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NMDA Receptor in Nucleus Accumbens is Implicated in Morphine Withdrawal in Rats*

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The purpose of the present study is to elucidate whether ketamine, a non-competitive antagonist of the NMDA receptor, can suppress the morphine withdrawal syndrome in rats at a dose without affecting motor functions and to identify its site of action in the central nervous system. Rats were made dependent on morphine by multiple injections of morphine hydrochloride for 5 days. They were then given ketamine at the following doses and routes of administration: (a) intraperitoneal (i.p.) injections (2–16 mg/kg), (b) intracerebroventricular (i.c.v.) injections (4–100 μg), and (c) intra-nucleus accumbens (NAc) or intra-amygdalar microinjections (0.4–10 μg). Naloxone HCl (1 mg/kg, i.p.) was administered 3 h after the last ketamine injection to precipitate withdrawal syndrome, which was scored within a period of 30 min. Results showed that some of the precipitated withdrawal signs were dose-dependently suppressed by repeated injections of ketamine at 8 and 16 mg/kg, i.p. or 100 μg, i.c.v. Dose-dependent suppression was observed by repeated microinjections (0.4–10 μg) of ketamine to NAc, but not to amygdala. These results indicate that the NMDA receptor antagonist ketamine has the ability to suppress morphine withdrawal syndrome in experimental settings without motor interference, and NAc could be the critical CNS site mediating such effect.

KEY WORDS: Ketamine; morphine dependence; NMDA receptor; withdrawal syndrome.

INTRODUCTION

Tolerance to and dependence on morphine have long been shown to be a key issue in the pharmacology of narcotic substances, with extreme theoretical and clinical interest. The involvement of excitatory amino acid and the NMDA receptor in mediating opiate tolerance and dependence have been documented by a series of findings, including the fact that NMDA receptor antagonists such as MK 801 have the ability to inhibit the development of opiate tolerance and physical dependence (1, 2). Several studies have also found that NMDA receptor antagonists can also inhibit the expression of morphine withdrawal syndrome (3,4). However, there have been arguments that these effects of NMDA receptor antagonists are due to their non-specific effects rather than a specific pharmacological action (5,6). One of the major concerns is the motor interference, such as ataxia, muscular weakness and even light general anesthesia, making it hard to determine whether the inhibition of withdrawal signs is owing to a motor dysfunction (7). This concern profoundly affected the development of NMDA receptor antagonists as a pharmacological agent for the treatment of opiate addiction.

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Ketamine, a non-competitive NMDA receptor antagonist, is widely used as an anesthetic in clinical practice, and is now used more and more extensively for pain treatment, especially for the treatment of chronic pain (8–10). To avoid adverse side effects, it is essential to use small repeated doses instead of large single dose in humans (11, 12). This strategy is also useful to increase the therapeutic effect. Thus, a single injection of ketamine (0.08, 0.4, 2, and 10 mg/kg) produced no significant analgesia, whereas repeated injections of 2 and 10 mg/kg showed a significant decrease of the pain scores (13).

Concerning the inhibition of opiate withdrawal signs, Trujillo and Akil (11) have reported that repeated administrations of ketamine (2–8 mg/kg) were able to delay the development of opiate withdrawal symptoms. Koyuncuoglu et al. (14) tried to use ketamine to block the expression of opiate withdrawal signs, but the dosage used (0.5 and 1.0 mg/kg, single injection) seemed too small to be effective.

The aims of the present study were: (a) to find an optimal dose of ketamine in order to obtain best therapeutic effect for suppressing morphine withdrawal signs with minimal aversive side-effects, (b) to identify the time window when the motor interference produced by ketamine, if any, is over, and (c) to locate the critical site of action in CNS for ketamine to exert a presumed pharmacological effect.

