Microinjection of M₅ muscarinic receptor antisense oligonucleotide into VTA inhibits FosB expression in the NAc and the hippocampus of heroin sensitized rats

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Abstract: Objective To investigate the effect of M₅ muscarinic receptor subtype on the locomotor sensitization induced by heroin priming, and its effect on the FosB expression in the nucleus accumbens (NAc) and the hippocampus in the heroin sensitized rats. **Methods** Locomotor activity was measured every 10 min for 1 h after subcutaneous injection of heroin. FosB expression was assayed by immunohistochemistry, and the antisense oligonucleotides (AS-ONs) targeting M₅ muscarinic receptor was transferred with the lipofectin. **Results** Microinjection of AS-ONs targeting M₅ muscarinic receptor in the ventral tegmental area (VTA) blocked the expression of behavioral sensitization induced by heroin priming in rats. Meanwhile, the expression of FosB-positive neurons in either the NAc or the dentate gyrus (DG) of the hippocampus increased in heroin-induced locomotor sensitized rats. The enhancement of FosB-positive neurons in the NAc or DG could be inhibited by microinjection of M₅ muscarinic receptor AS-ONs into the VTA before the heroin-induced locomotor sensitization was performed. In contrast, microinjection of M₅ muscarinic receptor sense oligonucleotide (S-ONs) into the VTA did not block the expression of behavioral sensitization or the expression of FosB in the NAc or DG in the heroin sensitized rats. **Conclusion** Blocking M₅ muscarinic receptor in the VTA inhibits the expression of heroin-induced locomotor sensitization, which is associated with the regulation of FosB expression in the NAc and hippocampus neurons. M₅ muscarinic receptor may be a useful pharmacological target for the treatment of heroin addiction.

Keywords: Heroin; locomotor activity; muscarinic receptor; FosB; nucleus accumbens; hippocampus

1 Introduction

Sensitization (a progressive enhancement of locomotor activity) to drugs of abuse is produced when the drugs are administered repeatedly in animals. Sensitization to morphine can be sustained for several months even after cessation of drug administration, and is thought to serve as a useful animal model of plasticity and neuroadaptation associated with repeated administration of opioids that have abuse potential. Recent studies show that sensitization has a close relationship with relapse, compulsive drug-seeking and drug-taking behaviors. In the incentive sensitization theory of addiction, it seems that sensitization plays an

important role in compulsive drug-seeking behavior and relapse^[1]. Therefore, investigation of sensitization may help us to better understand the relapse mechanisms and provide new strategies for the treatment of drug addiction.

Heroin-induced locomotor sensitization seems to be mediated by neuroplastic changes in the ventral tegmental area (VTA)^[2]. M₅ muscarinic receptor is the most recent member of the muscarinic acetylcholine receptor family (M₁–M₅) to be cloned. Interestingly, M₅ receptor mRNA has been identified as the only muscarinic receptor mRNA in dopamine-containing neurons of both the substantia nigra and the VTA^[3]. Evidence suggests that acetylcholine-dependent activation of M₅ receptors in dopamine-containing neurons of the VTA might modulate the manifestations of opiate reward and withdrawal^[4]. This hypothesis has been tested in a series of behavioral, biochemical, and neurochemical studies using M₅ receptor-deficient mice (M₅^{-/-} mice) as experimental tools^[4]. It has therefore been pro-

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posed that M_5 receptors may play a role in drug addiction. Owing to lacking ligands that can selectively stimulate or inhibit the M_5 receptor, the physiological roles of this receptor subtype remained obscure.

Oligonucleotides (oligos) are short DNA molecules that can be transported into neurons. Oligos could arrest the translation of new protein by hybridizing with the complementary mRNA^[5]. Injecting oligos into the brain can alter the production of selected proteins and alter the behaviors consistent with the functions of those proteins.

In the present study, an antisense oligonucleotide (AS-ON) of M_5 was infused into the VTA to inhibit the M_5 receptor production. The long-term neuroplastic changes as measured by the FosB protein expression in the nucleus accumbens (NAc) and the hippocampus, both of which were afferent mainly from VTA dopaminergic neurons, were examined.

