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Ionotropic glutamatergic neurotransmission in the ventral tegmental area modulates ΔFosB expression in the nucleus accumbens and abstinence syndrome in morphine withdrawal rats

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Abstract

The present study sought to assess whether the blockade of ionotropic glutamate receptors in the ventral tegmental area could modulate morphine withdrawal in morphine-dependent rats and the expression of stable ΔFosB isoforms in the nucleus accumbens during morphine withdrawal. Rats were injected (i.p.) with increasing doses of morphine for 1 week to develop physical dependence, and withdrawal was then precipitated by one injection of naloxone (2 mg/kg, i.p.). Abstinence signs such as jumping, wet-dog shake, writhing posture, weight loss, and Gellert-Holtzman scale score were recorded to evaluate naloxone-induced morphine withdrawal. Two ionotropic glutamate receptor antagonists, dizocilpine (MK-801) and 6, 7-dinitroquinnoxaline-2, 3-dione (DNQX), were microinjected unilaterally into the ventral tegmental area 30 min before naloxone precipitation. A second injection of naloxone (2 mg/kg i.p.) was given 1 h after the first naloxone injection to sustain a maximal level of withdrawal so that the expression of stable ΔFosB isoforms in the nucleus accumbens could be measured. This would enable determination of the correlation between the MK-801 or DNQX-induced decrease in somatic withdrawal signs and the change in neuronal activity in the nucleus accumbens. The results showed that both MK-801 and DNQX significantly alleviated all symptoms of morphine withdrawal except for weight loss and reduced the expression of stable ΔFosB isoforms within the nucleus accumbens. These data suggest that ionotropic glutamatergic neurotransmission in the ventral tegmental area regulates the levels of stable ΔFosB isoforms in the nucleus accumbens, which play a very important role in modulating opiate withdrawal.

Keywords: Morphine withdrawal; ΔFosB; Glutamate receptor; DNQX; MK-801; Nucleus accumbens; Ventral tegmental area

1. Introduction

In common with many other abused substances, the abrupt cessation of chronic opiate use results in a well-characterized withdrawal syndrome with symptoms of nausea, dysphoria, and anxiety. The severity of these symptoms is sufficient to drive opiate abusers into relapse. Studies have identified that the mesocorticolimbic dopaminergic pathway, which mainly consists of the ventral tegmental area, the nucleus accumbens and medial prefrontal cortex, is involved in opiate withdrawal (Harris and Aston-Jones, 1994; Koob and Bloom, 1988).

Dopaminergic neurons in the ventral tegmental area receive a glutamatergic input from the medial prefrontal cortex, the pedunculopontine region and the subthalamic nucleus (Kalivas, 1993; White, 1996). Pharmacological activation of ionotropic or metabotropic glutamate receptors in the ventral tegmental area elicits an increase in exploratory motor behavior with dopamine transmitter release in the nucleus accumbens and the medial prefrontal cortex (Suaud-Chagny et al., 1992; Swanson and Kalivas, 2000). Moreover, chronically intermittent

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administration of drugs of abuse, such as cocaine, morphine or alcohol, can increase extracellular levels of glutamate in the ventral tegmental area, and the expression of GluR1 (an α-amino-3-hydroxy-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunit 1) and NMDAR1 (an *N*-methyl-D-aspartic acid (NMDA) receptor subunit 1) in the ventral tegmental area (Fitzgerald et al., 1996; Kalivas and Duffy, 1998; Ortiz et al., 1995). Administration of both NMDA and AMPA receptor antagonists into the ventral tegmental area reduces heroin reinforcement (Xi and Stein, 2002), and the former also reduces the expression of morphine-induced conditioned place preference (Popik and Kolasiewicz, 1999). These data suggest that enhanced glutamate receptor function in the ventral tegmental area is involved in opiate reinforcement. Accumulating evidence suggests that the glutamatergic system might be hyperactive during opioid withdrawal (Hong et al., 1993; Rasmussen, 1995; Sepulveda et al., 1998). Systemic or intracerebroventricular injection of NMDA and AMPA receptor antagonists prevents certain withdrawal symptoms in morphine-dependent rodents (Akaoka and Aston-Jones, 1991; Cappendijk et al., 1993; Popik and Skolnick, 1996; Rasmussen, 1995; Rasmussen et al., 1996; Trujillo and Akil, 1991). However, some studies show that withdrawal behaviors are not reduced by the AMPA-receptor antagonists, GYKI52466 and LY293588 (Fundytus and Coderre, 1994; Mclemore et al., 1997). Because of conflicting information in the literature, further studies are needed to elucidate the function of glutamate in the ventral tegmental area on opiate withdrawal.

The present study examined the role of the nucleus accumbens in the process of opiate withdrawal by assessing whether the opiate withdrawal state was associated with changes in neuronal activity in the nucleus accumbens, and whether any of these changes were reversed if the Gellert-Holtzman Scale score for opiate withdrawal was improved by MK-801 or DNQX microinjection. To accomplish this, we measured withdrawal-induced changes in the expression of ΔFosB, an immediate early gene product, within nucleus accumbens cells. Changes in neuronal activity often result in the induction of immediate early genes that, in turn, promote the synthesis of various intracellular constituents including Fos or Fos-related proteins, as well as FosB, Fra-1, Fra-2 and ΔFosB. Previous studies have described that highly stable 35 and 37 kDa isoforms of ΔFosB, termed chronic Fos-related antigens, are induced in a region-specific manner in the brain in response to several chronic perturbations, but not to acute administration of cocaine (Hope et al., 1994b; Nye et al., 1995; Moratalla et al., 1996a,b), morphine (Nye and Nestler, 1996), nicotine (Pich et al., 1997), antipsychotic drugs (Nye et al., 1995), antidepressant drugs (Hope et al., 1994b), and electroconvulsive seizure (Hope et al., 1994a). The chronic Fos-related antigens are isoforms of ΔFosB, a stable truncated splice variant of FosB that accumulates in the brain after chronic treatment (Chen et al., 1997). These stable isoforms gradually accumulate in the brain with repeated treatment and mediate long-lasting neural and behavioral plasticity. Several studies have used immunohistochemical techniques successfully to identify regions of the central nervous system influenced by specific pharmacological or behavioral manipulations (Grande et al., 2004; Hollen et al., 1997; Morris et al., 2000; Murphy et al., 2003), including morphine withdrawal (Stornetta et al., 1993). We used ΔFosB immunohistochemistry to examine withdrawal-related neuronal activity within the nucleus accumbens, and to determine whether pharmacological stimulation of NMDA or AMPA receptors could interfere with this activity.

