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A

Ontogeny of Glutamate Receptors Mediated Morphine Tolerance and Withdrawal in Rats

by

Hongbo Zhu

A dissertation submitted to the Graduate Faculty in Psychology in partial
fulfillment of the requirement for the degree of Doctor of Philosophy
The City University of New York

2002

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This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK

ABSTRACT**Ontogeny of Glutamate Receptors Mediated Morphine Tolerance
and Withdrawal in Rats**

by

Hongbo Zhu

Advisor: Professor Gordon A. Barr

There is an increasing interest in the study and treatment of opiate tolerance and withdrawal in human infants. Glutamate receptors, especially NMDA receptors, are suggested to play key roles in the processes of opiate tolerance and withdrawal. Numerous studies in the past decade indicate that NMDA receptor antagonists inhibit opiate tolerance and withdrawal. Accumulating data also show that AMPA receptor antagonists and group II mGluR agonists attenuate opiate tolerance and withdrawal. However, most existing data are derived from adult animal models. All glutamate receptor types undergo dramatic developmental changes during early life. Thus, the author hypothesizes that the pharmacological effects on opiate tolerance and withdrawal of NMDA receptor antagonists, AMPA receptor antagonists and mGluR agonists in the developing organism may not be comparable to those in the adult. A combination of in vivo and in vitro assays were used in the present thesis to test the effect of treatment of agents acting on the NMDA, AMPA and group II mGluR on opiate tolerance and withdrawal in rats of 1-7, 8-14, and 15-21 days of

age, which are the critical periods when various glutamate receptor subunits experience differential change. NMDA receptor antagonists were found to be not effective in blocking either morphine tolerance or withdrawal in the 7-day-old rat, partially effective in the 14-day-old, and fully effective in the 21-day-old. There is a transition period around the second postnatal week for NMDA receptor antagonists to be effective in suppressing opiate tolerance and withdrawal. In contrast, the AMPA receptor antagonist and the group II mGluR agonist do not show such age effects, and were effective in attenuating opiate tolerance or withdrawal at all ages tested. These data demonstrate that age is a significant factor determining the effectiveness of NMDA receptor antagonists in suppressing morphine tolerance and withdrawal. The author concludes that NMDA receptor antagonists should not be used in human infant for the treatment of opiate tolerance and withdrawal. In contrast, AMPA receptors and mGluRs may prove to be good targets for the modulation of opiate tolerance and withdrawal at all ages.

Acknowledgments

I have been working on this thesis for a quite long time. Now I am both pleased that its present form finally satisfies myself and sad that it will soon close such an interesting chapter in my life. As said, behind every author stands a myriad of great supporters; there are many people deserving some special mention for their help in the creation of the present thesis.

Before anyone else, I would like to thank my mentor Dr. Gordon A. Barr. His insights and guidance have pushed this thesis far beyond what I could imagine five years ago when we first met. He has wanted to see the work summarized in the present thesis done since a long time ago and it is as much as his as anyone's.

Thanks go to all members of my dissertation committee, Drs. Victoria Luine, Jesus Angulo, Charles Inturrisi, and Jianren Mao. Thanks for your critical input regarding the thesis proposal from early on, your eagle eyes and cheery notes during the evolution of the thesis.

I would also like to thank Drs. Peter Moller, Phillip Zeigler, James Gordon, Martin Chodorow, Sheila Chase, and Sheryl Harding for their guidance and mentoring in various graduate classes.

Only with the hands-on training by Ms. Shirley Wong at Dr. Joan Kendig's world-class laboratory at Stanford University, those electrophysiological studies in the present thesis became possible.

I am grateful to all previous and current members of the labs at NYSPI and Hunter College, Dr. Christoph Wiedenmayer, Dr. Tamara King, Mr. Shaoning Wang, Ms. Jianxin Cheng, Jean Willi, Rebecca Smart, Estevan Limon, Anika McPhie, Arbi Nazarian, Anne Baliram. Your friendship and teamwork are to be cherished for the rest of my life.

Thank you!

H. Z.

Table of Contents

ACKNOWLEDGMENTS.....	VI
TABLE OF CONTENTS	VIII
LIST OF TABLES	XII
LIST OF FIGURES	XIV
PART I INTRODUCING	1
Chapter 1.....	2
The rising of a field - opiate tolerance and withdrawal.....	2
Opiate tolerance and withdrawal	2
Increasing interest in opiate tolerance and withdrawal in the developing organism	5
Opiate tolerance and withdrawal in the infant – behavioral evidence.....	13
<i>Tolerance.....</i>	<i>13</i>
<i>Withdrawal.....</i>	<i>16</i>
Opiate tolerance and withdrawal in the infant – electrophysiological evidence.....	19
<i>Tolerance.....</i>	<i>20</i>
<i>Withdrawal.....</i>	<i>21</i>
Opiate tolerance and withdrawal in the infant – Biochemical evidence.....	22
<i>Tolerance.....</i>	<i>22</i>
<i>Withdrawal.....</i>	<i>23</i>
Chapter 2.....	28
Mechanisms of opiate tolerance and withdrawal	28
Chapter 3.....	32
Glutamate receptors	32
Glutamate receptors: classification and properties	32
Glutamate receptors: pharmacology.....	36
The roles of glutamate receptors in opiate tolerance and withdrawal.....	40
Ontogeny of the AMPA receptor.....	45
Ontogeny of the NMDA receptor	47
Ontogeny of the metabotropic glutamate receptors	48
PART II HYPOTHESIZING.....	50
Chapter 4.....	51
A critical review of the literature.....	51

Chapter 5.....	54
Initial Studies.....	54
NMDA receptor and morphine withdrawal	54
NOS and morphine withdrawal.....	54
Chapter 6.....	56
Opiate tolerance and withdrawal: are NMDA receptors indispensable? .	56
PART III TESTING.....	62
Chapter 7.....	63
Experimental setup and conventions.....	63
Rationale of study.....	63
Subjects.....	65
Behavioral studies	66
<i>Tolerance</i>	68
<i>Withdrawal</i>	68
Electrophysiological studies.....	69
<i>The "isolated mammalian spinal cord"</i>	69
<i>Setup</i>	73
<i>Tolerance</i>	74
<i>Withdrawal</i>	75
Data analysis	75
<i>Behavior data statistics</i>	75
<i>Electrophysiology data statistics</i>	76
<i>Averaging technique</i>	77
Conventions	79
Chapter 8.....	80
Ontogeny of NMDA receptor mediated morphine withdrawal.....	80
Behavioral studies	80
Electrophysiological studies.....	81
Chapter 9.....	84
Ontogeny of NMDA receptor mediated morphine tolerance	84
Introduction	84
Material and methods.....	84
<i>Morphine treatment and tolerance assessment</i>	84
<i>In Vitro Electrophysiology</i>	85
<i>Application of chemicals</i>	85
<i>Behavior data analysis</i>	86
<i>Electrophysiology data analysis</i>	86
Results.....	87
<i>MK-801 did not attenuate morphine tolerance in the 7-day-old rat</i>	87
<i>MK-801 attenuated morphine tolerance in the 14-day-old rat</i>	88
<i>MK-801 attenuated morphine tolerance in the 21-day-old rat</i>	89
<i>Dextromethorphan did not attenuate morphine tolerance in the 7-day-old rat</i>	89
<i>Dextromethorphan attenuated morphine tolerance in the 21-day-old rat</i>	91

Discussion.....	92
Chapter 10	123
Ontogeny of AMPA receptor mediated morphine tolerance and withdrawal.....	123
Introduction	123
Material and methods.....	124
<i>Morphine treatment and behavior observation</i>	<i>124</i>
<i>Tolerance assessment</i>	<i>125</i>
<i>In vitro electrophysiology</i>	<i>125</i>
<i>Application of chemicals.....</i>	<i>126</i>
<i>Behavior data analysis.....</i>	<i>126</i>
<i>Electrophysiology data analysis.....</i>	<i>127</i>
Results.....	128
<i>NBQX attenuated morphine tolerance in the 7-day-old rat</i>	<i>128</i>
<i>NBQX attenuated behavioral signs of morphine withdrawal in the 7-day-old rat.....</i>	<i>129</i>
<i>NBQX attenuated behavioral signs of morphine withdrawal in the 14-day-old rat.....</i>	<i>129</i>
<i>NBQX attenuated behavioral signs of morphine withdrawal in the 21-day-old rat.....</i>	<i>130</i>
<i>NBQX's effect on sVRP area in the 7-day-old rat</i>	<i>131</i>
<i>Spinal cords from morphine treated rats demonstrated robust withdrawal in vitro.....</i>	<i>132</i>
<i>NBQX inhibited morphine withdrawal in the isolated spinal cord of the 7-day-old rat</i>	<i>133</i>
Discussion.....	135
Chapter 11	161
Ontogeny of mGluR mediated morphine withdrawal	161
Introduction	161
Material and methods.....	162
<i>Morphine treatment and behavior observation</i>	<i>162</i>
<i>In vitro electrophysiology</i>	<i>163</i>
<i>Application of chemicals.....</i>	<i>164</i>
<i>Behavior data analysis.....</i>	<i>164</i>
<i>Electrophysiology data analysis.....</i>	<i>165</i>
Results.....	165
<i>DCG-IV attenuated behavioral signs of morphine withdrawal in the 7-day-old rat....</i>	<i>165</i>
<i>DCG-IV attenuated behavioral signs of morphine withdrawal in the 14-day-old rat .</i>	<i>166</i>
<i>DCG-IV attenuated behavioral signs of morphine withdrawal in the 21-day-old rat..</i>	<i>167</i>
<i>DCG-IV's effect on sVRP area in the 7-day-old rat.....</i>	<i>167</i>
<i>Spinal cords from morphine treated rats demonstrated robust withdrawal in vitro.....</i>	<i>168</i>
<i>DCG-IV inhibited morphine withdrawal in the isolated spinal cord of the 7-day-old rat</i>	<i>169</i>
Discussion.....	171
PART IV CONCLUDING.....	191
Chapter 12	192
Different therapeutic approaches are needed for infant addicts	192
Summary of findings	192

The promise of NMDA receptor antagonism in treating opiate tolerance/withdrawal in the adult.....	194
NMDA receptors are inappropriate targets in treating opiate tolerance/withdrawal in the newborn.....	197
AMPA receptors and mGluRs are potential candidates for treating infant tolerance/withdrawal syndrome	198
Chapter 13	200
Mechanisms, potential pitfalls, and future directions.....	200
Age-related Ca^{2+} permeability of glutamate receptors may explain the age effect.....	200
Neonatal withdrawal involves neurotransmitters other than glutamate?	206
Morphine and NMDA receptor antagonists treatment altered the system?	210
Glutamate receptors' role in other related phenomena during ontogeny	212
The large picture.....	216
The final word	219
BIBLIOGRAPHY	221

List of Tables

Table 1-1. Evidence for tolerance to opiate drugs' analgesic effect in the infant rat. This list summarizes all reports on opiate's analgesic tolerance in the infant rat; references are sorted chronically in each category of mode of test.	26
Table 1-2. Evidence for opiate withdrawal in the infant rat. This list summarizes all reports on opiate withdrawal in the infant rat; references are sorted chronically in each category of mode of test.	27
Table 7-1. Morphine withdrawal behavior definitions.....	69
Table 9-1. Effects of chronic morphine or chronic morphine plus MK-801 treatment on baseline latency in the infant rat.	101
Table 9-2. Effects of chronic co-administration of MK-801 on ED50 of morphine in the infant rat.	102
Table 9-3. Effects of chronic morphine or morphine plus dextromethorphan on baseline latency in the infant rat.	103
Table 9-4. Effects of chronic co-administration of dextromethorphan with morphine on ED50 of morphine in the infant rat (Behavioral test).	104
Table 10-1. Statistical analyses results of the effect of chronic treatment of morphine or morphine plus NBQX on withdrawal latency from a hotplate in the 7-day-old rat.	143
Table 10-2. Effects of chronic co-administration of NBQX with morphine on ED50 of morphine in the infant rat.	143
Table 10-3. Behavior occurrences after treatment of NBQX but before treatment of naltrexone in the morphine-dependent rat of 7 days old.....	144
Table 10-4. Behavior occurrences after treatment of NBQX but before treatment of naltrexone in the morphine-dependent rat of 14 days old.....	144
Table 10-5. Behavior occurrences after treatment of NBQX but before treatment of naltrexone in the morphine-dependent rat of 21 days old.....	145
Table 10-6. Statistical analyses results for the dose effect of NBQX on baseline behaviors (pre-naltrexone period) and on morphine withdrawal behaviors (post- naltrexone period).	146

Table 11-1. Behavior occurrences after treatment of DCG-IV but before treatment of naltrexone in the morphine-dependent rat of 7 days old.....	178
Table 11-2 Behavior occurrences after treatment of DCG-IV but before treatment of naltrexone in the morphine-dependent rat of 14 days old.....	178
Table 11-3. Behavior occurrences after treatment of DCG-IV but before treatment of naltrexone in the morphine-dependent rat of 21 days old.....	179
Table 11-4. Statistical analyses results for the dose effect of DCG-IV on baseline behaviors (pre-naltrexone period) and on morphine withdrawal behaviors (post- naltrexone period).	180
Table 12-1. Summary of major findings of the present thesis.....	194

List of Figures

Figure 1-1. Opiate treatment and tolerance/withdrawal assessment paradigms. Solid circle: the subcategory of studies that the present thesis focuses on.....	8
Figure 7-1. Averaging multiple sVRP recordings to reduce random variability.....	78
Figure 8-1. Sample recordings demonstrating that chronic co-administration of dextromethorphan did not prevent morphine withdrawal in the isolated spinal cord in the 7-day-old rat.	82
Figure 8-2: Chronic co-administration of dextromethorphan failed to prevent the development of morphine dependence in the 7-day-old rat.....	83
Figure 9-1A. MK-801 did not prevent the development of morphine tolerance in the 7-day-old rat, forepaw.	108
Figure 9-1B. MK-801 did not prevent the development of morphine tolerance in the 7-day-old rat, Hindpaw.....	109
Figure 9-1C. MK-801 did not prevent the development of morphine tolerance in the 7-day-old rat, Tail.	110
Figure 9-2A. MK-801 prevented the development of morphine tolerance in the 14-day-old rat, forepaw.	111
Figure 9-2B. MK-801 prevented the development of morphine tolerance in the 14-day-old rat, Hindpaw.	112
Figure 9-2C. MK-801 prevented the development of morphine tolerance in the 14-day-old rat, tail.	113
Figure 9-3A. MK-801 prevented the development of morphine tolerance in the 21-day-old rat, Forepaw.	114
Figure 9-3B. MK-801 prevented the development of morphine tolerance in the 21-day-old rat, Hindpaw.	115
Figure 9-4A. Dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat, Forepaw.	116
Figure 9-4B. Dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat, Hindpaw.....	117

Figure 9-4C. Dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat, Tail.	118
Figure 9-5. Sample recordings demonstrating that chronic co-administration of dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat.	119
Figure 9-6. Dextromethorphan did not prevent the development of morphine tolerance at the spinal cord level in the 7-day-old rat.	120
Figure 9-7A. Dextromethorphan prevented the development of morphine tolerance in the 21-day-old rat, Forepaw.	121
Figure 9-7B. Dextromethorphan prevented the development of morphine tolerance in the 21-day-old rat, Hindpaw.	122
Figure 10-1A: NBQX dose-dependently attenuated morphine tolerance in the 7-day-old rat as assessed by the withdrawal time of the forepaw from a hotplate.	151
Figure 10-1B: NBQX dose-dependently attenuated morphine tolerance in the 7-day-old rat as assessed by the withdrawal time of the hindpaw from a hotplate.	152
Figure 10-1C: NBQX dose-dependently attenuated morphine tolerance in the 7-day-old rat as assessed by the withdrawal time of the tail from a hotplate.	153
Figure 10-2: NBQX dose-dependently suppresses morphine withdrawal in the 7-day-old rat.	154
Figure 10-3: NBQX dose-dependently suppresses morphine withdrawal in the 14-day-old rat.	155
Figure 10-4: NBQX dose-dependently suppresses morphine withdrawal in the 21-day-old rat.	156
Figure 10-5. NBQX's effect on the slow ventral root potential in the 7-day-old rat.	157
Figure 10-6A. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (without NBQX).	158
Figure 10-6B. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (with 3 μ M NBQX).	159
Figure 10-6C and D. Treatment effect of NBQX on morphine withdrawal at 40 and 60 min.	160
Figure 11-1: DCG-IV dose-dependently suppresses morphine withdrawal in the 7-day-old rat.	184

Figure 11-2: DCG-IV dose-dependently suppresses morphine withdrawal in the 14-day-old rat.	185
Figure 11-3: DCG-IV dose-dependently suppresses morphine withdrawal in the 21-day-old rat.	186
Figure 11-4. DCG-IV's effect on the slow ventral root potential in the 7-day-old rat.	187
Figure 11-5A. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (without DCG-IV).....	188
Figure 11-5B. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (with 30 nM DCG-IV).....	189
Figure 10-5C and D. Treatment effect of DCG-IV on morphine withdrawal at 40 and 60 min.	190
Figure 13-1. Morphine tolerance and withdrawal show differential effects for different stages of the sVRP.	209

Part I

Introducing

Chapter 1

The rising of a field - opiate tolerance and withdrawal in the infant

Opiate tolerance and withdrawal

Opiates have been used by humans for their potent pharmacological effects such as euphoria, analgesia, sleep, and relief from diarrhea for at least thousands of years (Julien, 1998; van Ree et al., 1999). When administered to human subjects who have not had previous or recent exposure to opiates, a variety of effects may be produced, including analgesia, sedation, respiratory depression, decreased gastrointestinal motility and a profound sense of well-being (Jaffe, 1975; Jaffe and Martin, 1975). Today, in clinical and hospital settings opiates are drugs of choice for the treatment of severe or chronic pain. For example, morphine has been used throughout the world as the premier agent for treating severe pain since it was isolated from opium in the early 1800s (Julien, 1998). It can be expected that opiates will continue to be used in medicine because they are irreplaceable as pain-relieving agents (Trujillo and Akil, 1991a). Outside the medical setting, opiates are powerful abused drugs due to their recreational and addictive properties. Because of their widespread medical use and nonmedical abuse, opiates have been the subject of intense research interest for many years (Trujillo and Akil, 1991a; Fundytus and Coderre, 1999; van Ree et al., 1999; Alvarez et al., 2001).

Chronic use of opiates for medical or nonmedical reasons leads to dramatic behavioral and neural changes known as tolerance and physical dependence (Jaffe, 1975; Jaffe and Martin, 1975). Tolerance refers to a decreased potency of the drug and results in a need for progressively higher doses to achieve the desired effect

(Fundytus and Coderre, 1999; van Ree et al., 1999). Stated in pharmacological terms, tolerance represents a shift to the right in the dose-response curve, whereby an increased dose of the drug is necessary to achieve a given effect (Trujillo and Akil, 1991a; Inturrisi, 1997). Tolerance develops to several opiate actions, including the analgesic, sedative, respiratory depressant, and euphorogenic effects (Trujillo and Akil, 1991a). Tolerance has also been called “neuroadaption”, reflecting the long-held idea that this phenomenon represents adaptive changes in neural systems to chronic drug exposure (Trujillo and Akil, 1991b). Physical dependence is a continued need for the drug, following repeated administration, to maintain a state of physiological equilibrium, and leads to a withdrawal syndrome on removal of the drug (Fundytus and Coderre, 1999; van Ree et al., 1999). Signs of withdrawal for a particular drug are typically opposite to the acute actions of that drug. Since the acute effects of opiates are generally depressant, withdrawal results in a hyperexcitable state that includes restlessness, irritability, tremor, diarrhea, gooseflesh, and muscle cramps (Trujillo and Akil, 1991a).

To help alleviate the symptoms of opiate withdrawal, two major approaches have been adopted. One extremely rapid method is termed rapid anesthesia-aided detoxification (RAAD) (Julien, 1998). A pure opioid antagonist, such as naloxone or naltrexone is administered intravenously to the addict while the patient is asleep under general anesthesia; the procedure goes on for several hours. After the procedure, the patient is maintained on orally administered naltrexone, allegedly to reduce opiate craving, and undergoes psychotherapy for relapse prevention. This approach is controversial, since it is expensive, it involves the risks of general anesthesia, and it is rather elaborate (Simon, 1997).

The alternative approach is the methadone maintenance program (Hutchings et al., 1976; Herman et al., 1995; Kreek, 1997). Methadone is a synthetic mu-opioid

agonist, the pharmacological activity of which is very similar to that of morphine. The outstanding properties of methadone are its effective analgesic activity, its efficacy by the oral route, its extended duration of action in suppressing withdrawal symptoms in physically dependent individuals, and its tendency to show persistent effects with repeated administration (Reisine and Pasternak, 1996; Kreek, 1997). To control opiate withdrawal, oral methadone is substituted for the injected opiate (e.g., heroin) to which the patient is addicted. Later, the dependent individual can be withdrawn slowly from the methadone, or the methadone can be continued indefinitely (Julien, 1998). Unfortunately, methadone has not proven to be a good medication for preventing the relapse to IV-administered opiates in individuals who have been medically withdrawn from this medication. It has been estimated that about 82% of opiate-dependent individuals previously maintained on methadone relapse to IV opiates about 1 year following cessation of methadone therapy (Herman et al., 1995). Furthermore, infants exposed to methadone prenatally via the placental blood supply or postnatally via the mother's milk have been shown to experience precipitated withdrawal both in human infant and in animal models (Hutchings et al., 1976; Hutchings et al., 1979; Hutchings, 1982; Barr et al., 1998). Other opioid-based alternatives to methadone have been proposed in recent years. For example, buprenorphine is under development as a medication for the treatment of opiate dependence in the United States, and is already marketed for that use in France (Strain et al., 2000). Similar to methadone, most of these opioids also have the potential to be abused (Strain et al., 2000).

Thus, although the value of opioid agonist or antagonist based medications for opiate dependence has been recognized, yet it is doubtful whether medication based on intervention of other non-opioid systems will prove superior. According to the Clinical Opioid Medications Program (COMP) branch of the National Institute on

Drug Abuse (NIDA), it is necessary for the development of new medications for opiate dependence in at least three directions. These are: 1.) seeking for medications that prevent the relapse to IV-administered opiates once medically supervised withdrawal from an opiate agonist; 2.) developing drugs that ameliorate the abstinence agony associated with opiate withdrawal; 3.) developing medications that can be administered with methadone gradually to decrease the dependence of the addicted individual to mu opiate agonist and help the individual achieve the transition from opiate agonist to opiate antagonist pharmacotherapy (Herman et al., 1995). In addition, since morphine is still the premier agent for treating severe pain clinically and it will be in the foreseeable future, medications co-administrated with morphine to prevent the development of tolerance and reverse tolerance is needed (Inturrisi, 1997; Suresh and Anand, 2001).

Increasing interest in opiate tolerance and withdrawal in the developing organism

Until recently, most anesthesiologists purposely withheld potent opiates when anesthetizing newborns, especially premature, for fear of hemodynamic compromise (Sukhani, 1989). Neonates frequently are denied the benefit of postoperative analgesia, the apparent reasons being: 1) they cannot experience pain, and 2) they are more susceptible to the respiratory depressant effects of narcotics (Sukhani, 1989). Much of the interest on anesthetic and analgesic needs of newborn and premature organism has been generated by the study that premature babies undergoing patent ductus arteriosus ligation with only nitrous oxide-oxygen-relaxant anesthesia exhibited a significant neuroendocrine response that could be attenuated by the

addition of potent opioid analgesic fentanyl to the anesthetic regime (Anand et al., 1987; Suresh and Anand, 2001).

Subsequent studies highlighted the need for opioid-based anesthesia and postoperative analgesia, and the resulting clinical benefit to the neonate. Opioid infusions promote respiratory synchrony, haemodynamic stability and decrease the incidence of neurological injury in ventilated preterm neonates (Suresh and Anand, 2001). Long-term follow-up studies of former preterm neonates further suggest improved behavioral and cognitive outcomes in children receiving morphine as compared with those randomized to nonopioid control groups (Suresh and Anand, 2001).

Improvements in the short-term and long-term clinical outcomes of critically ill neonates have promoted the widespread use of opioid drugs for analgesia and sedation. Consequently, large numbers of neonates are now receiving progressively increasing amounts of opioid therapy (Purcell-Jones et al., 1987; Thornton and Smith, 1997; Thornton et al., 1997; Suresh and Anand, 2001). These changes in clinical practice have led to an increasing incidence of opioid tolerance and dependence during therapy, with consequent increases in the number of patients showing the clinical signs of opioid withdrawal when therapy is discontinued (Suresh and Anand, 2001).

In addition to being exposed to opiates for the treatment of pain, a large number of infants are also passively exposed to opiates through their mothers (Barr and Jones, 1994). For example, detoxification of heroin-addicted mothers using methadone was instituted in the mid-1970s in the United States, which led to the use of methadone as a maintenance opioid (Hutchings et al., 1976; Hutchings and Dow-Edwards, 1991; Kreek, 1997). Neonates born to these mothers were reported to have withdrawal symptoms (Hutchings et al., 1993). These observations indicate that the

assessment and management of opioid tolerance, dependence and withdrawal deserve a great deal of attention clinically (Suresh and Anand, 2001).

Despite progress made in understanding the mechanisms of action of opiate drugs in the adult animal, the behavioral or physiological actions of these drugs in the infant remain largely a mystery (Barr, 1993). Although clinical reports of both acute neonatal and long-term behavioral effects are abundant and disturbing, these data are difficult to interpret because many of the women use other drugs concurrently, few receive adequate prenatal care, and some may even experience withdrawal during pregnancy (Lichtblau and Sparber, 1981). In addition, the passively addicted neonate is typically administered paregoric, diazepam, chlorpromazine or phenobarbital, depending on the severity, type and duration of withdrawal symptoms (Hutchings et al., 1993). Thus, to better understand both the acute and long-term risk in the human population, animal models of opiate tolerance and withdrawal need to be developed (Hutchings and Dow-Edwards, 1991; Barr and Jones, 1994).

To induce tolerance or withdrawal, the animal can be treated with opiates prenatally, postnatally but before it is weaned, or after it is weaned. Although theoretically feasible, the author does not know of any study that deliberately employs an opiate treatment regime across multiple developmental stages. Similarly, the assessment for tolerance/withdrawal can also be conducted prenatally, postnatally but before the animal is weaned, or after the animal is weaned, although the test obviously must be executed after the opiate treatment regime. Given these considerations, there are practically six combinations of treatment-test paradigms as summarized in Figure 1-1. These paradigms further fall into three major categories in that they are designed to test different aspects of opiate tolerance and/or withdrawal.

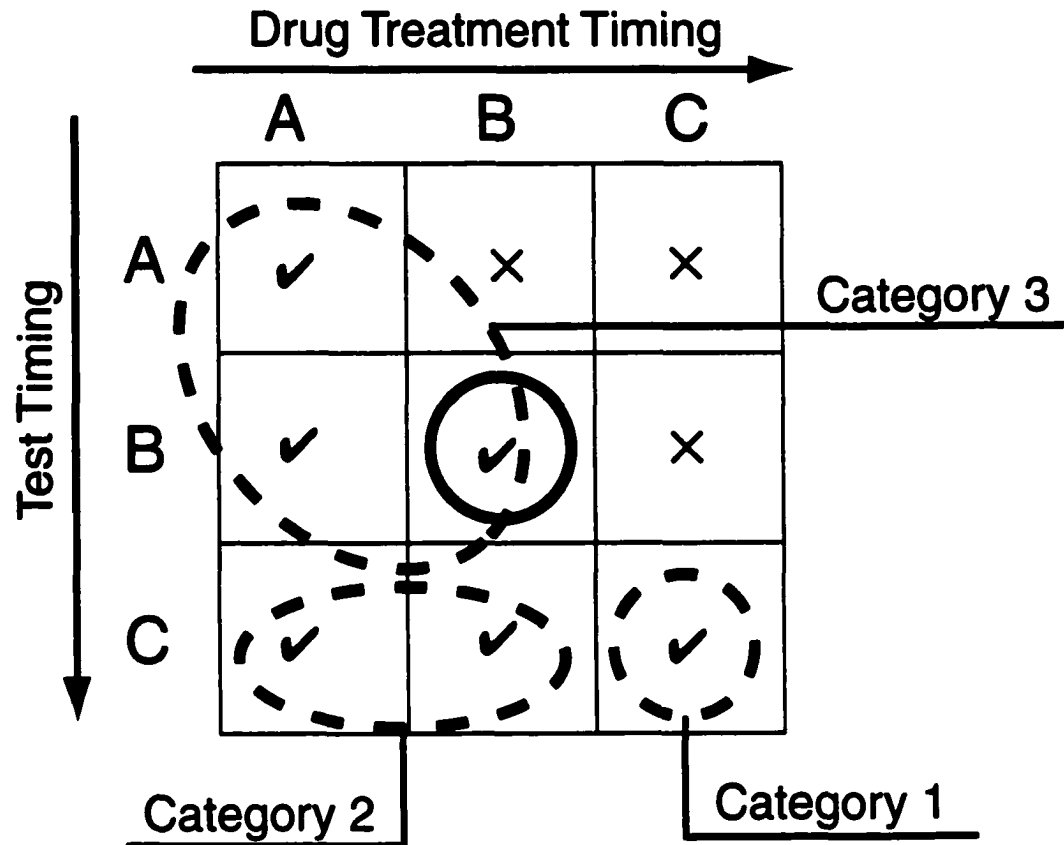


Figure 1-1: Three major category of opiate treatment - tolerance/withdrawal assessment paradigms depending on the timing of treatment and test.

- A: Pre-natal period
- B: Post-natal but pre-weanling
- C: Post-weanling
- ✓ This combination of treatment and test is possible
- X This combination of treatment and test is not possible

Figure 1-1. Opiate treatment and tolerance/withdrawal assessment paradigms. Solid circle: the subcategory of studies that the present thesis focuses on.

Category 1: Opiate tolerance/withdrawal in the weaned animals: both opiate treatment and tolerance/withdrawal assessment are conducted after the animal is weaned. This category of studies is intended to investigate the phenomena of tolerance and withdrawal in the more mature animal. Although this category of

studies helps us understand the mechanism of opiate tolerance and withdrawal, we should be cautious in applying the implications of data to the developing organism. The vast majority of existing data falls into this category.

Category 2: Opiates' protracted effects on tolerance/withdrawal: opiates are delivered perinatally, but tolerance/withdrawal assessment is conducted after the animal has weaned. Many earlier and current studies have examined prenatal or perinatal exposure to opiates on opiate tolerance and dependence after the rat has weaned (O'Callaghan and Holtzman, 1976, 1977; Sonderegger et al., 1977; Sonderegger et al., 1979; Bardo and Hughes, 1981; Koyuncuoglu and Aricioglu, 1993; Thornton and Smith, 1998; Thornton et al., 2000). The experiment parameters, such as dose of morphine, initial date of opiate treatment, varied widely, but the one common characteristic of all of these studies is that they tested tolerance and/or withdrawal after the rat weaned. This category of investigations falls into the broad field of studies examining the long-lasting effect of early perinatal experience on the behavior of the organism when it (or she/he) grows up (Kalinichev et al., 2001). This category of studies, if intended to study the effects of opiates, focuses on the nearly life-long effect of opiates. Despite that this class of studies certainly has shed important light on our understanding of opiates' long-lasting effects on neural plasticity, their implication in the phenomena of opiate tolerance and withdrawal *per se* is rather weak. During the prolonged opiate abstinence period, spontaneous withdrawal likely occurs and the effect of this abstinence on tolerance and withdrawal at later life is unknown. Furthermore, the interaction between earlier life opiate exposure and the later abstinence likely results in a lot complications for data interpretation. Indeed, in humans, the neonatal opiate withdrawal syndrome is present at birth or soon after and the symptoms are time limited and usually resolve within several weeks to months (Hutchings and Dow-Edwards, 1991).

Category 3: Both treatment and test are conducted perinatally. In contrast to the previous two classes of studies of tolerance and withdrawal, this third category of studies are intended to study the phenomena of tolerance and withdrawal in the developing organism. The author will review only this category of studies and all of the experiments in the present thesis focus on a subset of this category: both treatment and test are done postnatally but before weaning (the paradigm inside a solid circle in Figure 1-1).

Only a few years ago, there was a lack of animal data on either tolerance or withdrawal during the perinatal period. There are some reasons for this shortage of data. Firstly, as elaborated above, clinically tolerance in the infant did not attract enough attention until recently. This lack of clinical interest certainly has delayed the design and execution of animal studies examining tolerance in the developing organisms. Secondly, the traditional analgesic assay designed for adult animals could not test tolerance in the infant reliably, thus, earlier studies suffered from methodological difficulties (Fanselow and Cramer, 1988). Thirdly, opiate withdrawal behaviors in the developing animal are very different than the adult and not surprisingly, earlier studies intended to characterize typical adult rat withdrawal behaviors (e.g. wet dog shakes) in the developing rat failed to find such a correspondence (Fanselow and Cramer, 1988).

On the other hand, a clearer understanding of the mechanisms of opiate tolerance and withdrawal in the developing organisms is urgent: 1.) physicians are now more likely to use opiates to manage pain in neonates and infants than in the past (Suresh and Anand, 2001); considerable evidence indicates that tolerance and dependence can develop in this population; this tolerance is indicted when a given dose of fentanyl or morphine became ineffective and the patient required increasingly larger doses to provide the same level of analgesia observed with smaller doses

(Thornton and Smith, 1998); 2.) the effects of chronic opiate exposure on developing organisms may differ from adults if their capacity to develop tolerance is not as great as the adult, offspring would then be particularly vulnerable to opiates self-administered by their tolerant mother, as these doses may exert excessive action in the fetus (Little and Kuhn, 1995); thus, even for treatment under medical control and supervision, offspring of females continuing any kind of opiate intake during gestation would be regarded as prone to and most probable candidates for opiate addiction that should be more intense than normal (Koyuncuoglu and Aricioglu, 1993) or different than normal (Thornton and Smith, 1998; Thornton et al., 2000); 3.) there is a widespread use of illicit opiates like heroin or prescribed opiates such as methadone in child-bearing aged women, the infants' alertness, visual responsiveness, and sleep regulation are affected adversely (Hutchings and Dow-Edwards, 1991), possibly due to the acute effect of opiate withdrawal; 4.) Because neonatal withdrawal occur during the period when mother-infant interaction patterns are first established, withdrawal can induce far-reaching dysfunctional effects affecting not only attachment but also cognitive development as well (Hutchings and Dow-Edwards, 1991).

Fortunately, new methods and techniques have been developed over the years to test tolerance and withdrawal in the developing organism and the detailed characterization of withdrawal behaviors in the infant rat have provided us a stepping stone for addressing the mechanism of tolerance and withdrawal in the neonatal rat.

For these reasons, the present thesis will review studies that involves tests for opiate analgesic tolerance/withdrawal, no matter this is resulted from a single injection or chronic treatment of a opiate drug, no later than when the animal reaches 22 days of age. This cutoff age is chosen for three reasons: 1.) this is the weanling age for the rat, the species from which the vast majority of existing data are derived; 2.)

more importantly, the gist of the present study is the ontogenetic role of various glutamate receptors in tolerance and withdrawal; it is known that glutamate receptors undergo dramatic differential changes during the first three weeks of postnatal life in the rat, and after which become stable; 3.) besides theoretical reasons, from a clinical perspective, the sequence of events that take place in the neurological development in the rat and human CNS are similar, although it is difficult to make exact comparisons; the newborn rat CNS is approximately equivalent to a 24-week human fetus at birth, a full-term neonate at 7 days and a toddler at 3 weeks (Fitzgerald et al., 1988; Marsh et al., 1997); thus, this age range in rat is roughly corresponding to the a full-term human fetus to a child in human (Fitzgerald et al., 1988; Marsh et al., 1997) and tolerance and withdrawal during early human life represent a area of increasing concern yet remained largely unexplored. Although the attention of the review will focus on a thorough review of data from the rat, studies utilizing other species will also be included if relevant. Human infant withdrawal observations are not reviewed since these are mostly pseudo-experiments due to ethical constraints, are complicated by a myriad of confounding factors (Barr and Jones, 1994), and many reviews have been published on that topic (Hutchings, 1980, 1982; Hutchings and Dow-Edwards, 1991; Hutchings et al., 1993; Barr and Jones, 1994; Suresh and Anand, 2001).

This paper reviews the existing data on opiate tolerance and withdrawal in the infant rat model. Although Barr and Jones published a paper reviewing opiate withdrawal in infants (Barr and Jones, 1994), hardly any animal data existed at that time. A further limitation of that review is that it did not discuss tolerance. Lately, two review papers partially fulfilled this endeavor (Suresh and Anand, 2001; Zhu and Barr, 2001c). However, the Zhu and Barr review mainly focused on hypothesizing the interaction of glutamate receptors and the opioid receptors during ontogeny and

the Suresh and Anand review focused mainly on clinical observation rather than animal data, thus leaving the review of opiate tolerance and withdrawal data in the infant rat not complete. Therefore, the rapid development in the field in recent years and the lack of a thorough review paper warrant thus an effort.

Given that opioid system involves multiple receptors, receptor subtypes, and multiple endogenous opioid peptides, it is not surprising the opiates have a multitude of effects on the CNS and the behavior of the organism during development. The present study does not intend to address all these aspects of opiate actions on the developing organism, and readers looking for such information should go to other sources (Zagon et al., 1982; Zagon et al., 1984; Barr, 1992, 1993). Rather, the present thesis will focus attention on tolerance to morphine's analgesic effect and behavioral, electrophysiological, and biochemical changes following withdrawal from opiate drugs resulted from chronic opiates treatment during development. The literature cited in this thesis consists papers, available at the data of submission, considered as relevant by the author. The author would be grateful if readers could draw to his attention to any important material that may have been omitted through ignorance.

Opiate tolerance and withdrawal in the infant – behavioral evidence

Tolerance

With the exception of one report (Fanselow and Cramer, 1988), the earlier literature is consistent as to whether young infants demonstrate tolerance to the antinociceptive effects of opiates. Maternally-administered morphine resulted in tolerance to the analgesic effect of morphine in the rats when tested on postnatal day 12 or 13 (Johannesson and Becker, 1972); complete tolerance developed to morphine

following 24 hours of continuous morphine infusion at 14 days of age (Barr et al., 1986); and chronic pre- or postnatal exposure to morphine produced behavioral tolerance as evidenced by a decrease in the morphine-induced analgesic response (Tempel et al., 1988). In contrast, Faneslow and Cramer reported that they were unable to show tolerance to daily injections of morphine until after 15 days of age (Fanselow and Cramer, 1988).

One of the major design flaws in Faneslow and Cramer's (1988) experiment on tolerance is that the age effect may have confounded with tolerance effect to morphine's analgesic property. In fact, this group observed that the neonatal animals appeared to become increasingly sensitive to morphine over the drug administration period and incorrectly interpret this as evidence against the existence of tolerance to the analgesic effects of morphine in neonatal rats (Fanselow and Cramer, 1988). However, a later study showed that morphine was the least potent in postnatal day (PND) 3 rats, with potency reaching a peak on PND 9 and remaining essentially the same to PND 21 (Thornton et al., 1998). Thus, morphine's analgesic efficacy in the pre-weanling rat is a function of the age of the rat, and resembles a bell shape with PND9 at the peak (Thornton et al., 1998). Furthermore, Faneslow and Cramer tested pups with the same dose that had been injected chronically (Fanselow and Cramer, 1988). van Praag and Frenk (1991) reported that high doses of test morphine could overcome the tolerance. Thus, the conclusions of Faneslow and Cramer's (1988) study was compromised by confounding age effect with the effect of repeated morphine treatment on morphine's analgesic effect in the neonatal rat and they utilized too high doses of morphine to test tolerance, which could have masked the expression of morphine tolerance. Data in the present thesis demonstrated that 0.1 – 0.3 mg/kg of morphine are the most sensitive to tolerance effect. If the dose of

morphine goes higher than 0.3 – 1.0 mg/kg, tolerance effects will likely to be masked by ceiling effect of morphine's potent analgesic property (Chapter 9).

Later studies revealed that tolerance to the antinociceptive effects of morphine does develop in rats early in life following chronic treatment with morphine (Kitchen and Boswell, 1990; Van Praag and Frenk, 1991; Barr and Wang, 1992; Windh et al., 1995; Thornton and Smith, 1997; Thornton et al., 1997; Thornton and Smith, 1998; Choe and Smith, 2000; Thornton et al., 2000; Zhu and Barr, 2001a, d). The findings of these reports regarding whether opiate tolerance exists in the developing rat has been summarized into Table 1-2. The major conclusion from these data is that opiate tolerance can be established in rat at least as young as 7 day old. However, several experimental conditions (e.g., low test dose of morphine) need to be observed in order to reveal this tolerance effect reliably. The reason is at least partially because that tolerance to morphine's analgesic effect is relatively weak in the infant rat. This is not surprising since opioid receptors continue to proliferate during the first weeks of life and determine the potency of morphine's antinociceptive effects until day 15 (Marsh et al., 1997). This by itself should counteract opiate tolerance to multiple opiate administration during this period. In addition, it is possible that opioid receptors that develop in the middle or at the end of a tolerance-inducing schedule of morphine treatment regime will be less exposed to morphine, and hence develop less tolerance compared to those receptors that existed at the beginning of such a schedule. Thus, the proliferation of morphine receptors during the first two weeks of life may decrease functional tolerance in two ways when compared to the adult rat (Van Praag and Frenk, 1991).

Withdrawal

Just a decade ago, there were hardly any animal data on opiate withdrawal in the developing organism. Despite the lack of data, two lines of evidence suggested that opiate withdrawal exists in the infant. Firstly, existing data strongly support the argument that opiate drugs have rewarding effect in the infant animals (Barr, 1993). Secondly, it has long been known that human infants suffer deleterious effects from maternal opiate use (Happel, 1892; Tate, 1899; Shute and Davis, 1933), although until recently the only detailed description of opiate abstinence syndrome in neonates was for humans (Barr and Jones, 1994).

The human infant, passively exposed to opiates in utero through the therapeutic use of methadone, the illicit use of heroin, or postnatally through breast feeding, demonstrates characteristics of withdrawal including irritability, a high pitched cry, excessive sucking of fingers, sneezing, yawning, excessive weight loss, difficulty in regulating state, sleep disturbances and gastrointestinal disturbances (Finnegan, 1976; Kron et al., 1976). The addicted human infant also has a higher incidence of morbidity and mortality than do the offspring of nonaddicted women at least partly as a result of the fetus experiencing withdrawal during gestation (Kuwahara and Sparber, 1981a, b; Lichtblau and Sparber, 1981; Hutchings, 1982). But the complexity of the human setting make it difficult to separate those factors that are due to opiate use and those that are due to the abuse of other drugs, poor prenatal care, poor nutrition, or any other complications experienced by the mothers of these children (Barr and Jones, 1994). Animal models of opiate withdrawal thus were developed to control these confounding factors.

Quantification of specific behavioral changes unique to precipitated withdrawal in the infant is a necessary step to understand further the mechanisms of

morphine dependence in the infant rat. Fanselow and Cramer made the first attempt to thoroughly characterize opiate withdrawal behaviors in the developing rat in a controlled environment (Fanselow and Cramer, 1988). When morphine treatment was discontinued and the rats given a naloxone challenge, withdrawal symptoms were not observed in very young rats. Opiate withdrawal was first detected in rats that started to receive daily morphine treatment at 30 days of age and were then challenged with naloxone at 52 days of age. Based on these findings, the authors concluded that opiate withdrawal appear to be relatively late-developing phenomena in the rat (later than three weeks postnatally) (Fanselow and Cramer, 1988).

Unfortunately, this study set out to characterize withdrawal behaviors that only exist in the adult and neglected the possibility that infant rat would experience withdrawal but only express a developmental appropriate withdrawal repertoire, such as hyperactivity, vocalization, tonguing and mouthing behaviors as reported by another group (Enters et al., 1991a). Indeed, when age-appropriate behaviors such as ultrasonic vocalization were assessed as withdrawal behaviors, opiate withdrawal could be demonstrated in rat as earlier as 7 days of age (Barr and Wang, 1992). These earlier conflicting data suggest that although the developing rat experience withdrawal, the manifestation of this withdrawal syndrome could be quite different from that in the adult rat.

To test this hypothesis, two independent research groups subsequently examined opiate withdrawal in developing rats from 7 to 42 days of age (Jones and Barr, 1995; Windh et al., 1995).

Jones and Barr examined behavioral changes during precipitated morphine withdrawal in 7- to 42-day-old rat pups. One group of rats was injected with morphine sulfate twice daily for 6.5 days. Controls were saline injected or untreated litters. On Day 7, a target pup was injected with saline or naltrexone. Morphine-

treated pups tested with naltrexone showed significant alterations in behavior that varied at different ages. For example, rolling, stretching, and head and paw moves were observed at the younger ages, whereas burrowing, diarrhea, jumps, teeth chatter, and wet dog shakes occurred in the older rats (Jones and Barr, 1995).

Similarly, Windh et al. reported that both neonatal and weanling rats treated with an increasing dose regimen of morphine for 5 days (5-25 mg/kg b.i.d. s.c.) expressed morphine withdrawal after naloxone administration. Withdrawal in weanling rats was characterized by ptosis, piloerection, abnormal posture, forepaw treading, vocalization on touch and mastication. The behaviors constituting the withdrawal syndrome precipitated in neonates were distinct from those in weanling rats. Spontaneous vocalization, wall climbing, tremor, mouthing and increased locomotion were all observed (Windh et al., 1995).

Subsequent studies from these two groups and others, using different routes and dosing for opiate delivery, confirmed these findings (Little et al., 1996; Barr and Goodwin, 1997; Thornton and Smith, 1997; Thornton et al., 1997; Barr et al., 1998; Thornton and Smith, 1998; Ceger and Kuhn, 2000; Jones and Barr, 2000; McPhie and Barr, 2000a; Thornton et al., 2000; Zhu and Barr, 2000; Barr and Zhu, 2001; Jones and Barr, 2001; Lohmann and Smith, 2001; McPhie and Barr, 2001b; Zhu and Barr, 2001b; Jones et al., 2002). The findings of these studies regarding whether opiate withdrawal exists in the developing rat have been summarized into Table 1-2. Based on these data, two major conclusions can be drawn: 1.) when the behavioral repertoire appropriate to the age of the animal is examined, infant rat pups experience opiate withdrawal if the dams are exposed to opiates during their pregnancy or the pups are treated directly with opiates; and 2.) the withdrawal syndrome changes during development to the adult form following weaning.

Opiate tolerance and withdrawal in the infant – electrophysiological evidence

The study of tolerance in a specific region of the central nervous system can be accomplished by chronic opioid treatment of an animal followed by isolation of a portion of the central nervous system. One such region that has been utilized for in vitro electrophysiological study is the locus coeruleus (LC) (Andrade et al., 1983). In the LC slice, tolerance to morphine-induced decreases in the firing rate of spontaneously active isolated neurons has been demonstrated from morphine-treated rats (Andrade et al., 1983; Christie et al., 1987a; Nestler, 1992). The isolated spinal cord preparation from the neonatal rat (Otsuka and Konishi, 1974) could be similarly utilized as an in vitro model of spinal tolerance.

All existing electrophysiological data relevant to opiate tolerance and withdrawal in the infant rat came from studies utilizing the “isolated spinal cord model” (Otsuka and Konishi, 1974; Kerkut and Bagust, 1995). As an in vitro electrophysiological model of opiate tolerance and withdrawal, the spinal cord preparation from morphine-treated neonatal rats seems comparable to the LC slice preparation obtained from morphine treated adult rats. Whereas the LC represents a single cell model of tolerance and withdrawal, the ventral root potential from the isolated spinal cord represents activity of pre- and postsynaptic elements in an integrated synaptic circuitry amenable to in vitro study (Bell and Beglan, 1995b). Although these studies were proposed to study opiate tolerance or withdrawal phenomena per se regardless of age, all of them utilized the infant rat spinal cord since spinal cords from this age are the easiest to survive under the required experimental conditions. Thus, these experiments fit into our scope of discussion on tolerance and withdrawal in the infant rat.

In the isolated spinal cord from morphine-treated neonatal rats, tolerance is defined as a decreased depressant effect of acute morphine on an electrically evoked slow ventral root potential (Bell and Beglan, 1995b) or hyperanalgesic effect when opiate's depressant effect is antagonized (Feng and Kendig, 1995a). Withdrawal is represented by augmented spontaneous spinal cord neural activity (Bell and Beglan, 1995a) or increased sVRP area (Feng and Kendig, 1995b) following the introduction of an opioid antagonist such as naloxone or naltrexone into the bath. Excluding the present thesis and one of our abstracts presented at the ISDP meeting in the year of 2001 (Zhu and Barr, 2001d), there are eight published papers from two research groups reporting their findings on tolerance and withdrawal in the infant rat using the isolated spinal cord model. The conclusions regarding the existence of tolerance and withdrawal in the neonatal rat from these studies are summarized in Table 1-1 and 1-2, respectively.

Tolerance

Tolerance can be established in neonatal rats treated chronically with morphine. Morphine, in a concentration-dependent manner, depressed a slow ventral root potential produced by supramaximal electrical stimulation of an ipsilateral dorsal root. Chronic treatment of neonates with morphine produced tolerance, as manifested by a diminished acute morphine's effect in suppressing the sVRP (Bell and Beglan, 1995b).

Tolerance to morphine could also be demonstrated as the development of hyperalgesia following morphine treatment since hyperalgesic responses to noxious stimulation follow naloxone-precipitated withdrawal from opioids are suggested to be signs of tolerance (Feng and Kendig, 1995a). Naloxone-precipitated hyperalgesia can be observed following very brief exposure (less than seven minutes) to opiates. The

increase in sVRP area observed when an μ -opioid agonist is followed by naloxone thus may also be used as an in vitro manifestation of tolerance (Feng and Kendig, 1995a, b, 1996a, b; Feng and Kendig, 1997).

Withdrawal

Bell and Jaffe (Bell and Jaffe, 1986) first studied opiate withdrawal in the isolated spinal cord of the neonatal rat. Capsaicin depolarized the ventral root whereas morphine (10 μ M) abolished the capsaicin-induced ventral root depolarization. The capsaicin-induced depolarization of the ventral root was enhanced greatly (238%) when morphine was removed and naloxone (1 μ M) was added to the superfusion solution. Since the rebound effect upon administration of an opioid antagonist following treatment of morphine is a prominent sign of opioid withdrawal, these data provide the first electrophysiological evidence for the existence of morphine withdrawal in the neonatal rat spinal cord (Bell and Jaffe, 1986). The isolated spinal cord model was further validated as an in vitro preparation to assess physical opiate withdrawal at the spinal core level following chronic administration of morphine to neonates (Bell and Beglan, 1995a). Neonatal rats were treated chronically with injections of either morphine, or saline. Naloxone (10 μ M) induced more activity in spinal cords from morphine-treated neonates than in saline controls (Bell and Beglan, 1995a).