2 Materials and methods

2.1 Animals and housing conditions Male Sprague-Dawley rats (250–300 g, Experimental Animal Center of Zhejiang Province, China) were housed in a temperature- and humidity-controlled room with a 12/12-h light/dark cycle. All rats acclimated to the environment for 7 d before treatments. Drug treatment and behavioral monitoring began every morning after 8:00 am and ended before 12:00 am. All animal treatments were performed in strict accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. Diacetylmorphine (heroin) was obtained from the National Institute of Forensic Science, China.

The rats were divided randomly into 4 groups. The Her/Ver/Her group received heroin (0.25 mg/kg, s.c.) for consecutive 8 d and withdrawal for 10 d at home cages, then injected lipofectin (vehicle) into the VTA on day 18 and challenged with heroin (0.25 mg/kg, s.c.) on day 19. The Her/M5AS/Her group was treated with heroin as above, except only to inject M_5 muscarinic receptor AS-ONs instead of lipofectin into the VTA on day 18. Comparing the Her/M5AS/Her, the Her/M5S/Her group was treated with M_5 muscarinic receptor sense oligonucleotide (S-ONs) instead of AS-ONs on day 18. The NS/Veh/Her group was administered sterile saline instead of heroin for consecutive 8 d and challenged with heroin on day 19.

2.2 Stereotaxic surgery Rats were anesthetized with 60 mg/kg i.p. sodium pentobarbital before undergoing stereo-

taxic surgery. Animals were placed in stereotaxic instrument (ASI Instruments, USA). Guide cannulae (15 mm) were constructed from 23-gauge stainless steel tubing (Plastic One Inc., VA, USA) and implanted unilaterally into the VTA of cerebral hemisphere using the following stereotaxic coordinates (Paxinos and Watson, 1998^[6]) relative to bregma: AP, -6.0 mm; ML, -1.0 mm; DV, -7.3 mm. Stylets constructed from 30-gauge stainless steel tubing (Plastic One Inc., VA, USA) were inserted into the guide cannulae to maintain patency. Rats were allowed to recover from the surgery for at least 5 d before drug delivery and behavioral monitoring.

2.3 Oligos Oligos were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. The oligo sequences were based on a rat M_5 receptor cDNA sequence. The M_5 sense oligos was a copy of a 21-base sequence of the rat M_5 receptor gene, near the 5' end of the cDNA sequence, including the last 6 bases before the start codon and the next 15 bases in the coding region of the gene. The M_5 antisense oligos was the complement to the M_5 sense one, designed to prevent translation of the M_5 receptor protein by hybridizing with the initiation region of the mRNA transcribed from the M_5 receptor gene^[5]. All oligos had modified phosphodiester backbones. The sequences were as follows: M_5 sense, 5'-GCC AGG ATG GAA GGG GAG TCT-3'; M_5 antisense, 5'-AGA CTC CCC TTC CATCCTGGC-3'.

2.4 Drug administration and behavioral monitoring Rats were acclimated to the activity monitor for 15 min before drug injection. Locomotor activity over a 60-min period was quantified using 8-beam infrared animal activity cages (30 × 30 × 30 cm). In the following experiments, we examined the effect of intra-VTA administration of M_5 muscarinic receptor AS-ONs on the expression of heroin-induced motor sensitization. Rats received 3 treatments during this study: 1) a subcutaneous injection of either heroin (0.25 mg/kg) or sterile saline was administered on day 1–8; 2) unilateral intra-VTA injection of M_5 muscarinic receptor AS-ONs, S-ONs or lipofectin was administered respectively on day 18; 3) 24 h later (day 19), one challenging injection (heroin, 0.25 mg/kg, s.c.) was administered. All intra-VTA infusions were injected unilaterally via the injection cannulae in a volume of 0.5 μ L (2 μ g oligos) over 2 min using a micro-infusion pump (Bioanalytical Systems Inc., USA). The injection cannulae were kept in place for 2 min after infusion to avoid any backflow.

In all experiments, after the drug treatment, rats were placed in acrylic monitoring cages, and motor activity was assessed according to the number of successive infrared beam crossings in the horizontal plane within 10-min intervals over 60 min.