2. Materials and methods

2.1. Animals

The experimental protocol was approved by the Institutional Animal Center and Medical School of Xi'an Jiaotong University. Forty-two male Sprague–Dawley rats weighing 240–280 g were acquired from the Medical Experimental Animal Center of Shaanxi Province, China. Animals were housed four to five per cage with free access to food and water for at least 1 week before surgery, under a 12-h light/dark cycle (lights on at 20:00) with controlled temperature (22±2 °C) and humidity (55±5%). After surgery, rats were individually housed.

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Guide cannulae were placed 2 mm dorsal to the right ventral tegmental area (from bregma: AP −5.3 mm, ML+0.7 mm, DV −7.7 mm) (Paxinos and Watson, 1998) and anchored to the skulls. During surgery, the rectal temperature was monitored and kept between 37 and 38 °C by a thermostatically controlled heating pad. After stereotaxic surgery, rats were housed individually and allowed to recover for 7 days before any treatment. Rats were given sodium penicillin (0.2 million units per day, i.p.) during the first 3 days of recovery to prevent wound and intracerebral infections.

2.2. Drugs

Morphine hydrochloride was purchased from the Shenyang Pharmaceutical Factory (Shenyang, China). (+) MK-801 maleate, DNQX, and naloxone hydrochloride were obtained from Sigma (St. Louis, MO, USA). (+) MK-801 maleate, morphine hydrochloride and naloxone hydrochloride were dissolved in sterile saline. DNQX was dissolved in 30 μl 1 N NaOH, diluted with redistilled water, and then pH was balanced with 30 μl 1 N HCl to a final pH of 6.5. Morphine hydrochloride, naloxone hydrochloride and saline were injected i.p. at a volume of 2 ml/kg respectively. (+) MK-801, DNQX and saline were given 0.5 μl/side into the ventral tegmental area at a speed of 0.25 μl/min. The injectors were left in the place for 2 min after solution delivery.

2.3. Experimental procedure

2.3.1. Morphine dependence and withdrawal

An adapted method described by Chou et al. (2002) and Valverde and Roques (1998) was used. Rats received morphine i.p. injections twice a day at 08:30 and 16:30 for 7 days. The daily dosage was 10, 20, 30, 40, 50, 50 or 50 mg/kg/injection respectively. One hour after the last injection, rats were

microinjected with saline, (+) MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) into the ventral tegmental area. Thirty minutes later, rats were given naloxone hydrochloride (2 mg/kg i.p., Xavier et al., 1997; Aricioglu-Kartal et al., 2003) and immediately placed into an observation tank for behavior analysis. Animals were given an additional naloxone (2 mg/kg i.p.) injection 1 h later, which is required to sustain a maximal level of withdrawal for the assay of ΔFosB immunoreactivity. The saline control rats received saline injection instead of morphine on the same schedule. Animals were divided into seven treatment groups as shown in Table 1.

2.3.2. Behavior analysis

Withdrawal intensity was evaluated by assessing 10 withdrawal signs including wall clamber, jumping, wet-dog shakes, writhing posture, weight loss, genital grooming, teeth-chattering, ptosis, diarrhea, and irritability. The characteristics of these behaviors were described by Fernandez-Espejo et al. (1995). The rats were placed into a clear, Plexiglas observation tank (50 cm × 50 cm × 60 cm) and rated for withdrawal severity for 15, 30, 45 min by a rater blind to the treatment. The frequency of wall clamber, jumping, wet-dog shakes, and writhing posture was evaluated; the presence of ptosis, genital grooming, teeth-chattering, irritability, and diarrhea was recorded, and the percentage of weight loss was also calculated.

Furthermore, a score for each group was calculated using a modification of the scale described by Gellert and Holtzman (1978) in which two classes of signs were distinguished. (I) Graded signs were considered as: weight loss (per 1% of weight loss quantified as 1), number of jumps, writhing posture, and wall clamber (1–4 times quantified as 1, 5–9 quantified as 2, and 10 or more quantified as 3), number of body shakes (1–2 quantified as 2, 3 or more quantified as 4). (II) Checked signs, in which only the presence or absence was evaluated, were considered as: diarrhea (2), ptosis (2), teeth-chattering (2), irritability (3) and erection or genital grooming (3).

Non-parametric statistics were performed for behavioral patterns such as wet-dog shakes, jump and writhing posture. Kruskal–Wallis tests were used to assess the variance of the behavioral measures over groups using the medians for times. The two-tailed Mann–Whitney *U*-test was used to examine the differences between groups. Data generated with the Gellert-Holtzman scale and weight loss were analyzed by an analysis of variance (ANOVA) with one factor. When a significant interaction ($P<0.05$) was demonstrated, post hoc Newman–Keuls test was used. Differences were accepted as statistically significant at $P<0.05$.

