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## Naltrexone-precipitated morphine withdrawal in infant rat is attenuated by acute administration of NOS inhibitors but not NMDA receptor antagonists

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**Abstract** *Rationale:* There is increasing evidence that the *N*-methyl-D-aspartate (NMDA) receptor and the nitric oxide system are involved in opiate dependence in the adult rat, but whether these results in the adult apply to the infant rat is unknown. *Objectives:* Here we examined the effects of NMDA receptor antagonists and nitric oxide synthase (NOS) inhibitors, which reduce the opiate abstinence syndrome in adult animals, on morphine withdrawal in the infant rat. *Methods:* Neonatal rats were injected with morphine sulfate (10.0 mg/kg) twice daily for 6.5 days. On the 7th day, pups were injected with NOS inhibitors (L-NAME or 7-NI), NMDA receptor antagonists (MK-801 or AP-5), or vehicle. After 15 min, the pups were injected with naltrexone (1 mg/kg) to precipitate withdrawal. Behavior for each pup was identified and recorded every 15 s for 10 min before naltrexone injection and 15 min after naltrexone injection. *Results:* Both L-NAME and 7-NI significantly reduced most withdrawal behaviors in the infant rat, a result in line with previous studies in the adult rat. In contrast, AP-5 reduced some withdrawal behaviors but also increased others (e.g., moving paws). MK-801 was likewise ineffective in reducing most withdrawal behaviors and increased certain withdrawal behaviors (walking and wall climbing). *Conclusions:* In the infant rat, the production of nitric oxide is involved in opiate withdrawal whereas the NMDA receptor may not yet be functionally active or may play only a minor role.

**Key words** Opiate withdrawal · Neonatal · 7-NI · L-NAME · MK-801 · AP-5

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### Introduction

There is a large population of opiate-addicted childbearing-aged women in the United States and each year unknown numbers of infants are born to these women (Hutchings 1987). These human infants, born to mothers who are exposed to opiate drugs such as heroin during their pregnancy, have a higher incidence of morbidity and mortality than do the offspring of nonaddicted women (Naeye et al. 1973; Connaughton et al. 1977; Wilson et al. 1979). Although there is much research on opiate withdrawal in adult animals, there are few studies in the neonates (Barr and Jones 1994). Recent studies have shown that when the behavioral repertoire appropriate to the age of the animal is examined, infant rat pups experience opiate tolerance and dependence if the dams are exposed to opiates during their pregnancy or the pups are treated directly with opiates (Jones and Barr 1995; Windh et al. 1995; Thornton and Smith 1997; Thornton et al. 1997; Barr et al. 1998).

In spite of decades of research, the understanding of the mechanisms underlying opiate tolerance, physical dependence, and withdrawal is still very limited (Trujillo 1999). It has been suggested that multiple mechanisms operate in these processes (Thorat et al. 1994). Recently, there have been a number of reports from a variety of laboratories indicating that *N*-methyl-D-aspartate (NMDA) receptor antagonists (both noncompetitive and competitive) (Akaoka and Aston-Jones 1991; Trujillo and Akil 1991a, 1991b) and nitric oxide synthase (NOS) inhibitors (Adams et al. 1993; Cappendijk et al. 1993; Kimes et al. 1993; Vaupel et al. 1995a, 1995b) can inhibit the development and expression of mu opioid dependence in the adult rodent. It has been hypothesized that NMDA receptor, its second messenger system, and the production of nitric oxide (NO) via the activation of the NMDA receptor play pivotal roles (Elliott et al. 1995; Thorat et al. 1994; Mao et al. 1995; Inturrisi 1997; Mayer and Mao 1999; Trujillo 1995, 1999; Vaupel et al. 1995a, 1995b).

It is not known whether these mechanisms in adults apply to infants. The neonatal CNS is both structurally

and functionally different from that of the adult, and significant changes in opioid actions occur both prenatally and postnatally (Barr 1992, 1993; Fitzgerald 1995). At the same time, the NMDA receptor, which is believed to play an essential role in opiate dependence, undergoes qualitative and quantitative changes during development (Kalb et al. 1992; Hori and Kanda 1994; Kalb and Fox 1997). There are significant developmental alterations both in the density of the receptor (Tremblay et al. 1988; Morin et al. 1989; Represa et al. 1989) and in the sensitivity to magnesium (Ben-Ari et al. 1988; Bowe and Nadler 1990; Morrisett et al. 1990) of the NMDA receptor during the course of development. Thus, the pharmacological effects of NMDA antagonists and NOS inhibitors in the infant may not be necessarily comparable to those in the adult. In fact, there is at least one study (Bell and Beglan 1995) reporting negative results of the effects of an NMDA receptor antagonist (MK-801) on the development of tolerance to morphine in the neonatal rat, which is in contrast to reports in the adult rat (Trujillo and Akil 1991a, 1991b).

The present studies investigated the pharmacological effects of acute administration of MK-801, a noncompetitive NMDA receptor antagonist (Trujillo and Akil 1991a), AP-5, a competitive NMDA antagonist (Akaoka and Aston-Jones 1991), *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor (Kimes et al. 1993; Vaupel et al. 1995a, 1995b), and 7-nitroindazole (7-NI), a neural selective NOS inhibitor (Kimes et al. 1993; Vaupel et al. 1995a, 1995b), on the naltrexone-precipitated morphine withdrawal in the 7-day-old rat pup. Although these compounds were reported to inhibit both the development and the expression of opiate dependence, or withdrawal in the adult (Herman et al. 1995), the present studies focused attention on their potential in blocking various withdrawal signs in the neonatal rat. It was our hypothesis that these drugs should attenuate opiate withdrawal behaviors in the infant rat, even though any direct and meaningful comparison with the adult may be difficult since withdrawal behaviors displayed by the infant rat are very different from those of the adult rat (Jones and Barr 1995; Windh et al. 1995; Thornton et al. 1997).

## General materials and methods

### Subjects

All animal procedures were in accordance with the "Principles of laboratory animal care" (NIH publication, 1996). The subjects were the offspring of Long-Evans hooded rats bred in our laboratory. Parent animals were housed in plastic tubs with wood chips in a colony room maintained at 22–24°C on a 12-h light/12-h dark photocycle with light onset at 7 a.m. The breeding colony existed in a separate room with minimal disturbances except for normal cleaning, feeding and record keeping. Parent animals had Purina Lab Chow (Purina 5012) and water available ad libitum. Cages were checked twice daily, at approximately 10 a.m. and 6 p.m. Pups found at either time were termed 0 days of age. After parturition, litters were culled to ten pups without regard for the ratio of males to females.

