

# The role of AMPA and metabotropic glutamate receptors on morphine withdrawal in infant rats

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## Abstract

Glutamate receptors, especially *N*-methyl-D-aspartate (NMDA) receptors, are hypothesized to play key roles in opiate tolerance and withdrawal. There is also accumulating evidence that  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists and group II metabotropic glutamate receptor (mGluR) agonists attenuate opiate withdrawal. However, most existing data are derived from adult animal models. Glutamate receptor types undergo dramatic developmental changes during early life. Thus, the pharmacological effects on opiate withdrawal of NMDA receptor, AMPA receptor, and mGluR antagonists in the developing organism may not be comparable to those in the adult. Indeed, NMDA receptor antagonists do not block morphine tolerance or withdrawal in the 7-day-old rat, but are partially effective in the 14-day-old, and fully effective in the 21-day-old. Thus, there is a transition period around the second post-natal week for NMDA receptor antagonists to suppress opiate tolerance and withdrawal. A combination of *in vivo* and *in vitro* assays was used in the present studies to test the effect of drugs acting on AMPA and group II mGlu receptors on morphine withdrawal in rats at 7, 14, and 21 days of age. These ages represent the critical periods when various glutamate receptor subunits undergo differential change. In contrast to NMDA receptor antagonists' early ineffectiveness in suppressing morphine withdrawal, the AMPA receptor antagonist and the group II mGluR agonists were effective at all ages tested. Thus, for the human infant patient, pharmacotherapies to reduce opiate tolerance and withdrawal should focus on non-NMDA ionotropic and metabotropic receptors.

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## 1. Introduction

There is a population of opiate-addicted childbearing-aged women in the United States and each year a large numbers of infants are born to these women (Hutchings, 1987). These human infants have a higher incidence of morbidity and mortality than do the offsprings of non-addicted women (Connaughton et al., 1977; Naeye et al., 1973; Wilson et al., 1979). In addition, improvements in short- and long-term clinical outcomes of critically ill neonates have necessitated the widespread use of opioid drugs for analgesia and sedation (Suresh and Anand, 2001).

These changes in clinical practice have led to an increasing incidence of opiate tolerance and dependence during therapy, with consequent increases in the number of infant patients showing clinical signs of opioid withdrawal when therapy is discontinued (Suresh and Anand, 2001). Thus, there is an ever-increasing need for the study and treatment of opiate tolerance and withdrawal in the infant (Suresh and Anand, 2001).

Animal models of opiate tolerance and withdrawal have provided a better understanding of both the acute and long-term risk of opiate use in the human infant population (Barr and Jones, 1994; Hutchings and Dow-Edwards, 1991; Zhu and Barr, 2001a). In the infant rat, models for assessing opiate tolerance and withdrawal have shown that: (1) when the behavioral repertoire appropriate to the age of the animal

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is examined, infant rat pups develop opiate tolerance and dependence (Jones and Barr, 1995); (2) the in vitro “isolated rat spinal cord” (Otsuka and Konishi, 1974) can be adapted to examine opiate tolerance and withdrawal in the week-day-old rat pups (Bell and Beglan, 1995a,b; Feng and Kendig, 1995; Zhu and Barr, 2003). These models of opiate tolerance and withdrawal in the infant rat have provided a stepping stone for understanding the neural mechanisms of opiate tolerance and withdrawal in developing organisms (Jones and Barr, 2001).

In spite of decades of research, the precise mechanisms underlying opiate withdrawal remains elusive (Nestler, 2001; Trujillo, 1999); multiple mechanisms are likely involved (Redmond and Krystal, 1984; Thorat et al., 1994). One possibility is that chronic morphine treatment up-regulates the cyclic AMP pathway and withdrawal from opiates result in compensatory hyperactivity of this path (Nestler, 2001; Nestler and Tallman, 1988). A second likely possibility is that opiate withdrawal results in augmented central release of glutamate, which in turn contributes to the signs typifying opiate withdrawal (Aghajanian et al., 1994; Akaoka and Aston-Jones, 1991; Tokuyama et al., 1996). These two mechanisms may interact with each other; thus opiate withdrawal manifested by behavioral, electrophysiological, and biochemical indices may have dual-mechanisms (Aghajanian et al., 1992).

Glutamate is a major excitatory neurotransmitter in the mammalian CNS and exerts its function through two broad classes of receptors: ionotropic receptors, which include *N*-methyl-*D*-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors, and mGluRs that couple via G-proteins to intracellular second messenger cascades (Ozawa et al., 1998).

In adult animals, NMDA receptor antagonists inhibit the acquisition of opiate tolerance and dependence, and the expression of opiate withdrawal (for review, see (Herman et al., 1995; Mao, 1999; Trujillo, 1999)). Various drugs that act on the NMDA receptor or on  $\text{Ca}^{2+}$ -dependent second messenger systems have been studied recently in the infant rats. In contrast to the data in adults, NMDA receptor antagonists are neither effective in blocking the development of opiate tolerance or dependence (Bell and Beglan, 1995a,b; Zhu and Barr, 2001a, 2003), nor effective in suppressing the expression of opiate withdrawal (Zhu and Barr, 2000, 2001a) in infant rats. In particular, 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. In contrast, MK-801 reduces but does not eliminate withdrawal in the 14-day-old rat and fully abrogates withdrawal in the 21-day-old rat (Zhu and Barr, 2001a). Thus, the transition age for MK-801 to be effective in suppressing morphine withdrawal is around the second post-natal week (Zhu and Barr, 2001a). 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, in the infant, although opiate actions rely

on the same second messenger systems as in the adult, the mechanisms by which they are activated may differ (Zhu and Barr, 2001a,b).

Our more recent data (Barr and Zhu, 2001; Zhu and Barr, 2003) have further extended these findings to the establishment of morphine tolerance (Zhu and Barr, 2003). Neither of two NMDA receptor antagonists (MK-801 and dextromethorphan) was effective in blocking the development of morphine tolerance in the 7-day-old rat yet both were fully effective in the 21-day-old rat. Therefore, 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).

The AMPA receptor, another member of the glutamate receptor family, seems to fit such a role. First, that the AMPA receptor is co-localized with the NMDA receptor in many cell types (Gu et al., 1996) including NOS-positive neurons (Fedele and Raiteri, 1999) suggests that the AMPA receptor, similar to the NMDA receptor, is involved in neural circuitry that is activated during opiate withdrawal. Indeed, chronic morphine treatment elevates the AMPA receptor GluR 1 subunit levels in the rat ventral tegmental area (Fitzgerald et al., 1996). Morphine-induced tolerance and dependence are reduced in mice deficient in the AMPA receptor subunit A (Vekovischeva et al., 2001). Furthermore, morphine withdrawal increases the level of both AMPA receptor mRNA expression and [3H]AMPA binding in various brain sites in the rat (Jang et al., 2000). Secondly, in adult mice or rats, LY 293558, a competitive AMPA receptor antagonist, attenuates and reverses morphine analgesic tolerance (Kest et al., 1997; McLemore et al., 1997), inhibits the development of acute morphine dependence (McLemore et al., 1997), and decreases behavioral signs of morphine withdrawal (Rasmussen et al., 1996). Another AMPA receptor antagonist CNQX, when infused directly into the central nucleus of the amygdala or locus coeruleus (LC), attenuates behavioral signs of naloxone-precipitated withdrawal (Taylor et al., 1998). Furthermore, LY 293558 effectively suppresses morphine withdrawal-induced firing of locus coeruleus neurons (Rasmussen, 1995; Rasmussen et al., 1996) whereas NMDA receptor antagonists do not (Rasmussen et al., 1996). Thus, the AMPA receptor plays at least a role auxiliary to, if not parallel to and independent of, the role of the NMDA receptor in opiate tolerance and withdrawal in adult animals. Last but most importantly, the developmental profile of the AMPA receptor, in contrast to that of the NMDA receptor, suggests that the AMPA receptor rather than the NMDA receptor may play the dominant role in younger animals in these processes.

Although several authors have reported that AMPA receptor antagonists treatment reduce opiate tolerance and withdrawal (Cappendijk et al., 1993; Kest et al., 1997; McLemore et al., 1997; Rasmussen, 1995; Rasmussen et al., 1996; Taylor et al., 1998), these investigations were

exclusively done in vivo in the adult animals. It is not known whether these results 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 pre-natally and post-natally (Fitzgerald, 1995). At the same time, the AMPA receptor, which is believed to play an essential role in opiate tolerance and withdrawal, undergoes qualitative and quantitative changes during development.

Thus, the present study examined the effect of a competitive AMPA receptor antagonist NBQX in morphine tolerance and withdrawal in the infant rat using a combination of in vivo and in vitro methods. There are two reasons for such an approach: (1) AMPA receptors undergo developmental changes during earlier life, suggesting that a developmental difference would be possible, and therefore, conclusions based on information derived from adult animals models may not hold in the developing organism; (2) given that glutamate receptor neural transmission system is ubiquitous in the CNS, blockade or activation will undoubtedly generate a myriad of effects (Dingledine et al., 1999; Ozawa et al., 1998), the in vitro system would be more specific than the in vivo model in addressing the pharmacological effect of NBQX specific to opiate withdrawal.