Table 1
Test group

Groups	Day (1st–7th) administration (i.p.)	VTA administration	Test administration (i.p.)
Control groups			
Saline control (sal+sal+sal, $n=6$)	Saline	0.5 μl saline	3 ml/kg saline
Dependence control (mor+sal+sal, $n=6$)	Morphine	0.5 μl saline	3 ml/kg saline
Withdrawal control (mor+sal+nal, $n=6$)	Morphine	0.5 μl saline	2 mg/kg naloxone
MK-801 treatment groups ($n=6$, each group)			
(Mor+MK 2.5 μg+nal)	Morphine	2.5 μg/0.5 μl MK-801	2 mg/kg naloxone
(Mor+MK 5 μg+nal)	Morphine	5 μg/0.5 μl MK-801	2 mg/kg naloxone
DNQX treatment groups ($n=8$, each group)			
(Mor+DNQX 2 μg+nal)	Morphine	2 μg/0.5 μl DNQX	2 mg/kg naloxone
(Mor+DNQX 4 μg+nal)	Morphine	4 μg/0.5 μl DNQX	2 mg/kg naloxone

2.3.3. Immunohistochemistry for ΔFosB immunoreactivity

Twenty-four hours after the second injection of naloxone, rats were deeply anesthetized with 60 mg/kg sodium pentobarbital and underwent intracardiac perfusion with 200 ml physiological saline followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were then removed, post-fixed for 2 h in the same fixative, and cryoprotected overnight in 30% sucrose in phosphate buffer. The brains were dissected into two parts from the position of the guide cannula. The rostral parts were used for immunohistochemistry. The caudal parts were used for histology.

The rostral parts were subsequently frozen and sectioned coronally into 20-μm slices that were placed immediately into 0.1 M phosphate-buffered saline (PBS). The slices were later incubated with 0.3% hydrogen peroxide in PBS for 30 min at room temperature, rinsed thrice in PBS, and incubated again for 1 h at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton X-100 (Sigma). Then, the sections were incubated overnight at 4 °C in the primary antiserum directed against FosB (H-75) amino-terminus protein (Santa Cruz, sc-7203, USA) diluted in 0.1 M PBS to 1:600 (Hiroi et al., 2002; Hiroi and Graybiel, 1996; Moratalla et al., 1996a,b). The antibody is a rabbit polyclonal antibody raised against a recombinant protein corresponding to the amino acids 75–150 mapping at the amino terminus of FosB of human origin. The incubated sections were washed thrice in PBS and incubated with biotinylated goat anti-rabbit immune globulin G for 1 h at room temperature, then washed thrice in PBS and incubated for 30 min in Avidin–Biotin complex (Santa Cruz, USA). Finally, the sections were washed thrice in PBS and reacted in 0.02% 3,3′-diaminobenzidine (DAB; Santa Cruz, USA) for 15 min and were washed thrice in PBS to stop the staining reaction. The sections were mounted on gelatin-coated slides and air-dried, dehydrated through a graded alcohol series and finally cover-slipped with Permount mounting medium.

2.3.4. Quantitative analysis of immunostaining in the nucleus accumbens

As shown in Fig. 1, three coronal brain sections +2.2, +1.6, and +0.7 mm anterior to bregma (Paxinos and Watson atlas, 1998) were collected and analyzed. The most anterior section represented a level including the rostral pole of the nucleus accumbens. The two posterior sections represented midlevel

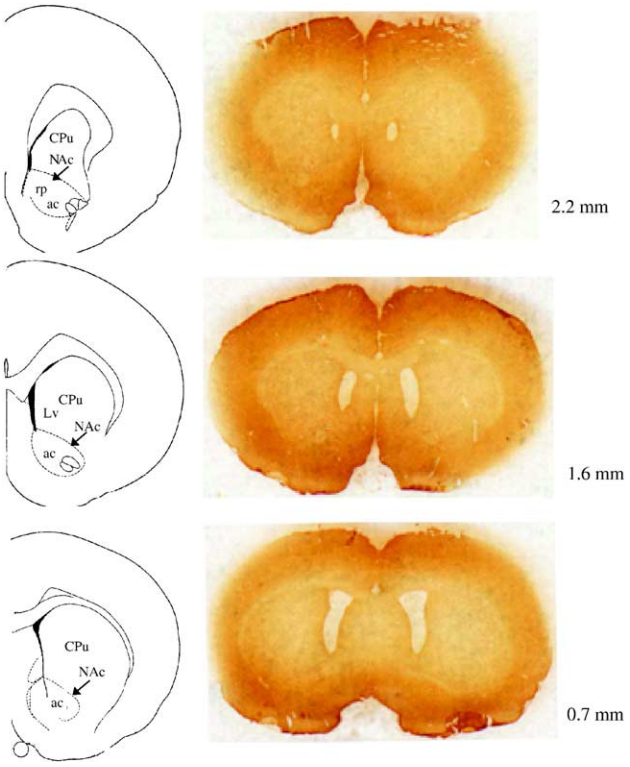


Fig. 1. Schematic (left) and coronal sections stained for the quantification of ΔFosB-positive nuclei. Dashed lines within the sections indicate the boundary of each quantified area. The number to the right of each section corresponds to the longitudinal coordinate in the atlas of Paxinos and Watson (1998). Abbreviations: ac, anterior commissure; CPu, central caudate putamen; NAc, nucleus accumbens.

and extreme caudal levels of the nucleus accumbens in which distinct core and shell regions could be anatomically differentiated under a light microscope with a 10× objective (Alheid and Heimer, 1996; Zahm and Brog, 1992; Carrie et al., 2000). All sections in this study were examined with bright-field illumination using a microscope (OLYMPUS-BX 51, Japan) under 10× objective. Images were captured using a sensicam digital camera (SPOT-Insight QE, American) and imported, as TIFF files, into SigmaScan Pro Image Analysis Software (American). The numbers of ΔFosB-immunoreactive nuclei (Fig. 3) were counted in a blinded manner within a 300 μm×300 μm area at the level of the rostral pole and cores of the nucleus accumbens or a 200 μm×200 μm area at the level of the nucleus accumbens shell (Hussain et al., 2002; Casu et al., 2002). The numbers of ΔFosB-positive nuclei were counted with the aid of SigmaScan Pro Image Analysis Software. Threshold was set at a level to include all labeled nuclei at this threshold. These counts were compared with manual counts carried out in five alternate sections per animal and four randomly selected areas through the nucleus accumbens core and shell. Treatment was considered as the only variable; the correlation between treatment and the number of ΔFosB-labeled nuclei was assessed using an analysis of variance. Data are given as means±S.E.M., where n is the number of sections in each group. Differences were accepted as statistically significant at $P<0.05$. All data were analyzed by one-way analysis of variance (ANOVA). When a significant interaction ($P<0.05$) was demonstrated, post hoc Newman–Keuls test was used.