Using the same model, another group independently found that opiate withdrawal could be demonstrated in the isolated spinal cord. Feng and Kendig reported that naloxone 0.5-200 nmol, which had no significant facilitatory effect on sVRP when the preparations had not received alfentanil, induced an obvious rebound increase in sVRP area after acute alfentanil treatment (Feng and Kendig, 1995a). This rebound effect was thought to be a sign of physical withdrawal on opioid analgesics.

The naloxone-induced rebound lasted for more than 30 min. A similar rebound was observed with naloxone after morphine (Feng and Kendig, 1995b)

The finding that the neonatal rat spinal cord support an opiate withdrawal manifestation was further confirmed in the follow-up studies by both groups and this in vitro model was used to examine the role of spinal glutamatergic (Bell and Beglan, 1995a; Feng and Kendig, 1995a, 1996b) and other (Feng and Kendig, 1996a; Feng and Kendig, 1997) neurotransmission in opiate withdrawal.

Opiate tolerance and withdrawal in the infant – Biochemical evidence

Tolerance

Due to the lack of a biochemical correlate of analgesia, which is subjective by nature and exclusively measured either by behavioral or electrophysiological indices, there are no reports examining the analgesic tolerance to opiates using the biochemical method in the infant rat. Instead, there are a few reports examining the effect of chronic opiate treatment on a variety of neuro-endocrine changes in the infant rat (Ignar et al., 1992; Little and Kuhn, 1995; Windh et al., 1995). The overall conclusion from these studies is that both neonatal and weanling rats develop tolerance to morphine-induced stimulation of the hypothalamic-pituitary-adrenal (HPA) axis. However, neonatal rats did not appear to develop tolerance to morphine-induced adrenocorticotrophic hormone (ACTH) release as robustly as that observed in weanling rats. Corticosterone release was abolished at both ages following chronic morphine treatment. Consistent with the behavioral evidence elaborated above, these findings suggest that neonatal rats can become tolerant to morphine, but that there may be differences in the relative sensitivity of tolerance development in neonatal rats compared to older rats (Little and Kuhn, 1995)

Although these data certainly provide further understanding for the mechanism of tolerance in a broader sense, these studies implication on analgesic tolerance is rather weak, since both in the adult and in the infant, tolerance to different properties of opiates have been seen as largely independently developed. For example, tolerance develops to the analgesic effect of morphine yet not to the vocalization suppression property of morphine in the neonatal rat (Barr and Wang, 1992). Unless a neurochemical variable is found to directly relate to analgesic levels (one good candidate could be cAMP level, as cAMP levels are greatly increased in the formalin test), biochemical approach does not seem to be a good measurement of analgesic tolerance.

Withdrawal

Little et al. (1996) made the first attempt to define neurochemical indices of opiate withdrawal in the infant rat. The activity of noradrenergic neurons was determined by measuring postmortem levels of 3-methoxy-4-hydroxyphenylglycol (MHPG) in the hypothalamus, hippocampus and cortex in opiate-abstinent 10-day-old rat pups. Treatment with morphine followed by naltrexone increased MHPG levels in all of the brain areas examined. Acute morphine treatment increased MHPG levels only in the hypothalamus. The results of this study provide neurochemical data supporting the hypothesis that noradrenergic hyperactivity plays a role in neonatal opiate abstinence (Little et al., 1996).

Although adult literature has shown that the opiate withdrawal results in biochemical changes in various site in the CNS including the ventral tegmental area (VTA), amygdala, nucleus accumbens (NAcc), periaqueductal gray area (PAG), LC, and spinal cord, little is known about the role of these areas in the infant rodent. Recently, McPhie and Barr reported that precipitated withdrawal induced by

naltrexone in morphine-exposed pups resulted in increased expression of the nuclear protein Fos in the PAG and spinal cord. In the PAG, there were no differences in the enhanced expression in different subregions. In the spinal cord, enhanced expression was mostly in the dorsal horn. In contrast, there was no significant increase in the number of Fos-LIR cells in the LC or the NAcc, as there are in the adult, where changes are seen in both the LC and the NAcc during opiate withdrawal. This discrepancy may be caused by a less developed system in the infant, where brain regions that interact in the adult have not yet begun to interact in the infant (McPhie and Barr, 2000b, 2001a).

Similarly, Jones et al. reported that there is an increased *c-fos* mRNA expression associated with acute morphine withdrawal in the 7-day-old rat. Rat pups were given a single dose of morphine (10 mg/kg) followed two hours later by a challenge of naltrexone (10 mg/kg) to precipitate withdrawal. Analysis by solution hybridization showed that *c-fos* mRNA expression in the brain and the spinal cord increased significantly subsequent to the acute withdrawal (Jones et al., 2002).

In summary, there is a ever-increasing interest in studying opiate tolerance and withdrawal in the developing organism using animal models. Behavioral, electrophysiological, and biochemical evidence accumulated in the past decade all points to the conclusion that the infant is not immune from either opiate tolerance or dependence or expression of withdrawal (refer to Table 1-1 and 1-2 for the complete list of studies). However, the characteristics of these phenomena (behavior repertoire, potency of tolerance, pharmacology etc.) in the infant are different from those in the adults. There are two possible reasons: 1.) central mechanisms (neurotransmitter systems, neural structure, neural substrate, pathway connection etc.) may differ; 2.) output mechanism may differ. The above reviewed studies thus provide a stepping-

stone for further analysis of the neural substrate and pharmacology of opiate tolerance and withdrawal in the developing organism that the author would like to pursue in the present thesis.

Since experiments are guided by current theoretical thinking and models on opiate tolerance and withdrawal, it is necessary to briefly review current models on opiate tolerance and withdrawal. All these models are based on data from the adult animals and they provide us a good starting point, although emerging data in the infant rat suggest that a different picture may exist in the immature animal.

Table 1-1. Evidence for tolerance to opiate's analgesic effect in the infant rat.

Mode of assay	1st week	2nd week	3rd week	Reference
Behavioral (Latency of withdrawal from a noxious stimulus)	NT	✓ (1)	NT	(Johannesson and Becker, 1972)
	NT	NT	✓	(Bardo et al., 1982)
	NT	✓	NT	(Barr et al., 1986)
	✓ (2)	NT	✓	(Tempel et al., 1988)
	×	×	✓	(Fanselow and Cramer, 1988)
	NT	✓	✓	(Kitchen and Boswell, 1990)
	NT	✓	NT	(Van Praag and Frenk, 1991)
	✓	NT	NT	(Barr and Wang, 1992)
	NT	×	✓	(Windh et al., 1995)
	NT	✓	NT	(Thornton and Smith, 1997)
	NT	NT	✓	(Thornton et al., 1997)
	NT	NT	✓	(Thornton and Smith, 1998)
	NT	NT	✓	(Thornton et al., 2000)
	NT	NT	✓	(Choe and Smith, 2000)
	✓	✓	✓	(Zhu and Barr, 2001a)
	✓	✓	✓	(Zhu and Barr, 2001d)
	✓	✓	✓	Zhu and Barr (this thesis)
Electrophysiology	✓	NT	NT	(Bell and Beglan, 1995b)
	✓	NT	NT	(Feng and Kendig, 1995b)
	✓	NT	NT	(Feng and Kendig, 1995a)
	✓	NT	NT	(Feng and Kendig, 1996b)
	✓	NT	NT	(Feng and Kendig, 1996a)
	✓	NT	NT	(Feng and Kendig, 1997)
	✓	NT	NT	(Zhu and Barr, 2001d)
	✓	NT	NT	Zhu and Barr (this thesis)

(1). Prenatal morphine treatment then tested on postnatal day 12-13

(2). Prenatal morphine treatment then tested on postnatal day 3.

✓: Tolerance to opiate's antinociceptive effect would be demonstrated at this age

×: Tolerance to opiate's antinociceptive effect would not be demonstrated at this age

NT: Tolerance to opiate's antinociceptive effect was not tested at this age

Table 1-2. Evidence for opiate withdrawal in the infant rat.

Mode of assay	1st week	2nd week	3rd week	Reference
Behavioral	X	X	✓	(Fanselow and Cramer, 1988)
	✓	NT	NT	(Enters et al., 1991a, b)
	✓	✓	✓	(Jones and Barr, 1995)
	NT	✓	✓	(Windh et al., 1995)
	NT	✓	NT	(Little et al., 1996)
	NT	✓	NT	(Thornton and Smith, 1997)
	NT	NT	✓	(Thornton et al., 1997)
	NT	NT	✓	(Thornton and Smith, 1998)
	✓	NT	NT	(Barr et al., 1998)
	NT	NT	✓	(Thornton et al., 2000)
	✓	NT	NT	(Zhu and Barr, 2000)
	NT	✓	NT	(Ceger and Kuhn, 2000)
	✓	NT	NT	(McPhie and Barr, 2000a)
	✓	NT	NT	(Jones and Barr, 2000)
	✓	NT	NT	(Jones and Barr, 2001)
	✓	✓	✓	(Zhu and Barr, 2001b)
	NT	NT	✓	(Lohmann and Smith, 2001)
	✓	NT	NT	(Jones et al., 2002)
	✓	✓	✓	(Barr and Zhu, 2001)
	✓	NT	NT	(McPhie and Barr, 2001b)
	✓	✓	✓	Zhu and Barr (this thesis)
Vocalization	✓	NT	NT	(Barr and Wang, 1992)
	✓	NT	NT	(Jones et al., 2002)
Conditioning	X	✓	NT	(Barr and Goodwin, 1997)
Electrophysiology	✓	NT	NT	(Bell and Jaffe, 1986)
	✓	NT	NT	(Bell and Beglan, 1995a)
	✓	NT	NT	(Feng and Kendig, 1995b)
	✓	NT	NT	(Feng and Kendig, 1995a)
	✓	NT	NT	(Feng and Kendig, 1996b)
	✓	NT	NT	(Feng and Kendig, 1996a)
	✓	NT	NT	(Feng and Kendig, 1997)
	✓	NT	NT	Zhu and Barr (this thesis)
Biochemical	NT	✓	NT	(Little et al., 1996)
	✓	NT	NT	(Jones et al., 2002)
	✓	NT	NT	(McPhie and Barr, 2000b)
	✓	NT	NT	(McPhie and Barr, 2001a)

- ✓: Opiate withdrawal would be demonstrated at this age
 X: Opiate withdrawal would not be demonstrated at this age
 NT: Opiate withdrawal was not tested at this age

Chapter 2

Mechanisms of opiate tolerance and withdrawal

In spite decades of research, the mechanisms underlying opiate tolerance, dependence, and withdrawal are still poorly understood (Mayer and Mao, 1999; Trujillo, 1999; Nestler, 2001). Several hypotheses have been proposed to explain the development of tolerance and dependence that results from chronic morphine administration (Fry et al., 1980; Christie et al., 1987a, b; Mao et al., 1995; Rohde et al., 1996; Nestler and Aghajanian, 1997; Fundytus and Coderre, 1999; Mayer and Mao, 1999). As a note, there are two major forms of opiate tolerance/dependence: 1.) the associative tolerance/dependence is closely linked with environmental cues such that the presence of these cues together with opiates result in a reduced effect or a withdrawal syndrome; 2.) nonassociative, or physical tolerance/dependence is linked with the opioid receptor and subsequent signal transduction processes. The present thesis will discuss only the second form of tolerance, dependence and withdrawal.

Opiates exert their powerful effects through opioid receptors (Martin et al., 1974). There is a general agreement on the existence of at least three types of opioid receptors: mu, kappa, and delta (Mansour et al., 1988; Yaksh, 1997). Each of these three major receptor types has been cloned (Evans et al., 1992; Meng et al., 1993; Wang et al., 1993). Physical morphine withdrawal is largely mediated by the μ -opioid receptor (MOR), a $G_{i/o}$ -coupled member of the heptahelical family of plasma membrane receptors (Matthes et al., 1996). As is the case in the adult animal, in the infant rat control of behavioral withdrawal signs is modulated primarily by the μ -opioid receptor (McPhie and Barr, 2000a), although infant rats experience opiate withdrawal behaviors that are qualitatively different than the withdrawal behaviors displayed by adult rats (Jones and Barr, 1995; Windh et al., 1995).

Not surprisingly, the discovery of opioid receptors in general and the progress made in elucidating the signal transduction pathways of opioid receptors in particular have profoundly influenced contemporary theoretical thinking on the mechanisms of opiate tolerance, dependence and withdrawal. As a result, all modern proposals modeling the mechanisms underlying opiate tolerance and withdrawal fall into two major categories: 1.) proposals that emphasize on opiates' direct effects on opioid receptors, including uncoupling of the opioid receptor from signal transduction (desensitization) and possibly a decrease in receptor number after prolonged exposure to opiates (downregulation); 2.) proposals that stress the change in the physiology of the cell resulted from adaptive processes in response to continued opioid signaling (Alvarez et al., 2001). Although both lines of hypotheses have gained considerable acceptance among researchers over the last decade, most recent and more conclusive evidence suggest that opioid receptor desensitization and downregulation evoke homeostatic regulation of important signaling cascades instead of contributing directly to the development of opiate tolerance and withdrawal (Finn and Whistler, 2001).

Thus, current thoughts on opiate tolerance and dependence suggest that these phenomena arise, at least in part, from a "functional decoupling" of opioid receptors from their associated signal transduction systems (Trujillo, 1999; Suresh and Anand, 2001), particularly the Ca^{2+} homeostasis (Mao, 1999; Smith et al., 1999). It has also been proposed that the increased neural activity seen during opiate withdrawal reflects a compensatory response in neurons that express the opioid receptor or of neural circuits in which those neurons participate (Nestler et al., 1993; Rohde et al., 1996; Inturrisi, 1997; Trujillo, 1999; Nestler, 2001). Finding a suitable explanation for this functional decoupling, however, has been difficult (Trujillo, 1999). Mao, Price, and Mayer have developed a model that provides a potential solution to this problem

(Mao et al., 1995; Mayer and Mao, 1999) by integrating recent molecular and behavioral findings. This model involves several steps. One of the critical steps is a μ -opioid receptor-mediated increase in protein kinase C (PKC) translocation and activation, leading to phosphorylation of the NMDA receptor and removal of the magnesium blockade results in influx of calcium and activation of several calcium-mediated processes. The final consequence is a functional decoupling of the μ receptor from transduction systems, thereby decreasing the cellular actions of opiates. In addition, influx of calcium causes activation of nitric oxide synthase (NOS) and liberation of nitric oxide (NO), which induces presynaptic release of glutamate, producing positive feedback of glutamatergic input and further activation of NMDA receptors. This model provides explanations for several known phenomena in the adult animal, including the ability of NMDA receptor antagonists, NOS inhibitors, and protein kinase inhibitors to inhibit the development of tolerance and dependence, and the potential roles of glutamate, calcium, PKC, and nitric oxide in tolerance and dependence (for review, see Inturrisi, 1997; Mao, 1999; Trujillo, 1999). Moreover, this model is consistent with the roles of these molecules in other forms of neural plasticity, such as learning, memory and LTP (Ozawa et al., 1998; Dingledine et al., 1999; Dingledine and McBain, 1999). The Mao et al. model has recently been expanded by Fundytus and Coderre to incorporate the latest finding (Fundytus and Coderre, 1999). A major change from the Mao et al. model is the prominence of mGluRs as the critical EAA receptors initiating the events leading to the development of opioid tolerance and dependence (Fundytus and Coderre, 1999). Thus, parallel mechanism may exist for the activation of the calcium dependent second messenger systems, including the activation of NOS and the production of NO.

It is important to note that, although the role of various glutamate receptors, particularly the NMDA receptor, has been firmly supported by a wealth of data, the vast majority of these data are from the adult animal. Whether these results apply to the infant is largely unknown and represents an unexplored area for further studies. Since glutamate receptors are believed to play key roles in opiate actions, a detailed description of the pharmacology and receptor properties of the various glutamate receptor types and their subunits is necessary. Thanks to the most recent advancement in cloning and gene knockout technology, an explosion of studies has characterized the molecular structure of the various glutamate receptor complex (for recent reviews, see Pin and Duvoisin, 1995; Conn and Pin, 1997; Danysz and Parsons, 1998; Michaelis, 1998; Nakanishi et al., 1998; Ozawa et al., 1998; Vallano, 1998; Dingledine et al., 1999; Yamakura and Shimoji, 1999). These breakthroughs have enabled us to address questions that we could not even a few years ago. The present thesis represents such an effort: by tapping into the insight provided by the most recent findings about the molecular structure and physiological properties of various glutamate receptors, we try to outline the dynamic functional role of the glutamate receptors in opiate tolerance, dependence and withdrawal.

Chapter 3

Glutamate receptors

Glutamate receptors: classification and properties

Glutamate receptors (GluRs) mediate most of the excitatory neurotransmission in the mammalian central nervous system (CNS) (Nakanishi, 1992; Ozawa et al., 1998; Yamakura and Shimoji, 1999). They also participate in plastic changes in the efficacy of synaptic transmission underlying memory and learning, and the formation of neural networks during development (Nakanishi et al., 1998). GluRs are categorized into two distinct classes, ionotropic and metabotropic receptors. The ionotropic glutamate receptors (iGluRs) contain cation-specific ion channels, and are further subdivided into three groups: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor channels. On the other hand, the metabotropic glutamate receptors (mGluRs) are coupled to GTP-binding proteins (G-proteins) and modulate the production of intracellular messengers (Michaelis, 1998; Ozawa et al., 1998). So far, at least 14 cDNAs of iGluR and 8 cDNAs of mGluR subunits have been identified in the mammalian CNS (Ozawa et al., 1998).

AMPA receptor channels are permeable not only to Na^+ and K^+ , but also to Ca^{2+} in some situations (Ozawa and Iino, 1993; Jakowec et al., 1995b; Jakowec et al., 1995a; Ozawa et al., 1998). The functional properties of recombinant AMPA receptors depend on their subunit compositions. Homomeric receptors assembled from GluR2 subunits display little permeability to Ca^{2+} and an outward rectification. In contrast, homomeric receptors assembled from either GluR1, GluR3 or GluR4

subunits are highly permeable to Ca^{2+} and display a strong inward rectification (Michaelis, 1998; Ozawa et al., 1998). In heteromeric receptors, GluR2 is dominant in determining both Ca^{2+} permeability and rectification properties. Coexpression of GluR2 subunits with GluR1, GluR3 and/or GluR4 results in the formation of recombinant receptors with little Ca^{2+} permeability and an outward rectification (Ozawa et al., 1998). The unique properties of GluR2 can be traced to a single amino acid residue in the M2 segment. This residue is a positively charged arginine (R) in GluR2, whereas it is a glutamine (Q) with a neutral charge in the other AMPA receptor subunits. This site in M2 has been referred to as the Q/R site. When the arginine in this site in GluR2 is replaced with glutamine using site-directed mutagenesis, the mutant GluR2 (Q) shows properties similar to those of the wild-type GluR1, GluR3 and GluR4 subunits both in the Ca^{2+} permeability and rectification properties. The arginine in the Q/R site is not encoded on the GluR2 genomic DNA, but is introduced by RNA editing to replace the gene-encoded glutamine (for review, see Michaelis, 1998; Ozawa et al., 1998). In adult brains, almost all GluR2 are expressed in the edited form. Thus, Ca^{2+} entry into the central neurons through AMPA receptors is prevented by the RNA editing. In embryonic brains, a small percentage of Glu2 mRNA does not undergo editing, and therefore unedited Ca^{2+} permeable AMPA receptor can exist during brain development (Pellegrini-Giampietro et al., 1992). AMPA receptors with high Ca^{2+} permeability also exist in other part of the CNS. For example, AMPA receptors in neonatal spinal cord can permit significant Ca^{2+} permeability due to the higher (GluR1 + GluR3 + GluR4)/Glu2 ratio than in the adult (Jakowec et al., 1995b; Jakowec et al., 1995a). Activation of AMPA receptors can produce a marked increase in cytoplasmic free Ca^{2+} (Ozawa and Iino, 1993; de Erausquin et al., 1994; Cebers and Liljequist, 1995; Akaike and Rhee, 1997). The role of Ca^{2+} entry through Ca^{2+} -permeable AMPA

receptor has been hypothesized to activate downstream Ca^{2+} dependent intracellular events (Jakowec et al., 1995a; Ozawa et al., 1998) such as NO production (Kest et al., 1997) and phosphorylation of cAMP response element-binding protein (Perkinton et al., 1999). There is evidence that NO synthesis can be suppressed by AMPA receptor blockade (Marin et al., 1993; Fedele and Raiteri, 1999).

The NMDA receptor is unique among all known neurotransmitter receptors in its requirement for the simultaneous binding of two different agonists for activation (Dingledine and McBain, 1999). In addition to the conventional agonists binding site typically occupied by glutamate, the binding of glycine appears to be required for receptor activation (Dingledine et al., 1999). The NMDA receptor is one of the most tightly regulated neurotransmitter receptors. These consist of two different agonist recognition sites (for glutamate and glycine) and a polyamine regulatory site that promote receptor activation, and separate recognition sites for Mg^{2+} and Zn^{2+} that act to inhibit ion flux through agonist-bound receptors. It is also voltage-dependent. For these reasons, it is believed that NMDA is involved in long-term changes and plasticity such as learning and memory (Michaelis, 1998; Ozawa et al., 1998; Dingledine et al., 1999; Yamakura and Shimoji, 1999), opiate tolerance and dependence (Trujillo and Akil, 1991a; Herman et al., 1995; Trujillo, 1995).

The NMDA receptor channel has characteristic ion permeation properties. The alkali-metal cations, Na^+ , K^+ and Cs^+ ions, permeate through the channel with a low selectivity, but major differences of the NMDA receptor channel from non-NMDA receptor channels exist in the permeation properties for Ca^{2+} and Mg^{2+} . Ca^{2+} is highly permeant, whereas Mg^{2+} is a potent blocker of the NMDA channel (Ozawa et al., 1998). Actually, a very important brake on NMDA receptor activation is provided by extracellular Mg^{2+} , which exerts a voltage-dependent block of the open ion channel of the NMDA receptor (Yamakura and Shimoji, 1999). Inspection of the

primary structures of the NMDA receptor subunits, NR1, NR2A-NR2D and NR3A has revealed that the positions corresponding to the Q/R site in the M2 segment of the AMPA receptor subunits are occupied by asparagine (N) in the NMDA receptor subunits. This N site governs both Ca^{2+} permeability and Mg^{2+} block in the NMDA channel. Replacing the asparagine in this site of the NR1 subunit with glutamine by site-directed mutagenesis leads to a reduction of Ca^{2+} permeability and a less degree of Mg^{2+} block. The importance of the asparagine is more convincingly supported by the result that replacing this amino acid with arginine almost completely abolishes both Ca^{2+} permeability and Mg^{2+} block. Asparagine in the corresponding site of the NR2 subunits (NR2A and NR2B) also plays an important role in the permeability of the divalent cations. Replacing the asparagine in this site of NR2 with glutamine markedly reduces the Mg^{2+} block and causes a permeability to Mg^{2+} . Thus, asparagine in the N site of the NMDA receptor subunits guarantees the hallmark of the NMDA receptor that the channel is highly permeable to Ca^{2+} with no permeability to Mg^{2+} . As described above, the selectivity of the Ca^{2+} permeable AMPA receptors for divalent cations is very low, and the receptors display a substantial permeability to Mg^{2+} . In the mutants of AMPA receptors containing an asparagine in the Q/R site, the relative permeability ratio between Ca^{2+} and Mg^{2+} , $P_{\text{Ca}}/P_{\text{Mg}}$, becomes higher than in those containing glutamine (for reviews, see Ozawa and Iino, 1993; Michaelis, 1998; Ozawa et al., 1998; Dingledine et al., 1999; Yamakura and Shimoji, 1999).

The mGluRs are different from ionotropic glutamate receptors (NMDA, AMPA and kainate receptors) in that they are G protein coupled (Schoepp and Conn, 1993; Riedel, 1996; Conn and Pin, 1997). Eight subtypes of mGluRs, from mGluR1 to mGluR8 have been cloned (Riedel, 1996; Michaelis, 1998; Dingledine et al., 1999). These subtypes of mGluRs can be divided into three groups (mGluR I, II and III) based on receptor pharmacology, signaling cascades and sequence similarities

(Ozawa et al., 1998). The group I mGluRs consists of mGluR1 and mGluR5, which are positively linked to the phosphatidylinositol second messenger system. Activation of these receptors leads to phospholipase C (PLC)-mediated phosphatidylinositol (PI) hydrolysis. The group II mGluRs consists of mGluR2 and mGluR3, which are negatively coupled, via adenylate cyclase, to the production of cAMP. The group III mGluRs consist of mGluR4, 6, 7 and 8, which are also negatively coupled to cAMP production, but which show a different receptor pharmacology than mGluR2, 3 in that they are selectively activated by L-amino-4-phosphonobutanoate (L-AP4) (Fundytus et al., 1997; Dingledine et al., 1999; Dingledine and McBain, 1999).

Glutamate receptors: pharmacology

The AMPA receptor has at least three separate binding sites at which agonists or antagonists can act: glutamate binding, desensitization and intra-ion channel binding sites. The glutamate binding site is the site for competitive antagonists such as 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX) and 6-(1 H-imidazol-1-yl)-7-nitro-2,3(1H, 4H)-quinoxalinedione (YM90K). AMPA receptors show a rapid desensitization. A number of substances such as cyclothiazide (CTZ) or aniracetam affect this desensitization mechanism. Another group of substances such as joro spider toxin (JSTX) and its analogues bind to the third site within the ion channel and block ion flows. Quinoxalinediones such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) are potent competitive antagonists for non-NMDA receptors. NBQX is a more selective competitive antagonist for AMPA receptors, which has approximately 30-fold selectivity for AMPA over kainate receptors (Sheardown, 1993; Sheardown et al., 1993). JSTX specifically blocks inwardly rectifying and Ca^{2+} permeable AMPA

receptors lacking the edited GluR2 subunit. At low concentrations ($<3 \mu\text{M}$), the toxin abolished almost completely responses of the Ca^{2+} permeable AMPA receptors without affecting outwardly rectifying Ca^{2+} impermeable AMPA receptors assembled with GluR2 (for reviews, see Ozawa et al., 1998; Dingledine et al., 1999; Dingledine and McBain, 1999). LY293558 is a novel systemically active competitive AMPA receptor antagonist (Ornstein et al., 1993). LY293558 has a greater than 10-fold selectivity for inhibiting the excitatory effects of AMPA vs. kainate, and a greater than 30-fold selectivity for inhibiting the excitatory effects of AMPA vs. NMDA (Schoepp et al., 1995). For its exceptional properties, LY293558 has recently been used to study the role of the AMPA receptor in opiate tolerance, dependence and withdrawal in adult rats and mice by several laboratories (Rasmussen et al., 1996; Kest et al., 1997; McLemore et al., 1997).

The NMDA receptor requires activation via two agonist sites, one of which binds glutamate and the other one of which binds glycine (Dingledine and McBain, 1999). Glycine is an obligatory co-agonists; activation of the NMDA receptor requires the simultaneous presence of glutamate and glycine (Dingledine et al., 1999). According to their site of actions on the NMDA receptor, drugs are divided into four groups, i.e. those acting at: (1) the intra-ion channel binding site; (2) the glutamate/NMDA recognition site; (3) the strychnine-insensitive glycine-binding site; and (4) modulatory sites such as the redox modulatory site, the proton sensitive site, the Zn^{2+} site, and polyamine site (Ozawa et al., 1998). According to this classification, there are four major types of NMDA receptor antagonists (Herman et al., 1995).

Example of each follow:

- 1.) Noncompetitive NMDA receptor antagonists. Noncompetitive NMDA receptor antagonists are thought to act by blocking the ion channel for

NMDA receptor complex. Once bound, the blocker can be trapped by channel closure and recovery from the trapped blocked state is generally slow (Dingledine et al., 1999). Examples of this category are: Phencyclidine (PCP), Ketamine, MK-801 (dizocilpine), dextrorphan, dextromethorphan, ADCI, FPL 12495, CNS 1102.

- 2.) **Competitive NMDA receptor antagonists.** Competitive NMDA receptor antagonists inhibit the binding of glutamate at the glutamate recognition site of the NMDA receptor. Examples of this category are: D-2-amino-5-phosphovalerate (AP5), 2-amino-7-phosphonoheptanoate (AP7), LY 274614, LY 233536, LY 233053, CGP 40016, CGP 43487, cis-4-(phosphonomethyl)piperidine-2-carboxylate (CGS 19755) 3-(2 carboxypiperazine-4yl)-propylphosphonate [D(-)CPP], D(-)-CPPene.
- 3.) **Glycine site antagonists.** Kynurenic acid, MDL 28469, 5, 7-DCKA, MDL 100748, MDL 29951, MDL 102288, MDL 100573, quinoxalinedione (e.g., ACEA-1328), 8-methyl-DDHB, L-689560 (tetrahydroquinolones), MDL 105572 (3-Aryl-4-hydroxy-2-quinolones), 7-CKA, felbamate (Felbatol), ACPC (partial glycine agonist).
- 4.) **Polyamine site antagonists.** Ifenprodil, eliprodil.

These pharmacological agents provide a rich array of drugs that can be used to address questions concerning the role of specific sites of the NMDA receptor and functions modulated by these receptors (Herman et al., 1995; Danysz and Parsons, 1998; Dingledine et al., 1999).

Glutamate, quisqualate and ibotenate have been shown to activate mGluRs as well as iGluRs (Michaelis, 1998). Some glutamate analogues, such as 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and L-AP4, are specific for mGluRs, but their potencies differ for each mGluR subtype (Riedel, 1996; Ozawa et

al., 1998). The most widely used agonist is ACPD, which is selective for mGluRs and devoid of any activity against ionotropic glutamate receptors. ACPD has been shown to act as an agonist on group I as well as on group II and group III mGluRs (Riedel, 1996).

3,5-dihydrophenylglycine (DHPG) has been shown to be a highly selective agonist for group I receptors (Ozawa et al., 1998). Recently, trans-azetidine-2,4-dicarboxylic acid (tADA) has been identified as a novel rigid glutamate analogue selectively activates group I mGluRs (Riedel, 1996). (+)- α -methyl-4-carboxyphenylglycine ((+)-MCPG) are antagonists of mGluR1 as well as of mGluR2 which belongs to group II receptors. (S)-4-Carboxyphenyl-glycine ((S)-4CPG) has been proposed as a relatively specific antagonist for group I (mGluR1a), although it acts on group II (mGluR2) at higher concentrations (Ozawa et al., 1998).

The most potent agonist of class II mGluRs is DCG-IV as revealed in brain slices, with very low activity on class I mGluRs (Riedel, 1996). (2S,1'S,2'S)-2-(carboxycyclopropyl)-glycine (L-CCG-I) were shown to selectively agonize group II mGluRs (Michaelis, 1998). LY354740 is a recently discovered selective group II mGluRs agonist, which shows no significant ionotropic (iGluR4 and iGluR6) nor mGlu1 and 5 or mGlu4, 6, 7 and 8 receptor activities (Bond et al., 1997). LY 354740 is systemically active and has been shown to decrease the evoked release of glutamate in the striatum (Vandergriff and Rasmussen, 1999). Recently, (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)-glycine (MCCG) has been proposed as a specific antagonist of group II mGluRs (Jane et al., 1994).

Group III mGluRs are selectively activated by 2-amino-4-phosphonobutyrate (AP4), which is ineffective against group I and group II mGluRs. Since AP4 depresses glutamate release from perforant-path synapses, class III mGluRs are widely believed

to be located presynaptically, thereby acting like autoreceptors (Riedel, 1996). Group III receptors are characterized by their sensitivity to L-AP4 as well as insensitivity to ACPD (Ozawa et al., 1998). It has also been proposed that α -methyl-L-AP4 (MAP4) is a selective antagonist of group III receptors (Ozawa et al., 1998).

The roles of glutamate receptors in opiate tolerance and withdrawal

There is broad and persuasive evidence supporting the role of glutamatergic receptors in mediating the phenomenon of opiate tolerance, dependence and withdrawal in brain and spinal cord (for reviews, see Trujillo and Akil, 1991a; Fundytus and Coderre, 1999; Mao, 1999; Trujillo, 1999). An increase in glutamate release is generally observed during opiate withdrawal (Rasmussen and Aghajanian, 1989; Akaoka and Aston-Jones, 1991; Desole et al., 1996; Jhamandas et al., 1996; Tokuyama and Ho, 1996; Hoshi et al., 1997; Huang et al., 1997). For example, naloxone-precipitated withdrawal from morphine 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 precipitated withdrawal signs in a dose-dependent manner, whereas prominent behavioral changes were not observed when animals continuously infused with saline were challenged with glutamate (Tokuyama et al., 1996). Hence, these results provide direct evidence that glutamate is essential for the expression of opiate withdrawal signs. The rapid release of glutamate from locus coeruleus and other regions under an opiate dependent state has been hypothesized to be triggers or key elements for the expression of opiate withdrawal signs (Rasmussen, 1991; Christie et al., 1997).

Although there is direct evidence supporting the role of glutamate, it is less clear which glutamate receptors are responsible for opiate tolerance, dependence and withdrawal. Some authors favor the NMDA receptor (Mao et al., 1995; Trujillo, 1995; Tokuyama et al., 1996; Mao, 1999), some others argue for a major role of the AMPA (Rasmussen, 1995), still others reported the role of mGluRs (Fundytus and Coderre, 1997; Fundytus et al., 1997). The following sections review the major evidence for the roles of the NMDA receptor, AMPA receptor and mGluRs in opiate tolerance, dependence and withdrawal.

There is evidence from numerous laboratories that a variety of noncompetitive NMDA receptor antagonists, competitive antagonists and glycine antagonists can inhibit mu opiate (morphine) tolerance in rodents (for reviews, see Trujillo and Akil, 1991a; Elliott et al., 1995a; Herman et al., 1995; Pasternak et al., 1995; Trujillo, 1995; Trujillo and Akil, 1995; Inturrisi, 1997; Danysz and Parsons, 1998; Mao, 1999). The potency and clinical potential of these agents on morphine tolerance are further strengthened by the findings of several studies indicating that both noncompetitive and competitive NMDA receptor antagonists are capable of “reversing” morphine tolerance (Bell and Beglan, 1995b; Trujillo and Akil, 1995; Grass et al., 1996; Hoffmann and Wiesenfeld-Hallin, 1996; for conflicting data, see Bhargava, 1997; for review, see Inturrisi, 1997). There is also increasing evidence that most of the symptoms of antagonist-precipitated opiate withdrawal observed in rodents exposed in a chronic fashion to a mu opioid agonist such as morphine can also be inhibited by noncompetitive NMDA receptor antagonists, competitive NMDA receptor antagonists, glycine antagonists, if these compound are co-administrated with morphine chronically (Trujillo and Akil, 1991a; Trujillo, 1999). There is also evidence from a number of laboratories that some of the withdrawal signs can be attenuated by acutely administered NMDA antagonists, although other

investigators have either obtained negative results or failed to obtain significant effects of some of these agents on opiate withdrawal (for review, see Herman et al., 1995).

Based on these findings, Mao, Price and Mayer provide a model on opiate tolerance and dependence (Mao et al., 1995; Mayer and Mao, 1999). The most prominent feature of this model is that it puts the role of the NMDA receptor in an indispensable position for the taking place of opiate tolerance and dependence. The Mao et al. model is the generally cited model due to the depth and strength of data supporting this model, as well as its consistency with models of other types of neuronal plasticity (Inturrisi, 1997; Trujillo, 1999), although competing models have been proposed recently (Fundytus and Coderre, 1999).

Compared with the wealth of literature supporting the role of the NMDA receptor in opiate tolerance and dependence, there are only a limited number of laboratories reporting the role of various mGluRs on these processes. This may be due to a combination of factors: 1.) mGluRs are more recently cloned than the NMDA receptor; 2.) there was a lack of selective agonists and antagonists until recently; (3) there is a lack of systemically active agonists and antagonists and this situation is still existing today; (4) current thinking favors the NMDA receptor because the NMDA receptor has an established status in neural adaptation like learning, memory, LTP and LTD (Michaelis, 1998; Ozawa et al., 1998; Dingledine et al., 1999; Dingledine and McBain, 1999; Klodzinska et al., 1999). In the past few years, several authors from different laboratories, using a variety methodology ranging from behavioral to electrophysiological, investigated the role of mGluRs on opiate tolerance (Popik et al., 2000a), dependence and withdrawal in rats and mice (Fundytus and Coderre, 1994, 1997; Fundytus et al., 1997; Klodzinska et al., 1999; Vandergriff and Rasmussen, 1999). These results are consistent across species, which is in contrast to the reports on various NMDA antagonists (Herman et al., 1995).

Chronic antagonism of mGluRs in the brain with the nonselective antagonist MCPG, as well as with 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 the group II selective agonist DCG-IV just prior to the precipitation of withdrawal dramatically reduced the severity of precipitated withdrawal symptoms (Fundytus and Coderre, 1994, 1997; Fundytus et al., 1997; for review, see Fundytus and Coderre, 1999). The novel systemically active group II mGluR agonist LY 354740 has also recently been shown to inhibit the naloxone induced symptoms of morphine withdrawal in morphine-dependent mice (Klodzinska et al., 1999). In addition, LY 354740 has been reported by another group to attenuate 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 also inhibited by metabotropic group II glutamate agonist LY 354740 in mice (Popik et al., 2000a). Thus, these recent data strongly suggest that, besides the NMDA receptor, mGluRs might also be involved in the development of opiate tolerance and dependence and expression of opiate withdrawal in the adult rat and mouse. In fact, at least one model has been proposed to emphasize the prominence of mGluRs as the critical EAA receptors initiating the events leading to the development of opioid tolerance and dependence (Fundytus and Coderre, 1999).

Although no model on opiate tolerance and dependence places the AMPA receptor at a prominent position, available evidence implies that the role of the AMPA receptor in these processes cannot be overlooked.

The role of the AMPA receptor in tolerance has been examined using the systemically active competitive antagonist LY293558 in mice (Kest et al., 1997;

McLemore et al., 1997). Tolerance was attenuated when mice received a continuous subcutaneous infusion of LY293558 at doses of 30, 45 or 60 mg/kg/24 hr via an osmotic pump concurrent with the morphine treatment. Continuous subcutaneous infusion of LY293558 (45 mg/kg/24 hr) also reversed established morphine tolerance. Thus, these data demonstrated that the AMPA receptor might play a role in the development and maintenance of morphine tolerance.

Besides its possible role in the development of opiate tolerance and dependence, AMPA receptor has been argued to play a more important role than the NMDA receptor in the expression opiate withdrawal. Rasmussen and colleagues has questioned the role that the NMDA receptor plays in opiate withdrawal (Rasmussen, 1995). Studies by this group lend support to the concept that NMDA receptors play only a minor role in the expression of opiate withdrawal. In opiate-dependent rats, there is a marked increase in firing of LC neurons during naltrexone-precipitated withdrawal; and opiate withdrawal increases glutamate and aspartate efflux in the LC (Rasmussen and Aghajanian, 1989; Rasmussen et al., 1996). This increase in activity of LC neurons has been hypothesized to be important in opiate withdrawal symptoms (Rasmussen et al., 1996). The excitatory effect of glutamate in the LC is mediated largely through AMPA receptors, whereas NMDA receptors mediate little, if any, of the withdrawal-induced excitation of the LC (Rasmussen et al., 1991a; Rasmussen, 1995; Rasmussen et al., 1996). Recently, another group reported that 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). These data suggest that, in the adult, although NMDA receptors may be important in the acquisition of morphine dependence, once the dependence is established, NMDA receptors may play a minor

role. Other glutamate receptors such as AMPA and metabotropic glutamate receptors (see discussion above) may play the major roles.

It is important to note that during ontogeny, opioid receptors undergo non-uniform developmental changes, and comprehensive reviews have been published on that aspect (Marsh et al., 1997; Suresh and Anand, 2001). Although the developmental profile of opioid receptors may explain some facts of opiate actions in the infant (e.g., the sensitivity of postnatal rats to mu receptor agonists) (Thornton et al., 1998), it cannot explain the qualitative difference of NMDA receptor antagonists' effect on morphine tolerance and withdrawal (Zhu and Barr, 2001b). Importantly, the transition age (second week postnatal) we observed for the effectiveness of NMDA receptor antagonists in suppressing morphine tolerance and withdrawal correlates well with the developmental profile of glutamate receptors rather than opioid receptors.

Ontogeny of the AMPA receptor

In the rat brain, the ratio of GluR-1 and GluR-3 to GluR-2 gene expression at various stages of development was high in postnatal CNS neurons but declined monotonically with age (Durand and Zukin, 1993; Brennan et al., 1997). For example, in the hippocampus, the subunits of the native AMPA receptors of Meynert neurons change at various developmental stages as the ratio of Ca^{2+} permeable GluR-1, 3, 4 subunits to that of the Ca^{2+} impermeable GluR2 subunit declines gradually during the development stages (Pellegrini-Giampietro et al., 1992; Akaike and Rhee, 1997). Developmental differences in expressions of two alternative splice variants of the AMPA receptor, flip and flop, have been studied using in situ hybridization histochemistry (for review, see Ozawa et al., 1998). AMPA receptor subunits are

expressed predominantly in the flip form in embryonic brains. The flop forms are expressed at low levels prior to postnatal day 8, and gradually increase throughout the brain, reaching adult levels by postnatal day 14 (Monyer et al., 1991). The GluR2 RNA editing to replace the gene-encoded Q to R is developmentally regulated. At the early developmental stage (embryonic day 14), a small percentage of the GluR2 does not undergo editing, and therefore the unedited form co-exists with the edited form (Sommer et al., 1990; Sommer et al., 1991). In postnatal stages, however, virtually all GluR2 exists in the edited form. The editing at the R/G site in GluR2-GluR4 is also developmentally regulated. The extent of R/G editing in the embryonic brain is generally small, but increases markedly during development up to 55% to ~ 100% in the adult brain (Lomeli et al., 1994).

In the developing rat spinal cord gray matter, the temporal expression of the various subunits of the AMPA receptor complex has been studied using receptor autoradiography, immunoblotting, immunohistochemistry and in situ hybridization (Furuyama et al., 1993; Tolle et al., 1993; Jakowec et al., 1995b; Jakowec et al., 1995a). During early postnatal life (around P7 to P14), AMPA binding sites are transiently expressed at high levels in the ventral horn (at least four-fold higher than those in the adult). Within the ventral horn, transcripts for GluR1 show the most dramatic changes in the level of expression, GluR2 and GluR4 go through a more modest developmental down-regulation in postnatal life, and GluR3 does not appear to change significantly. AMPA receptors expressed by adult spinal cord neurons are potentially composed of subunits encoded by GluR1 flop, GluR2 flip, GluR3 flip and flop, and GluR4 flip. This contrasts with AMPA receptors expressed by neonatal motor neurons that are potentially composed of all subunits (except GluR2 flop) although predominant subunits are likely to be the flip splice variants of GluR1, 2 and 4 and the GluR1 flop splice variant. With all AMPA receptor transcripts the

major developmental changes in subunit expression leading to the acquisition of the adult pattern of labeling occurs between P7 and P21. Based on the levels of expression of each subunit, the ratio of (GluR1 + GluR3 + GluR4)/GluR2 is higher in neonates than in adults, suggesting the possibility that AMPA receptors expressed by neonatal spinal cord may permit significant calcium permeability. The role of calcium flux through AMPA receptor in the neonatal rat is unknown and has been hypothesized to activate down-stream calcium-dependent intracellular events (Jakowec et al., 1995a; Gu et al., 1996; Kest et al., 1997; Albuquerque et al., 1999).

Ontogeny of the NMDA receptor

The various subunits of the NMDA receptor complex undergo dramatic differential maturation during the first few postnatal weeks of the animal (Pollard et al., 1993; Laurie and Seeburg, 1994; Zhong et al., 1994; Zhong et al., 1995; for review, see Danysz and Parsons, 1998; Guilarte and McGlothan, 1998), resulting in considerably different functions of the NMDA receptor (Colwell et al., 1998). Specifically, brain levels of NR1 and NR2A are lowest shortly after birth, increasing to reach peak levels by 14 or 21 days of age and subsequently decreasing at 28 days of age. NR2B levels are highest during early development and decrease as the animal ages. NR2C subunit is restricted to the cerebellum and its presence is undetectable until the second postnatal week (Guilarte and McGlothan, 1998). The expression of the NR3A subunit begins just prior to, or during the first postnatal week (Das et al., 1998). Thus, in neonatal brain the predominant NMDA receptor subunits are the NR1 subunit, the NR2B subunit and the NR2D subunit. Expression of the NR1-NR2B subunit complex is high at birth, peaks at about day 14 and then declines to day 21. The NMDA receptor containing the NR2A subunit is almost non-existent at

birth, increases gradually with age and reaches maximal level on day 21. Expression of the NR1 subunit also increases with age and peaks on day 21 (Sircar, 2000). It has recently been suggested that there are two types of NMDA receptors in the rat, an immature type and a mature type; and that there exists a temporal switching of the NMDA receptor from the immature form to the adult mature form around the second week postnatally; and that the pharmacological properties of the immature and mature NMDA receptor correlate well with the developmental profile of the NMDA receptor subunit configuration (Sircar, 2000).

Ontogeny of the metabotropic glutamate receptors

In the rat brain, mGluR subtypes undergo dynamic, regionally specific regulation during postnatal development (Conadorelli et al., 1992; Minakami et al., 1992; Ghosh et al., 1997; Liu et al., 1998; Elezgarai et al., 1999; Munoz et al., 1999). Developmentally, the age of maximal expression of mGluRs was dependent on the region (Ghosh et al., 1997). In general, the mRNA expression levels for mGluR1, mGluR2 and mGluR4 were low at birth and increased during postnatal development. In contrast, mGluR3 and mGluR5 were highly expressed at birth and decreased during maturation to adult levels of expression (Catania et al., 1994). It is important to note that mGluR5 plays a key role in release of Ca^{2+} from internal stores via inositol triphosphate mobilization (van den Pol et al., 1995). What is more, PKC may couple the postsynaptic mGluR5 to the NMDA-receptor potentiation during LTP (Ugolini et al., 1997; Jia et al., 1998; Holohean et al., 1999). In mice lacking mGluR5, the NMDA component of long-term potentiation is abolished (Jia et al., 1998). During early stages of development, there is a greatly enhanced expression of mGluR5 in rats compared with the adult in many brain areas (van den Pol et al.,

1995). Expression of the mGluR5 gene was reduced during development. For example, the hypothalamus showed a six-fold decrease in mGluR5 with development, whereas the cortex showed only a threefold decrease (van den Pol et al., 1995). The levels of mGluR7 are also differentially regulated across brain regions during postnatal development. In cortical regions (hippocampus, neocortex, and olfactory cortex), mGluR7 levels were highest at postnatal day 7 (P7) and P14, then declined in older rats. In contrast, mGluR7 levels were highest at P7 in pons/medulla and cerebellum and decreased markedly between P7 and P14 (Bradley et al., 1998).

The regional distribution of primary transcripts and splice variants of all mGluR subtypes have been studied in the lumbar enlargement of the rat spinal cord (Berthele et al., 1999; Silva et al., 1999). In adult animals, the messenger RNA of the mGluR subtypes 1, 5, 3, 4 and 7 were differentially expressed. The transcripts of mGluR1 and 5 were most abundant with mGluR5 messenger RNA concentrated in the superficial dorsal horn. In contrast, the mGluR2 transcript was not detectable with the sensitivity of the method. Age related changes (postnatal days 1, 7, 12, 21) in the postnatal expression of mGluR1-5 and 7 transcripts have been investigated. mGluR1 and 7 messenger RNA showed a general decrease in spinal expression from postnatal day 1 to day 21. High mGluR3 and 5 messenger RNA levels are expressed especially in the superficial dorsal horn at birth, and these levels decreased with age. In addition to changes in density, the regional distribution of mGluR3 messenger RNA was altered with postnatal development. Up to postnatal day 12, mGluR3 messenger RNA expression was almost exclusively restricted to the spinal gray matter. However, after postnatal day 21 a strong additional expression in the white matter occurred. Distribution of mGluR4 messenger RNA showed little developmental changes in the dorsal horn (Berthele et al., 1999).

Part II

Hypothesizing

Chapter 4

A critical review of the literature

As described in the introduction part, despite decades of effort, the mechanisms underlying opiate tolerance, physical dependence, and withdrawal remain largely unknown (Nestler, 1996; Fundytus and Coderre, 1999; Trujillo, 1999; Nestler, 2001). Multiple mechanisms may operate in these processes (Redmond and Krystal, 1984; Wuster et al., 1985; Thorat et al., 1994; Trujillo, 1999). In the adult animal, increasingly more data (Marek et al., 1991a; Marek et al., 1991b; Trujillo and Akil, 1991b; Elliott et al., 1994; Elliott et al., 1995a; Vaupel et al., 1995a, b; Bhargava and Thorat, 1996; Hall et al., 1996; Mao et al., 1996; Inturrisi, 1997; Vaupel et al., 1997; Hall et al., 1998; Kolesnikov et al., 1998; Mao et al., 1998; Zhu and Ho, 1998; Le Guen et al., 1999; Smith et al., 1999; Cuellar et al., 2000) suggest that the NMDA receptor and its second messenger systems play pivotal roles. Participation of other glutamate receptor types such as AMPA receptor (Rasmussen, 1995; Rasmussen et al., 1996; Kest et al., 1997; Taylor et al., 1998; Vandergriff and Rasmussen, 1999; Vekovischeva et al., 2001) and mGluRs (Fundytus and Coderre, 1994, 1997; Fundytus et al., 1997; Fundytus and Coderre, 1999; Klodzinska et al., 1999; Vandergriff and Rasmussen, 1999; Popik et al., 2000a) has also recently been suggested.