In addition to the AMPA receptor, mGluRs may also play a significant role in opiate withdrawal. To date, eight genes encoding for mGluRs have been cloned (mGlu1–8) (Dingledine et al., 1999). These receptors are generally classified into three groups (group I–III) based on their structural homology, signal transduction mechanism, and pharmacology. Group I (mGluR 1 and 5) receptors are coupled to phospholipase C and stimulate phosphoinositide hydrolysis and intracellular  $Ca^{2+}$  signal transduction. Group II (mGluR 2 and 3) and group III (mGluR 4 and 6–8) receptors are negatively coupled to adenylyl cyclase (Pin and Duvoisin, 1995). Activation of group II and group III mGluRs inhibits forskolin-induced increases in cAMP accumulation in brain slices and neuronal cultures, whereas stimulation of group I mGluRs does not (Conn and Pin, 1997). Through the decreased production of cAMP, group II mGluR activation has been hypothesized to play a critical role in opiate withdrawal (Fundytus and Coderre, 1994). Consistent with this hypothesis, recently, it has been shown that the mGlu2/3 receptor agonists, such as DCG-IV and LY 354740 decrease both behavioral signs of opiate withdrawal (Fundytus and Coderre, 1997; Klodzinska et al., 1999; Vandergriff and Rasmussen, 1999) and opiate withdrawal-induced activation of LC neuronal firing in the adult rat (Vandergriff and Rasmussen, 1999). In addition, the group II mGluR agonist LY 354740 attenuates tolerance to the analgesic effect of morphine in the adult mouse (Popik et al., 2000). However, these investigations were exclusively done in the adult animals and all of these studies employed in vivo models. It is not known whether these results in adults apply to infants.

Thus, in addition to investigating AMPA receptor's role in morphine dependence in infant rats, the present study further examined the effect of a group II mGluR agonist

DCG-IV on opiate withdrawal in the infant rat using a combination of in vivo and in vitro methods.

## 2. Material and methods

### 2.1. Subjects

Subjects were 1–21-day-old Long-Evans hooded male and female rat pups bred and born in our facilities (two males and two females per litter). Our previous studies have demonstrated that there is no sex differences in withdrawal behavior (Jones and Barr, 1995; Zhu and Barr, 2000). Dams were checked for new births twice daily at 9 AM and 5 PM. The day of birth was designated as age 0. All pups were housed with the dam and their siblings. Food and water were available at all times. The animals were maintained under a 12-h-light:12-h-dark cycle (lights on at 7:30 AM). Pups were removed from the cage and kept warm (30–32 °C) in an incubator until testing. All work was done in accordance with the provisions of the HHS "Guide for the Care and Use of Laboratory Animals" and the "Principles for the Utilization and Care of Vertebrate Animals".

In the behavioral tests, eight litters were used for each drug and each age; a total of 48 litters were tested for withdrawal behaviors.

### 2.2. Morphine treatment and behavior observation

Pups were tattooed with India ink injected into one or two paws to label individual pups permanently in each litter (Geller and Geller, 1966). To induce morphine dependence, starting at 1, 8, or 15 days of age, all pups were removed from the dam and individual rats were injected with morphine sulfate (IP, 10 mg/kg) twice daily (10 AM and 6 PM) for 6.5 days. The last injection was in the morning of the seventh day. In the afternoon of the seventh 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. In the AMPA receptor studies, the pups were treated with NBQX (IP, 5, 15, and 45 mg/kg, or saline as control for acute treatment in the withdrawal experiment;  $n = 8$  for each dose). In the mGluR studies, the pups were treated with DCG-IV (intracisternal injection, IC, 3, 15, and 75 pmol, or saline as control,  $n = 8$  for each dose). DCG-IV required IC injection because it does not pass the blood–brain barrier, and comparable studies used similar techniques to deliver it directly into the CNS (Fundytus and Coderre, 1997). The IC injection procedure followed a published protocol (Carden et al., 1991). Briefly, during the IC 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

Table 1  
Precipitated withdrawal behaviors in the infant rat

Burrow	Sliding the body under the shavings of the observation chamber
Head moves	Lateral and rotary motions of the head
Moving paws	Continuous movement of the hindpaws without walking
Quiet	Sedated appearance without movement. Decreased time spent “quiet” is indicative of withdrawal
Rolling	Turning the body over at least on one full rotation
Together	Bodily contact with one or more littermates.
Walking	Taking more than one step forward
Wall-climbing	Placing at both forepaws on the wall of the observation chamber

saline in a similar manner. After the drug treatment, the pup was placed back into the observation chamber with the remainder of the litter (without the dam). After 15 min, naltrexone (IP, 1.0 mg/kg) was injected to precipitate withdrawal. 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 NBQX and DCG-IV on the morphine-dependent rat without the influence of naltrexone. During these observation periods, the behavior of the pups was scanned sampled every 15 s and recorded on a checklist by an observer (see Table 1 for definitions of behaviors 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. Thus, except for during injection, the pup always remained with the litter to reduce possible stress. The next pup was then tested until all treatment groups were completed. Animals were sacrificed with sodium pentobarbital at the conclusion of the experiments. 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 (Thornton and Smith, 1997; Thornton et al., 1997; Windh et al., 1995) have demonstrated clearly that saline-treated rat pups of comparable age basically remain quiet and do not display withdrawal behaviors.

### 2.3. *In vitro* electrophysiology

The standard electrophysiology experimental setup (Zhu and Barr, 2003) was followed in the present studies. Spinal cords were obtained from 7-day-old rat pups only. These rats were treated similarly to those in the behavioral experiments from day 1 to day 7, except that on the seventh day, morphine withdrawal was assessed by the electrophysiological assay instead of the behavioral test. In electrophysiological studies of opiate withdrawal, spinal cords from pups treated with chronic saline or morphine were used. Dorsal

root evoked ventral root potentials (sVRP) were recorded from the ventral root for 30 min subsequent to the introduction of naltrexone (10  $\mu$ M) into the bath. The sVRP activity was digitized and the integral of the activity above baseline from 0 to 8 s computed and defined as the measure of physical withdrawal (Bell and Adler, 1988; Bell and Beglan, 1995a,b; Feng and Kendig, 1997). In studying the acute pharmacological effects of NBQX (3  $\mu$ M) or DCG-IV (30 nM) on morphine withdrawal, drugs were continuously applied to the ACSF starting 15 min before the introduction of naltrexone.

### 2.4. Application of chemicals

NBQX, DCG-IV, morphine, and naltrexone were made up as 1000 $\times$  stock solutions and were diluted in oxygenated ACSF solution immediately prior to use. They were applied to the spinal cord in known concentrations by addition to the superfusing medium. All compounds were applied via the bath, and each spinal cord served as its own control.

To construct concentration–response curves for the effects of these two drugs on the sVRP without naltrexone, NBQX (0.1, 1, 10, and 100  $\mu$ M) or DCG-IV (10 nM, 100 nM, 1  $\mu$ M, 10  $\mu$ M) was applied in the superfusate for 30 min after 15 min of baseline sVRP recording. For the withdrawal test, after 15 min of baseline sVRP recording, NBQX (3  $\mu$ M) or DCG-IV (30 nM) was applied in the superfusate and 15 min later, naltrexone (10  $\mu$ M) was introduced into the superfusate, after which, either NBQX or DCG-IV coexisted with naltrexone in the superfusate for the whole length of recording.

### 2.5. Behavior data analysis

Separate statistical tests were conducted for the three age groups and for each of the withdrawal behaviors. Multiple tests were necessary because there was no obviously way to combine behaviors. In addition, withdrawal behavior changes during development (Jones and Barr, 1995). Occurrences of each behavior were summed for the 15-min post-naltrexone observation period. All doses of NBQX or DCG-IV and their corresponding vehicles were injected within a single litter and thus treatment effect was treated as a within-subjects variable. A one-way ANOVA was conducted for the occurrence of each of the specific withdrawal behaviors as defined in Table 1. If significant results were detected, individual multiple comparisons were then conducted by Fisher's PLSD tests.

### 2.6. Electrophysiology data analysis

The effects of drug application in any individual spinal cord were determined by comparing the averaged sVRP area values of five consecutive sVRPs evoked immediately prior to any drug application (baseline sVRP area) to the area values of the sVRPs evoked during drug applications (treat-

ment sVRP area). All data points were normalized to the averaged baseline sVRP area and are expressed as percentage of control ( $(\text{treatment sVRP area}/\text{baseline sVRP area}) \times 100$ ). All values are expressed as means  $\pm$  one S.E.M. Statistical significance for the time effect of acute drug treatment was assessed relative to control responses by use of related samples Student's *t*-tests.

For withdrawal activity, baseline sVRP measurements were established before naltrexone was added to the spinal superfusate. The augmented sVRP due to morphine withdrawal is expressed as a percentage of the baseline sVRP. Three way factorial designs were used to analyze these data. The factors were acute treatment (e.g. NBQX versus saline), chronic treatment (morphine versus saline), and time (minutes of recording). There were no consistent time changes and they are not presented in the data analysis here. To test significance of treatment effects among different treatment groups, significant ANOVA's were further analyzed by Fisher's PLSD tests. For both behavioral and electrophysiological data statistical analyses, effects were considered significant if  $P < .05$ .