2.3.5. Histology

The caudal parts (as mentioned in Section 2.3.3) were placed into a buffered 30% sucrose solution for at least 2 days and cut on a cryostat into 50-μm coronal sections. Sections were stained with 0.5% Cresyl violet (Xi and Stein, 2002; Zhou et al., 2000) to determine locations of injector tips (Fig. 2).

3. Results

3.1. Effects of MK-801 and DNQX on morphine withdrawal symptoms in rats

Naloxone administration (2 mg/kg, i.p.) to morphine-dependent rats induced significant withdrawal signs. 15, 30 and 45 min after withdrawal precipitation: the Gellert-Holtzman scores were 25.92±0.62, 24.67±0.91 and 24.42±0.84 ($n=6$), respectively. In comparison, (+) MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) microinjected into the ventral tegmental area 30 min before the naloxone injection significantly reduced the Gellert-Holtzman scores to 14.1±1.17, 15.3±1.24, 14.3±1.15, 14.5±1.41, 13.67±0.80, 13.17+1.01 ($n=6$) and 17.0±1.11, 16.14±1.35, 15.57±1.00, 16.33±1.20, 15.33±0.97, 14.78±0.91 ($n=6$), respectively. Saline microinjection into the ventral tegmental area did not influence the Gellert-Holtzman score in either the morphine-dependent or non-dependent rats. The blockade of morphine

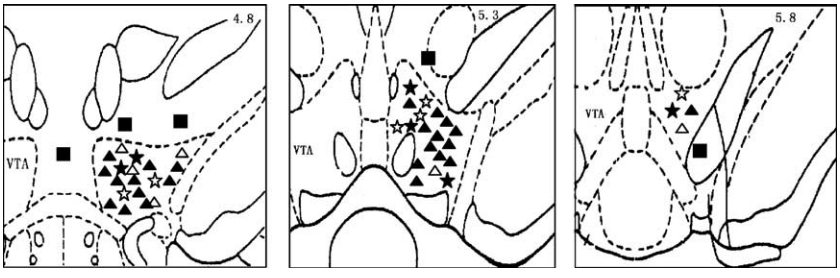


Fig. 2. The locations of injector tips for saline, (+) MK-801, or DNQX injection into the ventral tegmental area (VTA). The pentacles or white triangles represent the locations of saline microinjections in 3 treatment groups; ☆ saline control group, ★ dependence control group, △ withdrawal control group. ▲ represents the locations of MK-801 and DNQX injection. ■ represents injection locations outside of the VTA region, which were not included in statistical analyses. Abbreviations: VTA, ventral tegmental area; MK-801, dizocilpine; DNQX, 6, 7-dinitroquinoxaline-2, 3-dione.

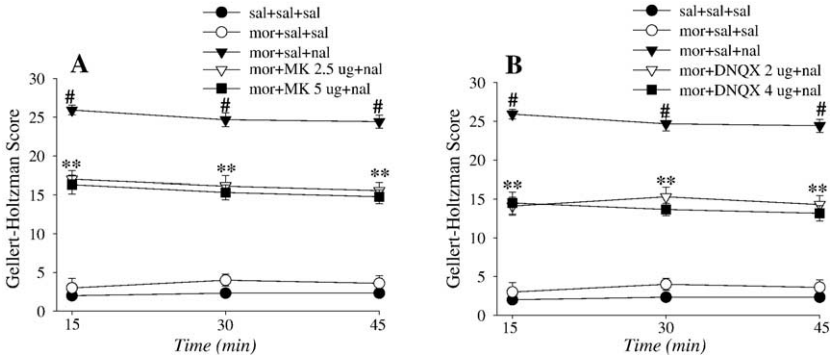


Fig. 3. Effects of MK-801 (A) and DNQX (B) microinjection into the VTA on the naloxone-precipitated increase in the mean Gellert-Holtzman Scale score in morphine-withdrawal rats. # ($P<0.001$) indicates a significant difference compared with the saline (sal+sal+sal) or morphine (mor+sal+sal) injection group; ** ($P<0.01$) indicates a significant difference compared with the naloxone (mor+sal+nal) injection group. Abbreviations: MK-801, dizocilpine; DNQX, 6, 7-dinitroquinoxaline-2, 3-dione.

withdrawal signs by (+) MK-801 or DNQX was not dose dependent, as shown in Fig. 3A, B.

Naloxone (2 mg/kg, i.p.) withdrawal precipitation induced wet-dog shakes in morphine-dependent rats with an incidence of 34.5 (29.0–39.0, $n=6$) within 45 min of the injection. In comparison, (+) MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) injected into the ventral tegmental area 30 min before naloxone precipitation significantly reduced the incidence to 11.5 (7.0–13.0), 6.0 (3.0–10.0) and 9.5 (6.0–10.0), 9.5 (4.0–15.0), respectively (Fig. 4A), though the effects were not dose dependent. No wet-dog shakes were observed after saline injection into the ventral tegmental area either in the morphine-dependent rats or the non-dependent rats.