EXPERIMENTAL PROCEDURE

Animals and Experimental Design. Adult male Sprague–Dawley (SD) rats weighing 200–250 g were obtained from the Experimental Animal Center, Peking University. They were housed five in a cage with food pellets and water *ad libitum*. The rats were divided into five batches. Thirty rats in the first batch were randomly divided into five groups of six each (one control group and four groups with single i.p. injection of ketamine). Forty-five rats in the second batch were randomly divided into five groups of nine rats (one control group and four groups with repeated i.p. injection of ketamine). Thirty-six rats in the third batch were randomly divided into four groups of nine rats (one control group and three groups with i.c.v. microinjection of ketamine). Sixty-five rats in the fourth batch were randomly divided into five groups of 13 rats (one control group and four groups receiving ketamine microinjection into NAc). Fifty rats in the fifth batch were randomly divided into five groups of 10 rats (one control group and four groups receiving ketamine microinjection into amygdala).

Rat Model for Induction of Morphine Dependence and Scoring of Withdrawal Signs. All rats with the exception of the first batch were injected intraperitoneally (i.p.) with morphine hydrochloride (Qinghai Drug House, China) three times a day (8, 16 and 24 h) at the following doses per injection: 5 mg/kg on the first day, followed by 10, 20, 40 and 50 mg/kg from day 2 to 5. At 8 h on day 6, the rats were given ketamine treatment as follows. Three hours after the last injection of ketamine (Sigma Inc. USA), naloxone hydrochloride (1 mg/kg, Sigma Inc., USA) was injected i.p. and four counted withdrawal signs (jumping, rearing, body shakes/wet-dog shakes, and hand shakes/flying) were scored for 30 min.

Intracerebroventricular (i.c.v.), Intra-NAc and Intra-amygdalar Catheterization and Injection. At 9 h on the fourth day of morphine administration, rats in the third, fourth and fifth batch were anesthetized with chlorohydrate (350 mg/kg, i.p.). Animals were then placed in a stereotaxic apparatus. The skull was exposed and a 0.8 mm gauge guide cannula was directed to (a) lateral ventricle (1.0 mm caudal to bregma, 1.5 mm lateral to the midline, 3.0 mm ventral to the skull surface), (b) NAc (2.5 mm posterior to bregma, 1.5 mm lateral to midline, 5.5 mm deep from the surface of the skull with the tip 1.5 mm above the intended site of injection) and (c) amygdala (5.5 mm posterior to bregma, 3.2 mm lateral to midline, 6.0 mm deep) (8). The guide cannulae were cemented in place. Cannulae were implanted bilaterally, but injections will be made unilaterally at alterative manner.

The rats in batch 2 were given four consecutive i.p. injections of ketamine (2–16 mg/kg), batch 3 i.c.v. injections (4–100 μg), batch 4 intra-NAc injections and batch 5 intra-amygdala injections (0.4–10 μg), respectively with 3 h apart. I.c.v. administration was performed by expelling 10 μl of solution through an injection cannula (0.45 mm gauge) inserted through the guide cannula and protruding an additional 2.0 mm into the ventricular space (15). Intracerebral injection was performed through the injection cannula extending 2.0 mm beyond the tip of cannula, the injection volume being 1 μl to be finished within 5 min via a slow injection apparatus (16).

The cannula placement was verified for each animal by histological examination of the brains after methylene blue injection. Only data obtained from rats with correctly placed cannulae were included for statistical analysis.

Evaluation of Motor Function after Ketamine Injection. To exclude the interference of motor function produced by ketamine, we used the following items of assessment to test the motor function of the rats: (a) Angle of Inclined-Plane (AIP): the inclined plane apparatus consists of two rectangular plywood boards connected at one end by a hinge. One of the boards serves as the base and the other as the movable inclined plane. Two protractor-like plywood side panels with degrees marked on them are fixed onto the base. A rubber mat with ridges 0.6 cm in height was fixed to the surface of the movable plane. For assessment, rats were placed in such a position on the mat that their body axis was perpendicular to the axis of the inclined plane. To maintain themselves on the plane the animals used both their fore and hind limbs. The maximum angle of the plane on which a rat could maintain itself for 5 s was recorded and taken to represent the rat's functional ability (17). (b) Tarlov Scale (TS): the spontaneous activity of the rat in an open field and its motor activity was assessed by the modified TS method described as follows: Grade 0 = complete paralysis of legs; Grade 1 = flicker of movement; Grade 2 = good movement at all joints but without walking or weight-bearing; Grade 3 = walking and weight-bearing, but not normally; Grade 4 = normal (18). The motor activity was assessed before and 3 h after the last injection of ketamine.