It is not known whether these mechanisms in adults apply to infants. On the one hand, opiate dependence and tolerance can be established in fetuses or infant rat pups if the dams are exposed to opiates during their pregnancy or the pups are treated directly with opiates (Fanselow and Cramer, 1988; Barr and Wang, 1992; Jones and Barr, 1995; Windh et al., 1995; Thornton and Smith, 1997; Thornton et al., 1997;

Barr et al., 1998; Jones and Barr, 2000). On the other hand, the neonatal CNS is both structurally and functionally different from that of the adult, and significant changes in opioid actions occur both prenatally and postnatally (Fitzgerald et al., 1988; Barr, 1992, 1993; Fitzgerald, 1995; Marsh et al., 1997). More importantly, the NMDA receptor, which is believed to play a crucial role in opiate tolerance, dependence and withdrawal, undergoes qualitative and quantitative changes during development (Kalb et al., 1992; Hori and Kanda, 1994; Kalb and Fox, 1997). These include significant developmental alterations in the density of the receptor (Tremblay et al., 1988; Morin et al., 1989; Represa et al., 1989), the sensitivity of the receptor complex to magnesium (Ben-Ari et al., 1988; Bowe and Nadler, 1990; Morrisett et al., 1990), and the rise and decay times of the NMDA EPSCs (Akaike and Rhee, 1997; Bardoni et al., 1998). Recent molecular studies revealed that various subunits (NR1, NR2A-D, NR3A) composing the native NMDA receptor complex undergo dramatic differential changes during the first few weeks of the postnatal life in the rat, resulting in functionally different NMDA receptors (for recent reviews, see Ozawa et al., 1998; Dingledine et al., 1999). It has been suggested that the NMDA receptor is not functionally mature until the second postnatal week in the rat (Sircar, 2000). Thus, the pharmacological effects for the various compounds acting on the NMDA receptor in the infant may not be necessarily comparable to those in the adult. In fact, some authors (Bell and Beglan, 1995a, b) reported negative results about the effects of MK-801 on morphine tolerance and withdrawal in the neonatal rat, which contrasted to reports in the adult rat (Marek et al., 1991a; Marek et al., 1991b; Trujillo and Akil, 1991b). Besides the NMDA receptor, other glutamate receptor types (mGluRs and AMPA receptor) also undergo significant developmental changes and their subunits mature differentially during early life (for review, see Ozawa et al., 1998). Therefore, although in adults, the role of glutamate receptors (especially the NMDA

receptor) in opiate tolerance, dependence and withdrawal has been widely supported, in neonates, their roles are empirically poorly studied and theoretically to a large degree questionable.

Following this line of reasoning, we designed and conducted several experiments to examine if the results in the adult apply to the infant. A large proportion of the initial studies has been reported in journal article and/or abstract forms (Zhu and Barr, 2000; Barr and Zhu, 2001; Zhu and Barr, 2001a, b, c, d). Being limited by the space, the following chapters will briefly summarize our major findings pertinent to the present thesis. For elaboration on the rationale of study, methods of experimentation, and full interpretation of data, please refer to the respective reprints and/or manuscripts attached to the end of each corresponding chapter.

Chapter 5

Initial Studies

NMDA receptor and morphine withdrawal

When acutely administered to morphine dependent 7-day-old rat, neither the noncompetitive NMDA receptor antagonist MK-801 nor the competitive NMDA receptor antagonist AP-5 was effective in attenuating the naltrexone precipitated morphine withdrawal. Furthermore, MK-801 exacerbated several withdrawal behaviors, including walking and wall climbing. The increase of these withdrawal behaviors was not due to the interaction of MK-801 with the specific doses of naltrexone we were using. Our finding about the effect of NMDA receptor antagonists on morphine withdrawal was inconsistent with the majority reports for the adult. On the other hand, in the available literature, there exists controversy about the effect of acute administered NMDA receptor on opiate withdrawal even in adult animals in the first place. Furthermore, our data are consistent with the available reports about the effects of NMDA receptor antagonist in the neonatal rat.

NOS and morphine withdrawal

Both the neural selective NOS inhibitor 7-NI and the non-selective NOS inhibitor L-NAME were very effective in suppressing most of the withdrawal behaviors in the 7-day-old rat pup. In addition, the relative effectiveness of these two NOS inhibitors was comparable to that in the adult rat. For example, all withdrawal behaviors suppressed by L-NAME were also attenuated by 7-NI, whereas walking behavior was inhibited by 7-NI but not L-NAME. Interestingly, Vaupel et al. compared the opiate withdrawal attenuation effect of 7-NI with other NOS

inhibitors (L-NNA, L-NAME, L-NIO) and found that 7-NI attenuated more signs of opiate withdrawal than did L-NNA, L-NAME or L-NIO in the adult rat (Vaupel et al., 1995a). Thus, our finding about the effects of NOS inhibitors on various withdrawal behaviors in infant rats extended the data in the adult to the neonate and suggest that opiate withdrawal in the infant relies on the same second messenger system as in the adult.

These data and their implications were summarized in a published journal paper (Zhu and Barr, 2000).

Chapter 6

Opiate tolerance and withdrawal: are NMDA receptors indispensable?

Our data of NMDA receptor antagonists' effects on morphine dependence do not support, at least in the neonatal rat, the Mao et al. model. As described earlier, this model places the NMDA receptor at an indispensable position for the acquisition of opiate tolerance and dependence (Mao et al., 1995; Inturrisi, 1997; Fundytus and Coderre, 1999; Trujillo, 1999). On the one hand, the role of the NMDA receptor in the establishment of opiate dependence in 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 the infant acquires tolerance, dependence and expresses withdrawal (Barr and Wang, 1992; Jones and Barr, 1995; Windh et al., 1995; Thornton et al., 1997) 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 activate the Ca^{2+} -dependent second messenger systems, thus leading to long lasting plasticity changes cascade (Inturrisi, 1997; Trujillo, 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 (for reviews, see Ozawa et al., 1998; Dingledine et al., 1999), the immature NMDA receptor seems to have low Ca^{2+} permeability (Frandsen et al., 1989; Frandsen and Schousboe, 1990; Resink et al., 1992; Didier et al., 1994; Barrios and Liljequist, 1996; Das et al., 1998; Goebel and Poosch, 1999). Furthermore, the dose

of MK-801 in our experiment should have effectively blocked the Ca^{2+} permeable ion pore of the immature NMDA receptor (Zhu and Barr, 2001b). Thus, it seems that the immature NMDA receptor is not a vital element for the development of opiate dependence in rats of this age. Our data showed that NOS inhibitors were very effective in suppressing withdrawal signs in the 7-day-old rat. These findings were consistent with the data from adult animals. Therefore, it seems that in the 7-day-old rat, the establishment of opiate dependence relies on the same second messenger systems as in the adult. The different pharmacological effects between the infant and adult we have seen for various NMDA receptor antagonists may be attributed to factors lying somewhere more “upstream”. One possibility is that other mechanisms activate the intracellular Ca^{2+} release and evoke the Ca^{2+} dependent second messenger system such as NO production. A second possibility is that channels besides the NMDA receptor allow significant Ca^{2+} influx in the infant CNS responsible for opiate actions. In any case, the essence of the Mao et al. model, which focuses on the role of the second messenger system cascades, including the increase of intracellular Ca^{2+} level 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 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 mGluRs. Because mGluRs are coupled to various second messenger cascades, they are ideal candidates for long-lasting intracellular changes such as learning and memory formation (Riedel, 1996; Ozawa et al., 1998). Particularly, group I mGluRs (mGluR1 and mGluR5) are positively coupled to phosphatidylinositol (PI) hydrolysis, and activation of these receptors stimulates phospholipase C (PLC), catalyzing the hydrolysis of inositol-4,5-

bisphosphate (PIP₂) to inosito-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Ambrosini and Meldolesi, 1989; Manzoni et al., 1990; Schoepp and Conn, 1993; Hayashi et al., 1994; Dingledine et al., 1999; Dingledine and McBain, 1999). This in turn leads to the IP₃-mediated release of Ca²⁺ from stores in the endoplasmic reticulum, and DAG-mediated translocation and activation of PKC (Kapcala et al., 1992; Schoepp and Conn, 1993; Dingledine et al., 1999; Dingledine and McBain, 1999). Recently, it has been suggested that, in addition to NMDA receptors, mGluRs may also be involved in opiate tolerance, dependence and withdrawal (Fundytus and Coderre, 1999). Chronic antagonism of mGluRs in the brain with nonselective, group I, group II or group III selective antagonists all reduced the severity of abstinence symptoms (Fundytus and Coderre, 1994, 1997; Fundytus et al., 1997; Fundytus and Coderre, 1999). Moreover, a single injection of group II selective agonists just prior to the precipitation of withdrawal dramatically reduced the severity of withdrawal symptoms (Fundytus and Coderre, 1997; Klodzinska et al., 1999; Vandergriff and Rasmussen, 1999). 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, whose anatomical distribution overlaps with the NMDA receptor and co-localizes with the NMDA receptor in many cell types (Ozawa et al., 1998), may also activate the Ca²⁺-dependent second messenger systems in neural circuits involved in opiate dependence in the newborn rat. Like NMDA receptors, AMPA receptors also undergo major developmental changes in subunit expression leading to the acquisition of the adult pattern between P7 and P21 (Jakowec et al., 1995b; Jakowec et al., 1995a). But unlike the immature NMDA receptor, which seems to have low Ca²⁺ permeability, dependent on the region in the CNS, the

immature AMPA receptor can have high Ca^{2+} permeability (Pellegrini-Giampietro et al., 1992; Ozawa and Iino, 1993; de Erausquin et al., 1994; Leinders-Zufall et al., 1994; Cebers and Liljequist, 1995; Goldstein et al., 1995; Garaschuk et al., 1996; Ozawa et al., 1998; Brorson et al., 1999; Grossman et al., 1999; Yin et al., 1999) and activation of AMPA receptors can produce a marked increase in cytoplasmic free Ca^{2+} (Ozawa and Iino, 1993; de Erausquin et al., 1994; Cebers and Liljequist, 1995; Ozawa et al., 1998). For example, AMPA receptors in neonatal spinal cord permit significant Ca^{2+} permeability due to the higher (GluR1 + GluR3 + GluR4)/GluR2 subunit ratio than in the adult (Jakowec et al., 1995b; Jakowec et al., 1995a). The role of Ca^{2+} entry through Ca^{2+} -permeable AMPA receptor has been hypothesized to activate downstream Ca^{2+} dependent intracellular events (Jakowec et al., 1995b; Jakowec et al., 1995a; Ozawa et al., 1998) such as NO production (Kest et al., 1997) and phosphorylation of cAMP response element-binding protein (Perkinton et al., 1999). There is both in vitro (Marin et al., 1993) and in vivo (Fedele and Raiteri, 1999) evidence that the AMPA receptor is linked to the production of NO. It has been recently suggested that AMPA receptor antagonism may block morphine tolerance in the adult mouse through mechanisms similar to those suggested for NMDA receptor antagonists (i.e., blockade of the Ca^{2+} /NO/cGMP cascade) (Kest et al., 1997). Thus, it is possible that AMPA receptor is involved in the development of morphine tolerance and dependence in the newborn rat.

There are other appealing reasons to consider the role of the mGluRs and the AMPA receptor in opiate tolerance, dependence and withdrawal in infants. The effectiveness of NMDA receptor antagonists in inhibiting opiate dependence varies not only ontogenetically but also phylogenetically (Herman et al., 1995). As Herman et al. (Herman et al., 1995) first pointed out, depending on species, the effect of MK-801 on opiate withdrawal is different. In guinea pigs, MK-801 consistently inhibits

various morphine withdrawal symptoms (Tanganelli et al., 1991; Brent and Chahl, 1993). In adult rats, the results are less consistent (Rasmussen, 1991; Bhargava et al., 1995). Most controversial are the effects of MK-801 in inhibiting morphine withdrawal symptoms in mice. There have been reports of both significant effects (Tanganelli et al., 1991) and observations that failed to obtain significant effects (Thorat et al., 1994). It has been suggested that the different development status of NMDA receptors parallels these observed differences of MK-801 in inhibiting opiate withdrawal in different species (Zhu and Barr, 2000). In contrast, the antagonism of both the mGluRs and the AMPA receptor has been consistently reported to inhibit opiate tolerance and dependence in both the rat and the mouse (Fundytus and Coderre, 1994, 1997; Fundytus et al., 1997; Kest et al., 1997; Klodzinska et al., 1999; Vandergriff and Rasmussen, 1999; Popik et al., 2000a). The implication is that the mGluRs and/or AMPA receptor may play the dominate role in the newborn rat, in which the NMDA receptor is not functional, and recede to a co-operative position as NMDA receptor takes effect. The ontogenetic and phylogenetic inconsistency seen for the effectiveness of NMDA receptor antagonists may reflect the different stages of the transition of roles between the mGluRs, AMPA receptor and NMDA receptor.

Based on these observations, we hypothesize that:

- 1.) Activation of Ca^{2+} -dependent second messenger systems, including the production of NO, is critical for opiate tolerance, dependence and withdrawal. Direct blockade of these pathways can inhibit these processes.
- 2.) In the newborn rat, the participation of the NMDA receptor is not indispensable to either the establishment of opiate tolerance and dependence or the expression of opiate withdrawal. When the immature NMDA receptor has been blocked by noncompetitive antagonist such as MK-801 or

competitive antagonist such as AP-5, opiate tolerance and dependence can still be established and opiate withdrawal expressed via other mechanisms.

- 3.) In the infant rat, the participation of various mGluRs may be important for opiate tolerance, dependence and withdrawal. Chronic antagonism of mGluRs should attenuate the development of opiate tolerance and dependence.
- 4.) In the infant rat, the participation of the AMPA receptor may be important for opiate tolerance, dependence and withdrawal. Chronic antagonism of AMPA receptor should attenuate the development of opiate tolerance and dependence. In addition, AMPA receptor may play a vital role for the expression of withdrawal in the infant rat, and this role of the AMPA receptor may be conserved into the adulthood.
- 5.) Parallel mechanisms for opiate tolerance, dependence and withdrawal may co-exist and other receptor types (e.g. GABA_A and Kainate receptor) may also be involved in these processes in the infant rat or even the fetal rat.
- 6.) The participation of the AMPA, metabotropic and NMDA receptors in opiate tolerance, dependence and withdrawal and their relative importance in these processes are developmentally regulated and are functions of time. There exist transitional periods for the various receptors to take dominant roles.
- 7.) These transition periods should correlate well with the maturational status of the receptors and reflect the functional (especially the ability to increase intracellular calcium levels) differences, between the immature receptor type and the mature receptor type, which is largely determined by the expression of various subunits of the native receptor complexes.

The detailed presentation of the comprehensive evidence leading to these hypotheses were summarized in a published journal paper (Zhu and Barr, 2001c).

Part III

Testing

Chapter 7

Experimental setup and conventions

Rationale of study

Clinically, there is a large population of opiate addicted childbearing-aged women in the United States and each year unknown numbers of infants are born to these women (Hutchings, 1987). These human infants, born to mothers who are exposed to opiate drugs such as heroin during their pregnancy, have a higher incidence of morbidity and mortality than do the offspring of nonaddicted women (Naeye et al., 1973; Connaughton et al., 1977; Wilson and Tonegawa, 1997) (see Chapter 1 for detail). Recent studies show that human infants develop opiate dependence and undergo withdrawal if they are exposed in utero to illicit drugs such as heroin, or to prescribed opiates such as methadone, or ex utero when treated with opiates for pain (for review, see Barr and Jones, 1994). In addition, since opiate withdrawal occurs during the period when mother-infant interaction patterns are first established, dysfunctional effects also can be produced that affect not only attachment but also cognitive development as well (Hutchings and Dow-Edwards, 1991). Particularly related is that physicians are now more likely to use opiates to manage pain in neonates and infants than in the past (Purcell-Jones et al., 1987; Yaster, 1987; Purcell-Jones et al., 1988; Sukhani, 1989; McLaughlin et al., 1993), and concerns has developed over the consequences with the increased use of opiates in this population (Thornton and Smith, 1997; Thornton et al., 1997). The costs of care for addicted infants contributed significantly to the cost of drug abuse in the United States, which was estimated to be \$100 billion in 1991 (Herman et al., 1995). Thus, no matter from an ethical, medical, or economical point of view, it is to the advantage of society

to search for new mediations for the treatment of opiate addiction for the infant. In addition, considering the importance of the opiates in the treatment of pain, information that can lead to strategies to reduce adverse effects (e.g. tolerance, dependence and withdrawal) of opiates associated with their use will obviously be valuable (Mayer and Mao, 1999).

Although there is strong preclinical evidence that the NMDA receptor plays a pivotal role in opiate tolerance, dependence and withdrawal and various NMDA receptor based mediation have been promoted to be clinically beneficial in treating these side effect of opiates (Inturrisi, 1997; Mao, 1999), especially, human subject clinical trials are being undertaken in the United States (Rosen et al., 1996; Bisaga et al., 2001) and other countries (Koyuncuoglu and Saydam, 1990; Koyuncuoglu, 1995), virtually all of these data are from the adult. Our data derived from experiments in the infant, a careful examination of available literature and some theoretical reasoning all suggest that quite different mechanisms of opiate tolerance, dependence and withdrawal may exist in the infant than those in the adult animal. If that is the case, very different pharmacological approaches are needed to treat the aversive effects of opiates in the infant.

Theoretically, despite decades of studies, the understanding of the mechanisms of opiate tolerance, dependence and withdrawal is still very limited. Although the majority of evidence implies a vital role of the NMDA receptor type, recently, it has also been suggested by several groups that the other glutamate receptor types, including AMPA receptor and mGluRs may also be involved in these processes. Thus, it is possible that multiple mechanisms are involved and the contribution of these parallel mechanisms differs depending on the species and developmental stages of the subject. The neonatal animal provides a good model system to study the

dynamic roles of various glutamate receptor types, as these receptors undergo differential development during first few weeks of the postnatal life.

Furthermore, the glutamate receptors mediate the vast majority of the excitatory transmission in the mammalian CNS and overly, inappropriate intervention of them results in pathological consequences (Michaelis, 1998; Ozawa et al., 1998; Dingledine et al., 1999; Yamakura and Shimoji, 1999). Thus, more targeted intervention specific to the functional subunit sites is therapeutically more desirable (Michaelis, 1998). The functional expression of the different subunits during the development of the organism provides us invaluable insights to link the functional role the receptor with the specific subunit responsible for that function. Therefore, useful information for the development of more specific pharmacological tools to intervene with the receptor complex can be drawn from such studies.

Thus, elucidation the role of various glutamate receptor types on morphine tolerance, dependence and withdrawal in the infant rat may lead to the unearthing of phenomenon overlooked by preceding studies done in the adult animal, the discovery of drugs that tailored specifically to the infant for alleviating various opiate adverse effects in this special population, the unfolding of precious information for the development of therapeutically more desirable prevention and interventions for these diseases, and above all, the further understanding of these events, which have been intriguing human since her record history.

Subjects

All animal procedures were in accordance with the “Principles of laboratory animal care” (NIH publication, 1996). The subjects were the offspring of Long-Evans hooded rats bred in our laboratory. Parent animals were housed in plastic tubs with

wood chips in a colony room maintained at 22–24°C on a 12-h light/12-h dark photocycle with light onset at 7 a.m. The breeding colony existed in a separate room with minimal disturbances except for normal cleaning, feeding and record keeping. Parent animals had Purina Lab Chow (Purina 5012) and water available ad libitum. Cages were checked twice daily, at approximately 10 a.m. and 6 p.m. Pups found at either time were termed 0 days of age.

Behavioral studies

For behavioral studies, three ages of rats are used: 1 to 7 day old, 8 to 14 day old, and 15 to 21 day old, corresponding to postnatal life of 1, 2, and 3 weeks. These three weeks are the time frames when all glutamate receptors undergo the most dramatic changes and after which become stabilized.

The present behavioral studies follow one of two basic paradigms: chronic study and acute study. In the chronic studies, animals are treated chronically with a pre-treatment drug (e.g. a NMDA receptor antagonist) followed 15 minutes later by morphine. This treatment paradigm continues for 6.5 days. In the afternoon of the 7th day, these chronically treated animals were challenged by naltrexone to precipitate withdrawal or injected with cumulative doses of morphine to assess tolerance. In the acute studies, animals are treated chronically only with morphine for 6.5 days. In the afternoon of the 7th day, subjects were treated with various doses of test drugs followed 15 minutes later by the test drug. Thus, in chronic test paradigm, the goal of the study is to study the role of glutamate receptors in the establishment, or the development, of morphine tolerance and withdrawal; in contrast, in the acute test paradigm, the goal of the study is then to study the role of glutamate receptors in the expression of morphine withdrawal.

Prominently, one common characteristic of the present studies is that most of them take an ontogenetic approach and for that there are several reasons. One strategy used to understand the neural bases of any behavior is to study its developmental history; and a complete understanding of behavior requires an understanding of its ontogeny (Tinbergen, 1963). The study of opiate tolerance, dependence and withdrawal is no exception. The CNS of the developing animal differs from that of the adult (Barr, 1992; Fitzgerald, 1995; Marsh et al., 1997). The use of maturing animals can provide information that cannot be easily obtained in adult animals (Barr, 1992), thus the immature organism provides a model system to analyze the relationships between neural systems and behavior. An ontogenetic approach helps to understand not only behaviors and processes unique to young animals but also how these behaviors evolve into the mature form, thus facilitating the understanding the adult behaviors too. Determining the changes in physiological and behavioral processes during ontogeny can provide clues about the neural organization of those functions. The appearance of neural circuits may correspond to the onset of specific behavioral functions, or neurobehavioral processes that are intertwined in adults may have separate developmental histories. This point is particularly pertinent to our study. The available literature on the role of various glutamate receptor types (NMDA, mGluRs and AMPA) in the adult seems to support parallel mechanisms. Unfortunately, it is difficult to tease the roles that various receptor types play apart in the adult animal model. That the various glutamate receptor types and their subunits undergo dramatic differential changes in the first few postnatal weeks provides us a window to investigate the dynamic roles these subunits play. Thus, only by taking a developmental perspective, can we obtain information on the mechanism of opiate action unique to the infant rat and on the understanding the mechanism as a whole.

Tolerance

The analgesia tolerance testing procedure will follow that of published protocols (Barr and Wang, 1992; Popik et al., 2000a). Morphine cumulative dose-response curves are used to reduce the number of animals required to assess the development of tolerance. After adaptation and baseline trials, each rat is i.p. injected with a low dose of morphine (0.1 mg/kg). Thirty minutes later, the rat is re-tested and injected with the next dose of morphine, and so on. For the specific test procedure, briefly, pups are removed from an incubator maintained a thermoneutral body temperature (33-35 °C), and gently held above a resistor. The temperature of the resistor is controlled by a precise electronic circuit to be 49 °C. The distal 2/3 of the forepaw, hindpaw and tail of the subject are sequentially put on the resistor. The latency for removal of the limb or tail from the resistor is recorded using a timer operated by a foot pedal. The timer is activated when the limb or tail hits the resistor surface and is terminated when the animal initiates withdrawal of the limb or tail from the resistor. Testing begins 20 minutes following the last administration of drugs. The withdrawal latency of the forepaw, hindpaw and tail of each animal are measured. The order of presentation of stimuli to each limb is randomly determined for each animal. A 20 second cutoff latency is used after which the stimulus will be discontinued if there is no response. In no case is there tissue damage. Pups are sacrificed by overdose of sodium pentobarbital after testing.

Withdrawal

For morphine withdrawal, behavior assessment protocol will follow that of published reports (Jones and Barr, 1995; Zhu and Barr, 2000, 2001b). Behavior of the pup will be observed for 10 minutes before the treatment of naltrexone (pre-naltrexone period) and 15 minutes after naltrexone injection (post-naltrexone period).

Data collected from the pre-naltrexone period are used to analyze the effects of chronic and/or acute effect of the test drug without the influence of naltrexone. Data collected from the post-naltrexone are used to analyze the effects of chronic and/or acute effect of the test drug on naltrexone precipitated withdrawal behaviors. Any potential order effect are minimized since the doses are randomly assigned and the observer is blind to doses and drugs. The following behaviors constitute the withdrawal repertoire of the pre-weaning rat.

Table 7-1. Morphine withdrawal behavior definitions.

Behavior	Definition
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 with no movement
Rolling	Turning the body over at least one full rotation
Together	Bodily contact with one or more littermates
Walking	Taking more than one step forward
Wall Climbing	Placing at least two forepaws on the wall of the observation chamber

Electrophysiological studies

The "isolated mammalian spinal cord"

The study of opiate tolerance and dependence in a specific region of the central nervous system can be accomplished by chronic opiate treatment of an animal followed by isolation of a portion of the central nervous system (Bell and Beglan, 1995b). One such region that has been utilized for in vitro electrophysiological study

is the locus coeruleus (Andrade et al., 1983). The isolated spinal cord preparation from the neonatal rat has also been similarly utilized as an in vitro model of spinal opiate tolerance (Bell and Beglan, 1995b), dependence and withdrawal (Bell and Beglan, 1995a; Feng and Kendig, 1995b).

Although the thought that “the brain is a spinal cord with knobs on” (Kerkut and Bagust, 1995) is too radical, it is true that much of the phenomena about opiate tolerance, dependence and withdrawal can be studied in the isolated spinal cord. The spinal cord is a very important locus for opiate actions (Yaksh and Rudy, 1976; Yaksh et al., 1977; Rohde et al., 1996; Marsh et al., 1997; Rohde et al., 1997b; Yaksh, 1997; Le Guen et al., 1999). Opiate tolerance and dependence can be established and withdrawal expressed in the spinal rat (Delander and Takemori, 1983; Rohde et al., 1996; Rohde et al., 1997a; Rohde et al., 1997b) and the spinal dog (Martin et al., 1976; Martin et al., 1980). The spinal cord has been believed to be intrinsically capable of generating opiate tolerance, dependence and withdrawal (Christie et al., 1997). Some authors even suggest that opiate withdrawal activation in brain regions such as LC is driven by the afferent stimulation from the dorsal horn neurons that are responsible for the initiation of withdrawal (Christie et al., 1997; Rohde et al., 1997b). Furthermore, neural circuitry in the spinal cord is simpler relative to the brain. Recently, it has been suggested that in the spinal cord, morphine tolerance and dependence involves a relatively limited number of neurons and occur in a spatially restricted region such as the lamina I of the superficial dorsal horn (Rohde et al., 1996; Rohde et al., 1997b). This makes the analysis of the factors that induce and maintain changes more amenable to study and particularly attractive considering that, in the brain, it is more or less controversial which loci is the major players of opiate action (Christie et al., 1997). In addition to the advantages resulted from simplicity of neural circuitry and relative better understanding of the anatomical locus of opiate

action, the isolated spinal cord preparation also eliminates associated withdrawal from physical withdrawal (Trujillo and Akil, 1991a), thus further simplifying the analysis of these complicated phenomenon. Lastly, the newborn rat spinal cord proves to be an excellent model that approximates the human infant spinal processing, comparable in developmental stage with the very preterm (24-25 weeks) human infant, maturing within the first postnatal weeks to compare with human infants and children (Fitzgerald et al., 1988; Fitzgerald, 1995; Marsh et al., 1997). Thus, the isolated spinal cord provides an excellent paradigm that can be used as a simple model system to study opiate actions. Its usefulness has long been recognized and still been appreciated: traditionally, spinally transected dogs were used as a model to classify agents acting at mu and kappa opioid receptors (Martin et al., 1974); recently, the spinal rat has been adapted as a model to study the role of NMDA receptor in morphine tolerance and dependence (Gutstein and Trujillo, 1993; Dunbar and Yaksh, 1996; Mao et al., 1998), to name only a few.

The spinal cord can be explored as a model system not only *in vivo* but also *in vitro*. The “isolated mammalian spinal cord” (Otsuka and Konishi, 1974) has been studied extensively in the past two decades (Otsuka and Yanagisawa, 1980, 1988; Thompson et al., 1990; Kendig et al., 1991; Thompson et al., 1992; Thompson et al., 1993; Thompson et al., 1994; for review, see Kerkut and Bagust, 1995; Feng and Kendig, 1996a, b; Feng et al., 1996; Feng and Kendig, 1997; Herrero et al., 2000). Recently, this preparation has been validated as an *in vitro* model for the study of opiate tolerance, dependence and withdrawal by two groups (Bell and Jaffe, 1986; Bell and Beglan, 1995a, b; Feng and Kendig, 1995a, b, 1996a, b; Feng et al., 1996; Feng and Kendig, 1997). Compared with the *in vivo* study, the *in vitro* model system provides a few additional advantages. Firstly, in the *in vitro* model, there is no protection of the Blood Brain Barrier (BBB), thus, chemicals can reach the CNS

readily. This is particularly appealing to our study, since most of the antagonists and agonists for mGluRs and AMPA receptors and some for NMDA receptors (e.g., AP-5) cannot pass the BBB easily. Secondly, drugs can be exchanged and washed out freely (especially important if anesthesia is used) in the bath. Thirdly, the dose or concentration of drugs and timing of drug application can be controlled precisely without complication resulting from drug absorption and metabolism as in most in vivo models. Lastly, the in vitro isolated spinal cord model is quite flexible, preparations have been made from the spinal cord of mice, rats and hamsters. In general, young animals (3-10 days old) have been used (Kerkut and Bagust, 1995), but good viable preparations can be obtained from older sexually mature rats (Long et al., 1988). In addition, a variety modified models such as the tail-pinch model (Yanagisawa et al., 1984) has been developed over the years to make this paradigm even more applicable to a wider range of studies such as opiate actions and pain processing mechanisms (for review, see Kerkut and Bagust, 1995; Herrero et al., 2000). Thus, the isolated mammalian spinal cord provides us an extremely simple yet powerful and flexible tool for the study of opiate actions. Not only will this model provide an independent verification for the data from the behavioral studies, but also it will shed light on the mechanism of opiate action that in vivo studies cannot provide. For example, due to the different behavioral repertoires in different species and different ages of the same species, across species and ages comparisons of opiate withdrawal is difficult, if not impossible in behavioral studies. Since the measurement of opiate tolerance, dependence and withdrawal is standardized (amplitude, area etc.) in electrophysiological studies, comparison across species and ages is practical.

Setup

The standard experimental setup has been described in several places (Otsuka and Konishi, 1974; Otsuka and Yanagisawa, 1980; Yanagisawa et al., 1982; Bell and Adler, 1988; Otsuka and Yanagisawa, 1988; Kendig et al., 1991; Bell and Beglan, 1995a; for review, see Kerkut and Bagust, 1995; Feng and Kendig, 1997; Herrero et al., 2000) and is followed in the present studies. Spinal cords are obtained from 7-day-old neonatal rats. These rats were treated similarly to those in the behavioral experiments from day 1 to day 7, except on the 7th day, they were tested using the electrophysiological assay instead the behavioral trials. The halothane anaesthetized or unanaesthetized animal is decapitated. The vertebral column and spinal cord is cut free from the animal and placed in ice-cold ACSF. The spinal cord is exposed by removing laminae from the ventral surface, using a pair of fine scissors working from rostral to caudal. After cutting the spinal nerves peripheral to the dorsal root ganglia, the cord is floated free from the vertebrae. Any adhering dura is carefully dissected away and the ventral and dorsal roots in the lumbar region are separated. In all experiments, the whole spinal cord is used. The ACSF consists of (in mM) NaCl 123, KCl 5, NaHCO₃ 26, NaH₂PO₄ 1.2, CaCl₂ 2, MgSO₄ 1.3, glucose 30, and is constantly bubbled with 95% O₂ and 5% CO₂ (Kendig et al., 1991; Wong et al., 1998). The spinal cord is exposed, and the pia and dura are carefully removed from the dorsal surface. The isolated spinal cord is placed in a 2 ml bath and superfused with ACSF at a rate of 2 ml/min. Inflow into the bath is by gravity feed from a reservoir filled with ACSF constantly bubbled with 95% O₂ and 5% CO₂. Perfusion fluid (ACSF) from the reservoir is feed into the chamber at a low level at one end and is sucked out by a negative pressure of a reservoir at the other end. The bath is maintained at room temperature.

Extracellular dorsal root stimulation and ventral root (L4 or L5) recording are made with glass capillary suction electrodes. These electrodes are pulled to fine tips with a microelectrode puller; the tips are broken back to 100-300 μm and are fire polished. Standard stimulating and recording techniques are used (Kendig et al., 1991). For stimulation, square-wave stimuli are supplied to the dorsal roots through a bipolar suction electrode made of fine platinum wires. For recording, the tip of the recording electrode fits tightly around the root, and the root is sucked into the suction electrode until the tip pressed lightly against the spinal cord to form a good seal. The suction electrode is connected through an AgCl pellet to a preamplifier. The slow ventral root potentials (sVRPs) (Kendig et al., 1991) are digitized through an InstruTech ITC-16 ADC interface card (InstruTech Inc., Long Island, NY). Signals are recorded 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 (Department of molecular and cellular pharmacology, University of Miami).

Tolerance

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

Withdrawal

Besides tolerance, the acutely isolated neonatal rat spinal cord is capable of generating withdrawal symptoms. In electrophysiological studies of opiate withdrawal, spinal cords from neonates treated with chronic saline, morphine or morphine co-administered with a interested drug (e.g. a NMDA receptor antagonist) are used. After baseline sVRP are recorded, naltrexone (10 μ M) is added into the superfusate. Spontaneous or dorsal root evoked ventral root activity is recorded from the ventral root for 15 min subsequent to the introduction of naltrexone into the bath. The sVRP activity is digitized and the integral of the activity above baseline is measured. The integral of spontaneous activity over time obtained is defined as the measure of physical dependence. In the studies of the acute pharmacological effects of the interested drug on morphine withdrawal, the drug can be dissolved in the ACSF and acutely co-administrated with naltrexone. The same methods of assessing physical dependence are used (Bell and Adler, 1988; Bell and Beglan, 1995a; Feng and Kendig, 1995b; Feng and Kendig, 1997).

Data analysis

Behavior data statistics

Most of the behavioral data meet the assumptions of ANOVA. Separate one-way (drug dose effect) or two-way (drug dose effect and time effect) repeated ANOVAs were conducted for the occurrences of each behavior (reaction time for tolerance). Separate analyses were conducted for different ages. These were then followed by post-hoc comparisons using the Fisher's PLSD to assess significance of drug dose effect on the occurrence of withdrawal behaviors and the reaction time (tolerance). For withdrawal data, the observed occurrences of withdrawal behaviors

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

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

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

Electrophysiology data statistics

For the tolerance experiments, baseline ventral root potential measurements were established before morphine is added to the spinal superfusate. Morphine-induced depression of the sVRP was expressed as a percentage depression of the baseline sVRP. A one-way ANOVA was used to assess the drug effect among different treatment groups.

For withdrawal activities, baseline sVRP measurements are established before naltrexone is added to the spinal superfusate. The naltrexone-induced sVRP is expressed as a percentage of the baseline sVRP. One-way ANOVAs were used to test significance of drug effects among different treatment groups.

For all statistical analyses, effect was considered significant if $P < .05$.

Averaging technique

Single recordings of the sVRP show quite small variability from time to time due to random factors such as the temperature and the flow rate of the of the superfusion fluid (Kerkut and Bagust, 1995). Although this variability is rather small and is not a problem for most experiments, several groups used the averaging technique to further reduce the sample-to-sample variability of sVRP recording (Woodley and Kendig, 1991; Sivilotti et al., 1995). This method of reducing variability along the temporal dimension by averaging several samples recorded from adjacent time points is relevant and valid only when the super perfusion fluid remains homogenous across time and the variability is random by nature. This technique should not be used when the superfusate is inhomogeneous (e.g., from the moment a drug added in to the time the drug takes full effect) and when time-related variability is not random and even may be the very effect the experimenter seeks to investigate (e.g., the time effect of morphine withdrawal). Thus, only when the situation calls for a further reduction of this variability, we will use the averaging technique. Depending on our needs and the experimental conditions, we can average an arbitrary number of original sVRP recordings into a single average trace. Figure 7-1 illustrates how this technique reduces the variability of the individual sVRP recordings by averaging 30 min of continuous recording (a total of 36 samples) into a single trace.

Averaging Technique

A single sVRP recording shows some variability. Averaging sVRP recordings over a period of 30 min into a single trace greatly reduced the variability. (a) 30 min recording of the sVRP in the spinal cord of a 7-day-old rat, order: left to right then top to bottom; (b) a single sVRP recording trace presented as full size; (c) the averaged sVRP trace of 30 min of recording (36 original traces). The stimulus is a constant electric square wave with a duration of 200ms, amplitude of 30v, stimulation frequency of 0.02 Hz.

(a) 30 min recording

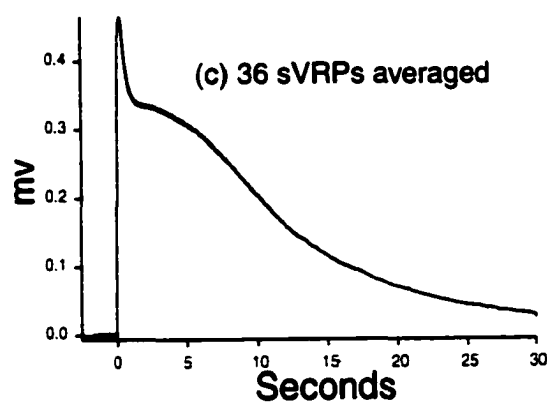
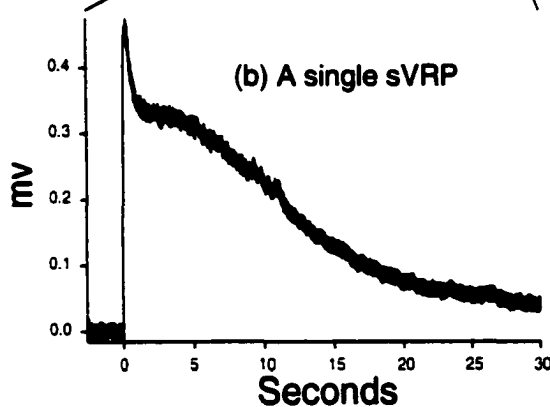
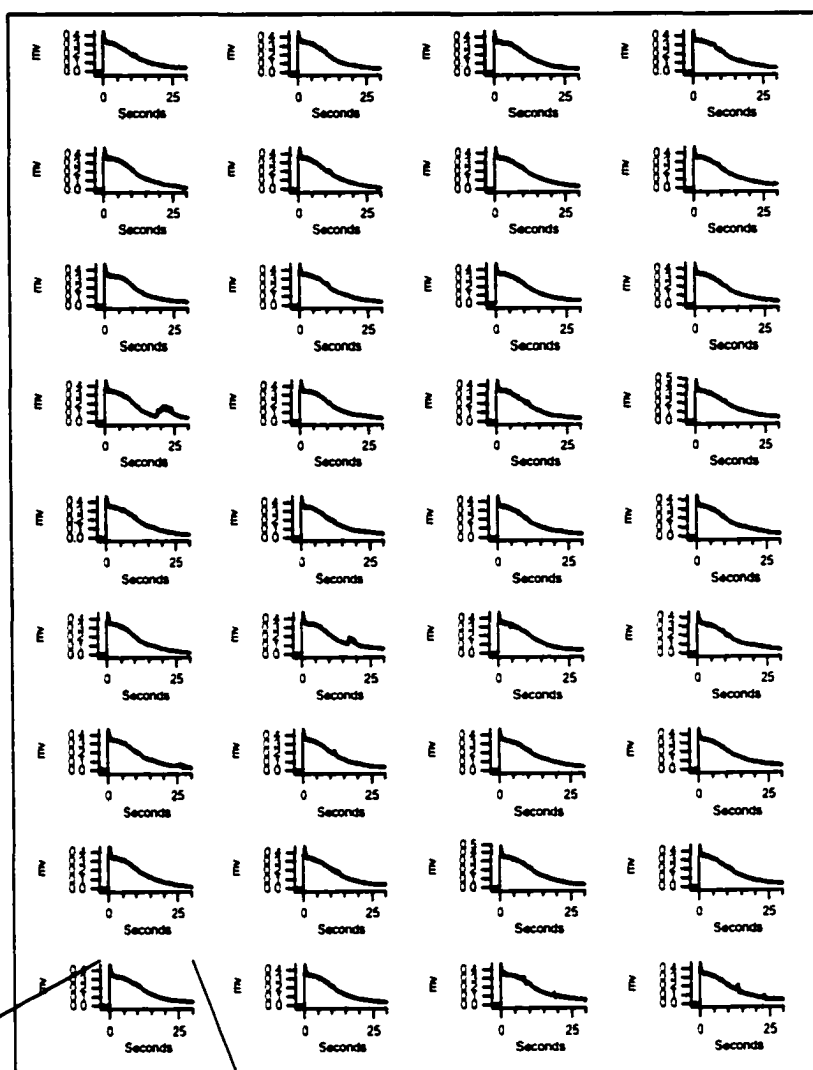


Figure 7-1. Averaging multiple sVRP recordings to reduce random variability.

Conventions

If not stated otherwise, morphine was injected i.p. with a dose of 10 mg/kg; naltrexone was injected i.p. with a dose of 1.0 mg/kg; morphine sulfate were obtained from the National Institute on Drug Abuse (Rockville, MD); all other chemicals were purchased from Tocris Cookson Inc. (Ballwin, MO); chemicals injected i.c.v. had a volume of 4 μ l per injection; rats were Long-Evans hooded rat pups bred in our laboratory, all animal care procedure followed published protocols (Zhu and Barr, 2000, 2001b); all chronic treatment lasted for 6.5 days (b.i.d.); the term *tolerance* refers to analgesic tolerance.

Chapter 8

Ontogeny of NMDA receptor mediated morphine withdrawal

Behavioral studies

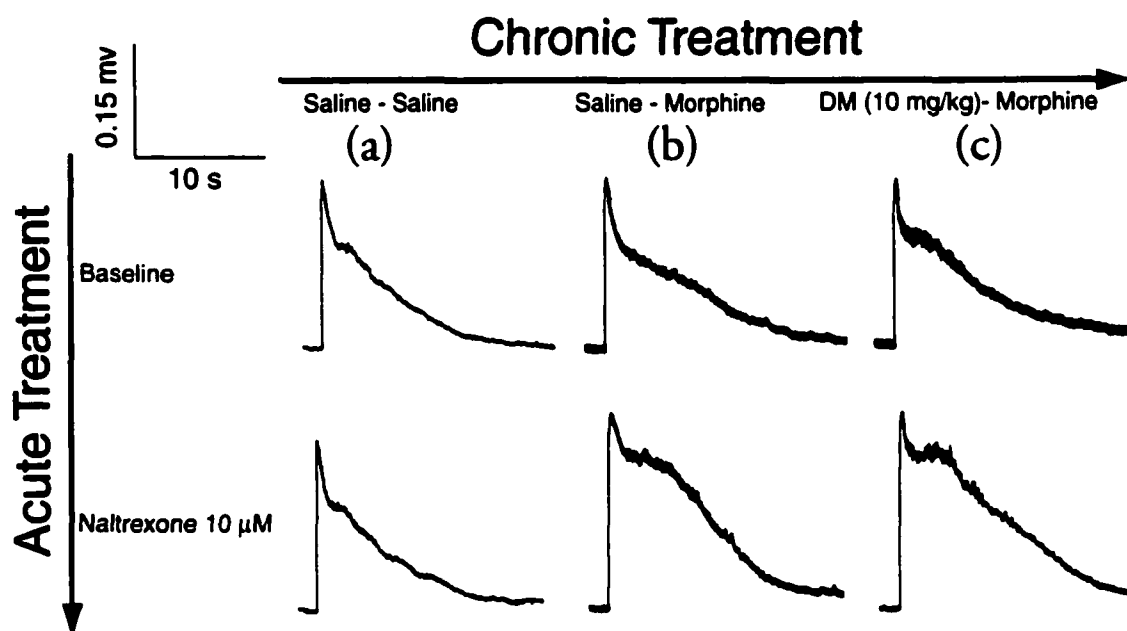
The behavioral data have been published in one of our recent papers (Zhu and Barr, 2001b). Here the author briefly outlines the major findings in this part of the study.

We tested the effect of chronic pre-treatment of MK-801 on the establishment of morphine dependence in the 7, 14 and 21-day-old rat. Chronic co-administration of MK-801 with morphine failed to suppress the acquisition of morphine dependence in the 7-day-old rat whereas it did significantly attenuate most withdrawal behaviors in the 14 and 21-day-old rat. Thus, there exists a clear transition period for MK-801 to take effect in inhibiting the development of morphine dependence 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 coincided well with the maturation of the NMDA receptor. Therefore, that MK-801 has different pharmacological properties in suppressing morphine withdrawal at different ages may reflect the different developmental stages of the NMDA receptor. Our finding that MK-801 was ineffective in the 7-day-old rat whereas effective in the 14 and 21-day-old rats may reflect the functional differences between the immature NMDA receptor type and the mature NMDA receptor type (Sircar, 2000).

Electrophysiological studies

The effects of chronic co-administration of dextromethorphan with morphine on the development of morphine dependence were assessed in the isolated spinal cord of the 7-day-old rat. Neonatal rats were treated chronically (6.5 days, b.i.d) with injections of either saline + saline, saline + morphine (10 mg/kg, i.p.), or dextromethorphan (10 mg/kg, i.p.) + morphine (10 mg/kg, i.p.). In the afternoon of the 7th day, an electrophysiological assay for assessing morphine withdrawal was conducted (for technical details, refer to Chapter 7 of the present thesis).

Naltrexone (10 μ M) did not significantly alter the area of the sVRP in the morphine naive rat. In contrast, naltrexone (10 μ M) increased the sVRP to about 200% compared with baseline sVRP (before naltrexone was added into the superfusate) in the rat that received chronic morphine treatment. Finally, chronic co-administration of dextromethorphan (10 mg/kg) did not prevent the development of morphine dependence in the 7-day-old rat, since the spinal cord of rats chronically received dextromethorphan + morphine also show about 200% augment of sVRP compared with baseline (Figure 8-1 and Figure 8-2).



Morphine withdrawal in the 7-day-old rat. Recordings in each column are from one individual rat. The electric stimulation was a constant square wave, with length 200 μ s, amplitude 30 V. Naltrexone (10 μ M) do not significantly alter the area of sVRP in the morphine naive rat (a.); in contrast, naltrexone (10 μ M) significantly increases the sVRP area in rats that received chronic morphine treatment, suggesting that morphine dependence was induced by chronic morphine treatment (b.); chronic co-administration of dextromethorphan failed to prevent the naltrexone precipitated morphine withdrawal in the 7-day-old rat (c.).

Figure 8-1. Sample recordings demonstrating that chronic co-administration of dextromethorphan did not prevent morphine withdrawal in the isolated spinal cord in the 7-day-old rat.

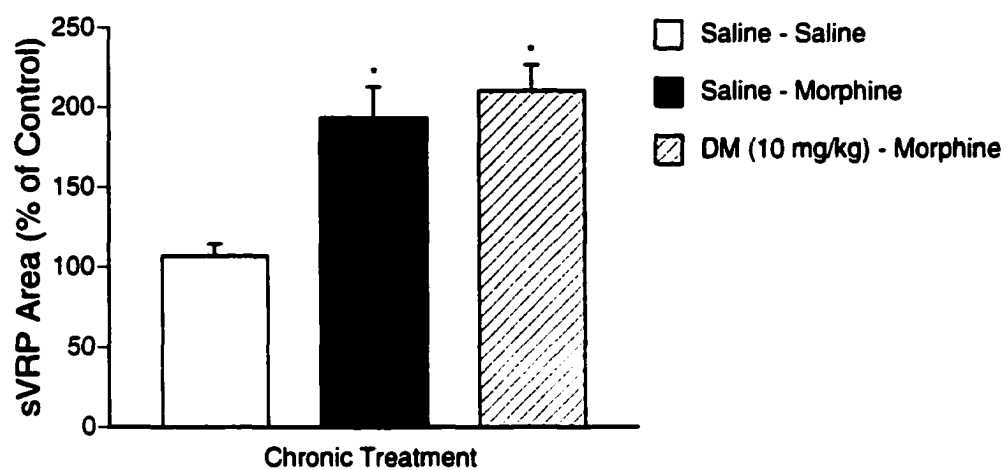


Figure 8-2: Chronic co-administration of dextromethorphan failed to prevent the development of morphine dependence in the 7-day-old rat.

Chapter 9

Ontogeny of NMDA receptor mediated morphine tolerance

Introduction

For clinical and theoretical reasons, there is increasing interest in the study of opiate tolerance in the infant rat model (Part I). Although data on NMDA receptors' critical role in opiate tolerance in the adult animal are abundant, it is unknown whether these results in adults apply to infants (Part I). That NMDA receptors undergo dramatic differential changes during earlier life (Chapter 3) suggests possible developmental differences (Part II). As a result, conclusions based on data derived from adult animals models should not be taken for granted that they would also hold in the developing organism.

Thus, the present study examined the effects of two non-competitive NMDA receptor antagonists MK-801 and dextromethorphan in morphine tolerance in the infant rat using a combination of in vivo and in vitro methods.

Material and methods

Morphine treatment and tolerance assessment

Pups were tattooed with India ink (Geller and Geller, 1966), which was injected into one or two paws to label individual pups permanently in each litter. To induce morphine tolerance, starting when the pups were 1, 8, or 15 days of age, all pups in a litter were removed from the dam and individual rats were injected with morphine sulfate (IP, 10 mg/kg) twice daily (10 a.m. and 6 p.m.) for 6.5 days. To determine NMDA receptor antagonists' effect on the development of morphine

tolerance, MK-801 (IP, 0.03, 0.1, 0.3 mg/kg, or saline as control, $n = 8$ for each treatment condition) or dextromethorphan (IP, 3, 10, 30 mg/kg, or saline as control, $n = 8$ for each treatment condition) were injected 15 min before each morphine (or saline for control) injection. The last injection was in the morning of the 7th day. In the afternoon of the 7th day, animals were transported from the animal facility to our laboratory in plastic tubs with wood chip bedding and placed in an incubator maintained at approximately 33°C for morphine's analgesic test. The tolerance assessment procedure followed a standard protocol (see Chapter 7 for detail).

In Vitro Electrophysiology

The standard electrophysiology experimental setup (see Chapter 7 for detail) was followed in the present studies. Spinal cords were obtained from 7-day-old rat pups. These rats 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.

Application of chemicals

In electrophysiological studies, morphine was made up as 1000× stock solution and were diluted in oxygenated recording solution immediately prior to use and 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.

For concentration-response curve construction of morphine's effect on the sVRP area, after 15 min of baseline sVRP recording, morphine (10 nM, 100 nM, 1 μM, and 10 μM) was applied in the superfusate for 30 min at each concentration.

Behavior data analysis

The raw behavioral data recorded by the experimenter were reaction time scores (in sec) and the data were converted to the percentage of maximum possible effect (%MPE) according to the method of Harris and Pierson (Harris and Pierson, 1964) (see Chapter 7 for detail)

The transformed %MPE data were then used to construct dose-response curves for morphine's analgesic effect. The ED₅₀ values and 95% confidence limits (95% C.L.) of morphine's analgesic effect were determined according to the method of Tallarida and Murray (Tallarida and Murray, 1987) using a Sigmoidal dose-response non-linear curve fitting module in the computer program Prism 3.0 (GraphPad Software Inc., San Diego, CA).

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:

$$\% \text{Suppression of baseline} = (\text{baseline area} - \text{treatment area}) / (\text{baseline area}) \times 100$$

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 the post-hoc Fisher's PLSD test to assess significance of drug effects on the areas under the individual concentration-response curves among different treatment groups (Bell and Beglan, 1995b).

For both behavioral and electrophysiological data statistical analyses, effects were considered significant if $P < .05$. All values are expressed as means $\pm$ one S.E.M.

