



# Ontogeny of NMDA receptor-mediated morphine tolerance in the postnatal rat

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## Abstract

*N*-methyl-D-aspartate (NMDA) receptor antagonists are effective in inhibiting the development of morphine tolerance in adult rats. But NMDA receptors undergo dramatic change during the first few weeks of the postnatal life in the rat, and it is unknown whether NMDA receptor antagonists can inhibit the acquisition of opiate tolerance in the developing organism. Here, we investigated the effects of two NMDA receptor antagonists MK-801 and dextromethorphan on the development of morphine tolerance in 7-, 14-, and 21-day-old rats. NMDA receptor antagonists are not effective in attenuating morphine tolerance in the neonatal rat whereas they were partially effective in the 14-day-old and fully effective in rats as old or older than 21 days of age. These data suggest that there exists a transition age, around the second postnatal week in the rat, for the NMDA receptor to play a role in the development of morphine tolerance.

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## 1. Introduction

In adult animals, *N*-methyl-D-aspartate (NMDA) receptor antagonists have been reported to inhibit the development of morphine tolerance, dependence, and the expression of opiate withdrawal (Herman et al., 1995). It is not known whether these results apply to infants. On the one hand, opiate tolerance can be established in infant rat pups if the pups are treated with opiates during ontogeny (Zhu and Barr, 2001b). On the other hand, the neonatal central nervous system (CNS) is both structurally and functionally different from that of the adult, and significant changes in opioid actions occur both prenatally and postnatally (Marsh et al., 1997). Furthermore, NMDA receptors undergo qualitative and quantitative changes during development (Ozawa et al., 1998). Recently, due to advances in cloning technology and gene knockout

technology, an explosion of studies have characterized the molecular structure of the NMDA receptor complex (Dingledine et al., 1999; Ozawa et al., 1998). The accumulating evidence indicates that the various subunits (NR1, NR2A–D, NR3A–B) composing the native NMDA receptor channels undergo dramatic differential changes during the first few weeks of postnatal life in the rat (Dingledine et al., 1999). It has been suggested that NMDA receptor is not functionally mature until the second postnatal week in the rat (Sircar, 2000).

Therefore, the pharmacological effects of NMDA antagonists in the infant may not necessarily be comparable to those in the adult. Indeed, in contrast to the data in adults, NMDA receptor antagonists are not effective in suppressing the expression of opiate withdrawal (Zhu and Barr, 2000, 2001a) in neonatal rats. On the other hand, in agreement with the adult data, NOS inhibitors suppress withdrawal in the 7-day-old rat (Zhu and Barr, 2000). Thus, that NMDA receptors undergo dramatic differential changes during earlier life suggests possible developmental differences. In the infant, although opiate actions rely on the same second messenger systems as in the adult, the factors that activate these systems differ (Zhu and Barr, 2001b). Particularly

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interesting is the recent finding that the NMDA receptor antagonist MK-801 is ineffective in blocking both the development of morphine dependence and the expression of morphine withdrawal in the 7-day-old rat whereas it is partially effective in the 14-day-old rat and fully effective in the 21-day-old rat (Zhu and Barr, 2001a). Thus, there is a transition age, around the second postnatal week, for MK-801 to be effective in suppressing morphine withdrawal (Zhu and Barr, 2001a). We proposed that from a developmental perspective, receptors other than NMDA receptors may link to the same second messenger system during early life and at a later time either confer this role to NMDA receptors or recede to an auxiliary role (Zhu and Barr, 2001b). As a result, conclusions based on data derived from adult animals models may not hold in the developing organism. Thus, the present study examined the effects of two NMDA receptor antagonists MK-801 and dextromethorphan in morphine tolerance in the infant rat using a combination of in vivo and in vitro methods.

## 2. Materials and methods

### 2.1. Subjects

All animal procedures were in accordance with the "Principles of Laboratory Animal Care, 1996 and the NIH guidelines (publication 86-23)" (Institute of Laboratory Animal Resources, 1996; National Institute of Health, 1985). 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. Food and water were available ad libitum. Cages were checked at 10 a.m. and 6 p.m. Pups found at either time were termed 0 days of age.

### 2.2. Morphine treatment and tolerance assessment

To induce morphine tolerance, starting when the pups were 1, 8, or 15 days of age, all pups in a litter were injected with morphine sulfate (IP, 10 mg/kg) twice daily (10 a.m. and 6 p.m.) for 6.5 days. To determine NMDA receptor antagonists' effect on the development of morphine tolerance, MK-801 or dextromethorphan were injected 15 min before each morphine injection. The last injection was in the morning of the 7th day.

Cumulative morphine dose–response curves were constructed at approximately 3 p.m. in the afternoon of the 7th day to assess the development of tolerance (Barr and Wang, 1992). After adaptation and baseline trials, each rat was injected with a low dose of morphine (0.1 mg/kg, i.p.). Thirty minutes later, the rat was re-tested and injected with the next dose of morphine. The dose was increased in a logarithmic manner, with a starting dose of 0.1 mg/kg, an ending dose of 10.0 mg/kg, and an increment of approxi-

mately half a log unit, such that each pup received 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg of morphine sequentially. Briefly, pups were removed from an incubator maintained a thermoneutral body temperature, and gently held above a hotplate. The temperature of the hotplate was controlled by a precise electronic circuit to be 49°C. The distal 2/3 of the forepaw, hindpaw, and tail of the subject were sequentially put on the hotplate. The latency for removal of the limb or tail from the hotplate was recorded using a timer operated by a foot pedal (due to the large variability from trial to trial, we did not test the tail withdrawal of the 21-day-old rat). Testing began 20 min following the last administration of drugs. The withdrawal latency of the forepaw, hindpaw, and tail of each animal were measured. A 20 s cutoff latency was used after which the stimulus was discontinued if there had been no responses. In no case was there tissue damage.

### 2.3. Spinal cord preparation

The standard electrophysiology experimental setup has been described before (Bell and Beglan, 1995; Feng and Kendig, 1995; Kerkut and Bagust, 1995) and was followed in present studies. Spinal cords were obtained from 7-day-old rat pups that were treated similarly to those in the behavioral experiments from day 1 to day 7, except that on the 7th day, morphine analgesic tolerance was assessed by the electrophysiological assay instead of the behavioral test. After decapitation, the vertebral column and spinal cord was cut free from the animal and placed in ice-cold (ACSF). The spinal cord was exposed by removing laminae from the ventral surface of the isolated column, using a pair of fine scissors working from rostral to caudal. After cutting spinal nerves peripheral to the dorsal root ganglia, the cord was floated free from the vertebrae. Any adhering dura was carefully dissected away and the ventral and dorsal roots in the lumbar region were separated. The normal superfusate ACSF consisted of (in mM) NaCl 123, KCl 5, NaHCO<sub>3</sub> 26, NaH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2, MgSO<sub>4</sub> 1.3, glucose 30, and was constantly bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (Kendig et al., 1991; Wong et al., 1998). The isolated spinal cord was then placed in a 2 ml bath and superfused with 95% O<sub>2</sub> and 5% CO<sub>2</sub> bubbled ACSF at a rate of 2 ml/min. The bath was maintained at a room temperature of approximately 25°C.