Data Analysis. The data were analyzed using the Prism 3.0 statistical software. Continuous data (AIP) are represented as mean ± SEM, and were tested for statistical significance with analysis of two-tail-paired t test or repeated measures ANOVA. Discrete data (scores of morphine withdrawal signs) are represented as median, and were treated with a

Kruscal–Wallis Test. A P value of less than 0.05 was considered statistically significant.

RESULTS

The Effects of i.p. ketamine on Motor Functions in Naive Rats (Batch 1)

(a) *Single Dose Study:* Thirty rats were randomly divided into five groups (n = 6). Four groups were given i.p. injection of ketamine (2, 4, 8 and 16 mg/kg, respectively) and one group with normal saline (NS). The rats were assessed by the AIP test and the TS score before and 10, 30 and 60 min after the injection. Results are shown in Table I. Single dose of 16 mg/kg ketamine significantly decreased the angle of inclined-plane (AIP) ($P < 0.001$) and 4, 8, 16 mg/kg decreased the Tarlov Score (TS) ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively) 10 min after the injection.

(b) *Repeated Doses Study*: Five days after the above-mentioned test, the 30 rats were randomly divided into five groups of six each. Four groups were given four consecutive i.p. injection of ketamine (2, 4, 8 and 16 mg/kg, respectively) with 3 h apart and one group with NS. The AIP test and the TS score were assessed before and 60, 90 min after the last injection. It is evident from Table I that four i.p. injections of 16 mg/kg ketamine produced a significant decrease in AIP ($P < 0.001$) 60 min after he last injection, which recovered 30 min thereafter.

Effect of Repeated i.p. and i.c.v. ketamine on Motor Functions in Morphine Dependent Rats (Batch 2 and 3, respectively)

(a) Forty-five morphine dependent rats were randomly divided into five groups of nine each. Four groups were given four consecutive i.p. injections of ketamine (2, 4, 8, and 16 mg/kg, respectively) and one group with NS, at 3 h intervals. The rats were assessed by the AIP test and the TS score 3 h after the last injection. Results were shown in Table II. There was no statistically significant difference observed between groups on the AIP and TS scores, suggesting that 3 h after the last i.p. administration, ketamine had no significant effect on the motor functions in morphine dependent rats.

(b) Thirty-six morphine dependent rats were randomly divided into four groups of nine each. Three groups were given four consecutive i.c.v. injections of ketamine (4, 20, and 100 μg/10μl) and one group with NS at 3 h intervals. Similar results were obtained (Table II).

Effect of Repeated i.p. and i.c.v. ketamine on Morphine Withdrawal Syndromes in Rats

The morphine dependent rats mentioned above were then given naloxone (1 mg/kg, i.p.), and the four withdrawal signs were scored for 30 min. (a) Figure 1a and 1b shows that repeated i.p. injection of 8 and 16 mg/kg ketamine attenuated jumping

Table I. Effect of Single-Dose and Repeated i.p. ketamine on AIP and TS in Normal Rats

		Dose of ketamine (mg /kg)				
Time after i.p. (min)		NS	2	4	8	16
Single-dose i.p.						
−5	AIP	64.8 ± 1.3	65.3 ± 1.5	63.9 ± 1.8	63.1 ± 2.3	63.2 ± 1.2
	TS	4	4	4	4	4
10	AIP	64.8 ± 0.9	65.5 ± 1.9	64.4 ± 2.2	62.6 ± 2.0	54.2 ± 4.1***
	TS	4	3.6 ± 0.6	3*	2.4 ± 0.6*	2**
30	AIP	64.2 ± 1.2	64.3 ± 1.8	64.3 ± 1.8	63.3 ± 1.6	60.2 ± 2.3
	TS	4	4	4	3.4 ± 0.6	3
60	AIP	64.0 ± 1.0	64.7 ± 1.3	64.4 ± 2.2	63.2 ± 1.8	63.3 ± 1.0
	TS	4	4	4	4	
Repeated i.p.						
−5	AIP	65.7 ± 1.4	66.1 ± 1.4	65.7 ± 1.5	66.1 ± 1.5	66.1 ± 1.6
	TS	4	4	4	4	4
60	AIP	65.7 ± 1.4	65.6 ± 1.3	65.0 ± 1.6	65.6 ± 1.2	64.3 ± 1.5***
	TS	4	4	4	3.6 ± 0.6	3.8 ± 0.5
90	AIP	65.4 ± 1.1	65.5 ± 1.0	65.5 ± 1.0	66.0 ± 1.5	65.7 ± 1.8
	TS	4	4	4	4	4