Results

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

Chronic treatment of morphine or MK-801 plus morphine did not significantly alter the baseline reaction time of the rat in withdrawing its appendages (forepaw, hindpaw, or tail) from the noxious thermal stimulus (Table 9-1, Figure 9-1A-C upper panels). 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 (Figure 9-1A-C lower panels) and a significantly increased ED_{50} of acute morphine in the chronic morphine groups compared with the morphine naive groups (Table 9-2). Importantly, maximal response was still obtained in the tolerant rat. Thus, this shift of morphine's dose-response curve to the right represents a potency rather than efficacy change to morphine's analgesic effect (Nies, 2001; Ross and Kenakin, 2001).

Chronic co-administration of lower and medium dose of MK-801 (0.03 mg/kg 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 (Figure 9-1A-C lower panels). Indeed, ED_{50} values of acute morphine of the groups received chronic co-administration of lower and medium dose of MK-801 with morphine were not significantly different from the ED_{50} of the group that received only chronic morphine treatment and both were significantly higher than that of the morphine naive group (Table 9-2).

Chronic co-administration of the high dose of MK-801 (0.30 mg/kg) with morphine also failed to prevent the development of morphine tolerance in the 7-day-old rat. On the contrary, for the high dose MK-801 treated group, the dose-response curves of acute morphine as assessed by both the forepaw and hindpaw shifted further to the right (Figure 9-1A-B lower panels) and ED_{50} values significantly increased compared with those of the chronic morphine only groups (Table 9-2), indicating that greater intensity of morphine tolerance was developed in this group.

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

Chronic treatment of morphine or MK-801 plus morphine did not significantly alter the baseline reaction time of the rat in withdrawing its appendages (forepaw, hindpaw, or tail) from the noxious thermal stimulus (Table 9-1, Figure 9-2A-C upper panels). 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 (Figure 9-2A-C lower panels) and a significantly increased ED_{50} of acute morphine compared with the morphine naive group (Table 9-2).

Chronic co-administration of MK-801 with morphine 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 mg/kg or 0.30 mg/kg) co-administration of MK-801 with morphine returned toward

that of the morphine naive group (Figure 9-2A-C lower panels) and decreased ED_{50} values of acute morphine compared with the groups that received only chronic morphine treatment (Table 9-2).

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

Chronic treatment of morphine or MK-801 plus morphine did not significantly alter the baseline reaction time of the rat in withdrawing its appendages (forepaw or hindpaw) from the noxious thermal stimulus (Table 9-1, Figure 9-3A-C upper panels). 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 (Figure 9-3A-B lower panels) and a significantly increased ED_{50} of acute morphine compared with the morphine naive groups (Table 9-2).

Chronic co-administration of MK-801 with morphine attenuated the development of tolerance, as reflected by that the morphine's dose-response curves for the groups that received chronic co-administration of MK-801 plus morphine returned toward those of the morphine naive groups (Figure 9-3A-B lower panels) and decreased ED_{50} values of acute morphine compared with the groups that received only chronic morphine treatment (Table 9-2).

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

Chronic treatment of morphine or dextromethorphan plus morphine did not significantly alter the baseline reaction time of the rat in withdrawing its appendages (forepaw, hindpaw, or tail) from the noxious thermal stimulus (Table 9-3, Figure 9-4A-C upper panels). 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 (Figure 9-4A-C lower panels) and a significantly increased ED_{50} of acute morphine in the chronic morphine groups compared with the morphine naive groups (Table 9-4).

Chronic co-administration of a low or medium dose of dextromethorphan (3 mg/kg 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 co-administration of dextromethorphan with morphine 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 (Figure 9-4A-C lower panels). 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 9-4).

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 (when the pups were one-day-old). Thus, further tests of this dose of dextromethorphan on morphine tolerance were terminated for this age group.

Similar to the behavioral data, chronic co-administration of dextromethorphan (10 mg/kg) with morphine also failed to prevent the development of tolerance, as assessed by a standard electrophysiological assays (Bell and Beglan, 1995b). The sample recording traces (Figure 9-5) clearly show that acute morphine (10 nM – 10 μ M) concentration-dependently suppressed the area of sVRP in spinal cord from morphine naive rats (Figure 9-5a). Acute morphine's suppression effect on sVRP area

was diminished in spinal cords from rats chronically treated with morphine (Figure 9-5b), suggesting that tolerance developed in rats that received chronic morphine treatment. Chronic co-administration of dextromethorphan with morphine failed to prevent this diminution of acute morphine's effect in suppressing sVRP (Figure 9-5c), indicating that pre-treatment of dextromethorphan failed to prevent the development of morphine tolerance in the 7-day-old rat.

Further statistical analysis of the concentration-response curves of acute morphine's effect in suppressing the pain-related sVRP area also confirmed this observation. Significant difference was detected among the three concentration-response curves (Figure 9-6) corresponding to the three different chronic treatment regimens (saline-saline, saline-morphine, or dextromethorphan-morphine), $F_{(2, 15)} = 26.343$, $P < .001$. Post-hoc multiple comparison 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 that of 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.

Dextromethorphan attenuated morphine tolerance in the 21-day-old rat

Chronic treatment of morphine or dextromethorphan plus morphine did not significantly alter the baseline reaction time of the rat in withdrawing its appendages (forepaw or hindpaw) from the noxious thermal stimulus (Table 9-3, Figure 9-7A-B upper panels). 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 (Figure 9-7A-B lower

panels) and a significantly increased ED_{50} of acute morphine (Table 9-4) compared with the morphine naive groups.

Chronic co-administration of dextromethorphan with morphine attenuated the development of tolerance, as reflected by that the morphine's dose-response curves for the groups that received chronic co-administration of dextromethorphan with morphine returned toward those of the morphine naive groups (Figure 9-7A-B lower panels) and decreased ED_{50} of acute morphine (Table 9-4) compared with the groups that received only chronic morphine treatment.

Discussion

Despite decades of research, the mechanism underlying opiate tolerance remains elusive. Current thoughts suggest that opiate tolerance arise, at least in part, from a "functional decoupling" of opioid receptors from their associated signal transduction systems (Nestler and Aghajanian, 1997; Mao, 1999; Trujillo, 1999), particularly the Ca^{2+} homeostasis (Mao, 1999; Smith et al., 1999). Finding a suitable explanation for this functional decoupling, however, has been difficult (Trujillo, 1999). Mao, Price, and Mayer have developed a model that provides a potential solution to this problem (Mao et al., 1995; Mayer and Mao, 1999) by integrating recent molecular and behavioral findings. This model involves several steps. One of the critical steps is a μ -opioid receptor-mediated increase in protein kinase C (PKC) translocation and activation, leading to phosphorylation of the NMDA receptor and removal of the magnesium blockade results in influx of calcium and activation of several calcium-mediated processes. The final consequence is a functional decoupling of the μ receptor from transduction systems, thereby decreasing the cellular actions of opiates. In addition, influx of calcium causes activation of nitric oxide synthase

(NOS) and liberation of nitric oxide (NO), which induces presynaptic release of glutamate, producing positive feedback of glutamatergic input and further activation of NMDA receptors. This model provides explanations for several known phenomena in the adult animal, including the ability of NMDA receptor antagonists, NOS inhibitors, and protein kinase inhibitors to inhibit the development of tolerance and dependence, and the potential roles of glutamate, calcium, PKC, and nitric oxide in tolerance and dependence (for review, see Inturrisi, 1997; Trujillo, 1999). Moreover, this model is consistent with the roles of these molecules in other forms of neural plasticity, such as learning, memory and LTP (Ozawa et al., 1998; Dingledine et al., 1999; Dingledine and McBain, 1999).

In supporting the Mao et al.'s model, there is broad and persuasive evidence from numerous laboratories that a variety of noncompetitive NMDA receptor antagonists, competitive antagonists and glycine antagonists can inhibit mu opiate (morphine) tolerance in the rodents (for reviews, see Trujillo and Akil, 1991a; Elliott et al., 1995a; Herman et al., 1995; Pasternak et al., 1995; Trujillo, 1995; Trujillo and Akil, 1995; Inturrisi, 1997; Danysz and Parsons, 1998; Mao, 1999). The potency and clinical potential of these agents on morphine tolerance are further strengthened by the findings of several studies indicating that both noncompetitive and competitive NMDA receptor antagonists are capable of "reversing" morphine tolerance (for reviews, see Trujillo and Akil, 1995; Inturrisi, 1997).

It is not known whether these mechanisms in adults 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 (Van Praag and Frenk, 1991; Barr and Wang, 1992; Bell and Beglan, 1995b; Windh et al., 1995; Thornton and Smith, 1997; Thornton et al., 1997). On the other hand, the neonatal CNS is both structurally and functionally different from that of the adult, and significant changes in opioid actions

occur both prenatally and postnatally (Barr, 1992, 1993; Fitzgerald, 1995; Marsh et al., 1997). More importantly, the NMDA receptor, which is believed to play a crucial role in the establishment of opiate tolerance, undergoes qualitative and quantitative changes during development (Kalb et al., 1992; Hori and Kanda, 1994; Kalb and Fox, 1997). These include significant developmental alterations both in the density of the receptor (Tremblay et al., 1988; Morin et al., 1989; Represa et al., 1989), the sensitivity of the receptor complex to magnesium (Ben-Ari et al., 1988; Bowe and Nadler, 1990; Morrisett et al., 1990), and the rise and decay times of the NMDA EPSCs (Akaike and Rhee, 1997; Bardoni 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 (Ozawa et al., 1998; Dingledine et al., 1999). The accumulating evidence indicates that the various subunits (NR1, NR2A-D, NR3A-B) composing the native NMDA receptor channel 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 (Hrabetova et al., 2000; Sircar, 2000). That NMDA receptors undergo dramatic differential changes during earlier life suggests possible ontogenetic differences. Indeed, 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, 2001c). As a result, conclusions based on data derived from adult animals models should not be taken for granted that they would also hold in the developing organism.

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, 1995b). Neonatal rats were treated chronically (3 or 4 days) with either

morphine, MK-801 + morphine, or saline. Morphine in a concentration-dependent manner depressed the sVRP produced by supramaximal electrical stimulation of an ipsilateral dorsal root. Chronic treatment of neonates with morphine alone produced tolerance to morphine's depression effect on the sVRP. Chronic co-treatment of morphine with MK-801 failed to prevent the development of morphine tolerance in the neonatal rat (Bell and Beglan, 1995b). 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.

Thus, 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 effective in rats with age of 14-day or older. 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 (Marek et al., 1991b; Trujillo and Akil, 1991b; Ben-Eliyahu et al., 1992; Elliott et al., 1994; Mao et al., 1996). 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.

The NMDA receptor is composed of various subunits (NR1, NR2A-D, and NR3A-B) (Dingledine et al., 1999; Andersson et al., 2001; Nishi et al., 2001; Chatterton et al., 2002). All native NMDA receptor has at least one NR1 subunit but functional channels are formed only when NR1 is expressed together with one of the four NR2 subunits (Yamakura and Shimoji, 1999). The NR3A subunit may serve a regulatory function in NMDA receptors (Das et al., 1998). The various subunits of the NMDA receptor complex undergo dramatic differential maturation during the first few postnatal weeks (Guilarte and McGlothan, 1998), resulting in considerably different functional states (Colwell et al., 1998). In the neonatal brain the predominant NMDA receptor subunits are the NR1, NR2B and NR2D subunits. Expression of the NR1-NR2B subunit complex is high at birth, peaks at about day 14 and then declines after day 21. The NMDA receptor containing the NR2A subunit is almost non-existent at birth, increases gradually with age and reaches maximal level on day 21. Expression of the NR1 subunit also increases with age and peaks on day 21 (Guilarte and McGlothan, 1998; Sircar, 2000). It has recently been suggested that there are two types of NMDA receptors in the rat – an immature type and a mature type, and that there exists a temporal switching of the NMDA receptor from the immature form to the adult mature form around the second week postnatally (Hrabetova et al., 2000; Sircar, 2000). Thus, the pharmacological properties of the

immature and mature NMDA receptor correlate well with the developmental profile of the NMDA receptor subunit configuration (Ozawa et al., 1998; Hrabetova et al., 2000; Sircar, 2000). Our finding that NMDA receptor antagonists were ineffective in the 7-day-old rat but effective in the 14 or 21-day-old rats may reflect the functional differences between the immature NMDA receptor type and the mature NMDA receptor type. It is important to note that although the NMDA receptor channel is not functional physiologically in infant rat, as a non-competitive NMDA receptor antagonist, MK-801 potently binds to the immature NMDA receptor and effectively blocks the ion-permeable channel (Ozawa et al., 1998; Dingledine et al., 1999; Yamakura and Shimoji, 1999). Thus the ineffectiveness of MK-801 in inhibiting the development of morphine dependence in the 7-day-old rat is not a failure to block the immature channel.

Our results do not support, at least in the neonatal rat, the widely cited Mao, Price and Mayer model on opiate tolerance and dependence (Mao et al., 1995; Mao, 1999; Mayer et al., 1999). This model places the NMDA receptor at an indispensable position for the acquisition of opiate tolerance and dependence (Fundytus and Coderre, 1999; 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 (Van Praag and Frenk, 1991; Barr and Wang, 1992; Windh et al., 1995; Thornton and Smith, 1997; Thornton et al., 1997) 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 activate the second messenger, thus

leading to long lasting plasticity changes cascade (Mao, 1999; Trujillo, 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; Sucher et al., 1995; Das et al., 1998), 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, 2001c). One possibility is that other mechanisms activate the intracellular Ca^{2+} release and evoke the Ca^{2+} dependent second messenger system such as NO production. A second possibility is that channels besides the NMDA receptor allow significant 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 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 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 Ca^{2+} cascades, they are ideal candidates for long-lasting intracellular changes such as learning and memory formation (Pin and Duvoisin, 1995; Conn and Pin,

1997; Ozawa et al., 1998). Recently, it has been reported that the development of antinociceptive morphine tolerance could be inhibited by a metabotropic group II glutamate agonist LY354740 in adult mice (Popik et al., 2000a). 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, whose anatomical distribution overlaps with the NMDA receptor, co-localizes with the NMDA receptor in many cell types (Gu et al., 1996; Ozawa et al., 1998), including NOS-containing neurons (Fedele and Raiteri, 1999), may also activate the Ca^{2+} -dependent second messenger systems in neural circuits involved in opiate dependence in the newborn rat. Like NMDA receptors, AMPA receptors also undergo major developmental changes in subunit expression leading to the acquisition of the adult pattern between P7 and P21 (Jakowec et al., 1995b; Jakowec et al., 1995a). But unlike the immature NMDA receptor, which has low Ca^{2+} permeability, dependent on the region in the CNS, the immature AMPA receptor has high Ca^{2+} permeability (Pellegrini-Giampietro et al., 1992; Geiger et al., 1995; Ozawa et al., 1998) and activation of AMPA receptor produces a marked increase in cytoplasmic free Ca^{2+} (Ozawa et al., 1998). For example, AMPA receptors in neonatal spinal cord permit significant Ca^{2+} permeability due to the higher (GluR1 + GluR3 + GluR4)/Glu2 subunit ratio than in the adult (Jakowec et al., 1995b; Jakowec et al., 1995a). The role of Ca^{2+} entry through Ca^{2+} -permeable AMPA receptor has been hypothesized to activate downstream Ca^{2+} dependent intracellular events (Jakowec et al., 1995b; Jakowec et al., 1995a; Ozawa et al., 1998) such as NO production (Kest et al., 1997) and phosphorylation of cAMP response element-binding protein (Perkinton et al., 1999). There is both in vivo and in vitro evidence that NO synthesis can be suppressed by AMPA receptor blockade (Marin et al., 1993;

Fedele and Raiteri, 1999). Thus, it is possible that AMPA receptor is involved in the development of morphine tolerance in the younger rat. This line of reasoning 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 AMPA receptors, resulting in greater levels of tolerance by these potential mechanisms other than the NMDA mediated mechanism.

In summary, the present studies examined the effects of a prototype of non-competitive NMDA receptor antagonist MK-801 and a clinically available NMDA receptor blocker dextromethorphan on morphine tolerance in rats aging from 7- to 21-day-old using both the in vivo and in vitro assays. We conclude that NMDA receptor antagonists are not effective in attenuating morphine tolerance in the neonatal rat whereas partially effect in the 14-day-old and fully effective in rats as old or older than 21 days of age. Thus, 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.

Table 9-1. Effects of chronic morphine or chronic morphine plus MK-801 treatment on baseline latency in the infant rat.

Age		Baseline Latency Statistics		
		Forepaw	Hindpaw	Tail
7do	F	0.72	2.45	0.30
	df	(4, 35)	(4, 35)	(4, 35)
	P	0.58	0.06	0.88
14do	F	0.38	0.12	0.32
	df	(4, 35)	(4, 35)	(4, 35)
	P	0.82	0.97	0.86
21do	F	0.20	0.94	NT
	df	(4, 35)	(4, 35)	NT
	P	0.93	0.45	NT

No significant results were found for any situations

NT: Not tested

Table 9-2. Effects of chronic co-administration of MK-801 on ED₅₀ of morphine in the infant rat.

Age	Treatment	ED ₅₀ mg/kg (95% C.L.)		
		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) *	2.25 (1.98-2.53) *	2.67 (2.46-2.89) *
	MK (0.03 mg/kg)-Mor	1.79 (1.52-2.08) *	2.38 (2.15-2.60) *	2.30 (2.12-2.48) *
	MK (0.10 mg/kg)-Mor	1.78 (1.63-1.94) *	2.14 (1.93-2.35) *	2.36 (2.12-2.60) *
	MK (0.30 mg/kg)-Mor	2.69 (2.48-2.90) *#	2.91 (2.72-3.09) *#	2.74 (2.53-2.95) *
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) *	3.17 (2.93-3.41) *	2.69 (2.23-3.14) *
	MK (0.03 mg/kg)-Mor	2.95 (2.46-3.44) *	2.98 (2.68-3.28) *	2.46 (1.85-3.06) *
	MK (0.10 mg/kg)-Mor	1.40 (0.39-2.41) &	1.34 (0.68-2.00) &	0.96 (0.61-1.31) &
	MK (0.30 mg/kg)-Mor	1.31 (0.94-1.68) &	1.37 (0.78-1.96) &	0.87 (0.51-1.22) &
21do	Sal-Sal	3.02 (2.43-3.61)	2.76 (1.99-3.52)	NT
	Sal-Mor	>10 *	> 10 *	NT
	MK (0.03 mg/kg)-Mor	3.60 (2.01-5.19) &	3.45 (2.23-4.68) &	NT
	MK (0.10 mg/kg)-Mor	3.34 (2.81-3.88) &	3.26 (2.78-3.74) &	NT
	MK (0.30 mg/kg)-Mor	3.23 (2.65-3.81) &	3.14 (2.61-3.68) &	NT

*: ED₅₀ significantly greater than Sal-Sal group

#: ED₅₀ significantly greater than Sal-Mor group

&: ED₅₀ significantly smaller than Sal-Mor group

NT: Not tested

Table 9-3. Effects of chronic morphine or morphine plus dextromethorphan on baseline latency in the infant rat.

Age		Baseline Latency Statistics		
		Forepaw	Hindpaw	Tail
7do	F	0.18	0.55	2.17
	df	(3, 28)	(3, 28)	(3, 28)
	P	0.91	0.65	0.12
21do	F	0.08	0.09	NT
	df	(4, 35)	(4, 35)	NT
	P	0.99	0.98	NT

No significant results were found for any situations

NT: Not tested

Table 9-4. Effects of chronic co-administration of dextromethorphan with morphine on ED₅₀ of morphine in the infant rat (Behavioral test).

Age	Treatment	ED ₅₀ 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) *	1.91 (1.72-2.10) *	2.63 (2.52-2.75) *
	DM (3 mg/kg)-Mor	1.39 (1.14-1.65) *	2.45 (2.27-2.64) * #	2.45 (2.36-2.54) *
	DM (10 mg/kg)-Mor	1.43 (1.18-1.67) *	2.48 (2.34-2.62) * #	2.89 (2.77-3.01) * #
21do	Sal-Sal	3.24 (2.64-3.83)	2.90 (2.15-3.64)	NT
	Sal-Mor	>10 *	> 10 *	NT
	DM (3 mg/kg)-Mor	3.71 (1.74-5.68) &	3.52 (2.65-4.40) &	NT
	DM (10 mg/kg)-Mor	3.45 (2.62-4.27) &	3.16 (2.52-3.81) &	NT
	DM (30 mg/kg)-Mor	3.27 (2.64-3.89) &	2.90 (2.30-3.50) &	NT

*: ED₅₀ significantly greater than Sal-Sal group

#: ED₅₀ significantly greater than Sal-Mor group

&: ED₅₀ significantly smaller than Sal-Mor group

NT: Not tested

Figure 9-1A-C: Upper panels: the effect of chronic treatment with morphine or MK-801 plus morphine on the baseline latency of the 7-day-old rat retracting its appendages from a hotplate. Ordinate: Mean latency of retraction of the appendage (Mean $\pm$ one S.E.M, in seconds). Abscissa: chronic treatment conditions). No significant results were found between any groups. A: forepaw; B: hindpaw; C: tail.

Lower panels: 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 $\pm$ one S.E.M.). Abscissa: acute morphine doses. A: forepaw; B: hindpaw; C: tail.

Figure 9-2A-C: Upper panels: the effect of chronic treatment with morphine or MK-801 plus morphine on the baseline latency of the 14-day-old rat retracting its appendages from a hotplate. Ordinate: Mean latency of retraction of the appendage (Mean $\pm$ one S.E.M, in seconds). Abscissa: chronic treatment conditions). No significant results were found between any groups. A: forepaw; B: hindpaw; C: tail.

Lower panels: 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 $\pm$ one S.E.M.). Abscissa: acute morphine doses. A: forepaw; B: hindpaw; C: tail.

Figure 9-3A-B: Upper panels: the effect of chronic treatment with morphine or MK-801 plus morphine on the baseline latency of the 21-day-old rat retracting its appendages from a hotplate. Ordinate: Mean latency of retraction of the appendage (Mean $\pm$ one S.E.M, in seconds). Abscissa: chronic treatment conditions). No significant results were found between any groups. A: forepaw; B: hindpaw.

Lower panels: 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 $\pm$ one S.E.M.). Abscissa: acute morphine doses. A: forepaw; B: hindpaw.

Figure 9-4A-C: Upper panels: the effect of chronic treatment with morphine or dextromethorphan plus morphine on the baseline latency of the 7-day-old rat retracting its appendages from a hotplate. Ordinate: Mean latency of retraction of the appendage (Mean $\pm$ one S.E.M, in seconds). Abscissa: chronic treatment conditions). No significant results were found between any groups. A: forepaw; B: hindpaw; C: tail.

Lower panels: 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 $\pm$ one S.E.M.). Abscissa: acute morphine doses. A: forepaw; B: hindpaw; C: tail.

Figure 9-5. Sample recording of the slow ventral root potential 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 μ 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 are 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.

Figure 10-6. Concentration-response curves of acute morphine's suppression effect on the pain-related slow ventral root potential 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 text for details about statistics. Ordinate: %Suppression of baseline sVRP (Mean $\pm$ one S.E.M.). Abscissa: acute morphine concentrations in the superfusate.

Figure 9-7A-B: Upper panels: the effect of chronic treatment with morphine or dextromethorphan plus morphine on the baseline latency of the 21-day-old rat retracting its appendages from a hotplate. Ordinate: Mean latency of retraction of the appendage (Mean $\pm$ one S.E.M, in seconds). Abscissa: chronic treatment conditions). No significant results were found between any groups. A: forepaw; B: hindpaw.

Lower panels: 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 $\pm$ one S.E.M.). Abscissa: acute morphine doses. A: forepaw; B: hindpaw.

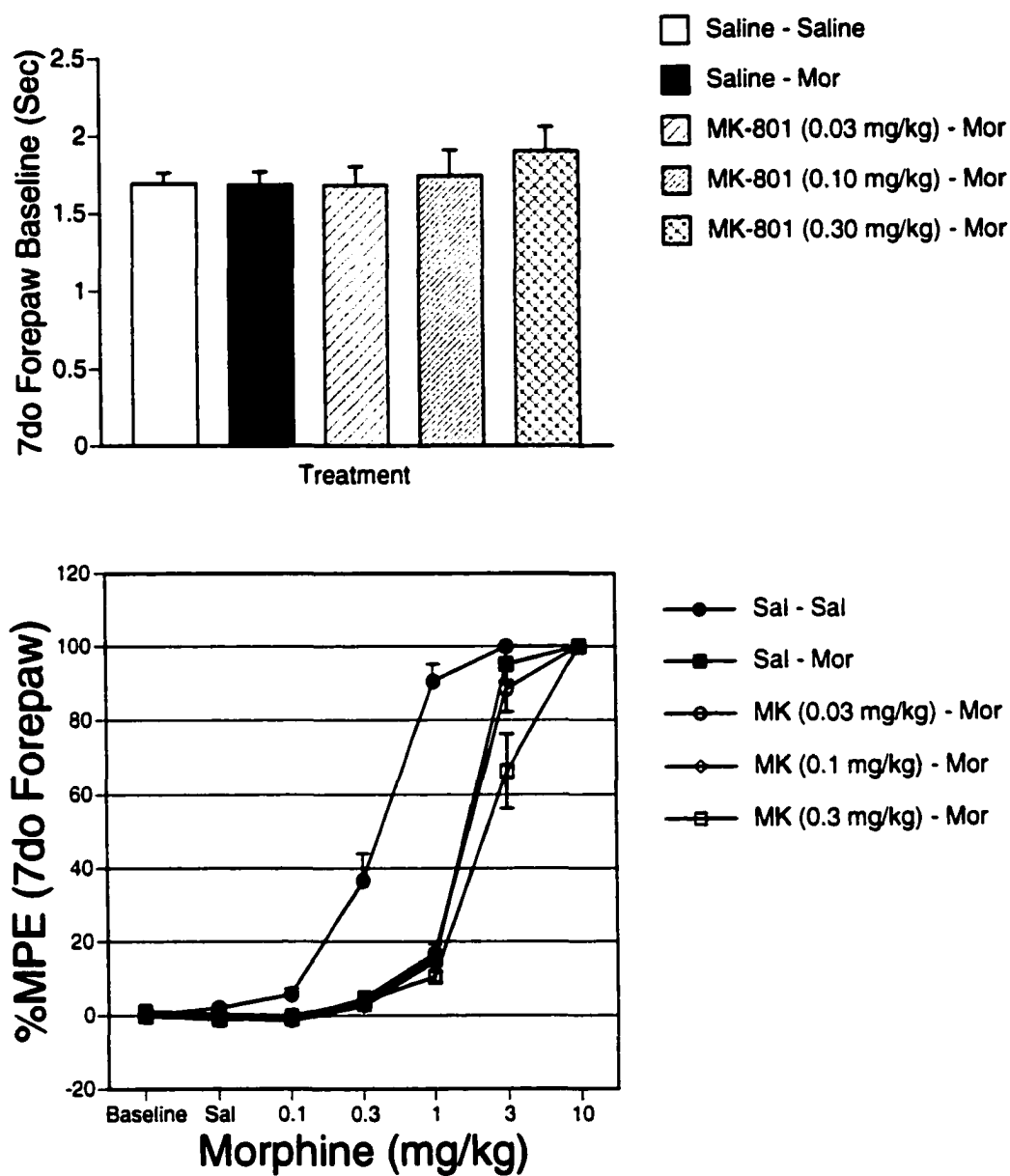


Figure 9-1A. MK-801 did not prevent the development of morphine tolerance in the 7-day-old rat, forepaw.

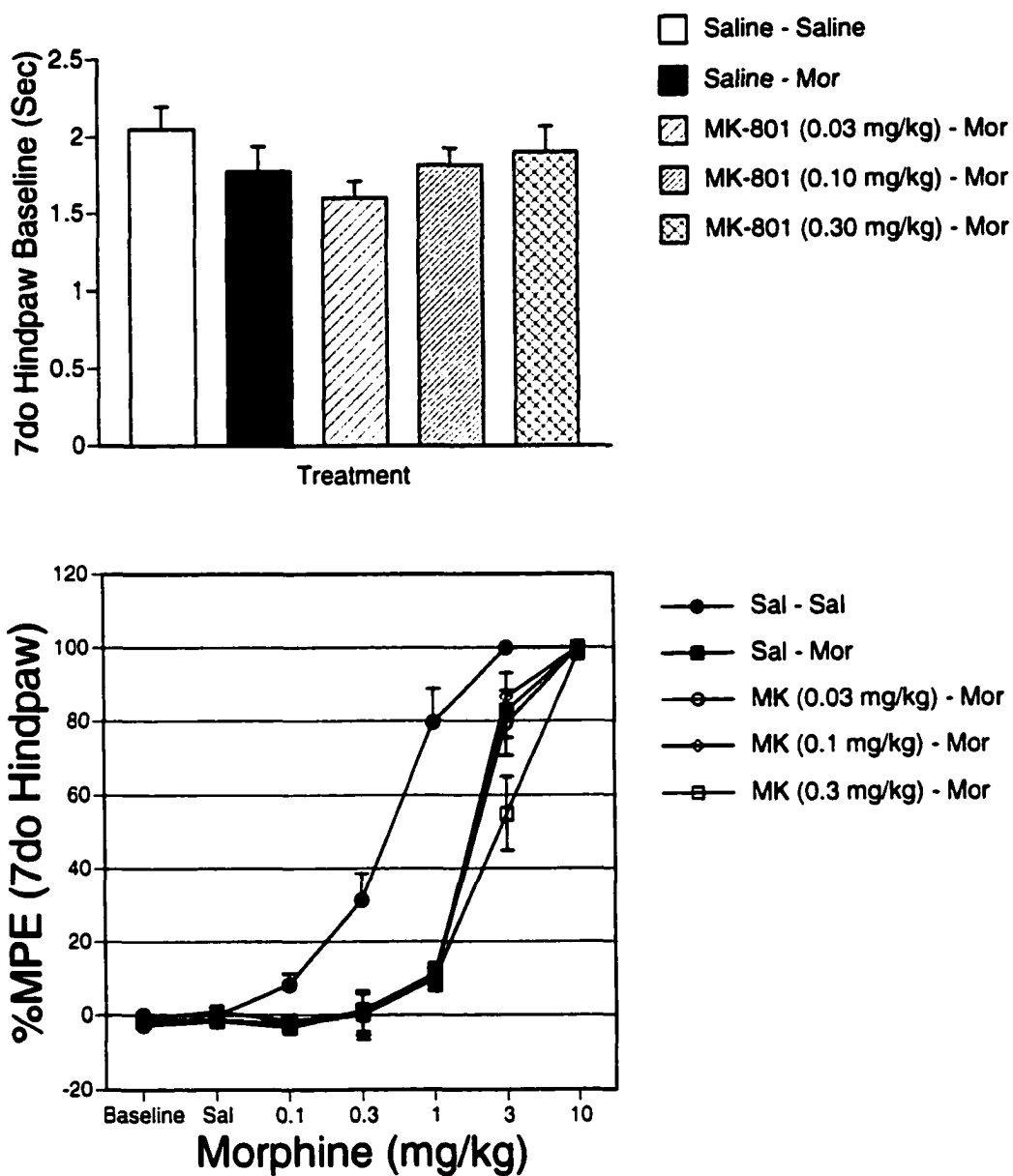


Figure 9-1B. MK-801 did not prevent the development of morphine tolerance in the 7-day-old rat, Hindpaw.

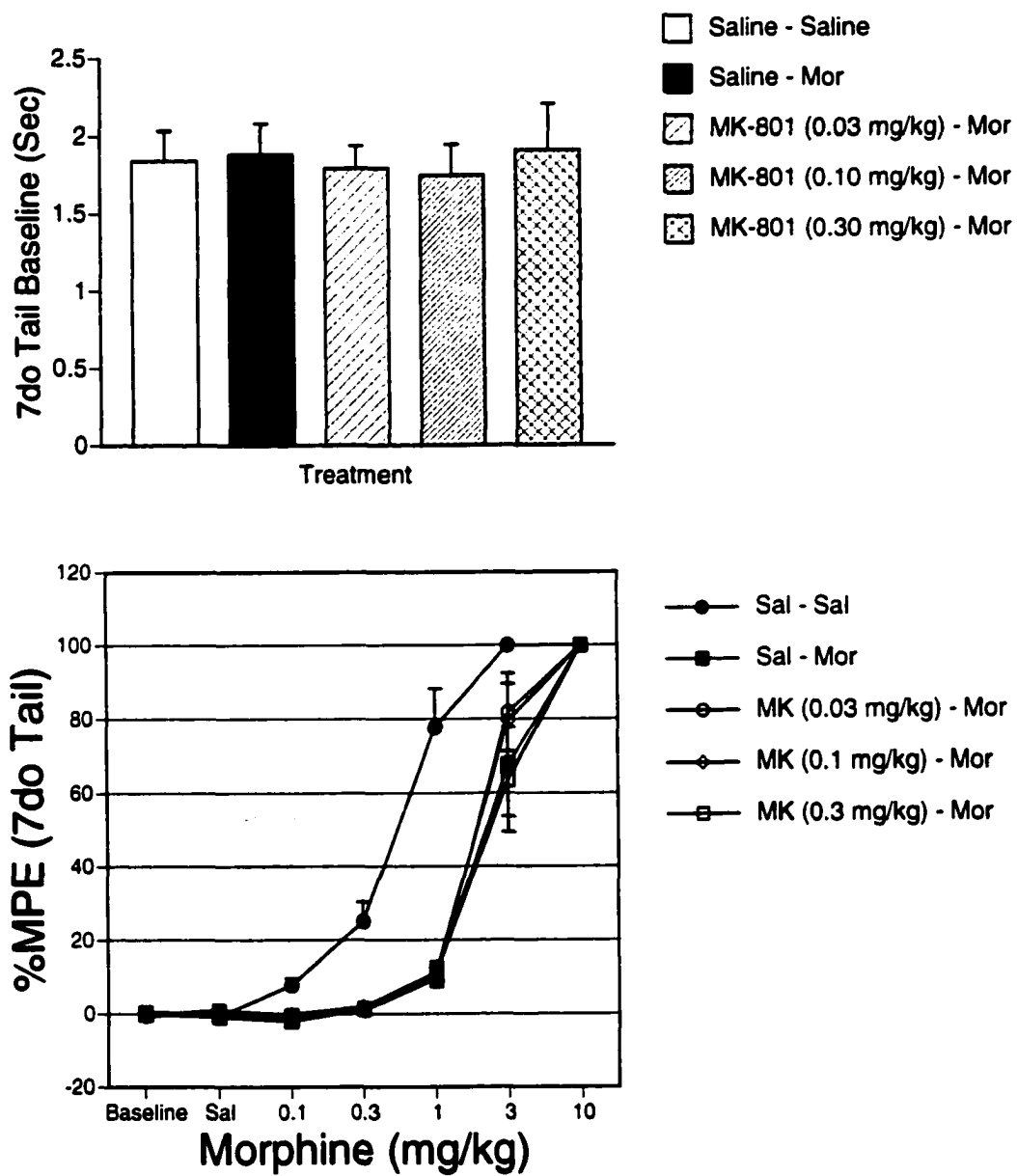


Figure 9-1C. MK-801 did not prevent the development of morphine tolerance in the 7-day-old rat, Tail.

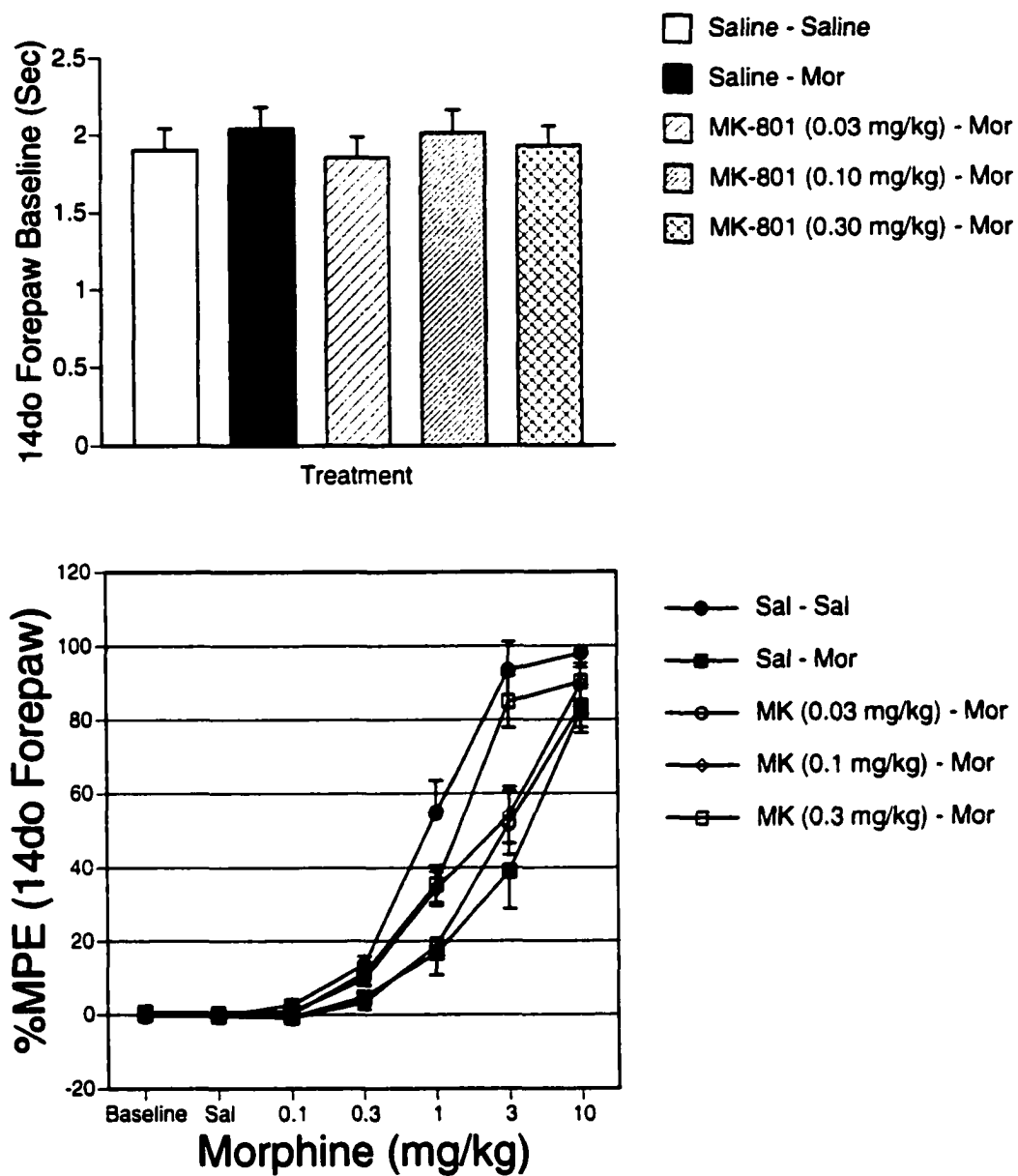


Figure 9-2A. MK-801 prevented the development of morphine tolerance in the 14-day-old rat, forepaw.

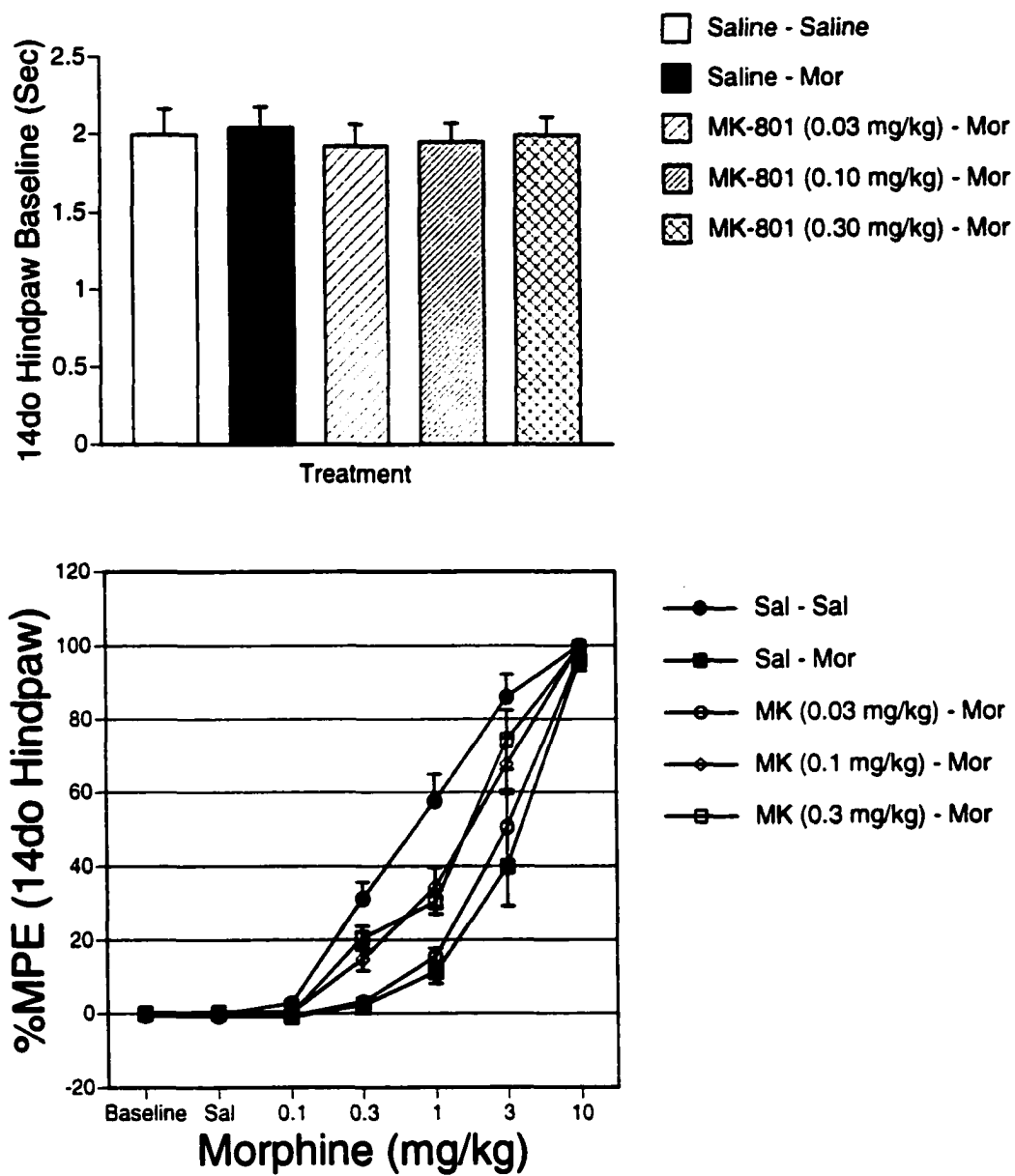


Figure 9-2B. MK-801 prevented the development of morphine tolerance in the 14-day-old rat, Hindpaw.

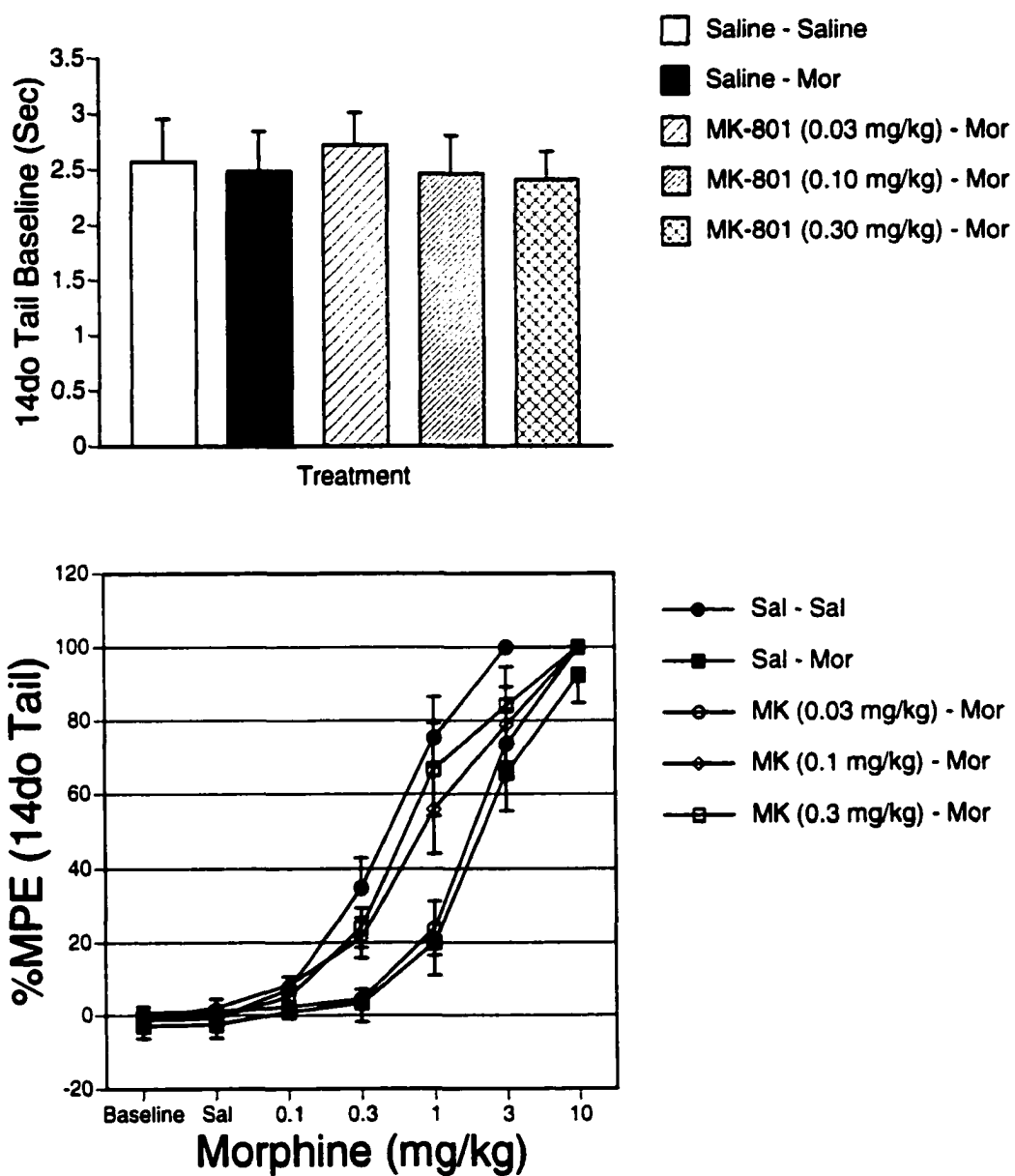


Figure 9-2C. MK-801 prevented the development of morphine tolerance in the 14-day-old rat, tail.

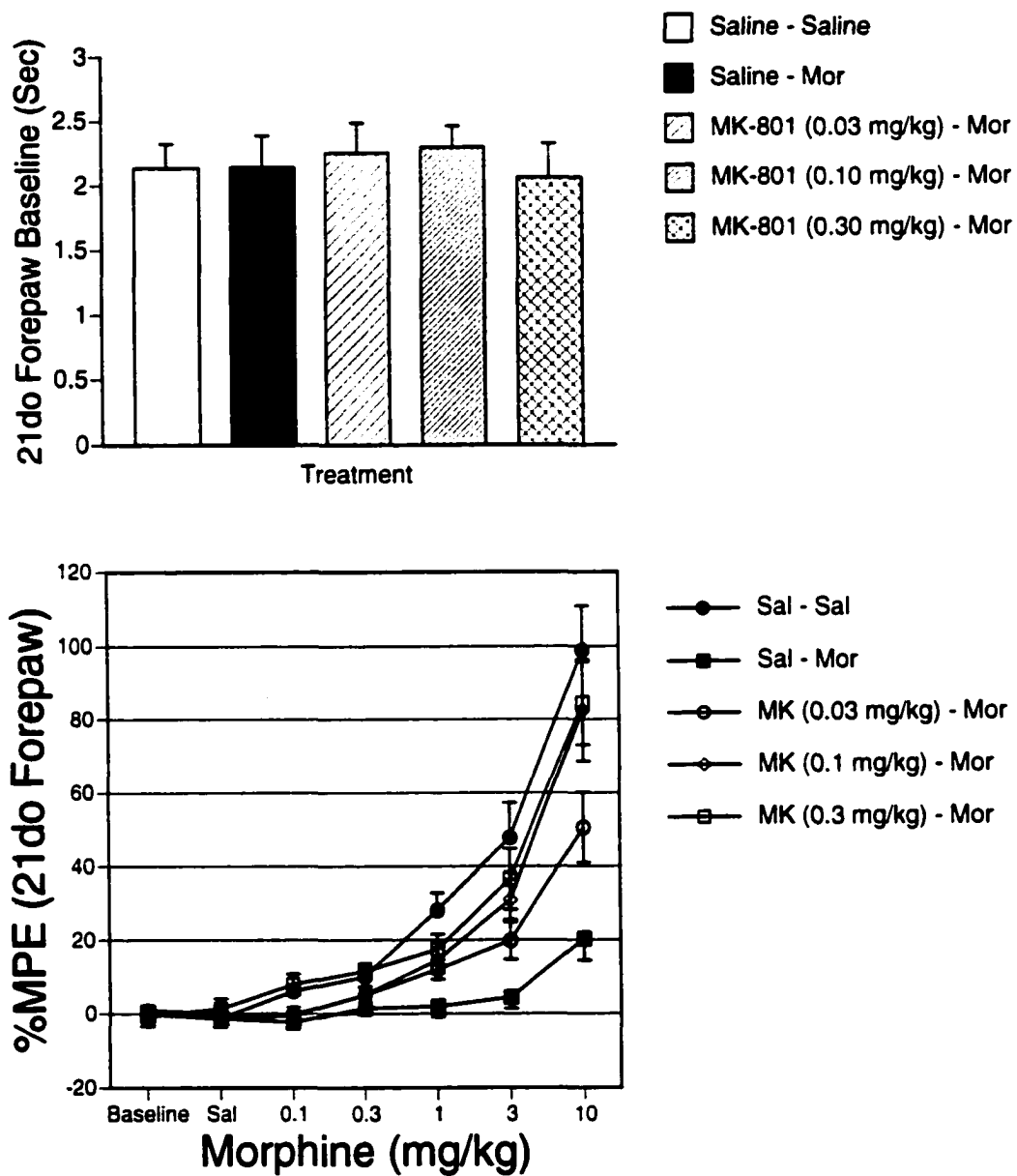


Figure 9-3A. MK-801 prevented the development of morphine tolerance in the 21-day-old rat, Forepaw.