### 2.4. Dorsal root stimulation and ventral root recording

Extracellular dorsal root stimulation and ventral root (L4 or L5) recordings, were made with glass capillary suction electrodes. These electrodes were pulled to fine tips with a microelectrode puller; the tips were broken back to 100–300 µm and were fire polished. Standard stimulating and recording techniques were used (Kendig et al., 1991). For stimulation, square-wave stimuli with an amplitude of 30 V, duration of 200 µs and a frequency of 0.02 Hz were applied to the dorsal roots through a bipolar suction electrode made of fine platinum wires. For recording, the tip of the recording

electrode fitted tightly around the ventral root, and the root was carefully sucked into the suction electrode until the tip pressed lightly against the spinal cord to form a good seal. The recording suction electrode was connected through an AgCl pellet to a preamplifier. The slow ventral root potentials (sVRPs) (Kendig et al., 1991) were digitized through a InstruTech ITC-16 ADC interface card (Instru-Tech Inc., Long Island, NY) and stored to the computer hard disk in real time by a PowerPC computer running the data analysis software Igor Pro 3.0 (Wavemetrics Inc., Lake Oswego, OR) with the ubiquitous data collection plug-in PulseControl (Bookman Laboratory, Department of Molecular and Cellular Pharmacology, University of Miami) for off-line analyses.

In the isolated spinal cord from morphine-treated neonatal rats tolerance is defined as a decreased depressant effect of acute morphine on an electrically sVRP (Bell and Beglan, 1995). The sVRP is associated with nociception by several criteria: elicitation by noxious stimuli, sensitivity to analgesics and C-fiber dependence (Feng and Kendig, 1995). Thus, that acute morphine induces diminished depression of the sVRP in neonatal rats chronically treated with morphine can be regarded as an electrophysiological manifestation of tolerance to morphine's analgesic effect at the spinal cord level (Bell and Beglan, 1995).

Spinal cords from rats older than postnatal day 11 do not display the nociception-related prolonged sVRP (personal communication with Dr Joan J. Kendig, Stanford University School of Medicine, also see Gibbs and Kendig, 1992). In addition, isolated spinal cords from older rats do not survive the above-described treatment (Kerkut and Bagust, 1995). Thus, we did not test the 14- or 21-day-old rat in the electrophysiological experiments.

### 2.5. Behavior data analysis

The raw behavioral data recorded by the experimenter were reaction time scores (in s) and the data were converted to the percentage of maximum possible effect (%MPE) according to the method of Harris and Pierson (1964) by the following formula:

$$\%MPE = \frac{\text{Test latency} - \text{Baseline latency}}{\text{Cutoff time} - \text{Baseline latency}} \times 100$$

where %MPE is the percentage of maximum possible effect, test latency the animal's reaction time to a hotplate maintained at a constant temperature of 49°C, baseline latency the animal's reaction time before any injection was conducted, and cutoff time is 20 s.

The transformed %MPE data were then used to construct dose–response curves for morphine's analgesic effect. The ED<sub>50</sub> values and 95% confidence limits (95% CL) of morphine's analgesic effect were determined according to the method of Tallarida and Murray (1987).

### 2.6. Electrophysiology data analysis

The effects of morphine application in any individual spinal cord were determined by comparing the averaged sVRP area values of five consecutive sVRPs evoked immediately prior to morphine application (baseline sVRP area) to the area values of the sVRPs evoked during any concentration of morphine applications (treatment sVRP area). All data points were normalized to the percentage of suppression of baseline sVRP area by the following formula:

$$\begin{aligned} \% \text{Suppression of baseline} \\ = \frac{\text{Baseline area} - \text{Treatment area}}{\text{Baseline area}} \times 100 \end{aligned}$$

The transformed %Suppression of baseline sVRP data were then used to construct concentration–response curves for morphine's analgesic effect. The area under the concentration–response curves produced by adding acute morphine to the spinal superfusate was calculated using the trapezoidal rule (Tallarida and Murray, 1987). The area values for each concentration–response curve were analyzed by a one-way ANOVA followed by post hoc Fisher's PLSD test to assess significance of drug effects (Bell and Beglan, 1995). Effects were considered significant if  $P < 0.05$ .

## 3. Results

### 3.1. MK-801 did not attenuate morphine tolerance in the 7-day-old rat

Chronic treatment of morphine or MK-801-morphine did not alter the baseline reaction time of the rat in withdrawing its appendages from the noxious thermal stimulus (baseline latency) (Table 1). In contrast, chronic treatment of morphine rendered the 7-day-old rat tolerant to morphine's acute analgesic effect, as indicated by a right-shift of the dose–response curve of acute morphine's analgesic effect (Fig. 1A–C) and a significantly increased ED<sub>50</sub> of acute morphine in the chronic morphine groups compared with the morphine naive groups (Table 2).

Chronic co-administration of low or medium dose of MK-801 (0.03 and 0.10 mg/kg) with morphine failed to prevent the development of tolerance, as reflected by that the morphine's dose–response curve for the groups that received chronic co-administration of MK-801 with morphine overlapped with that of the chronic morphine groups and did not return to those of the morphine naive groups (Fig. 1A–C). ED<sub>50</sub> values of acute morphine of the groups received chronic co-administration of low and medium dose of MK-801 with morphine were not significantly different from the ED<sub>50</sub> of the group that received only chronic

Table 1

The effect of chronic treatment with morphine or MK-801 plus morphine on the baseline latency of the 7-, 14-, and 21-day-old rat retracting its appendages from a hotplate