NS: normal saline; AIP: angle of inclined-plane (°); TS: Tarlov Score. n = 6.* $P < 0.05$,** $P < 0.01$ and*** $P < 0.001$, compared with the volume before injection.

Table II. Effect of Repeated i.p.and i.c.v. ketamine on AIP and TS in Morphine Dependent Rats

Time after injection (min)		Dose of ketamine								
		i.p. (mg/kg)					i.c.v. (μg/10μl)			
		NS	2	4	8	16	0	4	20	100
−5	AIP	63.1 ± 3.2	62.3 ± 3.6	64.5 ± 1.6	64.9 ± 1.9	65.3 ± 1.7	55.5 ± 2.2	56.0 ± 2.2	55.9 ± 2.0	6.0 ± 2.4
	TS	4	4	4	4	4	4	4	4	4
180	AIP	63.6 ± 3.5	61.2 ± 2.9	64.9 ± 1.7	64.3 ± 2.1	64.6 ± 1.8	55.5 ± 2.3	55.7 ± 2.4	56.0 ± 1.8	53.9 ± 3.3
	TS	4	4	4	3.9 ± 0.3	3.7 ± 0.5	4	4	4 ± 0	3.8 ± 0.4

NS: normal saline; AIP: angle of inclined-plane (°); TS: Tarlov Score. n = 9.

and rearing significantly ($P < 0.001$ and $P < 0.01$); 16 mg/kg ketamine attenuated body-shakes significantly (Fig. 1c, $P < 0.05$). (b) Repeated i.c.v. injection of 100 μg ketamine attenuated jumping and rearing significantly ($P < 0.05$); Body-shakes were slightly attenuated by ketamine at 100 μg dose, but the difference did not reach statistically significant level (Fig. 2a, 2b, 2c).

Effect of Repeated Intra-NAc and Intra-Amygdalar ketamine Injection on Morphine Withdrawal Syndromes

(a) Fifty-nine morphine dependent rats (batch 4) were randomly divided into five groups (n = 10–13). Four groups were given intra-NAc injections of ketamine (0.4, 2, 10, and 50 μg, respectively) and one group with NS for four times at 3 h intervals. The animals were then given naloxone, and withdrawal signs were observed as described previously. Results are shown in Fig 3. 10 and 2 μg ketamine attenuated jumping significantly (Fig. 3a; $P < 0.01$ and $P < 0.05$); 50 and 10 μg ketamine attenuated rearing significantly (Fig. 3b; $P < 0.05$ and $P < 0.05$); 50, 10 and 2 μg ketamine attenuated body-shakes significantly (Fig. 3c; $P < 0.01$, $P < 0.05$, $P < 0.05$); 50, 10 and 0.4 μg ketamine attenuated hand-shakes significantly (Fig. 3d; $P < 0.01$, $P < 0.001$, $P < 0.01$).

(b) Forty-seven morphine dependent rats were randomly divided into five groups (n = 9–10). Four groups were given *intra-amygdala* injections of ketamine (0.4, 2, 10, and 50 μg) and one group with NS for four times at 3 h intervals. The animals were then given naloxone, and withdrawal signs observed as described above. There were no significant differences observed between the groups during the 30 min observation period (Fig. 4).