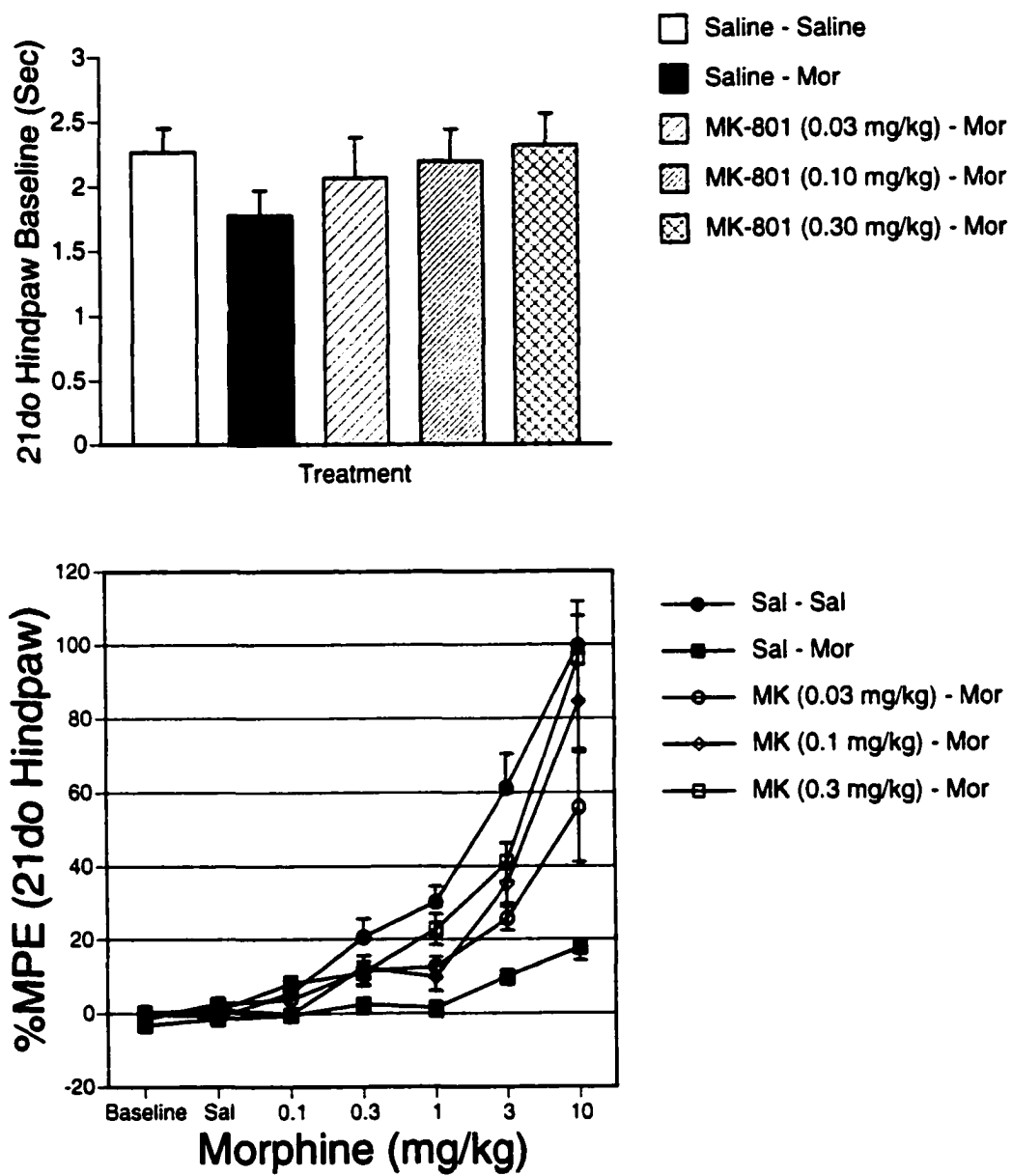


Figure 9-3B. MK-801 prevented the development of morphine tolerance in the 21-day-old rat, Hindpaw.

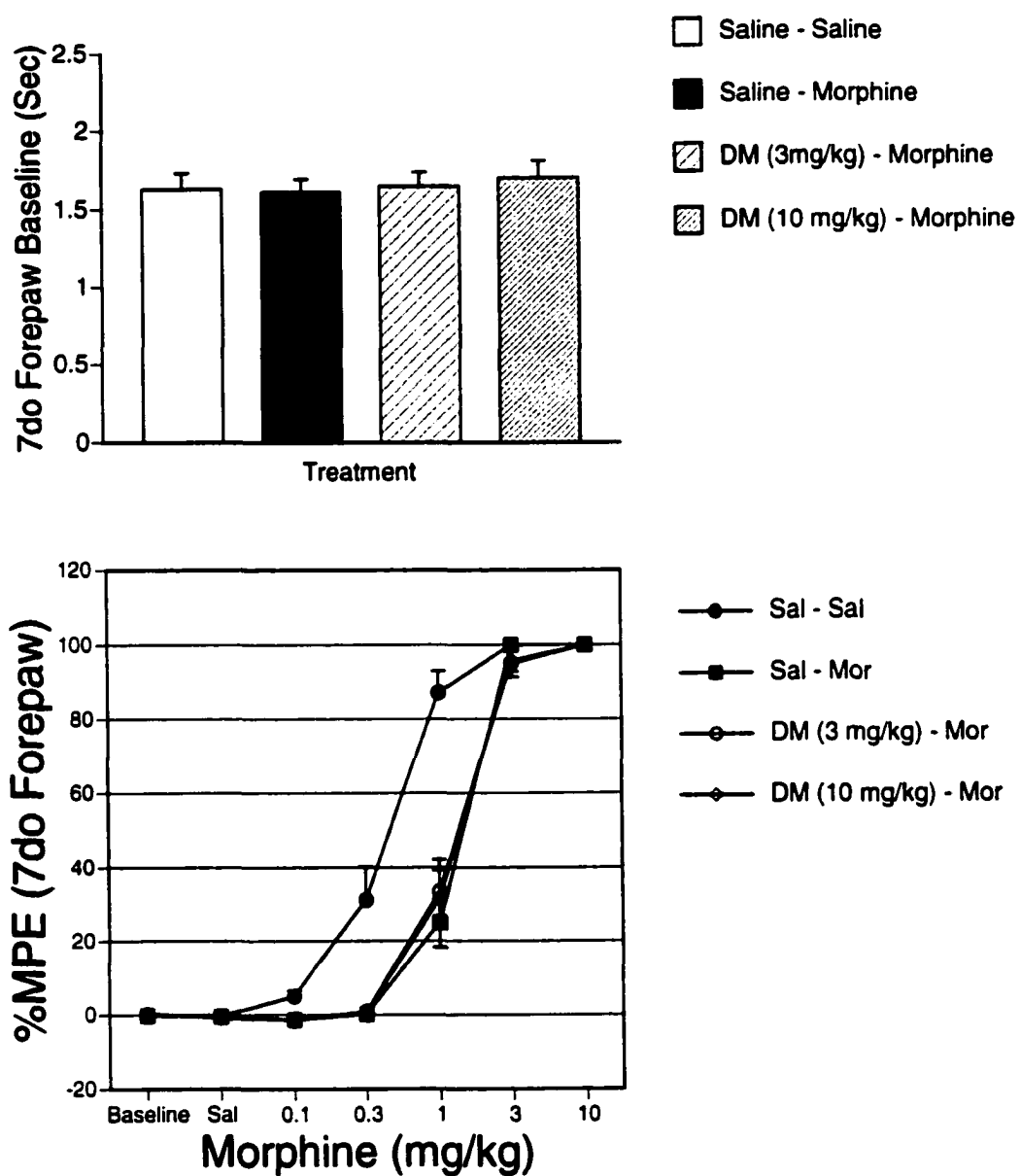


Figure 9-4A. Dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat, Forepaw.

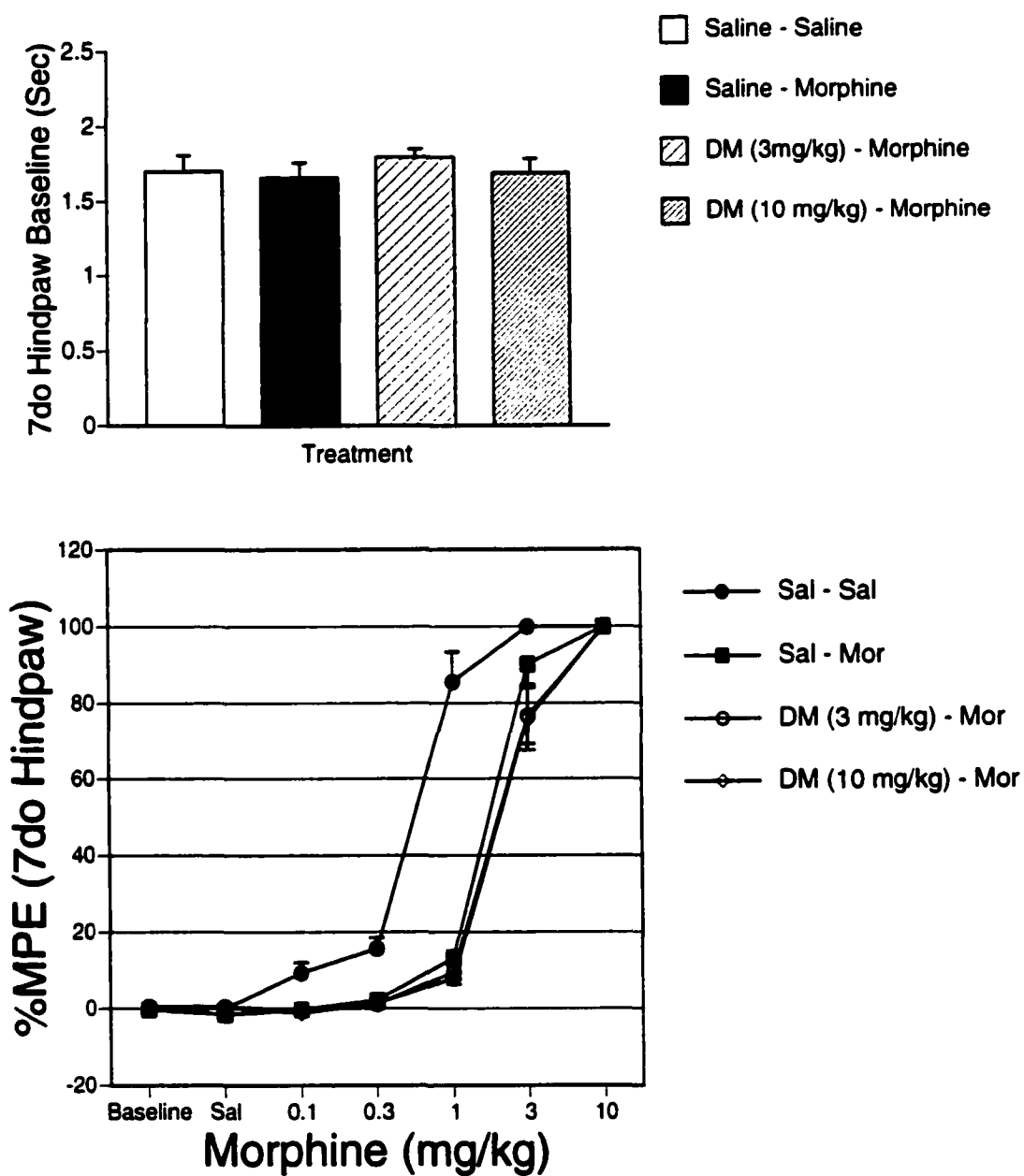


Figure 9-4B. Dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat, Hindpaw.

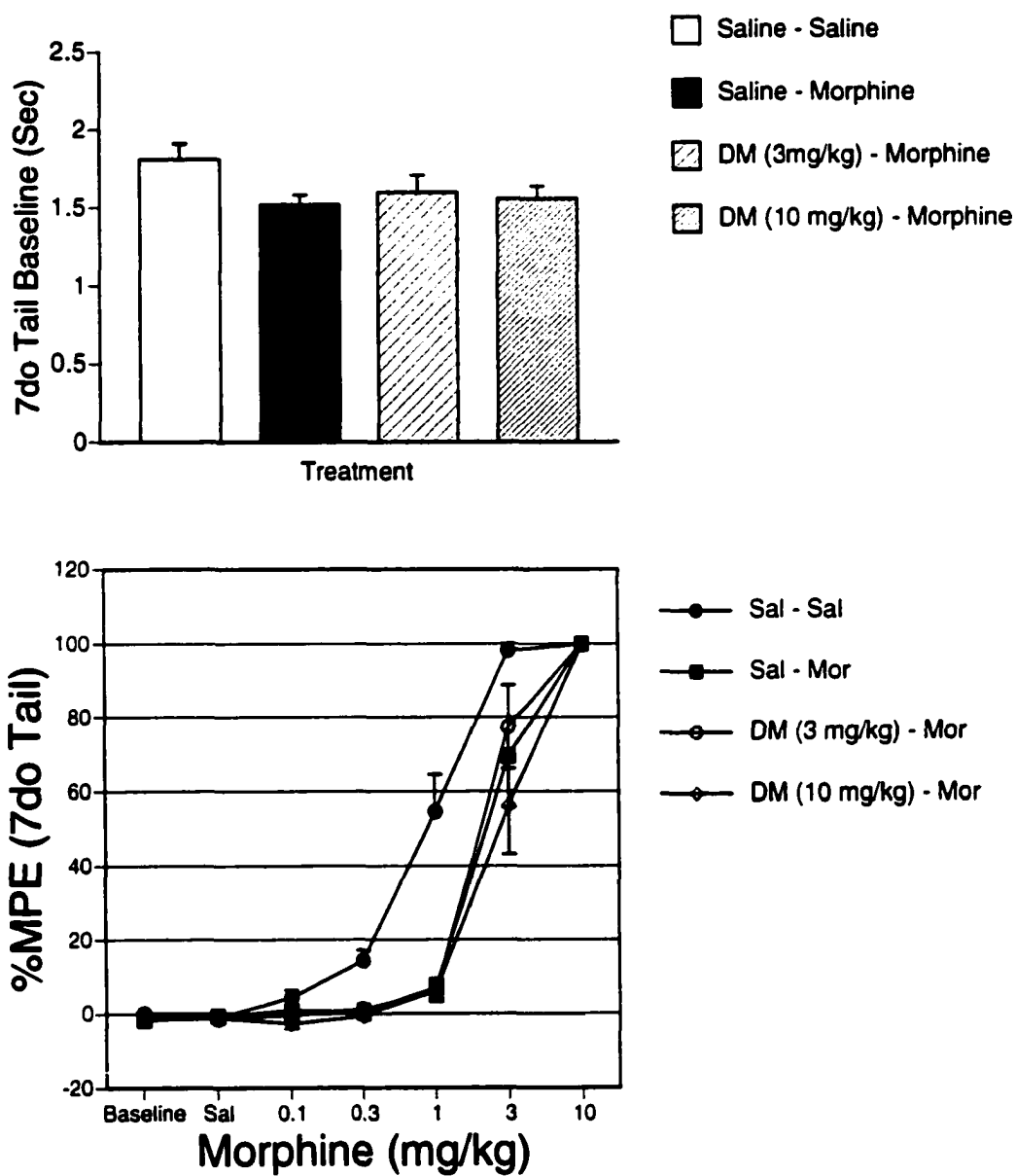
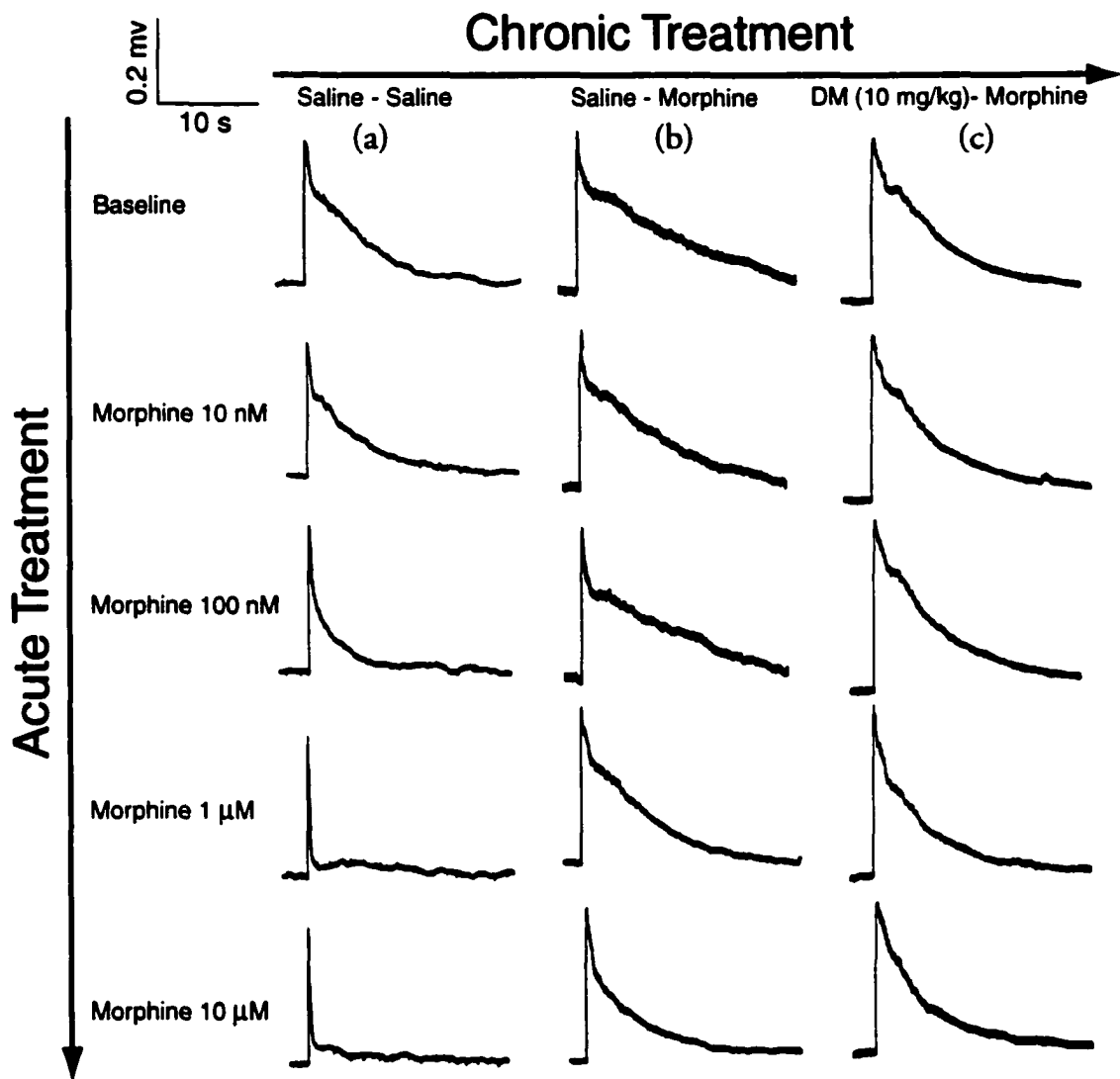


Figure 9-4C. Dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat, Tail.



Morphine tolerance in the 7-day-old rat. Recordings in each column are from one individual rat. The electric stimulation was a constant square wave, with length 200 μ s, amplitude 30 V. (a.) Acute morphine dose dependently suppressed the area of sVRP in morphine untreated rats. (b.) Acute morphine's suppression effect on sVRP area was diminished by chronic morphine treatment, suggesting that tolerance was developed by chronic morphine treatment. (c.) Pre-treatment of dextromethorphan failed to prevent the development of morphine tolerance in the 7-day-old rat.

Figure 9-5. Sample recordings demonstrating that chronic co-administration of dextromethorphan did not prevent the development of morphine tolerance in the 7-day-old rat.

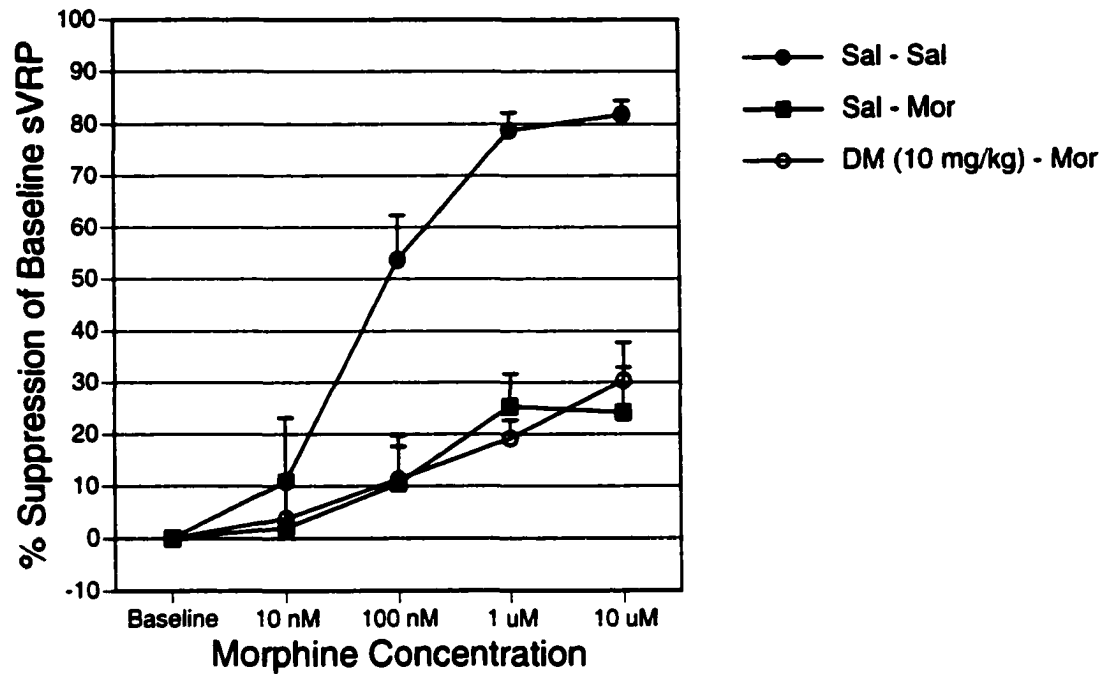


Figure 9-6. Dextromethorphan did not prevent the development of morphine tolerance at the spinal cord level in the 7-day-old rat.

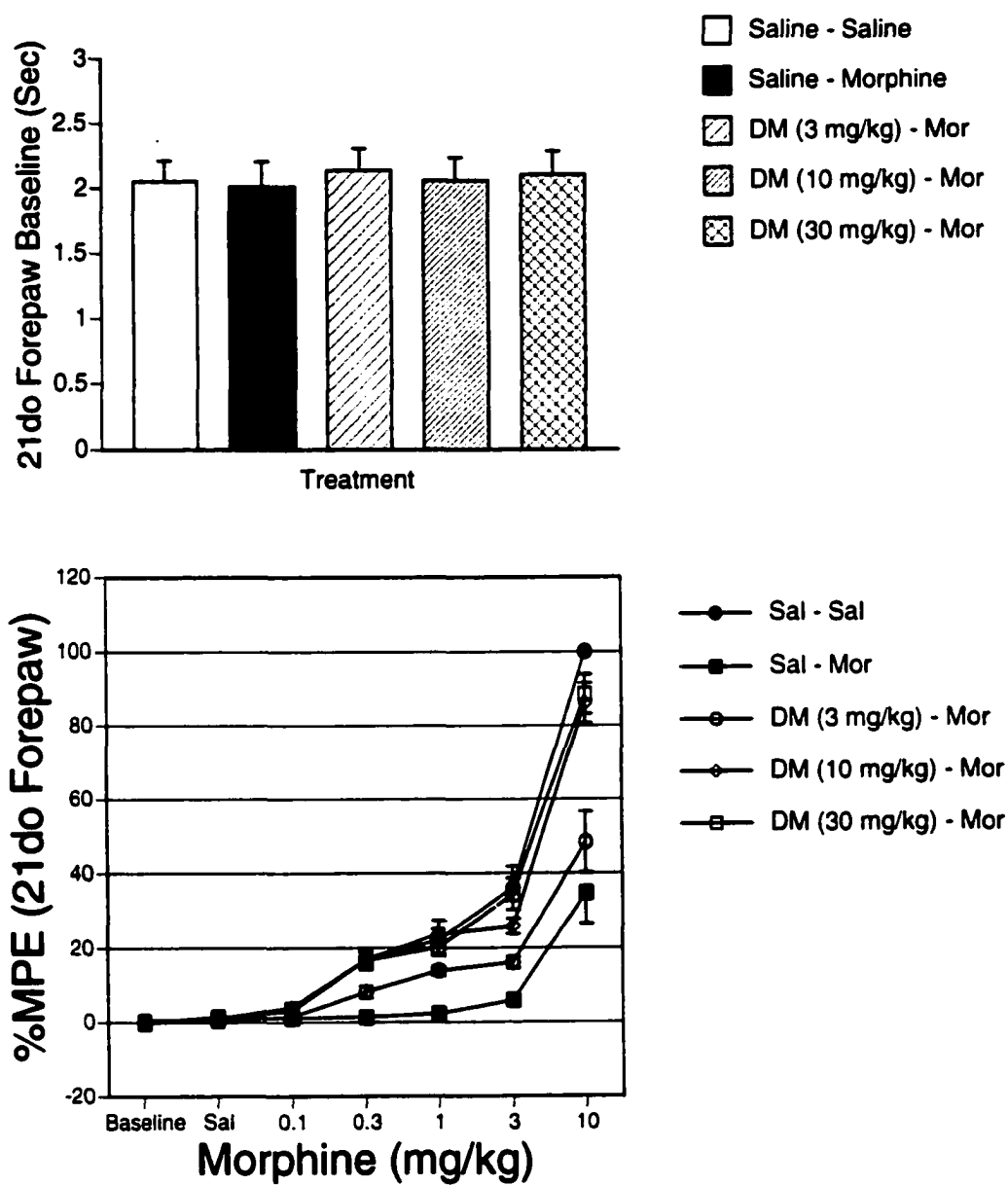


Figure 9-7A. Dextromethorphan prevented the development of morphine tolerance in the 21-day-old rat, Forepaw.

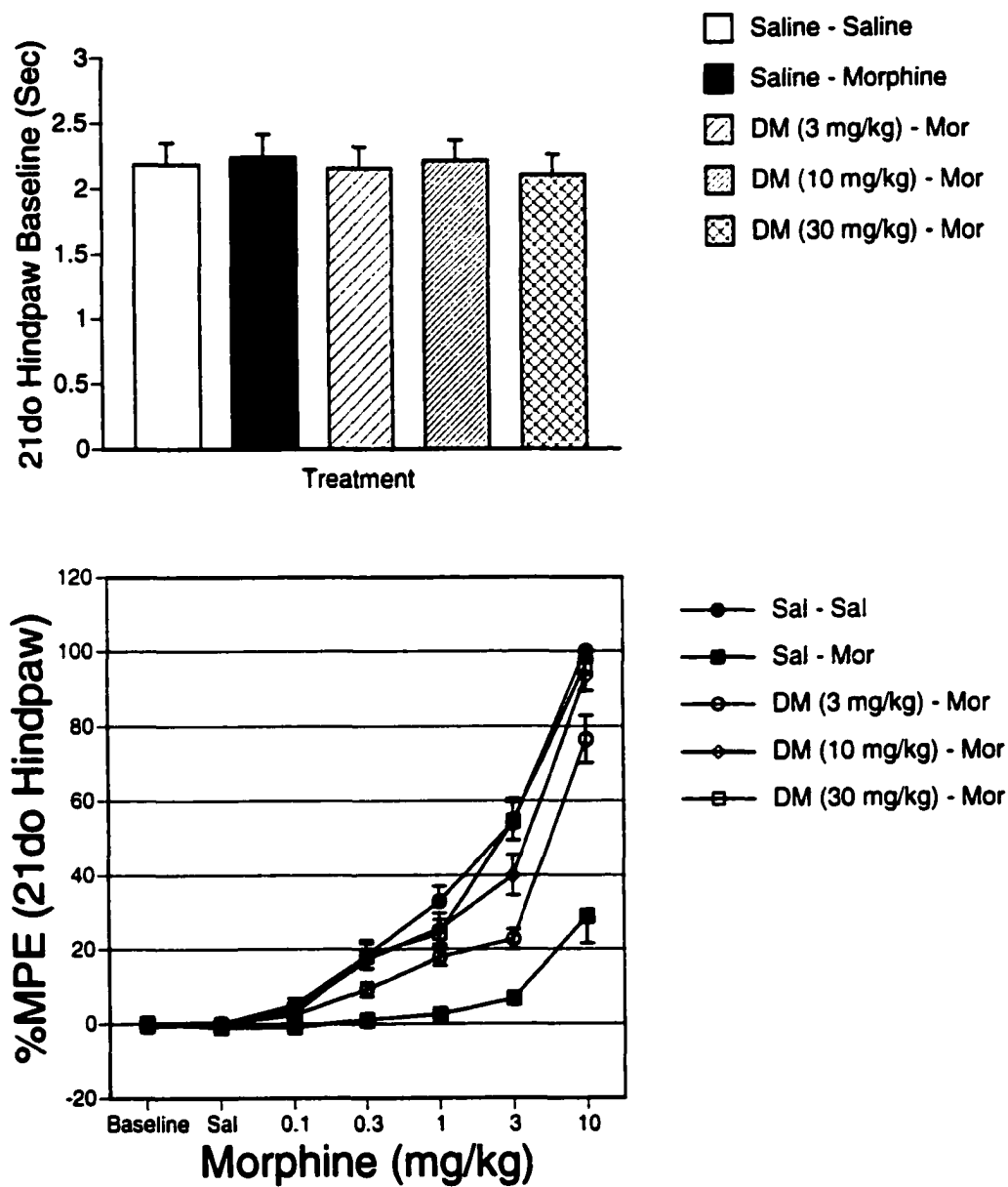


Figure 9-7B. Dextromethorphan prevented the development of morphine tolerance in the 21-day-old rat, Hindpaw.

Chapter 10

Ontogeny of AMPA receptor mediated morphine tolerance and withdrawal

Introduction

For clinical and theoretical reasons, there is increasing interest in the study of opiate tolerance and withdrawal in the infant rat model (Chapter 1). Although data on AMPA receptors' critical role in opiate tolerance and withdrawal in the adult animal are accumulating, it is unknown whether these results in adults apply to infants (Chapter 3). That AMPA receptors undergo dramatic differential changes during earlier life (Chapter 3) suggests possible developmental differences (Chapter 6). As a result, conclusions based on data derived from adult animals models should not be taken for granted that they would also hold in the developing organism.

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 dramatic differential changes during earlier life, suggesting that developmental difference would be possible, and therefore, conclusions based on information derived from adult animals models should not be taken for granted that they would also 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 (Ozawa et al., 1998; Dingledine et al., 1999), the in vitro system would be more appropriate than the in vivo model in addressing the pharmacological effect of NBQX specific to opiate withdrawal.

Material and methods

Morphine treatment and behavior observation

Pups were tattooed with India ink (Geller and Geller, 1966), which was injected into one or two paws to label individual pups permanently in each litter. To induce morphine dependence, starting when the pups were 1, 8, or 15 days of age, all pups in a litter were removed from the dam and individual rats were injected with morphine sulfate (IP, 10 mg/kg) twice daily (10 a.m. and 6 p.m.) for 6.5 days. The last injection was in the morning of the 7th day. In the afternoon of the 7th day, animals were transported from the animal facility to our laboratory in plastic tubs with wood chip bedding and placed in an observation chamber maintained at approximately 33°C. The pups were treated with NBQX (IP, 5, 15, 45 mg/kg, or saline as control for acute treatment in the withdrawal experiment, n=8 for each dose). After the drug treatment, the pup was then placed back into the observation chamber with the remainder of the litter (without the dam). Fifteen minutes later, naltrexone (IP, 1.0 mg/kg) was injected to precipitate withdrawal. Behavior of the pup was observed for 10 min before the treatment of naltrexone (prenaltrexone period) and 15 min after naltrexone injection (post-naltrexone period). Data collected from the prenaltrexone period were used to analyze the effects of the drugs on the morphine-dependent rat without the influence of naltrexone. During these observation periods, the behavior of the pups was identified every 15 s and recorded on a checklist by an observer (see Table 7-1 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. 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 (Windh et al., 1995; Thornton and Smith, 1997; Thornton et al., 1997) had clearly demonstrated that saline-treated rat pups of comparable age basically remain quiet and do not display withdrawal behaviors.

Tolerance assessment

To induce morphine tolerance, newborn rats were treated with morphine from day 1 to day 7 following the similar procedure as in the withdrawal experiment, except that in the study of NBQX's effect on tolerance, NBQX (IP, 0.5, 1.5, 5 mg/kg, or saline as control, $n = 8$ for each treatment condition) were injected 15 min before each morphine (or saline for control) injection. The tolerance assessment procedure followed a standard protocol (see Chapter 7 for detail).

In vitro electrophysiology

The standard electrophysiology experimental setup (see Chapter 7 for detail) was followed in the present studies. Spinal cords were obtained from 7-day-old rat pups. These rats were treated similarly to those in the behavioral experiments from day 1 to day 7, except that on the 7th day, morphine withdrawal was assessed by the electrophysiological assay instead of the behavioral test. In electrophysiological studies of opiate withdrawal, spinal cords from 7-day-old rats treated with chronic saline or morphine were used. After baseline sVRPs were recorded, naltrexone (10 μ M) is added into the superfusate. Dorsal root evoked ventral root activity is recorded from the ventral root for 30 min subsequent to the introduction of naltrexone into the

bath. The sVRP activity is digitized and the integral of the activity above baseline from 0 to 8 second was computed and defined as the measure of physical withdrawal (Bell and Adler, 1988; Bell and Beglan, 1995a; Feng and Kendig, 1995b; Feng and Kendig, 1997). In studying the acute pharmacological effects of NBQX (3 μ M) on morphine withdrawal, NBQX was applied to the ACSF 15 min before the introduction of naltrexone and after which acutely co-administrated with naltrexone.

Application of chemicals

NBQX, morphine, and naltrexone were made up as 1000 $\times$ stock solutions and were diluted in oxygenated recording solution immediately prior to use and 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.

For concentration-response curve construction of NBQX's effect on the sVRP area, after 15 min of baseline sVRP recording, NBQX (0.1 μ M, 1 μ M, 10 μ M, and 100 μ M) was applied in the superfusate for 30 min at each concentration. For withdrawal test, after 15 min of baseline sVRP recording, NBQX (3 μ M) was applied in the superfusate and 15 min later naltrexone (10 μ M) was introduced into the superfusate, after which, both NBQX and naltrexone co-existed in the superfusate for the whole length of recording.

Behavior data analysis

For withdrawal data, separate statistical tests were conducted for the three age groups and for each of the withdrawal behaviors. Occurrences of each behavior were summed for the 15-min postnaltrexone observation period. All doses of NBQX and vehicle 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 7-1. If significant results were detected, individual multiple comparisons were then conducted by Fisher's PLSD post-hoc test.

The raw behavioral tolerance data recorded by the experimenter were reaction time scores (in sec) and the data were converted to the percentage of maximum possible effect (%MPE) according to the method of Harris and Pierson (Harris and Pierson, 1964) (see Chapter 7 for detail).

The transformed %MPE data were then used to construct dose-response curves for morphine's analgesic effect. The ED₅₀ values and 95% confidence limits (95% C.L.) of morphine's analgesic effect were determined according to the method of Tallarida and Murray (Tallarida and Murray, 1987) using a Sigmoidal dose-response non-linear curve fitting module in the computer program Prism 3.0 (GraphPad Software Inc., San Diego, CA).

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 (treatment sVRP area). All data points were normalized to the averaged baseline sVRP area and were expressed as percentage of control: (treatment sVRP area)/(baseline sVRP area) × 100. All values are expressed as means ± 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 activities, baseline sVRP measurements were established before naltrexone being added to the spinal superfusate. The morphine withdrawal-induced sVRP augment is expressed as a percentage of the baseline sVRP. One-way ANOVAs were used to test significance of treatment effects among different treatment groups followed by Fisher's PLSD post-hoc test if significant results were detected.

For both behavioral and electrophysiological data statistical analyses, effects were considered significant if $P < .05$.

Results

NBQX attenuated morphine tolerance in the 7-day-old rat

Chronic treatment of morphine or NBQX plus morphine did not significantly alter the baseline reaction time of the rat in withdrawing its appendages (forepaw, hindpaw, or tail) from the noxious thermal stimulus (Table 10-1, Figure 10-1A-C upper panels). 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 (Figure 10-1A-C lower panels) and a significantly increased ED_{50} of acute morphine (Table 10-2) compared with the morphine naive group.

Chronic co-administration of NBQX with morphine dose-dependently attenuated the development of tolerance, as reflected by that the morphine's dose-response curve for the groups that received chronic co-administration of NBQX plus morphine returned toward that of the morphine naive group (Figure 10-1A-C lower panels) and decreased ED_{50} of acute morphine (Table 10-2) compared with the group that received only chronic morphine treatment.

NBQX attenuated behavioral signs of morphine withdrawal in the 7-day-old rat

The data (N = 32) for the pre-naltrexone period show that treatment with various doses NBQX in the 7-day-old rat did not significantly alter the baseline behaviors of the morphine-dependent rat (Table 10-6 for summary of statistical analysis results). Similar to the control group, except for some baseline activities, all experimental groups largely remained quiet (Table 10-3 for summary of data).

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 (Table 10-6 for summary of statistical analysis results). NBQX dose dependently decreased head moves, moving paws, walking, and wall climbing behaviors and increased quiet behavior. We failed to find any significant effect on together and rolling behavior for pretreatment with NBQX. The dose effects of pre-treatment of NBQX on naltrexone-precipitated morphine withdrawal in the 7-day-old rat are depicted in Figure 10-2.

NBQX attenuated behavioral signs of morphine withdrawal in the 14-day-old rat

The data (N = 32) for the pre-naltrexone period show that treatment with various doses NBQX in the 14-day-old rat did not significantly alter the behaviors of the morphine-dependent rat except for the walking behavior (Table 10-6 for summary of statistical analysis results). For the walking behavior, post-hoc multiple comparison analysis (Fisher's PLSD) revealed that only the lowest dose of NBQX (5 mg/kg) treatment significantly increased walking behavior compared with the vehicle control group, whereas the occurrences of walking behavior in rats received higher doses of NBQX (15 mg/kg and 45 mg/kg) were not significantly altered compared with the control group. Thus, in general, similar to the control group, except for

some baseline activities, all experimental groups remained quiet (Table 10-4 for summary of data).

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 14-day-old rat (Table 10-6 for summary of statistical analysis results). NBQX dose dependently decreased burrow, head moves, moving paws and walking behaviors and increased quiet behavior. Pretreatment with NBQX also had a trend to suppress together and wall-climbing 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 Figure 10-3.

NBQX attenuated behavioral signs of morphine withdrawal in the 21-day-old rat

The data (N = 32) for the pre-naltrexone period show that treatment with various doses NBQX in the 21-day-old rat did not significantly alter the behaviors of the morphine-dependent rat (Table 10-6 for summary of statistical analysis results). Similar to the control group, except for some baseline activities, all experimental groups largely remained quiet (Table 10-5 for summary of data).

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 21-day-old rat (Table 10-6 for summary of statistical analysis results). NBQX dose dependently decreased burrow, head moves, together and walking behaviors and increased quiet behavior. We did not find any significant effect on wall-climbing behavior for pretreatment with NBQX. The dose effects of pre-treatment of NBQX on naltrexone-precipitated morphine withdrawal in the 21-day-old rat are depicted in Figure 10-4.

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

Genesis of the sVRP relies on the functioning of many synaptic transmissions (Thompson et al., 1992; Thompson et al., 1993; Collins et al., 1995) whereas high concentrations of NBQX significantly inhibit normal synaptic transmission (Zeman and Lodge, 1992). Although there are several reports examining another AMPA receptor antagonist CNQX's effect on some components of the sVRP in the 8-12 day-old rats (King et al., 1992; Thompson et al., 1992), there is a lack of quantified dose-response studies of NBQX's effect on sVRP in the 7-day-old rat. Thus, the goal of this experiment was to determine a suitable concentration of NBQX that does not significantly alter the baseline sVRP for later experiments.

The spinal cords from 4 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 μM and 1 μM did not produce appreciable effect on the baseline sVRP ($98.92 \pm 3.45\%$ and $98.46 \pm 3.91\%$ of control, respectively). At 10 μM , NBQX slightly suppressed the sVRP area ($89.31 \pm 6.48\%$ of control, $P > .05$). Although statistically not significant, that is more likely due to that 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 μM , NBQX markedly suppressed sVRP ($66.26 \pm 8.48\%$ of control, $P < .05$). The concentration-response curve of NBQX's effect on the sVRP in the 7-day-old rat is depicted in Figure 10-5. These results 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 μM .

Spinal cords from morphine treated rats demonstrated robust withdrawal in vitro

Stable recording of the sVRP can be made from spinal cord of the neonatal rat for up to many hours (Kerkut and Bagust, 1995; Herrero et al., 2000). In our experiments, 7-day-old rats that did not receive chronic morphine treatment ($n = 4$) demonstrated excellent stability in generating sVRP over time (Figure 10-6Aa upper traces and Figure 10-6Ab). 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 ranging 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 (Figure 10-6Aa lower traces at 15 and 30 min). However, when naltrexone ($10 \mu\text{M}$) was added to the superfusate, sVRP of spinal cords from morphine treated rats rapidly increased to reach a peak in approximately 10 min after naltrexone was added in ($190.25 \pm 9.34\%$ of control, $P < .001$, $n = 6$). The intensity of this augment 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 in) (Figure 10-6Aa lower traces at 40 and 60 min and Figure 10-6Ab). 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. Thus, consistent with studies by others (Bell and Beglan, 1995a), we have 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.

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 (Figure 10-6Ba upper traces and Figure 10-6Bb), the addition of NBQX ($3 \mu\text{M}$) and the later addition of naltrexone ($10 \mu\text{M}$) to the superfusate did not significantly alter the sVRP area value at any time point during the recording (sVRP area values ranging 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 ($3 \mu\text{M}$) nor NBQX ($3 \mu\text{M}$) together with naltrexone ($10 \mu\text{M}$) significantly altered baseline sVRP in morphine naive rats.

Rats that received chronic morphine treatment ($n = 6$) demonstrated similarly stable recording in the normal perfusion medium and after NBQX ($3 \mu\text{M}$) was added in. When naltrexone ($10 \mu\text{M}$) was added to the superfusate, sVRP of spinal cords from morphine treated rats increased significantly in the first 20 min after naltrexone was added in (maximal $146.32 \pm 9.21\%$ of control, $P < .01$, $n = 6$). After which, the sVRP then gradually declined to reach the control level at 50 min and remained at that level for the rest length of recording (sVRP area values ranging from $105.97 \pm 3.49\%$ to $97.96 \pm 2.31\%$ of control, $P > .05$ at all time points, $n = 6$) (Figure 10-6Ba lower traces and Figure 10-6Bb). 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.

To better compare the treatment effect on morphine withdrawal among various chronic treatment (morphine 10mg/kg vs. saline) and acute treatment (NBQX 3 μ M vs. normal ACSF) groups, we have extracted data points from two temporal regions of the recording at 38-42 min and 58-62 min respectively; thus, data points in each of these two periods were averaged for treatment effect analyses. The time effect analyses detailed above demonstrated that these two temporal regions can probably best describe morphine withdrawal in the present protocol.

As expected, regardless of acute treatment conditions, spinal cords from rats that did not receive chronic morphine treatment did not show significant difference from each other at either 40 min or 60 min, indicating that the present electrophysiological assay was stable and NBQX's effect at a concentration of 3 μ M on the baseline sVRP is not significant (Figure 10-6C and D)

At 40 min, sVRP of the morphine treated spinal cord significantly increased to about 200% of control, indicating that chronic treatment of morphine induced morphine dependence in the rat spinal cord and the addition of naltrexone into the superfusate precipitated morphine withdrawal. Although pre-treatment of NBQX (3 μ M) significantly suppressed this naltrexone precipitated morphine withdrawal to approximately 140% of control, it did not completely suppressed morphine withdrawal since the averaged sVRP area value from this group was still significantly higher than the control groups that did not receive morphine treatment (Figure 10-6 D).

At 60 min, sVRP value of the morphine treated spinal cord still remained significantly higher than control at about 150% of control. In contrast to the data at 40 min, pre-treatment of NBQX (3 μ M) essentially completely suppressed this stage of naltrexone precipitated morphine withdrawal (Figure 10-6C).

Discussion

Despite decades of research, the mechanisms underlying opiate tolerance and withdrawal remain elusive (Trujillo, 1999; Nestler, 2001). It has been suggested that multiple mechanisms are involved (Thorat et al., 1994). One possibility is that chronic morphine treatment upregulate the cAMP pathway and withdrawal from opiates result in hyperactivity of the cAMP system (Nestler and Tallman, 1988; Nestler, 2001). Another possibility is that opiate withdrawal results in augmented central release of glutamate, which in turn contribute to the hyperactivity typifying opiate withdrawal (Akaoka and Aston-Jones, 1991; Aghajanian et al., 1994; Tokuyama et al., 1996). These two mechanisms may interact with each other, thus the hyperactivity during opiate withdrawal manifested by behavioral, electrophysiological and biochemical indices may have a dual-mechanism cause (Aghajanian et al., 1992).

Although there is direct evidence supporting the role of glutamate in opiate withdrawal syndrome (Tokuyama et al., 1996), it is less clear which glutamate receptors are responsible for 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 Trujillo and Akil, 1991a; Elliott et al., 1995a; Herman et al., 1995; Pasternak et al., 1995; Trujillo, 1995; Trujillo and Akil, 1995; Inturrisi, 1997; Danysz and Parsons, 1998; Mao, 1999). In contrast to these findings using the adult animal model, recent studies from our laboratory (Zhu and Barr, 2000, 2001b, c) and others (Bell and Beglan, 1995a, b) showed that NMDA receptor antagonists are not effective in suppressing morphine withdrawal in rats younger than a week of age, whereas partially effective in the 14-day-old rat and fully effective after 21-day-old (Zhu and Barr, 2001b). The reason for this age-dependent

effectiveness of NMDA receptor antagonists in suppressing opiate withdrawal has been attributed to the dramatic ontogenetic differential changes of the subunits of the NMDA receptor during earlier life (Zhu and Barr, 2001b, c).

In addition to acting on the NMDA receptor, glutamate also acts on other ionotropic glutamate receptors such as the AMPA receptor. There are several lines of evidence implicating a critical role of the AMPA receptor in opiate tolerance and withdrawal.

Firstly, 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.

Secondly, chronic morphine treatment elevates the AMPA receptor GluR1 subunit levels in the rat ventral tegmental area (Fitzgerald et al., 1996), morphine-induced tolerance and dependence are reduced in mice deficient in AMPA receptor subunit A (Vekovischeva et al., 2001) (note that the classification nomenclature of the AMPA receptor is different in the mouse than in the rat, subunit A in mice is similar to GluR1 in rats), and 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).

Thirdly, in the past few years, several laboratories, using a variety methodology ranging from behavioral to electrophysiological, investigated the role of AMPA receptor in opiate tolerance and withdrawal. The role of the AMPA receptor in analgesic tolerance has been examined using the systemically active competitive antagonist LY293558 in mice (Kest et al., 1997; McLemore et al., 1997). Analgesic tolerance was attenuated when mice received a continuous subcutaneous infusion of LY293558 at doses of 30, 45 or 60 mg/kg/24 hr via an osmotic pump concurrent

with the morphine treatment. Continuous subcutaneous infusion of LY293558 (45 mg/kg/24 hr) also reversed established morphine tolerance. Thus, these data demonstrated that the AMPA receptor may play a role in the development and maintenance of morphine tolerance. Besides its possible role in the development of opiate tolerance, AMPA receptor has been argued to play a more important role than the NMDA receptor in the expression opiate withdrawal. Rasmussen and colleagues has questioned the role that the NMDA receptor plays in opiate withdrawal (Rasmussen, 1995). Studies by this group lend support to the concept that NMDA receptors play only a minor role in the expression of opiate withdrawal. In opiate-dependent rats, there is a marked increase in firing of locus coeruleus (LC) neurons during naltrexone precipitated withdrawal; and opiate withdrawal increases glutamate and aspartate efflux in the LC (Rasmussen and Aghajanian, 1989; Rasmussen et al., 1996). This increase in activity of LC neurons has been hypothesized to be important in opiate withdrawal symptoms (Rasmussen et al., 1996). The excitatory effect of glutamate in the LC is mediated largely through AMPA receptors, whereas NMDA receptors mediate little, if any, of the withdrawal-induced excitation of the LC (Rasmussen et al., 1991a; Rasmussen, 1995; Rasmussen et al., 1996). Recently, another group reported that 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).

Although evidence suggesting that the AMPA receptor plays a critical role in opiate withdrawal is accumulating rapidly, 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. The neonatal CNS is both structurally and functionally different from that of the adult, and significant changes in opioid

actions occur both prenatally and postnatally (Barr, 1992, 1993; Fitzgerald, 1995). At the same time, the AMPA receptor, which is believed to play an essential role in opiate dependence, undergoes qualitative and quantitative changes during development (for details on the ontogenetic profile of AMPA receptor, refer to Chapter 2). Indeed, in contrast to the adult data, we previously reported that NMDA receptor antagonists were not effective in opiate tolerance (Chapter 9) and withdrawal (Zhu and Barr, 2000, 2001b) (Chapter 5 and Chapter 8) and 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, 2001c).

Thus, the present study examined the effect of a competitive antagonist of the AMPA receptor NBQX on morphine tolerance and withdrawal in the infant rat using a combination of *in vivo* and *in vitro* methods. The data of present study clearly show that NBQX was effective in attenuating tolerance and both behavioral signs and a electrophysiological measurement of morphine withdrawal in the 7-day-old; and as in the 7-day-old rat, NBQX was also effective in suppressing withdrawal behaviors in both the 14- and 21-day-old rats. Since morphine withdrawal involves both elevated cAMP level and augmented glutamate release in the CNS, NBQX may attenuate morphine withdrawal in two most likely mechanisms.