### 3. Results

#### 3.1. NBQX attenuates behavioral signs of morphine withdrawal

##### 3.1.1. Pre-naltrexone

As summarized in Table 2, control data for the pre-naltrexone period show that treatment with various doses

Table 2  
Statistical analyses results (*F* and *P* values) for the dose effect of NBQX on baseline behaviors (pre-naltrexone period) and on morphine withdrawal behaviors (post-naltrexone period)

Behavior	Age					
	7-Day-old		14-Day-old		21-Day-old	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Baseline/pre-naltrexone						
Burrow	ND	ND	ND	ND	0.64	0.60
Head moves	0.47	0.71	0.15	0.93	2.53	0.08
Moving paws	1.94	0.15	0.86	0.48	ND	ND
Quiet	2.35	0.10	2.91	0.06	0.20	0.89
Rolling	0.16	0.92	ND	ND	ND	ND
Together	1.00	0.41	2.42	0.09	0.39	0.76
Walking	0.64	0.60	<b>10.19</b>	<b>0.00</b>	0.28	0.84
Wall-climbing	ND	ND	0.83	0.49	0.10	0.96
Withdrawal/post-naltrexone						
Burrow	ND	ND	<b>8.10</b>	<b>0.00</b>	<b>5.80</b>	<b>0.00</b>
Head moves	<b>20.13</b>	<b>0.00</b>	<b>26.44</b>	<b>0.00</b>	<b>8.33</b>	<b>0.00</b>
Moving paws	<b>26.81</b>	<b>0.00</b>	<b>9.69</b>	<b>0.00</b>	ND	ND
Quiet	<b>60.92</b>	<b>0.00</b>	<b>36.85</b>	<b>0.00</b>	<b>14.84</b>	<b>0.00</b>
Rolling	3.04	0.05	ND	ND	ND	ND
Together	2.79	0.07	0.89	0.46	<b>4.66</b>	<b>0.01</b>
Walking	<b>8.38</b>	<b>0.00</b>	<b>11.98</b>	<b>0.00</b>	<b>11.73</b>	<b>0.00</b>
Wall-climbing	<b>11.91</b>	<b>0.00</b>	2.55	0.08	0.83	0.49

ND: this behavior was not displayed. Bold face: result is statistically significant. d.f. = (3, 28) in all cases.

of NBQX at any age did not significantly alter the baseline behaviors of the morphine-dependent rat, except for walking of 14 days of age (5 mg/kg only). Similar to the control group, except for some baseline activities, all experimental groups largely remained quiet before naltrexone treatment.

##### 3.1.2. 7-day-old

The results ( $N = 32$ ) for the post-naltrexone period show that treatment with NBQX significantly reduced most of the morphine withdrawal behaviors precipitated by naltrexone in the 7-day-old rat. NBQX dose dependently decreased head moves, moving paws, walking, and wall-climbing and increased quiet behavior. We failed to find any significant effect of NBQX on together and rolling. The dose effects of pre-treatment of NBQX on naltrexone-precipitated morphine withdrawal in the 7-day-old rat are depicted in Fig. 1.

##### 3.1.3. 14-day-old

The results ( $N = 32$ ) for the post-naltrexone period show that treatment with NBQX also significantly reduced most withdrawal behaviors in the 14-day-old rat. NBQX dose dependently decreased burrow, head moves, moving paws, and walking and increased quiet behavior. Pre-treatment with NBQX also tended to suppress wall-climbing and together behavior, although the result did not reach statistical significance. The dose effects of pre-treatment of NBQX on naltrexone-precipitated morphine withdrawal in the 14-day-old rat are depicted in Fig. 1.

##### 3.1.4. 21-day-old

As for the two younger ages ( $N = 32$ ), NBQX significantly reduced most of the morphine withdrawal behaviors precipitated by naltrexone in the 21-day-old rat. NBQX dose dependently decreased burrow, head moves, together, and walking and increased quiet behavior. We did not find any significant effect on wall-climbing. The dose effects of pre-treatment of NBQX on naltrexone-precipitated morphine withdrawal in the 21-day-old rat are depicted in Fig. 1.

#### 3.2. Spinal cords from morphine-treated rats demonstrate robust withdrawal in vitro

Stable recording of the sVRP can be made from spinal cord of the neonatal rat for up to at least 20 h (Herrero et al., 2000; Kerkut and Bagust, 1995). In our experiments, 7-day-old control rats that did not receive chronic morphine treatment ( $n = 4$ ), demonstrated very stable sVRP over time (Fig. 2). The addition of naltrexone (10  $\mu\text{M}$ ) to the superfusate did not alter the sVRP area value significantly at any time point of recording (sVRP area values from  $98.43 \pm 2.66\%$  of control to  $104.29 \pm 3.17\%$  of control;  $P > .05$  at all time points).

Rats that received chronic morphine treatment ( $n = 6$ ), demonstrated similarly stable recording in the normal perfusion medium (Fig. 2). However, when naltrexone (10  $\mu\text{M}$ )

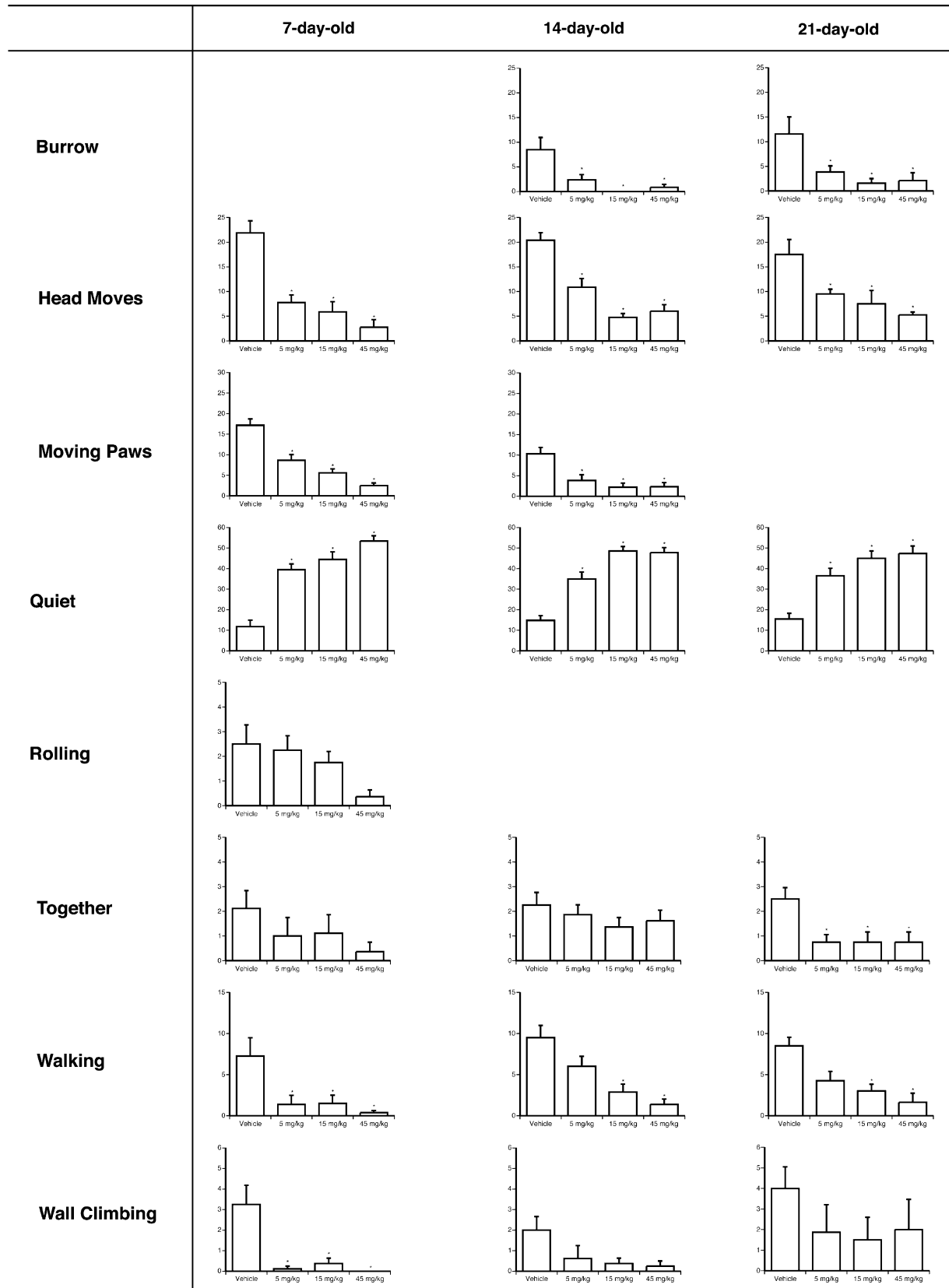


Fig. 1. The effect of pre-treatment with the competitive AMPA receptor antagonist NBQX (5, 15, and 45 mg/kg) on naltrexone-precipitated morphine withdrawal behaviors in the 7-, 14-, and 21-day-old rats. Ordinate: mean occurrences (mean  $\pm$  one S.E.M.) in 15 min of opiate withdrawal behaviors (definitions see Table 1). Abscissa: NBQX doses. Withdrawal was precipitated by naltrexone (1 mg/kg) in all groups. \*,  $P < 0.05$  compared to the saline control group. Note scale change.