**Table 1** Behavioral definitions

Behavior	Definition
Head moves	Lateral and rotary motions of the head
Moving paws	Continuous movement of the hindpaws without walking
Quiet	Sedated appearance with no movement
Rolling	Turning the body over at least one full rotation
Together	Bodily contact with one or more littermates
Walking	Taking more than one step forward
Wall climbing	Placing at least two forepaws on the wall of the observation chamber

### Morphine treatment and behavior observation

Pups were tattooed with India ink (Geller and Geller 1966), which was injected into one or two paws to label individual pups permanently in each litter. To induce morphine dependence, all pups in a litter were removed from the dam and individual rats were injected with morphine sulfate (IP, 10 mg/kg) twice daily (10 a.m. and 6 p.m.) for 6.5 days. The last injection was in the morning of the 7th day. In the afternoon of the 7th day, animals were transported from the animal facility to our laboratory in plastic tubs with wood chip bedding and placed in an observation chamber maintained at approximately 33°C. Pups were weighed and subjected to various drug treatment procedures (detailed in experiment 1 and experiment 2). The order of treatment conditions was assigned randomly within each experiment. After the drug treatment, the pup was then placed back into the observation chamber with the remainder of the litter (without the dam). Fifteen minutes later, naltrexone was injected to precipitate withdrawal. The behavior of the pups was observed for 10 min before the injection of naltrexone (5 min after the treatment with test drugs) and 15 min after the injection of naltrexone. During these observation periods, the behavior of the pups was identified every 15 s and recorded on a checklist by an observer (see Table 1 for definitions of behavior included in the checklist). When the observation for a specific pup ended, the pup was anesthetized and placed back into the litter to keep the litter size unchanged. The next pup was then tested until all treatment groups were completed. Animals were sacrificed with sodium pentobarbital at the conclusion of the experiments.

## Experiment 1

### Design and procedure

As described, in the afternoon of the 7th day, morphine-dependent rat pups were transported from the animal facility to our laboratory. The pups were treated with saline (IP, *n*=16, control for MK-801 and L-NAME, or ICV, *n*=8, control for AP-5), dimethylsulfoxide (DMSO) (IP, *n*=8, control for 7-NI), MK-801 (IP, 0.01, 0.05, 0.25 mg/kg, *n*=8 for each dose), AP-5 (ICV, 3, 30, 300 nmol, *n*=8 for each dose except for 300 nmol, which was terminated after four pups), L-NAME (IP, 15, 75, 375 mg/kg, *n*=8 for each dose), or 7-NI (IP, 5, 15, 45 mg/kg, *n*=8 for each dose), respectively. For each specific litter (*N*=16), eight pups were used. MK-801 and L-NAME, including the respective control groups, were tested concurrently in the same litter. AP5 and 7-NI, including the respective control groups, were also tested concurrently in the same litter. All drugs were delivered via IP injection except AP-5 (and its control group),

which was delivered via ICV injection (Carden et al. 1991). AP-5 is not effective in passing the blood-brain barrier, and comparable studies (Akaoka and Aston-Jones 1991) used similar techniques to deliver this compound directly into the CNS. During the ICV injection, the pup was held with its head gently bent forward and down. A beveled 30-gauge needle attached to a Hamilton syringe was introduced into the cisterna magna and 4  $\mu$ l of the drug was injected over a 30-s period. The needle was left in position for another 30 s, then removed. Control groups received the same volume of saline in a similar manner. We did not deliberately match the number of males and females in each treatment group since previous studies (Jones and Barr 1995) on the opiate withdrawal in the 7-day-old rat show that gender is not a factor in opiate withdrawal in rats of this age. Fifteen minutes after pre-treatment, the pup was injected with naltrexone (IP, 1.0 mg/kg) to precipitate withdrawal and returned to the litter. Behavior of the pup was observed for 10 min before the treatment of naltrexone (pre-naltrexone period) and 15 min after naltrexone injection (post-naltrexone period). Data collected from the pre-naltrexone period were used to analyze the effects of the drugs on the morphine-dependent rat without the influence of naltrexone. Any potential order effect was minimized since the doses were randomly assigned (Latin square design) and the observer was blind to doses and drugs. We did not include saline control groups for morphine treatment because previous reports by our laboratory (Jones and Barr 1995) and others (Windh et al. 1995; Thornton and Smith 1997; Thornton et al. 1997) had clearly demonstrated that saline-treated rat pups of comparable age basically remain quiet and do not display withdrawal behaviors.

## Statistics

Separate statistical analyses were conducted for the four drugs. For each behavior, a one-way analysis of variance (ANOVA) was conducted for the pre-naltrexone period and a two-way ANOVA was conducted for the post-naltrexone period. For the data collected in the pre-naltrex-

one period, behavior occurrences were summed for the 10-min observation period. For the data collected in the post-naltrexone period, the 15-min observation was divided into three different time periods, each consisting of 5 min; behaviors were summed in each time period, which was treated as a within-subjects variable. For both pre-naltrexone and post-naltrexone periods, all doses of the same drug were injected within a single litter, and the drug dose effect was treated as a within-subjects variable.

## Results

### *NOS inhibitors*

#### Pre-naltrexone

Treatment with various doses of 7-NI and L-NAME did not significantly alter the behaviors of the morphine-dependent rat (Table 2). Except for some baseline activities, all experimental groups largely remained quiet.

#### Post-naltrexone

The results for the post-naltrexone period show that treatment with both 7-NI and L-NAME significantly reduced most of the morphine withdrawal behaviors precipitated by naltrexone in infant rats (Table 3). We analyzed a total of three possible effects: dose effects, time effects and interaction between dose and time. Both 7-NI and L-NAME significantly dose dependently decreased head moves, moving paws, rolling and together behaviors and increased quiet behavior. In addition, pre-treatment with 7-NI significantly decreased walking behavior in the infant rat; pre-treatment with L-NAME had no significant effect on walking behavior. We failed to find any significant effect on wall-climbing behavior for pre-treatment with either 7-NI or L-NAME. The dose effects of 7-NI and L-NAME on naltrexone-precipitated morphine withdrawal in the 7-day-old rat are shown in Figs. 1 and 2, respectively.