Firstly, there is broad and persuasive evidence supporting the role of glutamatergic receptors in mediating morphine withdrawal in brain and spinal cord (for review, see Mao, 1999; Trujillo, 1999). An increase in glutamate release is generally observed during opiate withdrawal (Rasmussen and Aghajanian, 1989; Akaoka and Aston-Jones, 1991; Desole et al., 1996; Jhamandas et al., 1996; Tokuyama and Ho, 1996; Hoshi et al., 1997; Huang et al., 1997). For example, naloxone-precipitated withdrawal from morphine 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 precipitated withdrawal signs in a dose-dependent manner, whereas prominent behavioral changes were not observed when animals continuously infused with saline were challenged with glutamate (Tokuyama et al., 1996). Hence, these results provide direct evidence that glutamate is essential for the expression of opiate withdrawal signs. The rapid release of glutamate from locus coeruleus and other regions under an opiate dependent state has been hypothesized to be triggers or key elements for the expression of opiate withdrawal signs (Rasmussen, 1991; Christie et al., 1997). Since augmented glutamate release is a prominent characteristic of opiate withdrawal, it was possible that, by blocking the AMPA receptor, the acute treatment of NBQX in both the in vivo and in vitro assays have inhibited the possible excitatory effects of the exaggerated glutamate release typical of opiate withdrawal and thus attenuated both behavioral and electrophysiological manifestations of morphine withdrawal. Some potential sites of action of NBQX in suppressing withdrawal by blocking the excitatory effects of glutamate on AMPA receptor include those thought to be involved in opiate withdrawal and having the highest densities of AMPA receptors, especially the Ca^{2+} -permeable AMPA receptors. Such sites include the locus coeruleus, hippocampus, cortex areas, caudate-putamen (Jang et al., 2000) and the spinal cord (Jakowec et al., 1995b; Jakowec et al., 1995a). That the developing CNS expresses Ca^{2+} -permeable AMPA receptors (Pellegrini-Giampietro et al., 1992; Jakowec et al., 1995b; Jakowec et al., 1995a) may also explain our finding that NBQX was effective in preventing the development of tolerance. As detailed in Chapter 2, in the developing rat brain and spinal cord, NMDA receptors do not have high calcium permeability whereas AMPA receptors

have. 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 during chronic morphine treatment. 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, 2001a, d) (see also Chapter 9), possibly due to the low calcium permeability of these immature NMDA receptors (Zhu and Barr, 2001c)

Secondly, it is well known that whereas acute administration of morphine decreases cAMP production, during chronic administration cAMP system returns to control level (Sharma et al., 1975; Nestler and Tallman, 1988; Chieng and Williams, 1998; Bordi and Ugolini, 1999), suggesting that compensatory mechanisms are elicited. Withdrawal from morphine relieves the inhibition of cAMP by acute morphine and consequently unmasks the superactivation of the cAMP pathway produced by the cellular adaptive changes and results in the hyperactivity seen during morphine withdrawal (Nestler, 2001). Unlike the mGluRs, which share common pools of intracellular second messenger systems with opioid receptors (Fundytus and Coderre, 1997), and acute DCG-IV markedly decrease morphine withdrawal (Chapter 11), possibly by decreasing cellular cAMP levels (Pin and Duvoisin, 1995; Conn and Pin, 1997), there is no evidence that the AMPA receptor is directly related to the cAMP pathway. Thus, it is likely that the decreased withdrawal we have seen in the present behavioral studies were due to NBQX's effect on the glutamate pathway rather than the cAMP pathway.

In the in vitro study, we found that NBQX was more effective in suppressing the second stage than the first stage of morphine withdrawal in the spinal cord. Interestingly, in a parallel study (Chapter 11), we found that the metabotropic

glutamate receptor agonist DCG-IV was more effective in suppressing the first stage than the second stage of morphine withdrawal in the isolated spinal cord. By contrasting the pharmacological properties of these two agents and their different effect on morphine withdrawal and considering that morphine withdrawal involves dual mechanisms (Aghajanian et al., 1992), it seems that as in the LC (Aghajanian et al., 1992), in the in spinal cord, morphine withdrawal is due to the excitation resulted from a combination of at least two factors: elevated cAMP level and augmented glutamate release. We further hypothesize that: 1) the peak of morphine withdrawal we observed (10 min after naltrexone) is due to the temporal overlap of action from two excitatory systems, namely the cAMP second messenger system and the glutamate neural transmission system; 2) the 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 and thus, after the withdrawal peak, augmented glutamate release was mainly responsible for the elongated but attenuated morphine withdrawal plateau. Therefore, in the present study, NBQX could have effectively suppressed the excitatory effect of exaggerated glutamate release whereas not effective in suppressing the excitation due to the elevated cAMP level, resulting a differential suppression of the second stage of the in vitro withdrawal sign. In contrast, in our parallel studies (Chapter 11), 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 (Pin and Duvoisin, 1995; Conn and Pin, 1997). Further examination is necessary to differentiate these two processes.

In conclusion, treatment of the competitive AMPA receptor antagonist NBQX is effective in suppressing morphine tolerance and withdrawal in the infant rat and this effect is not age-dependent. Although great hopes for NMDA receptor

antagonists in the therapy of opiate withdrawal are promised by the overwhelming pre-clinical data (for review, see Herman et al., 1995; Inturrisi, 1997; Mao, 1999) and clinical evidence in both the United States (Rosen et al., 1996; Bisaga et al., 2001) and other countries (Koyuncuoglu et al., 1990; Koyuncuoglu, 1995), their future in the infant are hampered by the finding that NMDA receptor antagonists are not effective in suppressing opiate tolerance and withdrawal in the infant (Bell and Beglan, 1995a, b; Zhu and Barr, 2000, 2001b, c). In addition, that blockade of NMDA receptors results in apoptotic neurodegeneration in the developing CNS (Ikonomidou et al., 1999) further preclude the use of NMDA receptor antagonist for the treatment of opiate withdrawal in human infants. The AMPA receptor therefore provides a novel target for development of therapeutic agents that could have a dramatic impact on treatment of opiate withdrawal in human patients of all ages.

Table 10-1. Statistical analyses results of the effect of chronic treatment of morphine or morphine plus NBQX on withdrawal latency from a hotplate in the 7-day-old rat.

Age		Baseline Latency Statistics		
		Forepaw	Hindpaw	Tail
7do	F	1.07	0.47	0.56
	df	(4, 35)	(4, 35)	(4, 35)
	P	0.38	0.75	0.69

No significant results were found for any situations

Table 10-2. Effects of chronic co-administration of NBQX with morphine on ED₅₀ of morphine in the infant rat.

Age	Treatment	ED ₅₀ mg/kg (95% C.L.)		
		Forepaw	Hindpaw	Tail
7do	Sal-Sal	0.76 (0.43-1.09)	0.74 (0.43-1.05)	0.87 (0.56-1.18)
	Sal-Mor	2.62 (2.32-2.91) *	2.56 (2.35-2.78) *	3.42 (3.13-3.70) *
	NBQX (0.5 mg/kg)-Mor	1.34 (0.64-2.04) &	1.69 (1.10-2.47) *	2.94 (2.66-3.22) *
	NBQX (1.5 mg/kg)-Mor	1.33 (0.94-1.73) &	2.32 (1.76-2.88) *	2.99 (2.56-3.43) *
	NBQX (5 mg/kg)-Mor	1.00 (0.75-1.25) &	1.02 (0.78-1.26) &	1.34 (0.63-2.05) &

*: ED₅₀ significantly greater than Sal-Sal group

&: ED₅₀ significantly smaller than Sal-Mor group

Table 10-3. Behavior occurrences after treatment of NBQX but before treatment of naltrexone in the morphine-dependent rat of 7 days old.

Behavior (7do)	NBQX Dose			
	Vehicle	5 mg/kg	15 mg/kg	45 mg/kg
Head Moves	.625 ± .263	.750 ± .366	.500 ± .378	.375 ± .263
Moving Paws	.250 ± .164	1.125 ± .581	.500 ± .189	.625 ± .375
Quiet	58.875 ± .549	57.750 ± .701	58.625 ± .596	58.625 ± .680
Rolling	.250 ± .250	.250 ± .164	.125 ± .125	.250 ± .164
Together	0.000 ± 0.000	.125 ± .125	0.000 ± 0.000	0.000 ± 0.000
Walking	.125 ± .125	0.000 ± 0.000	.125 ± .125	0.000 ± 0.000
Wall Climbing	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

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

Table 10-4. Behavior occurrences after treatment of NBQX but before treatment of naltrexone in the morphine-dependent rat of 14 days old.

Behavior (14do)	NBQX Dose			
	Vehicle	5 mg/kg	15 mg/kg	45 mg/kg
Burrow	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Head Moves	.750 ± .491	1.250 ± .901	.875 ± .515	.750 ± .412
Moving Paws	.125 ± .125	1.000 ± .627	.625 ± .324	.500 ± .378
Quiet	56.875 ± 1.540	54.000 ± 1.871	58.625 ± .653	58.125 ± .666
Together	.875 ± .441	.750 ± .313	0.000 ± 0.000	.125 ± .125
Walking	1.125 ± .479	2.500 ± .655 *	.125 ± .125	.250 ± .164
Wall Climbing	.500 ± .378	.500 ± .378	0.000 ± 0.000	.250 ± .164

*: significantly different than control

Note: cell entries are mean occurrences of behavior ± SEM. Except for walking behavior, for which the 5 mg/kg group showed significant higher occurrences than the vehicle control group, there were no significant differences between any treatment condition and the corresponding control group ($N = 8$ per cell)

Table 10-5. Behavior occurrences after treatment of NBQX but before treatment of naltrexone in the morphine-dependent rat of 21 days old.

Behavior (21do)	NBQX Dose			
	Vehicle	5 mg/kg	15 mg/kg	45 mg/kg
Burrow	.125 ± .125	0.000 ± 0.000	0.000 ± 0.000	.125 ± .125
Head Moves	1.875 ± .743	.875 ± .350	3.625 ± 1.117	1.000 ± .732
Quiet	55.125 ± 1.619	56.625 ± 1.209	54.000 ± 1.763	55.375 ± 3.789
Together	.375 ± .183	.250 ± .164	.375 ± .375	.875 ± .743
Walking	1.500 ± .655	1.500 ± .463	1.000 ± .267	1.000 ± .627
Wall Climbing	.875 ± .479	.625 ± .420	.500 ± .378	.875 ± .875

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

Table 10-6. Statistical analyses results 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	
		F	P	F	P	F	P
Pre-Naltrexone	Burrow	ND	ND	ND	ND	0.6360	0.5999
	Head Moves	0.4670	0.7086	0.1500	0.9288	2.5330	0.0846
	Moving Paws	1.9360	0.1547	0.8620	0.4761	ND	ND
	Quiet	2.3500	0.1015	2.9080	0.0586	0.2020	0.8938
	Rolling	0.1580	0.9234	ND	ND	ND	ND
	Together	1.0000	0.4123	2.4210	0.0946	0.3860	0.7640
	Walking	0.6360	0.5999	10.1900	0.0002	0.2800	0.8392
	Wall Climbing	ND	ND	0.8280	0.4933	0.0960	0.9613
Post-Naltrexone	Burrow	ND	ND	8.0950	0.0009	5.8030	0.0047
	Head Moves	20.1280	0.0001	26.4400	0.0001	8.3340	0.0008
	Moving Paws	26.8060	0.0001	9.6930	0.0003	ND	ND
	Quiet	60.9240	0.0001	36.8470	0.0001	14.8440	0.0001
	Rolling	3.0440	0.0514	ND	ND	ND	ND
	Together	2.7850	0.0660	0.8860	0.4642	4.6560	0.0120
	Walking	8.3770	0.0007	11.9750	0.0001	11.7310	0.0001
	Wall Climbing	11.9080	0.0001	2.5510	0.0831	0.8250	0.4947

ND: this behavior was not displayed

Bold face: result is statistically significant

df = (3, 28) in all cases

Figure 10-1A-C: Upper panels: the effect of chronic treatment with morphine or NBQX plus morphine on the baseline latency of the 7-day-old rat retracting its appendages from a hotplate. Ordinate: Mean latency of retraction of the appendage (Mean $\pm$ one S.E.M, in seconds). Abscissa: chronic treatment conditions). No significant results were found between any groups. A: forepaw; B: hindpaw; C: tail.

Lower panels: The effect of pre-treatment with the competitive AMPA receptor antagonist NBQX (0.5, 1.5 , and 5 mg/kg) on the development of morphine tolerance in the 7-day-old rat. Ordinate: Mean %MPE (Mean $\pm$ one S.E.M.). Abscissa: acute morphine doses. A: forepaw; B: hindpaw; C: tail.

Figure 10-2. 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-day-old rat. Ordinate: Mean occurrences (Mean $\pm$ one S.E.M.) in 15 minutes of opiate withdrawal behaviors (definition see Table 7-1). Abscissa: NBQX doses. Withdrawal was precipitated by naltrexone (1 mg/kg) in all groups. *, $p < 0.05$ compared to the saline control group.

Figure 10-3. 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 14-day-old rat. Ordinate: Mean occurrences (Mean $\pm$ one S.E.M.) in 15 minutes of opiate withdrawal behaviors (definition see Table 7-1). Abscissa: NBQX doses. Withdrawal was precipitated by naltrexone (1 mg/kg) in all groups. *, $p < 0.05$ compared to the saline control group.

Figure 10-4. 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 21-day-old rat. Ordinate: Mean occurrences (Mean $\pm$ one S.E.M.) in 15 minutes of opiate withdrawal behaviors (definition see Table 7-1). Abscissa: NBQX doses. Withdrawal was precipitated by naltrexone (1 mg/kg) in all groups. *, $p < 0.05$ compared to the saline control group.

Figure 10-5. NBQX's effect on the slow ventral root potential area in the isolated spinal cord of the 7-day-old rat

(a) Sample recordings of different concentrations of NBQX's effect on the sVRP in the 7-day-old rat. All traces are from the recordings of one rat.

(b) Concentration-response of NBQX's effect on the slow ventral root potential area in the 7-day-old rat. 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).

Figure 10-6. Time and treatment effect of morphine withdrawal in the isolated spinal cord from the 7-day-old rat

A: 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 chronic 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)

B: Time course of morphine withdrawal in the isolated rat spinal cord (with 3 μ M NBQX)

(a) upper trace: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated chronic 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 (with 3 μ M NBQX). Ordinate: sVRP area expressed as the percentage of baseline sVRP area (Mean $\pm$ one S.E.M.). Abscissa: time of recording (min)

C: Treatment effect of NBQX (3 μ M) 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.

D: Treatment effect of NBQX (3 μ M) 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.

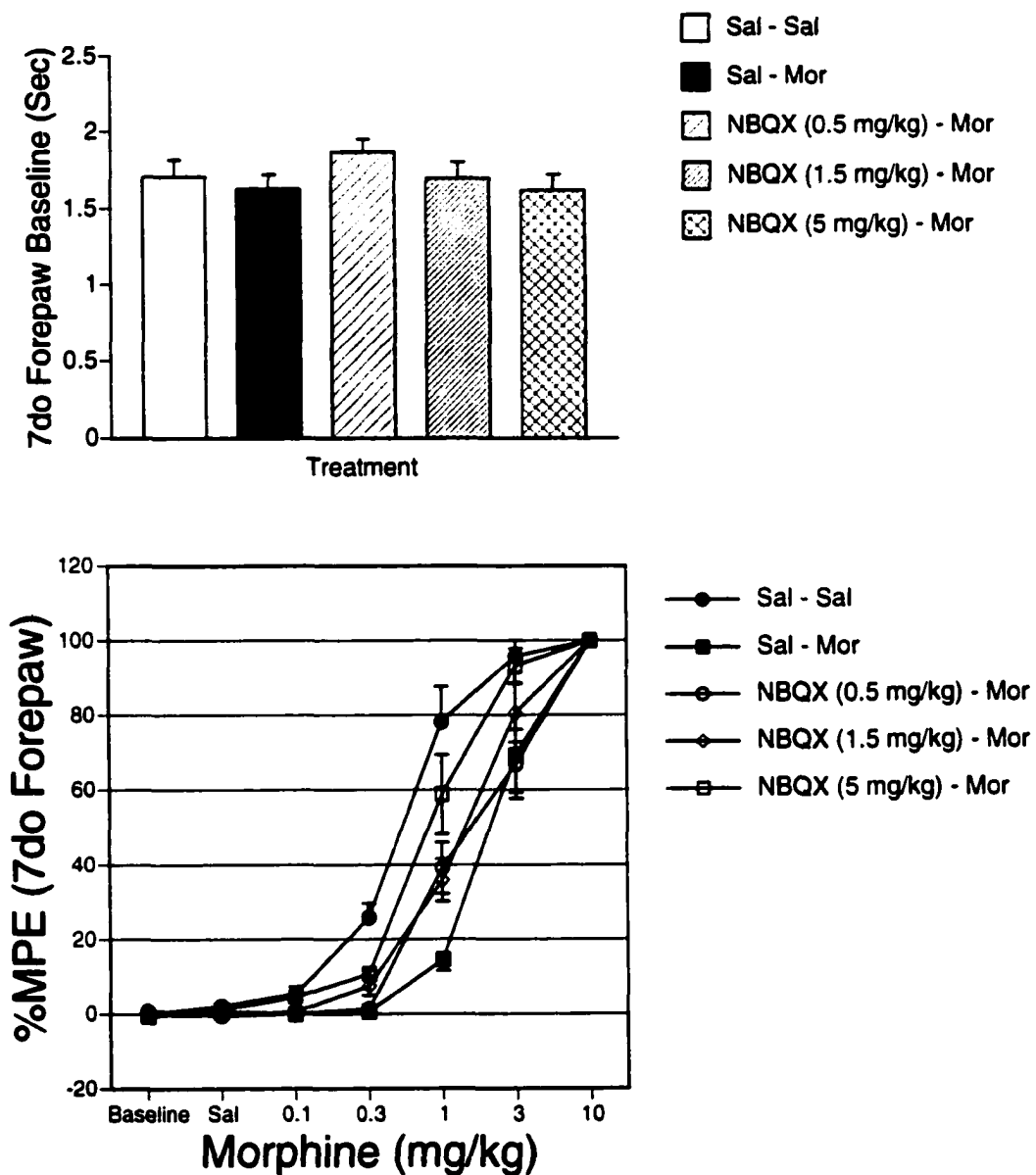


Figure 10-1A: NBQX dose-dependently attenuated morphine tolerance in the 7-day-old rat as assessed by the withdrawal time of the forepaw from a hotplate.

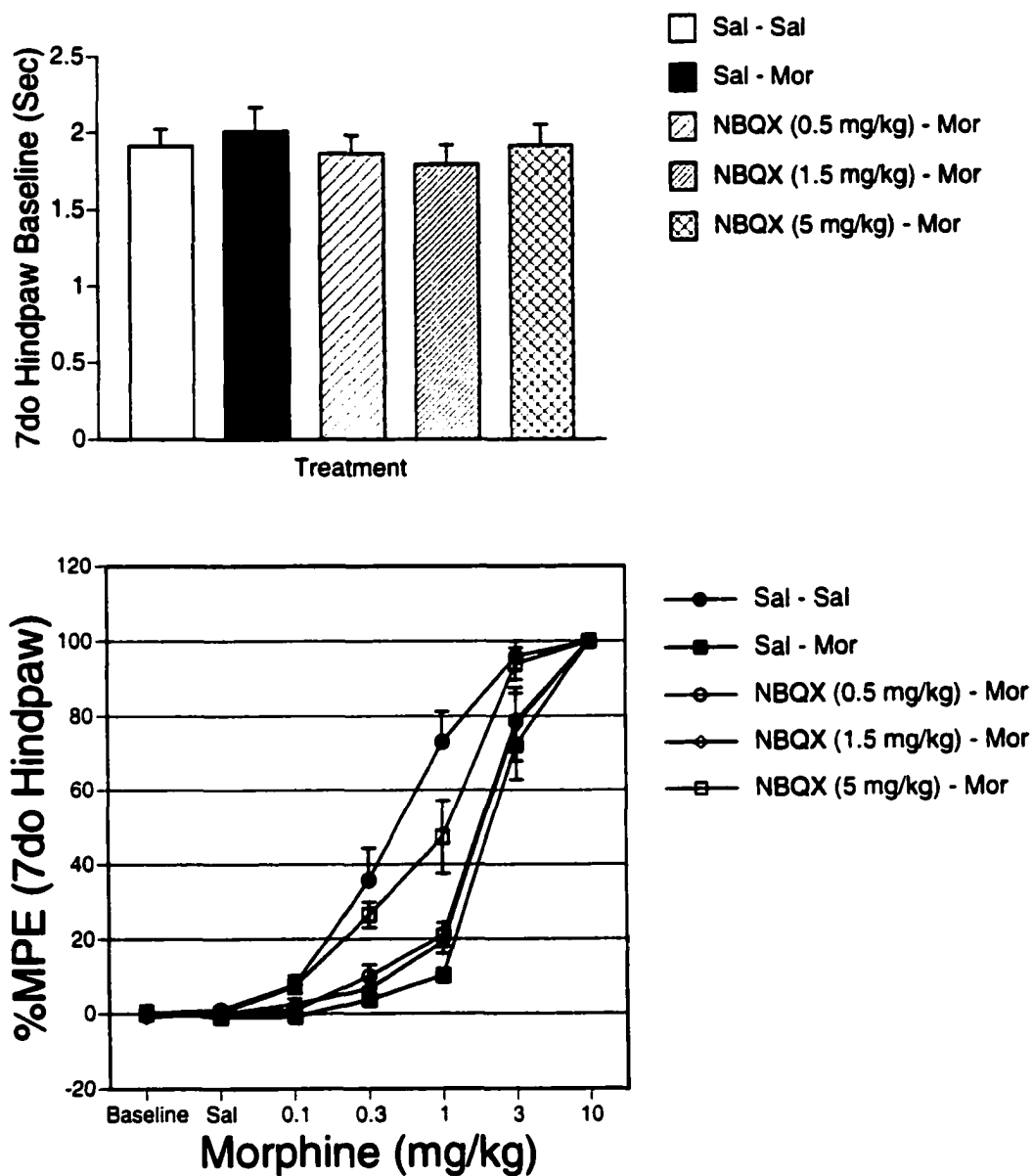


Figure 10-1B: NBQX dose-dependently attenuated morphine tolerance in the 7-day-old rat as assessed by the withdrawal time of the hindpaw from a hotplate.

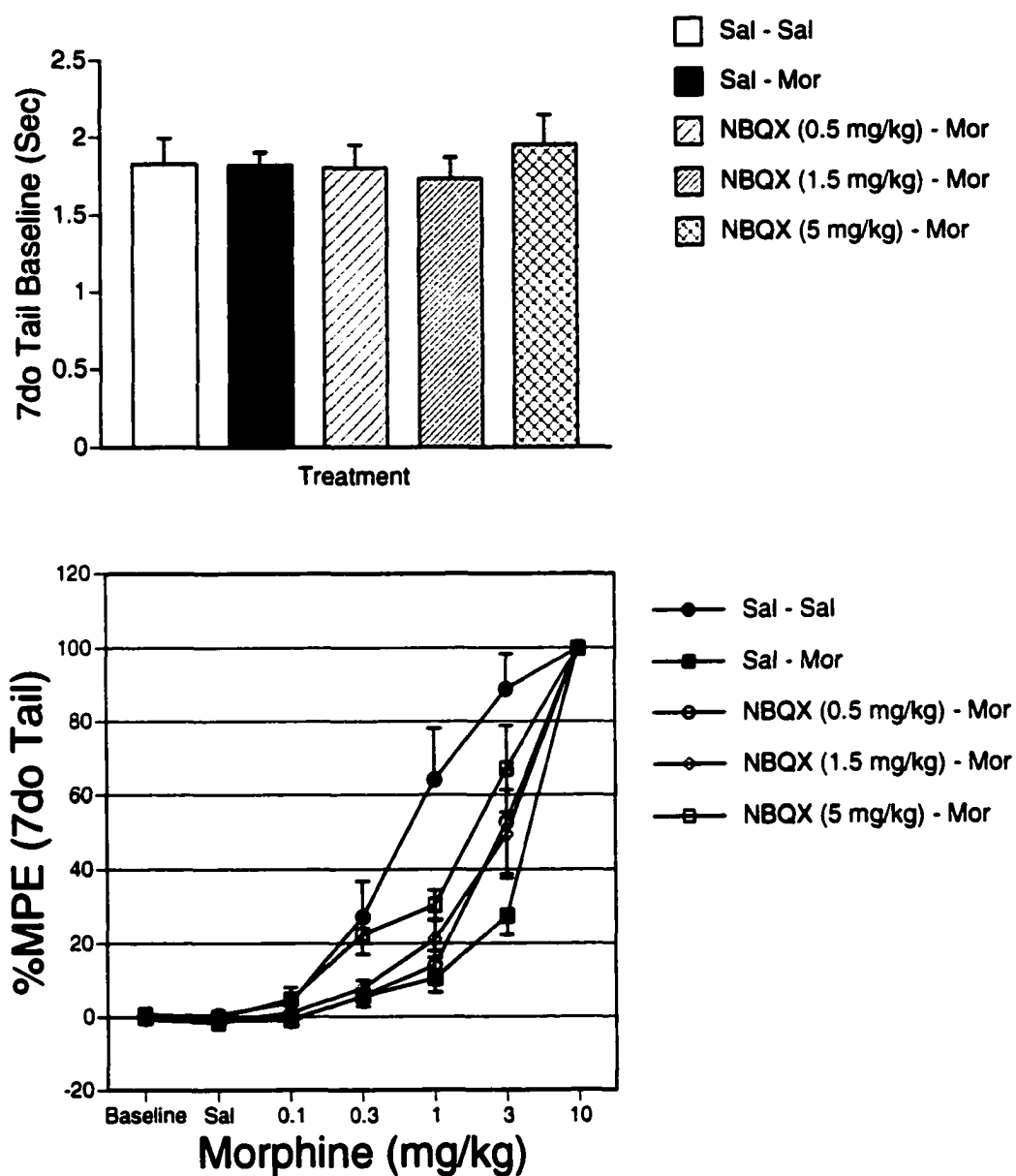


Figure 10-1C: NBQX dose-dependently attenuated morphine tolerance in the 7-day-old rat as assessed by the withdrawal time of the tail from a hotplate.

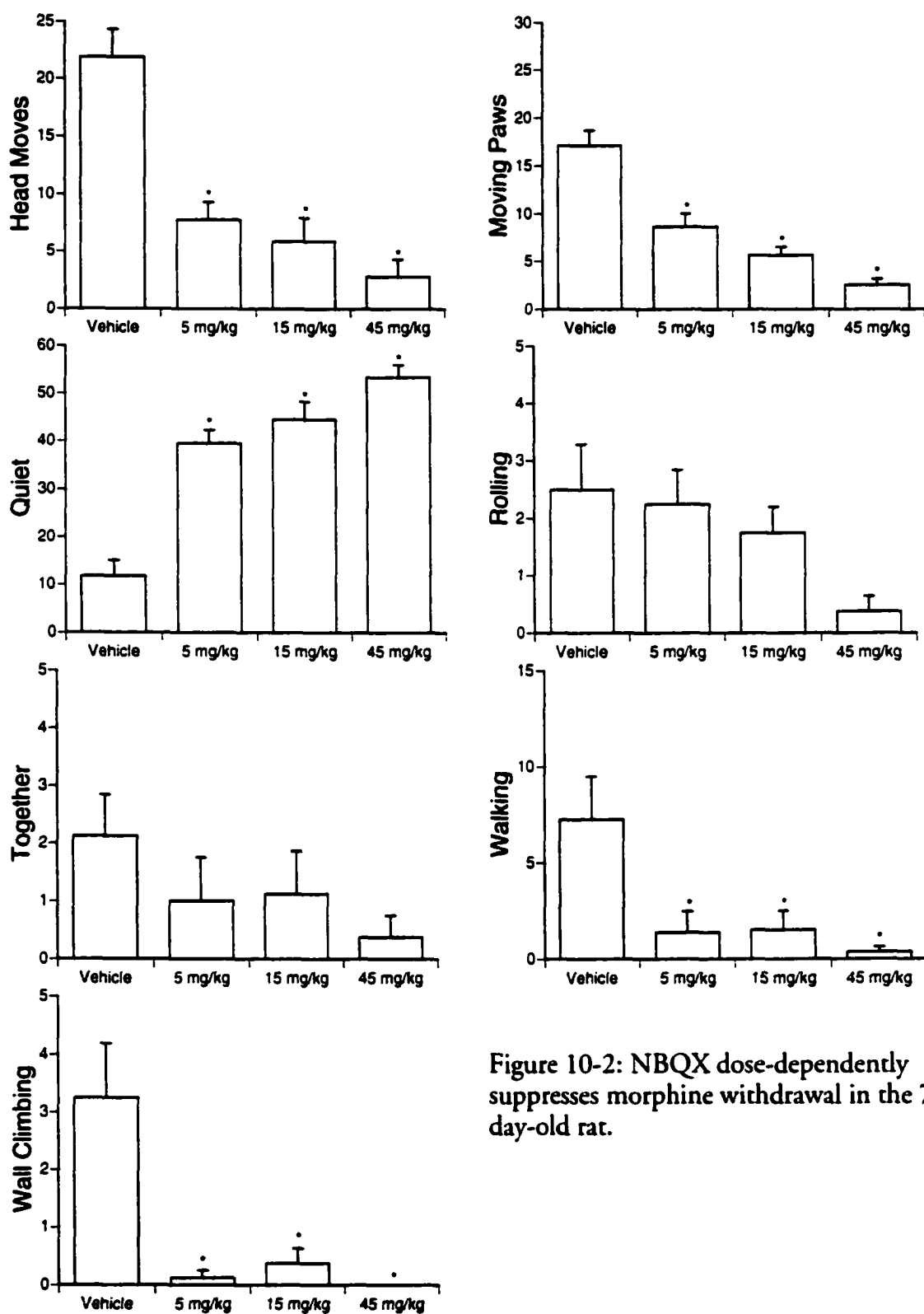


Figure 10-2: NBQX dose-dependently suppresses morphine withdrawal in the 7-day-old rat.

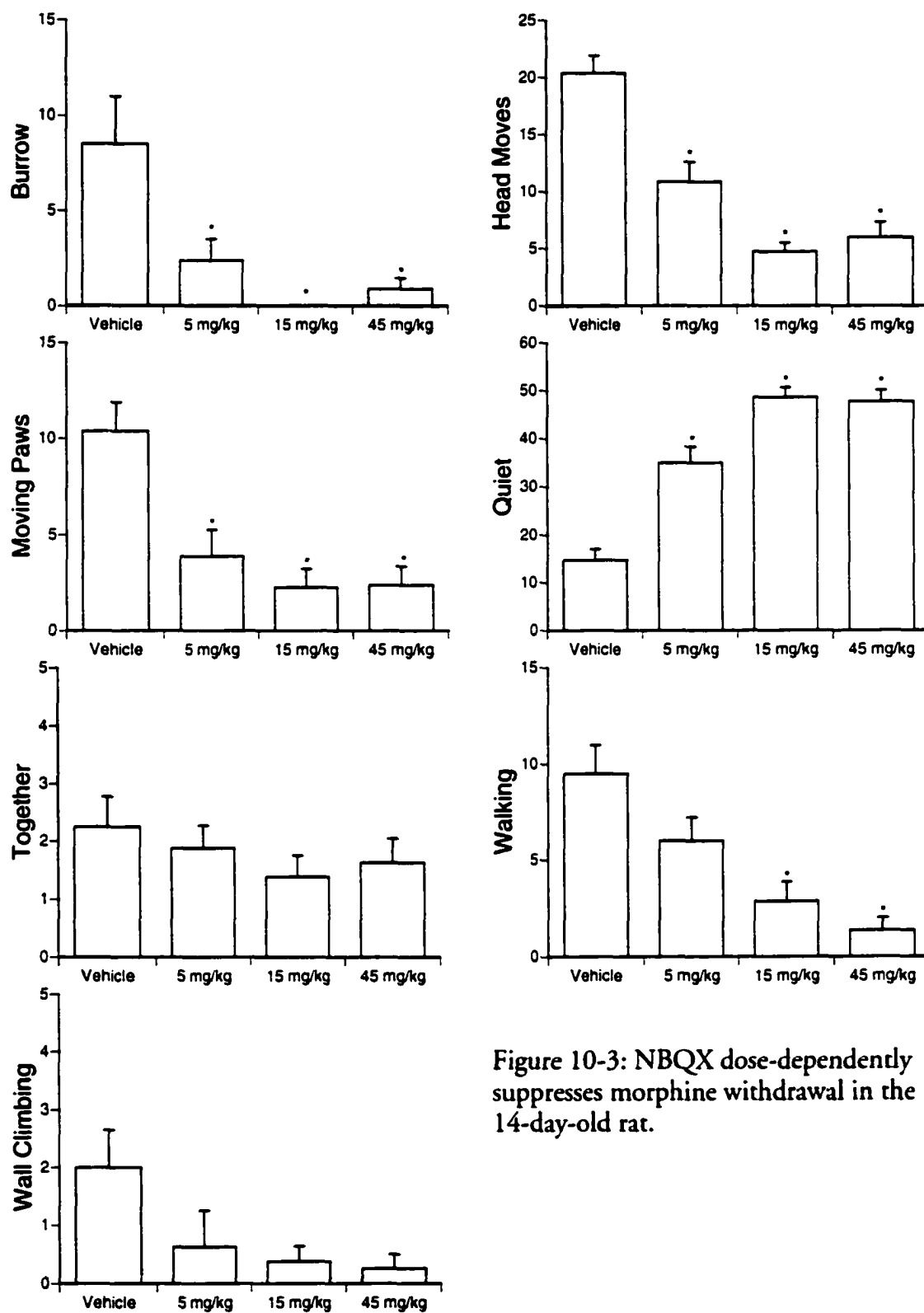


Figure 10-3: NBQX dose-dependently suppresses morphine withdrawal in the 14-day-old rat.

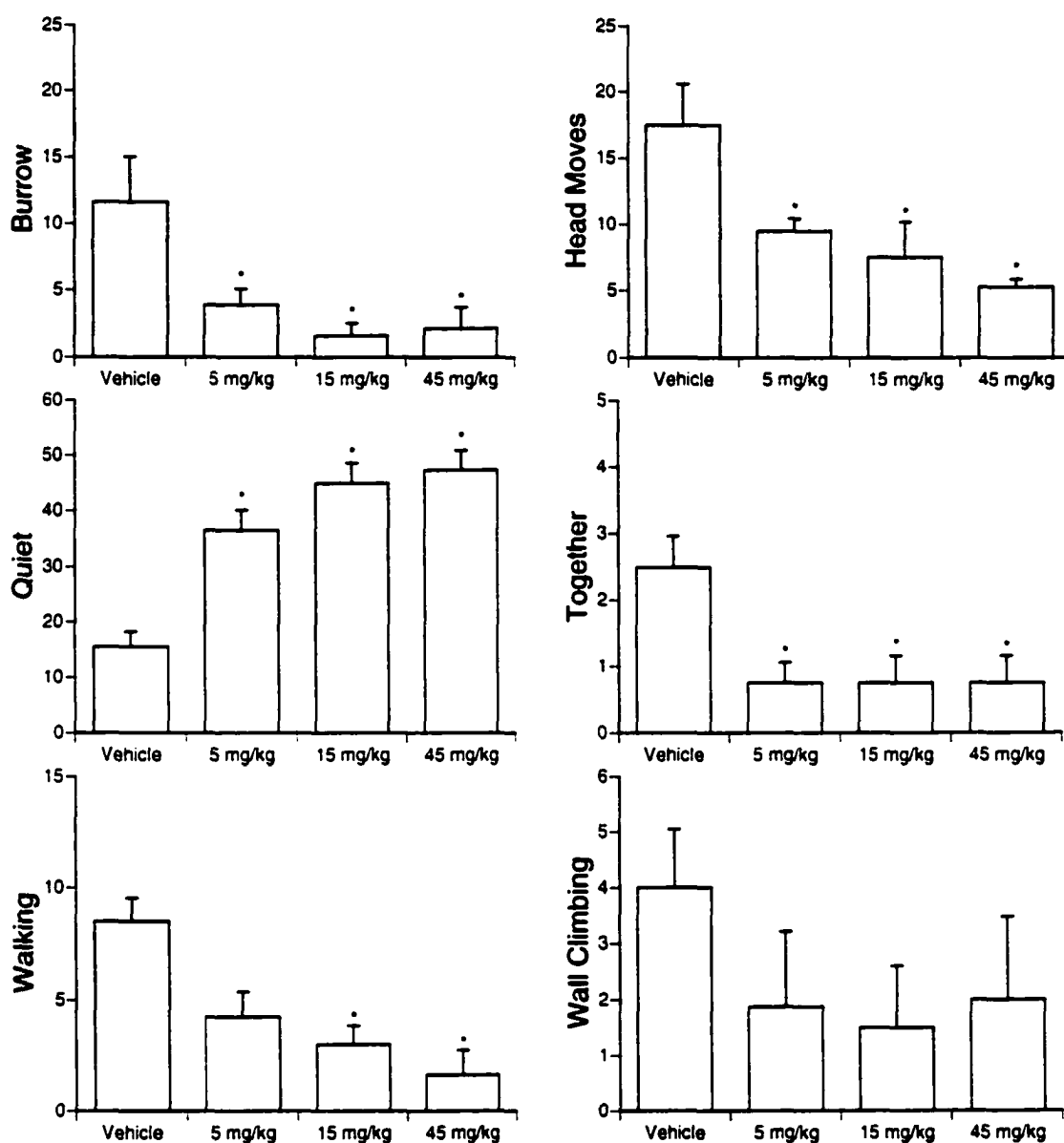
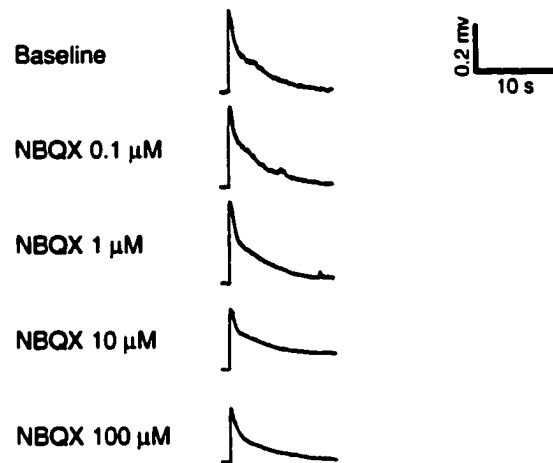


Figure 10-4: NBQX dose-dependently suppresses morphine withdrawal in the 21-day-old rat.

Figure 10-5 NBQX's effect on the sVRP in the 7-day-old rat

(a) Sample recordings NBQX's effect on the sVRP in the 7-day-old rat at different concentrations



(b) Concentration-response curve of NBQX's effect on the sVRP in the 7-day-old rat ($n = 4$)

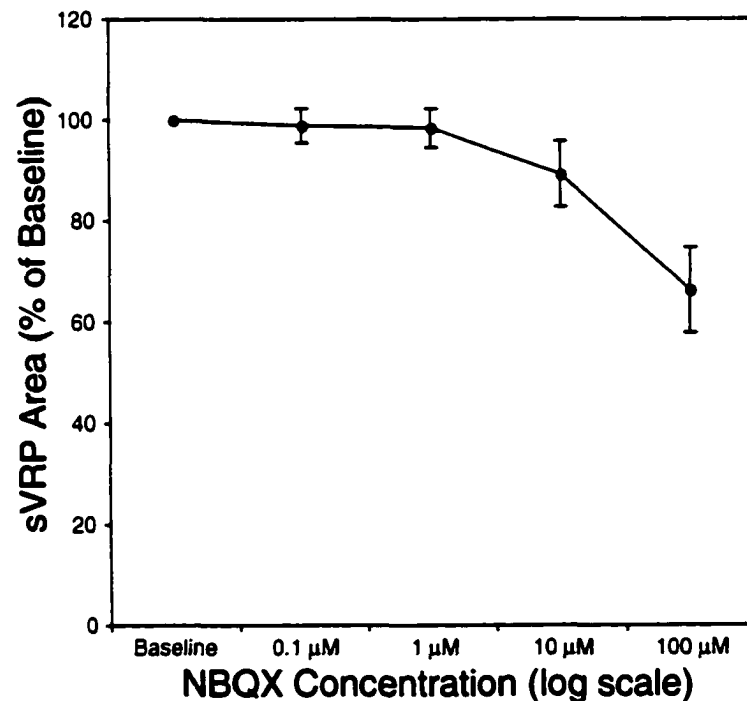


Figure 10-5. NBQX's effect on the slow ventral root potential in the 7-day-old rat.

Figure 10-6

Time and treatment effect of morphine withdrawal in the isolated spinal cord from the 7-day-old rat

A Time course of morphine withdrawal in the isolated rat spinal cord (without NBQX)

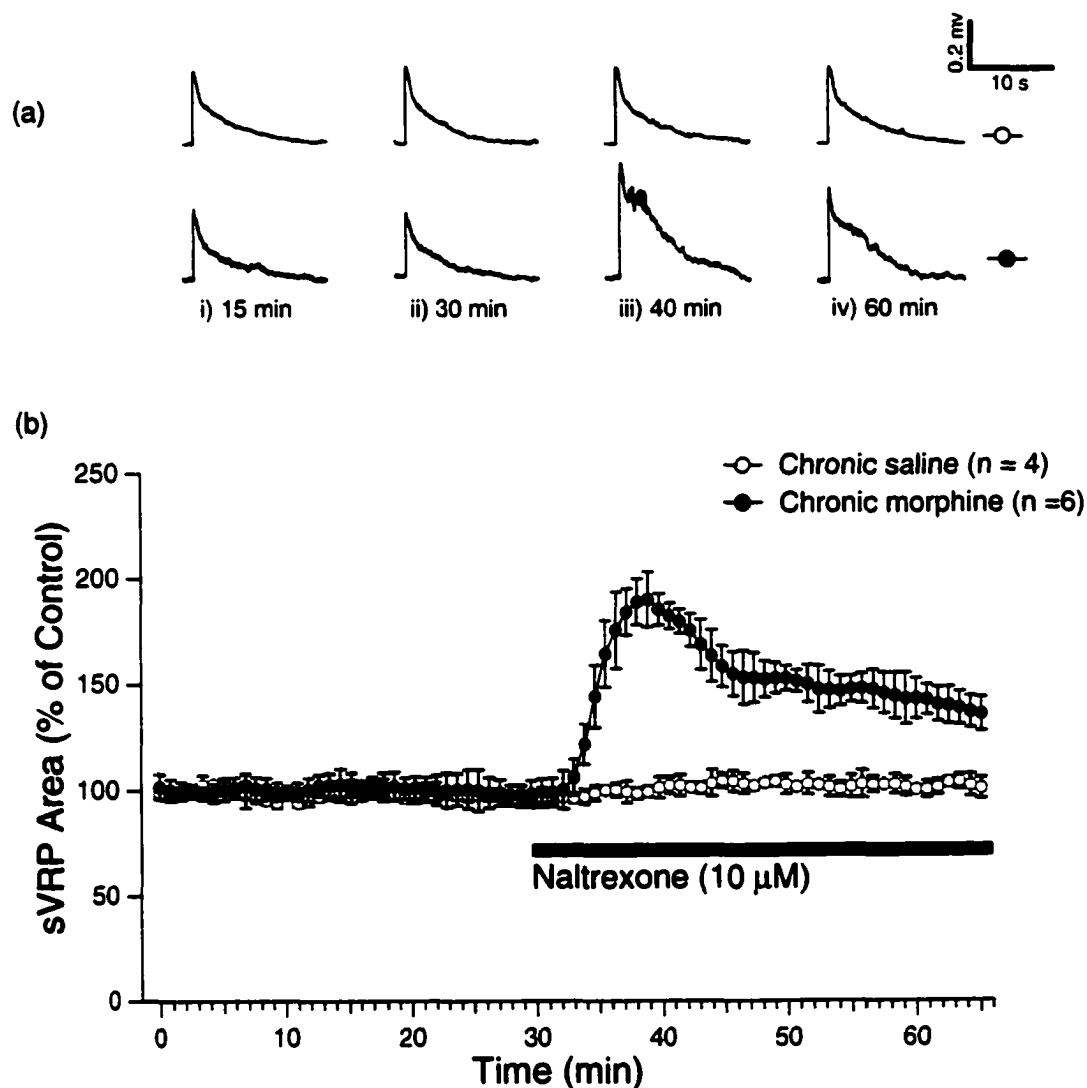


Figure 10-6A. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (without NBQX).

B Time course of morphine withdrawal in the isolated rat spinal cord (with 3 μ M NBQX)

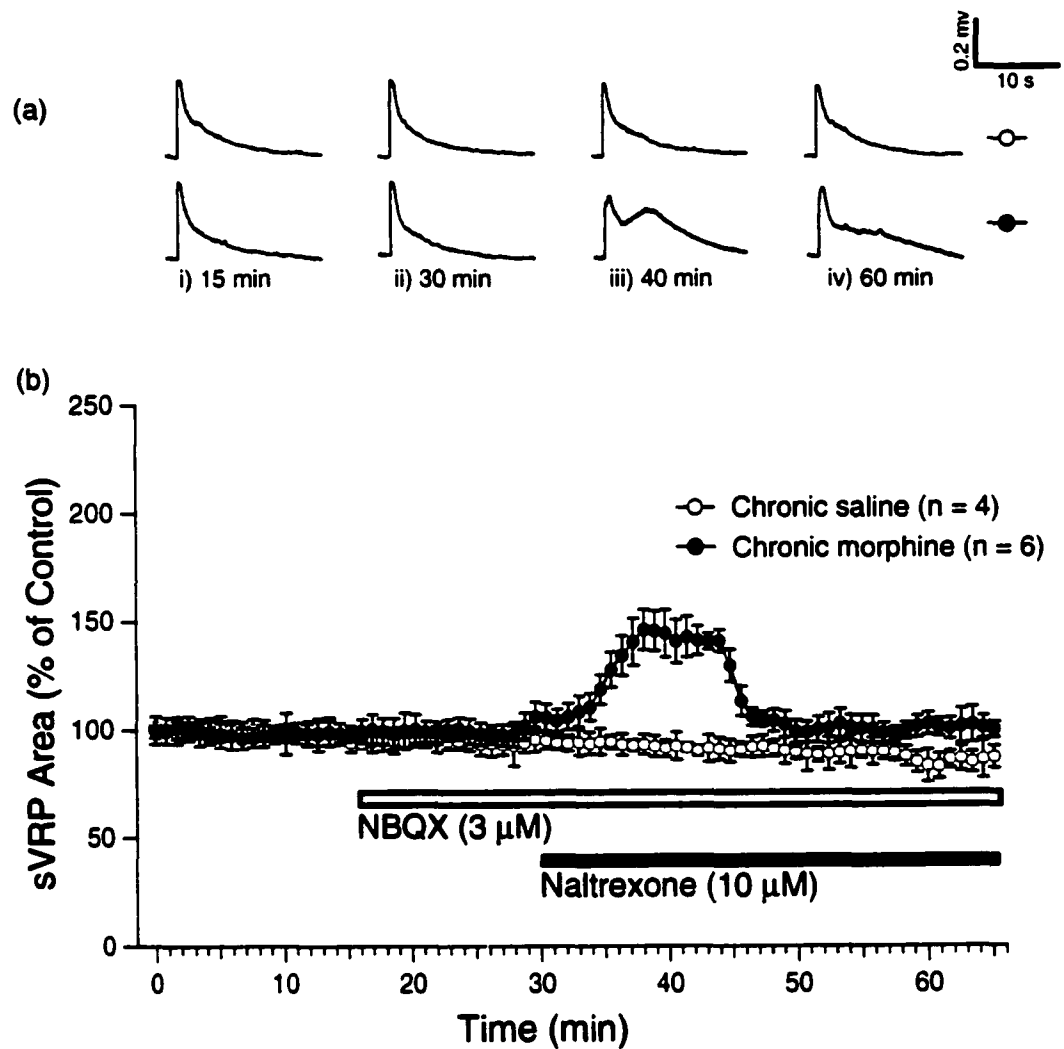
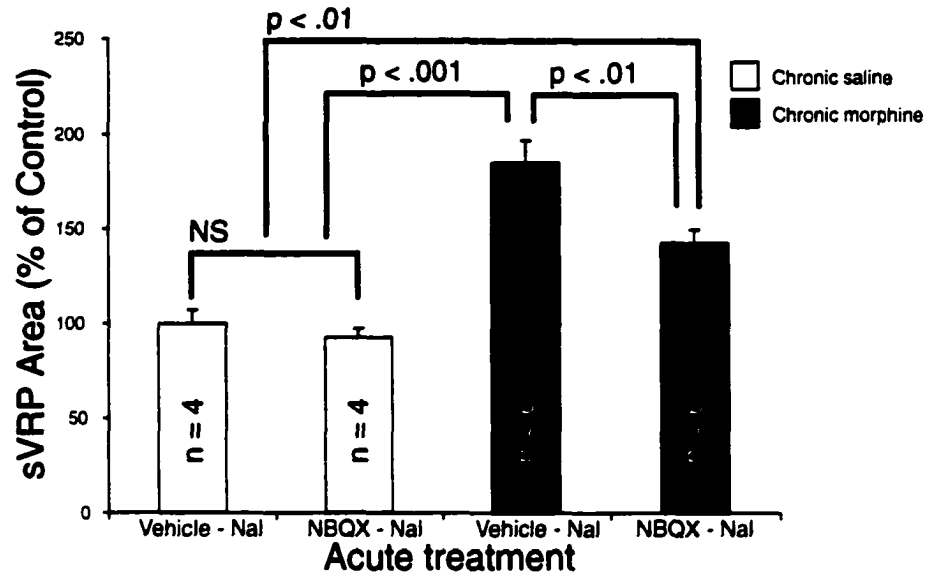


Figure 10-6B. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (with 3 μ M NBQX).

C Treatment effect of NBQX (3 μ M) on morphine withdrawal at 40 min



D Treatment effect of NBQX (3 μ M) on morphine withdrawal at 60 min

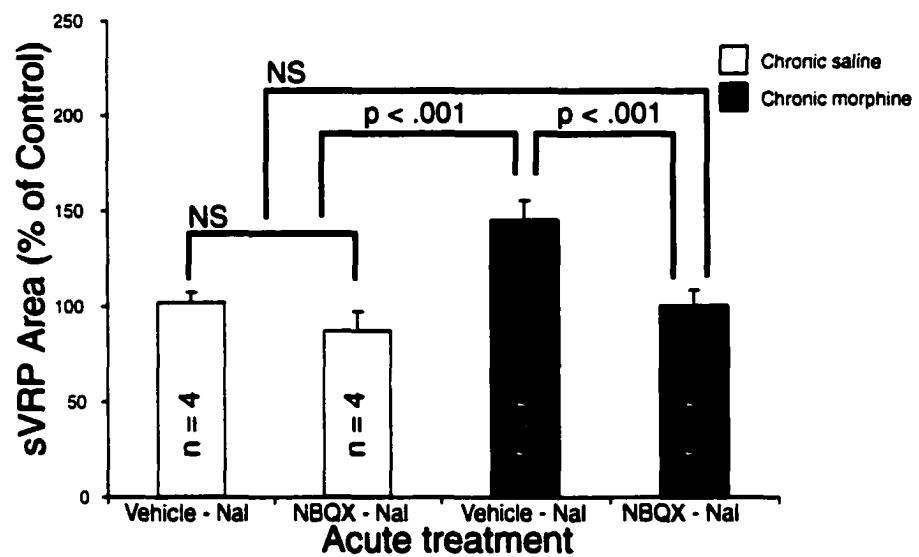


Figure 10-6C and D. Treatment effect of NBQX on morphine withdrawal at 40 and 60 min.

Chapter 11

Ontogeny of mGluR mediated morphine withdrawal

Introduction

For clinical and theoretical reasons, there is increasing interest in the study of opiate withdrawal in the infant rat model (Chapter 1). Although data on mGluRs' critical role in opiate withdrawal in the adult animal are accumulating, it is unknown whether these results in adults apply to infants (Part I). That mGluRs undergo dramatic differential changes during earlier life (Chapter 3) suggests possible developmental differences (Part II). As a result, conclusions based on data derived from adult animals models should not be taken for granted that they would also hold in the developing organism.