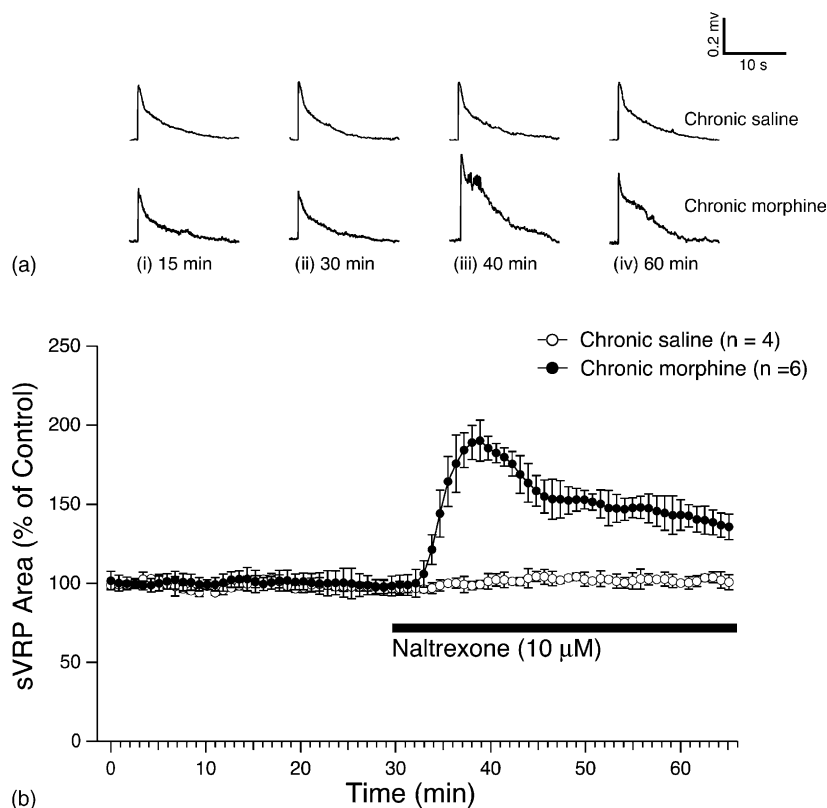


Fig. 2. Time and treatment effect of morphine withdrawal in the isolated spinal cord from the 7-day-old rat. The data represent the time course of morphine withdrawal in the isolated rat spinal cord (without NBQX): (a) upper trace: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated chronically with saline. Lower trace: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated with morphine; (b) time course of morphine withdrawal in the isolated rat spinal cord. Ordinate: sVRP area expressed as the percentage of baseline sVRP area (mean  $\pm$  one S.E.M.). Abscissa: time of recording (min).

was added to the superfusate, sVRP of spinal cords from morphine-treated rats rapidly increased to reach a peak approximately 10 min after naltrexone was added in ( $190.25 \pm 9.34\%$  of control;  $P < .001$ ;  $n = 6$ ). The intensity of this augmentation of sVRP then declined to reach a relatively stable elevated plateau (approximately 150% of control;  $P < .05$  at all time points;  $n = 6$ ) for the whole length of recording (65 min total, 35 min after naltrexone added) (Fig. 2). In contrast to spinal cords from morphine naive rats, which showed neither time nor treatment effect before or after naltrexone was added to the superfusate, spinal cords from morphine-treated rats demonstrated clear time and treatment effects in a naltrexone-containing medium. Consistent with studies by others (Bell and Beglan, 1995a), we demonstrated robust withdrawal in spinal cords from morphine-treated rats as assessed by the in vitro isolated spinal cord model. This experiment provided us a basis for further studying NBQX's effect in morphine withdrawal in the next experiment.

### 3.3. NBQX's effect on sVRP area in the 7-day-old rat

Genesis of the sVRP relies on the summation of many synaptic functions (Collins et al., 1995; Thompson et al., 1992; Thompson et al., 1993) and high concentrations of

NBQX significantly inhibit normal synaptic transmission (Zeman and Lodge, 1992). Although there are several reports examining the effects of another AMPA receptor antagonist, CNQX, on some components of the sVRP in the 8–12-day-old rats (King et al., 1992; Thompson et al., 1992), there are no quantified dose–response studies of NBQX's effect on sVRP in the 7-day-old rat. Thus, the first goal of this experiment was to determine a suitable concentration of NBQX that did not significantly alter the baseline sVRP for later experiments.

The spinal cords from four male naive 7-day-old rats were used to determine the concentration–response curve of NBQX's effect on the sVRP in at this age. NBQX at concentrations of 0.1 and 1  $\mu\text{M}$  did not produce an appreciable effect on the baseline sVRP ( $98.92 \pm 3.45\%$  and  $98.46 \pm 3.91\%$  of control, respectively). At 10  $\mu\text{M}$ , NBQX slightly suppressed the sVRP area ( $89.31 \pm 6.48\%$  of control;  $P > .05$ ). Although statistically not significant, that is likely because we used a relatively small sample size rather than a lack of significant effect of NBQX on the sVRP at this concentration. At a concentration of 100  $\mu\text{M}$ , NBQX markedly suppressed sVRP ( $66.26 \pm 8.48\%$  of control;  $P < .05$ ). The concentration–response curve for NBQX's on the sVRP in the 7-day-old rat is depicted in Fig. 3. These results

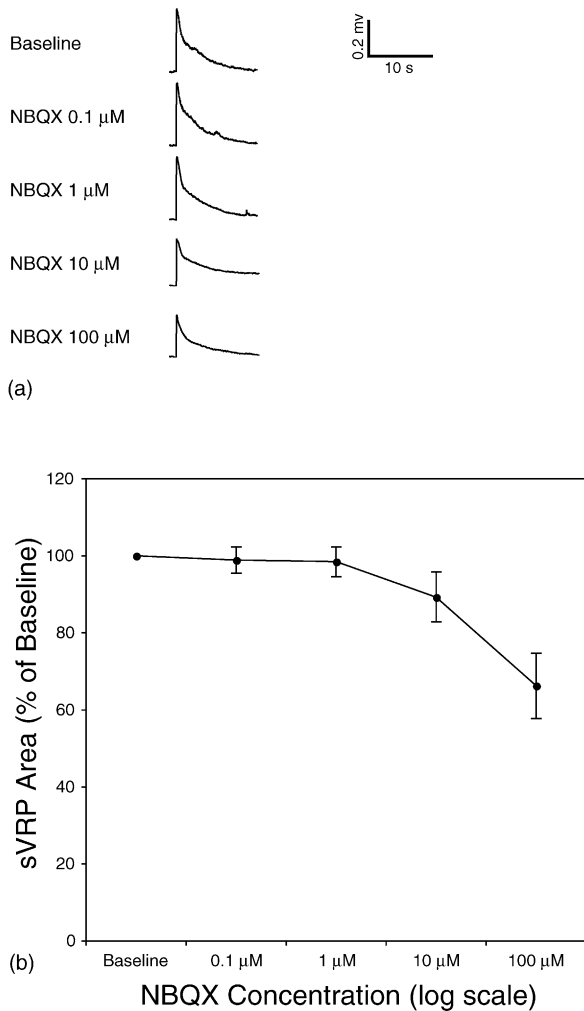


Fig. 3. NBQX's effect on the slow ventral root potential area in the isolated spinal cord 7 days of age: (a) sample recordings of different concentrations of NBQX's effect on the sVRP. All traces are from the recordings of one rat; (b) concentration–response of NBQX's effect on the slow ventral root potential area. Ordinate: sVRP area expressed as the percentage of baseline sVRP area (mean  $\pm$  one S.E.M.). Abscissa: concentration of NBQX in the superfusate medium (log scale).

agree with other comparable studies (King et al., 1992; Thompson et al., 1992; Zeman and Lodge, 1992). Based on the results of this experiment and reports by others (King et al., 1992; Thompson et al., 1992; Zeman and Lodge, 1992), we chose to study NBQX's effect on morphine withdrawal at a concentration of 3  $\mu$ M.

### 3.4. NBQX inhibited morphine withdrawal in the isolated spinal cord of the 7-day-old rat

Spinal cord from 7-day-old rats that did not receive chronic morphine treatment ( $n = 4$ ) demonstrated quite consistent sVRP over time (Fig. 4A(a) upper traces and Fig. 4A(b)), the addition of NBQX (3  $\mu$ M) and the later addition of naltrexone (10  $\mu$ M) to the superfusate did not significantly alter the sVRP area value at any time point

during the recording (sVRP area values from  $84.87 \pm 4.98\%$  of control to  $94.41 \pm 6.18\%$  of control;  $P > .05$  at all time points). Thus, neither NBQX (3  $\mu$ M) nor NBQX (3  $\mu$ M) together with naltrexone (10  $\mu$ M) significantly altered baseline sVRP in morphine naive rats.

Spinal cords from rats that received chronic morphine treatment ( $n = 6$ ) demonstrated similarly stable recording in the normal perfusion medium and after NBQX (3  $\mu$ M) was added. When naltrexone (10  $\mu$ M) was added to the superfusate, sVRP of spinal cords from morphine-treated rats increased significantly for the first 20 min (maximal  $146.32 \pm 9.21\%$  of control;  $P < .01$ ;  $n = 6$ ). Then, the sVRP gradually declined to reach the control level at 50 min and remained at that level for the rest duration of recording (sVRP area values from  $105.97 \pm 3.49\%$  to  $97.96 \pm 2.31\%$  of control;  $P > .05$  at all time points;  $n = 6$ ) (Fig. 4A(a) lower traces and Fig. 4A(b)). Thus, in contrast to spinal cords from morphine naive rats, which showed neither time nor treatment effect before or after naltrexone was added to the superfusate, spinal cords from morphine-treated rats demonstrated different effects in the naltrexone-containing medium.

To compare the treatment effect on morphine withdrawal among various chronic treatment groups (morphine 10mg/kg versus saline) and acute treatment (NBQX 3  $\mu$ M versus normal ACSF), we averaged data points at 38–42 min (“40”) and 58–62 min (“60”), respectively. These two times probably best describe morphine withdrawal in the present protocol.