**Table 2** Behavior occurrences after treatment of NOS inhibitors but before treatment of naltrexone in the morphine-dependent rat of 7 days old

Behavior	7-NI				L-NAME			
	Vehicle	5 mg/kg	15 mg/kg	45 mg/kg	Vehicle	15 mg/kg	75 mg/kg	375 mg/kg
Head moves	0.63±0.26	0.88±0.30	0.38±0.18	0.50±0.19	0.50±0.27	1.13±0.61	1.25±0.37	0.13±0.13
Moving paws	0.63±0.26	0.63±0.50	0.50±0.27	0.25±0.25	1.13±0.48	0.50±0.27	0.63±0.32	0.25±0.25
Quiet	38.75±0.45	39.25±0.25	39.00±0.54	38.50±0.63	38.13±0.92	37.38±1.34	36.50±0.91	39.25±0.41
Rolling	0.38±0.18	0.13±0.13	0.13±0.13	0.25±0.16	0.38±0.38	0.38±0.26	0.75±0.41	0.13±0.13
Together	0.38±0.26	0.63±0.50	0.25±0.16	0.00±0.00	0.38±0.38	0.38±0.26	0.88±0.44	0.00±0.00
Walking	0.38±0.26	0.5±0.27	0.13±0.13	0.25±0.16	0.38±0.38	0.25±0.25	0.38±0.26	0.13±0.13
Wall climbing	0.25±0.16	0.00±0.00	0.00±0.00	0.13±0.13	0.13±0.13	0.25±0.25	0.00±0.00	0.13±0.13

Note: cell entries are mean occurrences of behavior  $\pm$  SEM. There were no significant differences between any treatment condition and the corresponding control group ( $N = 8$  per cell)

**Table 3** Results of analyses of variance for withdrawal behaviors in NOS inhibitor treated 7-day-old rats (*bold type*  $P < 0.05$ )

	7-NI			L-NAME		
	Dose	Time	Dose×time	Dose	Time	Dose×time
Head moves						
<i>F</i>	27.514	3.128	1.219	10.798	0.843	0.978
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0001</b>	0.0753	0.3157	<b>0.0002</b>	0.4509	0.4519
Moving paws						
<i>F</i>	11.662	0.852	2.963	10.108	0.184	1.378
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0001</b>	0.4476	<b>0.0167</b>	<b>0.0003</b>	0.8337	0.2460
Quiet						
<i>F</i>	87.103	3.531	2.509	87.228	2.331	0.790
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0001</b>	0.0573	<b>0.0364</b>	<b>0.0001</b>	0.1337	0.5828
Rolling						
<i>F</i>	3.981	1.978	1.294	5.479	0.352	1.440
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0216</b>	0.1751	0.2807	<b>0.0061</b>	0.7095	0.2225
Together						
<i>F</i>	13.239	0.049	0.193	13.230	2.251	0.871
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0001</b>	0.9525	0.9771	<b>0.0001</b>	0.1420	0.5240
Walking						
<i>F</i>	9.284	0.919	0.467	2.171	1.528	1.881
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0004</b>	0.4216	0.8290	0.1216	0.2511	0.1067
Wall climbing						
<i>F</i>	2.333	0.467	0.467	0.371	0.218	1.395
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	0.1032	0.6365	0.8290	0.7745	0.8070	0.2393

### NMDA antagonists

#### Pre-naltrexone

Treatment with various doses of MK-801 and AP-5 did not significantly alter the behaviors of the morphine-dependent rat (Table 4). Except for some baseline activities, all experimental groups largely remained quiet. The highest dose of AP-5 (300 nmol) sedated all treated subjects (a total of four rat pups; simple reflexes disappeared in these subjects) and we terminated the test for this specific dose and the data are not included here.

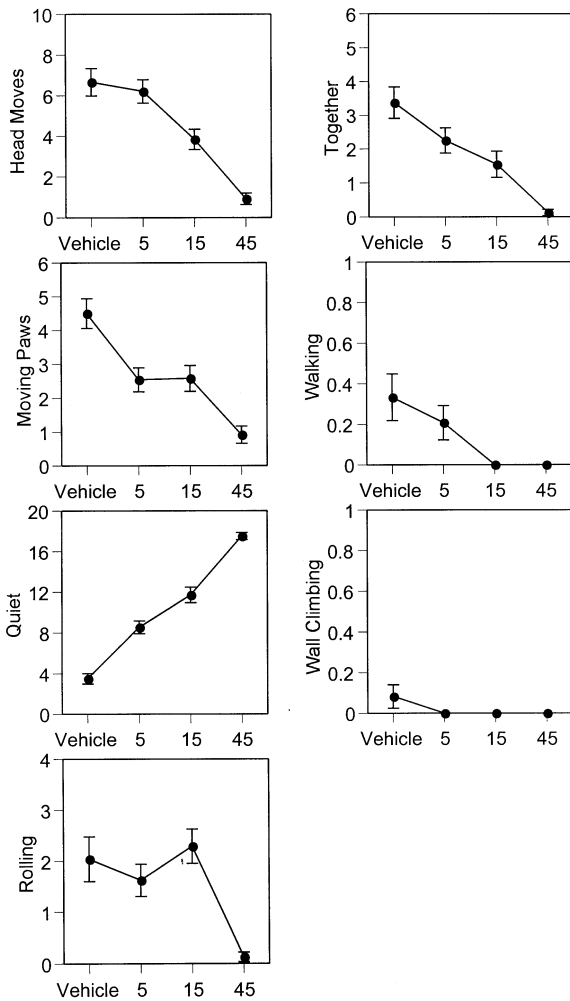
#### Post-naltrexone

The results for the post-naltrexone period show that treatment with either MK-801 or AP-5 had unexpected effects on the morphine withdrawal syndrome (Table 5). MK-801 significantly decreased head moves, moving paws, together and quiet behaviors. At the same time, pre-treatment with MK-801 significantly increased walking and wall-climbing behaviors. Treatment with AP-5