Naloxone (2 mg/kg, i.p.) precipitation induced writhing posture in morphine-dependent rats with an incidence of 30.0 (26.0–33.0, $n=6$) within 45 min of the injection. Injection of different doses of (+) MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) significantly decreased the incidence of writhing (Fig. 4B) to 18.0 (4.0–28.0), 7.0 (0.0–15.0) and 11.5 (1.0–20.0), 4.5 (0.0–11.0), respectively, but the reductions were not dose dependent. Saline injection into the ventral tegmental area did not induce writhing in either morphine-dependent rats or non-dependent rats.

Naloxone (2 mg/kg, i.p.) precipitation induced jumping behavior in morphine-dependent rats with a jumping incidence of 12.0 (11.0–14.0, $n=6$) at 45 min. Different doses of (+) MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) microinjected into the ventral tegmental area significantly attenuated the jumping behavior in morphine withdrawal rats (Fig. 4C). The incidence of jumping behavior was reduced to 3.0 (0.0–5.0), 3.0 (0.0–3.0) and 0.0 (0.0–3.0), 0.0 (0.0–2.0), respectively. In addition, the inhibiting effect of (+) MK-801 on jumping

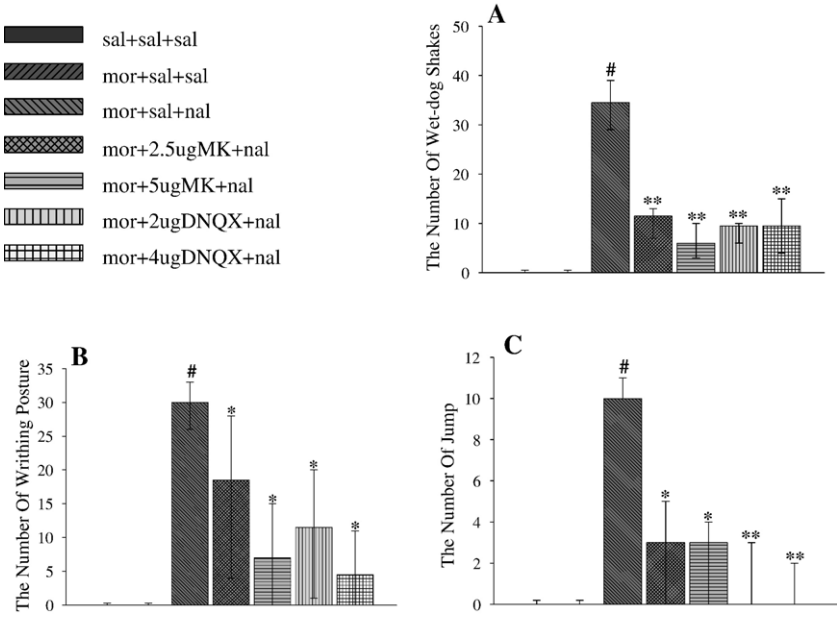
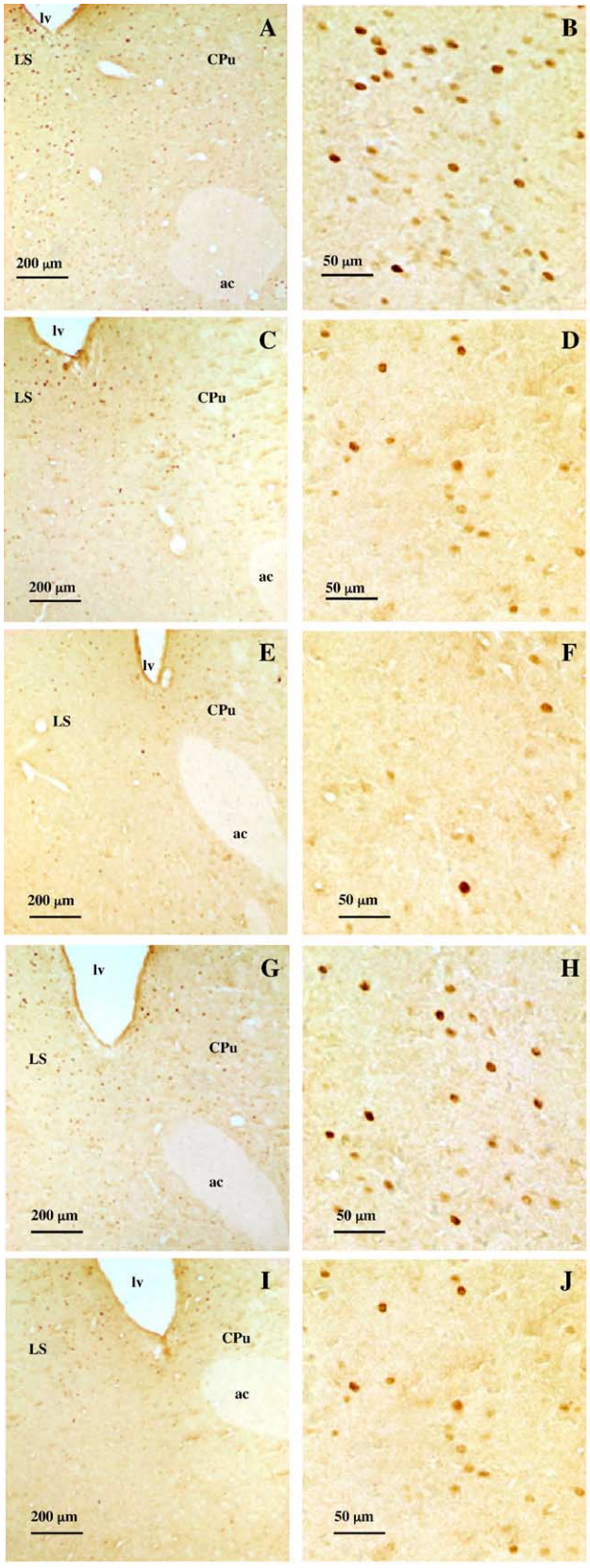


Fig. 4. Effects of MK-801 or DNQX microinjection into the VTA on the naloxone-precipitated increase in wet-dog shakes (A), writhing posture (B), jumping (C). # ($P<0.001$) indicates a significant difference compared with the saline (sal+sal+sal) or morphine (mor+sal+sal) injection group; * ($P<0.05$) or ** ($P<0.01$) indicates a significant difference compared with the naloxone (mor+sal+nal) injection group. Abbreviations: MK-801, dizocilpine; DNQX, 6, 7-dinitroquinoxaline-2, 3-dione.

behavior was dose dependent, as shown in Fig. 4C. No jumping was observed after saline injection into the ventral tegmental area either in the morphine-dependent rats or in the non-dependent rats.