As expected, regardless of acute treatment conditions, spinal cords from rats which did not receive chronic morphine treatment did not show significant difference from each other at either 40 or 60 min, indicating that the present electrophysiological assay was stable and NBQX's effect at a concentration of 3  $\mu$ M on the baseline sVRP was not significant (Fig. 4B and C)

At 40 min, the sVRP of morphine-treated animals increased significantly after naltrexone was added to about 200% of control. Pre-treatment of NBQX (3  $\mu$ M) significantly suppressed this naltrexone-precipitated morphine withdrawal to approximately 140% of control, but did not completely abolish it. The averaged sVRP area from this group was still significantly higher than the control groups that did not receive morphine treatment (Fig. 4B).

At 60 min, sVRP value of the morphine-treated spinal cord remained at about 150% of control. In contrast to the data at 40 min, pre-treatment of NBQX (3  $\mu$ M) essentially abrogated this stage of naltrexone-precipitated morphine withdrawal (Fig. 4C).

### 3.5. DCG-IV attenuates behavioral signs of morphine withdrawal

#### 3.5.1. Pre-naltrexone

As summarized in Table 3, the data ( $N = 32$ ) for the pre-naltrexone period show that treatment with various doses of

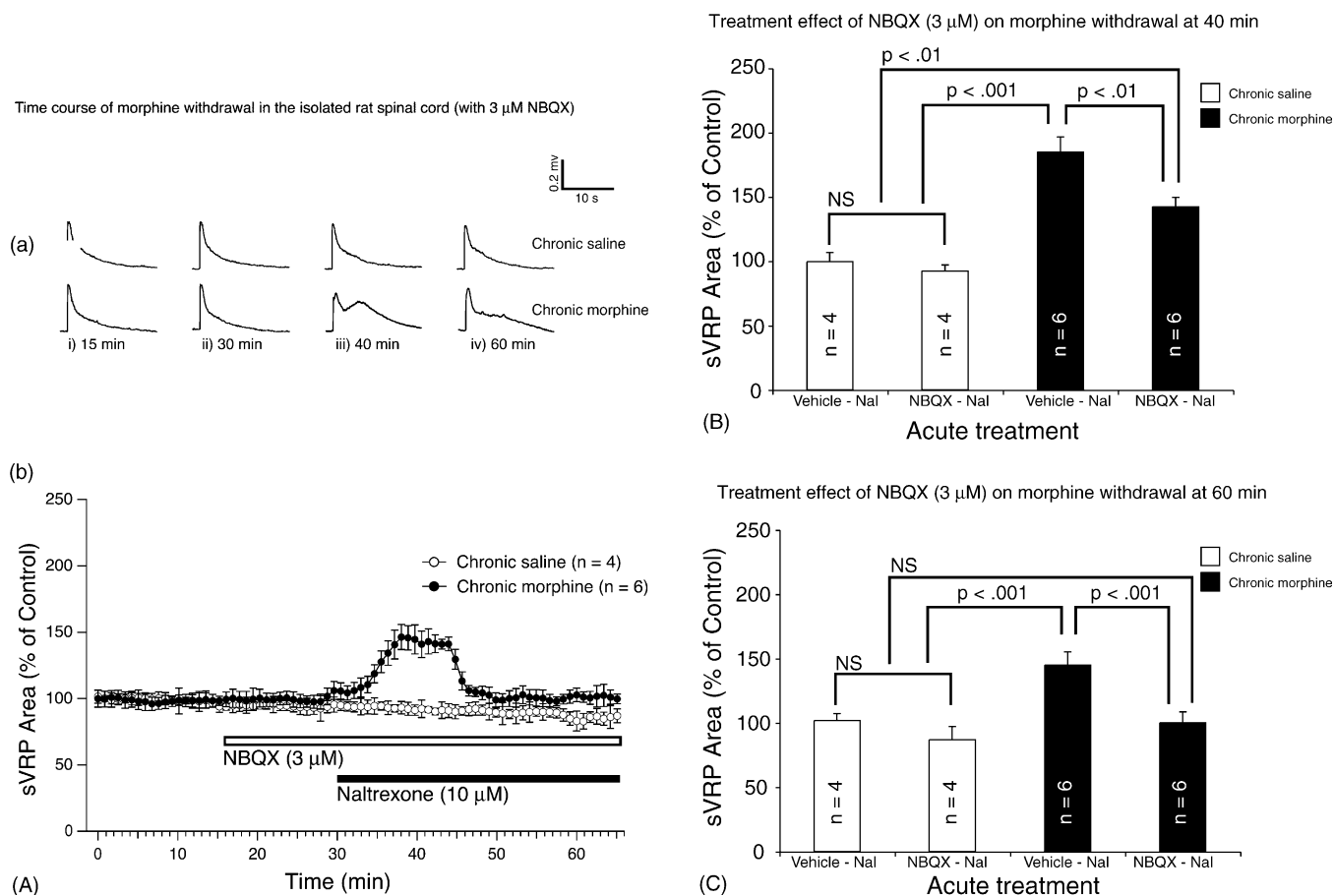


Fig. 4. Effects of NBQX on morphine withdrawal in the isolated spinal cord. (A) Time course of morphine withdrawal in the isolated rat spinal cord (with 3 μM NBQX): (a) upper trace of this panel: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated chronically with saline. Lower trace of this panel: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated with morphine; (b) time course of morphine withdrawal in the isolated rat spinal cord (with 3 μM NBQX). Ordinate: sVRP area expressed as the percentage of baseline sVRP area (mean ± one S.E.M.). Abscissa: time of recording (min). (B) Treatment effect of NBQX (3 μM) on morphine withdrawal in the isolated rat spinal cord at 40 min of recording. Ordinate: mean sVRP area expressed as the percentage of control (mean ± one S.E.M.). Abscissa: acute treatment conditions. (C) Treatment effect of NBQX (3 μM) on morphine withdrawal in the isolated rat spinal cord at 60 min of recording. Ordinate: mean sVRP area expressed as the percentage of control (mean ± one S.E.M.). Abscissa: acute treatment conditions.

DCG-IV in the 7- to 21-day-old rat did not significantly alter the behaviors of the morphine-dependent rat. All experimental groups largely remained quiet.

### 3.5.2. 7-day-old

The results ( $N = 32$ ) for the post-naltrexone period show that treatment with DCG-IV significantly reduced naltrexone-precipitated withdrawal behaviors in the 7-day-old rat. DCG-IV dose dependently decreased head moves, moving paws, together, and walking and increased quiet behavior. There was no significant effect on wall-climbing and rolling. The dose effects of pre-treatment of DCG-IV on naltrexone-precipitated morphine withdrawal in the 7-day-old rat are depicted in Fig. 5.

### 3.5.3. 14-day-old

The results ( $N = 32$ ) for the post-naltrexone period show that treatment with DCG-IV also reduced most of the

morphine withdrawal behaviors precipitated by naltrexone in the 14-day-old rat. DCG-IV significantly and dose dependently decreased burrow, head moves and walking and increased quiet behavior. There was no significant effect on moving paws, together, or wall-climbing behavior. The dose effects of pre-treatment of DCG-IV on naltrexone-precipitated morphine withdrawal in the 14-day-old rat are depicted in Fig. 5.

### 3.5.4. 21-day-old

As for the above two ages, DCG-IV significantly reduced most of the morphine withdrawal behaviors precipitated by naltrexone in the 21-day-old rat ( $N = 32$ ). DCG-IV dose dependently decreased burrow, head moves, together, and walking and increased quiet behavior. We did not find any significant effect on wall-climbing for pre-treatment. The dose effects of pre-treatment of DCG-IV on naltrexone-

Table 3

Statistical analyses results (*F* and *P* values) for the dose effect of DCG-IV on baseline behaviors (pre-naltrexone period) and on morphine withdrawal behaviors (post-naltrexone period)

Baseline/pre-naltrexone						
Burrow	ND	ND	ND	ND	1.00	0.41
Head moves	1.09	0.37	0.93	0.45	0.57	0.64
Moving paws	1.40	0.27	0.26	0.85	ND	ND
Quiet	1.07	0.38	1.15	0.35	1.40	0.27
Rolling	1.26	0.31	ND	ND	ND	ND
Together	ND	ND	0.16	0.92	0.89	0.46
Walking	ND	ND	0.26	0.85	1.76	0.19
Wall-climbing	ND	ND	0.64	0.60	1.50	0.24
Withdrawal/post-naltrexone						
Burrow	ND	ND	<b>5.98</b>	<b>0.00</b>	<b>7.24</b>	<b>0.00</b>
Head moves	<b>9.74</b>	<b>0.00</b>	<b>14.25</b>	<b>0.00</b>	<b>9.50</b>	<b>0.00</b>
Moving paws	<b>17.28</b>	<b>0.00</b>	1.41	0.27	ND	ND
Quiet	<b>52.73</b>	<b>0.00</b>	<b>11.51</b>	<b>0.00</b>	<b>16.98</b>	<b>0.00</b>
Rolling	0.69	0.57	ND	ND	ND	ND
Together	<b>4.07</b>	<b>0.02</b>	0.39	0.76	<b>4.06</b>	<b>0.02</b>
Walking	<b>7.99</b>	<b>0.00</b>	<b>4.64</b>	<b>0.01</b>	<b>7.75</b>	<b>0.00</b>
Wall-climbing	2.17	0.12	1.91	0.16	0.60	0.62

ND: this behavior was not displayed. Bold face: result is statistically significant. d.f. = (3, 28) in all cases.

precipitated morphine withdrawal in the 21-day-old rat are depicted in Fig. 5.