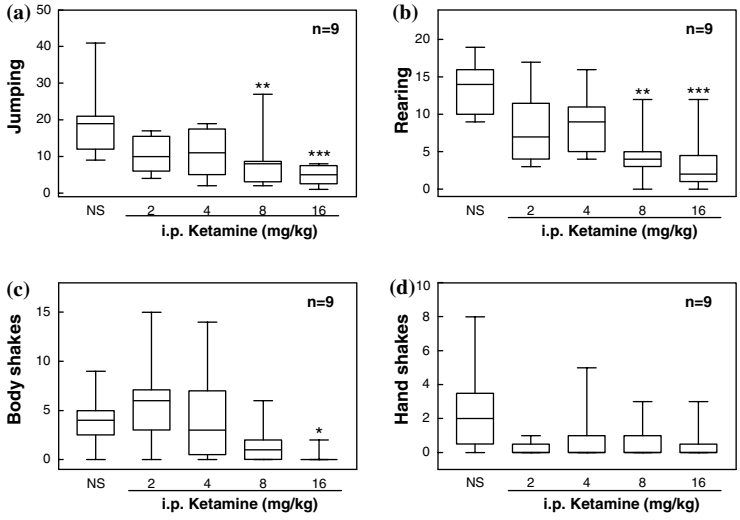

Fig. 1. Effect of repeated i.p. ketamine on naloxone-induced morphine withdrawal signs in rats. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ respectively, compared with NS group.

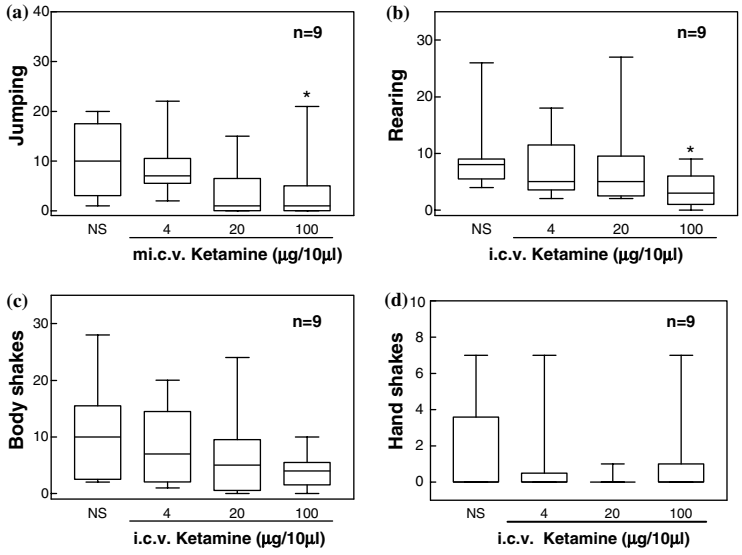

Fig. 2. Effect of repeated i.c.v. ketamine on naloxone-induced morphine withdrawal signs in rats. $*P < 0.05$ and $**P < 0.01$, respectively, compared with NS group.

DISCUSSION

One of the major concerns of using NMDA receptor antagonists for the treatment of opiate tolerance and physical dependence is their side-effects, especially those on motor system, such as stereotype, incoordination and ataxia (19), since it would be hard to differentiate whether the inhibition of withdrawal signs is due to its interference with motor functions. Furthermore, neurotoxicity has also been reported (20). On the other hand, however, clinical use of ketamine showed potential therapeutic efficacy for the treatment of addiction (21). So it is worthwhile to explore whether it is possible to dissociate the therapeutic effect of ketamine with its aversive side-effects.