Naloxone (2 mg/kg, i.p.) precipitation induced a significant loss of body weight by 3.67±0.42% (n=6) in morphine-dependent rats within 45 min. MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) pretreatment did not significantly reduce the weight loss caused by withdrawal. The weight loss of these groups was 3.5±0.34%, 3.5±0.34% and 2.5±0.43%, 2.67±0.33%, respectively. Saline microinjection into the ventral tegmental area did not cause weight loss in either morphine-dependent or non-dependent rats.

3.2. Effects of MK-801 and DNQX on withdrawal-induced ΔFosB expression in the nucleus accumbens

Rats subjected to naloxone-precipitated withdrawal after microinjection of saline into the ventral tegmental area showed strong somatic withdrawal responses that were similar to those described in Section 3.1 (Figs. 3 and 4). In contrast, rats receiving a microinjection of MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) into the ventral tegmental area showed significantly lower scores on the Gellert-Holtzman Scale, and a decreased incidence of wet-dog shakes, writhing posture and jump, as shown in Figs. 3 and 4.

Subsequent examination of immunohistochemically labeled sections taken from these animals indicated that the behavioral withdrawal responses were associated with significant increases in the expression of stable ΔFosB isoforms throughout the nucleus accumbens (Fig. 5A, B). Statistical analyses confirmed that opiate withdrawal rats had significantly higher numbers of ΔFosB-positive nuclei in each subregion of the nucleus accumbens examined (the rostral pole, and the core and the shell subregions of each middle level section and caudal section, Fig. 1) relative to the numbers found in sections taken from morphine-dependent rats (Fig. 5C, D) and non-dependent rats (Fig. 5E, F). The F values are: $F_{(1,\ 59)}$=103.86 and $F_{(1,\ 59)}$=47.67 in rostral pole, $F_{(1,\ 59)}$=136.68 and $F_{(1,\ 59)}$=79.58 in middle level core, $F_{(1,\ 59)}$=132.27 and $F_{(1,\ 59)}$=51.61 in middle level shell, $F_{(1,\ 59)}$=139.69 and $F_{(1,\ 59)}$=87.27 in caudal core, $F_{(1,\ 59)}$=154.10 and $F_{(1,\ 59)}$=88.57 in caudal shell; P<0.001 for each comparison. Furthermore, morphine-dependent rats even without naloxone precipitation also had significantly more ΔFosB-positive nuclei in each subregion of the nucleus accumbens bilaterally than did saline control rats. The F values are: $F_{(1,\ 59)}$=92.88 in

Fig. 5. ΔFosB expression within the nucleus accumbens of a morphine-dependent rat subjected to naloxone-precipitated withdrawal 10× (A); 40× (B), relative to that of a morphine-dependent rat not injected with naloxone 10× (C); 40× (D), a saline control group 10× (E); 40× (F), 2.5 μg/0.5μl MK-801 treatment group 10× (G); 40× (H) and 2 μg/0.5μl DNQX treatment group 10× (I); 40× (J). Each photomicrograph displays a coronal section of the dorsomedial nucleus accumbens from right hemisphere, at a level corresponding to the +1.6 mm longitudinal coordinate in the atlas of Paxinos and Watson (1998). Similar differences were observed in the nucleus accumbens sections corresponding to the +2.2 and +0.7 longitudinal coordinates, and when sections from morphine withdrawal rats were compared with sections from dependent- or non-dependent control rats and MK-801 or DNQX treatment rats. The photomicrographs were taken with a 10×, 40× objective, and each scale bar is equal to 200 μm, 50 μm respectively. Abbreviations: CPu, caudate putamen; LS, lateral septum; Lv, lateral ventricle; ac, anterior commissure.

Table 2
ΔFosB expression after treatment in the nucleus accumbens

	Rostral pole	Middle core	Middle shell	Caudal core	Caudal shell
Sal+sal+sal	14.19±0.58	15.61±1.32	4.48±0.39	11.19±1.17	5.29±0.05
Mor+sal+sal	22.45±0.90 [a]	30.32±2.28 [a]	15.39±0.82 [a]	23.94±2.25 [a]	13.26±1.27 [a]
Mor+sal+nal	61.68±5.61 [b]	88.03±6.05 [b]	35.35±2.66 [b]	84.87±6.12 [b]	46.03±3.24 [b]
Mor+MK 2.5 μg+nal	34.13±1.63 [c,d]	84.74±2.23 [e]	23.06±1.35 [d]	81.35±2.26 [e]	15.71±1.11 [d]
Mor+MK 5 μg+nal	38.06±2.69 [c,d]	81.77±2.04 [e]	19.48±1.32 [d]	82.00±2.26 [e]	16.81±1.39 [d]
Mor+DNQX 2 μg+nal	24.42±2.11 [d]	75.00±2.74 [e]	25.94±1.50 [f]	77.87±2.00 [e]	15.29±1.04 [d]
Mor+DNQX 4 μg+nal	25.77±2.19 [d]	86.74±1.45 [e]	17.06±1.15 [d]	82.65±2.04 [e]	15.61±1.20 [d]

