analyzed. The sections of interest initially selected for counting were those which had the largest number of nNOS positive neurons by visual inspection at ×40 magnification. The number and the average optical density of nNOS positive cell bodies were analyzed by pathological image analysis system PAS-5000.

Statistical analysis The data were presented by mean±SD and compared with one-way ANOVA.

RESULTS

Effects of intrathecal injection of M_1-AS and M_2-AS on the response scores of morphine withdrawal symptoms Intrathecal injection of M_2-AS could decrease the total scores of morphine withdrawal symptoms (P<0.01), but the effects of injection of M_1-AS were less than that of M_2-AS. Pretreatment of rats with M_2-AS (it) attenuated the expression of withdrawal symptoms as reflected by low scores. However, not all of the withdrawal signs were influenced by the treatment. For example, M_2-AS attenuated irritability, diarrhea, and weight loss (P<0.01), but had no effect on other withdrawal symptoms (P>0.05). M_1-AS only attenuated wet dog shakes (P<0.01) (Tab 1).

Effects of intrathecal injection of M_1-AS and M_2-AS on the number of nNOS positive neurons in LC during morphine withdrawal nNOS positive neurons in the LC increased in morphine-dependent (223.5 % from control, P<0.01) and in naloxone-precipitated withdrawal (243.2 % from control, P<0.01) rats. Intrathecal injection of M_2-AS inhibited the increase of nNOS-immunoreactive (IR) neurons in the LC during morphine-withdrawal (P<0.01), but there

was no effect in case of M_1-AS group (P>0.05) (Fig 1, 3).

Tab 1. Effects of intrathecal injection of M_1-AS and M_2-AS on the response scores of morphine withdrawal symptoms. n=6. Mean±SD. [a]P >0.05, [b]P<0.01 *vs* withdrawal group.

Signs and symptoms	Withdrawal	M_1-AS	M_2-AS
Wet dog shakes	2.9±2.1	0.4±0.8[b]	2.3±2.6[a]
Irritability	6.7±1.4	7.7±1.4[a]	4.9±1.0[b]
Teeth chatting	2.8±0.6	2.7±0.5[a]	2.8±0.8[a]
Abnormal position	2.7±0.4	2.3±0.7[a]	2.6±1.2[a]
Salivation	1.6±0.7	1.4±0.9[a]	1.1±1.0[a]
Diarrhea	7.6±1.3	7.5±1.7[a]	4.8±1.8[b]
Weight loss	12.5±2.7	11.7±2.6[a]	8.3±2.6[b]
Total withdrawal score	37±7	33±3[a]	27±5[b]

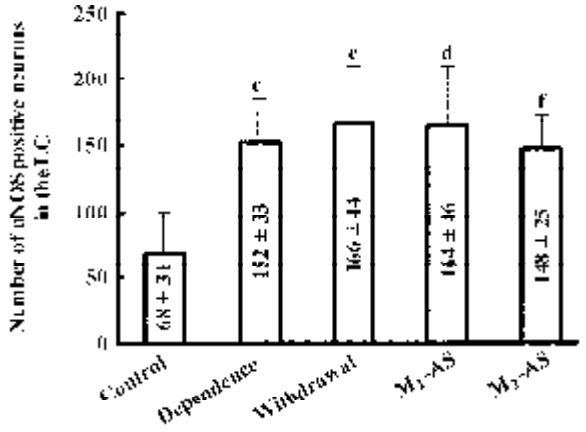

Fig 1. Effects of intrathecal injection of M_1 and M_2 antisense oligonucleotides on the number of nNOS positive neurons in LC during morphine withdrawal. n=6. Mean±SD. [c]P < 0.01 *vs* control. [d]P > 0.05, [f]P < 0.01 *vs* withdrawal group.

Effects of intrathecal injection of M_1-AS and M_2-AS on the average optical density of the nNOS positive cells in LC during morphine withdrawal

The expression of nNOS IR in the LC were more intense in morphine-dependent and morphine-withdrawal rats than in control animals (P<0.01). Pretreatment of rats with M_2-AS (it) decreased the average optical density during morphine withdrawal (P<0.01), but M_1-AS had no effect on the average optical density in the LC (P>0.05, from withdrawal) (Fig 2, 3)

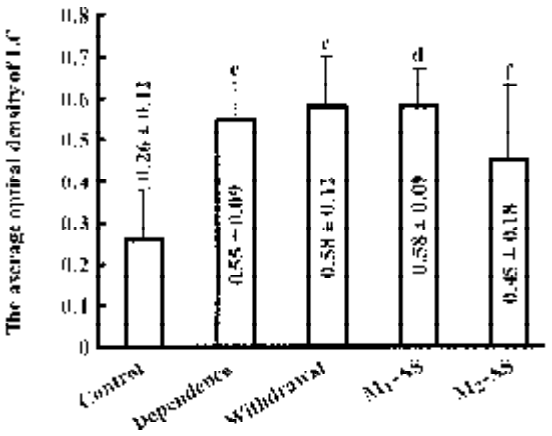

Fig 2. Effects of intrathecal injection of M_1 and M_2 antisense oligonucleotides on the average optical density of the nNOS positive cells in LC during morphine withdrawal. n=6. Mean ±SD. [c]P < 0.01 *vs* control. [d]P > 0.05, [f]P < 0.01 *vs* withdrawal group.