2.5 Location of microinjection cannula tips Rats underwent intracardiac perfusion (see below) immediately after activity monitoring. Brains were removed from the skull, rinsed in ice-cold PBS, and blocked on ice. The midbrain block was frozen separately and mounted in the Optimum Cutting Temperature Compound (Leica Instruments GmbH, Germany). Coronal sections through the VTA (50 μ m) were obtained and Nissl stained to verify correct placement of injection cannula tips. Behavioral and neurochemical data from rats with correctly positioned VTA cannulae were incorporated into the analysis.

2.6 Immunohistochemistry Immediately after behavioral testing, the rats were deeply anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with approximately 200 mL of saline, followed by 200 mL of 4% paraformaldehyde in 0.1 mol/L PB (pH 7.4). The brains were removed, post-fixed overnight, and then stored in 30 % sucrose at 4 °C. Coronal sections (based on Paxinos and Watson, 1998^[6]) of the NAc (30 μ m; from bregma 1.7 mm to 1.5 mm) and the hippocampus (30 μ m; from bregma -3.3 mm to -3.5 mm) were collected. All tissue sections used in this experiment were processed for FosB protein detection.

The sections were rinsed in 0.01 mol/L PBS (three times for 10 min each) and incubated in 0.01 mol/L PBS containing 5% normal goat serum (Vector Laboratories, USA) and 0.2% Triton X-100 (Sigma, USA) for 30 min. The sections were then incubated for 48 h at 4 °C in 0.01 mol/L PBS containing anti-FosB rabbit polyclonal antibody (1:500). The primary antibody used to recognize FosB/ Δ FosB was a polyclonal rabbit antibody (H-75, sc-7203; Santa Cruz Biotechnology Inc., USA). This antibody is raised against the N-terminus of FosB molecule and thus, recognizes both FosB and different isoforms of Δ FosB. After rinsed in 0.01 mol/L PBS, the sections were incubated in 0.01 mol/L PBS containing biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, USA). The sections were rinsed again using 0.01 mol/L PBS and incubated with avidin-biotinylated peroxidase complex (ABC kit, Vector Laboratories, USA) for 1 h. The reaction was terminated by rinsing the sections in 0.01 mol/L PBS. The sections were then incubated in diaminobenzidine for 5 min. Finally, the sections were

mounted onto gelatin-coated slides, dried and dehydrated before cover-slipped. FosB immunoreactivity was quantified using an Olympus Opticals (Olympus BX51, Olympus Optical Co., Japan) microscope attached to an image analysis system (Mcroimage, Olympus Optical Co.). The shape of the sampling area was varied to best conform to the structure being measured with the sampling area ranging 0.10 – 0.20 mm² rectangle. In addition, the dentate gyrus (DG) of the hippocampus was placed in a fixed rectangle area (0.60 mm²).

Results for total FosB-positive (FosB+) counts were presented as mean $\pm$ SEM. Statistical analysis was done by one-way ANOVA, post-hoc comparisons were conducted by using Newman–Keuls tests. $P < 0.05$ was considered significant.

3 Results

3.1 Location of microinjection cannula tips According to the result of sections stained with cresyl violet, it indicated that infusion-cannula tip placement was located in the VTA, generally at the rostral edge of the interpeduncular nucleus (Fig 1).

3.2 Drug diffusion into the VTA To examine the extent of drug diffusion and to verify that the drug was confined to the VTA, blue ink solution was microinfused unilaterally into the VTA according to the injection protocol as described previously. Coronal sections (50 μ m) through the VTA were visualized under a light microscopy (data not shown). That dye indicated that the extent of diffusion was confined to the VTA over study period, suggesting that the drug would remain within the VTA.

3.3 Repeated heroin administration produces locomotor sensitization The cumulative number of beam crossings over 60 min (mean $\pm$ SEM) for each treatment day is shown in Fig. 2A. A significant increase in locomotor activity was found on day 19 compared with day 1 ($P < 0.05$). It suggests that this treatment produces the locomotor sensitization.