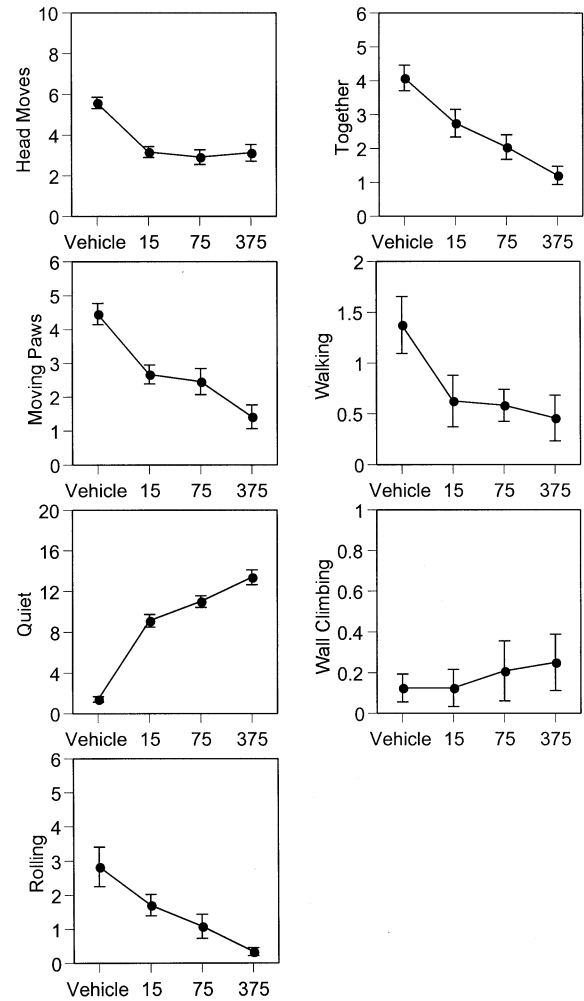
significantly attenuated the following behaviors: head moves, together and walking, but increased quiet behavior and intensified moving paws behavior. The dose effects of MK-801 and AP-5 on naltrexone-precipitated morphine withdrawal in the 7-day-old rat are shown in Figs. 3 and 4, respectively.

### Discussion

The results of experiment 1 suggest that neither of the two NOS inhibitors we tested significantly altered the occurrences of various behaviors in the 7-day-old rat prior to naltrexone injection, whereas both 7-NI and L-NAME were very effective in depressing various withdrawal behaviors precipitated by naltrexone. Thus, it seems that NOS inhibitors selectively attenuated withdrawal behaviors. Both 7-NI and L-NAME significantly decreased head moves, moving paws, rolling and together behaviors and increased quiet behavior. In addition, pre-treatment of 7-NI also significantly decreased walking behavior in the infant rats. Although we failed to find any significant results of wall-climbing behavior for ei-



**Fig. 1** Dose effect of 7-NI. *Ordinate* mean occurrences (mean  $\pm$  SEM) in 5 min of various opiate withdrawal behaviors (for definition see Table 1 ) in the 7-day-old rat. *Abscissa* treatment, log scale (units: mg/kg). Dose effects were significant for all withdrawal behaviors except wall climbing



**Fig. 2** Dose effect of L-NAME. *Ordinate* mean occurrences (mean  $\pm$  SEM) in 5 min of various opiate withdrawal behaviors (for definition see Table 1 ) in the 7-day-old rat. *Abscissa* treatment, log scale (units: mg/kg). Dose effects were significant for all withdrawal behaviors except walking and wall climbing

**Table 4** Behavior occurrences after treatment of NMDA antagonists but before treatment of naltrexone in the morphine-dependent rat 7 days old

Behavior	MK-801				AP-5			
	Vehicle	0.01 mg/kg	0.05 mg/kg	0.25 mg/kg	Vehicle	3 nmol	30 nmol	300 nmol
Head moves	0.88 $\pm$ 0.48	0.63 $\pm$ 0.18	0.13 $\pm$ 0.13	0.50 $\pm$ 0.27	0.50 $\pm$ 0.38	0.63 $\pm$ 0.38	0.88 $\pm$ 0.23	a
Moving paws	0.88 $\pm$ 0.52	0.00 $\pm$ 0.00	0.50 $\pm$ 0.33	0.13 $\pm$ 0.13	0.38 $\pm$ 0.26	0.88 $\pm$ 0.40	0.50 $\pm$ 0.27	a
Quiet	39.13 $\pm$ 0.30	38.13 $\pm$ 0.34	37.38 $\pm$ 0.35	39.13 $\pm$ 0.48	37.63 $\pm$ 0.91	36.88 $\pm$ 0.79	37.75 $\pm$ 1.35	a
Rolling	0.13 $\pm$ 0.13	0.13 $\pm$ 0.13	0.00 $\pm$ 0.00	0.25 $\pm$ 0.25	0.13 $\pm$ 0.13	0.38 $\pm$ 0.26	0.25 $\pm$ 0.25	a
Together	0.50 $\pm$ 0.19	0.25 $\pm$ 0.16	0.25 $\pm$ 0.25	0.25 $\pm$ 0.16	0.88 $\pm$ 0.74	0.38 $\pm$ 0.26	0.38 $\pm$ 0.26	a
Walking	0.25 $\pm$ 0.16	0.13 $\pm$ 0.13	0.25 $\pm$ 0.25	0.38 $\pm$ 0.18	0.50 $\pm$ 0.33	0.75 $\pm$ 0.41	0.25 $\pm$ 0.25	a
Wall climbing	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.13 $\pm$ 0.13	a

Note: Cell entries are mean occurrences of behavior  $\pm$  SEM. There were no significant differences between any treatment condition and the corresponding control group ( $N=8$  per cell)

<sup>a</sup>This dose of AP-5 sedated all treated subjects (a total of four rat pups; simple reflexes disappeared in these subjects) and the test for this specific dose was terminated