Age	Treatment	Forepaw	Hindpaw	Tail
7do	Sal–Sal	1.694 ± 0.069	2.053 ± 0.145	1.84 ± 0.192
	Sal–Mor	1.686 ± 0.084	1.778 ± 0.163	1.882 ± 0.196
	MK (0.03 mg/kg)–Mor	1.684 ± 0.121	1.604 ± 0.109	1.791 ± 0.147
	MK (0.10 mg/kg)–Mor	1.746 ± 0.165	1.819 ± 0.109	1.745 ± 0.2
	MK (0.30 mg/kg)–Mor	1.906 ± 0.152	1.903 ± 0.168	1.908 ± 0.295
14do	Sal–Sal	1.902 ± 0.14	1.994 ± 0.167	2.575 ± 0.381
	Sal–Mor	2.043 ± 0.139	2.043 ± 0.131	2.488 ± 0.361
	MK (0.03 mg/kg)–Mor	1.854 ± 0.132	1.922 ± 0.141	2.726 ± 0.286
	MK (0.10 mg/kg)–Mor	2.012 ± 0.151	1.95 ± 0.121	2.465 ± 0.344
	MK (0.30 mg/kg)–Mor	1.928 ± 0.129	1.992 ± 0.117	2.414 ± 0.255
21do	Sal–Sal	2.134 ± 0.19	2.267 ± 0.185	NT
	Sal–Mor	2.142 ± 0.248	1.775 ± 0.196	NT
	MK (0.03 mg/kg)–Mor	2.254 ± 0.236	2.068 ± 0.313	NT
	MK (0.10 mg/kg)–Mor	2.298 ± 0.166	2.196 ± 0.246	NT
	MK (0.30 mg/kg)–Mor	2.061 ± 0.27	2.322 ± 0.241	NT

NT: not tested. Data in each cell reflect the mean latency of retraction of the appendage (mean ± 1 SEM, in s). No significant results were found between any groups.

morphine and both were significantly higher than that of the morphine naive group (Table 2).

For the high dose MK-801 (0.30 mg/kg) treated group, the dose–response curves of acute morphine as assessed by both the forepaw and hindpaw shifted further to the right (Fig. 1A, B) and ED<sub>50</sub> values significantly increased compared with those of the chronic morphine only groups (Table 2), indicating that greater intensity of morphine tolerance was developed in this group.

### 3.2. MK-801 attenuated morphine tolerance in the 14-day-old rat

Chronic morphine or MK-801-morphine treatment did not significantly alter the baseline latency (Table 1). In contrast, chronic treatment of morphine rendered the 14-day-old rat tolerant to morphine's acute analgesic effect, as indicated by a right-shift of the dose–response curve of acute morphine's analgesic effect (Fig. 2A–C) and a

Table 2

Effects of chronic co-administration of MK-801 on ED<sub>50</sub> of morphine in the infant rat

Age	Treatment	Forepaw	Hindpaw	Tail
7do	Sal–Sal	0.64 (0.22–1.06)	0.72 (0.38–1.06)	0.77 (0.44–1.09)
	Sal–Mor	1.75 (1.61–1.89) <sup>a</sup>	2.25 (1.98–2.53) <sup>a</sup>	2.67 (2.46–2.89) <sup>a</sup>
	MK (0.03 mg/kg)–Mor	1.79 (1.52–2.08) <sup>a</sup>	2.38 (2.15–2.60) <sup>a</sup>	2.30 (2.12–2.48) <sup>a</sup>
	MK (0.10 mg/kg)–Mor	1.78 (1.63–1.94) <sup>a</sup>	2.14 (1.93–2.35) <sup>a</sup>	2.36 (2.12–2.60) <sup>a</sup>
	MK (0.30 mg/kg)–Mor	2.69 (2.48–2.90) <sup>a,b</sup>	2.91 (2.72–3.09) <sup>a,b</sup>	2.74 (2.53–2.95) <sup>a</sup>
14do	Sal–Sal	1.04 (0.79–1.28)	0.91 (0.56–1.27)	0.74 (0.40–1.07)
	Sal–Mor	3.19 (2.68–3.69) <sup>a</sup>	3.17 (2.93–3.41) <sup>a</sup>	2.69 (2.23–3.14) <sup>a</sup>
	MK (0.03 mg/kg)–Mor	2.95 (2.46–3.44) <sup>a</sup>	2.98 (2.68–3.28) <sup>a</sup>	2.46 (1.85–3.06) <sup>a</sup>
	MK (0.10 mg/kg)–Mor	1.40 (0.39–2.41) <sup>c</sup>	1.34 (0.68–2.00) <sup>c</sup>	0.96 (0.61–1.31) <sup>c</sup>
	MK (0.30 mg/kg)–Mor	1.31 (0.94–1.68) <sup>c</sup>	1.37 (0.78–1.96) <sup>c</sup>	0.87 (0.51–1.22) <sup>c</sup>
21do	Sal–Sal	3.02 (2.43–3.61)	2.76 (1.99–3.52)	NT
	Sal–Mor	> 10 <sup>a</sup>	> 10 <sup>a</sup>	NT
	MK (0.03 mg/kg)–Mor	3.60 (2.01–5.19) <sup>c</sup>	3.45 (2.23–4.68) <sup>c</sup>	NT
	MK (0.10 mg/kg)–Mor	3.34 (2.81–3.88) <sup>c</sup>	3.26 (2.78–3.74) <sup>c</sup>	NT
	MK (0.30 mg/kg)–Mor	3.23 (2.65–3.81) <sup>c</sup>	3.14 (2.61–3.68) <sup>c</sup>	NT

NT: not tested.

<sup>a</sup> ED<sub>50</sub> significantly greater than Sal–Sal group.

<sup>b</sup> ED<sub>50</sub> significantly greater than Sal–Mor group.

<sup>c</sup> ED<sub>50</sub> significantly smaller than Sal–Mor group.