DISCUSSION

Spinal pathways are important for the expression withdrawal signs following both local and systemic administration of naloxone in morphine dependent rats. Marshall *et al*[10] reported that spinal cholinergic pathways played a role in the expression of spinal mediated withdrawal symptoms such as mean arterial pressure (MAP). Pretreatment of dependent rats with intrathecal injection of atropine reduced the pressor and several behavioural responses elicited by naloxone. Further more, intrathecal injection of M_1 selective antagonist pirenzepine and M_2 selective antagonist methoctramine could attenuate morphine withdrawal symptoms[11]. As described above, the activation of spinal muscarinic receptor participated morphine withdrawal symptoms. The present study was consistent with their results. We also found that M_2 activation played a more important role than M_1 at the spinal cord level. Behaviour results showed that inhibiting M_2 gene expression could decrease the total and several response scores of morphine withdrawal symptoms. In contrast, inhibiting M_1 gene expression had no obvious effect during morphine withdrawal.

Previous studies have suggested that increased glutamatergic afferent neurons input to the LC is mainly responsible for its hyperactivity during opioid with-

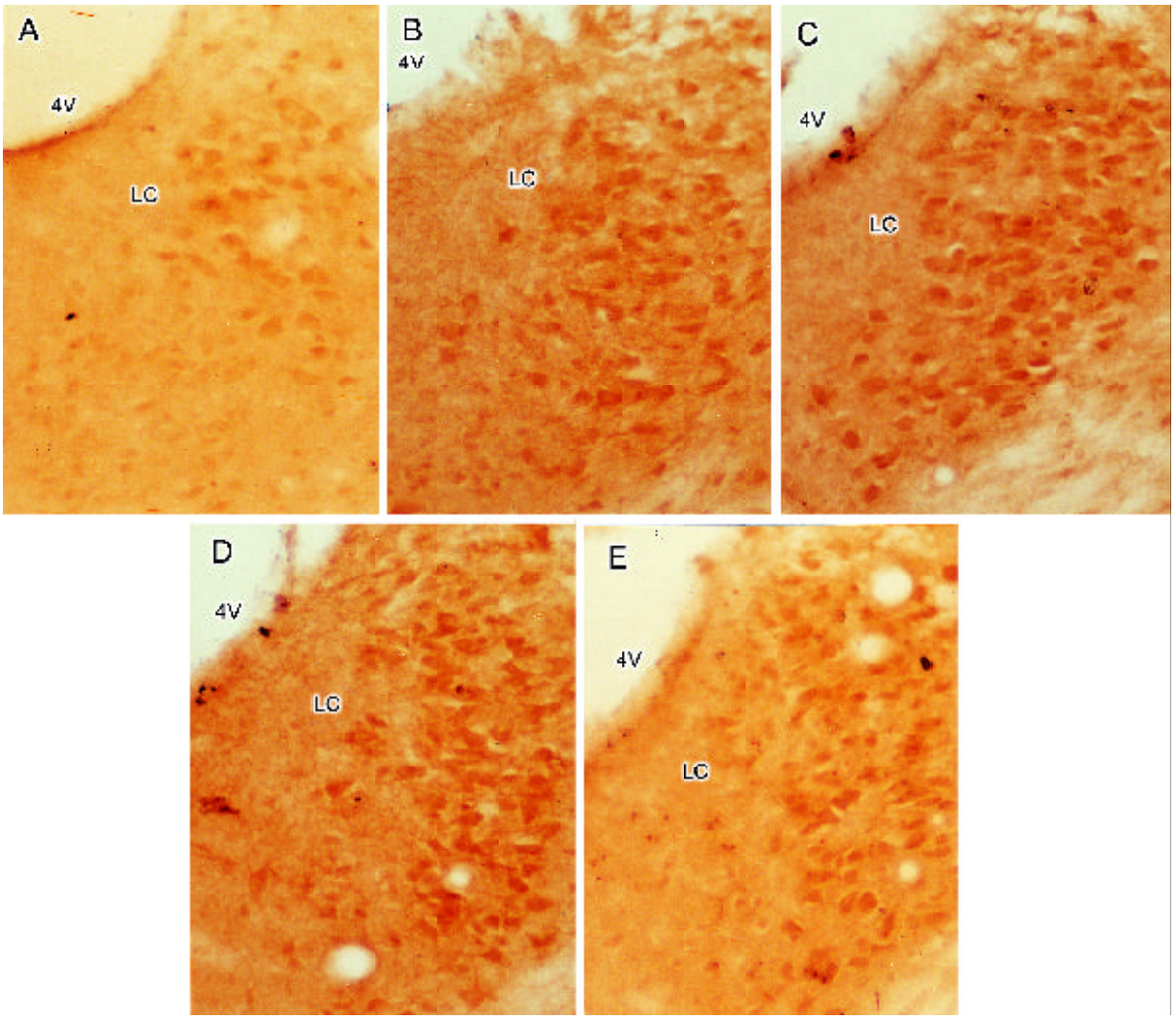

Fig 3. Effects of intrathecal injection of M_1 and M_2 antisense oligonucleotides on the expression of nNOS positive neurons in the LC during morphine withdrawal. A: Control group; B: Dependence group; C: Withdrawal group; D: M_1-AS group; E: M_2-AS group. ×120.