### 3.6. DCG-IVs effect on sVRP area in the 7-day-old rat

To determine a suitable concentration of DCG-IV that did not alter the baseline sVRP, the spinal cords from four male naive 7-day-old rats were used to determine the concentration–response curve of DCG-IVs effect on the sVRP. DCG-IV at 10 nM concentration did not produce appreciable effect on the baseline sVRP. At 100 nM, DCG-IV slightly suppressed the sVRP area ( $90.14 \pm 6.72\%$  of control;  $P > .05$ ) (Fig. 6(a) and (b)). Although statistically not significant, that is more likely due to the lack of statistical power rather than a lack of effect of DCG-IV. At a concentration of 1  $\mu\text{M}$ , DCG-IV markedly suppressed sVRP ( $56.15 \pm 12.02\%$  of control;  $P < .05$ ). Finally, at 10  $\mu\text{M}$ , DCG-IV essentially eliminated the sVRP ( $2.77 \pm 1.33\%$  of control;  $P < .001$ ). The concentration–response curve of DCG-IVs effect on the sVRP in the 7-day-old rat is depicted in Fig. 6. These results agree with other comparable studies (Ishida et al., 1993; Ross et al., 2000). Based on the results of this experiment and reports by others (Ishida et al., 1993; Ross et al., 2000), we chose a concentration of 30 nM.

### 3.7. DCG-IV inhibited morphine withdrawal in the isolated spinal cord of the 7-day-old rat

Similar to the NBQX study, the sVRP from 7-day-old rats that did not receive chronic morphine treatment ( $n = 4$ ) were stable. The addition of naltrexone (10  $\mu\text{M}$ ) to the superfusate did not alter the sVRP area value significantly at any

time point of recording (sVRP area values from  $94.55 \pm 5.53\%$  of control to  $102.98 \pm 1.78\%$  of control;  $P > .05$  at all time points).

Spinal cords from rats that did receive chronic morphine treatment ( $n = 6$ ) demonstrated similarly stable recording in the normal perfusion medium. However, when naltrexone (10  $\mu\text{M}$ ) was added to the superfusate, the sVRP rapidly increased to peak at approximately 10 min after naltrexone ( $206 \pm 22.24\%$  of control;  $P < .01$ ;  $n = 6$ ). This increase then declined to reach a relatively stable elevated plateau (approximately 150% of control;  $P < .05$  at all time points;  $n = 6$ ) for the whole length of recording (65 min total, 35 min after naltrexone added in). These data are virtually identical to those from the NBQX experiments.

Rats that received chronic morphine treatment ( $n = 6$ ) demonstrated a stable recording in the normal perfusion medium and after DCG-IV (30 nM). When naltrexone (10  $\mu\text{M}$ ) was added to the superfusate, sVRP from morphine-treated rats increased slightly but not significantly in the first 20 min (maximal  $113.83 \pm 13.15\%$  of control;  $P > .05$ ;  $n = 6$ ). The sVRP then gradually increased to reach a relatively stable elevated plateau (approximately 125% of control,  $P > .05$  at some time points but  $P < .05$  at some other time points due to the small differences and large variability;  $n = 6$ ) for the whole length of recording (65 min total, 35 min after naltrexone added in) (Fig. 7A(a) lower traces and Fig. 7A(b)). Thus, in contrast to spinal cords from morphine naive rats, which showed neither time nor treatment effect before or after naltrexone was added to the superfusate, spinal cords from morphine-treated rats demonstrated clear time and treatment effects in a naltrexone-containing medium.

As for the NBQX data, in the chronic morphine treatment and acute DCG-IV treatment group, we have assessed the treatment effects at two times: 38–42 min (40) and 58–62 min (60). As expected, regardless of acute treatment conditions, spinal cords from rats which did not receive chronic morphine treatment did not show significant differences from each other at either 40 or 60 min, indicating that the present electrophysiological assay was stable and the effects of DCG-IV on baseline sVRP were not significant (Fig. 7B and C).

At 40 min, the sVRP of the morphine-treated spinal cord significantly increased to about 200% of control, indicating morphine dependence after addition of naltrexone into the superfusate. Pre-treatment of DCG-IV almost completely suppressed this naltrexone-precipitated morphine withdrawal (Fig. 7B).

At 60 min, sVRP value of the morphine-treated spinal cord remained significantly higher than control at about 150% of control. In contrast to the data at 40 min, although DCG-IV significantly suppressed this naltrexone-precipitated morphine withdrawal (120%), it did not completely abolish it. The sVRP was still significantly higher than the control groups that did not receive morphine treatment (Fig. 7C).

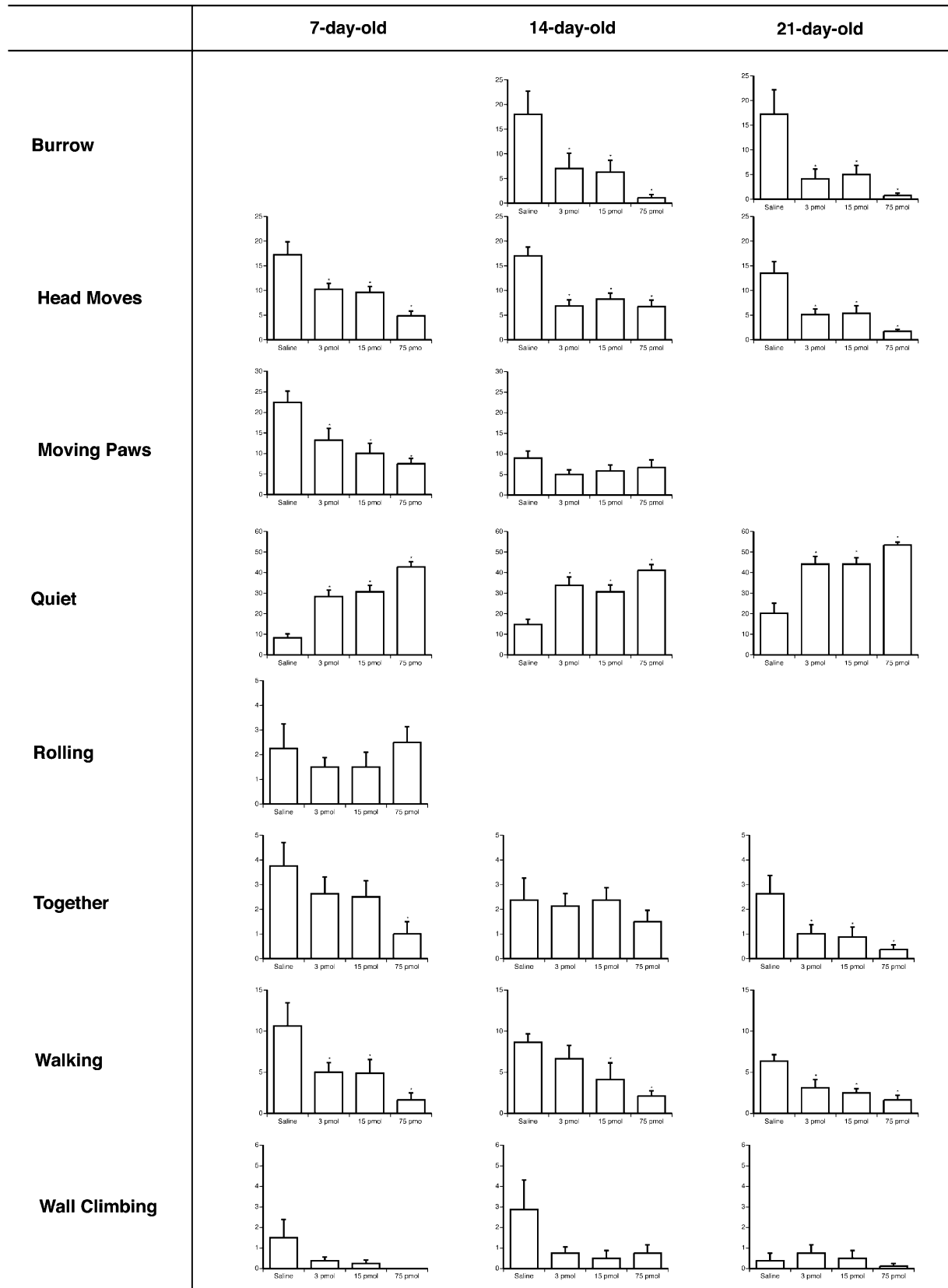


Fig. 5. The effect of pre-treatment with the group II mGluR receptor agonist DCG-IV (3, 15, and 75 pmol) on naltrexone precipitated morphine withdrawal behaviors in the 7-, 14-, and 21-day-old rats. Details as in Fig. 1.

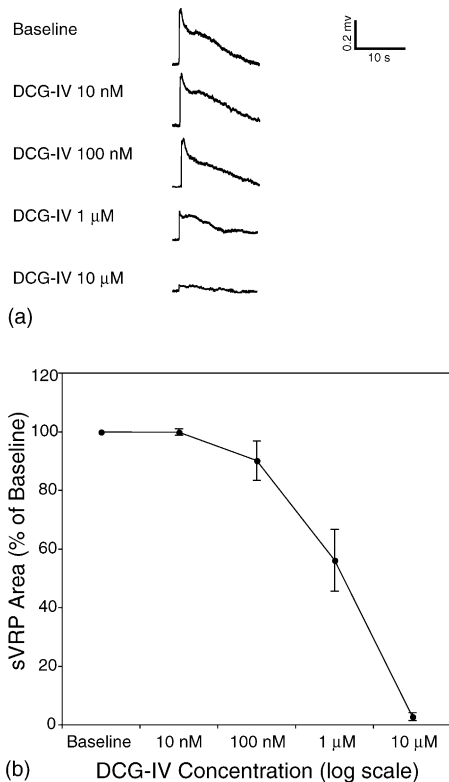


Fig. 6. DCG-IVs effect on the slow ventral root potential area in the isolated spinal cord at 7 days of age: (a) sample recordings of different concentrations of DCG-IVs effect on the sVRP. All traces are from the recordings of one rat; (b) concentration–response of DCG-IVs effect on the slow ventral root potential area. Ordinate: sVRP area expressed as the percentage of baseline sVRP area (mean  $\pm$  one S.E.M.). Abscissa: concentration of DCG-IV in the superfusate medium (log scale).