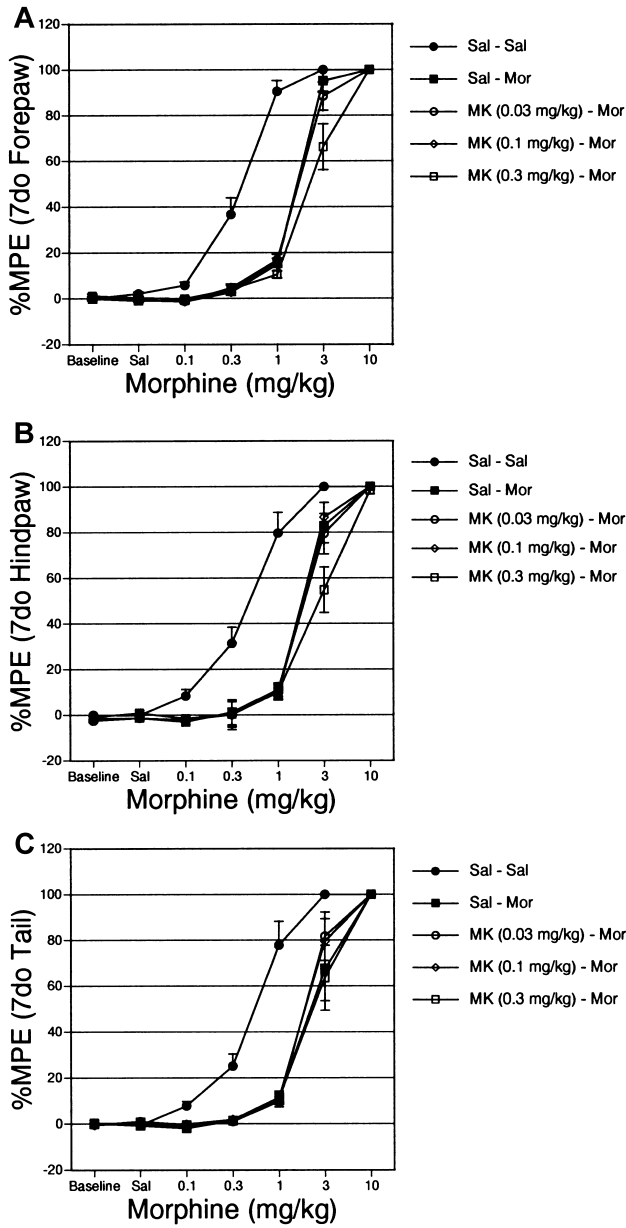


Fig. 1. The effect of pre-treatment with MK-801 (0.03, 0.10, and 0.30 mg/kg) on the development of morphine tolerance in the 7-day-old rat. Ordinate: mean %MPE (mean  $\pm$  1 SEM). Abscissa: acute morphine doses. (A) Forepaw, (B) hindpaw and (C) tail.

significantly increased  $ED_{50}$  of acute morphine compared with the morphine naive group (Table 2).

Chronic MK-801-morphine treatment dose-dependently attenuated the development of tolerance, as reflected by that the morphine's dose-response curves for the groups that received chronic medium or high dose (0.10 or 0.30 mg/kg) co-administration of MK-801 with morphine returned toward that of the morphine naive group (Fig. 2A–C) and decreased  $ED_{50}$  values of acute morphine compared with the groups that received only chronic morphine treatment (Table 2).

### 3.3. MK-801 attenuated morphine tolerance in the 21-day-old rat

Chronic morphine or MK-801-morphine treatment did not significantly alter baseline latency (Table 1). In contrast, chronic treatment of morphine rendered the 21-day-old rat tolerant to morphine's acute analgesic effect, as indicated by a right-shift of the dose-response curves of acute morphine's analgesic effect (Fig. 3A, B) and a significantly increased  $ED_{50}$  of acute morphine compared with the morphine naive groups (Table 2).

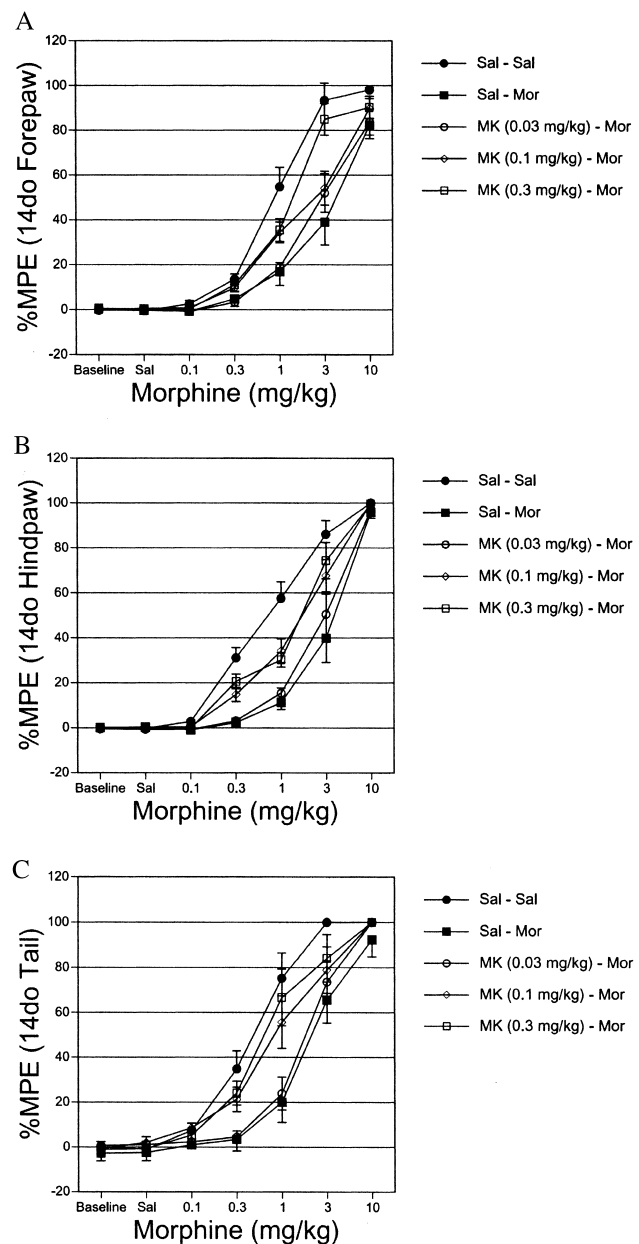


Fig. 2. The effect of pre-treatment with MK-801 (0.03, 0.10, and 0.30 mg/kg) on the development of morphine tolerance in the 14-day-old rat. Ordinate: mean %MPE (mean  $\pm$  1 SEM). Abscissa: acute morphine doses. (A) Forepaw; (B) hindpaw; (C) tail.