drawal[12]. There are many reports showing that antagonists of *N*-methyl-*D*-aspartate (NMDA) excitatory amino acid (EAA) receptors or inhibitors of EAA release modify tolerance and attenuate or prevent precipitated withdrawal syndrome in morphine-dependent animals[13]. Stimulation of NMDA receptor is the main synthesis pathway for the high expression of nNOS in the LC during morphine dependence and withdrawal. Influx of Ca^{2+} during depolarization of the postsynaptic cell activates a calmodulin-dependent enzyme nNOS, which generates NO. NO acts as a retrograde messenger by diffusing back to the presynaptic terminal where it activates the heme-bearing enzyme guanylate cyclase. The resultant increased level of cGMP then causes enhancement of the release of the transmitter, strengthening the synaptic signal. Consistent with results from our immunohistochemistry studies, morphine dependence and withdrawal induce high expression in both number and intensity of nNOS immunoreactive neurons in the LC.

One proposed hypothesis suggested that the behavioural symptoms of morphine withdrawal be partially attributed to a message wind-up phenomenon from spinal cord to brainstem. In morphine dependent rats, systemic injection of naloxone does provoke enhanced neuronal firing in dorsal root ganglion (DRG)/dorsal horn at spinal cord level, which can be inputted to the nucleus paragigantocellularis (PGi) and the LC by ascending

glutamatergic afferent neurons[14]. Therefore, the enhanced firing in LC neurons underlied the hyperactivity of LC and the expression of withdrawal symptoms during morphine withdrawal. In the present study, intrathecal injection of M_2-AS inhibited the increase of nNOS expression in both number and intensity in LC during morphine-withdrawal, but there was no effect in M_1-AS group. The results suggested that inhibiting M_2 gene expression could decrease the firing input of spinal ascending glutamatergic afferent neurons, and activating M_2 receptors of spinal cord could lead to the hyperactivity of LC during morphine withdrawal.

Zhou *et al* reported that pretreatment with scopolamine decreased the expression of NOS mRNA in spinal cord[6]. Previous behavioral studies showed that the manner of inhibiting morphine withdrawal symptoms with intrathecal injection of NMDA receptor antagonist was similar with muscarinic receptor antagonist[15,16]. It is possible, therefore, that some relationship exists between NMDA/NO/cGMP pathway and cholinergic system in spinal cord during morphine withdrawal. Spinal cholinergic neurons enhance glutamatergic tone to supraspinal regions. In both cases, muscarinic receptor-mediated morphine-withdrawal responses appear to be influenced by a local NO generating system. The most likely scenario is that during withdrawal there is an enhanced release of acetylcholine, which acts upon muscarinic receptor which may located on glutamatergic neurons. The release of glutamate, in turn, activates NMDA receptors on presynaptic terminal where it activates NMDA/NO/cGMP pathway[2]. Certain symptoms, such as wet dog shakes and diarrhea are most likely expressed in response to withdrawal-evoked enhanced autonomic nerve discharge, whereas other behaviour symptoms may be due to activation of ascending spinal pathways, including those to the LC. So, we considered that downregulation of M_2 subtype gene expression indirectly inhibited the activation of glutamatergic neurons in spinal cord, then blocked the firing input ascending to LC which could decrease the expression of nNOS in the LC. Further studies will be required to elucidate the precise mechanisms of communications between spinal muscarinic receptor and supraspinal regions during morphine withdrawal.

In conclusion, our results suggested that M_2 muscarinic receptor of spinal cord mediated increase of nNOS expression in LC during morphine withdrawal.

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吗啡戒断时 M_2 毒蕈碱受体介导大鼠蓝斑核中 nNOS 表达增强

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关键词 吗啡；物质禁断综合征；毒蕈碱受体；一氧化氮合酶；脊髓；蓝斑

目的：观察脊髓不同 M 受体亚型对纳络酮催促吗啡戒断大鼠戒断症状评分以及蓝斑核 nNOS 表达变化的影响．**方法**：采用 nNOS 免疫组织化学、鞘内注射和反义寡核苷酸技术．**结果**：鞘内注射 M_2-AS 可显著减少吗啡戒断症状评分，M_1-AS 虽可部分减轻戒断症状，但总的作用弱于 M_2-AS；吗啡依赖大鼠蓝斑核 nNOS 表达增加，用纳络酮催促戒断后 nNOS 表达进一步增加，鞘内注射 M_2-AS 可抑制在吗啡戒断时蓝斑核 nNOS 的表达增强，而 M_1-AS 对其无影响．**结论**：脊髓 M_2 受体介导吗啡戒断大鼠蓝斑核 nNOS 的表达增加．

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