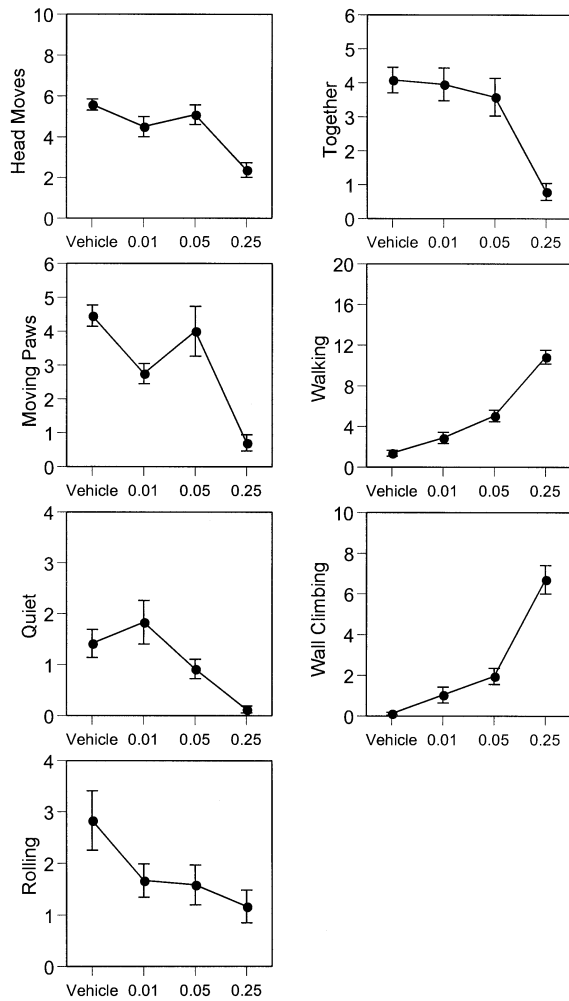
**Table 5** Results of analyses of variance for withdrawal behaviors in NMDA antagonist treated 7-day-old rats (*bold type*  $P < .05$ )

	MK-801			AP-5		
	Dose	Time	Dose×time	Dose	Time	Dose×time
Head moves						
<i>F</i>	7.992	9.183	2.607	15.766	0.346	0.645
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0010</b>	<b>0.0028</b>	<b>0.0307</b>	<b>0.0003</b>	0.7131	0.6352
Moving paws						
<i>F</i>	8.326	1.438	1.939	3.891	0.135	1.060
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0008</b>	0.2705	0.0968	<b>0.0453</b>	0.8750	0.3946
Quiet						
<i>F</i>	6.152	1.472	2.917	19.828	3.649	1.331
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0036</b>	0.2629	<b>0.0181</b>	<b>0.0001</b>	0.0530	0.2829
Rolling						
<i>F</i>	1.242	6.414	0.843	2.449	0.238	0.563
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	0.3196	<b>0.0105</b>	0.5441	0.1225	0.7913	0.6917
Together						
<i>F</i>	6.506	0.736	1.011	20.303	0.709	0.530
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0028</b>	0.4967	0.4313	<b>0.0001</b>	0.5091	0.7149
Walking						
<i>F</i>	34.402	8.950	3.078	5.009	2.212	0.978
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0001</b>	<b>0.0031</b>	<b>0.0137</b>	<b>0.0229</b>	0.1463	0.4351
Wall climbing						
<i>F</i>	43.942	23.764	14.592	2.519	3.092	0.588
<i>df</i>	(3,21)	(2,14)	(6,42)	(3,21)	(2,14)	(6,42)
<i>P</i>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	0.1163	0.0772	0.6743

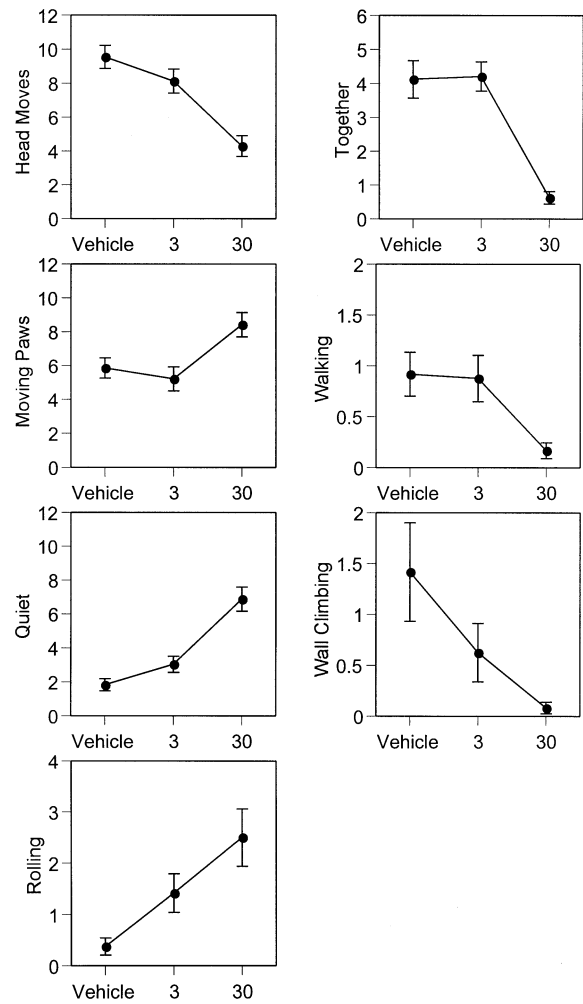
ther 7-NI or L-NAME and walking behavior for L-NAME, this was very likely due to the fact that walking and wall climbing are very rare withdrawal behaviors in the infant rat (Jones and Barr 1995), and thus the statistical power for these behaviors is considerably lower than for other withdrawal behaviors that occurred more frequently. Our results on the effect of NOS inhibitors in attenuating withdrawal are in line with the findings by others in adult rats (Kimes et al. 1993; Vaupel et al. 1995a, 1995b) and mice (Thorat et al. 1994).