[a] $P<0.01$, compared with non-dependent group by Newman–Keuls test.
[b] $P<0.01$, compared with morphine-dependent or non-dependent group by Newman–Keuls test.
[c] $P<0.05$, compared with morphine-dependent group by Newman–Keuls test.
[d] $P<0.01$, compared with morphine withdrawal group by Newman–Keuls test.
[e] $P<0.01$, compared with morphine-dependent group by Newman–Keuls test.
[f] $P<0.05$, compared with morphine withdrawal group by Newman–Keuls test.

rostral pole, $F_{(1, 59)}=31.08$ in middle level core, $F_{(1, 59)}=143.38$ in middle level shell, $F_{(1, 59)}=25.26$ in caudal core, $F_{(1, 59)}=34.36$ in caudal shell; $P<0.01$, as shown in Table 2.

MK-801 (2.5, 5 μg/0.5 μl) microinjection into the ventral tegmental area significantly reduced withdrawal signs scored on the Gellert-Holtzman Scale. Statistical analyses also confirmed that this ΔFosB expression within the rostral pole and shell of the nucleus accumbens was significantly reduced by microinjection of MK-801 into the ventral tegmental area relative to that in sections taken from the morphine withdrawal group (Fig. 5G, H and Table 2). The F values are: $F_{(1, 59)}=14.41$ and $F_{(1, 59)}=22.24$ in rostral pole, $F_{(1, 59)}=28.63$ and $F_{(1, 59)}=17.05$ in middle level shell, and $F_{(1, 59)}=68.53$ and $F_{(1, 59)}=78.21$ in caudal shell; $P<0.001$ for each comparison.

DNQX (2, 4 μg/0.5 μl) microinjection into the ventral tegmental area also significantly reduced ΔFosB expression within the rostral pole and shell of the nucleus accumbens relative to that found in sections taken from morphine withdrawal group (Fig. 5I, J and Table 2). The F values are: $F_{(1, 59)}=38.66$ and $F_{(1, 59)}=32.54$ in rostral pole, $F_{(1, 59)}=9.54$ and $F_{(1, 59)}=19.91$ in middle level shell, and $F_{(1, 59)}=81.49$ and $F_{(1, 59)}=77.35$ in caudal shell; $P<0.001$ (except the middle level in DNQX low-dose group, $P<0.01$) for each comparison.

4. Discussion

A major observation of the present study was that unilateral microinjection of either (+) MK-801 or DNQX into the ventral tegmental area significantly reduced naloxone-induced morphine withdrawal signs, as indicated by a decrease in total Gellert-Holtzman scale score and the incidence of jumping, wet-dog shakes and writhing posture. Our results provide strong evidence to support the involvement of enhanced glutamate activity in the tegmental areas in morphine withdrawal, which has repeatedly but only indirectly been suggested in previous studies. Morphine withdrawal induces glutamate release in the ventral tegmental area and in the nucleus accumbens (Aghajanian et al., 1994; Jhamandas et al., 1996; Tokuyama and Ho, 1996) and increases the expression of NMDA and non-NMDA receptor subunits in the ventral tegmental area (Fitzgerald et al., 1996; Ortiz et al., 1995). In addition, overexpression of GluR1 in the ventral tegmental area induced by viral-mediated gene transfer increases the stimulant and rewarding properties of morphine (Carlezon et al., 1997, 2000), while a lack of the gene-encoding epsilon subunit of the NMDA receptor in mice markedly reduces typical morphine withdrawal behaviors (Inoue et al., 2003). Furthermore, intracerebroventricular or systemic injections of NMDA receptor antagonists (MK-801, memantine and dextromethorphan, etc.) and non-NMDA receptor antagonists (DNQX, CNQX, LY293558 and LY215490) prevent some features of morphine withdrawal in rats and mice (Bristow et al., 1997; Fundytus and Coderre, 1994; Leal et al., 2003; Manning et al., 1996; Popik et al., 1998; Rasmussen and Vandergiff, 2003; Thorat et al., 1994). Taken together, our findings are in line with the hypothesis that endogenous glutamatergic transmission within the ventral tegmental area plays an important role in mediating opiate withdrawal.

The ventral tegmental area is a major source of dopamine in the brain, which is involved in drug abuse. The number and size of dopaminergic neurons in the ventral tegmental area are reduced in morphine-withdrawn rats (Spiga et al., 2003). Dopaminergic neurons in the ventral tegmental area receive glutamatergic inputs from multiple sources and mainly project into the nucleus accumbens and the medial prefrontal cortex, and also project into the lateral hypothalamus and the brainstem, including the locus coeruleus (Swanson, 1982). These target areas are involved in morphine withdrawal syndromes. It is conceivable that the influence of glutamate on morphine withdrawal is mediated by the dopaminergic neurons in the ventral tegmental area and their projections.

A second major finding of this study was that the naloxone-induced expression of stable ΔFosB isoforms within the nucleus accumbens was reduced by ionotropic glutamatergic receptor antagonists (+) MK-801 (2.5, 5 μg/0.5 μl) and DNQX (2, 4 μg/0.5 μl). Unilateral injection of (+) MK-801 or DNQX into the ventral tegmental area suppressed stable ΔFosB isoform expression within the nucleus accumbens. We also found that intermittent escalating doses of morphine without naloxone precipitation produced a significant increase in ΔFosB expression in the nucleus accumbens. These data are consistent with previous reports on the role of morphine in ΔFosB expression (Muller and Uniterwald, 2005; Nye and Nestler, 1996).