3.4 Microinjection of M₅ muscarinic receptor AS-ONs into the VTA blocks the expression of heroin-induced locomotor sensitization Effect of M₅ muscarinic receptor on the expression of heroin-induced locomotor sensitization (Fig. 2B, C) was evaluated by comparing the treatment group (Her/M5AS/Her) with the sensitized rats (Her/Veh/Her). In the Her/M5AS/Her group, M₅ muscarinic receptor AS-ONs treatment reduced heroin-induced locomotor ac-

Fig. 1 Confirmation of cannula placement at the VTA. Coronal sections through the VTA were obtained from all rats. **A:** the representative section stained with cresyl violet acetate. Unilateral guide cannula tracks and arrowhead represents the drug injection site at the VTA. **B:** schematic illustration of the cannula tips (black dots) in the VTA. Numbers to the left of the schematics indicate the distance from bregma (mm).

Fig. 2 Effects of M5 muscarinic receptor AS-ONs on the expression of heroin-induced locomotor sensitization. **A:** Rats received a subcutaneous injection of heroin (0.25 mg/kg) on day 1–8, and day 19, followed by behavioral monitoring for 60 min every day. * $P < 0.01$ vs day 1 ($n = 18$). **B:** Rats ($n = 5–6$) received intra-VTA injection of M5 muscarinic receptor AS-ONs (2 μ g), S-ONs (2 μ g) or vehicle (Veh) respectively on day 18 after subcutaneous injection of heroin or saline (NS) on day 1–8, and challenged on day 19. **C:** Time course of the cumulative number of locomotor activity per 10 min. # $P < 0.05$ vs Ns/Veh/Her; * $P < 0.05$, ** $P > 0.05$ vs Her/Veh/Her. Data were shown as mean $\pm$ SEM.

tivity on day 19 compared with the Her/Veh/Her group ($P < 0.05$, Fig. 2B). At 10, 20, and 30 min time point, the cumulative number of locomotor activity of the Her/M5AS/Her group on day 19 were significantly lower than that of the Her/Veh/Her group ($P < 0.05$, Fig. 2C). But there are no differences between the Her/M5S/Her group and the Her/Her group ($P > 0.05$; Fig. 2C). This result suggests that

blocking M₅ muscarinic receptor in the VTA inhibits the expression of heroin-induced locomotor sensitization.

3.5 Treatment of M₅ muscarinic receptor AS-ONs during the expression of heroin sensitization blocks FosB immunoreactivity in the NAc and DG of the hippocampus Effect of repeated heroin treatment and unilateral intra-VTA injection of M₅ muscarinic receptor AS-ONs on the FosB immu-

Fig. 3 FosB immunoreactivity on the representative coronal sections through the NAc on the challenge day (day 19). Rats received intra-VTA injections of either M₅ muscarinic receptor AS-ONs (2 μ g), S-ONs (2 μ g) or vehicle (Veh) respectively on day 18, following heroin (Her; 0.25 mg/kg, s.c.) or saline (NS) injected on day 1–8. A: NS/Veh/Her group, B: Her/Veh/Her group, C: Her/M₅AS/Her group, D: Her/M₅S/Her group. Note the differences between the groups in FosB immunoreactivity in the NAc shell (NAcS) and NAc core (NAcC) (see Fig. 4 for quantitative analysis). VTA, ventral tegmental area; aCa, anterior commissure. Scale bar, 50 μ m.

noreactivity in the NAc and DG was examined in the rats on day 19 of treatment. Figure 4D and E is a stereotaxic illustration of a coronal section through the DG and NAc. Figure 3 illustrates the distribution pattern of FosB-positive nuclei in the shell (NAcS) and the core (NAcC) regions of the NAc. Cumulative FosB immunoreactivity from multiple subjects for each treatment group is shown in Fig. 4A, B and C.