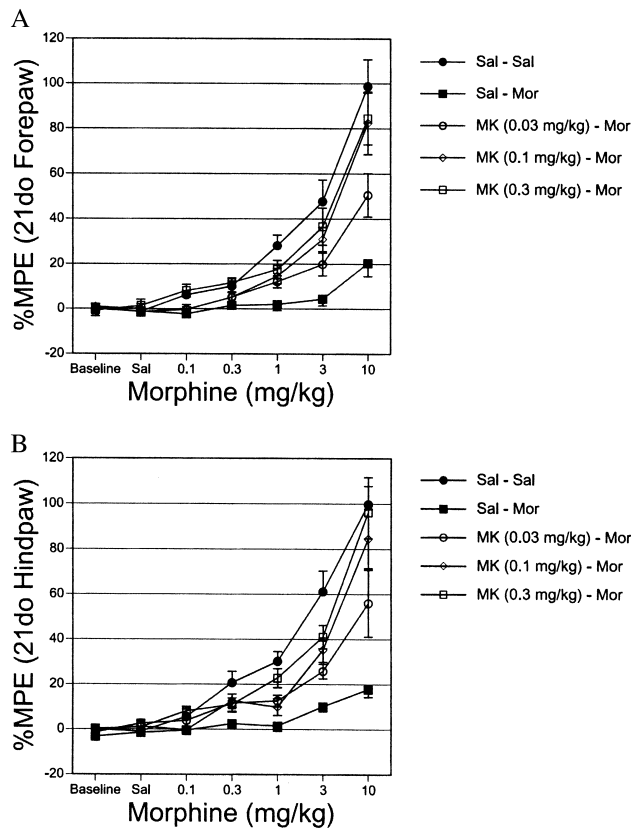


Fig. 3. The effect of pre-treatment with MK-801 (0.03, 0.10, and 0.30 mg/kg) on the development of morphine tolerance in the 21-day-old rat. Ordinate: mean %MPE (mean  $\pm$  1 SEM). Abscissa: acute morphine doses. (A) Forepaw, (B) hindpaw.

Chronic MK-801-morphine treatment attenuated the development of tolerance, as reflected by that the morphine's dose-response curves for the groups that received chronic MK-801-morphine treatment returned toward those of the morphine naive groups (Fig. 3A, B) and decreased  $ED_{50}$  values of acute morphine compared with the groups that received only chronic morphine treatment (Table 2).

Table 3

The effect of chronic treatment with morphine or dextromethorphan plus morphine on the baseline latency of the 7- and 21-day-old rat retracting its appendages from a hotplate

Age	Treatment	Forepaw	Hindpaw	Tail
7do	Sal-Sal	1.631 $\pm$ 0.103	1.702 $\pm$ 0.107	1.811 $\pm$ 0.1
	Sal-Mor	1.614 $\pm$ 0.082	1.661 $\pm$ 0.099	1.52 $\pm$ 0.063
	DM (3 mg/kg)-Mor	1.651 $\pm$ 0.09	1.797 $\pm$ 0.054	1.599 $\pm$ 0.11
	DM (10 mg/kg)-Mor	1.705 $\pm$ 0.105	1.691 $\pm$ 0.095	1.559 $\pm$ 0.081
21do	Sal-Sal	2.053 $\pm$ 0.158	2.185 $\pm$ 0.167	NT
	Sal-Mor	2.012 $\pm$ 0.193	2.239 $\pm$ 0.176	NT
	DM (3 mg/kg)-Mor	2.138 $\pm$ 0.17	2.155 $\pm$ 0.163	NT
	DM (10 mg/kg)-Mor	2.058 $\pm$ 0.18	2.213 $\pm$ 0.157	NT
	DM (30 mg/kg)-Mor	2.104 $\pm$ 0.182	2.107 $\pm$ 0.155	NT

NT: not tested. Data in each cell reflect the mean latency of retraction of the appendage (mean  $\pm$  1 SEM, in s). No significant results were found between any groups.

### 3.4. Dextromethorphan did not attenuate morphine tolerance in the 7-day-old rat

Chronic morphine or dextromethorphan-morphine treatment did not significantly alter the baseline latency (Table 3). In contrast, chronic treatment of morphine rendered the 7-day-old rat tolerant to morphine's acute analgesic effect, as indicated by a right-shift of the dose-response curve (Fig. 4A–C) and a significantly increased  $ED_{50}$  of acute morphine in the chronic morphine groups compared with the morphine naive groups (Table 4).

Chronic co-administration of a low or medium dose of dextromethorphan (3 or 10 mg/kg) with morphine failed to prevent the development of tolerance, as reflected by that the morphine's dose-response curves for the groups that received chronic dextromethorphan-morphine treatment either overlapped with or shifted further to the right of those of the chronic morphine only groups and failed to return to that of the morphine naive groups (Fig. 4A–C). Indeed, dependent on appendage tested, the  $ED_{50}$  values of acute morphine of the groups received chronic co-administration of lower and medium dose of dextromethorphan with morphine were either not significantly different from or significantly higher than the  $ED_{50}$  values of the groups that received only chronic morphine treatment and all were significantly higher than that of the morphine naive groups (Table 4).

Co-administration of a high dose of dextromethorphan (30 mg/kg) with morphine killed all rat pups tested ( $n = 4$ ) on the first day of treatment. Thus, further test of this dose of dextromethorphan on morphine tolerance was terminated for this age group.

Chronic dextromethorphan-morphine treatment also failed to prevent the development of tolerance in the electrophysiological assay. The sample recording traces (Fig. 5) clearly show that acute morphine concentration-dependently suppressed the area of sVRP in spinal cord from morphine naive rats (Fig. 5a). Acute morphine's