Concerning the side effects on motor system, first of all, one should determine the dose of katamine to be used. Hetzler et al. (22) observed locomotor effect of ketamine on rats with open

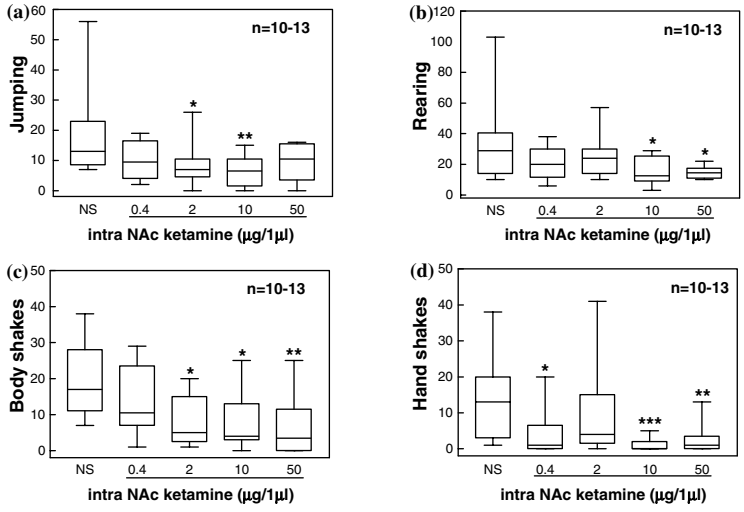

Fig. 3. Effect of repeated microinjection of ketamine to NAc on naloxone-induced morphine withdrawal signs in rats. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, respectively, compared with NS group.

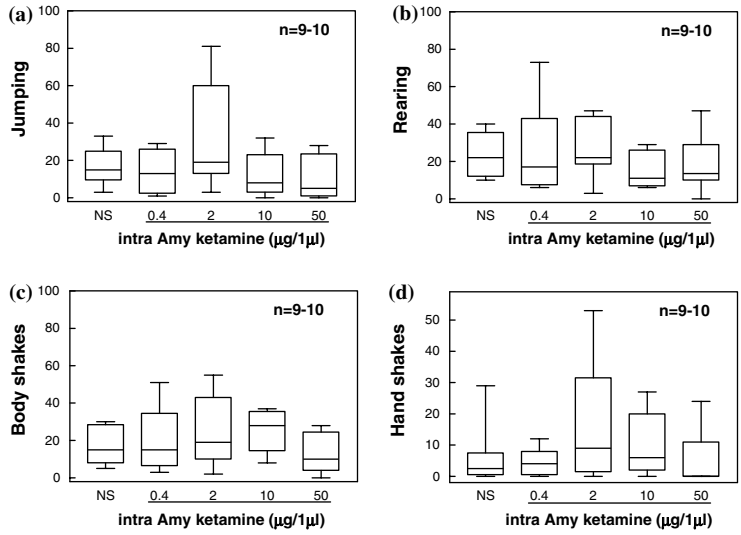

Fig. 4. Effect of repeated microinjection of ketamine to Amygdala on naloxone-induced morphine withdrawal signs in rats.

field setup. They found that single injection of 50 mg/kg produced an increase in locomotion, which peaked at 30 min, whereas 100 mg/kg produced cataleptic immobility. Repeated administration of ketamine at 10 mg/kg dose five times a week over 4 weeks showed no apparent locomotor effects in rats (13,23). We have thus selected a dose range between 2 and 16 mg/kg and made a careful survey on its effect on motor system. Since the effect of ketamine is short lasted and the phenomenon of drug withdrawal is relatively long lasting, we also tried repeated injections for four times at 3 h intervals.

Motor function is assessed via two approaches, AIP test and the scores on a modified TS (24, 25). The AIP test was developed for the assessment of motor function in rats after experimental spinal cord injury. It has some advantages as follows: (a) providing a quantitative assessment to motor function, (b) being extremely easy to construct and inexpensive, (c) requiring less than 1 min to perform on each animal, (d) being completely non-invasive and being capable of repeating as frequently as desired. The AIP test could thus be a valuable assessment for this purpose (17). To assess limbs deficit during locomotion, a modified method of the scale described by Tarlov was also used (18).

Our results present evidence that single dose of i.p. ketamine at 4–16 mg/kg showed very brief impairment of motor functions in normal rats 10 min after injection, which disappeared in 30 min. The results of repeated administration of ketamine (four times with interval of 3 h) indicate that the motor functions recovered 90 min after the last injection (Table I). These findings suggested that choosing a proper time period after administration of ketamine (3 h after the last injection in the present case) could be a good way to exclude the locomotive side effects.