#### 4. Discussion

The mechanisms underlying opiate tolerance and withdrawal remain elusive and multiple mechanisms are involved (Thorat et al., 1994). One hypothesis is that chronic morphine treatment upregulates cAMP and withdrawal from opiates results from that upregulation (Nestler, 2001; Nestler and Tallman, 1988). Another possibility is that opiate withdrawal augments central release of glutamate, which in turn contributes to opiate withdrawal (Aghajanian et al., 1994; Akaoka and Aston-Jones, 1991; Tokuyama et al., 1996). These two mechanisms are not mutually exclusive and opiate withdrawal likely has more than a single cause (Aghajanian et al., 1992).

Although there is a direct evidence supporting the role of glutamate in opiate withdrawal (Tokuyama et al., 1996), it is less clear which glutamate receptors modulate opiate withdrawal. There is a wealth of data suggesting that a variety of NMDA receptor antagonists inhibit mu opiate (morphine) withdrawal in the adult rodents (for review, see (Mao, 1999; Trujillo, 1995)). In contrast to these findings in the adult animal, recent studies from our laboratory (Zhu and Barr, 2000, 2001a,b) and others (Bell and Beglan, 1995a,b) show

that NMDA receptor antagonists are not effective in suppressing morphine withdrawal in rats younger than a week of age, partially effective at 14 days of age and fully effective in the 21-day-old (Zhu and Barr, 2001a). The reason for this age-dependent effectiveness of NMDA receptor antagonists in suppressing opiate withdrawal may be due to the dramatic ontogenetic differential changes of the subunits of the NMDA receptor during earlier life (Zhu and Barr, 2001a,b).

There are several lines of evidence for a critical role of the AMPA receptor in opiate tolerance and withdrawal. First, the AMPA receptor is co-localized with the NMDA receptor in many cell types (Gu et al., 1996) including NOS-positive neurons (Fedele and Raiteri, 1999) suggest that the AMPA receptor, similar to the NMDA receptor, is involved in neural circuitry that is activated during opiate withdrawal. Second, chronic morphine treatment elevates the AMPA receptor GluR 1 subunit levels in the rat ventral tegmental area (Fitzgerald et al., 1996). Third, morphine-induced tolerance and dependence are reduced in mice deficient in the AMPA receptor subunit A (Vekovischeva et al., 2001). Fourth, morphine withdrawal increases both the level of AMPA receptor mRNA expression and [3H]AMPA binding in various brain sites in the rat (Jang et al., 2000). Finally, analgesic tolerance development is attenuated and established morphine tolerance reversed in mice when receiving a continuous infusion of LY 293558 concurrent with or after morphine treatment. Thus, these data demonstrate that the AMPA receptor plays a role in the development and maintenance of morphine tolerance.

Besides its possible role in the development of opiate tolerance, the AMPA receptor has been argued to play a more important role than the NMDA receptor in the expression of opiate withdrawal. Rasmussen and coworkers questioned the role that the NMDA receptor plays in opiate withdrawal (Rasmussen, 1995). There is a marked increase in firing of locus coeruleus (LC) neurons during naloxone-precipitated withdrawal; 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 receptors, whereas NMDA receptors mediate little, if any, of the withdrawal-induced excitation of the LC (Rasmussen, 1995; Rasmussen et al., 1996; Rasmussen et al., 1991). Moreover, infusion of the non-NMDA excitatory amino acid antagonist CNQX into the LC or the central nucleus of the amygdala significantly attenuated most signs of naloxone-precipitated morphine withdrawal in rats (Taylor et al., 1998). Thus, there may be circuits that are more responsive to AMPA blockade than to NMDA blockade.

In addition to AMPA receptors, mGluRs may also be involved in opiate withdrawal. In the past few years, several laboratories, also using a variety of methods, investigated the role of mGluRs on opiate tolerance (Popik et al., 2000), dependence, and withdrawal in rats and mice (Fundytus and

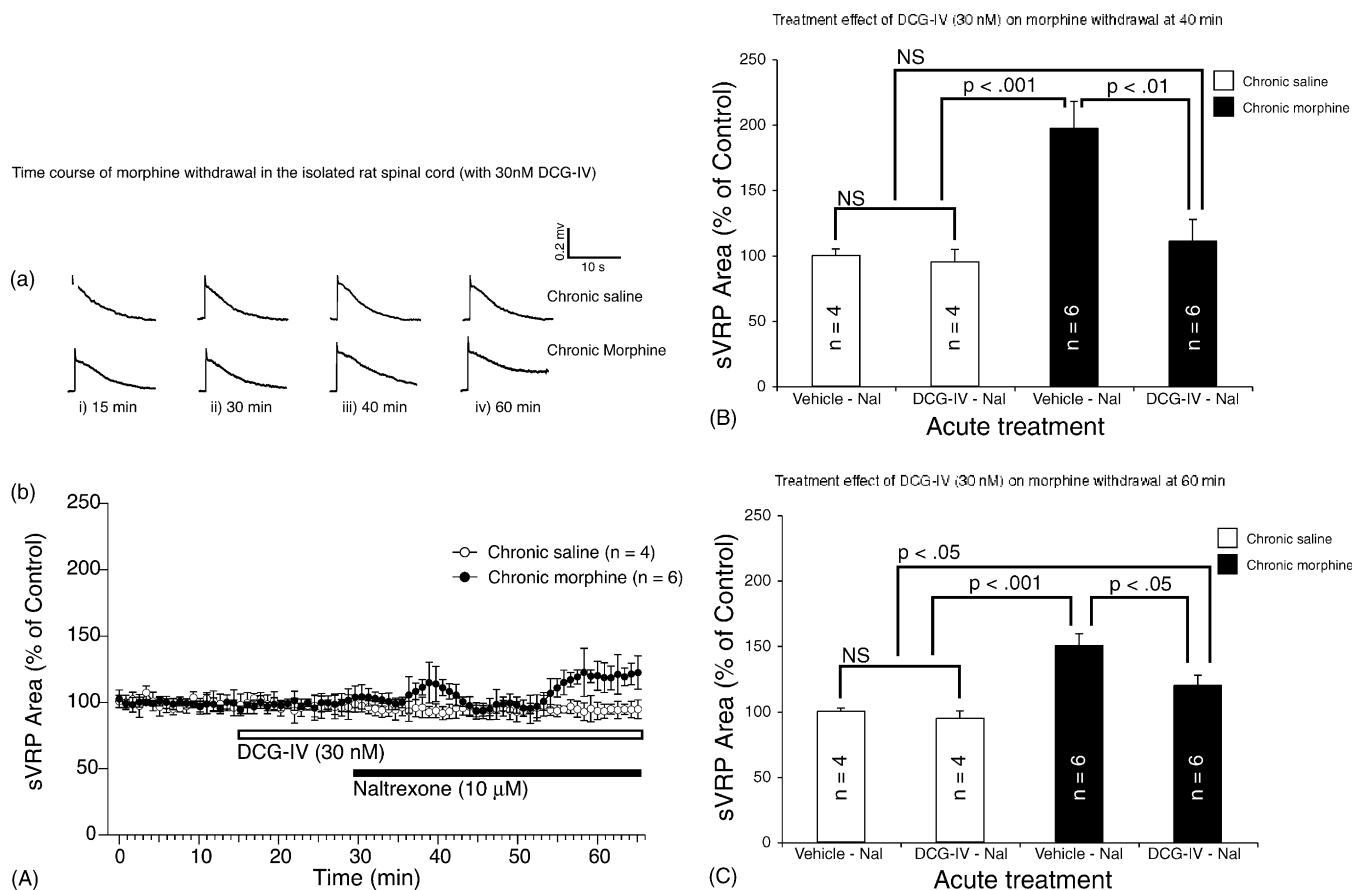


Fig. 7. Effects of DCG-IV on morphine withdrawal in the isolated spinal cord. (A) Time course of morphine withdrawal in the isolated rat spinal cord (with 30nM DCG-IV): (a) upper trace of this panel: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated chronically with saline. Lower trace of this panel: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated with morphine; (b) time course of morphine withdrawal in the isolated rat spinal cord (with 30nM DCG-IV). Ordinate: sVRP area expressed as the percentage of baseline sVRP area (mean  $\pm$  one S.E.M.). Abscissa: time of recording (min). (B) Treatment effect of DCG-IV (30 nM) on morphine withdrawal in the isolated rat spinal cord at 40 min of recording. Ordinate: mean sVRP area expressed as the percentage of control (mean  $\pm$  one S.E.M.). Abscissa: acute treatment conditions. (C) Treatment effect of DCG-IV (30 nM) on morphine withdrawal in the isolated rat spinal cord at 60 min of recording. Ordinate: mean sVRP area expressed as the percentage of control (mean  $\pm$  one S.E.M.). Abscissa: acute treatment conditions.