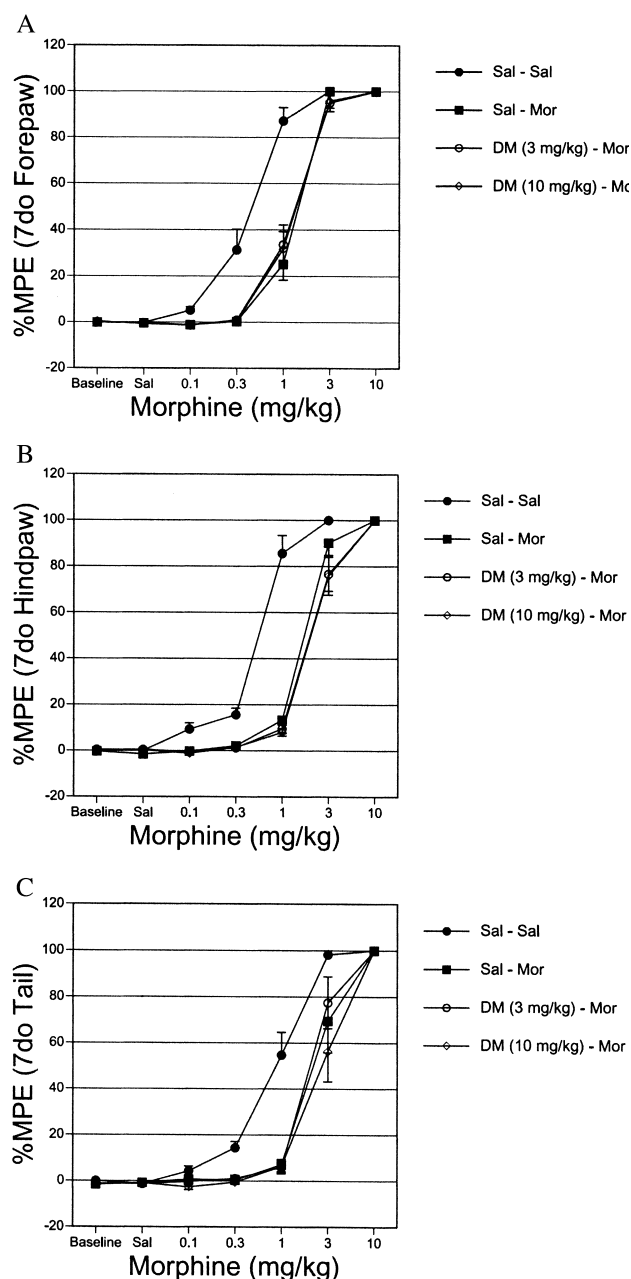


Fig. 4. The effect of pre-treatment with dextromethorphan (3 or 10 mg/kg) on the development of morphine tolerance in the 7-day-old rat. Ordinate: mean %MPE (mean  $\pm$  1 SEM). Abscissa: acute morphine doses. (A) Forepaw; (B) hindpaw; (C) tail.

suppression effect on sVRP area was diminished in spinal cords from rats chronically treated with morphine (Fig. 5b), suggesting that tolerance developed in rats that received chronic morphine treatment. Chronic dextromethorphan–morphine treatment failed to prevent this diminution (Fig. 5c), indicating that pre-treatment with dextromethorphan failed to prevent the development of morphine tolerance in the 7-day-old rat. Significant differences were detected among the three concentration–response curves (Fig. 6) corresponding to the three different chronic treatment regimens (saline–saline, saline–morphine, or dextro-

methorphan–morphine),  $F_{(2,15)} = 26.343$ ,  $P < 0.001$ . Post hoc tests revealed that the concentration–response curves of saline–morphine and the dextromethorphan–morphine were not significantly different from each other and both were significantly shifted to the right than compared to the saline–saline group. Thus, chronic morphine treatment induced tolerance at the spinal cord level in the 7-day-old rat and chronic co-administration of dextromethorphan failed to prevent the development of morphine tolerance at the spinal cord level.

### 3.5. Dextromethorphan attenuated morphine tolerance in the 21-day-old rat

Chronic morphine or dextromethorphan–morphine treatment did not alter the baseline latency (Table 3). In contrast, chronic treatment of morphine rendered the 21-day-old rat tolerant to morphine's acute analgesic effect, as indicated by a right-shift of the dose–response curves (Fig. 7A, B) and a significantly increased  $ED_{50}$  of acute morphine (Table 4) compared with the morphine naive groups.

Chronic dextromethorphan–morphine treatment attenuated the development of tolerance, as reflected by that the morphine's dose–response curves for the groups that received chronic dextromethorphan–morphine treatment returned toward those of the morphine naive groups (Fig. 7A, B) and decreased  $ED_{50}$  of acute morphine (Table 4) compared with the groups that received only chronic morphine treatment.

## 4. Discussion

Bell and Beglan published the first piece of evidence implying that the NMDA receptor antagonist be ineffective in suppressing tolerance in the infant (Bell and Beglan, 1995). Chronic co-treatment of morphine with MK-801 failed to prevent the development of morphine tolerance in the neonatal rat (Bell and Beglan, 1995). However, this earlier study is limited in that it investigated only one NMDA receptor blocker MK-801's effect on morphine tolerance; tolerance was assessed solely by the in vitro paradigm; and it did not address the age issue since it used rats of only one age. Therefore, it is unclear whether the discrepancy between this study and the vast available adult literature was a result of the age effect, a special effect of MK-801, or simply different tolerance assessment paradigms.

In the present studies, we examined the pharmacological effects of two NMDA receptor antagonists on the development of morphine tolerance at different ages, using both the in vivo and the in vitro paradigms for tolerance assessment. Our results clearly show that co-administration of NMDA receptor antagonists MK-801 or dextromethorphan did not inhibit the acquisition of morphine tolerance in the 7-day-old rat, but that both MK-801 and dextromethorphan were

Table 4  
Effects of chronic co-administration of dextromethorphan with morphine on ED<sub>50</sub> of morphine in the infant rat (behavioral test)

Age	Treatment	ED <sub>50</sub> mg/kg (95% C.L.)		
		Forepaw	Hindpaw	Tail
7do	Sal–Sal	0.69 (0.28–1.11)	0.76 (0.34–1.18)	1.03 (0.81–1.25)
	Sal–Mor	1.53 (1.30–1.76) <sup>a</sup>	1.91 (1.72–2.10) <sup>a</sup>	2.63 (2.52–2.75) <sup>a</sup>
	DM (3 mg/kg)–Mor	1.39 (1.14–1.65) <sup>a</sup>	2.45 (2.27–2.64) <sup>a,b</sup>	2.45 (2.36–2.54) <sup>a</sup>
	DM (10 mg/kg)–Mor	1.43 (1.18–1.67) <sup>a</sup>	2.48 (2.34–2.62) <sup>a,b</sup>	2.89 (2.77–3.01) <sup>a,b</sup>
21do	Sal–Sal	3.24 (2.64–3.83)	2.90 (2.15–3.64)	NT
	Sal–Mor	> 10 <sup>a</sup>	> 10 <sup>a</sup>	NT
	DM (3 mg/kg)–Mor	3.71 (1.74–5.68) <sup>c</sup>	3.52 (2.65–4.40) <sup>c</sup>	NT
	DM (10 mg/kg)–Mor	3.45 (2.62–4.27) <sup>c</sup>	3.16 (2.52–3.81) <sup>c</sup>	NT
	DM (30 mg/kg)–Mor	3.27 (2.64–3.89) <sup>c</sup>	2.90 (2.30–3.50) <sup>c</sup>	NT

NT: not tested.

<sup>a</sup> ED<sub>50</sub> significantly greater than Sal–Sal group.

<sup>b</sup> ED<sub>50</sub> significantly greater than Sal–Mor group.

<sup>c</sup> ED<sub>50</sub> significantly smaller than Sal–Mor group.