Previous withdrawal studies demonstrated robust induction of c-*fos*, FosB, Fra-1, Fra-2 and ΔFosB (33kDa) and stable ΔFosB isoforms (35–37 kDa) within the nucleus accumbens (Rasmussen et al., 1995; Hayward et al., 1990; Carrie et al., 2000; Nye and Nestler, 1996). The expression of c-*fos* and other acute Fos-related antigens is induced rapidly and transiently in the nucleus accumbens since they have short (10–12 h) half-lives (Chen et al., 1997; Nestler et al., 2001). We examined the expression of stable ΔFosB isoforms only because tissue samples were taken from animals at 24 h after the last injection of naloxone hydrochloride. Our results are consistent with the previously reported increase in stable ΔFosB isoforms 6 h after morphine withdrawal (Nye and Nestler, 1996), and the induction of c-*fos* (Rasmussen et al., 1995; Hayward et al., 1990) and Fos-related antigens (Carrie et al., 2000) within the nucleus accumbens during morphine withdrawal.

Our microinjection data together with these previous observations indicate that the opiate withdrawal-associated induction of ΔFosB in the nucleus accumbens is regulated by glutamate activity in the ventral tegmental area, likely mediated by dopaminergic projections. Recent evidence suggests that the NMDA receptor antagonists MK-801, LY274614 and clonidine block c-*fos* expression during morphine withdrawal (Rasmussen et al., 1995; Maeda et al., 2002). Furthermore, GluR1 and GluR2 overexpression (AMPA receptor subunit) reduces the electrophysiological sensitivity of nucleus accumbens neurons to AMPA receptor agonists after chronic cocaine administration. The reduced responsiveness of these neurons to excitatory input may then enhance the response to a drug of abuse (White et al., 1995; Sutton et al., 2003; Tang et al., 2004). Taken together, our data support that glutamatergic and dopaminergic signaling in the brain mediates the expression of immediate early genes in neurons during morphine withdrawal.

Although we have stressed the importance of the ventral tegmental area in the regulation of stable ΔFosB expression, the contribution of other brain regions should not be neglected. Opiate withdrawal stimulates neuronal activation within regions that send excitatory amino acid projections to the nucleus accumbens, such as the amygdala, the prefrontal cortex, and the hippocampus (Hayward et al., 1990; Stornetta et al., 1993; Rasmussen et al., 1995). Increased activity within these projections would lead to heightened excitatory amino acid activity within the nucleus accumbens, and the resulting increase in cellular activation could be responsible for the induction of the stable ΔFosB isoforms in the nucleus accumbens during opiate withdrawal.

The naloxone-induced increase in stable ΔFosB isoforms following withdrawal in morphine-treated rats was observed within the rostral pole and the shell of the nucleus accumbens but not in the core. Coincidently, Kozell and Meshul (2004) recently showed that withdrawal from cocaine enhances glutamate immunoreactivity mainly in the shell of nucleus accumbens. The differential response of the shell and core may be caused by differences in the distribution of dopaminergic and glutamatergic projections and receptor subtypes within the nucleus accumbens. Evidence shows that D1 and D2 receptors on the shell, which receive input from the ventral tegmental area, primarily regulate the neuronal activity of the nucleus accumbens (Heimer et al., 1995; Genfen et al., 1990; Le Moine et al., 1990). This may explain why the nucleus accumbens shell is the main target of other drugs of abuse such as stimulants and alcohol (Bassareo and Di Chiara, 1997; Di Chiara and Imperato, 1988; Pettit and Justice, 1989; Weiss et al., 1992). Another report (Hemby, 2004) also showed that there are differences between nucleus accumbens shell and core in the expression of Calbinded-D28 gene and the regulation of GRIA1 (glutamate receptor ionotropic) and GABA-A α1 receptors induced by morphine.

Glutamate also regulates the expression of c-*fos* family of immediate genes in other brain regions. The glutamatergic regulation of immediate early gene family expression is involved in a more complex regulation like mu opioid receptor–dopamine D1 receptors (Georges et al., 2000; Carrie et al., 2000; Nye et al., 1995) and NMDA or AMPA receptor–dopamine D1 receptor (Liu et al., 1994; Sutton et al., 2003) interactions in striatonigral neurons during withdrawal. Injection of quinolinic acid (an NMDA receptor agonist) into the striatum induced rapid and transient c-*fos* expression, FosB expression from 4 to 18 h, and ΔFosB from 6 h to beyond 30 h in the nucleus accumbens. At 24 h following quinolinic acid injection, there was no immunoreactivity with carboxy-terminal antibody, and less immunoreactivity with amino-terminal antibodies compared with those at earlier times, while ΔFosB was the only species left (Hollen et al., 1997). Further studies are needed to determine whether the effects of glutamate on the expression of these genes are mediated by similar mechanisms in the accumbens and striatum.

The stable ΔFosB isoform expression in the nucleus accumbens induced by morphine withdrawal could be related to a variety of functional changes that occur during withdrawal. It has been shown that the stable ΔFosB isoforms gradually accumulate in the brain with repeated treatment and enhance sensitivity to the behavioral effects of drug of abuse and increase drug-seeking behavior (Kelz et al., 1999; Chen et al., 1998; Hiroi et al., 1997; Nestler et al., 2001). However, the precise roles and mechanisms for ΔFosB expression remain unclear. The expression of ΔFosB may also be coupled with an increase in excitatory amino acid transmission within the nucleus accumbens.

5. Conclusion

MK-801 (2.5, 5 μg/0.5 μl) or DNQX (2, 4 μg/0.5 μl) unilaterally injected into the ventral tegmental area significantly decreased the intensity of most of the physical signs of withdrawal in morphine-dependent rats and reduced stable ΔFosB isoform expression in the nucleus accumbens. Our data support that the ventral tegmental area glutamatergic transmission plays a crucial role in mediating opiate withdrawal syndrome and modulating stable ΔFosB isoform expression in the nucleus accumbens during opiate withdrawal. Our findings suggest the usefulness of NMDA and/or AMPA/KA antagonists in the therapeutic treatment of opiate abuse.

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