Coderre, 1994; Fundytus and Coderre, 1997; Fundytus et al., 1997; Klodzinska et al., 1999; Vandergriff and Rasmussen, 1999). Chronic antagonism of mGluRs in the brain with the non-selective antagonist MCPG, the group I mGluR selective antagonist (S)-4CPG, the group II selective antagonist MCCG, or the group III antagonist MAP4 all reduced the severity of abstinence symptoms. Moreover, a single intracerebral injection of DCG-IV just prior to the precipitation of withdrawal dramatically reduced the severity of precipitated withdrawal symptoms ((Fundytus and Coderre, 1994; Fundytus and Coderre, 1997), for review, see (Fundytus and Coderre, 1999; Fundytus et al., 1997)). The novel systemically active group II mGluR antagonist LY 354740 also inhibited naloxone-induced symptoms in morphine-dependent mice and attenuated naltrexone-precipitated, morphine-withdrawal-induced activation of locus coeruleus neurons and behavioral signs of morphine withdrawal in adult rat (Vandergriff and Rasmussen, 1999). Finally, development of antinociceptive morphine tolerance was inhibited by the metabotropic group II glutamate agonist LY 354740 in mice

(Popik et al., 2000). Thus, these recent data strongly suggest that, besides the NMDA receptor, mGluRs might also be involved in opiate tolerance and withdrawal in the adult rat and mouse. The reason both the mGluR agonists and antagonists are effective remains to be determined.

Although evidence suggesting that the AMPA receptor and mGluRs play critical roles in opiate withdrawal is accumulating rapidly, these investigations were exclusively done in the adult animals and all employed in vivo models. It was not known whether these results in adults apply to infants. AMPA receptors and mGluRs undergo qualitative and quantitative changes during development. Indeed, in contrast to the adult data, NMDA receptor antagonists are not effective in opiate tolerance (Zhu and Barr, 2003) and withdrawal (Zhu and Barr, 2000, 2001a). We proposed that the AMPA receptor rather than the NMDA receptor may play the dominant role in younger animals in these processes due to the developmental profile of the AMPA receptor, in contrast to that of the NMDA receptor (Zhu and Barr, 2001b).

Thus, the present study examined the effect of a competitive antagonist of the AMPA receptor NBQX and a group II mGluR agonist DCG-IV on morphine withdrawal in the infant rat using a combination of *in vivo* and *in vitro* methods. The data clearly show that the NBQX and DCG-IV were effective in attenuating both behavioral signs and an electrophysiological measurement of morphine withdrawal in the 7-day-old; and as in the 7-day-old rat, NBQX and DCG-IV were also effective in suppressing withdrawal behaviors in both the 14- and 21-day-old rats. Since morphine withdrawal both elevates cAMP level and augments glutamate release, NBQX and DCG-IV may attenuate morphine withdrawal by either mechanism.

During opiate withdrawal, there is an increase in glutamate release (Rasmussen and Aghajanian, 1989; Tokuyama and Ho, 1996). For example, naloxone-precipitated morphine withdrawal is associated with increased extracellular concentrations of glutamate within the pontine locus coeruleus (Aghajanian et al., 1994; Zhang et al., 1994) and nucleus accumbens (Sepulveda et al., 1998). Direct *i.c.v.* injection of glutamate to the brains of morphine- or butorphanol-dependent rats precipitates withdrawal signs in a dose-dependent manner, but not when animals are not opiate-dependent (Tokuyama et al., 1996). Hence, these results provide direct evidence that glutamate can elicit signs of opiate withdrawal. The rapid release of glutamate from locus coeruleus and other regions under an opiate-dependent state has been hypothesized to be a trigger or key element for the expression of opiate withdrawal signs (Christie et al., 1997; Rasmussen, 1991). Since augmented glutamate release is a prominent characteristic of opiate withdrawal, it is possible that blocking the AMPA receptor, both *in vivo* and *in vitro*, inhibited the excitatory effects of the exaggerated glutamate release. This would attenuate morphine withdrawal. Some potential sites by which NBQX could suppress withdrawal are those hypothesized to be involved in opiate withdrawal and to have the highest densities of AMPA receptors, especially the  $\text{Ca}^{2+}$ -permeable AMPA receptors. Such sites include the locus coeruleus, hippocampus, cortex, caudate-putamen (Jang et al., 2000), PAG, and the spinal cord (Jakowec et al., 1995a,b). Some of these sites are involved in morphine withdrawal in the infant (Jones and Barr, 2001). That the developing CNS expresses  $\text{Ca}^{2+}$ -permeable AMPA receptors (Jakowec et al., 1995a,b; Pellegrini-Giampietro et al., 1992) may also explain our finding that NBQX was effective in preventing the development of tolerance. In the developing rat brain and spinal cord, NMDA receptors are not highly permeable to calcium whereas AMPA receptors are. Since the influx of calcium into the cell plays critical role in the development of opiate tolerance and dependence, it is possible that NBQX attenuated the development of morphine tolerance in the 7-day-old rat by preventing calcium influx into the cell. In contrast, NMDA receptor antagonists were not effective in preventing the development of morphine tolerance in the 7-day-old rat (Bell and Beglan, 1995b; Zhu and Barr, 2003), possibly due

to the low calcium permeability of these immature NMDA receptors (Zhu and Barr, 2001b).

Consistent throughout the CNS, one of the most prominent physiologic effects of mGluR agonists is the reduction of transmission at glutamatergic synapses (Anwyl, 1999). Thus DCG-IV likely suppresses opiate withdrawal by inhibiting that transmission. Pre-synaptic mGluRs serve as autoreceptors to reduce glutamate release (Miller, 1998). Since augmented glutamate release is a prominent characteristic of opiate withdrawal, the acute treatment of DCG-IV in both the *in vivo* and *in vitro* assays likely inhibited the exaggerated glutamate release typical of opiate withdrawal and thus attenuated both behavioral and electrophysiological manifestations of morphine withdrawal.

It is well known that whereas acute administration of morphine decreases cAMP production, during chronic administration cAMP system returns to control level (Nestler and Tallman, 1988; Sharma et al., 1975), suggesting compensatory mechanisms. Withdrawal from morphine relieves the acute inhibition of cAMP by morphine and consequently unmasks the superactivation. This results in morphine withdrawal signs (Nestler, 2001). Both opioid receptors and mGluRs share common pools of intracellular second messengers (Fundytus and Coderre, 1997), and acute DCG-IV may decrease morphine withdrawal by decreasing intracellular cAMP levels (Conn and Pin, 1997; Pin and Duvoisin, 1995). In contrast, there is no evidence that the AMPA receptor is directly linked to the cAMP pathway. The sites proposed to be involved in opiate withdrawal and that have the highest densities of group II mGluRs include the locus coeruleus, hypothalamus, amygdala, periaqueductal grey area, and the spinal cord (Maldonado et al., 1992; Petralia et al., 1996). In the infant, the locus coeruleus, periaqueductal grey area, and spinal cord have been demonstrated to be involved in morphine withdrawal (Conn and Pin, 1997). Since DCG-IV acts on both mGlu2 and mGlu3 receptor subtypes of the group II mGluRs (Conn and Pin, 1997), further studies are needed to determine which subtype is more important in opiate withdrawal.

In the *in vitro* study, we found that NBQX was more effective in suppressing morphine withdrawal at earlier stages than later stages. In contrast, DCG-IV was more effective in suppressing the later stage than the earlier stage of morphine withdrawal. Because these two drugs may act on two different mechanisms to reduce withdrawal, elevated cAMP level and augmented glutamate release, we hypothesize that the: (1) peak of morphine withdrawal at 10 min after naltrexone is due to the temporal overlap of these two mechanisms; (2) excitation due to the action of a elevated cAMP system has a relatively shorter duration than the excitation due to the action of the augmented glutamate system. Thus, after the peak of withdrawal, augmented glutamate release would be mainly responsible for the elongated but attenuated plateau of morphine withdrawal. Therefore, we propose that NBQX may suppress withdrawal by decreasing exaggerated glutamate release but not by

altering elevated cAMP levels. This would explain the differential suppression of the later stages of the withdrawal. In contrast, DCG-IV differentially suppressed the first stage of morphine withdrawal. Because DCG-IV is directly and negatively coupled to the cAMP system whereas only indirectly coupled to the glutamate system as autoreceptors (Conn and Pin, 1997; Pin and Duvoisin, 1995), we propose that its action is largely by dampening the overcompensation of cAMP activation. Further examination is necessary to differentiate these two processes.

In conclusion, treatment with the competitive AMPA receptor antagonist NBQX or the group II mGluR agonist DCG-IV effectively suppressed the expression of morphine-induced tolerance and dependence in the infant rat. These effects were not age-dependent. There are no data that we are aware of, however, on whether these drugs can reduce the establishment of dependence and tolerance when given concurrently with morphine in the infant.

Although NMDA receptor antagonists have been proposed as a therapy of opiate withdrawal as evidenced by promising pre-clinical (for review, see (Herman et al., 1995; Inturrisi, 1997; Mao, 1999)) and clinical data (Bisaga et al., 2001; Koyuncuoglu, 1995; Koyuncuoglu et al., 1990; Rosen et al., 1996), the therapeutic potential in the infant is limited by the fact that NMDA receptor antagonists are not effective in suppressing either the establishment or expression opiate tolerance and dependence in the infant (Bell and Beglan, 1995a,b; Zhu and Barr, 2000, 2001a,b). In addition, that blockade of NMDA receptors by very high doses of NMDA receptor antagonists results in apoptotic neurodegeneration in the developing CNS (Ikonomidou et al., 1999) further precludes the use of NMDA receptor antagonist for the treatment of opiate withdrawal in human infants. AMPA and metabotropic glutamate receptors, therefore, provide novel targets for the development of therapeutic agents that could have a dramatic impact on treatment of opiate withdrawal in human patients of all ages.

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