Results of the present study demonstrated that some withdrawal signs were dose-dependently suppressed by repeated i.p. ketamine (8 and 16 mg/kg × 4) or i.c.v. ketamine (100 μg × 4). It suggests that ketamine may act at the brain to suppress withdrawal signs (Figs. 1 and 2). Biochemical, behavioral, and electrophysiologic studies indicate that activation of the noradrenergic cells in the locus coeruleus (LC) plays an important role in the expression of opiate withdrawal (26–28). For example, in opiate-dependent rats, there is a marked increase in the firing of LC neurons during naloxone-precipitated withdrawal. There are several lines of evidence supporting a role for excitatory amino acid afferents to the LC in the withdrawal activation of LC neurons (27,29). However, NMDA receptors seem to play at most a minor role in the withdrawal-induced activation of the LC by glutamate whereas AMPA receptors play an important role. This explains why the naloxone-induced increase in norepinephrine turnover and LC neuronal firing is not reversed by NMDA receptor antagonists (30). Therefore, it has been proposed that excitatory amino acid released elsewhere in the brain might mediate the morphine withdrawal syndrome (29). For the reasons

mentioned above, LC was not chosen as a target site in the present study.

If NMDA antagonists suppress some morphine withdrawal symptoms without affecting the activity of the LC, where would be the NMDA antagonists acting to inhibit morphine withdrawal symptoms? It is interesting to note that the NAc and the amygdala have been suggested to play important roles in the aversive effects of opiate withdrawal (31–33), and pretreatment with noncompetitive NMDA antagonist MK801, the competitive NMDA antagonist LY274614 or the alpha2-adrenergic agonist clonidine blocks morphine withdrawal-induced increased *c-fos* expression in the amygdala or/and NAc (34, 35). So we chose these two nuclei as the candidate sites to elucidate, at least partially, the neuroanatomy and neurophysiology underlying morphine withdrawal.

Morphine injections increase the expression of the immediate early genes c-*fos* and *ju*n-B in several brain areas including NAc (36) and chronic morphine administration augmented the expression of Fos-related antigens and decreased cAMP responsive element binding protein (CREB) in the NAc in rats (37–40). A single injection of morphine decreased glutamate and aspartate in NAc, and this effect disappeared after repeated morphine injections. In contrast, naloxone injections to morphine-dependent rats increased glutamate and aspartate release in the NAc (41). All these evidence suggest that excitatory amino acid release in the NAc might play a role in morphine withdrawal. To our knowledge, experiments using direct administration of NMDA receptor antagonist to the NAc and amygdala have not been reported in morphine-dependent animals.

Figure 3 shows that repeated microinjections of ketamine (0.4, 2, 10, and 50 μg) to NAc dose-dependently suppressed some of the withdrawal signs. The mean effective dose (4 μg) is about 1% of the dose of i.p. administration (2–4 mg/rat i.p. versus 4 μg/rat in NAc), suggesting that NAc could be the critical CNS site for ketamine to suppress withdrawal signs. No such effect however was observed in amygdala (up to 50 μg of ketamine), indicating that NMDA receptors in amygdala may not be involved in the inhibition of the expression of morphine withdrawal sings.

While ketamine may be developed as a useful means to treat physical dependence to morphine, it is premature to recommend it for clinical use at the present stage, because of the potential damage to the brain. Compared to MK-801 that was reported to cause serious brain damage (42, 43), ketamine is much milder in this regard. Recently Hayashi et al. (44) reported that six doses of ketamine (25 mg/kg) administered at 90-min intervals over 9 h increased degenerating neurones in seven out of 10 brain regions, suggesting that repeated administration of ketamine may lead to neuronal degeneration in the developing rat brain. More studies are needed to clarify this point before consideration of its extensive clinical application.

Results obtained in the present study show that (a) ketamine is effective in suppressing the expression of morphine withdrawal symptoms in rats, (b) it is unlikely that this is due to the interference on motor functions, (c) NMDA receptors in nucleus accumbens, but not amygdale, seem to play an important role in mediating the therapeutic effect of ketamine on inhibiting morphine withdrawal symptoms in the rat.

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