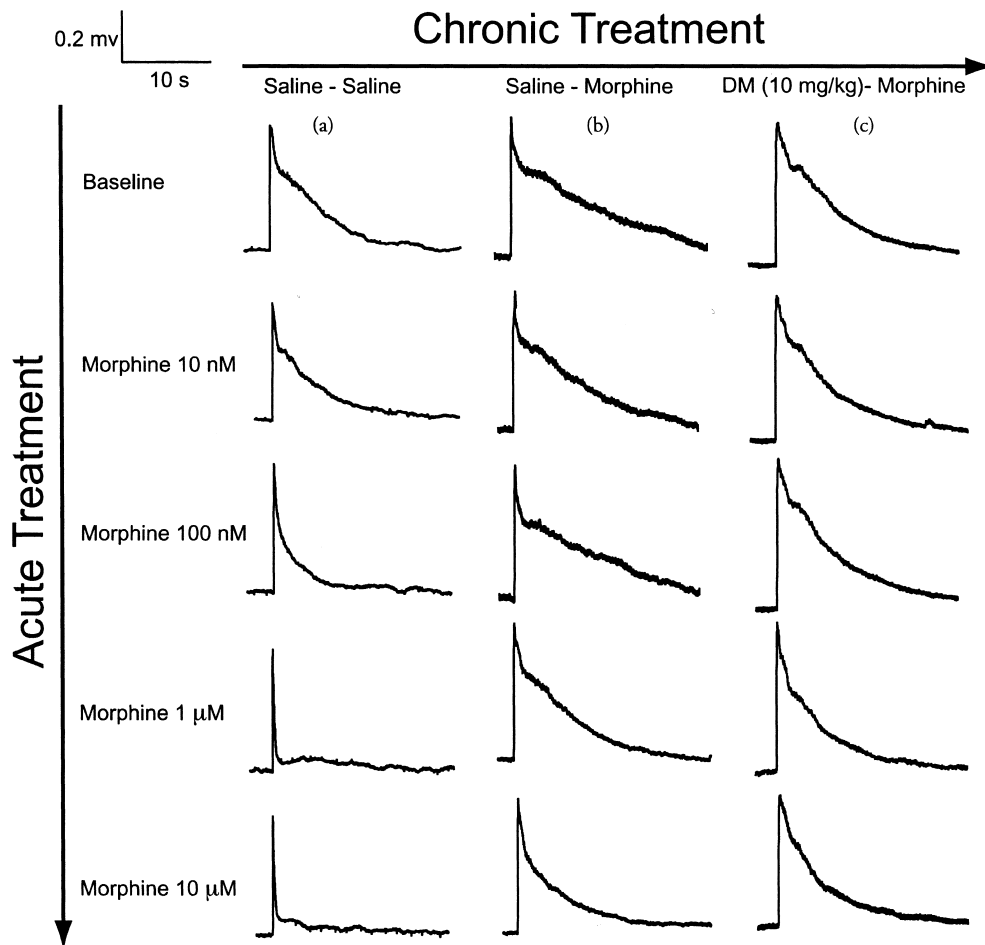


Fig. 5. Sample recording of the sVRP in the isolated spinal cord from 7-day-old rats. Recordings in each column are from one individual rat. The electric stimulus was a constant square wave, with a duration of 200  $\mu$ s, amplitude of 30 V. (a) Acute morphine concentration-dependently suppressed the area of sVRP in morphine untreated rats. (b) Acute morphine's suppression effect on sVRP was diminished by chronic morphine treatment, suggesting that tolerance was developed by chronic morphine treatment. (c) Pre-treatment of dextromethorphan (10 mg/kg) failed to prevent the development of morphine tolerance in the 7-day-old rat.

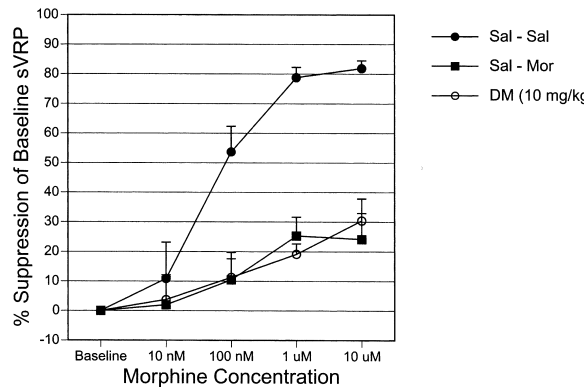


Fig. 6. Concentration–response curves of acute morphine's suppression effect on the pain-related sVRP area in isolated spinal cord from rats chronically treated with saline–saline, saline–morphine, and dextromethorphan–morphine injections. The DM (10 mg/kg)–Mor curve and the Sal–Mor curves are not significantly different from each other and both are significantly shifted to the right compared with the Sal–Sal curve. See

effective in rats with age of 14 days or older. The inhibition of tolerance by NMDA receptor antagonists is selective for the acquisition of tolerance rather than an enhancement of acute morphine's analgesic effect (Elliott et al., 1994, 1995; Manning et al., 1996; Mao et al., 1996). We did not test older rats since the effectiveness of MK-801 and dextromethorphan in attenuating morphine tolerance in the adult animals is well documented. At the same time, as shown in the present studies, the effects of chronic MK-801 in the 14- and 21-day-old rats and the effects of chronic dextromethorphan in the 21-day-old rat were quite comparable, suggesting that the underlying mechanisms by which NMDA receptor antagonists reduce tolerance are already stable at these ages. Our data thus suggest that there is a transition period for NMDA receptor antagonists in attenuating the development of morphine tolerance in the rat and this period seems to be somewhere during the second postnatal week. The timing of the effectiveness of MK-801 in inhibiting the acquisition of morphine coincides well with the maturation of the NMDA receptor (Hrabetova et al., 2000; Sircar, 2000). Therefore, it is possible that the different pharmacological properties of MK-801 and dextromethorphan in different aged rats may reflect the different developmental stages of the NMDA receptor.

Our data in the 7-day-old rat do not support a role of the NMDA receptor in tolerance in the infant, whereas our data in the 14- and 21-day-old rats are consistent with the widely cited Mao, Price, and Mayer model on opiate tolerance and dependence (Mao, 1999; Mao et al., 1995; Mayer et al., 1999). This model places the NMDA receptor at an indispensable position for the acquisition of opiate tolerance and dependence (Trujillo, 1999). On the one hand, the role of the NMDA receptor in the development of opiate tolerance in the adult rodents has been firmly supported (Trujillo, 1999) and our data for the 14- and 21-day-old rats also support the role of the NMDA receptor in rats of these ages. Nevertheless, this model may need to be expanded to

take into consideration the fact that opiate tolerance can be established in the infant (Barr and Wang, 1992; Thornton and Smith, 1997; Thornton et al., 1997; Van Praag and Frenk, 1991; Windh et al., 1995) in the absence of functionally mature NMDA receptors.