The effects of the NMDA antagonists are more difficult to interpret. On the one hand, both MK-801 and AP-5 attenuated some of the withdrawal behaviors; on the other hand, both of them increased some. The effects of MK-801 were especially unexpected. MK-801 dose dependently intensified walking and wall climbing and reduced the quiet behavior. Because we did not record head moves and moving paws behaviors if the rat pup was walking or wall climbing, for MK-801, the attenuation of head moves and moving paws is most likely due to the robust walking and wall-climbing behavior exhibited in the rat pups pre-treated with various dose of MK-801. Thus, overall withdrawal behaviors appeared to in-

crease for pups pre-treated with MK-801. MK-801 was reported to increase locomotion activities in adult rats ("PCP like" effect) (Herman et al. 1995). It is possible that the observed increase in walking and wall climbing in the 7-day-old rat was due to the "PCP like" effect of MK-801 independent of withdrawal. There are several reasons why this is unlikely. First, MK-801 did not significantly alter the occurrence of walking and wall-climbing behavior in the 7-day-old rat which was not experiencing opiate withdrawal (Table 4); instead, MK-801 only increased these behaviors after the naltrexone challenge. Therefore, it seems that MK-801 selectively increased withdrawal behaviors in the 7-day-old rat. Second, in an ongoing study we also failed to find any significantly increased activity elicited by comparable doses of MK-801 alone, MK-801 with chronic morphine but not naltrexone, or MK-801 with naltrexone but not chronic morphine in the 7-day-old neonatal rat. Third, MK-801 induced locomotion activities only at considerably higher doses (Trujillo 1995). Lastly, when MK-801 was administered to preweanling rats (12- to 19-day-old), the "PCP like" effects of increased locomotion were much less robust in the younger pups even within



**Fig. 3** Dose effect of MK-801. Ordinate mean occurrences (mean  $\pm$  SEM) in 5 min of various opiate withdrawal behaviors (for definition see Table 1) in the 7-day-old rat. Abscissa treatment, log scale (units: mg/kg). Dose effects were significant for all withdrawal behaviors except rolling. Note ordinate scale changes compared to Figs. 1 and 2



**Fig. 4** Dose effect of AP-5. Ordinate mean occurrences (mean  $\pm$  SEM) in 5 min of various opiate withdrawal behaviors (for definition see Table 1) in the 7-day-old rat. Abscissa treatment, log scale (units: nmol). Dose effects were significant for all withdrawal behaviors except rolling and wall climbing

that age range (Scalzo and Burge 1994). Since we found no increased locomotion or wall climbing in pups given MK-801, and our subjects were much younger than the youngest pups demonstrating a "PCP like" effect, it is not probable that the increased walking and wall climbing seen after MK-801 administration in pups undergoing withdrawal is due to the MK-801 alone.

Since we used only one dose of naltrexone to precipitate withdrawal in experiment 1, there was a possibility that the intensification of walking and wall-climbing behaviors was an interaction between the MK-801 and the test concentration of the naltrexone (1 mg/kg). Therefore, we tested the interaction between MK-801 and multiple doses of naltrexone in experiment 2.

## Experiment 2

### Design and procedure

Morphine dependence was established in infant rats as described in experiment 1, and in the afternoon of day 7 rats were injected with saline (IP,  $n=32$ ) or MK-801 (IP, 0.05 mg/kg,  $n=32$ ) as pre-treatment. Fifteen minutes later, each pup from both the saline pre-treated group and the MK-801 pre-treated group was injected with naltrexone (IP, 0.03, 0.1, 0.3 or 1 mg/kg,  $n=8$  for each dose) to precipitate withdrawal. All of the doses were randomly assigned and the observer was blind to each dose and drug. The pup that received the naltrexone injection was then returned to the litter and its behavior observed for a total of 15 min. A total of eight pups from each litter were injected and tested in this manner.

**Table 6** Results of the analysis of the interaction between MK-801 and naltrexone (*bold type*  $P < 0.05$ ) (*MK-801* × *naltrexone* indicates the interaction between MK-801 and naltrexone)

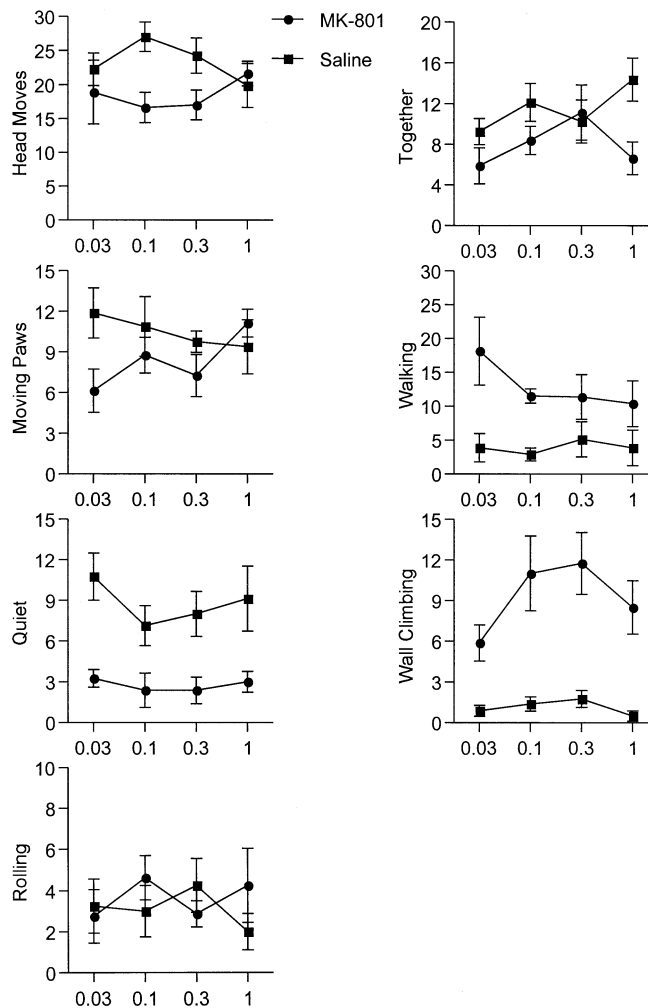
	MK-801	Naltrexone	MK-801 × naltrexone
<b>Head moves</b>			
<i>F</i>	5.686	0.160	0.667
<i>df</i>	(1,7)	(3,21)	(3,21)
<i>P</i>	<b>0.0486</b>	0.9222	0.2044
<b>Moving paws</b>			
<i>F</i>	3.156	0.499	2.430
<i>df</i>	(1,7)	(3,21)	(3,21)
<i>P</i>	0.1189	0.6872	0.0936
<b>Quiet</b>			
<i>F</i>	25.438	0.941	0.318
<i>df</i>	(1,7)	(3,21)	(3,21)
<i>P</i>	<b>0.0015</b>	0.4387	0.8123
<b>Rolling</b>			
<i>F</i>	0.675	0.236	1.439
<i>df</i>	(1,7)	(3,21)	(3,21)
<i>P</i>	0.4385	0.8704	0.2598
<b>Together</b>			
<i>F</i>	3.668	1.298	4.017
<i>df</i>	(1,7)	(3,21)	(3,21)
<i>P</i>	0.0970	0.3013	<b>0.0209</b>
<b>Walking</b>			
<i>F</i>	18.168	1.459	1.977
<i>df</i>	(1,7)	(3,21)	(3,21)
<i>P</i>	<b>0.0037</b>	0.2543	0.1483
<b>Wall climbing</b>			
<i>F</i>	46.427	1.666	1.325
<i>df</i>	(1,7)	(3,21)	(3,21)
<i>P</i>	<b>0.0003</b>	0.2048	0.2928