ANOVA demonstrated significant differences in the number of FosB-positive nuclei between different drug treatment groups in the DG, NAcS and NAcC on day 19 of treatment. *Post-hoc* testing of individual treatment groups in the DG, NAcS and NAcC showed significant differences between treatment groups. Heroin treatment (Her/Veh/Her) produced a significant increase in the number of FosB-

Fig. 4 Quantitative analysis of FosB-positive nuclei in the DG (A), NAcS (B) and NAcC (C) on the challenge day (day 19). Rats received intra-VTA injections of either M₅ muscarinic receptor AS-ONs (2 μ g), AS-ONs (2 μ g / right side, M₅S-oligo) or vehicle (Veh) respectively on day 18 following the subcutaneous injection of heroin (Her, 0.25mg/kg) or saline (NS) on day 1–8. FosB-positive nuclei / 0.1 μ m² on coronal sections (Fig. 3) in brain regions was quantified using a computer-assisted method. Data were shown as mean $\pm$ SEM. A, B and C: [#] $P < 0.05$ vs NS/Veh/Her; ^{*} $P < 0.05$, ^{**} $P > 0.05$ vs Her/Veh/Her. D, E: Schematic coronal section through the DG (D) and NAc (E) illustrating the placement, the rectangle in the DG, NAcS and NAcC, where FosB-positive nuclei were counted. aca, anterior commissure.

positive nuclei compared with rats received saline treatment ($P < 0.05$; Fig. 3A, B). M₅ muscarinic receptor AS-ONs treatment significantly attenuated heroin-induced FosB immunoreactivity in the DG, NAcS and NAcC in the Her/M₅AS/Her group compared with the Her/Veh/Her group ($P < 0.05$; Fig. 4, Fig. 3B, C), while the M₅ muscarinic receptor S-ONs had no effect ($P > 0.05$; Fig. 4, Fig. 3D, B). The number of FosB-stained neurons in the control group (NS/Veh/NS) was at baseline levels (data not shown).

4 Discussion

The finding in the present study was that the repeated heroin administration produced the sensitization of locomotor activity. The expression of sensitization induced by heroin can be blocked by microinjection of M₅ muscarinic receptor AS-ONs, but not by injection of M₅ muscarinic receptor S-ONs, into the VTA. Meanwhile, the expression of FosB in the NAc and the hippocampus could be induced by chronic treatment with heroin, and the enhancement of FosB in the NAc and the hippocampus was inhibited by blocking M₅ muscarinic receptor. The cholinergic system has been implicated in the reinforcing effect of drugs

of abuse^[7]. The cholinergic modulation of midbrain dopaminergic neuron activity may be mediated by the M₅ subtype of muscarinic cholinergic receptors^[3,8]. Dopaminergic VTA neurons receive cholinergic input from the lateral-dorsal tegmental (LDT) and pedunculopontine (PPT) nuclei. Infusion of M₅ receptor AS-ONs into the VTA temporarily increased brain stimulation reward thresholds in the rats^[7]. The generation of M₅^{-/-} mice has provided evidence that M₅ receptors facilitate striatal dopamine release. The prolonged accumbal dopamine release, observed in intact animals after electrical stimulation of the LDT, was absent in M₅^{-/-} mice^[9-11]. The M₅^{-/-} mice exhibited decreased dopamine release and conditioned place preference in response to morphine and blunted withdrawal symptoms compared with wild-type mice^[12], and also decreased cocaine-conditioned place preference and cocaine self-administration in M₅^{-/-} mice^[13,14]. Moreover, the abuse-related effects of morphine were attenuated in the M₅^{-/-} mice relative to the wildtype mice, whereas morphine-induced analgesia remained unchanged^[12]. The present results further support the idea that M₅ muscarinic receptors in the VTA are important for the cholinergic activation of dopamine neurons in

modulating drug addiction.