One of the crucial assumptions in this model is that the activation of NMDA receptor results in  $Ca^{2+}$  influx into the cell and activates the second messenger, thus leading to long-lasting plasticity changes (Mao, 1999). Because of the combination of factors, such as low-level expression of NR1 subunit, lack of NR2A subunit and the expression of NR3A subunit (Ciabarra et al., 1995; Das et al., 1998; Sucher et al., 1995), the immature NMDA receptor seems to have low  $Ca^{2+}$  permeability (Barrios and Liljequist, 1996). The dose of MK-801 and dextromethorphan in our experiment should have effectively blocked the  $Ca^{2+}$  permeable ion pore of the immature NMDA receptor. Thus, it seems that the immature NMDA receptor is not a vital element to the development of opiate dependence in rats of this age. We have previously proposed that in the 7-day-old rat, the establishment of opiate tolerance and dependence relies on the same second messenger system as in the adult. The difference between the infant and adult may lie somewhere more “upstream” (Zhu and Barr, 2001b). One possibility is that other

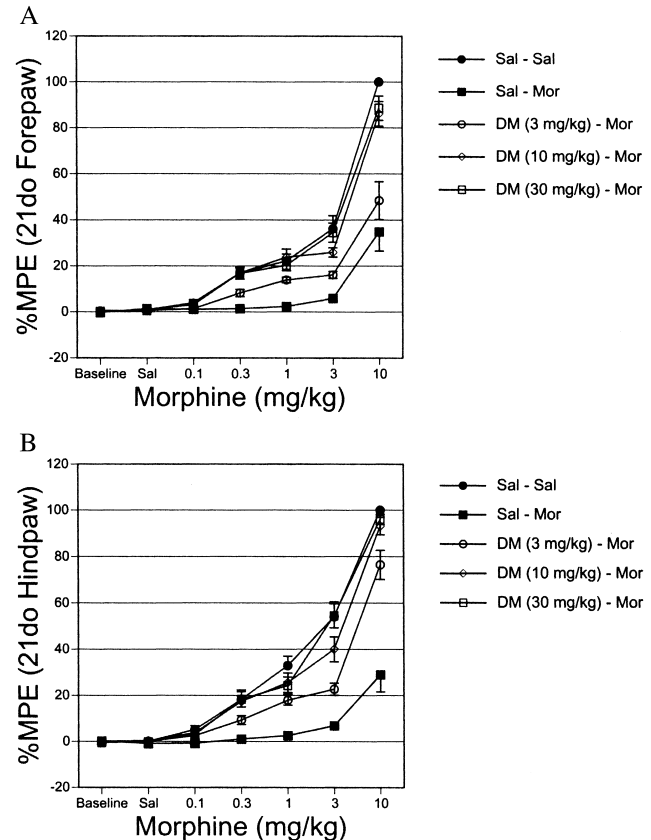


Fig. 7. The effect of pre-treatment with dextromethorphan (3 or 10 mg/kg) on the development of morphine tolerance in the 21-day-old rat. Ordinate: mean %MPE (mean  $\pm$  1 SEM). Abscissa: acute morphine doses. (A) Forepaw; (B) hindpaw.

mechanisms activate the intracellular  $\text{Ca}^{2+}$  release and evoke the  $\text{Ca}^{2+}$ -dependent second messenger system such as NO production. A second possibility is that channels besides the NMDA receptor allow significant  $\text{Ca}^{2+}$  influx in the infant CNS responsible for opiate actions. If so, the essence of the Mao et al. model, which focuses on the role of the second messenger system, including the influx of  $\text{Ca}^{2+}$  into the cell and the production of NO, may still hold in the infant. But, in the infant, there may exist some other mechanisms fulfilling the role of coupling to the activation of  $\text{Ca}^{2+}$ -dependent second messenger system and at a later developmental time either confer this role to the NMDA receptor or play an adjunct role to the NMDA mediated mechanism.

One of these mechanisms may be mediated by the metabotropic glutamate receptors (mGluRs). Because mGluRs are coupled to various second messenger including  $\text{Ca}^{2+}$  cascades, they are ideal candidates for long-lasting intracellular changes such as learning and memory formation (Conn and Pin, 1997; Ozawa et al., 1998). Recently, it has been reported that the development of morphine tolerance could be inhibited by a group II mGluR agonist LY354740 in adult mice (Popik et al., 2000). Therefore, it is possible that in the infant rat, the mGluRs play a more important role than the NMDA receptor and that these parallel mechanisms may co-exist even in the adult, although NMDA receptor's importance increases after the early transition period.

The AMPA receptor may also activate the  $\text{Ca}^{2+}$ -dependent second messenger systems in neural circuits involved in opiate tolerance in the newborn rat. The immature AMPA receptor has high  $\text{Ca}^{2+}$  permeability (Geiger et al., 1995; Ozawa et al., 1998; Pellegrini-Giampietro et al., 1992) and activation of AMPA receptor produces a marked increase in cytoplasmic free  $\text{Ca}^{2+}$ , which further activates downstream  $\text{Ca}^{2+}$ -dependent intracellular events (Jakowec et al., 1995a,b; Ozawa et al., 1998) such as NO production (Kest et al., 1997). Indeed, AMPA receptor antagonists have been reported to reverse morphine's analgesic tolerance in adult mice (Kest et al., 1997). Thus, it is possible that AMPA receptor is involved in the development of morphine tolerance in the younger rat at a period when the NMDA receptor is not functional.

This possible involvement of other glutamate receptor types may also explain the exacerbation of morphine tolerance seen in the 7-day-old rat by chronic co-administration of higher dose of MK-801 or dextromethorphan with morphine. Since the vast majority of excitatory transmission in the CNS is conducted by glutamate receptors (Dingledine et al., 1999), chronic blockade of the NMDA receptor may activate compensatory mechanisms such as up-regulation of the mGluRs and AMPA receptors, resulting in greater levels of tolerance by these potential mechanisms other than the NMDA mediated component. However, we know of no data addressing this possibility.

In summary, the present study is the first to provide conclusive evidence that NMDA receptor antagonists are not effective in attenuating tolerance in the newborn rat; and there is a transition age, around the second postnatal week, for NMDA receptor antagonists to be effective in inhibiting the development of morphine tolerance in the rat.

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