## Statistics

A two-way ANOVA was conducted for each behavior, which reflected its occurrences for 15 min. Unlike in experiment 1, the time factor was not analyzed in experiment 2. All doses of naltrexone were injected within a single litter, and the drug dose effect was treated as a within-subjects variable. The control group (four pups) and the MK-801 group (four pups) were from the same litter and the MK-801 treatment was treated as a within-subjects variable.

## Results

Compared to the control group (saline pre-treatment), pre-treatment with MK-801 had significant effects on morphine withdrawal behaviors precipitated by naltrexone in infant rats. We analyzed three possible effects: the effects of the pre-treatment with MK-801 vs saline, the dose effects of the naltrexone and the interaction between naltrexone and pre-treatment with MK-801



**Fig. 5** Interaction between dose of naltrexone and pre-treatment of MK-801. *Ordinate* mean occurrences (mean  $\pm$  SEM) in 15 min of various opiate withdrawal behaviors (for definition see Table 1) in the 7-day-old rat. *Abscissa* naltrexone dose, log scale (units: mg/kg)

(Table 6). Treatment of MK-801 significantly decreased head moves and quiet behaviors, and, at the same time, significantly increased walking and wall-climbing behaviors. We failed to find any significant effect of pre-treatment with MK-801 for the following behaviors: moving paws, rolling and together. For no behavior was there a significant dose effect of naltrexone. There was a significant interaction between MK-801 and naltrexone for the together behavior only.

The effects of pre-treatment with MK-801 at different doses of naltrexone on withdrawal behaviors in the infant rat are depicted in Fig. 5.

## Discussion

The results of experiment 2 are generally in agreement with the results on the effects of MK-801 in experiment 1. The interaction between treatment of MK-801 and nal-

naltrexone only exists for the together behavior, which declined with the high dose of MK-801 in experiment 1. Unlike experiment 1, we failed to find any significant effect of pre-treatment of MK-801 on the moving paws behavior. This difference may be due to the fact that, in experiment 2, we adopted a medium dose of MK-801 (0.05 mg/kg) and the dose effect of MK-801 in decreasing moving paws behavior exhibited in experiment 1 was significant only for the highest dose (0.25 mg/kg). As in experiment 1, regardless of the dose of naltrexone, MK-801 decreased head moves and quiet behavior whereas it increased the occurrences of walking and wall climbing. MK-801 did not have any significant effect on the rolling behavior. For the same reasons described in experiment 1, we believe that the reduced head moves is likely to be confounded by increased walking and wall climbing and cannot be regarded as a sign of attenuation of withdrawal for MK-801. Together with the results from experiment 1, the results of experiment 2 confirmed that MK-801 is ineffective in suppressing the naltrexone-precipitated morphine withdrawal in the neonatal rat.

## General discussion

There are two conclusions that can be drawn from these experiments. First, acute administration of NOS inhibitors is quite effective in attenuating naltrexone-precipitated morphine withdrawal in 7-day-old rat pups. Second, acute administration of NMDA antagonists is not effective in inhibiting most of the naltrexone-precipitated morphine withdrawal behaviors in 7-day-old rat pups.

### NOS inhibitors

In the adult rat, L-NAME, 7-NI and other NOS inhibitors have been widely reported to reduce opiate withdrawal symptoms such as wet dog shakes, diarrhea, and grooming (Kimes et al. 1993; Vaupel et al. 1995a, 1995b). However, wet dog shakes, diarrhea, grooming and other reported attenuated opiate withdrawal behaviors are characteristic only for adult animals and are not present in the infant rat (Jones and Barr 1995; Windh et al. 1995; Thornton and Smith 1997; Thornton et al. 1997). The effect of these drugs on opiate withdrawal behaviors unique to infants was unknown. We found that, when behavioral measures appropriate for the infant rat are adopted, acute administration of NOS inhibitors effectively reduced a variety of the opioid abstinence symptoms in 7-day-old rats, including head moves, moving paws, rolling together, walking, and increased quiet behavior.

Vaupel et al. reported that, although NOS inhibitors produced significant decreases in weight loss, diarrhea, wet dog shakes and grooming in adult rats, some withdrawal signs (e.g., exploratory activity) were increased. They attributed this to the relatively low level of opiate dependence of the subjects due to short morphine treat-

ment period (2–4 days) (Vaupel et al. 1995a, 1995b). In our experiment 1, the rat pups had been treated with morphine considerably longer (6.5 days) before NOS inhibitor pre-treatment and naltrexone challenge, and we did not find any increased withdrawal signs for either 7-NI or L-NAME. Although we failed to find any significant effects on walking and wall-climbing behaviors for L-NAME and on wall-climbing behavior for 7-NI, as explained earlier, it is likely because these are rarely occurring behaviors. Though statistically nonsignificant, the mean occurrences of both wall-climbing behavior for the 7-NI pre-treatment group and walking behavior for the L-NAME pre-treatment group dose dependently declined.

Vaupel et al. compared the opiate withdrawal attenuation effect of 7-NI with other NOS inhibitors (L-NNA, L-NAME, L-NIO) and found that 7-NI attenuated more signs of opiate withdrawal than did L-NNA, L-NAME or L-NIO in the adult rat (Vaupel et al. 1995a). The results of experiment 1 extend their data to the neonatal rat. All withdrawal behaviors suppressed by L-NAME were also attenuated by 7-NI, and walking behavior was inhibited by 7-NI but not L-NAME. Unlike other NOS inhibitors, 7-NI selectively inhibits neuronal NOS (Moore et al. 1993; Vaupel et al. 1995a, 1995b) and, therefore, it lacks the capacity to elevate blood pressure in both untreated and morphine-dependent rats (Vaupel et al. 1995a, 1995b). The implication of these studies is that compounds like 7-NI should rank high on the list of drugs that have potential as a treatment for alleviating clinical signs of opiate withdrawal in human infants.