Thus, the present study 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. There are two reasons for such an approach: 1.) mGluR undergo dramatic differential changes during earlier life, suggesting that developmental difference would be possible, and therefore, conclusions based on information derived from adult animals models should not be taken for granted that they would also 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 (Conn and Pin, 1997), the in vitro system would be more appropriate than the in vivo model in addressing the pharmacological effect of DCG-IV specific to opiate withdrawal.

Material and methods

Morphine treatment and behavior observation

Pups were tattooed with India ink (Geller and Geller, 1966), which was injected into one or two paws to label individual pups permanently in each litter. To induce morphine dependence, starting when the pups were 1, 8, or 15 days of age, all pups in a litter were removed from the dam and individual rats were injected with morphine sulfate (IP, 10 mg/kg) twice daily (10 a.m. and 6 p.m.) for 6.5 days. The last injection was in the morning of the 7th day. In the afternoon of the 7th day, animals were transported from the animal facility to our laboratory in plastic tubs with wood chip bedding and placed in an observation chamber maintained at approximately 33°C. The pups were treated with DCG-IV (ICV, 3, 15, 75 pmol, or saline as control, n=8 for each dose). DCG-IV was delivered via ICV injection since this compound 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 ICV injection procedure followed a published protocol (Carden et al., 1991). Briefly, during the ICV injection, the pup was held with its head gently bent forward and down. A beveled 30-gauge needle attached to a Hamilton syringe was introduced into the cisterna magna and 4 µl of the drug was injected over a 30-s period. The needle was left in position for another 30 s, then removed. Control groups received the same volume of saline in a similar manner. After the drug treatment, the pup was then placed back into the observation chamber with the remainder of the litter (without the dam). Fifteen minutes later, naltrexone (IP, 1.0 mg/kg) was injected to precipitate withdrawal. Behavior of the pup was observed for 10 min before the treatment of naltrexone (prenaltrexone period) and 15 min after naltrexone injection (post-naltrexone period). Data collected from the prenaltrexone period were used to

analyze the effects of the drugs on the morphine-dependent rat without the influence of naltrexone. During these observation periods, the behavior of the pups was identified every 15 s and recorded on a checklist by an observer (see Table 7-1 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. 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 (Windh et al., 1995; Thornton and Smith, 1997; Thornton et al., 1997) had clearly demonstrated that saline-treated rat pups of comparable age basically remain quiet and do not display withdrawal behaviors.

In vitro electrophysiology

The standard electrophysiology experimental setup (see Chapter 7 for detail) was followed in the present studies. Spinal cords were obtained from 7-day-old rat pups. These rats were treated similarly to those in the behavioral experiments from day 1 to day 7, except that on the 7th day, morphine withdrawal was assessed by the electrophysiological assay instead of the behavioral test. In electrophysiological studies of opiate withdrawal, spinal cords from 7-day-old rats treated with chronic saline or morphine were used. After baseline sVRPs were recorded, naltrexone (10 μ M) is added into the superfusate. Dorsal root evoked ventral root activity is recorded from the ventral root for 30 min subsequent to the introduction of naltrexone into the bath. The sVRP activity is digitized and the integral of the activity above baseline

from 0 to 8 second was computed and defined as the measure of physical withdrawal (Bell and Adler, 1988; Bell and Beglan, 1995a; Feng and Kendig, 1995b; Feng and Kendig, 1997). In studying the acute pharmacological effects of DCG-IV (30nM) on morphine withdrawal, DCG-IV was applied to the ACSF 15 min before the introduction of naltrexone and after which acutely co-administrated with naltrexone.

Application of chemicals

DCG-IV, morphine, and naltrexone were made up as 1000× stock solution and were diluted in oxygenated recording solution immediately prior to use and 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.

For concentration-response curve construction of DCG-IV's effect on the sVRP area, after 15 min of baseline sVRP recording, DCG-IV (10nM, 100nM, 1μM, 10μM) was applied in the superfusate for 30 min each concentration. For withdrawal test, after 15 min of baseline sVRP recording, DCG-IV (30 nM) was applied in the superfusate and 15 min later naltrexone (10 μM) was introduced into the superfusate, after which, both DCG-IV and naltrexone co-existed in the superfusate for the whole length of recording.

Behavior data analysis

Separate statistical tests were conducted for the three age groups and for each of the withdrawal behaviors. Occurrences of each behavior were summed for the 15-min postnaltrexone observation period. All doses of DCG-IV and vehicle 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 7-1. If significant results were detected, individual multiple comparisons were then conducted by Fisher's PLSD post-hoc test.

Electrophysiology data analysis

The effects drugs in any individual spinal cord were determined by comparing the averaged sVRP area 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 (treatment sVRP area). All data points were normalized to the averaged baseline sVRP area and were expressed as percentage of control: (treatment sVRP area)/(baseline sVRP area) $\times$ 100. All values are expressed as means $\pm$ 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 activities, baseline ventral root potential measurements were established before naltrexone being added to the spinal superfusate. The morphine withdrawal-induced ventral root potential augment is expressed as a percentage of the baseline sVRP. One-way ANOVAs were used to test significance of treatment effects among different treatment groups followed by Fisher's PLSD post-hoc test if significant results were detected.

For both behavioral and electrophysiological data statistical analyses, effects were considered significant if $P < .05$.

Results

DCG-IV attenuated behavioral signs of morphine withdrawal in the 7-day-old rat

The data (N = 32) for the pre-naltrexone period show that treatment with various doses DCG-IV in the 7-day-old rat did not significantly alter the behaviors of

the morphine-dependent rat (Table 11-4 for summary of statistical analysis results). Similar to the control group, except for some baseline activities, all experimental groups largely remained quiet (Table 11-1 for summary of data).

The results (N = 32) for the post-naltrexone period show that treatment with DCG-IV significantly reduced most of the morphine withdrawal behaviors precipitated by naltrexone in the 7-day-old rat (Table 11-4 for summary of statistical analysis results). DCG-IV dose dependently decreased head moves, moving paws, together and walking behaviors and increased quiet behavior. We failed to find any significant effect on wall-climbing and rolling behavior for pretreatment with DCG-IV. The dose effects of pre-treatment of DCG-IV on naltrexone-precipitated morphine withdrawal in the 7-day-old rat are depicted in Figure 11-1.

DCG-IV attenuated behavioral signs of morphine withdrawal in the 14-day-old rat

The data (N = 32) for the pre-naltrexone period show that treatment with various doses DCG-IV in the 14-day-old rat did not significantly alter the behaviors of the morphine-dependent rat (Table 11-4 for summary of statistical analysis results). Similar to the control group, except for some baseline activities, all experimental groups largely remained quiet (Table 11-2 for summary of data).

The results (N = 32) for the post-naltrexone period show that treatment with DCG-IV significantly reduced most of the morphine withdrawal behaviors precipitated by naltrexone in the 14-day-old rat (Table 11-4 for summary of statistical analysis results). DCG-IV dose dependently decreased burrow, head moves and walking behaviors and increased quiet behavior. We did not find any significant effect on moving paws, together wall-climbing behavior for pretreatment with DCG-IV. The dose effects of pre-treatment of DCG-IV on naltrexone-precipitated morphine withdrawal in the 14-day-old rat are depicted in Figure 11-2.

DCG-IV attenuated behavioral signs of morphine withdrawal in the 21-day-old rat

The data (N = 32) for the pre-naltrexone period show that treatment with various doses DCG-IV in the 21-day-old rat did not significantly alter the behaviors of the morphine-dependent rat (Table 11-4 for summary of statistical analysis results). Similar to the control group, except for some baseline activities, all experimental groups largely remained quiet (Table 11-3 for summary of data).

The results (N = 32) for the post-naltrexone period show that treatment with DCG-IV significantly reduced most of the morphine withdrawal behaviors precipitated by naltrexone in the 21-day-old rat (Table 11-4 for summary of statistical analysis results). DCG-IV dose dependently decreased burrow, head moves, together and walking behaviors and increased quiet behavior. We did not find any significant effect on wall-climbing behavior for pretreatment with DCG-IV. The dose effects of pre-treatment of DCG-IV on naltrexone-precipitated morphine withdrawal in the 21-day-old rat are depicted in Figure 11-3.

DCG-IV's effect on sVRP area in the 7-day-old rat

Genesis of the sVRP relies on the functioning of many synaptic transmissions (Thompson et al., 1992; Thompson et al., 1993; Collins et al., 1995) whereas high concentrations of DCG-IV significantly inhibit normal synaptic transmission (Ishida et al., 1993; Watkins and Collingridge, 1994). Although there is one report examining DCG-IV's effect on some components of the sVRP in the 3-4 day old rat (Ishida et al., 1993), there is a lack of quantified dose-response studies of DCG-IV's effect on sVRP in the 7-day-old rat. Age is a major concern since the effect of DCG-IV in regulating synaptic transmission is highly age-dependent (Ross et al., 2000). Thus, the goal of this experiment was to determine a suitable concentration of DCG-IV that does not alter the baseline sVRP for later experiments.

The spinal cords from 4 male naive 7-day-old rats were used to determine the concentration-response curve of DCG-IV's effect on the sVRP in at this age. 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, $P > .05$) (Figure 11-4a and b). Although statistically not significant, that is more likely due to that we used a relatively small sample size rather than a lack of effect of DCG-IV at this concentration. 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-IV's effect on the sVRP in the 7-day-old rat is depicted in Figure 11-4. 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 to study DCG-IV's effect on morphine withdrawal at a concentration of 30 nM.

Spinal cords from morphine treated rats demonstrated robust withdrawal in vitro

Stable recording of the sVRP can be made from spinal cord of the neonatal rat for up to many hours (Kerkut and Bagust, 1995; Herrero et al., 2000). In our experiments, 7-day-old rats that did not receive chronic morphine treatment ($n = 4$) demonstrated excellent stability in generating sVRP over time (Figure 11-5Aa upper traces and Figure 11-5Ab), 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 ranging from $94.55 \pm 5.53\%$ of control to $102.98 \pm 1.78\%$ 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 (Figure 11-5Aa lower traces at 15

and 30 min). However, when naltrexone (10 μ M) was added to the superfusate, sVRP from spinal cords from morphine treated rats rapidly increased to reach a peak in approximately 10 min after naltrexone was added in ($206 \pm 22.24\%$ of control, $P < .01$, $n = 6$). The intensity of this augment 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 in) (Figure 11-5Aa lower traces at 40 and 60 min and Figure 11-5Ab). 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. Thus, consistent with studies by others (Bell and Beglan, 1995a), we have 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 DCG-IV's effect in morphine withdrawal in the next experiment.

DCG-IV 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 (Figure 11-5Ba upper traces and Figure 11-5Bb), the addition of DCG-IV (30 nM) and the later addition of naltrexone (10 μ M) to the superfusate did not alter the sVRP area value significantly at any time point of recording (sVRP area values ranging from $91.84 \pm 5.02\%$ of control to $100.53 \pm 6.57\%$ of control, $P > .05$ at all time points). Thus,

neither DCG-IV (30nM) nor DCG-IV (30nM) together with 10 μ M naltrexone significantly altered baseline sVRP in morphine naive rats.

Rats that received chronic morphine treatment ($n = 6$) demonstrated similarly stable recording in the normal perfusion medium and after DCG-IV (30 nM) was added in. When naltrexone (10 μ M) was added to the superfusate, sVRP from spinal cords from morphine treated rats increased slightly but not significantly in the first 20 min after naltrexone was added in (maximal $113.83 \pm 13.15\%$ of control, $P > .05$, $n = 6$). After which, the sVRP then gradually increased to reach a relatively stable elevated plateau (approximately 125% of control, $P > .05$ at some time points whereas $P < .05$ 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) (Figure 11-5Ba lower traces and Figure 11-5Bb). 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.

To better compare the treatment effect on morphine withdrawal among various chronic treatment (morphine 10mg/kg) and acute treatment (DCG-IV 30nM) groups, we have extracted data points from two regions at 38-42 min and 58-62 min respectively; thus, data points in each of these two periods were averaged for treatment effect analyses. The time effect analyses detailed above demonstrated that this two temporal regions can probably best describe morphine withdrawal in the present protocol.

As expected, regardless of acute treatment conditions, spinal cords from rats that did not receive chronic morphine treatment did not show significant difference

from each other at either 40 min or 60 min, indicating that the present electrophysiological assay was stable and DCG-IV (30 nM) on baseline sVRP is not significant (Figure 11-5C and D)

At 40 min, sVRP of the morphine treated spinal cord significantly increased to about 200% of control, indicating that chronic treatment of morphine induced morphine dependence in the rat spinal cord and addition of naltrexone into the superfusate precipitate morphine withdrawal. Pre-treatment of DCG-IV (30 nM) almost completely suppressed this naltrexone precipitated morphine withdrawal (Figure 11-5C).

At 60 min, sVRP value of the morphine treated spinal cord still remained significantly higher than control at about 150% of control. In contrast to the data at 40 min, although pre-treatment of DCG-IV (30 nM) significantly suppressed this naltrexone precipitated morphine withdrawal to 120% of control, it did not completely suppressed morphine withdrawal since the sVRP area value from this group is still significantly higher than the control groups that did not receive morphine treatment (Figure 11-5 D).

Discussion

Despite decades of research, the mechanism underlying opiate withdrawal remains elusive (Trujillo, 1999; Nestler, 2001). It has been suggested that multiple mechanisms are involved (Redmond and Krystal, 1984; Thorat et al., 1994). One possibility is that chronic morphine treatment upregulate the cAMP pathway and withdrawal from opiates result in hyperactivity of the cAMP system (Nestler and Tallman, 1988; Nestler, 2001). Another possibility is that opiate withdrawal results in augmented central release of glutamate, which in turn contribute to the hyperactivity

typifying opiate withdrawal (Akaoka and Aston-Jones, 1991; Aghajanian et al., 1994; Tokuyama et al., 1996). These two mechanisms may interact with each other, thus the hyperactivity during opiate withdrawal manifested by behavioral, electrophysiological and biochemical indices may have a dual-mechanism cause (Aghajanian et al., 1992).

Although there is direct evidence supporting the role of glutamate in opiate withdrawal syndrome (Tokuyama et al., 1996), it is less clear which glutamate receptors are responsible for 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 Trujillo and Akil, 1991a; Elliott et al., 1995a; Herman et al., 1995; Pasternak et al., 1995; Trujillo, 1995; Trujillo and Akil, 1995; Inturrisi, 1997; Danysz and Parsons, 1998; Mao, 1999). In contrast to these findings using the adult animal model, recent studies from our laboratory (Zhu and Barr, 2000, 2001b, c) and others (Bell and Beglan, 1995a, b) showed that NMDA receptor antagonists are not effective in suppressing morphine withdrawal in rats younger than a week of age, whereas partially effective in the 14-day-old rat and fully effective after 21-day-old (Zhu and Barr, 2001b). The reason for this age-dependent effectiveness of NMDA receptor antagonists in suppressing opiate withdrawal has been attributed to the dramatic ontogenetic differential changes of the subunits of the NMDA receptor during earlier life (Zhu and Barr, 2001b, c).

In addition to acting on ionotropic glutamate receptors such as the NMDA receptor, glutamate also acts on the metabotropic glutamate receptors. In the past few years, several laboratories, using a variety methodology ranging from behavioral to electrophysiological, investigated the role of mGluRs on opiate tolerance (Popik et al., 2000a), dependence and withdrawal in rats and mice (Fundytus and Coderre, 1994, 1997; Fundytus et al., 1997; Klodzinska et al., 1999; Vandergriff and Rasmussen,

1999). Chronic antagonism of mGluRs in the brain with the nonselective antagonist MCPG, as well as with 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 the group II selective agonist DCG-IV just prior to the precipitation of withdrawal dramatically reduced the severity of precipitated withdrawal symptoms (Fundytus and Coderre, 1994, 1997; Fundytus et al., 1997; for review, see Fundytus and Coderre, 1999). The novel systemically active group II mGluR agonist LY 354740 has also recently been shown to inhibit the naloxone induced symptoms of morphine withdrawal in morphine-dependent mice (Klodzinska et al., 1999). In addition, LY 354740 has been reported by another group to attenuate 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 also inhibited by metabotropic group II glutamate agonist LY 354740 in mice (Popik et al., 2000a). 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.

Although evidence suggesting that mGluRs play a critical role in opiate withdrawal are accumulating rapidly, 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. The neonatal CNS is both structurally and functionally different from that of the adult, and significant changes in opioid actions occur both prenatally and postnatally (Barr, 1992, 1993; Fitzgerald, 1995). At the same time, the mGluR, which is believed to play an essential role in opiate dependence, undergoes qualitative and quantitative changes during development (Chapter 3).

Thus, the present study 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. The data of present study clearly show that the group II mGluR agonist DCG-IV was 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, DCG-IV was also effective in suppressing withdrawal behaviors in both the 14- and 21-day-old rats. Since morphine withdrawal involves both elevated cAMP level and augmented glutamate release the CNS, DCG-IV attenuate morphine withdrawal in two most likely mechanisms.

Firstly, it is well known that whereas acute administration of morphine decreases cAMP production, during chronic administration cAMP system returns to control level (Sharma et al., 1975; Nestler and Tallman, 1988; Chieng and Williams, 1998; Bordi and Ugolini, 1999), suggesting that compensatory mechanisms are elicited. Withdrawal from morphine relieves the inhibition of cAMP by acute morphine and consequently unmasks the superactivation of the cAMP pathway produced by the cellular adaptive changes and results in the hyperactivity seen during morphine withdrawal (Nestler, 2001). Both opioid receptors and mGluRs share common pools of intracellular second messenger systems (Fundytus and Coderre, 1997), and acute DCG-IV decreases cellular cAMP levels (Pin and Duvoisin, 1995; Conn and Pin, 1997). Thus, it is possible that the marked decrease of morphine withdrawal we have seen was due to the effectiveness of DCG-IV in suppressing the cAMP level during morphine withdrawal.

Secondly, there is broad and persuasive evidence supporting the role of glutamatergic receptors in mediating morphine withdrawal in brain and spinal cord (for review, see Mao, 1999; Trujillo, 1999). An increase in glutamate release is generally observed during opiate withdrawal (Rasmussen and Aghajanian, 1989;

Akaoka and Aston-Jones, 1991; Desole et al., 1996; Jhamandas et al., 1996; Tokuyama and Ho, 1996; Hoshi et al., 1997; Huang et al., 1997). For example, naloxone-precipitated withdrawal from morphine 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 precipitated withdrawal signs in a dose-dependent manner, whereas prominent behavioral changes were not observed when animals continuously infused with saline were challenged with glutamate (Tokuyama et al., 1996). Hence, these results provide direct evidence that glutamate is essential for the expression of opiate withdrawal signs. The rapid release of glutamate from locus coeruleus and other regions under an opiate dependent state has been hypothesized to be triggers or key elements for the expression of opiate withdrawal signs (Rasmussen, 1991; Christie et al., 1997). Consistent throughout the CNS, one of the most prominent physiologic effects of mGluR agonists is the reduction of transmission at glutamatergic synapses (Anwyl, 1999). This effect is typically mediated by presynaptic mGluRs that serve as autoreceptors to reduce glutamate release (Miller, 1998). Since augmented glutamate release is a prominent characteristic of opiate withdrawal, it was possible that the acute treatment of DCG-IV in both the in vivo and in vitro assays have inhibited the exaggerated glutamate release typical of opiate withdrawal and thus attenuated both behavioral and electrophysiological manifestations of morphine withdrawal.

Some potential sites of action of DCG-IV in suppressing withdrawal by either decreasing cAMP level or attenuating glutamate release or combination of both mechanisms include those thought to be involved in opiate withdrawal and having the highest densities of group II mGluRs. Such sites include the locus coreleus,

hypothalamus, amygdala, periaqueductal grey area, and the spinal cord (Maldonado et al., 1992; Petralia et al., 1996). 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 elucidate which subtype is more important in opiate withdrawal.

In the *in vitro* study, we found that DCG-IV was more effective in suppressing the first stage than the second stage of morphine withdrawal in the spinal cord. Interestingly, in a parallel study (Chapter 10), we found that the competitive AMPA receptor antagonist NBQX was more effective in suppressing the second stage than the first stage of morphine withdrawal in the isolated spinal cord. By contrasting the pharmacological properties of these two agents and their different effects on morphine withdrawal and considering that morphine withdrawal involves dual mechanisms (Aghajanian et al., 1992), it seems that as in the locus coeruleus (Aghajanian et al., 1992), in the spinal cord morphine withdrawal is due to the excitation resulted from a combination of at least two factors: elevated cAMP level and augmented glutamate release. We further hypothesize that: 1.) the peak of morphine withdrawal we observed (10 min after naltrexone) is due to the temporal overlap of action from two excitatory systems, namely the cAMP second messenger system and the glutamate neural transmission system; 2.) the 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 and thus, after the initial withdrawal peak due to the dual-action of both the cAMP and glutamate systems, augmented glutamate release was then mainly responsible for the elongated but attenuated morphine withdrawal plateau. Therefore, in the present study, DCG-IV could have effectively suppressed the excitatory effect of elevated cAMP levels whereas not effective in suppressing the excitation due to the augmented glutamate release, resulting a differential suppression of the first stage of the *in vitro* withdrawal sign. In

contrast, in our parallel studies (Chapter 10), NBQX differentially suppressed the second stage of morphine withdrawal because NBQX directly blocked one of the major receptor types on which glutamate acts on whereas do not directly modulate the cAMP second messenger system. Further examination is necessary to differentiate these two processes.

In conclusion, acute treatment of the group II mGluR agonist DCG-IV is effective in suppressing morphine withdrawal in the infant rat and this effect is not age-dependent. Although great hopes for NMDA receptor antagonists in the therapy of opiate withdrawal are promised by the overwhelming pre-clinical data (for review, see Herman et al., 1995; Inturrisi, 1997; Mao, 1999) and clinical evidence in both the United States (Rosen et al., 1996; Bisaga et al., 2001) and other countries (Koyuncuoglu et al., 1990; Koyuncuoglu, 1995), their future in the infant are hampered by the finding that NMDA receptor antagonists are not effective in suppressing opiate withdrawal in the infant (Bell and Beglan, 1995a; Zhu and Barr, 2000, 2001b, c). In addition, that blockade of NMDA receptors results in apoptotic neurodegeneration in the developing CNS (Ikonomidou et al., 1999) further preclude the use of NMDA receptor antagonist for the treatment of opiate withdrawal in human infants. The mGluRs therefore provide a novel target for development of therapeutic agents that could have a dramatic impact on treatment of opiate withdrawal in human patients of all ages.

Table 11-1. Behavior occurrences after treatment of DCG-IV but before treatment of naltrexone in the morphine-dependent rat of 7 days old.

Behavior (7do)	DCG-IV Dose			
	Vehicle	3 pmol	15 pmol	75 pmol
Head Moves	.125 ± .125	.500 ± .327	.375 ± .183	.125 ± .125
Moving Paws	.250 ± .164	.250 ± .164	.500 ± .267	.500 ± .189
Quiet	59.375 ± .324	59.250 ± .412	58.875 ± .479	59.250 ± .313
Rolling	.125 ± .125	0.000 ± 0.000	.250 ± .164	.250 ± .164
Together	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Walking	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Wall Climbing	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

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

Table 11-2 Behavior occurrences after treatment of DCG-IV but before treatment of naltrexone in the morphine-dependent rat of 14 days old.

Behavior (14do)	DCG-IV Dose			
	Vehicle	3 pmol	15 pmol	75 pmol
Burrow	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Head Moves	.750 ± .491	1.125 ± .639	1.250 ± .526	2.125 ± .875
Moving Paws	.250 ± .250	.125 ± .125	.125 ± .125	.250 ± .164
Quiet	58.625 ± .625	57.625 ± 1.179	56.875 ± 1.469	55.875 ± 1.231
Together	.500 ± .378	.250 ± .164	.375 ± .183	.375 ± .183
Walking	.500 ± .378	.625 ± .420	1.000 ± .567	.875 ± .479
Wall Climbing	0.000 ± 0.000	.250 ± .250	.250 ± .250	0.000 ± 0.000

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

Table 11-3. Behavior occurrences after treatment of DCG-IV but before treatment of naltrexone in the morphine-dependent rat of 21 days old.

Behavior (21do)	DCG-IV dose			
	Vehicle	3 pmol	15 pmol	75 pmol
Burrow	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	.125 ± .125
Head Moves	.875 ± .295	1.250 ± .620	1.625 ± .460	1.500 ± .732
Quiet	58.000 ± .500	56.750 ± 1.176	56.875 ± .854	55.750 ± 1.411
Together	.250 ± .164	.250 ± .164	.125 ± .125	.625 ± .375
Walking	.375 ± .183	.750 ± .313	1.000 ± .378	1.750 ± .901
Wall Climbing	.375 ± .263	.750 ± .412	.250 ± .164	0.000 ± 0.000

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

Table 11-4. Statistical analyses results for the dose effect of DCG-IV 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	
	F	P	F	P	F	P
Pre-Naltrexone	Burrow	ND	ND	ND	ND	ND
	Head Moves	1.0920	0.3741	0.9270	0.4452	0.5680
	Moving Paws	1.4000	0.2706	0.2590	0.8539	ND
	Quiet	1.0680	0.3840	1.1520	0.3514	1.3970
	Rolling	1.2620	0.3128	ND	ND	ND
	Together	ND	ND	0.1630	0.9202	0.8870
	Walking	ND	ND	0.2630	0.8511	1.7620
	Wall Climbing	ND	ND	0.6360	0.5999	1.5040
Post-Naltrexone	Burrow	ND	ND	5.9770	0.0041	7.2370
	Head Moves	9.7380	0.0003	14.2540	0.0001	9.5000
	Moving Paws	17.2820	0.0001	1.4090	0.2680	ND
	Quiet	52.7270	0.0001	11.5050	0.0001	16.9820
	Rolling	0.6910	0.5680	ND	ND	ND
	Together	4.0670	0.0200	0.3940	0.7583	4.0580
	Walking	7.9870	0.0010	4.6350	0.0122	7.7470
	Wall Climbing	2.1710	0.1216	1.9070	0.1595	0.6030

ND: this behavior was not displayed

Bold face: result is statistically significant

df = (3, 28) in all cases

Figure 11-1. 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-day-old rat. Ordinate: Mean occurrences (Mean $\pm$ one S.E.M.) in 15 minutes of opiate withdrawal behaviors (definition see Table 7-1). Abscissa: DCG-IV doses. Withdrawal was precipitated by naltrexone (1 mg/kg) in all groups. *, $p < 0.05$ compared to the saline control group.

Figure 11-2. 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 14-day-old rat. Ordinate: Mean occurrences (Mean $\pm$ one S.E.M.) in 15 minutes of opiate withdrawal behaviors (definition see Table 7-1). Abscissa: DCG-IV doses. Withdrawal was precipitated by naltrexone (1 mg/kg) in all groups. *, $p < 0.05$ compared to the saline control group.

Figure 11-3. 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 21-day-old rat. Ordinate: Mean occurrences (Mean $\pm$ one S.E.M.) in 15 minutes of opiate withdrawal behaviors (definition see Table 7-1). Abscissa: DCG-IV doses. Withdrawal was precipitated by naltrexone (1 mg/kg) in all groups. *, $p < 0.05$ compared to the saline control group.

Figure 11-4. DCG-IV's effect on the slow ventral root potential area in the isolated spinal cord of the 7-day-old rat

(a) Sample recordings of different concentrations of DCG-IV's effect on the sVRP in the 7-day-old rat. All traces are from the recordings of one rat.

(b) Concentration-response of DCG-IV's effect on the slow ventral root potential area in the 7-day-old rat. 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).

Figure 11-5. Time and treatment effect of morphine withdrawal in the isolated spinal cord from the 7-day-old rat

A: Time course of morphine withdrawal in the isolated rat spinal cord (without DCG-IV)

(a) upper trace: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated chronic 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)

B: Time course of morphine withdrawal in the isolated rat spinal cord (with 30nM DCG-IV)

(a) upper trace: sample recordings at 15, 30, 40, and 60 min of the sVRP from a rat treated chronic 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 (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)

C: 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.

D: 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.

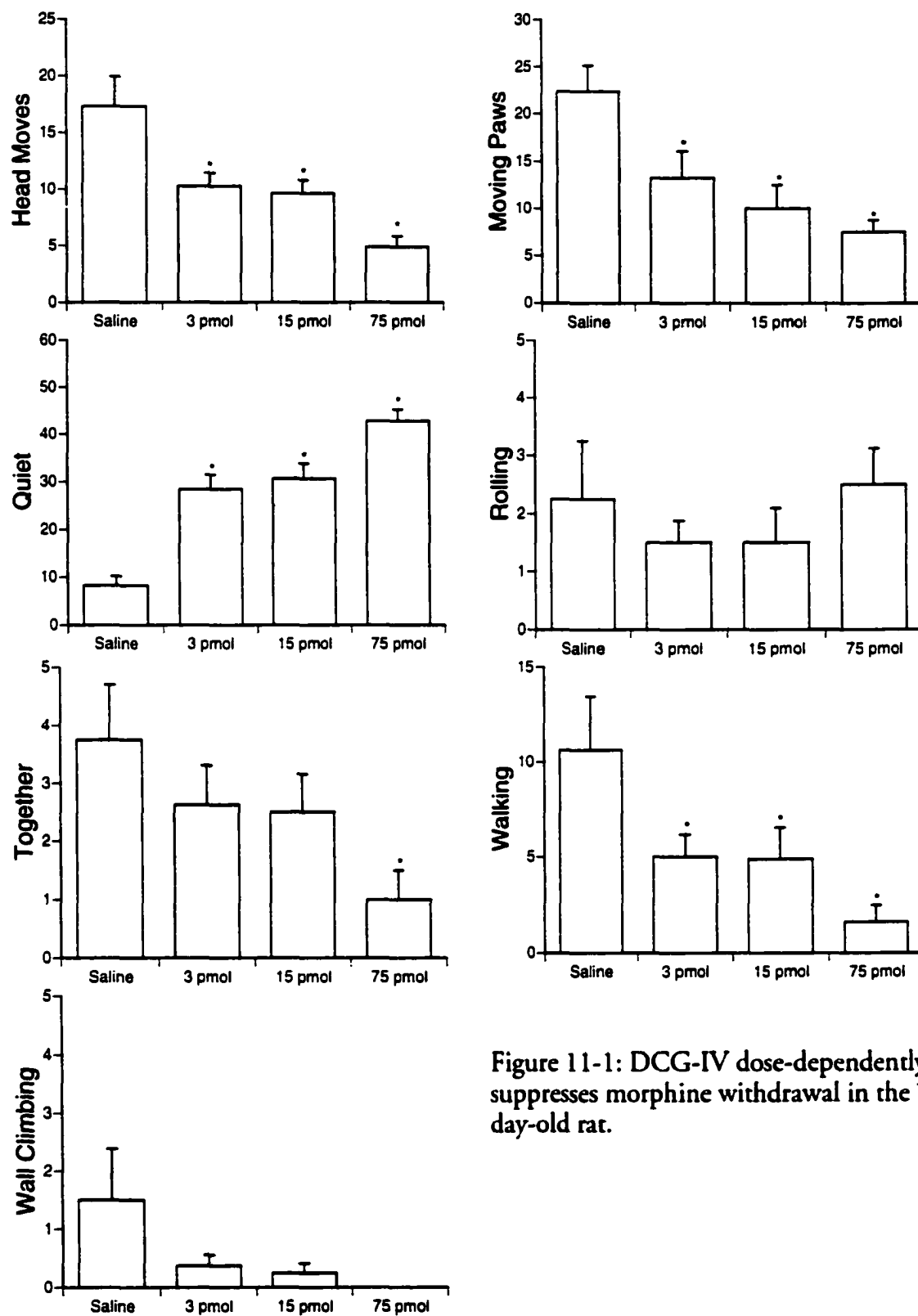


Figure 11-1: DCG-IV dose-dependently suppresses morphine withdrawal in the 7-day-old rat.

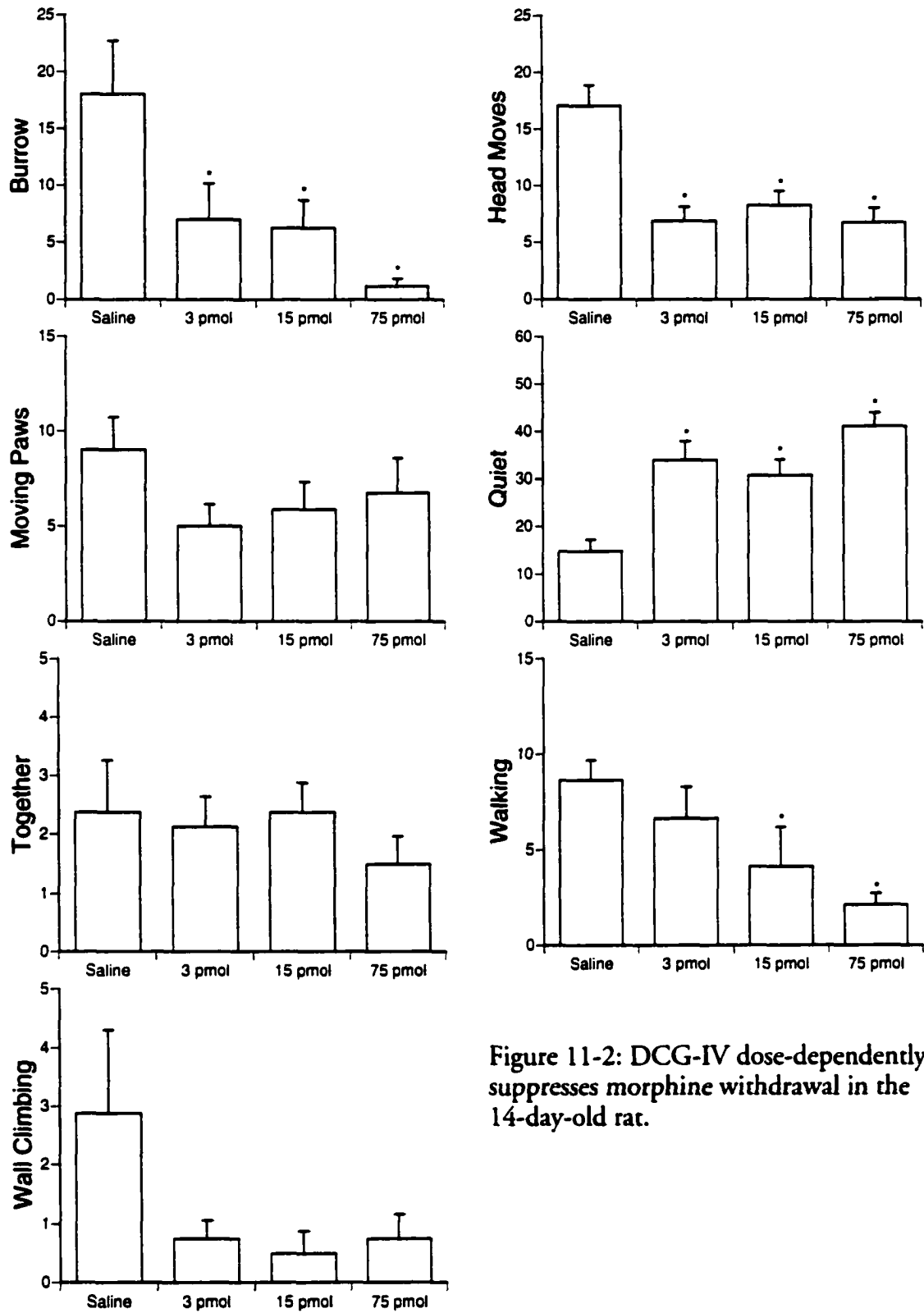


Figure 11-2: DCG-IV dose-dependently suppresses morphine withdrawal in the 14-day-old rat.

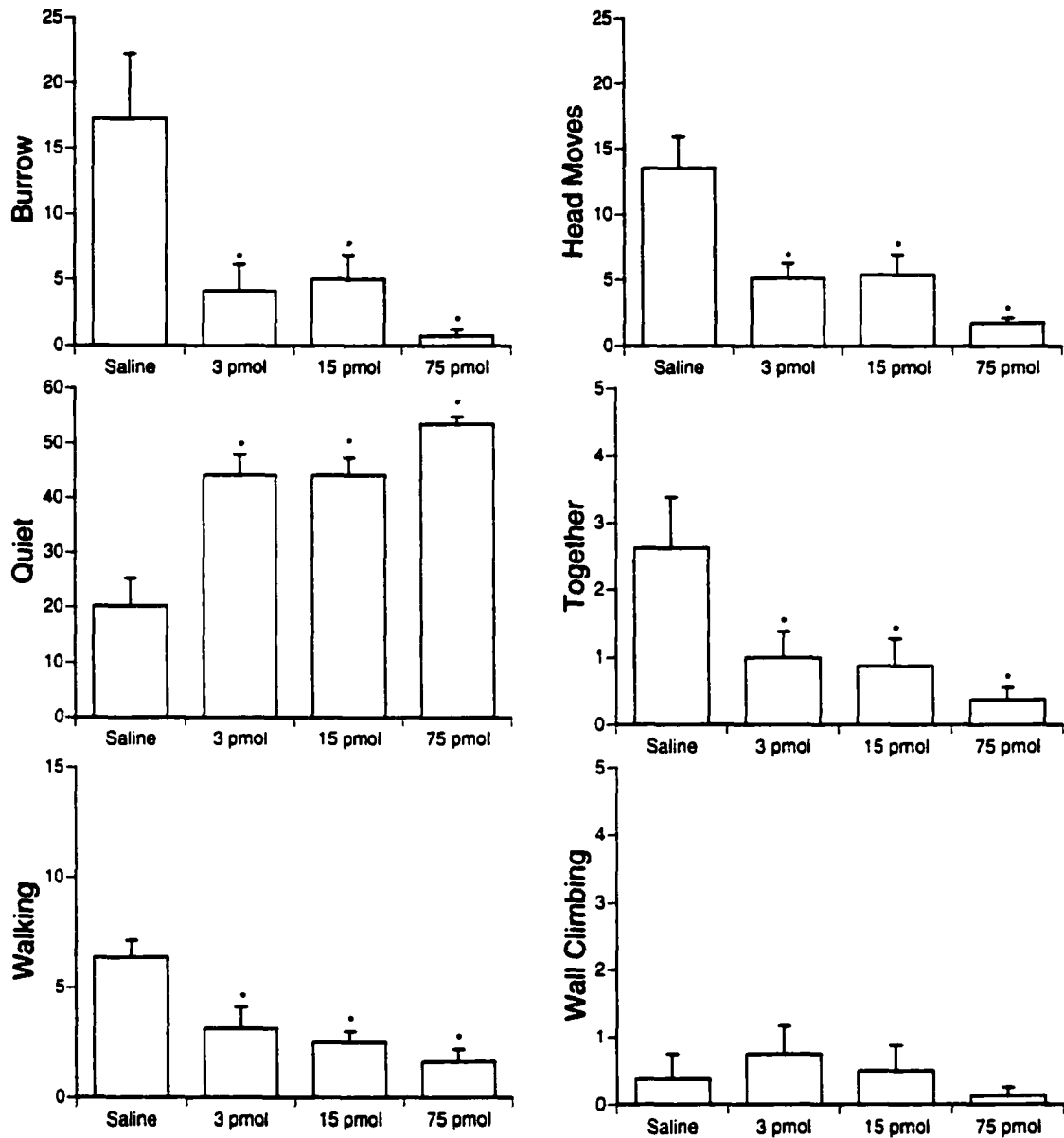
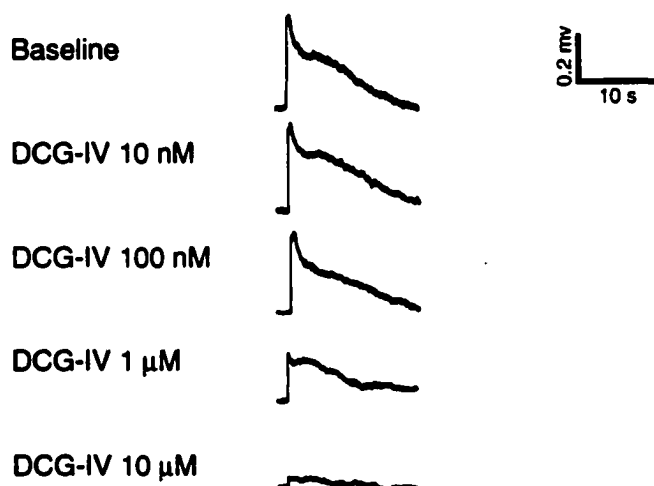


Figure 11-3: DCG-IV dose-dependently suppresses morphine withdrawal in the 21-day-old rat.

Figure 11-4 DCG-IV's effect on the sVRP in the 7-day-old rat

(a) Sample recordings of different concentrations of DCG-IV's effect on the sVRP in the 7-day-old rat



(b) Concentration-response curve of DCG-IV's effect on the sVRP in the 7-day-old rat ($n = 4$)

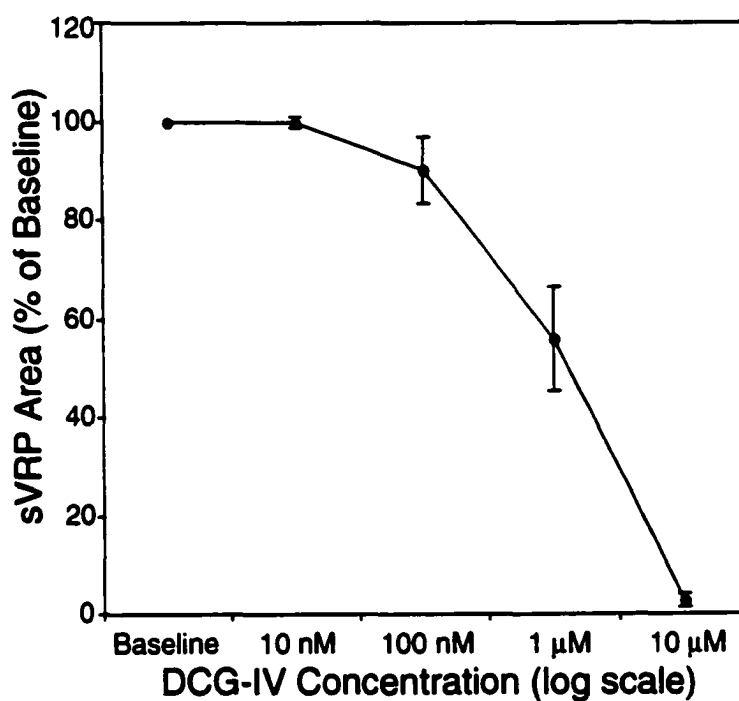


Figure 11-4. DCG-IV's effect on the slow ventral root potential in the 7-day-old rat.

Figure 11-5

Time and treatment effect of morphine withdrawal in the isolated spinal cord from the 7-day-old rat

A Time course of morphine withdrawal in the isolated rat spinal cord (without DCG-IV)

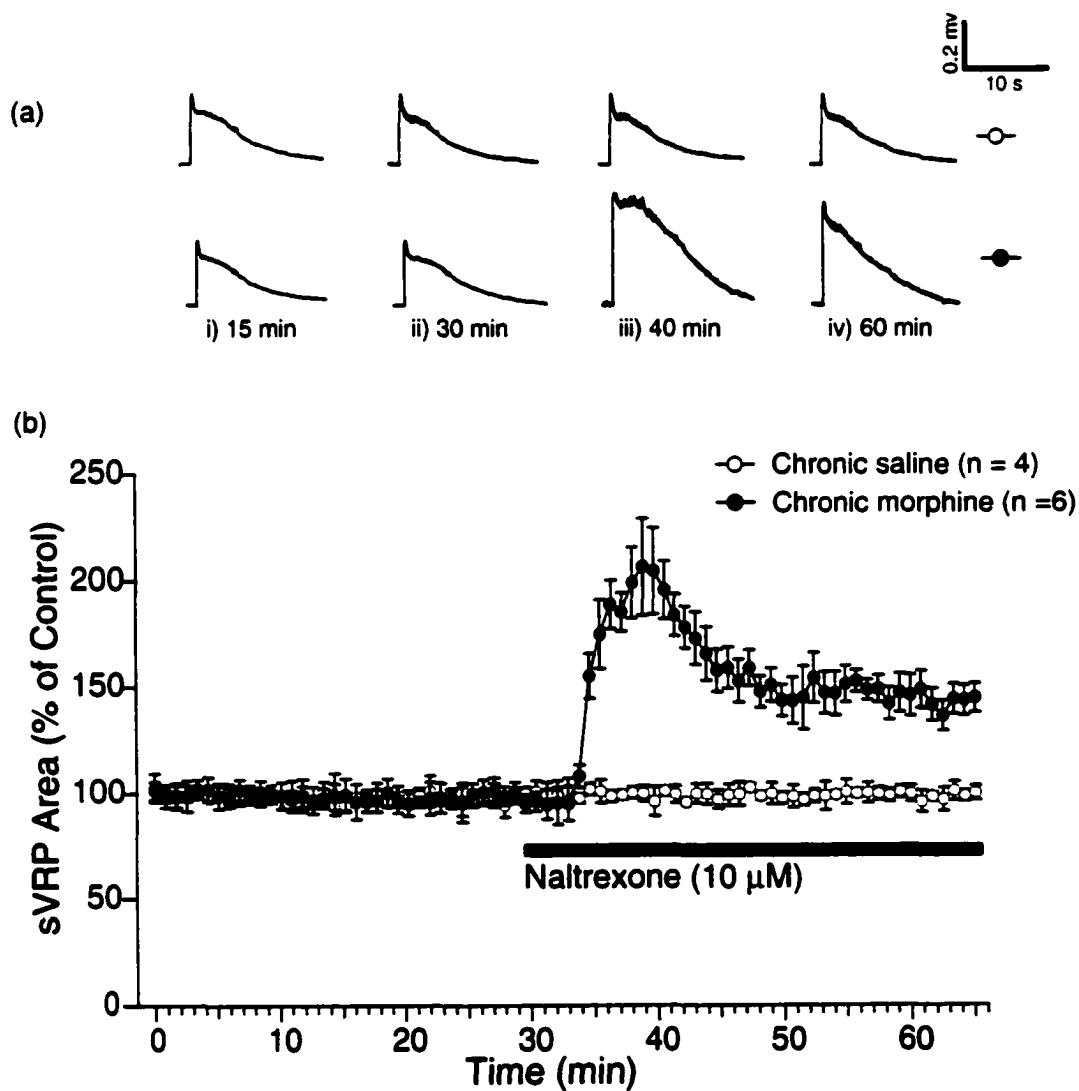


Figure 11-5A. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (without DCG-IV).

B Time course of morphine withdrawal in the isolated rat spinal cord (with 30nM DCG-IV)

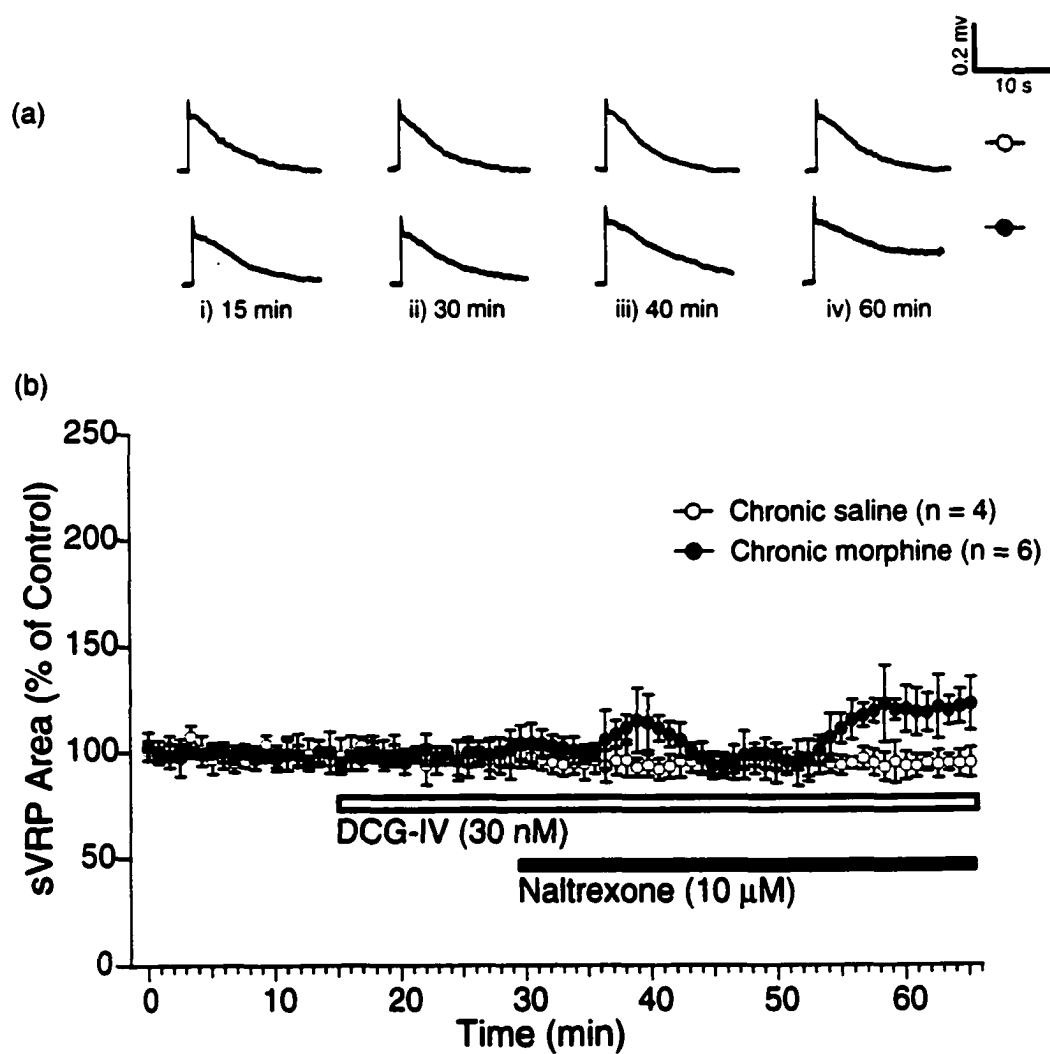


Figure 11-5B. Time course of morphine withdrawal in the isolated spinal cord from 7-day-old rat (with 30 nM DCG-IV).