The analysis of immediate-early gene (IEG) expression provides a useful tool to identify the brain regions that are influenced by a particular environmental or pharmacologic treatment, and has been used extensively in studies addressing the biological bases of animals' responses to drugs of abuse^[15,16]. The various IEGs (*e.g.* *c-fos*, FosB, JunB) studied in this way are characterized by different regional sensitivities to specific stimuli as well as differing time courses of expression^[17,18]. It has been previously reported that the expression of Δ FosB and the chronic Fos-related antigens (FRAs) that are derived from the Δ FosB protein is augmented by chronic treatment with cocaine^[19]. Similarly, other drugs of abuse, as well as other types of chronic treatment, can elicit increased neuronal expression of Δ FosB^[20]. Increasing evidence suggests that Δ FosB is an important transcription factor induced in the brain's reward pathways after chronic exposure to drug abuse. It appears to be a molecular switch for long-term adaptation in the brain^[21,22]. Our data showed the enhancement of FosB expression in the NAc and the hippocampus during heroin repeated administration, suggesting that the transcriptional regulation by FosB gene plays a critical role in heroin-induced behavioral responses. Here, an antibody directed against the N-terminus of the FosB (46 kDa) molecule, from which the smaller Δ FosB fragment is derived, was used in the immunohistochemical analysis to assess the chronic changes of FosB gene expression in the specific nuclei^[23,24]. The Δ FosB protein (33 kDa) and the chronic FRAs (35 – 37 kDa) were not differentiated in the present experiment, both of which can be definitively identified only by using gel-electrophoresis techniques.

The mesolimbic dopamine system is thought to be involved in morphine-induced hyperactivity^[25,26]. The fibers of the NAc and the hippocampus were projected from the dopaminergic neurons of VTA. The present study demonstrated that the heroin-sensitized rats produced a significant increase in the number of FosB-positive nuclei in the DG, NAcS and NAcC. Because FosB is one of the key molecules for neural plasticity induced by chronic external stimuli^[27], the data indicate that the high level expression of FosB-positive nuclei in the DG and NAc are associated with heroin-induced sensitization in rats. The M₅ receptor is the only muscarinic acetylcholine receptor subtype detected in the dopaminergic neurons of VTA, which ascend to the NAc and the hippocampus. The present results found

that infusion M₅ muscarinic receptor AS-ONs in the VTA inhibits the expression of heroin-induced locomotor sensitization, and attenuates heroin-induced FosB immunoreactivity in the DG, NAcS and NAcC. The results suggest that M₅ muscarinic receptor may exert important effect in sensitized responses to drug exposure, possibly by modulating FosB activity in postsynaptic neurons.

In conclusion, both the expression of heroin sensitization and the expression of FosB in the NAc and the hippocampus were inhibited by blocking M₅ muscarinic receptor expression in the VTA. The data provided the evidence that M₅ muscarinic receptor may represent a useful treatment target for heroin addiction.

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中脑腹侧被盖区注射 M₅ 受体反义寡核苷酸抑制海洛因敏化大鼠伏隔核和海马中 FosB 表达

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摘要: **目的** 探讨 M₅ 毒蕈碱受体亚型对海洛因诱导的大鼠行为敏化以及敏化后大脑伏隔核 (NAc) 和海马中 FosB 蛋白表达的影响。**方法** 建立海洛因诱导的大鼠行为敏化模型, 测定大鼠的自主活动量 (locomotor activity, LA), 观察 M₅ 毒蕈碱受体反义寡核苷酸 (M₅AS-ONs) 对行为敏化表达的影响。用免疫组化法测定大鼠 NAc 及海马齿状回 (DG) FosB 蛋白表达。**结果** 海洛因处理组与盐水处理组相比, 大鼠在 1 小时内的 LA 显著增加, 表明这些大鼠已稳定建立了海洛因诱导的敏化。中脑腹侧被盖区 (VTA) 中注射 M₅AS-ONs 能抑制大鼠海洛因行为敏化的表达。海洛因诱导的行为敏化大鼠中 NAc 及 DG 中的 FosB 免疫反应阳性神经元的表达增加, 而在 VTA 内注射 M₅AS-ONs 能明显抑制 NAc 及 DG 中 FosB 阳性神经元表达的增加; 但 VTA 中注射有义寡核苷酸 (M₅S-ONs) 不能明显抑制大鼠行为敏化的表达, 也不能抑制海洛因敏化大鼠 NAc 和 DG 中 FosB 蛋白的表达。**结论** 阻断 VTA 中 M₅ 毒蕈碱受体可抑制海洛因诱导的行为敏化的表达, 其机制可能和抑制大脑 NAc 和 DG 神经元中 FosB 蛋白的激活有关。M₅ 毒蕈碱受体可作为改变海洛因行为学效应的有效药理学靶点之一。

关键词: 海洛因; 自主活动; 毒蕈碱受体; FosB; 伏隔核; 海马