### NMDA antagonists

Since Trujillo and colleagues (Trujillo and Akil 1991a) first reported that the noncompetitive NMDA antagonist MK-801 inhibits the development of morphine tolerance and dependence in the adult rat, their findings have been widely replicated (Marek et al. 1991a, 1991b; Ben-Eliyahu et al. 1992; Trujillo and Akil 1994; Elliott et al. 1995; Inturrisi 1997; Trujillo 1995, 1999). It is now generally agreed that chronic coadministration of MK-801 and other NMDA antagonists prevents the development of opiate tolerance and dependence in rodents (Herman et al. 1995; Trujillo 1995, 1999). More controversial are the reports about the effects of MK-801 and other NMDA antagonists on the expression of morphine withdrawal (Herman et al. 1995). Some laboratories report that NMDA antagonists inhibit withdrawal symptoms (Brent and Chahl 1993; Cappendijk et al. 1993; Tanganelli et al. 1991); others report that they do not attenuate opiate withdrawal (Trujillo and Akil 1991a, 1991b), and still others report mixed results (Rasmussen et al. 1991; Thorat et al. 1994). At first glance, it seems that our results are in line with the last group, because in our experiments both MK-801 and AP-5 failed to attenuate some opiate withdrawal behavior in infant rats, and both intensified several others. However, it is not clear if our nega-

tive results for MK-801 are replicating those negative results from the adult or are revealing true differences between infants and adults.

The reason for the difference between chronic treatment and acute treatment of MK-801 in adult animals is unclear (Trujillo 1995). There may exist fundamental differences between the mechanisms of the development or acquisition of opiate dependence and the physical expression of dependence withdrawal. Although the NMDA receptor plays a vital role in the development of opiate dependence, once the dependence is established, its expression may not rely on the functioning of the NMDA receptor. Studies by Rasmussen and colleagues lend support to the concept that NMDA receptors play only a minor role in the expression of opiate withdrawal. In opiate-dependent rats, there is a marked increase in firing of locus coeruleus (LC) neurons during naltrexone-precipitated withdrawal; and opiate withdrawal increases glutamate and aspartate efflux in the LC (Rasmussen and Aghajanian 1989; Rasmussen et al. 1996). This increase in activity of LC neurons has been hypothesized to be important in opiate withdrawal symptoms (Rasmussen et al. 1996). The excitatory effect of glutamate in the LC is mediated largely through AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, whereas NMDA receptors mediate little, if any, of the withdrawal-induced excitation of the LC (Rasmussen 1995; Rasmussen et al. 1991, 1996). Recently, this group also reported that the selective metabotropic glutamate receptor (mGlu2/3 receptor) agonist LY354740 attenuated morphine withdrawal induced activation of LC neurons and behavioral signs of morphine withdrawal (Vandergriff and Rasmussen 1999). These data suggest that, although NMDA receptors are important in the acquisition of morphine dependence, once the dependence is established, NMDA receptors may play a minor role. Other glutamate receptors such as AMPA and metabotropic glutamate receptors may play the major roles.

On the other hand, the reason that we obtained negative results for the NMDA receptor antagonists in the neonatal rat may be because NMDA receptors function differently in neonatal rats than in adult rats. During development, NMDA receptors undergo qualitative and quantitative changes (Kalb et al. 1992; Hori and Kanda 1994; Kalb and Fox 1997). These include alterations in density of the receptor (Morin et al. 1989; Represa et al. 1989; Tremblay et al. 1988) and sensitivity to magnesium (Ben-Ari et al. 1988; Bowe and Nadler 1990; Morrisett et al. 1990) of NMDA receptors during the course of development. At the same time, the various subunits (R1, R2A–D, etc.) of the NMDA receptor complex also undergo dramatic differential maturation during the first few postnatal weeks of the animal (Pollard et al. 1993; Laurie and Seeburg 1994; Zhong et al. 1994, 1995; Guilarte and McGlothan 1998), resulting in considerably different function of the NMDA receptor (Colwell et al. 1998). The effectiveness of MK-801 in inhibiting opiate withdrawal varies not only ontogenetically

but also phylogenetically. As Herman et al. (1995) first pointed out, depending on species of rodent, the effect of MK-801 on opiate withdrawal is different. In guinea pigs, MK-801 is consistently reported to inhibit various morphine withdrawal symptoms (Tanganelli et al. 1991; Brent and Chahl 1993). In adult rats, the results are less consistent (Rasmussen et al. 1991; Bhargava et al. 1995). Most controversial are the effects of MK-801 in inhibiting morphine withdrawal symptoms in mice. There have been reports of both significant effects (Tanganelli et al. 1991) and observations that failed to obtain significant effects (Thorat et al. 1994). These differences among species may indicate a relationship between the level of brain evolution and the degree of MK-801 response (Herman et al. 1995). In our experiments, a possible reason for the ineffectiveness of MK-801 in inhibiting opiate withdrawal behaviors in the infant rat is that neural systems of the neonatal rat are ontogenetically less evolved than that of the adult rat and phylogenetically less evolved than that of the guinea pig. Therefore, the mechanism by which MK-801 takes effect in reducing various opiate withdrawal symptoms in guinea pigs and adult rats is ineffective in neonatal rats. Whether the different development status of NMDA receptors is responsible for these observed differences of MK-801 in inhibiting opiate withdrawal in different species and differently aged rodents is unknown and deserves further study.

It is important to note that our data do not support, at least in the infant rat, the notion that the production of NO is linked to the activation of NMDA receptors (Mao et al. 1995; Mayer and Mao 1999) during opiate withdrawal. When NOS inhibitors were applied, withdrawal behaviors were inhibited, but withdrawal syndromes were still manifest after the blockade of NMDA receptors by NMDA receptor antagonists. Our data thus suggest that the activation of NMDA receptors is not necessarily the single trigger of the activation and production of NO. It seems that once opiate dependence is established, the activation of NO during withdrawal is triggered by some factors that are “downstream” to the NMDA receptor.

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