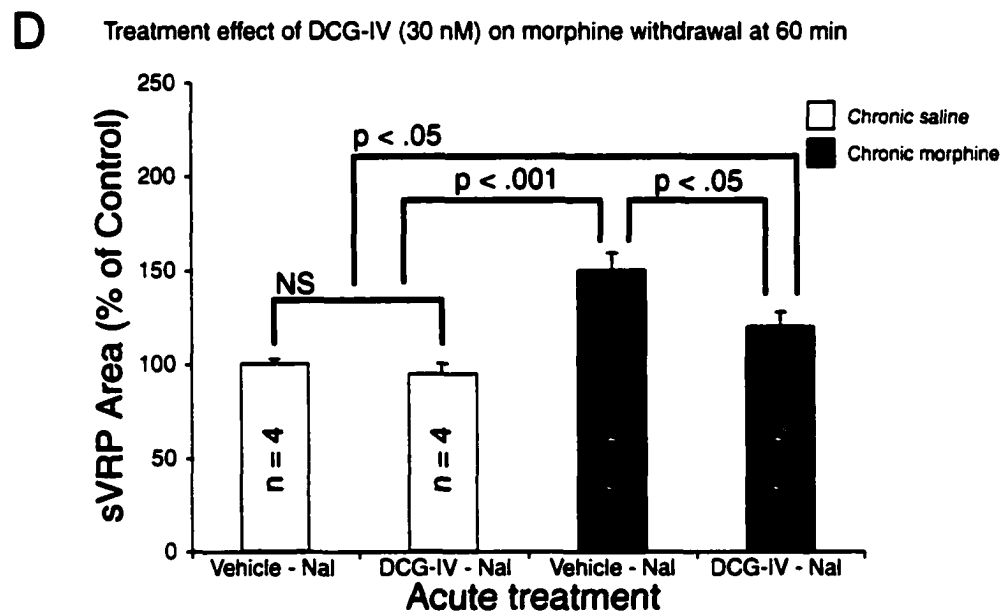
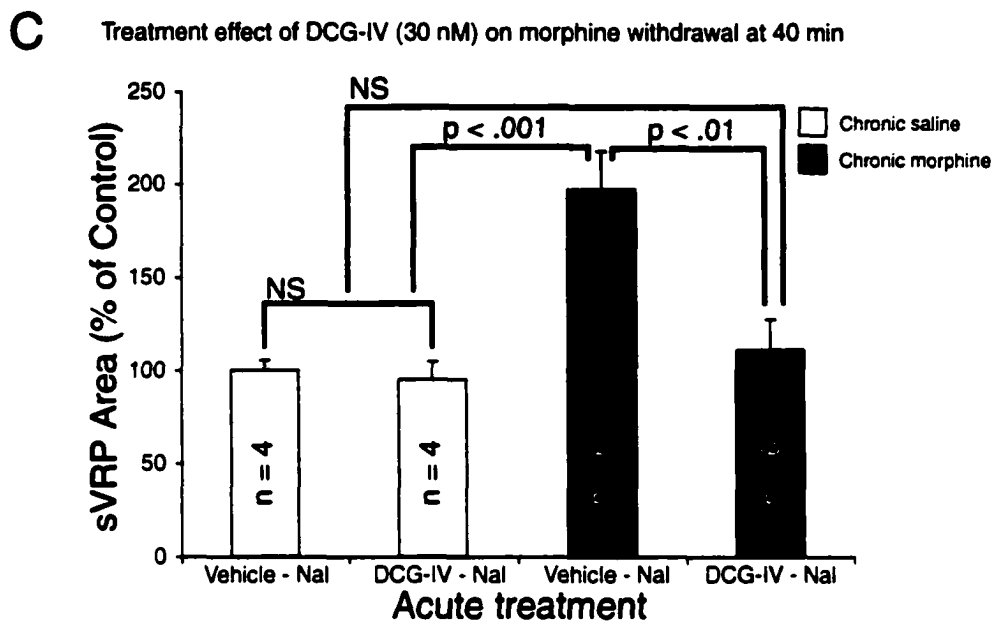


Figure 10-5C and D. Treatment effect of DCG-IV on morphine withdrawal at 40 and 60 min.

Part IV

Concluding

Chapter 12

Different therapeutic approaches are needed for infant addicts

Summary of findings

As detailed in previous chapters, there were many parameters that changed from experiment to experiment in the studies of the present thesis. For instances, different NMDA receptor antagonist including MK-801, AP-5, and dextromethorphan were used to test NMDA receptor's role in opiate tolerance and withdrawal; both chronic injection and acute injection paradigms were utilized to test some drugs' effect on development of morphine dependence and expression of morphine withdrawal; different appendages including forepaw, hindpaw and tail were examined to test morphine tolerance. Since the goal of the present thesis was to investigate the age effects on glutamate receptor's role in morphine tolerance and withdrawal using both in vivo and in vitro assays, these four dimensions can best grasp the gist and reveal the structure of the present thesis: 1.) glutamate receptor types (NMDA, AMPA, or mGluR); 2.) morphine actions (tolerance or withdrawal); 3.) methods of test (in vivo or in vitro assays); 4.) subject age (7-, 14-, or 21-day-old). Thus, without elaborating all of these minutiae of experimentation once again, the author will focus attention on these four key dimensions and summarize our major findings as following:

- 1) NMDA receptor antagonists were not effective in suppressing morphine withdrawal in the 7-day-old rat whereas they were effective in the 14- and 21-day-old rat; there was a transition age for the effectiveness for NMDA receptor

antagonist in suppressing morphine withdrawal; this age was in the second postnatal week in the rat.

- 2.) NMDA receptor antagonists were not effective in preventing the development of morphine tolerance in the 7-day-old rat whereas they were effective in the 14- and 21-day-old rat; there was a transition age for the effectiveness for NMDA receptor antagonist in preventing the development of morphine tolerance; this age was in the second postnatal week in the rat.
- 3.) The competitive AMPA receptor antagonist NBQX was effective in attenuating morphine withdrawal in the infant rat at 7, 14, and 21 days of age; the effectiveness of NBQX in suppressing morphine withdrawal in the infant rat was consistent across age.
- 4.) The competitive AMPA receptor antagonist NBQX was effective in preventing the development of morphine tolerance in the 7-day-old rat.
- 5.) The group II mGluR agonist DCG-IV was effective in attenuating morphine withdrawal in the infant rat including 7, 14, and 21 days of age; the effectiveness of DCG-IV in suppressing morphine withdrawal in the infant rat was consistent across age.

Table 12-1 further summarizes our major findings along with these four key dimensions.

Table 12-1. Summary of major findings of the present thesis.

Action	Assay	Age	NMDA Receptor	AMPA Receptor	Group II mGluR
Tolerance					
	In Vivo	1-7 do	⊖No	Yes	NT
		8-14 do	Yes	NT	NT
		15-21 do	Yes	NT	NT
	In Vitro	1-7 do	⊖No	NT	NT
Withdrawal					
	In Vivo	1-7 do	⊖No	Yes	Yes
		8-14 do	Yes	Yes	Yes
		15-21 do	Yes	Yes	Yes
	In Vitro	1-7 do	⊖No	Yes	Yes

NT: Not tested in the present thesis.

Yes: Suppress tolerance or withdrawal.

No: Do not suppress tolerance or withdrawal.

⊖ When present, age effect is significant.

The promise of NMDA receptor antagonism in treating opiate tolerance/withdrawal in the adult

The early discovery that NMDA receptor antagonists inhibit opiate tolerance and withdrawal in adult rodents (Marek et al., 1991a; Marek et al., 1991b; Trujillo and Akil, 1991b) stimulated an explosion of research on the role of NMDA receptors in these phenomena in the following decade. Despite later studies found that non-competitive NMDA receptor channel blockers have many adverse side effects (e.g., PCP-like effects) that contraindicate their clinical use (Herman et al., 1995; Frantz and Van Hartesveldt, 1999a, b), today the eager quest of NMDA receptor's great promise in treating opiate tolerance and withdrawal has yet to show any signs of

losing momentum, if not some gain. There are appealing reasons for such an enthusiasm. Firstly, all existing major therapeutic approaches for the treatment of opiate addiction are opioid-based and thus have inherent adequacy in eliminating addiction in the host and may evoke complications (e.g., methadone maintenance program may render the offspring dependent (Barr et al., 1998); furthermore, despite their distinct side-effects, opiates such as morphine and fentanyl remain as the most popular analgesics in the hospital setting (Marsh et al., 1997; Thornton and Smith, 1997; Thornton et al., 1997; Suresh and Anand, 2001); these strong clinical demands undoubtedly have generated a forceful impetus driving the zealous search of non-opioid based therapies for many years (Herman et al., 1995; Julien, 1998; Alvarez et al., 2001) (see also Chapter 1). Secondly, current thoughts on opiate tolerance and withdrawal suggest that these phenomena are instances of neural plasticity and have common underlying physical substrates as other forms of neural plasticity, such as LTP (Nestler et al., 1993; Nestler, 2001). Thirdly, the NMDA receptor, with its unique molecular structure and rich interaction with various second messenger systems, has a firmly established role in neural plasticity (Ozawa et al., 1998; Dingledine et al., 1999). Fourthly, breakthroughs in molecular biology have shed important light on the functional of the NMDA receptor; and advances in pharmaceutical technology have provided researchers more selective pharmacological tools to better investigate the function of the NMDA receptor.

As a result, the body of evidence accumulated over the past decade suggests that various NMDA receptor antagonists can attenuate opiate tolerance and withdrawal is overwhelming (see Chapter 1). It can be expected that the NMDA receptor antagonism approach is highly likely to be employed in treating opiate tolerance and withdrawal in the human patients in the near future. Besides broad and persuasive pre-clinical data (Herman et al., 1995; Inturrisi, 1997; Mao, 1999), this

trend is further reflected in three lines of observations: 1) to overcome NMDA receptor channel blockers' side effects, more selective NMDA receptor antagonists such as the glycine site antagonist's effect on opiate tolerance and withdrawal are actively being tested (Kolesnikov et al., 1994; Kosten et al., 1995; Lutfy et al., 1995; Bristow et al., 1997; Popik et al., 1998; Christensen et al., 2000; Kotli et al., 2001); 2) clinically available NMDA receptor antagonists such as memantine and dextromethorphan are being examined in animal models of opiate tolerance and withdrawal (Elliott et al., 1994; Elliott et al., 1995b; Manning et al., 1996; Mao et al., 1996; Popik and Skolnick, 1996; Popik and Danysz, 1997; Popik et al., 2000b); 3) human subject clinical trials investigating the effect of clinically available NMDA receptor antagonists (memantine and dextromethorphan) on opiate withdrawal have obtained quite positive results in both the United States (Rosen et al., 1996; Bisaga et al., 1997; Bisaga et al., 2001) and other countries (Koyuncuoglu et al., 1990; Koyuncuoglu, 1995).

In spite of the extensive and comprehensive evidence supporting NMDA receptor antagonists' effectiveness in inhibiting opiate tolerance and withdrawal in the adult organism, there is a lack of data in the infant. Conclusions based on adult data should not be applied to the infant without scrutiny due to a combination of factors: 1) although opiate tolerance and dependence can be established in infant rat pups, the withdrawal repertoire is different in the infant than that in the adult (Jones and Barr, 1995; Windh et al., 1995); 2) the neonatal CNS is both structurally and functionally different from that of the adult, and significant changes in opioid actions occur both prenatally and postnatally (Barr, 1992, 1993; Fitzgerald, 1995; Marsh et al., 1997); 3) most importantly, the NMDA receptor, which is believed to play a crucial role in the establishment of opiate tolerance, undergoes qualitative and quantitative changes during development (see Chapter 3).

The present thesis thus systematically investigated the pharmacological effect of NMDA receptor antagonists in opiate tolerance and withdrawal in the developing rat.

NMDA receptors are inappropriate targets in treating opiate tolerance/withdrawal in the newborn

Our results clearly show that a variety of NMDA receptors are not effective in either preventing the development of morphine tolerance or dependence, nor effective in suppressing morphine withdrawal. Although there existed two reports by another group implying that the NMDA receptor antagonist MK-801 were ineffective in preventing the development of either morphine tolerance or morphine withdrawal in the neonatal rat (Bell and Beglan, 1995a, b), these earlier studies are limited in that they investigated only one NMDA receptor blocker (MK-801) on morphine tolerance and withdrawal; tolerance and withdrawal were assessed solely by the in vitro paradigms; and they did not address the age issue since only one age of rats were tested. Therefore, it is unclear whether the discrepancy between these studies and the vast available adult literature was a result of the age effect, a special effect of MK-801, or simply different tolerance and withdrawal assessment paradigms.

Thus, in the present thesis, we examined the pharmacological effects of two NMDA receptor antagonists on morphine tolerance and withdrawal at different ages, using both the in vivo and the in vitro paradigms for tolerance and withdrawal assessment. Our results clearly show that for both morphine tolerance and withdrawal suppression, NMDA receptor antagonist were not effective in the week-old rat pups whereas they were in rats 14-day or older. Therefore, for NMDA receptor's role in opiate tolerance and withdrawal, in rats older than two weeks of age, our data agree

with the adult literature, but for the week-old rat pups, NMDA receptor antagonists are not effective for either tolerance or withdrawal, regardless of in vivo or in vitro test. More importantly, we systematically tested the age effect using multiple age groups, thus having eliminated potential experimental confounding factors, and convincingly demonstrated that there is a transition age for NMDA receptor to be effective for both tolerance and withdrawal in the rat.

Based on these results and earlier reports from another group (Bell and Beglan, 1995a, b), we thus predict that NMDA receptor antagonist will not be effective in attenuating opiate tolerance and withdrawal in the newborn human infant. Furthermore, we recommend against using NMDA receptor antagonists in the human infant for the treatment of opiate tolerance and withdrawal. Such a proposal is further strengthened by the recent finding that blockade of NMDA receptors results in apoptotic neurodegeneration in the developing brain, at least at high doses (Ikonomidou et al., 1999).

AMPA receptors and mGluRs are potential candidates for treating infant tolerance/withdrawal syndrome

Not only have we studied the effect of NMDA receptor antagonists in opiate tolerance and withdrawal in the infant, but also we have examined the role of AMPA receptors and mGluR in opiate tolerance/withdrawal in the developing organism.

Although increasingly more data appeared in the past few years suggesting that both AMPA receptor antagonists and group II mGluR agonists are effective in inhibiting opiate tolerance and withdrawal, caution should be taken when extrapolating conclusions based on the adult data to the infant. The reasons are similar to those as in the NMDA receptor case, especially since both AMPA receptors

and mGluRs undergo qualitative and quantitative changes during development (Ozawa et al., 1998; Dingledine et al., 1999) (see also Chapter 3).

Our results clearly show that for both the AMPA receptor antagonist and the group II mGluR agonist, their pharmacological effects on opiate tolerance and/or withdrawal in the infant rat are consistent with the adult literature. Unlike NMDA receptor antagonists, AMPA receptor antagonist and group II mGluR agonist do not show age effect, suggesting that these agents may be effective in attenuating opiate tolerance and withdrawal at all ages, which makes them as good candidates for the treatment of opiate tolerance and withdrawal in the developing organism as in the mature organism.

In conclusion, although great hopes for NMDA receptor antagonists in the therapy of opiate tolerance and withdrawal are promised by the overwhelming pre-clinical evidence and accumulating clinical data, their potential application in the infant are hampered by the finding by ours and others that NMDA receptor antagonists are not effective in suppressing opiate tolerance or withdrawal in the very young infant. In addition, that blockade of NMDA receptors results in apoptotic neurodegeneration in the developing CNS (Ikonomidou et al., 1999) has further precluded the use of NMDA receptor antagonist for the treatment of opiate tolerance and withdrawal in human infants. In contrast, the AMPA receptor and mGluRs may provide novel targets for the development of therapeutic agents that could have a dramatic impact on the treatment of opiate withdrawal in human patients of all ages.

Chapter 13

Mechanisms, potential pitfalls, and future directions

Chapter 12 discussed the potential clinical impact of our finding; this chapter will focus on the possible theoretical implication for our data. The most significant conclusion of our studies is that age is a key factor for NMDA receptor modulation of opiate tolerance and withdrawal whereas AMPA receptor and mGluR modulation do not show such an age effect. Although we have fairly thoroughly described these phenomena, empirically examining the underlying mechanism accountable for such an age effect falls out the scope of the present thesis. Despite that, several alternative hypotheses exist. The author would like to explore these possibilities tentatively and point out potential future research directions to test these possibilities experimentally.

Age-related Ca^{2+} permeability of glutamate receptors may explain the age effect

Our data clearly show that there is a transition period around the second postnatal week for NMDA receptor antagonists to attenuate morphine tolerance and withdrawal in the rat. The timing of the effectiveness of NMDA receptor antagonists in inhibiting morphine tolerance and withdrawal thus coincides well with the maturation of the NMDA receptor (Hrabetova et al., 2000; Sircar, 2000), especially the degree of Ca^{2+} permeability of the NMDA receptor (Das et al., 1998). Therefore, it is possible that the different pharmacological properties of NMDA receptor in rats of different age may reflect the functional differences related to different developmental stages of the NMDA receptor.

The NMDA receptor is composed of various subunits including NR1, NR2A-D, NR3A (Ozawa et al., 1998; Dingledine et al., 1999; Dingledine and McBain, 1999), and the recently discovered NR3B (Andersson et al., 2001; Nishi et al., 2001; Chatterton et al., 2002) subunits. All native NMDA receptor has at least one NR1 subunit but functional channels are formed only when NR1 is expressed together with one of the four NR2 subunits (Yamakura and Shimoji, 1999). The NR3A subunit may serve a regulatory function in NMDA receptors (Das et al., 1998). The various subunits of the NMDA receptor complex undergo dramatic differential maturation during the first few postnatal weeks (Guilarte and McGlothan, 1998; Vallano, 1998), resulting in considerably different functional states (Colwell et al., 1998). In the neonatal brain the predominant NMDA receptor subunits are the NR1, NR2B and NR2D subunits. Expression of the NR1-NR2B subunit complex is high at birth, peaks at about day 14 and then declines after day 21. The NMDA receptor containing the NR2A subunit is almost non-existent at birth, increases gradually with age and reaches maximal level on day 21. Expression of the NR1 subunit also increases with age and peaks on day 21 (Guilarte and McGlothan, 1998; Vallano, 1998; Sircar, 2000). It has recently been suggested that there are two types of NMDA receptors in the rat – an immature type and a mature type, and that there exists a temporal switching of the NMDA receptor from the immature form to the adult mature form around the second week postnatally (Hrabetova et al., 2000; Sircar, 2000). Thus, the pharmacological properties of the immature and mature NMDA receptor correlate well with the developmental profile of the NMDA receptor subunit configuration. Our finding that NMDA receptor antagonists were ineffective in the 7-day-old rat but effective in the 14 and 21-day-old rats may reflect the functional differences between the immature NMDA receptor type and the mature NMDA receptor type.

In the adult, one of the crucial roles of the NMDA receptor in opiate tolerance and withdrawal is that the activation of NMDA receptor results in Ca^{2+} influx into the cell and activates second messengers, thus leading to long lasting plasticity changes cascade (Mao, 1999; Smith et al., 1999; Trujillo, 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 (Das et al., 1998; Dingledine and McBain, 1999), the immature NMDA receptor seems to have low Ca^{2+} permeability (Das et al., 1998; Goebel and Poosch, 1999). Thus, it seems that the immature NMDA receptor is not a vital element to the development of opiate tolerance and dependence in rats of this age. Rather, in the infant, there may exist other mechanisms fulfilling the role of coupling to the activation of 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. Indeed, such a proposal can explain not only our data on the effects of the AMPA receptor antagonist and the group II mGluR agonist in morphine tolerance and withdrawal but also several known facts regarding opiate tolerance and withdrawal in the infant.

Firstly, like NMDA receptors, AMPA receptors also undergo major developmental changes in subunit expression leading to the acquisition of the adult pattern between P7 and P21 (Pellegrini-Giampietro et al., 1992; Jakowec et al., 1995b; Jakowec et al., 1995a). But unlike the immature NMDA receptor, which has low Ca^{2+} permeability, dependent on the region in the CNS, the immature AMPA receptor has high Ca^{2+} permeability (Pellegrini-Giampietro et al., 1992; Ozawa et al., 1998) and activation of AMPA receptor produces a marked increase in cytoplasmic free Ca^{2+} (Ozawa et al., 1998). For example, AMPA receptors in neonatal spinal cord permit significant Ca^{2+} permeability due to the higher (GluR1 + GluR3 + GluR4)/GluR2 subunit ratio than in the adult (Jakowec et al., 1995b; Jakowec et al.,

1995a). The role of Ca^{2+} entry through Ca^{2+} -permeable AMPA receptor has been shown to activate downstream Ca^{2+} dependent intracellular events (Jakowec et al., 1995b; Jakowec et al., 1995a; Ozawa et al., 1998). These events include NO production (Fedele and Raiteri, 1999) and phosphorylation of cAMP response element-binding protein (Perkinton et al., 1999) – processes known to be key steps in the establishment of opiate tolerance and withdrawal (Lane-Ladd et al., 1997; Mao, 1999; Trujillo, 1999). This explains well our data that NMDA receptor antagonists are not effective in inhibit morphine tolerance and withdrawal whereas the AMPA receptor antagonist NBQX is in the 7-day-old rat. Furthermore, this line of reasoning may further explain the exacerbation of certain withdrawal behaviors and augmented tolerance seen in the 7-day-old rat induced by high doses of NMDA receptor antagonists. 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 AMPA receptors, resulting in greater levels of dependence by these potential mechanisms other than the NMDA mediated mechanism.

Secondly, similarly, these calcium-dependent mechanisms may be mediated by the metabotropic glutamate receptors (mGluRs). Because mGluRs are coupled to various second messenger cascades, they are ideal candidates for long-lasting intracellular changes such as learning and memory formation (Pin and Duvoisin, 1995; Conn and Pin, 1997; Ozawa et al., 1998). Indeed, activation of the group II mGlu receptors inhibits calcium influx, thereby reducing the probability of transmitter release (Kilbride et al., 1998). Thus, acute treatment of the group II mGluR agonist DCG-IV inhibited morphine withdrawal in the 7-day-old rat. 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.

Thirdly, there is another appealing reason to consider the role of both the mGluRs and the AMPA receptor in opiate dependence. The effectiveness of NMDA receptor antagonists in inhibiting opiate withdrawal varies not only ontogenetically as we summarized in the present thesis but also phylogenetically as reported by others (Herman et al., 1995). As Herman et al. (1995) first pointed out, depending on species, the effect of MK-801 on opiate withdrawal is different. In guinea pigs, MK-801 consistently inhibits various morphine withdrawal symptoms (Tanganelli et al., 1991; Brent and Chahl, 1993). In adult rats, the results are less consistent (Rasmussen et al., 1991b; Bhargava et al., 1995). Most controversial are the effects of MK-801 in inhibiting morphine withdrawal symptoms in mice. There have been reports of significant effects (Tanganelli et al., 1991) and observations that failed to obtain significant effects (Thorat et al., 1994). It then possible that the different developmental status of NMDA receptors parallels these observed differences of MK-801 in inhibiting opiate withdrawal in different species. In contrast, the antagonism of both the mGluRs and the AMPA receptor has been consistently reported to inhibit opiate tolerance and dependence in both the rat and the mouse (Kest et al., 1997; McLemore et al., 1997; Fundytus and Coderre, 1999; Klodzinska et al., 1999; Vandergriff and Rasmussen, 1999). The implication is that mGluRs and AMPA receptors may play the dominate role in the newborn rat, in which the NMDA receptor is not functional, and recede to a co-operative position as NMDA receptor takes effect. The ontogenetic and phylogenetic inconsistency seen for the effectiveness of NMDA receptor antagonists may reflect the different stages of the transition of roles between mGluRs, AMPA receptors and NMDA receptors.

Fourthly, although tolerance can be established in the infant rat, the degree of tolerance in younger rats is less than that in the older ones (Windh et al., 1995). Given that multiple mechanisms accounting for opiate tolerance and withdrawal co-exist (Redmond and Krystal, 1984; Thorat et al., 1994; Crain and Shen, 1998; Fundytus and Coderre, 1999; Trujillo, 1999; Nestler, 2001), our proposal that age-related Ca^{2+} permeability of glutamate receptors determines their relative role in opiate tolerance and withdrawal may also provide an explanation to these age-related differences. Thus in the very young infant rat, mechanisms other than the NMDA receptor such as calcium-permeable AMPA receptors (Pellegrini-Giampietro et al., 1992; Gu et al., 1996; Ozawa et al., 1998) are coupled to the activation of Ca^{2+} dependent second messenger system. When the animal matures, the NMDA receptor mediated mechanism together with these adjunct mechanisms take a collective role in mediating the Ca^{2+} influx regulation, thus resulting in greater intensity of morphine tolerance.

Finally yet importantly, care must be taken when applying these developmental changes in the receptor functional and molecular properties to any model on opiate tolerance, dependence and withdrawal. The functional levels of expression of various subunits and their specific combinations vary markedly in different regions of CNS (Ozawa et al., 1998; Vallano, 1998; Dingledine and McBain, 1999) and the anatomical locus for opiate actions is multiple (Aghajanian et al., 1992; Christie et al., 1997). Therefore, simple model systems to combine behavioral, electrophysiological and molecular studies may be useful to elucidate the underlying mechanisms of opiate tolerance, dependence and withdrawal. The isolated spinal cord model we utilized in the present thesis is an excellent candidate for such a model system (see Chapter 7 for a list of benefit of this model). If appropriately combined with the use of various glutamate receptor subunit knock-out animals (Jia

et al., 1996; Ito, 1997; Das et al., 1998; Jia et al., 1998; Sprengel and Single, 1999; Rampon et al., 2000; Vekovischeva et al., 2001), this in vitro model system will generate reliable and conclusive data regarding the roles of glutamate receptors in opiate tolerance and dependence and withdrawal in the infant.

Neonatal withdrawal involves neurotransmitters other than glutamate?

Besides glutamate, other neurotransmitter systems have also been suggested be involved in the rebound effects seen during opiate withdrawal (Redmond and Krystal, 1984). For example, substance P (SP) may play a role in the opiate withdrawal syndrome (Bell and Jaffe, 1986). Opiates inhibit the release of SP from the spinal cord (Yaksh et al., 1977); acute or chronic morphine treatment causes an increase in SP levels in the dorsal horn (Bell and Jaffe, 1986); and increased release of SP is involved in the production of opiate withdrawal in the isolated neonatal rat spinal cord (Bell and Jaffe, 1986). Particularly relevant to our data is the finding that there exists dramatic developmental change in the role of SP and glutamate excitation in the genesis sVRP during the postnatal period from 1 to 21 days (Gibbs and Kendig, 1992).

The nociception-related sVRP consists of two phases: an early phase comprising a monosynaptic and a short polysynaptic response and a very long late phase (Thompson et al., 1992). Through 9 days, the sVRP has a typical peak amplitudes at 3-4 s, decay time constants of 18-20 s, and durations > 20 s. After 11 days, peak amplitude shortens to < 1 s, decay time constant 4-5 s, and duration < 10 s (Gibbs and Kendig, 1992). The neural transmitter basis of the sVRP has been examined extensively (Konishi and Otsuka, 1974; Nussbaumer et al., 1989; Woodley and Kendig, 1991; Gibbs and Kendig, 1992; Thompson et al., 1992; Guo et al.,

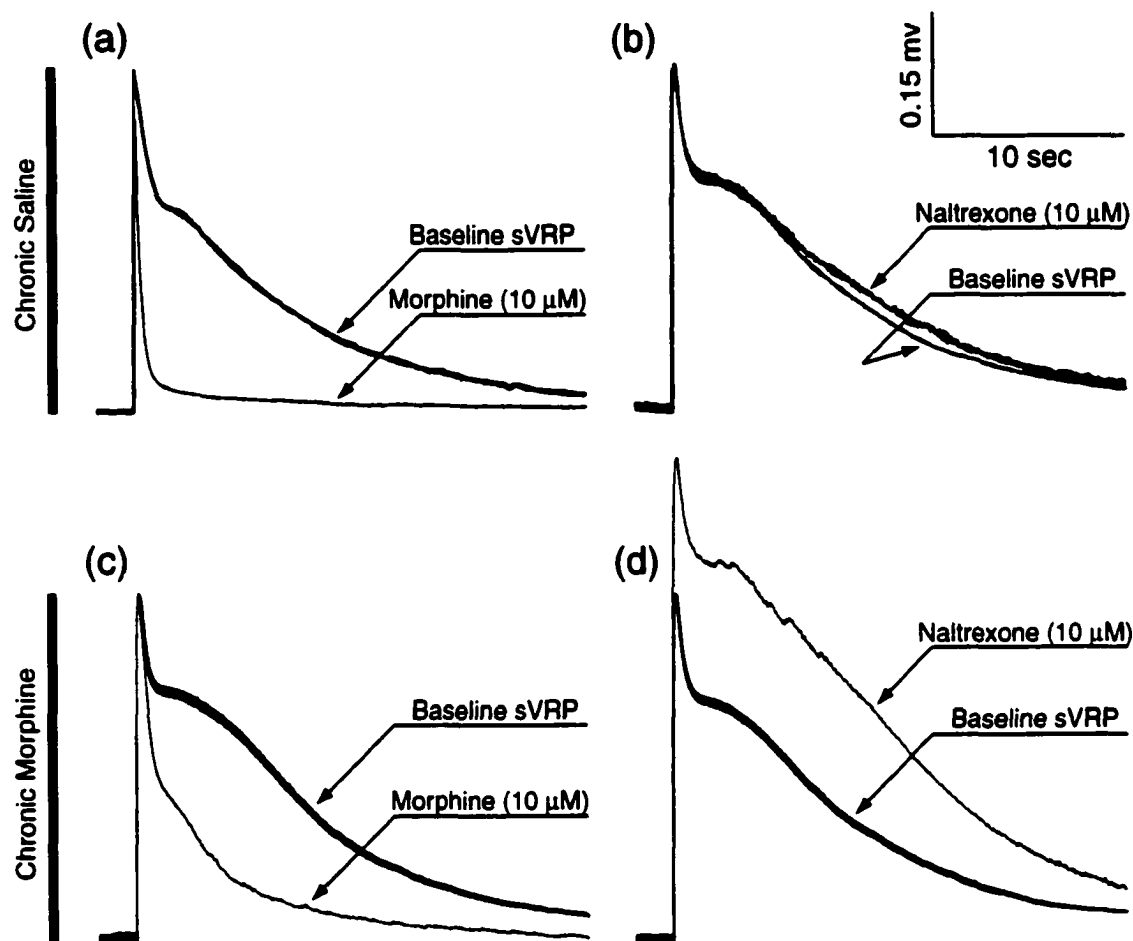
1993; Fox et al., 1996). At 1-6 days, sVRP's are sensitive to glutamate receptor antagonists and the SP antagonists. After 11 days, glutamate receptor antagonist sensitivity is retained, but sensitivity to SP antagonist is diminished. Thus, abbreviated sVRPs in post-11-day spinal cords appear to correspond to the early glutamate receptor antagonism sensitive component of long-duration sVRPs in younger animals. These results suggest that a SP response, some of which is mediated by NK1 receptors, is lost with maturation of the cord and there is a developmental role played by SP changes with maturity (Woodley and Kendig, 1991; Gibbs and Kendig, 1992; Thompson et al., 1992; Nagy et al., 1994; Fox et al., 1996).

Thus, in rat younger than 9 to 11 days of age, the sVRP consists of a brief initial component (0.1-1 s) which has a basis of glutamate receptor system excitation and is sensitive to glutamate receptor antagonism (Woodley and Kendig, 1991; Gibbs and Kendig, 1992; Thompson et al., 1992) and a very long lasting second component (> 20 s) which is resistant to these antagonists but sensitive to significantly reduced by both NK1, NK2, and probably NK3 tachykinin receptor antagonists (Nagy et al., 1994; Fox et al., 1996). In contrast, in rats older than 11 days of age, the sVRP is abbreviated to consist only the early glutamate receptor antagonist sensitive component of long-duration sVRP's in younger animals (Gibbs and Kendig, 1992). Thus, proportionally speaking, the sVRP in the younger rat has a relatively small glutamate receptor dependent component but a large SP dependent mechanism; in contrast, the sVRP in the older rat has almost an exclusively glutamate receptor dependent component. Particularly interesting are that these processes also occur in the second postnatal week (9-11 days of age) and that nociception in neonates does not require the NK1 receptor and that within the spinal cord, NK1 receptors start to play a role in the pain response to formalin between 10 and 21 days (King et al., 2000a; King et al., 2000b). Whether this developmental shift from a SP-dominant

mechanism to a glutamate-dominant mechanism of the nociception-related sVRP accounts for some of our major findings deserves further investigation.

Thus, one of our future directions would be studying the neural transmitter basis for the genesis of morphine withdrawal (and maybe also tolerance, see below) using the isolated spinal cord model. After more than two decades of study since the model was first established (Otsuka and Konishi, 1974), now we have a very good understanding of the genesis of the sVRP, both anatomically (Collins et al., 1995) and neurochemically (Otsuka and Yanagisawa, 1980; Evans, 1989; Otsuka and Yanagisawa, 1990; Kerkut and Bagust, 1995). This knowledge will greatly help us “narrow down” the neurotransmitters accounting for either the hyperactivity seen during morphine withdrawal or the tolerant component of the sVRP following chronic morphine. For example, that morphine preferentially suppresses the later component of sVRP can be interpreted as that morphine preferentially suppresses the glutamate receptor independent component of the sVRP (Sivilotti et al., 1995). Indeed, our preliminary data also suggest that morphine’s analgesic effect, morphine tolerance, and morphine withdrawal seems to be differential for different stages of the sVRP, which may be interpreted as the differential responses of different neurotransmitter and receptor systems to chronic morphine. As illustrated in Figure 13-1, when combined with the averaging technique (Chapter 7), the quantification of different stages of morphine tolerance and withdrawal can be expected to be highly reliable. However, caution should also be taken in pursuing such a line of research, since all existing pharmacological studies on the neurochemical basis of the sVRP used morphine-naive animals. Whether the neurochemical substrate of the sVRP generated in spinal from chronically morphine treated rat is comparable to that from the morphine-naive animal is unknown, although we did not see any difference

between the sVRPs generated from the morphine treated rat and the naive animal (Figure 13-1).



Morphine tolerance and withdrawal in isolated spinal cords from 7-day-old rats. (a) Acute morphine effectively suppressed the sVRP in the spinal cord from a morphine naive 7-day-old rat; notice morphine's differential effect in suppressing the different stages of the sVRP; (b) acute naltrexone only slightly increased the baseline sVRP in the spinal cord from a morphine naive 7-day-old rat; (c) morphine demonstrated diminished effect in suppressing the sVRP in the spinal cord from a 7-day-old rat treated chronically with morphine (10 mg/kg); (d) acute naltrexone greatly increased the sVRP in the spinal cord from a 7-day-old rat treated chronically with morphine (10 mg/kg). Each graph was from one individual rat. Each trace represents the average of 18 to 54 individual recordings.

Figure 13-1. Morphine tolerance and withdrawal show differential effects for different stages of the sVRP.

Morphine and NMDA receptor antagonists treatment altered the system?

The previous two hypotheses are similar in that both of them pursue the assumption that the mechanisms of opiate tolerance and withdrawal in the neonate and in the adult are different. The former hypothesizes that glutamate receptor other than the NMDA receptor plays a more dominant role in the neonate whereas the latter assumes that other neurotransmitter systems such as the SP system plays a dominant role in the neonatal rats. Although both lines of reasoning can explain our data well, there is yet another possibility that can potentially be a pitfall here: chronic morphine treatment, chronic NMDA receptor antagonists treatment, or the combination of both in the younger rat may have altered the very mechanisms on which morphine tolerance and dependence are established and morphine withdrawal is expressed. Thus, the opiate tolerance and withdrawal mechanism per se may not be different in the neonatal than that in the adult, rather, the regimen of chronic treatment of both morphine and NMDA receptor antagonists altered the immature CNS differently than it did to the mature CNS and should be responsible for the age effect we have seen.

For example, NMDA receptors are down-regulated in hypothalamus, midbrain and the spinal cord in the rat chronically treated with morphine (Bhargava et al., 1995), indicating there exists interaction between the opioid receptor system and NMDA receptor system. Whether chronic treatment of morphine down-regulates NMDA receptor in brain regions involved in opiate withdrawal in the neonatal rat is unknown. Similarly, chronic NMDA receptor antagonists treatment may also change the NMDA receptor system (Dravolina et al., 1999). However, whether NMDA receptors are unchanged, upregulated, or downregulated significantly depends on a myriad of experimental parameters including the type of

antagonists used, duration of exposure to the antagonist, dosing, and schedule of administration (Koyuncuoglu et al., 1992; Koyuncuoglu et al., 1994; Dravolina et al., 1999). The NMDA receptor is one of the most tightly regulated ion channels, suggesting that the normal functional NMDA receptors is essential for its role in many physiological and developmental processes such as cell migration, survival, and synaptic maturation (Vallano, 1998; Contestabile, 2000). Thus, it is likely that chronic antagonism of NMDA receptors in the infant have a plethora of direct and indirect effects on the later developmental process of the CNS. Indeed, a recent study found that blockade of NMDA receptors by high doses of NMDA receptor antagonists for only a few hours during late fetal or early neonatal life triggered widespread apoptotic neurodegeneration in the developing rat brain (Ikonomidou et al., 1999). Although the threshold dose of NMDA receptor antagonists to induce the apoptotic process was significantly higher than the doses employed in our studies, how the side effects of chronic NMDA receptor antagonism on the developing CNS are related to this antagonism's interaction with opiate tolerance and withdrawal needs further examination.

The potential side effect of altering the normal development process of the opioid system, the glutamate receptor system, and maybe many other aspects of the CNS by chronic morphine and NMDA receptor antagonists treatment may explain some data in the infant rat. For example, although NMDA receptor antagonists are not effective in suppressing chronic morphine withdrawal induced by chronic morphine treatment in the neonatal rat in vivo (Zhu and Barr, 2000, 2001b) and in vitro (Bell and Beglan, 1995a) (see also Chapter 8), they are at least partially effective in attenuating acute morphine withdrawal induced by a single injection of morphine in vivo (Jones et al., 2002) or a short period of opiate infusion in vitro (Feng and Kendig, 1995a, 1996b). These observations led us to hypothesize that either opiate

withdrawal resulted from acute and chronic morphine treatment are mechanistically different from each other (Jones et al., 2002); or, as elaborated in this section, chronic treatment of morphine or NMDA receptor antagonists plus morphine may significantly altered the system we intended to study.

The microarray technology (for review, see Marcotte et al., 2001) may help us gain insight into these complicated dynamic interaction between opioid and glutamate receptor systems during development. The ability of DNA microarrays (often referred to as “gene chips”) to monitor gene expression simultaneously in a large-scale format is expected to stimulate a revolution in the functional understanding of gene expression in living organisms (Marcotte et al., 2001). At present, highdensity DNA microarrays allow researchers to quickly and accurately quantify gene-expression changes in a massively parallel manner, although the need of standardization is serious (Marcotte et al., 2001). By employing the gene chips technology in analyzing the changes of gene and receptor protein expression, we may be able to launch a large-scale analysis on how the opioid receptor system and the glutamate receptor dynamically interact with each other to result in opiate tolerance and withdrawal manifestations unique to the infant.

Glutamate receptors' role in other related phenomena during ontogeny

Although we conclude that NMDA receptors are not involved in morphine tolerance or withdrawal induced by chronic morphine treatment during early development, NMDA receptors are critical for many normal physiological functions in the infant rat and are functional in several phenomena closely related to opiate tolerance and withdrawal.

In the adult rat, glutamate receptors play an important role in transmitting nociceptive signals within the spinal cord. This is indicated by several lines of research: dependence on spinal AMPA and NMDA receptors of inflammation-induced hyperalgesia; reduction of sensitization of the nociceptive neurons within the dorsal horn after exposure to prolonged nociceptive stimulation following NMDA and AMPA antagonists administration; altered expression of the protein and mRNA levels of AMPA and NMDA receptors after exposure to nociceptive stimulation (Mao, 1999; Urban and Gebhart, 1999). In addition, the NMDA receptor play a crucial role in the generation of windup and the maintenance of central hyperexcitability (Urch et al., 2001). Primary afferents in the dorsal horn can produce fast EPSPs that are non-NMDA dependent, and slower prolonged EPSPs produced by small afferent fibers that are NMDA dependent. These slow potentials temporally summate to produce large depolarization, a phenomenon termed windup (Thompson et al., 1990). Windup is believed to share common mechanisms with central sensitization and hyperalgesia and has been examined intensively as a model system for the study of nociception (for review, see Herrero et al., 2000). The selective competitive NMDA receptor antagonist AP5 has been shown to reduce the primary afferent evoked input and windup evoked responses of dorsal horn neurons in both infant and adult rats (Urch et al., 2001).

King and colleagues recently demonstrated that spinal administration of both AMPA and NMDA receptor antagonists attenuate nociceptive response to formalin in rat pups as young as 3 days old (King et al., 2002) whereas the neurokinin NK1 receptor becomes functional during the second postnatal week (King et al., 2000a; King et al., 2000b), although in vitro studies suggest that both glutamate and neurokinin neurotransmissions are involved in pain transmission at the spinal cord level in neonatal rats (Woodley and Kendig, 1991; Gibbs and Kendig, 1992;

Thompson et al., 1992; Nagy et al., 1994; Fox et al., 1996). These studies demonstrated that the NMDA receptor appears to play a crucial role in nociception at all ages and does not show a functional transition as it does in its role in morphine tolerance and withdrawal induced by chronic morphine treatment.

The involvement of NMDA receptors are also implicated in acute morphine withdrawal. In isolated neonatal rat spinal cord, naloxone administered after an opioid increases sVRP to levels above pre-drug controls. Naloxone (200 nM) alone produced no significant effect on sVRP area. Following 200 nM alfentanil, naloxone (200 nM) produced a significant increase in sVRP area to $138 \pm 18.0\%$ of control level. MK-801 (20 nM) had no effect on sVRP area when applied alone, although higher concentrations produced irreversible depression. MK-801 (20 nM) co-applied with 200 nM alfentanil blocked the rebound increase in sVRP area following naloxone 200 nM and also the increase following naloxone alone (560 nM) (Feng and Kendig, 1995a). Similarly, naloxone following morphine produced a significant increase in sVRP area above pre-morphine control values. MK-801 co-applied with morphine blocked the rebound increase in sVRP area following naloxone. Thus, NMDA receptors play a role in determining naloxone-precipitated hyperresponsiveness following acute alfentanil or morphine treatment (Feng and Kendig, 1996b). In addition to the in vitro studies supporting a role of the NMDA receptor in acute opiate withdrawal, LY235959, a competitive NMDA receptor antagonist, attenuates behaviors and c-fos mRNA expression associated with acute morphine withdrawal in the neonatal rat (Jones et al., 2002). Nevertheless, although NMDA receptor antagonists suppress acute opiate withdrawal as assessed in vitro and the in vivo, NMDA receptor antagonists are not effective in attenuating opiate withdrawal induced by chronic morphine treatment either in vitro or in vivo

(Chapter 8, 9). These results thus suggest that acute and chronic morphine withdrawal may be mechanistically different (Jones et al., 2002). Indeed, the mechanisms underlying acute and chronic actions of opiates may be qualitatively different from, and in some cases opposing to, each other (Sharma et al., 1975; Nestler and Aghajanian, 1997). Notably, acute morphine inhibits adenylate cyclase and results in a reduction of cAMP production in the cell whereas repeated treatment of morphine increases adenylate cyclase and cAMP-dependent protein kinase (Duman et al., 1988; Nestler and Tallman, 1988; Nestler and Aghajanian, 1997). However, the different effects of NMDA receptor antagonists in suppressing opiate withdrawal may be developmentally regulated, since NMDA receptor antagonists are effective in suppressing opiate withdrawal induced by both acute (McLemore et al., 1997) and chronic (Trujillo and Akil, 1991b) opiate treatment in the adult animals.

The reason that the NMDA receptor is functional in pain-related phenomena and acute morphine withdrawal but not involved in morphine tolerance and withdrawal during early life is unknown. This discrepancy may be due to the fact that NMDA receptors have different functions in different neural pathways. NMDA receptors are ubiquitously distributed throughout neurons in the central and peripheral nervous system where they directly modulate, or contribute to, a myriad of physiological and pathophysiological processes in developing and mature neurons. These include migration, survival, growth and differentiation, learning and memory, and neurodegeneration (Vallano, 1998). Especially during development, the activity of the NMDA receptor and the production of NO are key steps of neural development such as synaptogenesis, formation of neural maps, refinement of connections, neural cell proliferation, migration, neuronal survival or elimination through programmed cell death (Contestabile, 2000). A central role of NMDA receptors in such diverse processes is expected due to their unique properties.

However, dependent on the specific subunit composition, NMDA receptors may have very different properties (Chapter 3) and assume very different functions (Dingledine et al., 1999). In addition, subunit composition is subject to activity-dependent modification (Ozawa et al., 1998). Adding to this level of complexity, the function of a NMDA receptor is further dependent on the process of alternative splicing (Ozawa et al., 1998; Dingledine et al., 1999). Thus, determining NMDA receptors' functions in neurons is a formidable task, because 1600 combinations may exist (Vallano, 1998). In this light, it is not impossible that although NMDA receptors are functional in other related phenomena, including pain transmission, windup hyperalgesia, and acute morphine withdrawal, they are not involved in morphine tolerance and withdrawal during early life, in developing organisms.

The large picture

Although developmentally regulated calcium permeability property of the NMDA receptor may be a key factor in understanding the transition age we have seen, other alternative mechanisms are possible. For example, one of the more “upstream” steps before the influx of calcium through the NMDA receptor channel involves the activation of protein kinase C (PKC) (Chen and Huang, 1992; Mao, 1999). It is known that the activation of PKC influences both the conductance and clustering properties of NMDA receptors in a manner that depends upon NR1 subunit splicing and NR2 subunit association, as the carboxy-terminus of NR1, located intracellularly, contains several consensus phosphorylation sites for PKC (Vallano, 1998). Thus, the link between the mu-opioid receptor and the NMDA receptor are also developmentally regulated (Vallano, 1998; Liu and Anand, 2001). If the link between the mu-opioid receptor and the NMDA receptor is “broken” during

early life, the blockade of the NMDA receptor channel by administration of NMDA receptor antagonists is redundant since without the phosphorylation of PKC, the NMDA receptor is already blocked by Mg^{2+} . However, this line of reasoning will strengthen our most important proposal in the present thesis - that the NMDA receptor is not functionally involved in morphine tolerance and withdrawal during early life. According the Mao et al. model, without the activation of the NMDA receptor due to whatever reasons (e.g., the link between the mu-opioid and the NMDA receptor via PKC is not established during ontogeny), neonatal rats would be expected to not acquire morphine tolerance and dependence and express morphine withdrawal. However, as detailed in Chapter 1, neonatal rats do develop tolerance and dependence and express opiate withdrawal. How are tolerance and dependence established in these organisms without the participation of the NMDA receptor, either due to the blockade of antagonists approach as employed in the present thesis or as our alternative hypothesis suggests?

We believe that these seeming contradictory data are the results of the fact that the phenomena of opiate tolerance and withdrawal rely on multiple mechanisms. A parallel processing perspective is the key conceptual underpinning to understanding these perplexing phenomena. Although the present thesis explored the parallel roles of glutamate receptors in morphine tolerance and withdrawal during ontogeny, many other valid models on opiate actions exist. Nestler and colleagues have made considerable progress in identifying the mechanisms that contribute to long-lived neural and behavioral plasticity related to opiate and cocaine dependence, including drug- induced changes in gene transcription (e.g., CREB activation), in RNA and protein processing, and in synaptic structure (Nestler, 2001). Crain and Shen have provided evidence and improved our understanding on GM1 ganglioside-regulated excitatory opioid receptor functions in opiate tolerance and withdrawal (Crain and

Shen, 1998). Finn and Whistler recently demonstrated a critical role for receptor endocytosis in the development of opiate tolerance and withdrawal associated with prolonged opiate use (Finn and Whistler, 2001). Given the convincing data available supporting most of these models, it is likely that parallel mechanisms exist for opiate tolerance and withdrawal both in the adult and in the infant. How the development of the CNS and major receptor systems involved in opiate action affect these mechanisms is largely unknown at the present time (Marsh et al., 1997) and deserves further investigation.

Given the multiple steps necessary for the final successful expression of opiate withdrawal, it is not surprising that a great diversity of agents can modulate opiate withdrawal (Bhargava, 1994). Indeed, a specific drug can act on at least three major components that are indispensable to the final successful expression of withdrawal: 1) acquisition, various stages needed the establishment process of morphine dependence; 2) storage, the memory-like storage mechanism on which opiate dependence rely to last temporally; 3) retrieval, various affecting neural pathways necessary for the successful expression of withdrawal.

This understanding provides us a roadmap to the understanding of many puzzling data. A case in point is the role of mGluR in opiate tolerance and withdrawal. Chronic treatment of group II mGluR antagonists attenuates opiate withdrawal (Fundytus and Coderre, 1994; Fundytus et al., 1997). Surprisingly, acute treatment of group II mGluR agonists also suppresses opiate withdrawal (Fundytus and Coderre, 1997; Vandergriff and Rasmussen, 1999) (also see Chapter 11). This could be understood, however, given that chronic mGluR antagonism counteracts the various second messenger system alterations otherwise evoked by morphine treatment (Fundytus and Coderre, 1994; Fundytus et al., 1997) whereas acute mGluR agonism

offsets acute cAMP overshoot. Thus, the former approach blocks the acquisition mechanisms and the latter suppresses the retrieval mechanism of opiate withdrawal.

Thus, there are many sites that could be targeted to achieve a final goal of attenuating the intensity of final expression opiate withdrawal. Indeed, a great variety of agents have been reported to suppress opiate withdrawal (Bhargava, 1994). Some (e.g., Alpha 2-adrenoceptors, on which clonidine acts on) block the final common pathway of opiate withdrawal; some others (glutamate receptors) counterbalance the central mechanism of morphine's chronic effect; still others act on multiple components simultaneously (e.g., NMDA receptor antagonists). Given that glutamate transmission is the major excitatory neurotransmission mechanism (Dingledine et al., 1999), it is reasonable to assume that glutamate receptors are involved in all of the three stages for opiate withdrawal: acquisition, storage, and retrieval. This explains why data in the literature are so inconsistent (Chapter 1), since the majority of them focused only on NMDA receptor's role on a subset of one component of the three components of opiate withdrawal.

The final word

Like most other branches of sciences, the major conceptual approaches to complex problems in neuroscience that neural scientists have in command fall into one of two: 1) reductionist or a bottom-up approach attempts to analyze the nervous system in terms of its elementary components, by examining one molecule, one cell, or one circuit at a time, or 2) holistic or top-down approach attempts to focus on higher functions in alert behaving human beings and in intact experimentally accessible animals and attempt to relate these behaviors to the higher-order features of large systems of neurons (Albright et al., 2000). The phenomena of opiate tolerance,

dependence and withdrawal, certainly belong to the complex problem category and the final understanding of these phenomena needs both reductionist and holistic approaches to analyze processes encompassing psychological, physiological, cellular, molecular, and genetic levels.

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